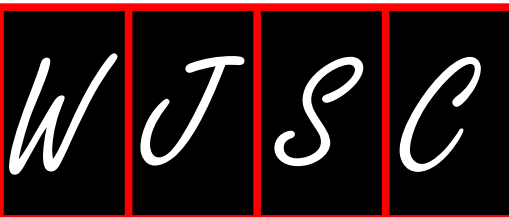


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REVIEW

- 1 Alternative splicing: An important mechanism in stem cell biology
Chen K, Dai X, Wu J

- 11 Augmenting peripheral nerve regeneration using stem cells: A review of current opinion
Fairbairn NG, Meppelink AM, Ng-Glazier J, Randolph MA, Winograd JM

- 27 Cancer stem cell plasticity and tumor hierarchy
Cabrera MC, Hollingsworth RE, Hurt EM

- 37 Control of stem cell fate by engineering their micro and nanoenvironment
Griffin MF, Butler PE, Seifalian AM, Kalaskar DM

- 51 Current progress in use of adipose derived stem cells in peripheral nerve regeneration
Zack-Williams SDL, Butler PE, Kalaskar DM

- 65 Mesenchymal stem cell tracking in the intervertebral disc
Handley C, Goldschlager T, Oehme D, Ghosh P, Jenkin G

- 75 *In vivo* imaging of endogenous neural stem cells in the adult brain
Rueger MA, Schroeter M

- 84 Identify multiple myeloma stem cells: Utopia?
Saltarella I, Lamanuzzi A, Reale A, Vacca A, Ria R

- 96 Stem cells for the treatment of musculoskeletal pain
Labusca L, Zugun-Eloae F, Mashayekhi K

- 106 Advances in cellular technology in the hematology field: What have we learned so far?
Souza GT, Maranduba CP, Souza CM, Amaral DLAS, Guia FC, Zanette RSS, Rettore JVP, Rabelo NC, Nascimento LM, Pinto IFN, Farani JB, Neto AEH, Silva FS, Maranduba CMC, Atalla A

- 116 Methods of induced pluripotent stem cells for clinical application
Seki T, Fukuda K

- 126 Adult human neural stem cell therapeutics: Current developmental status and prospect
Nam H, Lee KH, Nam DH, Joo KM

- 137** Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries
Wongtrakoongate P

- 149** Perinatal stem cells: A promising cell resource for tissue engineering of craniofacial bone
Si JW, Wang XD, Shen SG

MINIREVIEWS

- 160** Stem cell therapy for retinal diseases
Garcia JM, Mendonça L, Brant R, Abud M, Regatieri C, Diniz B
- 165** Obesity and weight loss could alter the properties of adipose stem cells?
Baptista LS, Silva KR, Borojevic R
- 174** Advances and challenges in the differentiation of pluripotent stem cells into pancreatic β cells
Abdelalim EM, Emara MM
- 182** How do resident stem cells repair the damaged myocardium?
Hayashi E, Hosoda T
- 186** Stem cells for spine surgery
Schroeder J, Kueper J, Leon K, Liebergall M

ORIGINAL ARTICLE

Basic Study

- 195** Early gestation chorionic villi-derived stromal cells for fetal tissue engineering
Lankford L, Selby T, Becker J, Ryzhuk V, Long C, Farmer D, Wang A

- 208** Regulated genes in mesenchymal stem cells and gastric cancer
Tanabe S, Aoyagi K, Yokozaki H, Sasaki H

Clinical Trials Study

- 223** Fetal vs adult mesenchymal stem cells achieve greater gene expression, but less osteoinduction
Santiago-Torres JE, Lovasz R, Bertone AL

Contents

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ABOUT COVER

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Alternative splicing: An important mechanism in stem cell biology

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Abstract

Alternative splicing (AS) is an essential mechanism in post-transcriptional regulation and leads to protein diversity. It has been shown that AS is prevalent in metazoan genomes, and the splicing pattern is dynamically regulated in different tissues and cell types, including embryonic stem cells. These observations suggest that AS may play critical roles in stem cell biology. Since embryonic stem cells and induced pluripotent stem cells have the ability to give rise to all

types of cells and tissues, they hold the promise of future cell-based therapy. Many efforts have been devoted to understanding the mechanisms underlying stem cell self-renewal and differentiation. However, most of the studies focused on the expression of a core set of transcription factors and regulatory RNAs. The role of AS in stem cell differentiation was not clear. Recent advances in high-throughput technologies have allowed the profiling of dynamic splicing patterns and cis-motifs that are responsible for AS at a genome-wide scale, and provided novel insights in a number of studies. In this review, we discuss some recent findings involving AS and stem cells. An emerging picture from these findings is that AS is integrated in the transcriptional and post-transcriptional networks and together they control pluripotency maintenance and differentiation of stem cells.

Key words: Alternative splicing; Stem cell; Pluripotency; Differentiation; Splicing factor

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Core tip: Alternative splicing (AS) produces multiple transcript isoforms from a single gene, and the regulation of cell-type-specific splicing pattern is crucial for the properties and functions of cells, including pluripotent stem cells. A better understanding of the role of AS in stem cell pluripotency maintenance and differentiation will offer potential new approaches for enhancing the production of induced pluripotent stem cells and/or better control of cell differentiation for research or therapeutic purposes. In this brief review, we provide a timely update of recent studies related to stem cell regulation and splicing in a genome-wide scale.

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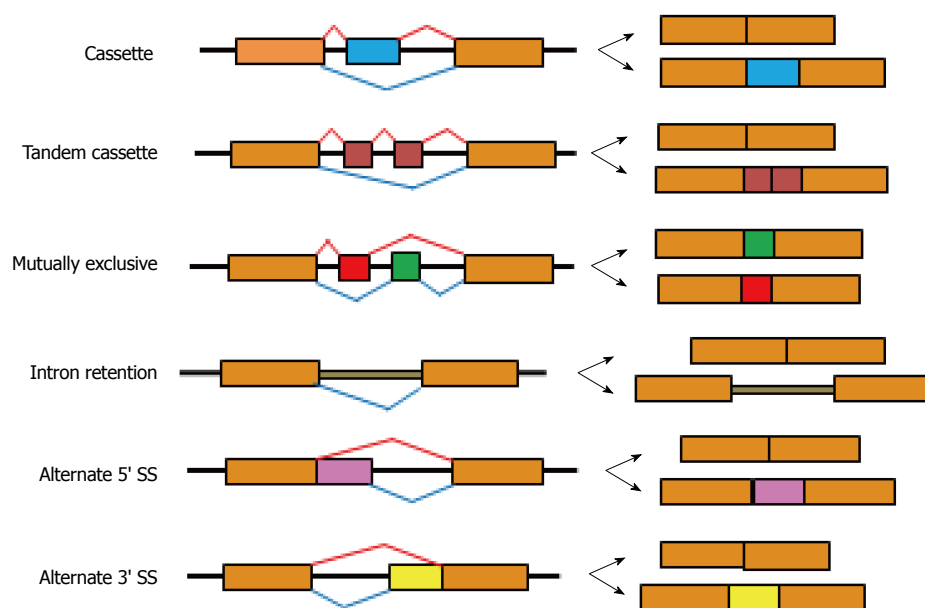


Figure 1 Major alternative splicing events in the metazoan transcriptome. Major types of alternative splicing events are shown. Brown boxes indicate constitutive exons, while boxes in other colors indicate alternative spliced exons.

INTRODUCTION

The splicing of messenger RNA precursors, namely the precise removal of introns and the joining of exons, is a crucial yet highly dynamic and flexible process in the synthesis of mature eukaryotic mRNAs. Alternative splicing (AS), the inclusion of different exons in mature mRNA by selecting different splice sites in pre-mRNA, can result in different transcript isoforms from a single gene, and give rise to a much larger number of proteins compared to the number of genes encoded in metazoan genomes^[1-3]. AS regulation plays an important role in almost every aspect of eukaryotic biological processes, including cell growth, death, pluripotency maintenance, differentiation, development, circadian rhythms, response to external changes, and disease^[4,5]. Recent advances in high-throughput RNA sequencing technology revealed that a greater number of multi-exon genes can produce alternatively spliced transcripts than previously thought^[6,7]. In humans, more than 90% of genes were estimated to undergo AS in different tissues and/or cell types. Compared with other RNA processing mechanisms such as alternative transcription initiation, RNA editing and alternative poly(A) site selection, AS is the most prominent in generating mRNA complexity. In addition, AS events can introduce premature termination codons in mature mRNAs, triggering mRNA degradation by the process of nonsense-mediated mRNA decay (NMD)^[8,9]. AS events can also cause mRNA untranslated region (UTR) variation, which affects mRNA translation efficiency, stability and localization^[10-12].

Splicing of pre-mRNA involves the formation of active splicing complexes on pre-mRNAs *via* a stepwise assembly process. The basal splicing machinery (spliceosome) is comprised of five small nuclear ribonucleoprotein particles

(snRNP), such as U1, U2, U4/U6 and U5 in the case of the major spliceosome, and U11, U12, U4atac/U6atac and U5 in the case of the minor spliceosome. AS is primarily regulated by approximately 200 RNA-binding proteins (splicing factors) together with a basal spliceosome through direct recognition of short sequence motifs near exon/intron boundaries^[13]. Depending on the pattern of exon inclusion/skipping, AS events can be categorized into at least six major types, including cassette exon skipping, mutually exclusive exons, alternative 5' splice site selection, alternative 3' splice site selection, alternative retained intron, and tandem cassette (Figure 1). There are more complex patterns but they are much fewer in number than these major types, therefore most analyses of AS events focus on these six types, particularly cassette exon skipping which represents the majority of AS events.

The knowledge of the crosstalk between splicing and other layers of gene regulatory network is fundamentally important for understanding biological processes such as cell differentiation, development, and pluripotency maintenance. In this review, we will highlight recent progress related to these themes, with an emphasis on studies involving both AS and stem cell research, to provide timely insight into AS regulation and its important roles in the determination of cell fate. The general principles of splicing regulation have been covered in detail in a number of excellent reviews, and readers who are interested in the mechanisms of splicing regulation can refer to these^[9-11,14-21].

GENOME-WIDE METHODS APPLIED IN AS RESEARCH

Our understanding and knowledge about AS has increased rapidly during last decade, thanks to the advancement

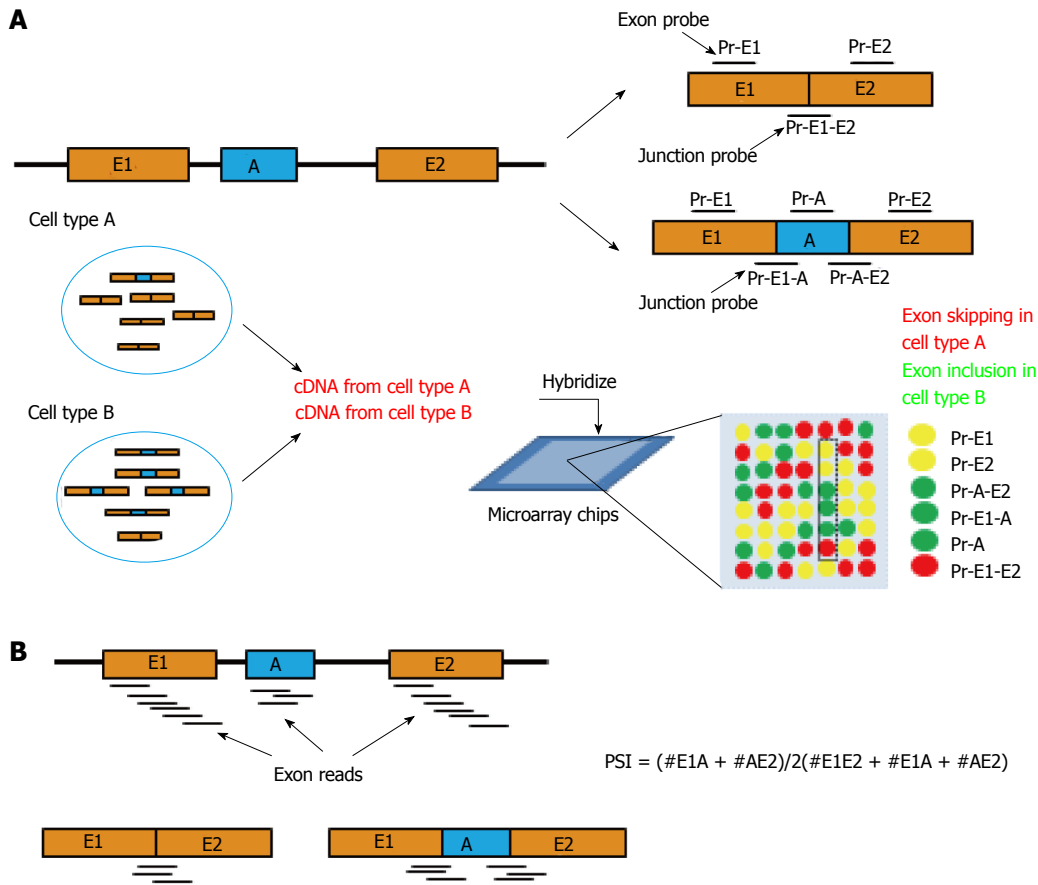


Figure 2 Methods for profiling alternative splicing events. The diagram depicts methods used for profiling AS events. The cassette exon AS event is used as an illustration for simplicity. "E1" and "E2" are constitutively spliced exons, and "A" is an alternative exon. Probes designed for specific regions are noted with a "Pr-" prefix. A: Splicing sensitive microarray for AS profiling. Adopted from^[2]. B: RNA sequencing for AS profiling. Percent spliced-in value (PSI) #E1A, #E1E2, #AE2 are the number of reads mapped to the spliced junctions. AS: Alternative splicing.

of several high-throughput technologies. To better understand AS regulation, it is necessary to be familiar with the basic principles of these technologies. Here, we summarized some of the technologies that were applied to study AS in a genome-wide scale.

The first genome-scale AS study was carried out using microarray platform. Traditional microarrays have been designed to measure the total level of expression of a gene, without discrimination of its different isoforms^[22-24]. To probe AS events, several splicing-sensitive microarray platforms have been developed^[3,25,26]. Although there are variations between them, these splicing-sensitive microarrays all utilize short oligonucleotide probes designed to cross exon-exon junctions. cDNA samples were derived from mRNA and hybridized to the probes (Figure 2A). The signal intensity of these junction probes can then be used to infer exon inclusion ratios by sophisticated algorithms^[27-37]. These microarrays have been applied in a number of studies to generate genome-scale profiling of AS, and provided quantitative measurements of AS at different time points of development, across tissues, and upon perturbation of interesting splicing factors^[28,31,32,34,35,37]. From these pioneering studies, genome-level regulatory mechanisms of AS have been better understood, and have largely transformed our

view about AS in every aspect including their evolution, dynamic regulation, and their organization in global transcription networks^[2,19].

Recently, RNA sequencing technology has been evolving rapidly, and has become the method of choice for genome-wide AS analysis. In RNA sequencing methodology, cDNA fragments derived from poly(A) selected RNA population are sequenced from the ends and generate a large number of short sequence tags (reads). These reads can then be mapped (aligned) back to the reference transcriptome and splice-mapped reads can reveal the exon-exon junctions (Figure 2B)^[38,39].

Compared with microarrays, RNA sequencing (RNA-Seq) does not rely on probes pre-designed across exon-exon junctions based on prior knowledge about the transcriptome under study, thus novel exons and splice junctions can be detected in an unbiased manner. RNA sequencing also has other advantages such as no cross-hybridization issues, higher sensitivity and broader dynamic range^[40-46]. As the technology keeps improving and costs continue to decline, longer read length and more extensive sequencing coverage can lead to more accurate AS detection at a reasonable price.

High-throughput reverse transcription-polymerase chain reaction (RT-PCR) has also been developed and used

for monitoring AS changes^[47-49]. Although the number of AS events monitored is limited by prior knowledge from the reference AS database, in theory, it has the advantage of avoiding bias towards the highly expressed genes, and can quantify AS of medium- and extremely low-expressed genes^[50]. There is also a very good correlation between percent spliced-in (PSI, the percent of transcripts that include a specific AS exon; Figure 2) values obtained with RNA-Seq data and the PCR-based method for events in which RNA-Seq data had enough coverage to produce confident PSI estimates, suggesting that the PCR-based method is consistent with RNA-Seq and these two methods can complement each other^[48,51].

Methods for directly mapping RNA-binding protein (RBP) and the mRNA interaction transcriptome-wide *in vivo* have also been developed, complementing AS event profiling to decipher the regulatory network of splicing by RBP. To identify binding targets, a specific RBP together with its associated RBP complex is immunoprecipitated from cell lysate, and bound RNA transcripts are then purified and subjected to high-throughput sequencing^[52-54]. After mapping the reads back to the reference genome sequence, potential binding locations of RBPs can then be inferred by computer algorithms. RBP complexes can be immunoprecipitated under native condition; however, this can increase the risk of losing low-affinity yet specific *in vivo* binding or of obtaining artificial binding following cell extraction^[55]. A cross-linking step is usually performed to circumvent these problems. Several methods have been developed in this area. The CLIP-Seq (cross-linking immunoprecipitation and high-throughput sequencing, or HITS-CLIP) method uses UV light to crosslink proteins with RNAs^[56]. In PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking immunoprecipitation), photoreactive ribonucleotide analogs are used to treat cells and are incorporated into RNAs before UV treatment^[57]. And individual-nucleotide resolution cross-linking and immunoprecipitation (iCLIP) employs a self-circularization strategy to achieve individual-nucleotide resolution^[58]. RBP mapping combined with AS profiling can be used for constructing “RNA maps” which correlate binding site positions with splicing regulatory differences upon perturbation of specific splicing factors.

ALTERNATIVE SPLICING IN STEM CELLS

Advanced technologies have recently been adopted to profile AS in stem cells. Extensive AS patterns were observed in stem cells and their contribution to pluripotency maintenance and differentiation has been noted.

Pervasive splicing in embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells which can self-renew and has the ability to differentiate into all three germ layers^[59,60]. As ESCs can generate most if not all of the cell types of a human body, they serve as an excellent model for studying early embryonic development. ESC is also a valuable source for producing differentiated

cells for potential cell therapeutic purposes^[61,62]. Thus, intensive efforts have been devoted to stem cell gene expression profiling, and genes associated with pluripotency were discovered^[63,64]. However, only recent advances in next generation sequencing technology made it possible to profile the AS pattern of a given cell/tissue in a global scale. A number of genome-wide studies showed specific transcriptome changes during the differentiation of ESCs into different lineages^[65-70].

In 2005, Pritsker *et al.*^[65] started using expressed sequence tag collections derived from stem cells to identify splice variants in ESCs and hematopoietic stem cells, and this was one of the first AS analyses in stem cells on a genome-wide scale. AS was detected in > 1000 genes. Although the technology is outdated nowadays, it showed that AS generates a large diversity in the stem cell molecular repertoire.

Further studies using advanced technologies confirmed the pervasive AS in ESC. A study by Wu *et al.*^[66] adopted three types of RNA sequencing technologies and profiled the transcriptome changes during the differentiation of human ESCs (hESCs) into the neural lineage. The authors combined Illumina single and paired-end reads (sequence reads from both ends of cDNA fragments; 35 bp reads) and longer Roche 454 FLX and titanium sequencing reads (250-450 bp reads) to discern transcript structure and analyze transcriptome complexity. Transcriptome profiles of cells in the ESC stage, N1 (early neural initiation) stage, N2 (neural progenitor) stage, and N3 (early glial-like) stage were reconstructed from mapped sequencing reads. Utilizing the unique spliced junction reads detected from each gene across all four stages, the authors then calculated a “junction complexity index” and found that splicing isoform diversity is highest in undifferentiated hESCs and decreases upon differentiation, a phenomenon they named “isoform specialization”. Observations like this can only be achieved with a genome-scale study, demonstrating the power and potential of RNA sequencing in AS research. In 2010, Revil *et al.*^[71] applied splicing-sensitive exon microarray technology to profile alternative isoform expression in embryonic day 8.5, 9.5 and 11.5 embryos and placenta. Although the profiling was not performed using pure ESCs, their results revealed frequent AS during embryonic developmental stages. Intriguingly, a number of RBPs, including putative splicing factors, are differentially expressed and spliced across developmental stages, suggesting these RBPs may be involved in regulating tissue and temporal variations in isoform expression.

During reprogramming, the AS profile of induced pluripotent stem cells is reversed to an ESC-like state

It is well known that when somatic cells are reprogrammed to pluripotent stem cells (PSCs), the transcription of most genes reverted to an ESC-like state. An interesting question is whether this is also true for AS?

Several recent studies answered this question by profiling both induced PSC (iPSC) and ESC AS patterns in a

genome-wide scale. Ohta *et al.*^[72] combined RNA-seq and high-throughput absolute qRT-PCR to analyze splicing pattern changes during the reprogramming process. The somatic cell splicing profiles reverted to a pluripotent-like state during reprogramming. In addition, to determine whether alterations in splicing patterns are specific for PSCs, the authors identified 27 genes which undergo alterations during the reprogramming process, and profiled the splicing pattern of these genes across multiple tissues by qRT-PCR. Interestingly, the splicing patterns in iPSCs were most similar in the testes compared with other tissues, suggesting an intriguing hypothesis that PSCs regulate AS using the same mechanism as the testes does. Other work also showed that the splicing pattern is similar in iPSCs and ESCs^[48,51]. These observations raised the possibility that manipulating specific splicing regulators can potentially fine tune the reprogramming process.

ALTERNATIVE SPLICING INFLUENCES PLURIPOTENCY

In addition to investigating AS patterns during ESC differentiation, efforts have also been made to determine the functional impact of AS in ESCs^[65]. In the study of Pritsker *et al.*^[65], splicing complexity in ESCs was observed, and they also found that AS can modify multiple components of signaling pathways which are important for stem cell function. The distribution of splice variants across different classes of genes indicated that tissue-specific genes have a higher tendency to undergo AS than ubiquitously expressed genes. Comparisons between all orthologous genes which undergo AS in human and mouse transcriptomes showed that the patterns of AS are only weakly conserved, supporting that AS patterns evolve fast^[73,74]. Because multiple genes in stem cells undergo AS and these genes are enriched in regulatory proteins, stem cell molecular networks are highly dependent on AS.

Salomonis *et al.*^[67] investigated the roles of AS and alternative promoter selection in differentiating mouse ESCs using the Affymetrix exon-exon junction microarray. Among approximately 7500 genes and 40000 putative exon-exon junctions represented in the microarray, the authors identified 170 unique alternative exons (AEs) corresponding to 144 genes. It was predicted that 67% of these AEs altered the protein sequence and domain composition. Pathway analysis of these genes showed enrichment in genes associated with Wnt and transforming growth factor-beta receptor signaling pathways, the actin cytoskeleton, lipid transport, muscle contraction, mRNA metabolism, and embryonic development. Most of these 170 AEs were conserved between mouse and human, suggesting their functional importance. In order to examine the functional impact of AS, two genes, *SERCA2* and *Tcf3* that showed large differences in the expression level of alternative isoforms, were selected for examination. *SERCA2* is a Ca^{2+} pump that hydrolyzes ATP during the translocation of calcium from the cytosol to sarco/endoplasmic reticulum^[75]. PCR confirmed that one of its isoform (SERCA2b), with

an additional 44 amino acids and a longer 3'UTR region, was expressed in both ESCs and embryoid bodies (EBs), whereas another isoform with a shorter alternative 3'UTR (SERCA2a) was mainly expressed in EBs. Interestingly, the 3'UTR of SERCA2b was predicted to be targeted by many microRNAs (miR-200b, miR-214, *etc.*) but not SERCA2a in both mice and humans. This is consistent with the observation that SERCA2b mRNA is more degraded than SERCA2a in an experiment *in vitro*^[76], indicating 3'UTR of SERCA2b can inhibit protein expression. Using a library of miRNA mimics, the authors further confirmed that miR-200b and miR-214 among other miRNAs were indeed targeting SERCA2b. Bioinformatics analysis showed that miR-214 and miR-200b binding sites were enriched for inhibitor genes of cardiac differentiation, indicating they play functional roles in cardiac development by repressing cardiac inhibitor gene expression. In addition, previous studies showed that miR-200b and SERCA2a are both highly induced upon cardiac differentiation^[75,77,78]. Taken together, this study suggests that *SERCA2* can avoid direct repression by miRNAs through selectively expressing one of its isoforms (SERCA2a) which has no miRNA target sites in differentiated ESCs. This observation implies the ability of AS to regulate protein expression, without affecting gene or miRNA transcription. The other gene studied is *Tcf3* (TCF7L1 in humans), a Wnt signaling transcription factor and a repressor of ESC self-renewal. *Tcf3* inhibits ESC self-renewal through repression of Nanog and Oct4 transcription^[79,80]. The authors identified a longer isoform of *Tcf3* [Tcf3(l)] which is enriched in ESCs but downregulated upon differentiation. Tcf3(l) includes a 42-bp cassette exon which encodes an additional 14 amino acids overlapping with the Groucho binding domain^[81,82]. This domain is necessary for *Tcf3* to repress Nanog expression^[79]. Tcf3(l) is upregulated in ESCs compared with EBs, while a shorter isoform of *Tcf3*, Tcf3(s), is expressed at a constant level. Selective knockdown of Tcf3(l) in ESCs revealed several distinct targets of transcriptional repression compared with Tcf3(s). For example, knocking down any *Tcf3* isoform increased Nanog expression whereas Oct4 was upregulated only during knockdown of Tcf3(l). Knockdown of one or both *Tcf3* isoforms can lead to delayed differentiation in ESCs. Interestingly, Tcf3(l) knockdown and Tcf3(s) knockdown inhibit distinct differentiation pathways, raising the intriguing hypothesis that isoform-specific regulation of *Tcf3* targets affects distinct lineage commitment decisions. In short, these examples demonstrate that specific AS events can modulate transcriptional networks involved in pluripotency maintenance *vs* differentiation.

ALTERNATIVE SPLICING IS INTEGRATED IN THE CORE REGULATORY CIRCUIT OF ESCs

During the last decade, a core set of transcription factors (TFs), including OCT4 (POU5F1), NANOG and SOX2

among others which control the pluripotency of ESCs, has been uncovered^[83]. Together with specific microRNAs and long non-coding RNAs, these TFs control the expression of gene cohorts required for establishment and maintenance of ESC pluripotency^[84-86].

The AS of the core TFs can directly influence pluripotency control. One classic example is the pluripotency gene *OCT4*. The *OCT4* gene was identified to encode three isoforms which were named *OCT4A*, *OCT4B* and *OCT4B1*^[87]. Two isoforms, *OCT4A* and *OCT4B*, were shown to encode different binding domains which resulted in different target genes. While *OCT4A* can regulate genes that are responsible for stemness^[83,88], *OCT4B* does not have the ability to maintain ESC self-renewal and it regulates genes that are responsive to cell stress.

Not only can the AS of core TFs affect pluripotency control, but the AS of several other genes is also linked to stem cell self-renewal and lineage specification^[89-93]. Genes that have ESC-specific isoforms are particularly intriguing. A study conducted by Gabut *et al.*^[94] used microarray profiling to compare patterns of AS in undifferentiated and differentiated hESCs, and identified an evolutionarily conserved ESC-specific AS event of the gene *FOXP1* (Forkhead box transcription factor 1). Experimental validation showed that inclusion of *FOXP1* exon 18b is specific to self-renewing, pluripotent hESCs, thus this transcript isoform was named “*FOXP1-ES*”. The inclusion of exon 18b within *FOXP1-ES* changes the DNA-binding specificity of *FOXP1*, and allows *FOXP1-ES* to regulate distinct programs of gene expression in hESCs. Knockdown of *FOXP1-ES* results in a significant decrease in the expression of the pluripotency genes *OCT4*, *NANOG*, *NR5A2*, *GDF3*, and *TDGF1*, and an increase in the expression of differentiation-associated genes, including *GAS1*, *HESX1*, *SFRP4*. Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) was performed to identify genes that are directly regulated by *FOXP1-ES* and *FOXP1* in hESCs. The *FOXP1-ES* binding target genes significantly overlap with the set of genes that are dependent on *FOXP1-ES* expression in hESCs and a set of genes that are regulated by *OCT4* in hESCs. Overexpression of *FOXP1-ES* in mESC also promotes mESC self-renewal and pluripotency. Collectively, this study provided evidence that an AS switch regulating the *FOXP1-ES* isoform is integrated into the core circuit of the transcriptional regulatory network required for ESC pluripotency and iPSC reprogramming.

SPLICING FACTORS INVOLVED IN THE MAINTENANCE OF PLURIPOTENCY

Compared with transcription factors, little is known about splicing factors that may contribute to stem-cell self-renew and lineage specification. Technology advancement allows the identification of functional RNA cis-elements related to AS and splicing factors in stem cells recently.

In 2007, Yeo *et al.*^[68] studied the AS events in hESCs and neural progenitors using exon array analysis combined with sophisticated algorithms to identify exons undergoing AS. The analysis showed that RBFOX binding motif GCAUG was enriched proximal to a set of exons that are alternatively spliced in hESCs, suggesting that RBFOX splicing factors may play a critical role in hESCs. Following this study, the same group constructed an RNA map for RBP RBFOX2 to identify functional RNA elements in the human genome in hESCs^[91]. RBFOX2 is expressed abundantly in hESC cell lines, whereas RBFOX1 is not. Using CLIP-seq technology, thousands of RBFOX2 RNA targets were uncovered, representing approximately 7% of human genes in hESCs. Many RBFOX2 targets are themselves splicing factors, suggesting that RBFOX2 might act as an upstream regulator of many splicing factors. Interestingly, RBFOX2 pre-mRNA is also the target of itself, supporting the autoregulation of RBFOX2. It is possible that AS of RBFOX2 pre-mRNA may result in distinct proteins that can target different pre-mRNAs. RBFOX2 depletion in hESCs led to rapid cell death, indicating that RBFOX2 is important in maintaining hESC viability. However, RBFOX2 depletion in neural progenitor cells or primary human fetal neural stem cells did not cause cell death, suggesting that RBFOX2 has a different set of targets in different cell types.

Several recent studies have also used CLIP-seq to map binding sites of specific splicing factors. Combined with AS profiling, these studies revealed several splicing factors that are potentially associated with pluripotency maintenance. The work of Han *et al.*^[51] demonstrated a systematic strategy to study the function of specific splicing factors. They combined RNA-seq, CLIP-seq datasets, and “splicing code” analysis (a computational method which predicts cis-elements that promote or repress specific splicing events) to identify splicing regulators that are differentially expressed between stem cells and differentiated cells and control cell-specific AS. In particular, MBNL1 and MBNL2 were found to have the lowest relative expression levels in stem cells (ESCs and iPSCs) compared with differentiated cells in both humans and mice, suggesting that these proteins performed their function by repressing ESC-specific AS events. The authors tested the hypothesis using the *FOXP1* transcription factor. As mentioned previously, isoform *FOXP1-ES* contains an ESC-specific exon which allows *FOXP1* to bind and activate genes (*OCT4* and *NANOG*, *etc.*) required for pluripotency. Supporting this hypothesis, the experiments showed that the *FOXP1-ES*-specific exon was retained in differentiated cells in which MBNL1 and MBNL2 were knocked down, whereas, over-expression of MBNL1 and MBNL2 in ESCs promoted differentiated cell-like splicing patterns. Furthermore, MBNL knockdown enhanced the efficiency of reprogramming from fibroblasts into iPSCs by about 2-fold. Taken together, the study revealed that MBNL protein expression plays a functional role in differentiation

by promoting FOXP1-ES specific exon skipping, and their knockdown can facilitate reprogramming of somatic cell into iPSCs.

Another study by Venables *et al.*^[48] found that splicing factors MBNL1 and RBFOX2 cooperate to control pluripotency in stem cells. The authors adopted high-throughput RT-PCR technology to monitor splicing changes of more than 3000 AS events annotated in the RefSeq database during the induction of fibroblasts into iPSCs, and their subsequent re-differentiation. Comparing the AS profiles in fibroblasts and iPSCs, and fibroblasts re-differentiated from iPSCs, the authors observed that AS changes are reversible during these processes. Their finding uncovered a program of concerted AS changes involved in late mesoderm differentiation. The authors selected 47 AS regions (in different genes) whose splicing profiles showed an equivalent near-perfect anti-correlation in reversible stem cell induction and re-differentiation, and used these AS regions to investigate splicing mechanisms involved in stemness and maintenance of pluripotency. To identify the splicing factors involved in pluripotency and reprogramming, the authors knocked down 81 potential splicing factors in various cell lines and monitored these 47 AS regions using RT-PCR. The differences in PSI values (between iPSCs and original fibroblasts used for inducing pluripotent cells) of these 47 AS events were then compared with the differences in PSI values before and after 81 splicing factors were knocked down individually during reprogramming of fibroblasts into iPSCs. They found that MBNL1 knockdown correlated most strongly with the splicing profile change in the induction of pluripotency. Splicing factor RBFOX2 knockdown showed the second highest correlation with the induction of pluripotency. By knocking down both MBNL1 and RBFOX2 in fibroblasts, a significant correlation between splicing changes and the induction of pluripotency was observed, and the correlation was even higher than knocking down MBNL1 and RBFOX2 individually, suggesting that MBNL1 and RBFOX2 cooperate to establish the splicing program involved in stem cell differentiation.

Ohta *et al.*^[72] used siRNA screening in PSCs to identify RBPs involved in the reprogramming process by enhancing stem cell specific AS. After a screen of 92 RBPs, 9 RBP-coding genes that had a major effect on the splicing patterns were examined to assess the impact on somatic cell reprogramming using shRNA knockdown. The downregulation of U2af1 and Srsf3 was found to suppress both the efficiency of alkaline phosphatase-positive colony formation and ESC marker gene expression.

AS can also affect RNA stability through NMD. Most recently, Jangi *et al.*^[95] performed a genome-wide analysis of RBFOX2 activity in mESCs by mapping RBFOX2 binding sites to transcriptome changes after loss of RBFOX2. Using iCLIP and RNA-seq technologies, the authors identified more than 200 AS-NMD (AS-coupled nonsense mediated decay) splicing events that were mediated by RBFOX2 in mESCs. These events showed

minimal splicing changes but appreciable changes in gene expression upon RBFOX2 knockdown due to the degradation of the NMD-inducing isoform. About 70 of these AS-NMD events were within genes encoding RBPs. Many of these RBPs were also autoregulated. A large fraction of bound but apparently unregulated events likely generated the NMD isoforms. This led to the hypothesis that RBFOX2 can control the gene expression level by regulating AS-NMD. The authors further demonstrated that RBFOX2 determines a threshold for the ratio of NMD to non-NMD isoforms for several of these RBPs. These findings uncovered an unexpectedly broad multilayered regulatory network controlled by RBFOX2, and established a model for how autoregulatory splicing networks are tuned.

CONCLUSION

Induced PSCs hold the promise of future cell-based therapy. A thorough understanding of the mechanisms underlying stem cell pluripotency and differentiation is critical for harnessing the cell reprogramming process. In this review, we have summarized recent progress in the field of AS and its role in stem cell pluripotency maintenance and differentiation. It was found that AS is pervasive in stem cells, and reprogramming reverts the splicing pattern to an ESC-like state. AS has a fundamental impact on stem cell differentiation by regulating different isoforms of the core pluripotency transcription factors. AS of genes other than the core factors is also linked to stem cell self-renewal and lineage specification. Additionally, splicing factors can regulate pluripotency by affecting stem cell-specific AS. In summary, these findings present a picture in which AS is integrated in the transcriptional and post-transcriptional networks, and the crosstalk between AS and other layers of the gene regulatory network have a fundamental effect on stem cell pluripotency maintenance and differentiation. These findings can lead to novel approaches for improving iPSC development and a better control of cell differentiation.

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Augmenting peripheral nerve regeneration using stem cells: A review of current opinion

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Abstract

Outcomes following peripheral nerve injury remain frustratingly poor. The reasons for this are multifactorial, although maintaining a growth permissive environment in the distal nerve stump following repair is arguably the most important. The optimal environment for axonal regeneration relies on the synthesis and release of many biochemical mediators that are temporally and spatially regulated with a high level of incompletely understood complexity. The Schwann cell (SC) has emerged as a key player in this process. Prolonged periods of distal

nerve stump denervation, characteristic of large gaps and proximal injuries, have been associated with a reduction in SC number and ability to support regenerating axons. Cell based therapy offers a potential therapy for the improvement of outcomes following peripheral nerve reconstruction. Stem cells have the potential to increase the number of SCs and prolong their ability to support regeneration. They may also have the ability to rescue and replenish populations of chromatolytic and apoptotic neurons following axotomy. Finally, they can be used in non-physiologic ways to preserve injured tissues such as denervated muscle while neuronal ingrowth has not yet occurred. Aside from stem cell type, careful consideration must be given to differentiation status, how stem cells are supported following transplantation and how they will be delivered to the site of injury. It is the aim of this article to review current opinions on the strategies of stem cell based therapy for the augmentation of peripheral nerve regeneration.

Key words: Peripheral nerve; Augmentation; Regeneration; Stem cells

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Core tip: Outcomes following peripheral nerve injury remain poor. Stem cells may increase Schwann cell numbers and longevity, prolonging their ability to support regeneration. At the level of the cell body, they may have the ability to rescue populations of neurons destined for apoptosis. They may also be used in non-physiologic ways in order to protect muscle targets from the detrimental effects of denervation. Aside from stem cell type, consideration must be given to differentiation status, scaffolding support and the mechanism of delivery. This article reviews current opinion on the strategies of stem cell-based therapy for the augmentation of peripheral nerve regeneration.

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Winograd JM. Augmenting peripheral nerve regeneration using stem cells: A review of current opinion. *World J Stem Cells* 2015; 7(1): 11-26 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i1/11.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i1.11>

INTRODUCTION

Peripheral nerve injury, repair and challenges to recovery

Following nerve transection, the cell bodies of axons swell in anticipation of the increased metabolic demand of regeneration. Some nerve cells, particularly in response to proximal sectioning of axons, will then go on to apoptosis, lowering the number of regenerating neurons permanently. The Schwann cells (SCs), the principal glial cells of the peripheral nervous system and orchestrators of regeneration, switch from a myelinating to a phagocytic phenotype and, through chemotactic signaling, recruit circulating macrophages into the zone of injury. This heralds the beginning of Wallerian degeneration and serves to clear the distal stump of axonal and myelin debris in preparation for the reception of the regenerating growth cone. Through complex and incompletely understood cell signaling, arborized daughter axons extending from the proximal stump are attracted to columns of SCs known as the Bands of Büngner. These scaffolds nurture and guide regenerating axons towards their distal basal lamina tubes. Once SCs and the regenerating axon have been reunited, a growth promoting symbiosis is established. Any interruption in this process results in chronic SC senescence, down-regulation, a loss of axonal support and compromised recovery.

The gold standard method of nerve repair involves re-approximation using suture. Even with meticulous tissue handling and accurate, tensionless re-orientation of fascicular anatomy, outcomes are limited by suture inflammation, intra- and extra neural scar tissue, escape of axons and neurotrophic factors into the surrounding tissues, axonal mis-direction and the extremely slow rates of regeneration. These limitations are amplified following proximal limb injuries and in nerve gap reconstructions when axons must traverse two coaptation sites bridged by nerve grafts.

Autologous nerve grafts represent the gold standard bridging material for nerve gaps but these are limited by the morbidity of harvesting an “expendable” nerve. In addition, following severe trauma involving multi-limb amputation, demand for autologous material may exceed supply. In these situations, alternatives such as conduits or acellular nerve allograft (ANA) must be considered. The efficacy of hollow conduits is limited to short distances due to the lack of cellular components and extracellular matrix (ECM) to aid axonal guidance. Despite increasingly elaborate designs, these fail to replicate the efficacy of autogenous nerve. The use of ANA provides the most accurate representation of ECM, however, these grafts lack SCs and must be

repopulated with endogenous SCs migrating from adjacent nerve. As a result, outcomes following the use of these grafts for large gaps have typically been poor in comparison to autografts, though better than conduits. Improving the performance of conduits and allografts by adding stem cells, ECM components and growth factors is an area of intensive research, one that may have enormous clinical implications.

The overarching goal of any stem cell based approach to peripheral nerve injury is to establish a more favorable environment for regenerating axons and perhaps more importantly, maintain this support for an extended period of time. Repopulation of the depleted motor or sensory neuron pools with stem cells would potentially provide higher numbers of neurons for regeneration, which could aid regeneration in an additive fashion to the creation of a favorable environment. Preventing SC senescence and down-regulation during prolonged recovery periods, such as occurs in proximal injuries and long nerve gaps, and expediting the re-innervation of distal targets should translate to improvements in recovery. Finally, stem cell transplantation into denervated muscle may provide a means of preventing the expected sequelae of denervation, preventing atrophy and leaving the tissue more receptive to reinnervation over longer periods. It is the aim of this article to discuss how stem cell-based strategies have attempted to overcome these problems and how they may provide solutions in the future.

AUGMENTING NERVE REGENERATION USING STEM CELLS

The ideal stem cell for clinical application, including peripheral nerve repair, must be easily accessible, rapidly expandable in culture, capable of *in vivo* survival and integration into host tissue and must be amenable to stable transfection and expression of exogenous genes^[1]. If the process of nerve regeneration is deconstructed into a sequence of individual events, a strategy for optimizing outcome can be formulated. Emphasis has been placed on the importance of stem cell type, differentiation, cell scaffold and method of cell delivery^[2]. The influence on regeneration of each of these components has been thoroughly investigated. An overview of each of these, in addition to proposed mechanisms of action behind the therapeutic effect, will now be provided. Table 1 supplements the section on stem cell type, summarizing outcomes following the application of different stem cells in animal models.

Mechanism of action

Stem cells have the potential to replace lost neurons or increase the number of glial support cells. Replacing lost neurons has been widely demonstrated *in vitro* and has great potential in the CNS. Although not yet demonstrated in the dorsal root ganglia or ventral horn of the spinal cord, stem cells have the potential to rescue and replenish axotomized neurons as well as to manipulate the

Table 1 Summary of current evidence assessing the efficacy of different types of stem cell on peripheral nerve regeneration

Stem cell source	Author	Experimental model	Stem cell Diff.	Scaffold	Delivery system	Outcome
Embryonic	Cui <i>et al</i> ^[71]	Rat sciatic transection (10 mm gap)	D	Culture medium	Epineurium natural conduit	Cell survival and differentiation into SCs after 3-mo; superior regeneration of myelinated axons in comparison to culture media alone
	Lee <i>et al</i> ^[75]	Mouse sciatic transection (2 mm gap)	U	Matrigel	Direct injection of microspheres	ESC-derived MSC sphere-treated nerves recovered significantly greater CMAP, SFI and histological parameters than ESC-MSC single cell suspension
	Kubo <i>et al</i> ^[77]	Mouse tibial	D	PBS	Direct injection into gastrocnemius muscles following nerve transection and repair	Co-culture identified formation of new NMJs; muscle transplanted with stem cells experienced less atrophy 7 and 21-d post injury; cells transplanted after 2-wk were unable to provide any protective effect; motor recovery following repair superior in those muscles receiving stem cells
	Craff <i>et al</i> ^[76]	Rat sciatic + gastrocnemius muscle	D	PBS	Direct injection into gastrocnemius muscles following nerve transection	Muscles injected with stem cells retained muscle weight preservation and myocyte cross sectional area in comparison to control muscle after 7-d post injury. New NMJs observed. Benefits lost after 21 d
Neural	Fu <i>et al</i> ^[15]	Rat sciatic transection (15 mm gap; gene transfection)	U (gene therapy)	Culture medium (cells seeded directly onto conduit wall)	Poly(D,L-Lactide) conduit	Enhanced expression of BDNF and GDNF in transfected cells; conduits with transfected cells led to larger myelinated axons and improved functional and electrophysiological outcome
	Zhang <i>et al</i> ^[62]	Rabbit facial nerve transection (5 mm gap)	U	Collagen medium	HA-collagen composite conduit	Conduits with NSCs and NT-3 experienced superior outcomes in comparison to NSC alone; results equivalent to normal nerves after 12-wk
	Murakami <i>et al</i> ^[86]	Rat sciatic transection (15 mm gap)	U	Collagen gel	Silicone conduit	NSCs differentiated into astrocytes, oligodendrocytes and schwann cell-like cells; dNCs implanted into 15 mm defects; Improved axon number, diameter and myelination compared with controls; labeled cells present after 10-wk; expressed markers of SC phenotype
	Guo <i>et al</i> ^[87]	Rabbit facial nerve transection (10 mm gap)	U	Collagen sponge	Chitosan conduit	Electrophysiological and histological outcomes and immunohistochemistry superior in chitosan conduits seeded with NSCs and NGF in comparison to conduit+NGF alone. Results comparable to standard autograft
	Liard <i>et al</i> ^[88]	Pig nervus cruralis transection (30 mm gap)	U	Neurosphere in culture medium	Autologous vein graft	Grafts containing NSCs recovered superior EMG recording and immunohistochemistry profiles in comparison to empty conduits; NSCs identifiable after 240 d follow-up.
	Johnson <i>et al</i> ^[89]	Rat sciatic crush, transection, (10 mm gap)	U (C17.2)	Culture medium	Direct injection	12/45 rodents developed neuroblastomas
	Zhao <i>et al</i> ^[160]	Rat sciatic transection (15 mm gap)	U	Fibrin glue	ANA	Survival of BMSCs within fibrin glue; growth factor secretion preserved (NGF, BDNF); equivalent results when cells injected directly into nerve compared with around nerve
Bone marrow	Hu <i>et al</i> ^[165]	Monkey median transection (50 mm gap)	U	Culture medium	Chitosan conduit with longitudinally aligned PGLA fibers	Functional and electrophysiological recovery and FG retrograde tracing after 1-year with BMSC-laden conduits equivalent to autograft and superior to empty conduits
	Dezawa <i>et al</i> ^[19]	Rat sciatic transection (15 mm gap)	U + D	Matrigel	Matrigel graft	Successful differentiation into SC phenotype; Axon number and elongation superior with dBMSCs
	Jia <i>et al</i> ^[32]	Rat sciatic transection (10 mm gap)	U	Gelatin	Acellular xenograft	Neurotrophic factor expression elevated in BMSC xenografts; regeneration and functional recovery significantly better than empty xenografts and equivalent to autograft
	Mohammadi <i>et al</i> ^[34]	Rat sciatic transection (10 mm gap)	U	Culture medium	Vein graft	Veins filled with uBMSCs had significantly improved functional, histological and immunohistochemical outcomes compared with veins filled with PBS
	Nijhuis <i>et al</i> ^[36]	Rat sciatic transection (15 mm gap)	U	Culture medium	Vein graft +/- muscle	BMSC identifiable after 6 and 12-wk follow-up; vein graft + muscle + BMSCs outperformed vein graft+muscle but inferior to autograft
	Salomone <i>et al</i> ^[39]	Rat facial nerve transection (3 mm gap)	U + D	Matrigel	Silicone conduit	Histological outcomes superior in conduits containing uBMSCs and dBMSCs compared to empty and matrigel containing conduits; functional outcomes superior using uBMSCs

	Wang <i>et al</i> ^[41]	Rat sciatic transection (15 mm gap)	U	Culture medium	Direct injection into ANA	BMSC produced NGF and BDNF; CSPGs reduced in grafts treated with ChABC Allograft containing BMSCs and ChABC resulted in superior functional, electrophysiological and histological outcome compared with BMSC alone
Adipose	di Summa <i>et al</i> ^[28]	Rat sciatic transection (10 mm gap)	D	Culture medium	Fibrin glue conduit	Reduced muscle atrophy in autograft, dADSC and dBMSC groups in comparison to empty conduits; dADSCs recovered greatest axon and fiber diameter, evoked potentials and regeneration of motoneurons; results comparable to autograft
	Tomita <i>et al</i> ^[21]	Rat common peroneal nerve transection (no gap)	D	Culture medium	Direct injection into distal nerve	dADSCs survived for at least 10 wk <i>in vivo</i> ; dADSCs associated with axons and participated in re-myelination; dADSCs resulted in regeneration superior to cultured SCs
	Zhang <i>et al</i> ^[31]	Rat sciatic transection (10 mm gap)	D	Collagen gel	Xenogeneic acellular graft	dADSCs formed columns resembling bands of Büngner and expressed NGF, BDNF and GDNF; axon regeneration, retrograde labeling and electrophysiology were similar between dADSCs and SC supplemented grafts, superior to empty grafts but inferior to standard autograft
	Mohammadi <i>et al</i> ^[33]	Rat sciatic transection (10 mm gap)	U	Culture medium	Vein graft	No difference in functional, morphometric or immunohistochemistry between ADSCs and BMSCs
	Erba <i>et al</i> ^[45]	Rat sciatic transection (10 mm gap)	U	Fibrin	PHB conduit	Lack of sufficient quantities of viable cells 14-d after transplantation; conclusion that regenerative effect due to initial growth factor boost or paracrine effect on resident cells
	Sun <i>et al</i> ^[51]	Rat facial transection (8 mm gap)	D	Matrigel	Decellularized allogeneic artery	dADSCs persisted at repair site and integrated with regenerated tissue; conduits containing dADSCs achieved results comparable to those of SC-containing conduits and superior to matrigel-containing conduits alone; results inferior to autograft
Fetal	Pan <i>et al</i> ^[106]	Rat sciatic crush	U	Fibrin glue	Direct injection at site	High expression of BDNF, CNTF, NGF and NT-3 found in AFMSCs; motor function, CMAP and conduction velocity improved in those nerves augmented with AFMSCs; high levels of S-100 and GFAP and reduced fibrosis found at repair site
	Pan <i>et al</i> ^[111]	Rat sciatic crush	U	Fibrin glue	Direct injection at site	HBO therapy reduced production of inflammatory cytokines and macrophage chemokines following crush injury; when administered with AFMSCs, HBO reduced apoptosis of AFMSCs in comparison to AFMSCs alone; myelination and motor recovery superior in HBO + AFMSC group
	Pan <i>et al</i> ^[110]	Rat sciatic crush	U	Fibrin glue	Direct injection at site	Anti-apoptotic, anti-inflammatory agent G-CSF, when administered with AFMSCs, reduced crush-induced inflammation, and apoptosis in comparison to AFMSCs alone; myelination and motor function superior with AFMSCs + G-CSF in comparison to AFMSCs alone
	Matsuse <i>et al</i> ^[109]	Rat sciatic nerve transection (8 mm gap)	U + D	Matrigel	“Transpermeable” tube	Differentiated UC-MSCs regenerated greater number of myelinated axons and thicker nerve fibers compared with undifferentiated UC-MSCs; number of labeled cells greater in dUC-MSC nerves; results comparable to SC group
	Cheng <i>et al</i> ^[113]	Rat sciatic crush	U	Matrigel	Direct injection at site	AFMSCs successfully transfected; high expression of GDNF detected for 4 wk before subsiding; GDNF-modified AFMSCs recovered greatest SFI, conduction velocity, CMAP and muscle weight in comparison to AFMSCs alone
	Gärtner <i>et al</i> ^[108]	Rat sciatic crush	D	Culture medium	Cells seeded onto PLC wrap	Wraps seeded with UC-MSCs resulted in superior increased myelin thickness, motor and sensory function in comparison to unseeded wraps
Skin	McKenzie <i>et al</i> ^[120]	Mouse sciatic crush	D/ U	Culture medium	Direct injection at site	SKPs successfully induced into SKP-SCs; SKP and SKP-SCs associated with and myelinated axons
	Marchesi <i>et al</i> ^[123]	Rat sciatic transection (16 mm gap)	D	PBS	(1) Synthetic co-polymer L-lactide and trimethylene carbonate; and (2) collagen conduit	SFI and CMAPs were significantly better in conduits filled with SDSCs; number of regenerated myelinated axons significantly greater in SDSC conduits; no significant difference in neurotrophic factor expression
	Walsh <i>et al</i> ^[124]	Rat sciatic transection (12 mm gap)	D	Culture medium	Direct injection into acellular freeze-thawed nerve graft	SKP-SCs maintained differentiation up to 8-wk; outcomes significantly improved in comparison to cell free grafts and comparable to cultured SCs; neurotrophic factor release greater in SKP-SCs
	Walsh <i>et al</i> ^[121]	Rat CP/tibial (Immediate <i>vs</i> chronic repair; no gap)	D	Culture medium	Direct injection into distal nerve	Muscle weight and CMAPs superior in SKP-SC group in comparison to media injected controls; significantly higher counts of axon regeneration in SKP-SC group equivalent to immediate suture group
	Walsh <i>et al</i> ^[22]	Rat sciatic transection [acute <i>vs</i> chronic <i>vs</i> ANA (12 mm gap)]	U/ D	Culture medium	Direct injection into nerve ends and ANA	SKP-SCs maintained <i>in vivo</i> viability and differentiation better than uSKP; viability poorest in normal nerve, best in acutely injured nerve; SKP-SCs remain differentiated over time and myelinate axons; neuregulin able to prevent apoptosis following transplantation
	Khuong <i>et al</i> ^[122]	Rat sciatic and tibial (12 mm gap)	D	Culture medium	Direct injection into ANA	SKP-SCs containing allografts resulted in superior functional and histological outcomes in both acute and delayed injury models compared with SCs and media controls

Hair follicle	Amoh <i>et al</i> ^[135]	Mouse sciatic and tibial transection (no gap)	U	Culture medium	Direct injection at site	HFSC transplanted nerves recovered significantly greater function compared with untreated nerves; GFP-labeled cells differentiated into GFAP positive schwann cells and were involved with myelination
	Amoh <i>et al</i> ^[133]	Mouse sciatic crush	U	Culture medium	Direct injection at site	HFSCs transplanted around crushed nerve differentiated into SC-like cells and participated in myelination; gastrocnemius muscle contraction significantly greater compared with untreated crushed nerves
	Amoh <i>et al</i> ^[134]	Mouse sciatic transection (2 mm gap)	U	Culture medium	Direct injection at site	HFSCs differentiated into GFAP expressing SCs and were able to myelinate axons; gastrocnemius muscle contraction significantly greater compared with untreated nerves
	Lin <i>et al</i> ^[136]	Rat sciatic transection (40 mm gap)	D	PBS	Direct injection into acellular xenograft	Differentiation into neurons and SCs maintained for 52-wk; number of regenerated axons, myelin thickness and ratio of myelinated axons to total nerve count significantly higher in dHFSCs compared with acellular grafts; conduction velocity slower in dHFSC nerves
Induced pluripotent stem cell	Ikeda <i>et al</i> ^[146]	Mouse sciatic nerve (5 mm gap)	D	Microsphere seeded into conduit	Mixed PLA/PCL conduit +/- iPSC microspheres +/- bFGF	Regeneration was accelerated by combination of iPSCs + bFGF within conduits in comparison to iPSCs and bFGF alone; outcomes remained inferior to autograft controls; empty conduits performed least well
	Uemura <i>et al</i> ^[148]	Mouse sciatic nerve (5 mm gap)	D	Microsphere seeded into conduit	Mixed PLA/PCL conduit +/- iPSC microspheres	Motor and sensory recovery was superior in iPSC group at 4, 8 and 12 wk in comparison to empty conduits. Axonal regeneration superior in iPSC group. Conduit structurally stable after 12 wk
	Wang <i>et al</i> ^[149]	Rat sciatic nerve (12 mm gap)	D	Matrigel	PLCL/PPG/sodium acetate copolymer electrospun nanofiber conduit	Conduits filled with either (1) matrigel; (2) matrigel + NCSCs differentiated from ESCs; and (3) matrigel + NCSCs differentiated from iPSCs; NCSC differentiated into SCs and integrated into myelin sheaths; electrophysiology and histology showed equivalent regeneration in all NCSC containing conduits; no teratoma formation observed after 1-yr

ADSC: Adipose derived stem cell; ANA: Acellular nerve allograft; AFMSC: Amniotic fluid derived mesenchymal stem cell; BDNF: Brain derived neurotrophic factor; BDGF: Brain derived growth factor; bFGF: Basic fibroblast growth factor; BMSC: Bone marrow derived mesenchymal stem cell; CP: Common peroneal; CMAP: Compound muscle action potential; CSPG: Chondroitin sulphate proteoglycan; ChABC: Chondroitinase ABC; D: Differentiated; DMEM: Dulbecco's Modified Eagle's Medium; ECM: Extracellular matrix; EMG: Electromyography; ESC: Embryonic stem cell; FG: Fluorogold; GFAP: Glial fibrillary acidic protein; GDNF: Glial cell derived neurotrophic factor; G-CSF: Granulocyte colony stimulating factor; GFP: Green fluorescent protein; HA: Hyaluronic acid; HFSC: Human fetal derived stem cell; HBO: Hyperbaric oxygen; iPSC: Induced pluripotent stem cell; MSC: Mesenchymal stem cell; NCSC: Neural crest stem cell; NGF: Nerve growth factor; NMJ: Neuromuscular junction; NSC: Nerve stem cell; NT-3: Neurotrophin-3; PBS: Phosphate buffered saline; PCL: Poly-ε-caprolactone; PFTE: Polytetrafluoroethylene; PGLA: Polyglycolic lactic acid; PHB: Polyhydroxybutyrate; PLA: Poly-L-lactide; PLCL: Poly(L-lactide-co-caprolactone); PPG: Polypropylene glycol; SC: Schwann cell; SFI: Sciatic function index; SKP: Skin derived precursor; SKP-SC: Skin precursor derived Schwann cell; SDSC: Skin derived stem cell; U: Undifferentiated; USW: Ultra-short wave therapy.

microenvironment of the neurons during regeneration. In the peripheral nervous system, emphasis has been placed mostly on increasing SC number and activity. This approach is driven by the pragmatic difficulties associated with autologous SC culture. Achieving sufficient autologous SC numbers requires explantation of healthy nerve, extended periods of purification and expansion and consequently, delays to repair. Prolonged axotomy and denervation only exacerbate central cell body apoptosis and loss of SC-mediated axonal support in the distal nerve^[3,4]. As a result, the use of cultured SCs is considered impractical.

Exogenous stem cells differentiated into SC-like phenotype may integrate into Bands of Büngner and aid axonal guidance and re-myelination. Transplanted cells enhance growth factor secretion and ECM production. These factors may be released directly or, through cell-to-cell contact and paracrine signaling, can stimulate endogenous SCs to upregulate secretory activity^[5]. Increased recruitment of circulating macrophages into the area can potentiate this effect^[6].

ECM proteins such as collagen I, collagen IV, fibronectin

and laminin, and neurite guidance proteins such as netrin and ninjurin-2 have pro-regenerative effects^[7,8]. Other ECM components such as chondroitin sulphate proteoglycan are potent inhibitors of axonal regeneration and the degradation of this protein by liberated matrix metalloproteinase-2 has been shown to support regeneration^[9,10].

The production of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor and neurotrophin-3 (NT-3) by endogenous SCs and transplanted stem cells has been described^[5,7,8,11-17]. Angiogenic factors such as vascular endothelial growth factor, basic fibroblast growth factor (bFGF), hepatocyte growth factor and angiopoietin-1 may also be released^[12]. Hypoxia has been found to potentiate the angiogenic and neurogenic influence of some stem cells, leading to improved blood vessel and nerve fiber formation^[7]. This has led some investigators to attempt pre-conditioning and differentiation in hypoxic environments^[7]. The expression of leukemia inhibitory factor and insulin-like growth factor has also been detected^[8]. These factors are thought to improve neuronal survival, promote corticospinal tract growth,

increase astrogliosis and potentiate the inflammatory response^[8,18]. The observed immunomodulatory effects of stem cells are believed to be due to the secretion of factors such as granulocyte and macrophage colony stimulating factor (G-CSF, M-CSF), interleukin-6, 7, 8, and 11 (IL-6, IL-7, IL-8, IL-11) and TNF- α ^[8]. Suppression of the host immune response may help reduce the detrimental impact of inflammation and fibrosis following nerve repair and is obviously desirable in the context of allotransplantation of cells. The relative contribution of each of these mechanisms and therefore the levels of ECM protein and growth factor production may vary not only with stem cell type but also with the differentiation status of transplanted cells.

Differentiation

Stem cells can be transplanted in their undifferentiated state or can undergo a period of *in vitro* differentiation into SC-like cells. This is commonly achieved with exposure to β -mercaptoethanol, all-trans retinoic acid, fetal bovine serum, forskolin, recombinant human bFGF, recombinant human platelet derived growth factor-AA, and recombinant human heregulin β -1^[19,20]. The purported benefits of differentiation include superior *in vivo* viability and an enhancement of neurotrophic factor secretion and myelinating ability^[14,21,22]. Many investigators have shown the beneficial effects on regeneration of these cells^[19-21,23-31]. Some claim that transplanting undifferentiated cells risks *in vivo* differentiation along unwanted, non-neuronal lines in response to other dominant cells in the area.

Interestingly, however, the positive effects of undifferentiated cells have also been widely reported^[5,7,13,32-51]. These investigators claim that *in vitro* differentiation incurs an unnecessary delay, limiting clinical applicability. In one study, the process of differentiation actually reduced the secretion of neurotrophic factors^[52]. Others have reported difficulties with maintaining *in vivo* neuronal differentiation, claiming that once *in vitro* differentiation cues are removed, cells tend to revert back to their original phenotype^[53]. Evidence suggests that undifferentiated cells may undergo *in vivo* differentiation in response to local stimuli^[20,40,54-56], although others have been unable to show this^[45,49,57]. A small number of direct comparisons have been performed and have shown either a small advantage for differentiated cells^[20,23], a small advantage for undifferentiated cells^[39] or no significant difference^[45,48].

Stem cell source

Embryonic stem cells: Thomson *et al.*^[58] described the isolation of pluripotent cell lines from human blastocysts in 1998. Since this discovery, stem cells have been harvested from a variety of different fetal and adult tissues (Figure 1). Embryonic stem cells (ESCs) can form derivatives of all three embryonic germ layers and can provide an unlimited source of cells with superior differentiation potential and long-term proliferation capacity in comparison to mesenchymal stem cells (MSCs)^[59]. Unlike adult sources, these cells are homogenous, are not susceptible to the

detrimental impact of age and disease and can be harvested without physical discomfort^[60]. However, differentiation down specialized neural cell lines is challenging and established protocols only exist for a limited number of lines^[61,62]. Additional disadvantages include immunogenicity, tumorigenicity and potential ethical controversy due to the fact that cells are harvested during the blastocyst stage of development and consequently result in the destruction of embryos.

ESCs have been shown to migrate and differentiate into neurons, and glial cells of the central and peripheral nervous system and have the capacity for *in vivo* myelination^[61,63-70]. Studies reporting the successful application of these cells to peripheral nerve repair are limited, perhaps reflecting the lack of facile clinical translatability^[71]. In recognition of the limitations, investigators have derived mesenchymal precursors from ESC^[59,72-74]. Augmentation of peripheral nerve regeneration following the transplantation of spheres of human MSCs derived from ESCs has been reported^[75]. Our group has shown in small animal models of sciatic and tibial nerve transection that ESCs transplanted directly into muscle can preserve short-term muscle mass and myocyte cross-sectional area following injury and repair^[76,77].

Nerve stem cells: Nerve stem cells (NSCs) naturally differentiate into neurons and glial cells. However, the process of neurogenesis almost exclusively occurs during embryogenesis and continues in the adult in response to injury only within very limited locations of the CNS^[78]. NSCs were first isolated from adult murine brain in the early 1990s^[79,80]. This was followed by similar discoveries in humans and non-human primates^[81,82]. The subventricular zone and the subgranular zone in the brain represent the primary sites of NSC differentiation in adult mammals (Figure 1). Most recently, populations of NSCs and neurogenesis have been discovered in adjacent areas of the adult human striatum^[83]. Neural crest stem cells have been isolated from fetal rat sciatic nerve but the existence of such cells in peripheral nerves of the adult human is unknown^[84,85].

Many have reported positive results following NSC implantation into peripheral nerve injury^[15,62,86-88]. Heine *et al.*^[9] showed that the beneficial effects of NSC transplantation are not confined to acute peripheral nerve injury. Transplantation of NSCs into chronically denervated nerve had a beneficial effect on morphological and electrophysiological recovery in comparison to controls. Immortalized murine C17.2 NSCs have become commercially available and are commonly used for *in vivo* animal studies. However, experience with C17.2 NSCs in the PNS has not been uniformly positive. Following the implantation of these cells into rat models of crush, transection and graft, high rates of neuroblastoma formation have been encountered^[89]. In addition to these concerns, pragmatic difficulties associated with cell harvest have limited their use.

MSCs: MSCs are multipotent stromal cells that originate from the bone marrow and a variety of non-marrow

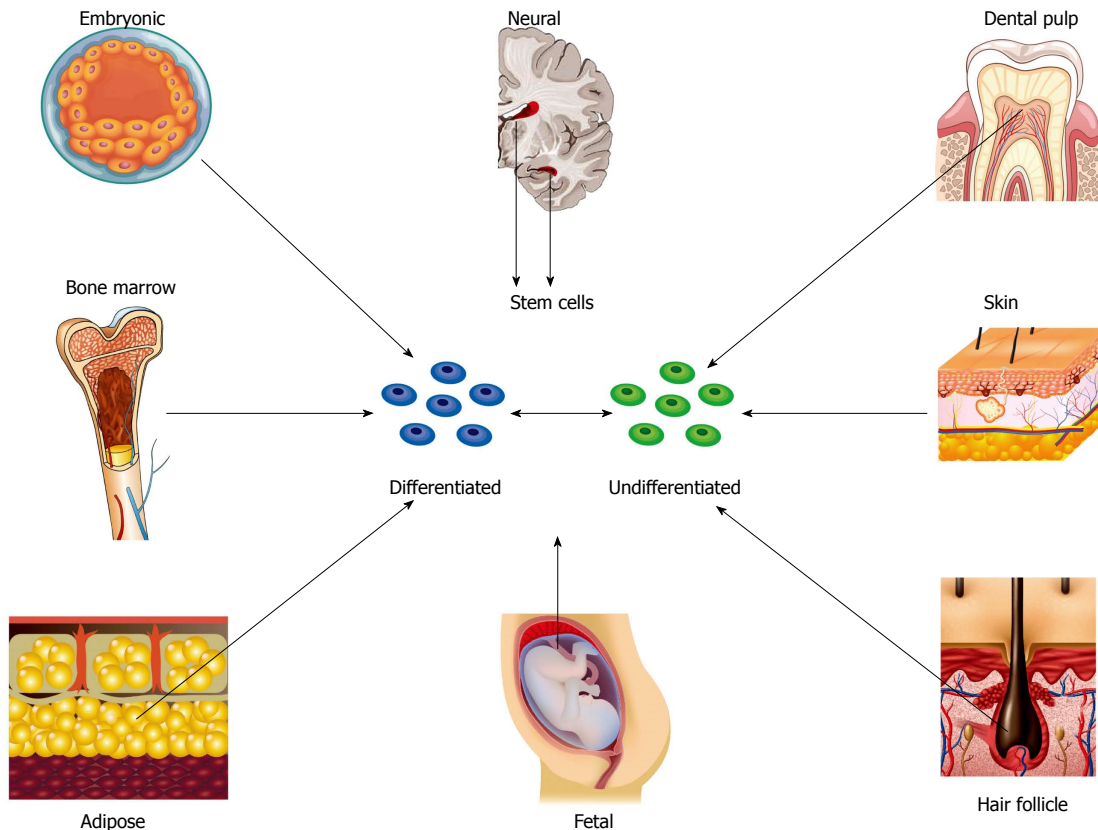


Figure 1 Different stem cell sources. Embryonic stem cells are obtained from the inner cell mass of the blastocyst and therefore require destruction of the embryo. Nerve stem cells are harvested from the subventricular layer of the lateral ventricle and the subgranular layer of the hippocampus. Bone marrow derived stem cells are harvested from the marrow cavity of long bones. Adipose derived stem cells are derived from subcutaneous fat and are abundantly available following commonly performed procedures such as liposuction. Skin derived precursors are harvested from the dermis and represent a related population of cells harvested from hair follicles. Fetal tissue provides populations of cells from amniotic membrane, amniotic fluid, umbilical cord blood, umbilical cord tissue and Wharton's jelly. Dental pulp stem cells can be harvested from deciduous teeth.

sources such as adipose tissue, fetal tissue, skin, hair follicle and dental pulp (Figure 1). Although the potency of these cells was originally considered to be limited to tissues of mesodermal origin, differentiation into non-mesodermal lineage is now generally accepted. It is precisely this characteristic that endows MSCs with the ability to support neuroregeneration. An overview of the main sub-types of MSC and the evidence behind their application to peripheral nerve regeneration will now be discussed.

Bone marrow derived stem cells: In contrast to embryonic and NSCs, bone marrow derived stem cells (BMSCs) are readily accessible and have no potential ethical concerns. Under appropriate conditions BMSCs can differentiate into non-mesodermal lineages such as neurons, astrocytes and SC-like cells^[90]. Many studies have shown that the addition of BMSCs to conduits and acellular grafts results in superior outcomes when compared with empty or cell deplete channels^[24,36-38,40,41]. Although some have failed to show that BMSCs can match outcomes achieved with cultured SCs^[20], the majority of reports show that performance is at least equivalent^[27,43,91]. There is a dearth of studies showing superiority over gold standard autograft although some report equivalent outcomes^[42,44]. Evidence suggests that these outcomes may be dose dependent^[92].

Although BMSCs are more clinically applicable than ESCs, NSCs and cultured SCs, stem cell fraction is low and in comparison with other sources, BMSCs generally have inferior proliferation capacity and differentiation potential. In addition, harvest is invasive and painful. This has driven the pursuit of less invasive MSC sources such as fetal tissue, skin, hair follicle and adipose tissue.

Adipose derived stem cells: Adipose tissue is abundant, easily harvested and is a frequent waste product of commonly performed procedures such as liposuction. It has a superior stem cell fraction in comparison to bone marrow and adipose derived stem cells (ADSCs) have superior proliferation and differentiation potential in comparison to BMSCs^[93,94]. Unlike BMSCs, donor age and site of harvest does not seem to significantly influence the therapeutic effect of ADSCs^[50].

Multiple neuronal and SC markers have been expressed from differentiated ADSCs^[7,30,95-97]. The expression of myelin protein zero, peripheral myelin protein-22 and myelin basic protein also suggest the retention of myelinating capability^[98]. Although some reports have found no difference in outcome between ADSC-filled conduits and cell deplete products^[49], the vast majority of studies show an augmented effect^[13,28,29,31,99]. Several studies have shown that

ADSCs are at least as effective as autologous SCs^[21,45,51]. As with BMSCs, a small number of investigators have reported equivalent outcomes in comparison to autograft^[13,28,29] with even fewer reporting superior outcomes^[48]. Studies directly comparing the performance of ADSCs to BMSCs have found no significant differences between the two^[33,45]. Low morbidity of harvest, wide availability and superior stem cell characteristics have helped establish ADSCs as the preferred option for pre-clinical studies.

Fetal derived stem cells: Fetal tissues represent the most primitive source of MSCs. These tissues are ordinarily discarded following birth and are therefore in abundant supply. Tissue age rarely exceeds 42-wk and therefore, in comparison to other adult sources, cells have accumulated less genetic damage due to age, environment and disease^[100]. Stem cells can be harvested from amniotic membrane, amniotic fluid, umbilical cord cells, umbilical cord blood and Wharton's jelly. These cells are readily expandable in culture and possess the ability to differentiate into neural phenotype^[101,102].

Wharton's jelly derived mesenchymal stem cells can differentiate into functional SC-like cells, produce neurotrophic factors such as NGF, BDNF and NT-3 and stimulate neurite growth *in vitro*^[103]. Amniotic fluid derived stem cells (AFDSCs) express characteristics of both mesenchymal and NSCs^[104], can differentiate into neural tissue^[105] and, following transplantation into rat sciatic nerve gap models, have been shown to promote peripheral nerve regeneration^[106,107]. Umbilical cord-derived mesenchymal stem cells have also been shown to improve outcomes following crush and transection injuries in rodent models^[108,109].

Survival of AFDSCs following transplantation can be limited and as a result, efforts have been made to improve *in vivo* survivability. Inhibition of inflammatory mediators can attenuate the apoptotic cascade^[110-112]. Cells can be genetically modified in order to over-express therapeutic genes. GDNF-modified human AFMSCs have been shown to improve viability, regeneration and motor outcomes in rodent models^[113]. Stromal cell-derived factor-1 α (SDF-1 α) is chemotactic towards MSCs and is expressed in several tissues in the rat, including injured nerve. By timing intravenous administration of AFDSCs with peak expression of SDF-1 α , this novel approach has been shown to improve AFDSC numbers at the repair site and nerve regeneration^[114].

Fetal tissue represents a promising alternative source of stem cells. However the use of autologous cells following injury is currently impractical. The use of allogeneic cells and their associated immunoreactivity are obstacles that are not encountered with other adult sources such as adipose tissue. Widespread banking of fetal products following birth offers a solution to this problem.

Skin derived precursors: Skin derived precursors (SKPs) reside in the dermis and are an accessible source of adult multipotent cells that are easily expandable in culture. SKPs

exhibit a remarkable similarity to embryonic neural crest cells, a migratory population of multipotent cells that gives rise to a diverse array of cell types such as melanocytes, craniofacial cartilage, bone and connective tissue, smooth muscle of vasculature, endocrine cells, and neurons and glial cells of the autonomic and peripheral nervous system^[115]. Neuregulin is known to promote proliferation and differentiation of SCs from embryonic neural crest precursors^[115-117] and is also believed to be mediate SC proliferation during Wallerian degeneration^[118]. When cultured with neuregulin-1 β , rodent and human SKPs express markers consistent with functioning SCs^[115-120]. Exogenous neureglins and heregulin-1 β may also improve *in vivo* viability by reducing apoptosis^[22].

Both undifferentiated and differentiated SKPs (SKP-SCs) have been shown to have positive effects on regeneration. In rodent models, SKP-SCs have been shown to maintain differentiation and viability, myelinate axons, and exhibit superior outcomes following de-myelination and crush injury^[117,120], acute and chronic transection injury^[22,120-122] and following implantation into ANAs^[122-124].

Hair follicle stem cells: Hair follicles are dynamic dermal appendages and are abundant and readily accessible sources of pluripotent stem cells. The hair follicle is derived from the neural crest and harbors NCSC-like cells^[125]. Hair follicle stem cells (HFSCs) were discovered following the detection of nestin, a marker for neural progenitor cells, in the cells of the hair follicle bulge in mice^[126] and humans^[127]. They are readily expanded in culture, although, as with SKPs, clinical applicability may be limited by prolonged periods of cell expansion. HFSCs are positive for ESC transcription factors Nanog, Oct4 and nestin, and have the ability to differentiate into adipocytes, smooth muscle cells, melanocytes, neurons and glial cells^[116,128,129]. Differentiation into epithelial and vascular cells emphasizes the important role HFSCs have in vascularizing the skin and dermis^[130-132].

A small number of studies have reported the use of undifferentiated HFSCs in murine models of sciatic and tibial nerve crush and transection injuries^[133-135]. HFSCs were found to differentiate into glial fibrillary acidic protein positive SC-like cells and participated in myelination. Functional outcomes were significantly improved in those nerves receiving HFSCs. The efficacy of differentiated HFSCs seeded into acellular xenografts has also been investigated^[136]. Cells were able to maintain differentiation long term and the number of regenerated axons and myelination in cell-supplemented xenografts was higher than cell-free xenografts.

Dental pulp stem cells: Dental pulp is neural crest-derived tissue that can be harvested from exfoliated deciduous teeth and arguably represents the most convenient source of multipotent stem cells. As with fetal tissue, widespread application of autologous cells would require harvest and storage of teeth at an early age. Although experience is limited with this newly described population of cells,

evidence shows that these cells may have a role in supporting peripheral nerve regeneration. In avian embryonic models, dental pulp stem cells (DPSCs) have been shown to chemo-attract trigeminal ganglion axons^[137]. DPSCs have also been shown to successfully differentiate into SCs *in vitro* and were able to support DRG neurite outgrowth^[138]. When implanted into a collagen gel matrix, DPSCs were able to form aligned columns and had the ability to guide and myelinate neurites^[138]. Rat DPSCs have also been shown to provide axonal support following hemisection of the spinal cord^[139,140].

Induced pluripotent stem cells: In 2006, Takahashi *et al*^[141] described the derivation of induced pluripotency in somatic mouse cells following ectopic co-expression of transcription factors^[141]. This ability to reprogram cells questioned the stability of cellular identity and injected new optimism into the quest to develop a patient specific pluripotent stem cell that could circumvent the limitations of ESCs. Although multiple similarities between ESCs and induced pluripotent stem cells (iPSCs) exist, subtle differences have also been detected^[142]. Currently it is believed that these cells are neither identical nor distinct populations and instead represent cells with overlapping identity. These differences do not abrogate their potential. Since their discovery, iPSCs have furthered the understanding of many disease mechanisms and have advanced *in vitro* drug screening and the assessment of therapeutic efficacy following pharmaceutical and cell-based interventions^[142].

In addition to differentiation into various somatic cell lines, established protocols now exist for the *in vitro* differentiation of iPSCs along neural lineages^[143,144]. Although evidence suggests that differentiation occurs with reduced efficiency and increased variability^[145], iPSCs have been shown to have a pro-regenerative effect in small animal models of central and peripheral nervous system injury^[146-149].

These cells possess several advantages over embryonic sources such as the avoidance of ethical and immunosuppressive issues. However, concerns surrounding these cells exist. Retained epigenetic “memory” from their somatic cells of origin, coupled with chromosomal aberrations resulting from re-programming, can render these cells even more tumorigenic than ESCs^[150].

The preferred option: Adipose derived stem cells represent the most practical source of cells for transplantation. In light of easier harvest, superior stem cell fraction, differentiation potential and proliferation capacity, they have supplanted bone marrow derived cells. Other alternatives such as fetal, hair follicle and tooth pulp have great potential but are currently limited by the lack of autogenous supply and the requirements for cell expansion. Whether these cells are best differentiated prior to transplantation is a contentious issue although the use of undifferentiated cells obviates the requirement for *in vitro* differentiation. The beneficial effect of these cells on regeneration has been widely reported

and, until clear evidence emerges that differentiation is superior, the use of undifferentiated cells is the most pragmatic and clinically translatable option. Optimizing the effect of the preferred option is heavily reliant on appropriate delivery and support.

Cell scaffolds and methods of delivery

Cell scaffolds aim to maintain cell viability, support proliferation and permit intercellular communication and growth factor elution. N-methacrylate glycol chitosan, alginate, hyaluronic acid, polyethylene glycol, fibrin and Matrigel have all been shown to successfully fulfill these requirements^[151-157]. In addition to their supportive role, scaffolds also help prevent unwanted cell dissipation from the site of injury and, when used in combination with nerve conduits, have emerged as useful adjuncts for improving the delivery of cells.

In cases where nerves are repaired primarily or when autograft or allograft nerve is going to be used, stem cells can be delivered by a variety of different methods (Figure 2). Cells suspended in culture medium can be microinjected into nerve ends or grafts (Figure 2A)^[28,29,32,40,158-160]. Although this delivers cells directly to the site of injury, the process of micro-injection can be traumatic to the delicate intra-neural architecture and can result in unpredictable cell distribution. Evidence suggests that intra-neural injection may be unnecessary. Cells suspended in fibrin matrix that were injected around repair sites maintained neurotrophic factor secretion and cell migration and resulted in outcomes that were not significantly different in comparison to injected nerves (Figure 2B)^[160].

Cells can be injected within the lumen of a conduit (Figure 2C) or can be seeded onto conduit matrix (Figure 2D). Natural conduits including vein and artery grafts are rich in ECM proteins such as collagen and laminin and provide a useful substrate for cell adhesion, though only at the periphery of the constructs^[33,34 37,161-163]. Vessels can remain empty or can be pre-filled with tissue such as muscle to support axonal guidance^[36]. Commercially available natural conduits are usually composed of ECM components such as collagen^[38] and fibrin^[28]. Synthetic materials include polyglycolic acid^[57], Poly(lactic-co-glycolic) acid^[164], silicone tube^[39,43], polyhydroxybutyrate^[20], polytetrafluoroethylene^[24] silk fibroin^[42] and chitosan^[91,165]. The degradation profile of conduits must be carefully considered when used with cell therapy. Natural conduits tend to degrade in a predictable, non-toxic fashion, though the rate of degradation may not be sufficiently slow in some cases to allow for adequate regeneration time. Some synthetic polymers can acidify the microenvironment during degradation and can have a detrimental impact on cellular activity^[166].

In recognition of the importance of basal lamina and other ECM framework for axonal guidance, conduits with internal structure have become more popular than hollow, single lumen tubes. These structures may be organized arrangements of multiple fibers^[23,164,165] or less orderly structures such as collagen sponge^[24]. However,

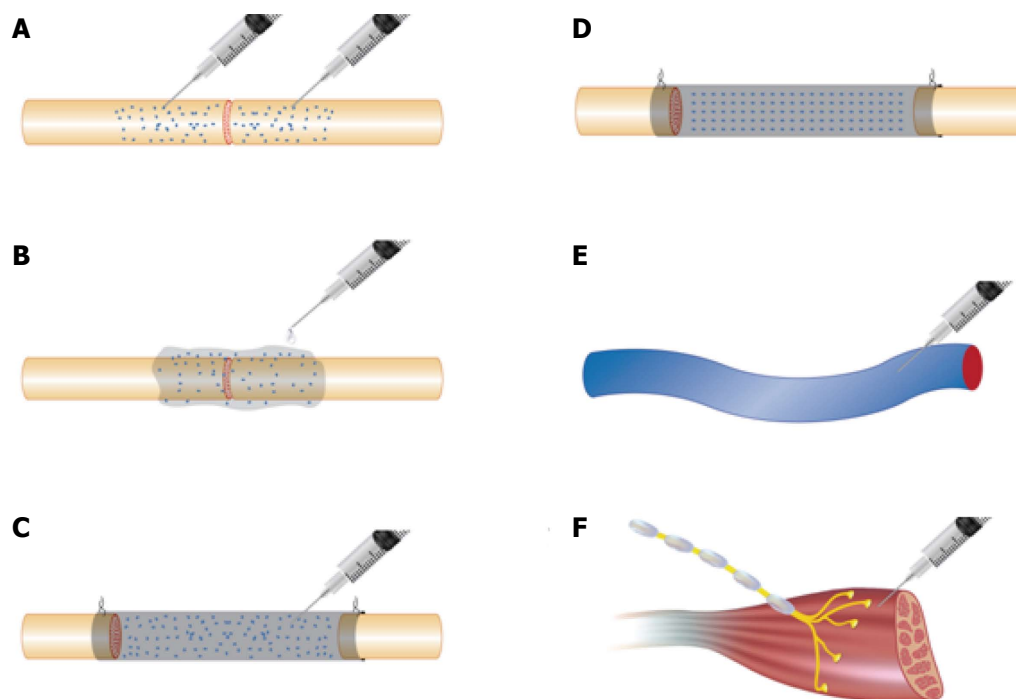


Figure 2 Methods of stem cell delivery. A: Direct injection into nerve ends or bridging nerve graft; B: Injection of cells suspended in matrix around nerve rather into the substance of the nerve; C: Nerve gap model using a conduit as the bridging method. Conduit lumen can be injected and filled with cells suspended in scaffold; D: Nerve gap model using conduit as bridging method. Conduit has been cultured and seeded with stem cells. Lumen of conduit may also be filled with cells. Hollow conduits may have internal framework added. This framework may also be seeded with cells; E: Systemic injection of cells. Stem cells have been shown to home to the site of injury; F: Cells may also be injected into neuromuscular junctions of peripheral muscles.

in the absence of cells, attempts to replicate the structural complexity of the nerve have led to disappointing results.

Recent evidence exists that systemic administration of stem cells following peripheral nerve injury can be efficacious (Figure 2E)^[5,114]. With further refinement, it may become possible to sustain regenerative support through regular systemic dosing. There is also evidence that stem cells may be efficacious when administered at the level of neuromuscular junction (NMJ) (Figure 2F) or when distributed within denervated muscle^[76,77,167].

Future

Nerve regeneration and re-innervation is a protracted and dynamic process. Any attempts to improve the speed and integrity of this process must be equally enduring and migratory. Local delivery of biochemical mediators to the site of repair is a common approach, though this provides only finite support that is unable to follow the advancing growth cone, not to mention recapitulate the elegant temporal coordination of myriad different factors. Development of systemic administration and cell homing to bring these cells to the advancing neural growth cone and the regenerating axons may help sustain regenerative support.

A fusion of cell based therapy and optogenetics may also hold promise by restoring motor function in those that have lost central control. Murine embryonic stem cell derived motor neurons have been induced to express the light-sensitive ion channel channelrhodopsin-2. When engrafted into mouse sciatic nerve, not only did axons re-

innervate hind limb muscles, but function was restored in a controllable manner through optical stimulation and did not require connection to the central nervous system^[168].

CONCLUSION

Despite *in vitro* and pre-clinical success, the application of stem cells to peripheral nerve repair has yet to make an impact in the clinical arena. Translation is currently limited by justified concerns regarding genetic manipulation, cell instability and the risks of tumorigenesis. However, perhaps the most pertinent issue is that despite great effort to manipulate the regenerative process, the use of stem cells simply has not led to outcomes that significantly and consistently surpass those achieved with conventional techniques. Augmentation of peripheral nerve repair with stem cells must consider stem cell type, differentiation, cell scaffold and method of delivery in order to offer effective new therapies. These factors, in addition to animal and injury models, vary widely in the current literature, making it very difficult to draw definitive conclusions as to which combination is most efficacious.

The ideal strategy would involve administration of stem cells immediately, or as soon as possible, following injury and for this to be sustained throughout the period of regeneration and recovery. This may require a shift away from the development of methods of local delivery and a re-focus on developing cells that can be administered systemically and that have the ability to specifically target and support not only the migrating growth cone, but also

centrally located cell bodies and peripherally located NMJs.

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Cancer stem cell plasticity and tumor hierarchy

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Abstract

The origins of the complex process of intratumoral heterogeneity have been highly debated and different cellular mechanisms have been hypothesized to account for the diversity within a tumor. The clonal evolution and cancer stem cell (CSC) models have been proposed as drivers of this heterogeneity. However, the concept of cancer stem cell plasticity and bidirectional conversion between stem and non-stem cells has added additional complexity to these highly studied paradigms and may help explain the tumor heterogeneity observed in solid tumors. The process of cancer stem cell plasticity in which cancer cells harbor the dynamic ability of shifting from a non-CSC state to a CSC state and vice versa may be modulated by specific microenvironmental signals and cellular interactions arising in the tumor niche. In addition to promoting CSC

plasticity, these interactions may contribute to the cellular transformation of tumor cells and affect response to chemotherapeutic and radiation treatments by providing CSCs protection from these agents. Herein, we review the literature in support of this dynamic CSC state, discuss the effectors of plasticity, and examine their role in the development and treatment of cancer.

Key words: Cancer stem cells; Stem cell; Plasticity; Tumor hierarchy; Microenvironment; Immune-mediated therapies; Epithelial-to-mesenchymal transition

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Core tip: The origins of the complex process of intratumoral heterogeneity have been highly debated and different cellular mechanisms have been hypothesized to account for the diversity within a tumor. The clonal evolution and cancer stem cell (CSC) models have been proposed as drivers of this heterogeneity. However, the concept of cancer stem cell plasticity and bidirectional conversion between stem and non-stem cells has added additional complexity to these highly studied paradigms and may help explain the tumor heterogeneity observed in solid tumors. Herein, we review the literature in support of this dynamic CSC state, discuss the effectors of plasticity, and examine their role in the development and treatment of cancer.

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MODELS OF TUMOR CELL HETEROGENEITY

The concept of tumor heterogeneity has evolved in the last few decades into a complex picture of phenotypic,

functional, and genetic heterogeneity not only within tumors but also between primary cancers and metastases. Adding to this multifaceted heterogeneity are the continuously changing extracellular influences that can modulate tumor development and progression including, but not limited to, inflammatory stimuli, microenvironmental signals, and immune cell interactions.

Recent technological advances have permitted in depth and rapid analysis of individual cancer genomes at the single-nucleotide level. These advances have shed light on intratumoral heterogeneity both within tumor biopsies and between spatially separated biopsies of the same tumor^[1,2]. Sequential tumor sampling and analysis has also revealed that intratumoral heterogeneity temporally evolves during the course of disease^[3]. This observed heterogeneity may present major challenges in the development of biomarkers, therapeutics, and personalized-medicine approaches.

CLONAL EVOLUTION, CANCER STEM CELLS, AND MOVEMENT TO A UNIFIED MODEL

The origins of intratumoral heterogeneity have been highly debated and different cellular mechanisms have been hypothesized to account for the diversity within a tumor. The clonal evolution theory, first introduced by Peter Nowell in a landmark article published almost four decades ago, proposed cancer to be an evolutionary process where most neoplasms arise from a single cell of origin, and tumor progression results from a stepwise acquisition of mutations within the original clone allowing sequential selection of more aggressive subclones. He hypothesized that cells in the dominant subclone populations would possess similar tumorigenic potential^[4]. The second theoretically opposing hypothesis is the cancer stem cell (CSC) paradigm. Unlike clonal evolution, where subclones possess tumorigenic potential, the CSC hypothesis postulates that only small subpopulations of the tumor, the CSCs, are capable of self-renewal and the have potential to give rise to a tumor-the rest of the tumor consists of phenotypically diverse cells with limited proliferation and tumorigenic potential. The benchmark work that helped to establish the CSC theory in solid tumors also showed that no clear morphological distinction was apparent between tumorigenic and nontumorigenic breast cancer cells, with the two populations displaying equal cell kinetics-yet the tumors appeared to be hierarchically organized when tested functionally. The group identified the CD44+/CD24- cells as putative CSCs. When these isolated cells were injected into immunodeficient mice, tumors arose in 89% of cases-and only as few as 100 CD44+/CD24- were needed to recapitulate the tumor^[5]. Further studies by other groups consistently verified that the frequencies of tumorigenic cells were low, variable, and were able to recapitulate some of the heterogeneity of the original tumors^[6].

Proving that cancers strictly follow either the clonal

evolution or CSC model is limited by experimental methodologies. The elucidation of a CSC model has been dependent on xenograft limiting dilution assays and tumor-specific CSC markers to isolate the tumor initiating cells. This assumes that the resulting increased tumorigenicity lies with an intrinsic trait of CSCs but does not rule out the that these cells are just somehow better suited to growth within an immunocompromised mouse that bears little resemblance to the normal human environment. It also relies heavily on the use of surface markers that may not adequately delineate the CSCs from non-CSCs. However, elegant lineage tracing experiments have clearly demonstrated that CSCs are a tumorigenic reservoir, capable of surviving chemotherapy, and drive relapse in mouse models of cancer^[7-9]. Even with these experiments, direct evidence that unmanipulated human solid tumors harbor cells with self-renewing properties that fuel sustained tumor expansion is lacking. Further, the CSC model alone cannot account for functional heterogeneity in all tumors. Despite these complexities, recent studies illustrate that a stem cell-like gene expression signature is related to relapse in glioblastoma (GBM) and is predictive of patient outcome in human leukemia, breast cancer, GBM, ovarian cancer-lending support to the clinical relevance of cancer stem cells^[10-13]. Furthermore, ample evidence exists that the prevalence of cells with a CSC phenotype can predict response and that these cells persist even after chemotherapy or radiotherapy. Despite experimental, analytical, and theoretical caveats for both the clonal evolution and CSC models, clinical studies in leukemia have shown that in a cancer distinctly driven by stem cells, such as in chronic myelogenous leukemia, clonal evolution is observed when tyrosine-kinase inhibitors are administered^[14,15]. This initially successful therapy can result in the emergence of subclones that harbor mutations in the *BCR-ABL* fusion gene, the target of imatinib, giving rise to a tumor-resistant phenotype^[15,16]. Moreover, it has also been demonstrated that CSCs harbor the *BCR-ABL* fusion gene but remain insensitive to imatinib. Instead, these CSCs revert to a normal dependence on cytokines for survival and proliferation^[17]. These cells could therefore be the ones that survive the initial therapy and sustain further mutations giving rise to a fitter clone.

These observations highlight fact that the progression of tumor heterogeneity is a wildly complex process and support the possibility that clonal evolution and the CSC model are not mutually exclusive. A single tumor may comprise several cancer stem cell clones that have a common ancestor (the cell that sustained the first oncogenic mutation) yet are genetically distinct. These cancer stem subclones with self-renewal capabilities would persist over time and accumulate epigenetic and genetic changes required for cancer initiation and progression. Each different CSC subclone would give rise to intermediate progenitors as well as more differentiated, non tumorigenic cancer cells. The intermediate transit-amplifying cells would lack self-renewal capabilities, but could continue to accumulate genetic changes and

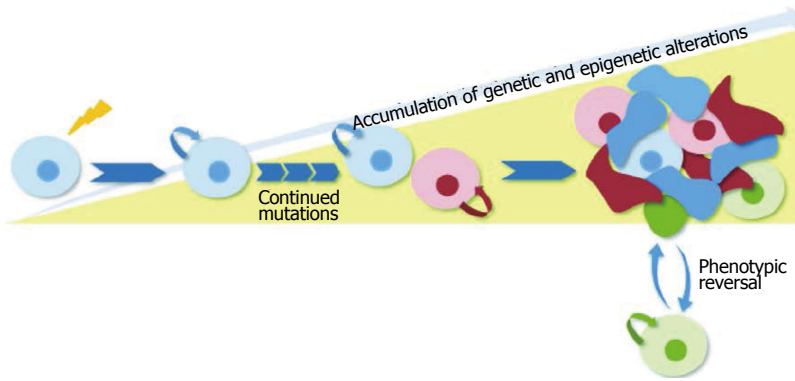


Figure 1 Schematic of unified model of clonal evolution and cancer stem cells. The proposed unified model depends on dynamic hierarchical organization and clonal mutations for tumor heterogeneity. In this depiction, the originating CSC that sustained the first oncogenic mutation gives rise to subclones with self-renewal capabilities that accumulate epigenetic and genetic changes over time. Each different CSC subclone gives rise to intermediate transit-amplifying progenitors that lack self-renewal capabilities. A subset of these progenitors (shown in green) follows a model of tumor cell plasticity and bidirectional conversion between non-CSC to CSC states. This phenotypic change is modulated by microenvironmental stimuli which confer CSC self-renewal capacities to the differentiated cell. CSC: Cancer stem cell.

possibly a mutation conferring self-renewal capabilities to the cell. Alternatively, these cells could follow a model of tumor cell plasticity in which microenvironmental stimuli could co-opt self-renewal mechanisms and acquire CSC characteristics—a process that is inherently transitory and would also allow the conversion from cancer stem cell to non-self-renewing progenitor (Figure 1).

The concept of cancer stem cell plasticity and bidirectional conversion between stem and non-stem cells has added additional complexity to the CSC and clonal evolution models and may help explain the tumor heterogeneity observed in solid tumors. Recent studies provide evidence that a select group of cancer cells can readily switch between non-tumorigenic and tumorigenic cell states in response to appropriate stimuli and that this conversion may be modulated by endogenous transcription factors^[18–20]. This suggests that both CSCs and non-CSCs are highly adaptable populations capable of transient evolution and plasticity. This review will focus on the evidence for this transient state and discuss how these observations impact our understanding of the evolution of cancer and its treatment.

EPITHELIAL-TO-MESENCHYMAL TRANSITION AND CANCER STEM CELLS

Epithelial-to-mesenchymal transition (EMT) is a process integral to early embryogenesis and development where epithelial cells transdifferentiate into motile mesenchymal cells^[21]. During the process of conversion into mesenchymal cells, epithelial cells lose their cellular junctions and apico-basal polarity, reorganize their cytoskeleton, and reprogram their signaling patterns and gene expression to gain the ability to migrate, increase motility, and invade adjacent tissue^[22,23]. During embryogenesis, EMT allows epithelial cells to travel through the embryo and participate in the formation of internal organs. The processes underlying EMT can also be reactivated for wound healing and cancer progression^[24,25]. The mechanism of EMT is transient

in nature and allows transformed mesenchymal cells the capability to reacquire their epithelial phenotype upon arriving at their organ or tissue of destination where they proliferate and differentiate into organs, a process termed mesenchymal to epithelial transition (MET)^[22,23]. The ability of epithelial cells to transform into mesenchymal cells and back into epithelial cells illustrates the inherent plasticity of the epithelial phenotype. These EMT and MET programs are coordinated by pleiotropic EMT transcription factors (TFs) and a multitude of extracellular signals^[26,27]. Three families of transcriptional regulators that are essential during EMT events have been identified as core EMT regulators: The zinc finger E-box binding homeobox members *ZEB1* and *ZEB2*^[28], the SNAIL zinc finger family^[29], and the TWIST family of basic helix-loop-helix transcription factors^[30].

Recent studies have elucidated molecular links between EMT/MET-TFs and self-renewal, one of the defining traits of cancer stem cells, suggesting that EMT processes play a role in the development of the CSC state. Several groups have demonstrated that EMT activation can induce CSC generation^[18,19]. Using a mammary tumor progression model, Morel and others showed that EMT induction can drive mammary epithelial cells to acquire stem and tumorigenic characteristics of CSCs following the activation of the Ras-mitogen-activated protein kinase pathway^[18]. In a correlative study, Mani *et al.*^[19] illustrated that cells that have undergone an EMT behave similarly to stem cells isolated from normal or neoplastic cell populations. The group induced EMT in nontumorigenic, immortalized human mammary epithelial cells (HMLEs) by ectopic expression of either the TWIST or SNAIL transcription factors (capable of inducing MET in epithelial cells). They found that most of the HMLEs that underwent MET acquired a CD44^{high}/CD24^{low} antigenic phenotype representative of mammary CSCs and that their mammosphere-forming ability was increased by 30-fold. Additionally, they demonstrated that CD44^{high} stem-like cells are more mesenchymal than their CD44^{low} counterparts^[19].

More recently, a study using a model of basal breast carcinoma elucidated a new role of EMT transcription factors in regulating CSC plasticity during tumor progression. Chaffer and group showed that neoplastic non stem cells (CD44^{low}) can readily convert to a stem-like state (CD44^{high}) and that this transition is mediated by *ZEB1*, a well-characterized EMT-TF. Further, they demonstrated that TGFβ, a recognized EMT-inducing stimulus can efficiently promote non-CSC-to-CSC conversion in their basal cell model^[20]. Taken together, these studies highlight the role of EMT regulators TWIST1, SNAIL2, and *ZEB1* in regulating CSCs in some tumor models. Importantly, complementary studies by these groups indicate that EMT transcription factors and extracellular signals cannot universally induce a CSC phenotype in all models, highlighting the heterogeneity of tumor progression and its microenvironment.

A DYNAMIC CSC STATE

Cancer stem cells are characterized as having an intrinsically determined state of unrestricted proliferative potential that permit self-renewal and give rise to progenitors with limited proliferative potential^[31]. Recent studies have proposed the concept of cancer stem cell plasticity in which these two states may not be definitive but instead have a transitional capability of shifting from a non-CSC state to a CSC state and vice versa^[20,32-36]. In experimental models of melanoma, tumorigenic cells display considerable plasticity transiently acquiring stemness properties depending on the tumor context. An early study revealed that both CD133+ and CD133- melanoma cells have the ability to form tumors, suggesting that CD133 is reversibly expressed by tumorigenic melanoma cells rather than identifying cells at a static level in a hierarchy^[37]. This group further characterized 22 markers and found that none of them robustly distinguished tumorigenic from non tumorigenic cells; instead, they observed phenotypically distinct melanoma cells having the capacity to form tumors that recapitulated the parent tumor^[33]. A complementary study further corroborates the plasticity of melanoma cells. The group showed that JARID1B histone demethylase is a regulator of tumorigenicity in melanoma cell lines and that JARID1B negative cells can become positive and acquire self-renewal potential. They proposed the hypothesis that the tumorigenicity of a single tumor-initiating cell is reversible and that some stem-like cells from solid tumors may not actually be static, but rather have a transient nature and acquire stem-cell-like properties depending on the tumor context^[34]. These results are compatible with the concept that tumorigenic potential might reflect a reversible state in cancer.

There is additional evidence supporting the model of dynamic stemness in breast cancer studies^[20,32,35]. Gupta and group developed and validated a theoretical quantitative Markov model of phenotypic transitions that predicts evolution toward equilibrium in cancer cell populations. Their model predicts that cancer cells could

interconvert between different states in a manner that maintains equilibrium in the proportions of cellular states; more specifically, it predicts that cancer stem-like cells can arise from non-stem-like cells. To test this prediction, they evaluated the ability of stem-like, basal, and luminal cells of seeding tumors. They demonstrated that the luminal and basal fractions could indeed generate functional stem-like cells *in vivo* supporting their hypothesis that convergence toward equilibrium cell-state proportions could be occurring due to cell-state interconversion within tumors^[35]. Additional work by the Weinberg group proposes that not all cancers adhere to a unidirectional model of cancer stem cell hierarchy. They identified subpopulations of non-cancer stem cells that could readily switch from a non-CSC to a CSC state. They found that the non-CSC population could give rise to aggressive CSCs^[32]. Further, they found that this plasticity is enabled by the transcription factor *ZEB1* (a well-characterized EMT transcription factor) and that microenvironmental stimuli could enhance the rate of non-CSC to CSC conversion. Importantly, they note that this plasticity and stimulus-mediated conversion is readily observed in basal-type cells, but not in luminal-type cells^[20]. Together, these studies suggest that certain cancer types may be following a bi-directional CSC model; however, they are not without limitations. The experiments described above rely on cell sorting to isolate and characterize cells, yet definitive surface markers are lacking for CSCs and their progenitors in undisturbed human tumors. Additionally, the results are obtained using artificial experimental systems, however delineation of functional plasticity will require minimally manipulated human cells or clinically relevant models of human disease. Further, if CSCs are present in a dynamic equilibrium, their respective isolation will be confounded by this fluctuating phenotype.

Two recent investigations in glioblastoma and colon cancer further validate the model of dynamic stemness and elucidate potential reprogramming factors responsible for the plastic phenotype^[36,38]. Suva and colleagues identified a set of core neurodevelopmental transcription factors (*POU3F2*, *SOX2*, *SALL2*, and *OLIG2*) that are essential for GBM propagation. They introduced each transcription factor individually into differentiated glioblastoma cells (DGCs) and monitored enhanced sphere formation ability. Next, they coinfecting DGCs with different combinations of TFs in a stepwise fashion and observed that the combined induction of *POU3F2* + *SOX2* + *SALL2* + *OLIG2* yielded cells capable of tumor initiation in 100% of animals. They showed that this set of TFs coordinately bind and activate CSC-specific regulatory elements and are sufficient to fully reprogram differentiated GBM cells to “induced” stem-like cells, recapitulating the epigenetic landscape and phenotype of native CSCs^[39]. Similarly, a study in colorectal cancer introduced a set of defined factors (*OCT3/4*, *SOX2* and *KLF4*) into human colon cancer cells and observed an enhancement in CSC properties such as sphere formation capability, marker gene expression, chemoresistance, and tumorigenicity. The tumors derived from these induced

CSCs had immunohistological similarities to human colon cancer tissue and their phenotypes were reproducible in serial transplantation experiments^[38].

Tumor suppressors have also been shown to play a role in CSC maintenance and plasticity. A study using an *in vivo* model of breast cancer showed knockdown of the *PTEN* tumor suppressor in normal mammary epithelial cells enriched for the stem/progenitor compartment leading to the generation of premalignant lesions^[40]. In cell lines, overexpression of *Mir-7*, a micro-RNA with tumor suppressor-like characteristics, decreased the population of breast cancer stem cells and partially reversed EMT by directly targeting the oncogene *SETDB1*^[41]. In prostate, *in vitro* analyses showed that *PTEN* and *TP53* play a role in regulating self-renewal and differentiation of prostate stem/progenitor cells^[42]. Further, using clonal epithelial cell lines the researchers showed that prostate epithelial *PTEN/TP53* loss led to transformation of multipotential progenitors, EMT, and increased plasticity of tumor cells^[43].

TUMOR MICROENVIRONMENT AND CSC PLASTICITY

The tumor niche or microenvironment is a complex network of cells, signaling molecules, soluble factors, and the extracellular matrix that plays a crucial role in tumor development, metastasis, and response to therapy^[44]. During embryonic development, niche factors affect stem cell gene expression and regulate cellular differentiation for fetal development. After birth, stem cells continue to be highly regulated for tissue homeostasis and during response to injury by the surrounding microenvironment^[45]. A tumor is comprised of neoplastic cancer cells together with microenvironmental factors that include hematopoietic cells, stroma, inflammatory cells, vasculature, and the extracellular matrix (a network of polysaccharides and proteins secreted by cells that serves as a structural tissue element and influences development and physiology)^[46,47]. Each of these cellular and non-cellular factors contributes to the transformation of tumor cells and may also affect response to chemotherapeutic and radiation treatments by providing protection from these agents^[46]. Similarly, cancer stem cell function and plasticity may be induced by specific microenvironmental signals and cellular interactions arising in the tumor niche^[48].

As discussed in the EMT section above, stromal cells secrete signaling factors that are received by epithelial cells and generate a signaling cascade that can orchestrate an epithelial to mesenchymal transition. A correlative interplay is observed between neoplastic cancer cells, CSCs, and the stroma in pancreas, breast, and colon cancer models. In the pancreas, stellate cells (myofibroblast-like cells found in the stromal compartment) remain activated after chemotherapy and radiation and impart a protective effect on cancer cells^[49-51]. Stellate cells have been shown to enhance spheroid-forming ability of CSCs through

induced expression of CSC-related genes and to promote the cancer stem cell phenotype through paracrine Nodal/Activin signaling at the tumor-stromal interface^[52,53]. Additionally, pancreatic stellate cells isolated from patients and co-cultured with pancreatic cancer cells enhanced cancer cell migration, and increased mesenchymal gene expression suggestive of an EMT phenotype^[54]. Parallel studies in breast and colon cancer revealed that CSC plasticity can be regulated by cytokine networks and growth factors^[55,56]. Vermeulen and group showed that hepatocyte growth factor can activate β -catenin-dependent transcription, CSC clonogenicity, and restore the CSC phenotype in more differentiated tumor cells both *in vitro* and *in vivo*^[56].

The interaction between immune cells and cancer cells has been shown to promote tumor development and progression and results in tumor immune evasion^[57]. In colorectal cancer, analysis of the type, density, and location of tumor-infiltrating immune cells within patient tumor samples revealed that this immunological data was a better predictor of patient survival than current histopathology methodologies used for staging that disease type^[58]. Some tumor cells escape immune system detection by decreasing the expression of specific antigen-presenting proteins on their cell surface, allowing them to evade cytotoxic T lymphocytes. Tumor cells can also secrete factors that inhibit effector T cell activity and promote the production of regulatory T cells that suppress immune responses^[57,59]. Similarly, stem-like cells in melanoma have been shown to preferentially inhibit T-cell activation and to support induction of regulatory T cells in order to evade immune system recognition^[60]. In GBM, CSCs suppress T cell response by producing immunosuppressive cytokines- they inhibit T cells through the *STAT3* pathway, and induce T cell apoptosis mediated by inhibitory molecules^[61,62]. A recent study has elucidated a novel role for how the immune system affects CSCs through paracrine signaling in colorectal cancer. This study revealed that CD4+ T cells secreted interleukin (IL)-22 which acted on cancer cells through *STAT3* and induced the core stem cell genes *NANOG*, *SOX2*, and *POU5F1*, resulting in increased cancer stemness and tumorigenic potential^[63].

The reciprocal communication and interplay between cancer stem cells and the immune niche can also induce CSC plasticity. This bidirectional phenotypic change may be driven by pro-inflammatory mediators such as tumor necrosis factor (TNF) and IL-6 that are secreted by various immune cells in the tumor microenvironment. Examples of cytokine-driven tumor cell plasticity have been demonstrated in melanoma, breast, and lung cancer where TNF and IL-6 can affect the differentiation state of tumor cells by the upregulation of mesenchymal genes- resembling an EMT-type switch. Similar changes have been observed in a study using an experimental model of colon cancer where elevated inflammatory nuclear factor kappa B signaling enhanced Wnt activation and induced dedifferentiation of non-stem cells that acquire tumor-initiating capacity. In an important complementary

study, a group at the Mayo Clinic observed that CD8+ T cells promoted EMT in an intact *in vivo* model of breast cancer. These transformed cells had characteristics of cancer stem cells that included potent tumorigenicity, the ability to reestablish an epithelial tumor, and enhanced resistance to drugs and radiation^[64]. In support of the hypothesis that cancer stem cell plasticity is driven by pro-inflammatory mediators induced by therapy regimens, gene expression signatures and histological studies in patients revealed an increase in mesenchymal markers in chemotherapy-resistant tumors^[65-67]. Taken together, these results suggest that immune cells and their related cytokines and growth factors can directly modulate and enhance the CSC phenotype. However, the niche effect likely will differ for different tumor subtypes with CSCs displaying diverse levels of dependency on these extrinsic microenvironmental interactions.

These experimental observations underscore the role of the tumor microenvironment, including immune cells, the stromal compartment, growth factors, and cytokines in phenotypic plasticity of tumor cells and CSCs and highlight potential novel targets to exploit therapeutic synergies to target multiple factors in the tumor niche.

CSC TARGETED THERAPIES

The data reviewed herein describe the hierarchical, molecular, extracellular, and plastic complexities of cancer and highlight the need for more comprehensive therapeutic strategies that address and target the multiple stages and branches of a single tumor. The renewed interest and deeper understanding of the CSC field have led to new ideas about therapeutic strategies including CSC signaling pathway inhibitors, immune mediated therapies targeting CSCs, and combination treatments targeting the microenvironment. However, CSC plasticity induced by microenvironmental and immune-related signals may limit the effect of CSC treatments. Therapies targeting CSCs would only be temporarily effective in eliminating this population since new CSCs may arise from non-CSCs left untargeted. Therefore, targeting each subpopulation or subclone within the tumor will be the most effective way of achieving a successful clinical response.

Data from high-throughput screening studies revealed that EMT programs rely on classic embryonal signaling pathways such as Notch, Wnt, Shh, and TGFβ^[68]. As reviewed, EMT processes play a role in the development of the CSC state and may be an attractive therapeutic target for inhibiting CSC plasticity. Because aberrant signaling in these pathways has been recognized to cause tumorigenesis in a broad variety of tissues, significant investment has been made by the industry to develop inhibitors towards them^[48]. It is possible that these agents already in development could be used to induce differentiation of CSCs in combination with drugs that would target the newly-differentiated cells. A number of experimental agents are being evaluated in the clinic for their ability to inhibit Wnt signaling that could be studied for this purpose^[69-72]. Novartis is currently testing

a Wnt-specific acetyltransferase inhibitor, LGK974, in a phase I study on malignancies dependent on Wnt ligands^[70]. Interestingly, their pre-clinical studies showed that cell lines with loss-of-function mutations in the Notch signaling pathway responded well to this inhibitor, suggesting an underlying mechanism of action for increased sensitivity to LGK974 in the Notch1 mutant carcinomas and highlighting the potential to target a subset of patients^[73]. One caveat of these therapies is the fact that the pathways they target are not exclusive to CSCs but are shared with normal stem cells. Identifying a threshold of killing activity of normal cells will be necessary to prevent deleterious effects. Additionally, it is unlikely that a single pathway will be operative in all of the CSCs in a given tumor; therefore, concurrent use of drugs that can affect multiple pathways that are essential to CSCs and EMT-mediated plasticity are likely to be obligatory to achieve effective therapies.

It is well-accepted that tumors deregulate certain immune-checkpoint pathways as a major mechanism of evasion and immune-mediated therapies (IMT) have been developed that utilize the immune system to target cancer cells. Cancer cells that undergo EMT develop a mesenchymal phenotype yet retain some epithelial characteristics^[64]. A recent study demonstrated that mesenchymal cells overexpress genes for immune inhibitory molecules including programmed cell death protein 1 (PD1)/PD-L1 and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)^[74]. It is also possible that mesenchymal cells resulting from EMT during bidirectional CSC conversion also express these molecules and that IMTs may have the potential to target this subgroup of dynamic cells. The two immune-checkpoint receptors that have been most actively studied in the context of clinical cancer immunotherapy are CTLA-4 and PD1. Both are inhibitory receptors and regulate immune responses at different levels and through different mechanisms^[75]. In 2010, a crucial Phase III trial using an anti-CTLA4 antibody, ipilimumab, demonstrated statistical survival benefit in patients with melanoma^[76]. This was the first immune-mediated therapy to demonstrate survival benefit in patients with advanced melanoma in a randomized trial. Other drug companies have accelerated preclinical studies to advance their immune-mediated therapies to the clinic^[77-79]. The observations that mesenchymal cells in non-small-cell lung cancer are associated with distinct immune phenotypes with increased expression of inhibitory molecules provide a potential mechanism for EMT-associated immunosuppression utilizing the therapies that are currently being tested in the clinic. Therefore, the IMTs described above may have the potential to target newly transformed CSCs as well as cancer cells that have not undergone a transformation thereby simultaneously eliminating both subpopulations leading to a more complete therapeutic response.

Cancer stem cells are characterized by immune suppressive activity and a low immunogenicity that enhance their ability to survive. CSCs need to evade immune reactions mounted by the host and adapt to the dynamic microenvironment altered by radiation and chemotherapy^[80]. Phenotypic

analysis in GBM, colorectal cancer, and melanoma revealed a potential impairment of antigen presenting function by CSCs helping to shield them from T-cells^[81]. Additionally, CSCs enhance immunosuppression by down-regulation of major histocompatibility complex MHC- I and - II (a milieu of proteins that regulate cell-mediated adaptive response) and through the release of immunosuppressive cytokines such as IL-10 and TGF β ^[60,61,81,82]. These cytokines can also affect the differentiation state of tumor cells by upregulating EMT genes. Therefore, Identifying targets that will inhibit and reverse the CSC escape from immunosurveillance may also elucidate targets for CSC plasticity and could prove necessary to successfully eliminate tumors.

PERSPECTIVE AND FUTURE DIRECTIONS

Additional understanding of the role of tumor cell dynamics will continue to inform our therapeutic strategies. Answers to important questions remain to be determined in the characterization of patient tumors and advances in technologies like single cell analysis are likely needed to identify a resolution. Perhaps the most important questions that remain with respect to therapeutic targeting are (1) whether plasticity causing non-CSCs to develop the tumorigenic properties ascribed to a CSC truly exists in human patient tumors and, even more importantly; (2) whether this interconversion results in a CSC clone that is different from the original clone and requires a different therapeutic approach. Additionally, (3) whether the extent of heterogeneity found within CSCs results in the need for independent therapies targeting of multiple CSC clones. Despite these questions, an overwhelming body of literature points to a need to develop therapeutics specifically targeted to CSCs as they are clearly implicated in disease relapse.

Continued research is needed to identify CSC-specific targets and to understand the effects of the stem cell niche on plasticity, survival, and tumorigenicity in order to develop novel therapeutic regimens. Identification of additional immuno-modulating therapies that can inhibit or reverse the ability of CSCs and their dynamic subclones to escape from immunosurveillance will contribute to the comprehensive cancer therapy approach that is likely necessary to achieve successful eradication of a tumor. Further, in order to achieve more complete therapeutic responses it will be necessary to pursue treatments against CSCs in each of their dynamic states or to first halt the interconversion between non-CSC to CSC and then eliminate each subpopulation therapeutically, and ultimately to combine these specialized CSC therapies with strategies that target the bulk population.

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Control of stem cell fate by engineering their micro and nanoenvironment

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Abstract

Stem cells are capable of long-term self-renewal and differentiation into specialised cell types, making them an ideal candidate for a cell source for regenerative medicine. The control of stem cell fate has become a major area of interest in the field of regenerative medicine and therapeutic intervention. Conventional methods of chemically inducing stem cells into specific lineages

is being challenged by the advances in biomaterial technology, with evidence highlighting that material properties are capable of driving stem cell fate. Materials are being designed to mimic the clues stem cells receive in their *in vivo* stem cell niche including topographical and chemical instructions. Nanotopographical clues that mimic the extracellular matrix (ECM) *in vivo* have shown to regulate stem cell differentiation. The delivery of ECM components on biomaterials in the form of short peptides sequences has also proved successful in directing stem cell lineage. Growth factors responsible for controlling stem cell fate *in vivo* have also been delivered *via* biomaterials to provide clues to determine stem cell differentiation. An alternative approach to guide stem cells fate is to provide genetic clues including delivering DNA plasmids and small interfering RNAs *via* scaffolds. This review, aims to provide an overview of the topographical, chemical and molecular clues that biomaterials can provide to guide stem cell fate. The promising features and challenges of such approaches will be highlighted, to provide directions for future advancements in this exciting area of stem cell translation for regenerative medicine.

Key words: Biomaterials; Scaffold; Stem cells; Surface topography; Tissue engineering

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Core tip: Stem cells receive instructions from their niche environment, which guide their survival and phenotype. Stem cells receive physical and biochemical clues from their extracellular matrix where they reside *in vivo*. This paper will discuss the utilization of biomaterial surface topography and elasticity and delivery of chemical and genetic clues *via* scaffold materials to mimic the extracellular matrix to guide stem cell fate. The understanding of the parameters that guide stem cell differentiation is of great interest for the tissue-engineering field.

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INTRODUCTION

Stem cells have the ability to differentiate into several tissue types and have captured a great interest for regenerative medicine due to their ability to regenerate and repair injured tissues^[1]. Stem cells are broadly classified into embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are pluripotent stem cells capable of regenerating into cells types from all three germ layers (ectoderm, mesoderm and endoderm), being themselves derived from the inner cell mass of blastocysts^[2]. ESCs have a long term self-renewal capacity, ability to expanded for extended culture time and multi-lineage differentiation potential making them a very attractive tissue-engineering cell resource for regenerative medicine^[2]. However, efficient and safe isolation and differentiation protocols are required for their optimal clinical translation^[3]. In addition, ESCs have shown to differentiate into tumour cells, limiting their translation to clinical trials^[4]. The second type of stem cells that have attracted extensive research interest is ASCs. These cells are multi-potent cells derived from adult somatic tissues with the potential to differentiate into many specific cell types^[5]. Mesenchymal stem cells (MSCs) are the commonest type of ASCs investigated for tissue-engineered applications, which are found to be isolated from several tissue sources such as the bone marrow and adipose tissue^[6-8]. Controlling both the proliferative and differentiation abilities of stem cells has been the focus of utilizing stem cells for a therapeutic platform^[9]. In their *in vitro* environment stem cell fate is determined by a reservoir of biochemical and biophysical clues^[6]. The instructions stem cells receive from their stem cell niche will control their stem-ness, multi-potency and determine their phenotype of differentiation^[6].

Stem cells have the ability to differentiate spontaneously *in vitro* but this is uncontrolled and inefficient for tissue-engineering applications^[10]. Therefore, over the last decade it has become important to understand how to control stem cell fate effectively for regenerative medicine^[10]. A common approach to determine stem cell fate *in vitro* is by adding medium growth components including chemokines and hormones, to differentiate stem cells into a particular lineage^[7-9]. Chemical patterning has shown to be very successful in the differentiation of ASCs down osteogenic, chondrogenic and adipogenic lineages^[7-9]. Whilst understanding chemical clues of stem cell fate is important, with the advances in knowledge that stem cells are highly sensitive to their topography, stiffness and molecular environment, it may only be part of the strategy for controlling stem cell fate for regenerative medicine^[10]. The extracellular matrix (ECM) in the *in vitro* stem cell niche is arranged into complex topographic features,

which stem cells have shown to be sensitive to and provide clues to guide their phenotype^[11]. This evidence has prompted materials to be manufactured with surface topographical clues, which has shown to greatly influence cell behavior altering cell morphology, adhesion, motility proliferation and gene regulation^[12]. The *in vitro* mechanical clues from the ECM (stress, shear and strain) have also shown to play a part in the precise control of stem cell fate^[13]. With this knowledge material substrate stiffness is now taken into consideration to guide stem cell fate^[14]. As biochemical clues provide important instructions to stem cells *in vitro* growth factors have been immobilised onto material surfaces to guide stem cell behaviour^[15,16]. Further understanding of these environmental instructions on stem cell differentiation is emerging, with the hope to be able to better control stem cell fate for tissue-engineering purposes.

This unique review, aims to provide an update on the three main biomaterial parameters that are being explored to govern stem cell fate, with a focus on stem cell differentiation. The parameters discussed in this review will be the effect of topographical, chemical and genetic clues on guiding stem cell differentiation. In this review, both ASCs and ESCs will be considered to provide an overview of the different parameters that are being investigated to determine stem cell differentiation. The emphasis of discussion will be placed on the techniques used to govern stem cell fate rather than exploring their effect on different stem cell types. This review aims to highlight the successful strategies to control stem cells fate for regenerative medicine applications.

EFFECT OF STEM CELL FATE AND SURFACE TOPOGRAPHICAL CLUES

In their natural niche environment, cells are interacting with various nanotopographically sized clues^[17]. Cells will encounter different topographies sized clues *in vivo* from macro- (bone and ligament) to micro- (other cells) and nanotopography (proteins and ligands), all of which will influence cell behavior and consequently cell fate^[17]. The most important is the basement membrane, a vital structure of the ECM, which provides tissue support and organization^[17]. The basement membrane contains pores, ridges and fibers, which are all in the nanometer range^[17].

The use of topographically patterned surfaces for culturing cells has a very important advantage over chemical induction of stem cells, the disregard to using chemicals in the body yet still tailor stem cell for particular clinical applications. There is now growing evidence that topographical clues alone can produce the same effect as chemical induction^[18]. Extensive research has been attempted to understand the interaction of cells with their substrate topography using *in vitro* studies^[18]. With the advancement in micro and nanofabrication techniques, numerous studies have been able to be carried out to understand the effect of nanotopography on a wide range of materials. Patterning techniques such as soft lithography, photolithography, electrospinning, layer-by-

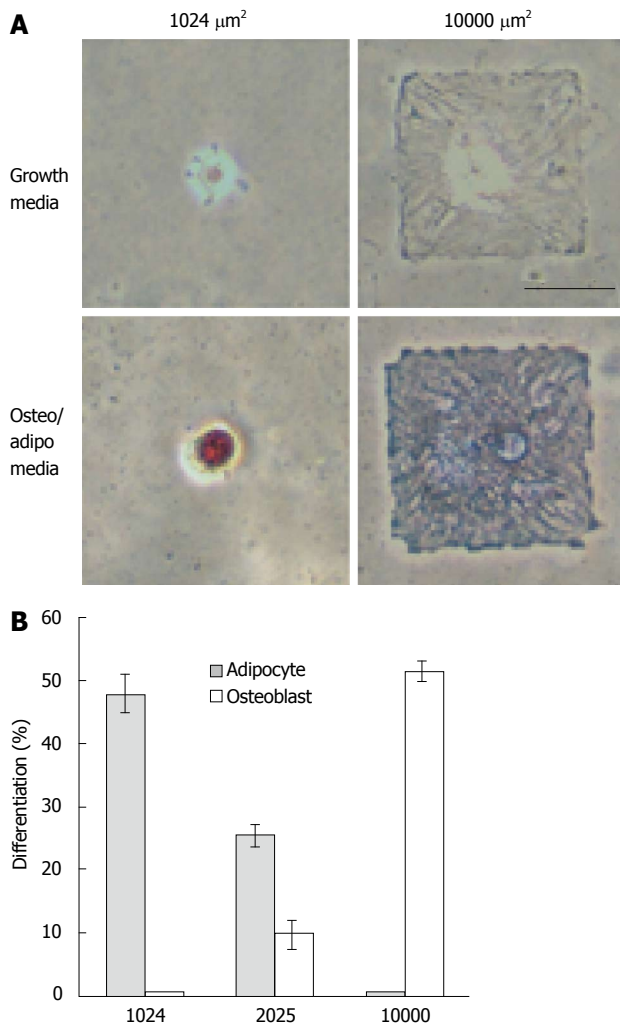


Figure 1 The effect of nanotopographies on stem cell shape and its consequential effect on the differentiation of stem cells. **A:** Brightfield images of human mesenchymal stem cells (hMSCs) plated onto small (1024 μm^2) or large (10000 μm^2) fibronectin islands after 1 wk in growth or mixed media. Note hMSCs plated on large islands showed differentiation for osteogenic differentiation and those on small islands showed differentiation for adipogenic differentiation. Lipids stain red; alkaline phosphatase stains blue. Scale bar = 50 μm ; **B:** Percentage differentiation of hMSCs plated onto 1024, 2025, or 10000 μm^2 islands after 1 wk of culture in mixed media. Taken with permission from McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004; **6**: 483-495.

layer microfluidic patterning, three-dimensional (3D) printing, ion milling and reactive ion etching have all made it possible to create scaffolds materials with precise controlled geometry, texture, porosity and rigidity^[19-23]. Different types of surface topographies have been generated and investigated for their effect on stem cell differentiation including the topographical scale (micro or nano), type (ridges, pillar, pit or groove) and distribution (random or regular distributed features).

Stem cells have shown to rely on spatial information for differentiation, as this controls their cell shape and size, elongation and positioning of the focal adhesion (FA) formation and lastly cell-cell interactions^[24]. The effect of surface topography on cell shape and size, which will later determine stem cell fate has been explained by many researchers^[25-27]. For example, MSCs cultured

on large 10000 μm^2 islands allow spreading and permit differentiation down the osteoblastic lineage but MSCs on small 1024 μm^2 islands remain rounded and as a consequence differentiated into adipocytes (Figure 1)^[25]. Similarly MSCs cultured on small 30 nm nanotubes showed no obvious differentiation whereas 70-100 nm nanotubes showed cytoskeletal stress and osteoblastic lineage differentiation^[28]. Overall, it has been assumed that geometric changes causes an increase in actomyosin contractility leading to osteogenesis due to the activation of RhoA and its effector ROCK kinase^[28]. The surface topography also has significant effects on the alignment of the nuclei. The nuclear shape will be distorted during cell extension on material surfaces as the nucleus is mechanically integrated to the rest of the cell through filaments^[29]. Nuclear elongation has shown to influence osteoblastic stem cell differentiation^[29]. Geometric clues will also alter the cell-cell interactions. This is very clear with the example of directing differentiation of ESCs. Aggregation is an important step for the differentiation of ESCs. This was demonstrated by the enhancement of ESCs differentiation into cell aggregates called embryoid bodies (EBs), which recapitulate early stages of embryonic development. By creating microfabricated cell-repellent poly(ethylene glycol) (PEG) wells, EBs cell aggregates could be formed with controlled sizes and shaped defined by the geometry of the microwells^[30].

A seminal paper showed that the order of the nanotopography is also important in controlling stem cell fate. Dalby *et al*^[31] compared the osteogenesis of osteoprogenitor cells and human mesenchymal stem cells (hMSCs) on polymethylmethacrylate substrate with ordered square and hexagonal pattern, disordered square and random nanopits using electron beam lithography (EBL) (Figure 2). Osteogenesis was the highest on the disordered square array without the presence of any induction medium and the lowest on the surfaces with high symmetry^[31]. Quantitative Polymerase Chain Reaction and microarray data showed an increase in expression of genes responsible for osteogenic differentiation, which was of a comparable level to those cells on a flat planar surface in osteogenic medium^[31]. Stein *et al*^[32] followed up this work, by illustrating that skeletal stem cells cultured on these surfaces had a normal differentiation profile according to the osteogenic differentiation model explained by Stein and Lian. The authors provided further evidence that differentiation of skeletal stem cells cultured on nanotopographical surfaces, provided an equal and effective technique of differentiation of stem cells compared to chemical induction^[33]. Zouani *et al*^[34] similarly showed that hMSCs cultured on polyethylene terephthalate substrates with nano-depths of varying degrees, had higher osteogenic differentiation on the 100 nm patterns compared to the 10 nm patterns. The organization of the hMSCs on the 100 nm was believed to induce FA contacts, generating stress in the actin filaments. Actin tension was found to be important in the osteogenic differentiation of the cells as the osteoblastic phenotype was inhibited by cytochalasin D^[34].

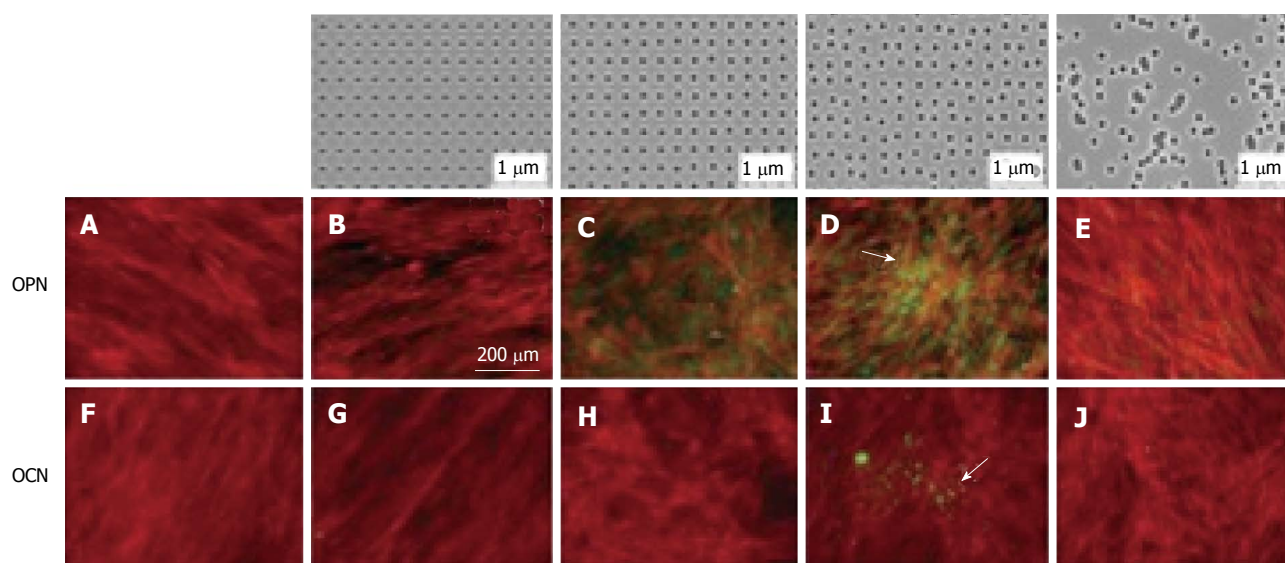


Figure 2 The effect of nanopits on the differentiation of stem cells. The top row showed the nanotopographies produced using electron beam lithography. All have 120 nm diameter pits (100 nm deep, absolute or average 300 nm centre-centre spacing) with square (B, G), displaced square 20 (± 20 nm from true centre), (C, H), displaced square 50 (± 50 nm from true centre) (D, I) and random placements (E, J). There is lack of positive staining for osteocalcin (OCN) and osteopontin (OPN) on control nanotopographies (A, F) but good positive staining for expression for OPN and OCN for cells grown on displaced square 50 nanotopographies (D, I). Actin = red, OPN/OCN = green. Taken with permission from Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CD, Oreffo RO. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat Mater* 2007; 6: 997-1003.

Nanogrooves have found to be particularly useful for controlling cellular morphology, orientation and direct cell migration in a mechanism called “contact guidance”, proposed by Harrison in 1911^[35]. Harrison illustrated that ESCs cultured on a spider followed the pattern of the fibers of the web^[35]. It has emerged that phenotypic changes can occur using nanoscale grooves to direct stem cell differentiation towards the neuronal lineage^[36,37]. For example, bone marrow derived stem cells were cultured on micro and nanoscale ridges and found to undergo neurogenesis without the presence of chemical induction medium. In this study, hydrogenated amorphous carbon groove topographies with width/spacing ridges ranging from 80/40 μm , 40/30 μm and 30/20 μm and depths of 24 nm were used^[37].

Grating like surfaces have also shown to be a clue for ESC differentiation. Smith *et al.*^[38] demonstrated that mouse ESC differentiation was induced on poly-L-lactide (PLLA) matrices with a grating morphology, a fibrous nano-architecture with diameters of 50-500 nm. ESCs were cultured on PLLA nanofibrous scaffolds and compared to elastin-coated tissue-culture polystyrene and PLLA films and demonstrated an increase in osteogenic markers on the nanofibrous matrix than the other substrates^[38]. Grating like surfaces have also demonstrated to induce the differentiation of ASCs^[39]. Human MSCs have shown to differentiate into the neuronal lineage on polydimethylsiloxane (PDMS) with and without retinoic acid induction with nanogratings of 350 nm width^[39].

Vertically standing nanotubes illustrate another topographical control of stem cell fate^[11]. The diameter size of vertically aligned titanium dioxide (TiO_2) nanotubes formed by anodization has showed to be crucially vital to determine stem cell response^[11]. For example, 15 nm TiO_2

nanotubes allowed integrin clustering and cell spreading but this was suppressed when grown on 100 nm TiO_2 nanotubes^[11]. This morphological effect resulted in less differentiation of the MSCs into smooth muscles and endothelial cells on the 10 nm compared to the 15 nm tubes in chemical induction medium^[11].

Stem cells will respond to the mechanical properties of the material, which they are adhering and growing^[40]. A influential paper by Engler *et al.*^[40] showed that hMSCs on polyacrylamide hydrogels of varying stiffness, coated with collagen I were capable of differentiation without any inducing medium (Figure 3). Soft gels mimicked the rigidity of brain tissue (0.1-1.0 kPa), enabled the hMSCs to undergo neurogenic differentiation, stiffer gels which resembled muscle tissue provided clues for myogenic differentiation and very stiff gels which resembled bone tissue enabled osteogenic commitment (25-40 kPa). Zouani *et al.*^[41] similarly found that culturing hMSCs on poly(acrylamide-co-acrylic acid) surfaces and then varying the stiffness of the gels depending on the % of cross-linker bis-acrylamide could cause myogenic (13-17 kPa) or osteogenic differentiation (45-49 kPa). Other researchers have also reported that stiffness of the cell culture substrate is important for stem cell differentiation in 2D-culture^[42-44]. The mechanism by which the elasticity of ECM on culture substrates induces specific stem cell differentiation is still under debate. However, it is thought that the elasticity of the ECM induces changes in the FA protein activity and remodeling. Furthermore, as the growth and elongation of the FA will change according to the substrate stiffness, which means the elasticity regulates the FA assembly^[45,46]. Integrins have been suggested to be the starting point for cells to sense the mechanical stimuli of the biomaterial to which they are adhered^[45,46].

Table 1 Examples of studies, which have demonstrated surface topographies can direct stem cell differentiation

Stem cell source	Scaffold type	Description of topographical feature	Differentiation outcome	Ref.
hMSC	PDMS	Islands	Large 10000 μm^2 islands promoted osteogenesis. Small 1024 μm^2 island promoted adipogenesis	McBeath <i>et al.</i> ^[25]
hMSC	TiO ₂ nanotubes	30-, 50, -70 and 100 nm nanotubes	70-100 nm nanotubes promoted osteogenesis	Oh <i>et al.</i> ^[28]
hMSC	PMMA	Ordered and disordered square or hexagonal pattern and Nanopits	Disordered squared promoted osteogenesis	Dalby <i>et al.</i> ^[31]
hBMDSC	Hydrogenated amorphous carbon	Grooves	Neurogenesis	D'Angelo <i>et al.</i> ^[37]
mESC	PLLA	Grating	Osteogenesis	Smith <i>et al.</i> ^[38]
hMSC	PDMS	Grating	Neurogenesis	Yim <i>et al.</i> ^[39]
hESC	PDMS	Micropatterned fibronectin with square shape surrounded by Pluronic F127	Myogenesis and chondrogenesis	Gao <i>et al.</i> ^[49]
hMSC	PDMS	Micropatterned with striped groove morphology coated with collagen type I.	Myogenesis	Kurpinski <i>et al.</i> ^[50]
hNSC	PDMS	Micropatterned with striped groove morphology coated with PLL and laminin.	Neuronal	Bédier <i>et al.</i> ^[51]
hMSC	PDMS	Micropatterned with striped groove morphology coated with collagen type I	Neuronal	Biehl <i>et al.</i> ^[52]
hESC	PDMS	Groove	Neuronal	Lu <i>et al.</i> ^[53]
hMSC	PCL	Nanopillar, nanohole and nanogrill	Nanopillar and nanohole topography enhanced	Wu <i>et al.</i> ^[54]
hNSC	PUA	Groove and Pillar	MSC chondrogenesis and facilitated hyaline cartilage.	Yang <i>et al.</i> ^[55]
rMSCs	Polystyrene	Groove	Neuronal Myogenesis and adipogenesis	Wang <i>et al.</i> ^[56]

hESC: Human embryonic stem cell; hMSC: Human mesenchymal stem cell; rMSCs: Rat mesenchymal stem cell; hNSC: Human neural stem cell; hBMDSC: Human bone marrow derived stem cell; mESC: Mouse embryonic stem cell; PMMA: Polymethyl methacrylate; PDMS: Polydimethylsiloxane; PCL: Polycaprolactone; PUA: Polyurethane acrylate; PLL: Poly-L-lysine; PLLA: Poly(L-lactide).

Modification to the FA complexes can cause a cascade of signaling pathways and allow differentiation of stem cells^[45,46].

Elasticity of scaffolds has also shown to cause specific lineage differentiation modifications to stem cells in 3D-culture. For example, Banerjee *et al.*^[47] showed that the proliferation and differentiation of rat neural stem cells encapsulated in 3D-alginate hydrogels showed enhanced expression of neuronal marker β -tubulin II in the softer hydrogels, which resembled brain tissue than the stiffer hydrogels. Murphy *et al.*^[48] found that cross-linked collagen-glycosaminoglycan scaffolds with three varying stiffness of 0.5, 1 and 1.5 kPa showed difference in their chondrogenic differentiation capacity. The scaffolds with the lowest stiffness showed an upregulation of sox expression and MSCs on the stiffest scaffolds showed differentiation down the osteogenic lineage^[48]. This study highlighted that MSC fate in 3D-culture can be controlled even in the absence of differentiation supplements^[48]. Table 1 provides the summary of the key research studies highlighting the effect of nanotopographies on directing stem cell fate^[49-56].

EFFECT OF STEM CELL FATE AND CHEMICAL CLUES

As previously highlighted, stem cells in their niche environment interact with their ECM. The stem cells will recognise and attach to specific amino acid sequences *via* integrins, which are surface receptors composed of heterodimers of α - and β -subunits^[57,58]. Cells will receive chemical signals from the

ECM that will initiate signaling cascades, which will determine critical cell functions including proliferation, differentiation, migration and apoptosis^[57,58]. Therefore, an important goal of regenerative medicine is to mimic the critical chemical clues of the ECM to control cell function and direct cell fate (see Table 2)^[15,16,59-79].

Growth factors are one key chemical clue that is provided by the ECM, which regulates a key number of cellular functions including adhesion, proliferation and differentiation^[80]. Kuhl *et al.*^[80] were the first to illustrate that providing growth factors in their matrix-bound mode to regulate cell response rather than providing it in a soluble mode allowed effective cell behavior control. They demonstrated that mouse epidermal growth factor covalently tethered to a glass substrate *via* star poly (ethylene oxide) could induce DNA synthesis^[80]. Whole proteins are usually denatured or degraded during the biomaterial modification step and as a consequence short peptide sequences are often implemented to control cell response, which provides increased specificity, stability and the capability for high concentrations^[81]. The specific peptide sequence arginine-glycine-aspartic acid (RGD) sequence present in ECM proteins [*i.e.*, fibronectin (FN)] has been extensively attached to several natural and synthetic biomaterials and found to regulate MSC behaviour^[81]. Surfaces functionalised with RGD ligands has demonstrated to increase the osteogenic and chondrogenic lineage compared to unmodified surfaces^[82-85]. Other peptide sequences have also shown to influence stem cell differentiation for example, Martino *et al.*^[86] investigated the effect of fibronectin's central binding

Table 2 Example of studies, which have demonstrated that the use of different chemical inducers can direct stem cell differentiation

Immobilised chemical inductor	Scaffold type	Functional group	Differentiation outcome	Ref
Chemical group	Silk fibroin	-COO-	hMSC osteogenic differentiation	Murphy <i>et al</i> ^[59]
	Silk fibroin	=C=O	hMSC osteogenic differentiation	Murphy <i>et al</i> ^[59]
	Silk fibroin	SO ₃ H	hMSC osteogenic differentiation	Murphy <i>et al</i> ^[59]
	PEG	PO ₃	Increase in hMSC osteogenic markers at gene and protein level	Benoit <i>et al</i> ^[60] , Nuttelman <i>et al</i> ^[61] , Nuttelman <i>et al</i> ^[62] , Gandavarapu <i>et al</i> ^[63]
	Silk fibroin	NH ₂	hMSC osteogenic differentiation	Murphy <i>et al</i> ^[59]
	Silk fibroin	CH ₃	hMSC osteogenic differentiation	Murphy <i>et al</i> ^[59]
	Glass	COOH, CH ₃ , OH, NH ₂	-NH ₂ support hMSC osteogenic differentiation	Liu <i>et al</i> ^[64]
	Glass	OH, SO ₃ H, NH ₂ , COOH, SH and CH ₃	-NH ₂ support hNSC differentiation	Ren <i>et al</i> ^[65]
	Glass	CH ₃ , NH ₂ , SH, OH and COOH	NH ₂ and -SH- promoted and maintained hMSC Osteogenesis, -OH and -COOH- promoted and maintained chondrogenesis	Curran <i>et al</i> ^[66]
	Glass	CH ₃ , NH ₂ , SH, OH and COOH	NH ₂ and -SH- promoted and maintained hMSC Osteogenesis, -OH and -COOH- promoted and maintained chondrogenesis	Curran <i>et al</i> ^[66]
Peptide sequence	Alginate	Osteopontin peptide	Increase in hMSC osteogenic markers	Lee <i>et al</i> ^[67]
	HA-PLG	Osteocalcin peptide	Increase hMSC osteogenic markers	Lee <i>et al</i> ^[68]
	PLGA	BMP-2 peptide	Increase rMSC ALP expression in osteogenic medium and promotion of ectopic bone formation <i>in vivo</i>	Lin <i>et al</i> ^[15]
Molecule	RGD	BCP/PLA	hMSC osteogenic differentiation	Shin <i>et al</i> ^[70]
	PLGA	BMP-2	hMSC osteogenic differentiation	Ko <i>et al</i> ^[71]
	PLLA	BMP-2	hMSC osteogenic differentiation	Beazley <i>et al</i> ^[16]
	Collagen-PLGA hybrid	Collagen-binding domain derived from fibronectin (CBD-BMP4)	hMSC osteogenic differentiation	Lu <i>et al</i> ^[72]
	Methacrylamide chitosan hydrogel coated glass substrates	Laminin and collagen	Supported hNSC differentiation	Wilkinson <i>et al</i> ^[73]
	PMMA-g-PEG	EGF	MSC osteogenic differentiation	Platt <i>et al</i> ^[74]
	Agarose	PDGF-AA	MSC neural differentiation	Aizawa <i>et al</i> ^[75]
	PCL/PCL-PEG	NGF	MSC neural differentiation	Cho <i>et al</i> ^[76]
	Chitosan/collagen IV	VEGF	Endothelial differentiation	Chiang <i>et al</i> ^[77] , Poh <i>et al</i> ^[78] , Rahman <i>et al</i> ^[79]
	Chitosan/collagen IV	VEGF	Endothelial differentiation	Chiang <i>et al</i> ^[77] , Poh <i>et al</i> ^[78] , Rahman <i>et al</i> ^[79]

hESC: Human embryonic stem cell; hMSC: Human mesenchymal stem cell; rMSCs: Rat mesenchymal stem cell; hNSC: Human neural stem cell; PEG: Polyethylene glycol; HA-PLG: Hydroxyapatite (HA)/poly(lactic-co-glycolic acid); BMP: Bone morphogenetic protein; PLGA: Poly(lactic-co -g lycolic acid); PLA: Poly(lactic acid); PLLA: Poly(L-lactide); PCL: Polycaprolactone; PMMA-g-PEG: Poly(methyl methacrylate)-graft-poly(ethylene glycol); BCP: Biphasic calcium phosphate; EGF: Epidermal growth factor; NGF: Nerve growth factor; PDGF-AA: Platelet-derived Growth Factor AA; VEGF: Vascular endothelial growth factor.

domains (FN III9-10) variants with varying specificities to integrin $\alpha 5 \beta 1$ on stem cell differentiation on fibrin matrices. The data illustrated that MSC underwent osteogenic differentiation on the modified surfaces. The spatial density of RGD peptide has also illustrated to influence stem cells differentiation^[85]. Frith *et al*^[84] showed that as the lateral spacing of RGD peptide increased, the ability of the MSCs to spread was reduced and that their morphology changed from well-spread cells with normal fibroblastic morphology and defined stress-fibres, to less-spread cells with numerous cell protrusions and few stress fibres. Using qRT-PCR to determine gene expression levels and a quantitative alkaline phosphatase assay, they showed that MSC osteogenesis was reduced on surfaces with increased lateral spacing while adipogenic differentiation was increased^[84].

Peptide sequences that resemble several growth factors and chemokines have also been immobilised onto

substrates to induce specific stem cell differentiation. Bone morphogenic proteins have shown to play an important role in stem cell activity and control of differentiation of stem cell *in vitro* for osteoblastic differentiation and bone formation^[87]. RGD and RKIPKASSVPTLSAISMLYL peptide sequence, which mimicked BMP-2 was grafted onto the poly(acrylamide-co-acrylic acid) surfaces of different stiffness through carbodiimide chemistry^[41]. The study illustrated that hMSCs showed commitment to the osteoblastic lineage in the presence of the BMP-2. Interestingly, the osteogenic differentiation did not occur on the softer scaffolds, highlighting the important interaction of biochemical and mechanical stimuli to guide stem cell fate^[41]. Several other chemical inducers have also been investigated to control stem cell fate, including collagen type I (COL-1), bone sialoprotein (BSP) and osteopontin (OSP)^[88-92]. The $\alpha 2 \beta 1$ integrin is highly

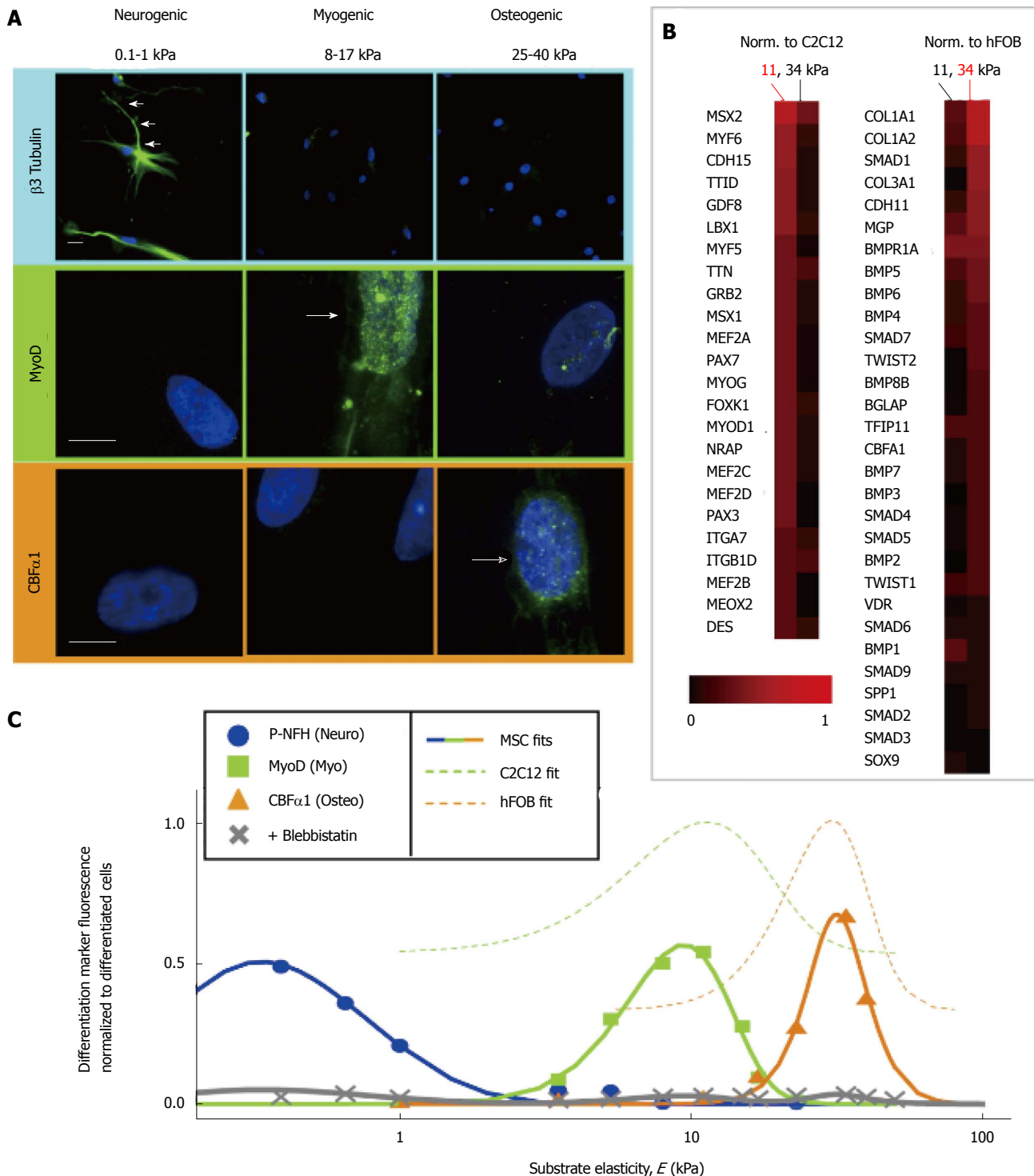


Figure 3 Effect of substrate stiffness on the differentiation of stem cells. A: The neuronal cytoskeletal marker $\beta 3$ tubulin is expressed in branches (arrows) of initially naive mesenchymal stem cells (MSCs) (> 75%) and only on the soft, neurogenic matrices. The muscle transcription factor MyoD1 is upregulated and nuclear localised (arrow) on myogenic matrices and osteoblast transcription factor CBF- α 1 (arrow) is expressed only on stiff, osteogenic gels. Scale bar is 5 μ m; B: The microarray profiles of MSCs cultured on 11 or 34 kPa matrices. The expression was normalised first to actin and then to expression of committed C2C12 myoblasts and hFOB osteoblasts; C: The highest fluorescent intensity of differentiation markers was observed at the substrate elasticity of the elastic modulus typical of each tissue type. Taken with permission from Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006; **126**: 677-689.

expressed on osteoblasts and a major adhesion receptor for COL-1, playing an important role in the activation of FAs kinase and pathways for osteoblastic differentiation^[88]. BSP is another important molecule involved in the bone mineralization process by inducing hydroxyapatite

nucleation^[89]. OSP is a glycosylated phosphoprotein highly expressed in bone and shown to be responsible for the formation and remodelling of bone^[90]. COL-1 and OSP peptides have demonstrated to promote osteogenesis of stem cells when immobilised onto scaffolds materials^[91,92].

Applying growth factors to scaffold materials has shown not only to induce osteogenesis of stem cells but demonstrated beneficial effects on several differentiation pathways. NSCs cultured on agarose scaffold immobilised with RGD and platelet derived growth factor AA (PDGF-AA) showed differentiation into oligodendrocytes, being confirmed at a protein and gene level^[75]. Nerve growth factor (NGF) immobilised onto nanofibrous polycaprolactone- polyethylene glycol (PCL/PCL-PEG) scaffolds also induced the neural differentiation of MSCs^[76]. Vascular endothelial growth factor (VEGF) has also demonstrated to induce differentiation of mouse ESCs and human MSCs into endothelial cells^[77-79].

The modification of materials with specific chemical functional groups is a common strategy to induce specific stem cell behaviour^[93]. Surface chemistries have shown to sufficiently induce differentiation of MSCs alone^[94]. Benoit *et al*^[60] has provided and modelled the effect of unique chemical environments on the differentiation of stem cells (Figure 4). In this study, PEG hydrogels were functionalised with carboxylic groups to mimic the glycoaminoglycans in cartilage, phosphate groups for their role in bone mineralization and tert-butyl to mimic the lipid rich environment in adipose tissue^[60]. After functionalisation, the gels were able to direct the differentiation of the stem cells down the chondrogenic, osteogenic and adipogenic pathways as hypothesised^[60].

Self assembled monolayers (SAMs) have been an effective tool for reviewing the effect of specific surface chemistries for stem cell differentiation^[93]. SAMs are highly organised substrates that are adsorbed onto a solid surface^[94]. Phillips *et al*^[95] illustrated using SAMs functionalised with four different functional groups, methyl (-CH₃), hydroxyl (-OH), carboxyl (-COOH) and amino (-NH₂), effected the adsorption of fibronectin onto the surfaces, which consequently allowed the osteogenic differentiation of hMSCs. The study illustrated that -NH₂ allowed the highest level of osteogenic differentiation in osteoconductive medium, with up regulation of Runt-related transcription factor 2 (Runx2), BSP and osteocalcin (OCN) expression and alizarin red staining^[95]. Similarly, Curran *et al*^[66] investigated methyl (-CH₃), hydroxyl (OH), carboxyl (-COOH), amino (-NH₂) and thiols (-SH) and also observed that hMSCs cultured on -NH₂ surfaces showed a higher capability for osteogenic differentiation than other cell lineages or to remain in an undifferentiated state. Valamehr *et al*^[96] have also used the SAM technique to create different surface hydrophobicities and found the increase in surface hydrophilicities could increase hESC proliferation and differentiation. With the emergence of plasma surface modification over the last ten years, which alters the surface chemistry with different chemical functional groups, controlling stem cell fate using chemical is likely to receive extensive research^[64].

DELIVERY OF GENETIC CLUES FOR STEM CELLS

The last technique is to provide genetic clues to direct

stem cell fate decisions. Traditionally the expression of specific genes has been forced in the stem cells by using viral and non-viral delivery systems. Viral transfection are highly efficient but their size limits restricts its transfection efficiency^[97]. Adenoviruses have been illustrated to have higher transfection efficiency but their poor *in vivo* responses including early failures has led to a decrease in use^[97]. Several studies have shown to use viral gene therapy to induce the differentiation of stem cells. Chuang *et al*^[98] found that BMP-2 transduced using the baculovirus into human BMMSCs successfully induced differentiation into osteoblasts after two weeks when seeded onto alginate scaffolds in nude mice. Li *et al*^[99] similarly investigated the effect of BMP-7 adenovirus on the mandibular damage in a rabbit model. The addition of BMP-7 induced early bone regeneration compared to scaffold hydroxyapatite/ polyamide (nHA/PA) only and scaffold with non-transduced BMMSCs^[99]. Adenoviruses with Runx2 and fibroblast growth factor-2 have also shown to effectively enhance the osteogenic differentiation of stem cells^[100,101]. Despite viral strategies promising effect on stem cell fate, non viral techniques have been employed to overcome the limitations of viral strategies including transposons (mobile DNA elements), plasmids (circular, self-replicable double-stranded DNA), short-interfering RNA molecules (siRNAs), cell-penetrating peptides, or bacterial artificial chromosomes (DNA constructs) to deliver a transgene^[102].

An exciting area of research is non-viral gene delivery *via* biomaterials to direct stem cell function. The gene delivery could be in the form of DNA plasmids or siRNAs. This would allow localised and sustained delivery of the genetic siRNA clues to the stem cells to direct their fate more precisely^[102-105]. Furthermore, by delivering genetic clues *via* a scaffold they are protected from biodegradation^[102-104]. Delivering the gene *via* scaffolds also allows the potential of prolonged delivery of the genetic material^[102-105].

DNA plasmids can be incorporated into the scaffold *via* encapsulation or surface mobilisation^[106]. Few studies have highlighted the delivery of DNA plasmids *via* scaffold to control stem cell fate^[104-107]. Im *et al*^[106] showed successful chondrogenic differentiation of adipose derived stem cells (ADSCs) by the plasmid DNA (pDNA) containing SOX trio (SOX-5, -6, and -9) genes into poly(lactic-co-glycolic acid) PLGA scaffolds after 21 d. Furthermore, after implanting the ADSCs seeded scaffold *in vivo* gross and histological findings confirmed cartilage regeneration^[106]. Furthermore, porous chitosan/ collagen scaffold prepared using freeze drying process and loaded with the plasmid vector encoding human bone morphogenetic protein-7 (BMP-7) gene, enabled human dental pulp stem cells to undergo odontoblastic differentiation confirmed by PCR studies^[107].

An emerging tool for controlling stem cell fate and biology is the use of RNA interference (RNAi) by which delicate control of gene expression is created^[102-105]. The focus of RNAi has already been utilised extensively in treating genetic disease or cancerous cells by combining it with chemical conjugates^[102-105]. However, recently there has been a shift in focus of using the RNAi to stem

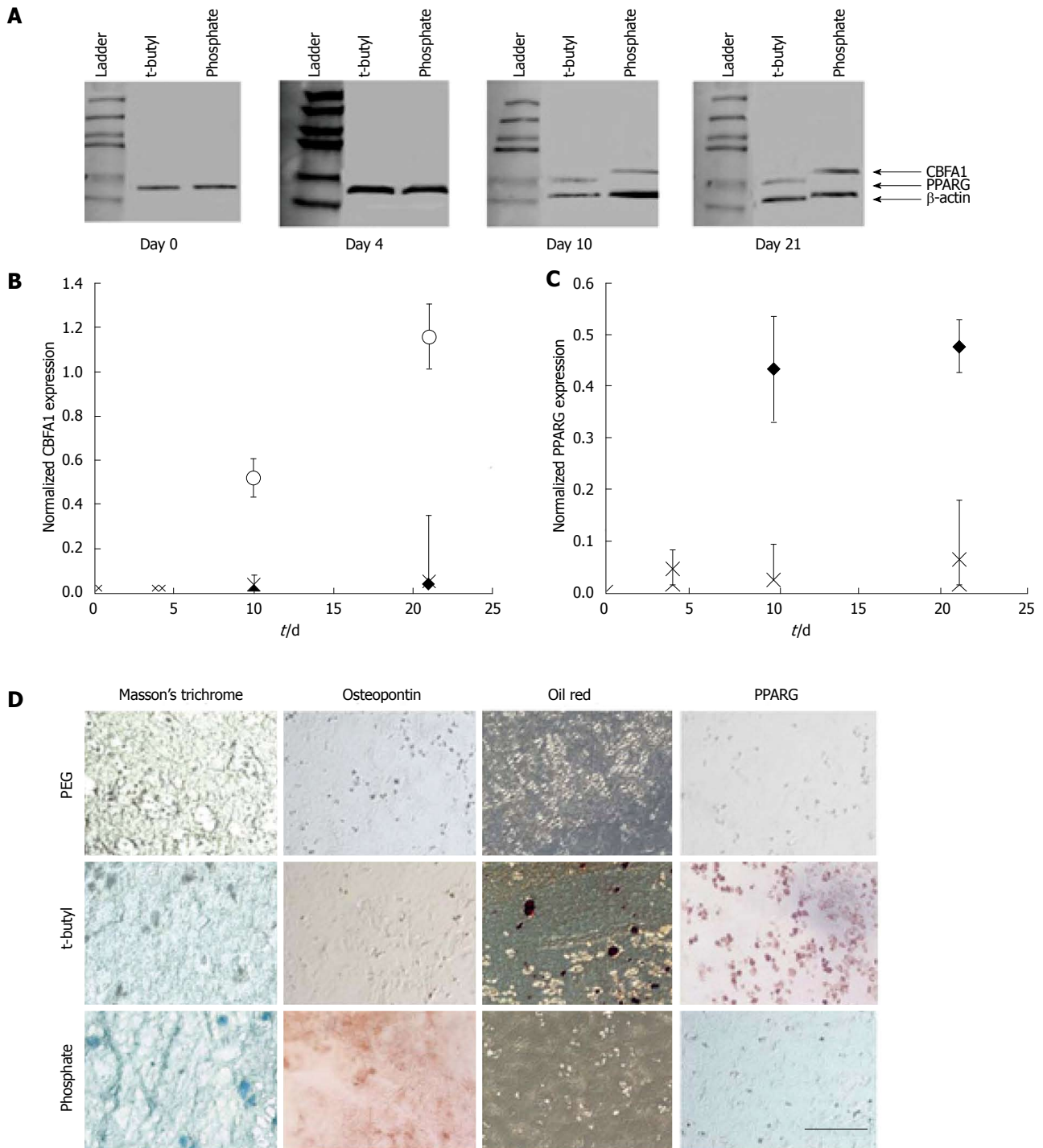


Figure 4 Effect of surface chemical functional groups on the differentiation of stem cells. Encapsulation of human mesenchymal stem cells (hMSCs) in phosphate and t-butyl functionalised polyethylene glycol hydrogels affected the CBFA1 and PPARG expression. A: CBFA1, PPARG, and β -actin expression of hMSCs encapsulated in control, t-butyl, and phosphate-functionalised PEG hydrogels was performed. Immunoblots were quantified with ImageJ software and CBFA1 (B) and PPARG (C) expression levels over the 21-d culture period were normalised to β -actin expression; D: Differentiation was confirmed using Masson's trichrome stains collagen blue (left column) and Oil Red stains intracellular lipid deposits red (third column). (bar = 100 μ m). Taken with permission from Benoit DS, Schwartz MP, Durney AR, Anseth KS. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. *Nat Mater* 2008; 7: 816-823.

cell differentiation and regenerative medicine. In 2003, Hribal *et al*^[108] was the first to induce the pluripotent P19 teratocarcinoma cells by using small interfering RNAs (siRNA) targeting the pan-Foxo genes, without the induction agent dimethyl sulfoxide (DMSO). RNAi works by silencing any gene of interested by eradication of

the target mRNAs by the introduction of siRNAs^[102-105]. Several areas of research have endeavoured to use siRNA to induce specific differentiation of stem cells. Studies examining the effect of siRNA have shown to influence stem cell fate in the presence and in the absence of chemical induction medium^[109-112]. One of the main

limitations of using siRNA in controlling stem cell fate is the low transfection efficacy of stem cells. It has been demonstrated that stem cells are not very sensitive to the common transfection agents, which cause transfection using “proton-sponge” effect, which causes disruptions the nucleic acid complexes in the endosomes^[109]. Some specific transfection reagents are being designed for stem cell transfection including Oligofectamine™ and siPORT™, which may begin to increase the use of siRNA for controlling stem cell fate^[109-111]. Alternatively nanoparticle-based delivery of siRNA is already well developed for therapeutic purposes in tumour cells but have yet to be tested extensively on stem cells. Nanoparticle based delivery hold promise for stem cell transfection due to their less disruption on genetic modification compared to viral mediated transduction^[112]. Few studies are now emerging to investigate this area of research^[112]. Poly-ethylenimine (PEI) modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles were used to deliver SOX5, SOX6, and SOX9 into human MSCs to enhance chondrogenesis. The delivery of the genes was capable of inducing chondrogenic differentiation of stem cells^[112].

An exciting area of research is scaffold based siRNA transfer. Over the last few years, few studies have now utilised siRNA to guide stem cell fate^[113]. PCL scaffolds functionalised with TransIT-TKO/siRNA nanoparticles enabled the successful gene silencing of tribbles homolog 2 and BCL2L2 causing an increase in expression of adipogenic and osteogenic differentiation respectively^[113]. The group further implanted the scaffold *in vivo* and demonstrated the capability of stem cells to commit to different differentiation pathways in specific locations within the same implant, by placing different siRNAs in distinct locations^[113]. Nguyen *et al.*^[114] recently highlighted that siRNA delivered *via* a hydrogel was able to ensure the osteogenic differentiation of stem cells. Low *et al.*^[115] showed that RE-1-silencing transcription factor (REST) siRNAs could successfully enhance the neuronal commitment of mouse neural stem cells. As there are many benefits of using scaffold for gene delivery to control stem cells, it is likely that extensive research will be carried out to exploit the RNA-functionalised scaffolds in the future. Furthermore, the added benefit of priming the scaffold with topographical and chemical inductors as discussed earlier means gene delivery *via* scaffolds shows exciting advantages, which deserves further research interest.

FUTURE PERSPECTIVES

Nanotopographical clues have shown to provide important clues for stem cells and surface patterning has presented to be an important tool to control specific stem cell responses. Using nanotopographies of biomaterials to guide stem cell fate holds great promise, as surface modifications are not affected by degradation over time unlike chemical medium components. Furthermore, nanotopographies could be varied on a single biomaterial to induce different stem cell responses by inducing

gradients on the surfaces, creating further scope for therapeutic applications. One of the main challenges in using nanotopographical clues in the medical field is the application of the required tightly controlled nanofeatures for large scale production that have been shown to control stem cell fate. Newer technologies such as colloidal lithography could provide the solutions for the large-scale production but further work is required.

The immobilisation of growth factors and chemical groups to biomaterials has proved to be successful in guiding stem cell differentiation. However, despite growth factors immobilisation proving to be better than the delivery of soluble factors in the stem cell's culture medium to control stem cell fate, there are difficulties that must be overcome for successful clinical translation. A greater control of the bioactivity, orientation and spacing, of the growth factor must be found to yield the desired control of stem cell differentiation. Another important question is the response of the stem cells to the growth factors and functional groups in the long term due to the continuous activation of the cells. Greater control of the inductive chemical clues needs to be found to ensure the desired response is specific for the intended purpose. Similarly, the response of the other cells *in vivo* to the chemical clues provided may cause unwanted effects, which warrants further investigation.

Despite the emerging studies of gene delivery *via* scaffolds to stem cells to control stem cell fate currently it is very difficult to translate these results to human clinical practice. Further work, is required to find optimal transfection and safe gene transfer to fully appreciate the impact of molecular control of stem cells for tissue engineering and regenerative medicine. Extensive safety reports will need to be undertaken to fully appreciate the effectiveness of gene therapy for future regenerative medical applications.

Many studies have investigated the effect of different environmental parameters on stem cell behaviour; however few studies have taken into account more than one environmental clue to control stem cell fate. In the future, as technology advances allow microarrays to test the effect of more than one environmental parameter on stem cell behaviour, greater knowledge will be obtained. For biomaterial design it will be important to understand the biophysical and biochemical properties that are optimal to guide stem cell response.

CONCLUSION

There is accumulating evidence to demonstrate that stem cell fate could be regulated by surface topography and chemical and molecular clues delivered using biomaterials. It is clear that this field of research is still emerging but there is great promise that stem cell fate could be controlled by creating advanced biomaterials, which are responsive to their environment for intended applications.

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Current progress in use of adipose derived stem cells in peripheral nerve regeneration

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have been used with varying successes to augment the natural regenerative processes which occur following nerve injury. Stem cell therapy in peripheral nerve injury may be an important future intervention to improve the best attainable clinical results. In particular adipose derived stem cells (ADSCs) are multipotent mesenchymal stem cells similar to bone marrow derived stem cells, which are thought to have neurotrophic properties and the ability to differentiate into multiple lineages. They are ubiquitous within adipose tissue; they can form many structures resembling the mature adult peripheral nervous system. Following early *in vitro* work; multiple small and large animal *in vivo* models have been used in conjunction with conduits, autografts and allografts to successfully bridge the peripheral nerve gap. Some of the ADSC related neuroprotective and regenerative properties have been elucidated however much work remains before a model can be used successfully in human peripheral nerve injury (PNI). This review aims to provide a detailed overview of progress made in the use of ADSC in PNI, with discussion on the role of a tissue engineered approach for PNI repair.

Key words: Peripheral nerve injury; Adipose derived stem cells; Cell based therapies; Stem cells

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Core tip: Adipose derived stem cell differentiation is an area of important active research at present. Since adipose tissue is ubiquitous throughout the body it is an ideal source of cells for regeneration of damaged body parts. In the peripheral nervous system there are currently significant limitations in the methods of treatment and subsequent rehabilitation. Adipose stem cells can express proteins which are similar to schwann cells and are termed schwann like cells. In this review we provide an update on the current methods used in peripheral nerve reconstruction using adipose stem cells.

Abstract

Unlike central nervous system neurons; those in the peripheral nervous system have the potential for full regeneration after injury. Following injury, recovery is controlled by schwann cells which replicate and modulate the subsequent immune response. The level of nerve recovery is strongly linked to the severity of the initial injury despite the significant advancements in imaging and surgical techniques. Multiple experimental models

Zack-Williams SDL, Butler PE, Kalaskar DM. Current progress in use of adipose derived stem cells in peripheral nerve regeneration. *World J Stem Cells* 2015; 7(1): 51-64 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i1/51.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i1.51>

INTRODUCTION

Background

The peripheral nervous system (PNS) consists of 3 principle cell types: axonal, glial and supporting stromal tissue^[1] arranged in long efferent (motor) and afferent (sensory) fibres relaying information to the CNS. Peripheral nerve injury (PNI) is common occurring in 2.8%-5% of poly trauma patients^[2,3] and may lead to long term functional deficiencies.

Traction related injuries are the most common pathophysiology seen during PNI^[4]. The natural elasticity of the endoneurium and perineurium of the nerve permits stretching to occur within the planes of the collagen fibres. If a sufficient traction force is applied to the nerve a route avulsion may lead to disruption of anatomy and complete loss of function. The perineurium has much greater elastic strength than the endoneurium^[4]. Therefore, elasticity is maintained within the nerve undergoing a stretching injury until the perineurium is devitalised. Peripheral nerve lacerations related to by penetrating trauma from knives and glass account for up to 7% of all brachial plexus lesions^[5]. These injuries are usually partial injuries where a segment of the injured nerve remains in continuity. When a clean laceration occurs this is usually amenable to a primary repair^[6]. Another common cause of peripheral nerve injuries is direct compression onto the nerve^[4]. This pressure on the nerve results in both mechanical compression and ischaemia. Initial microvascular damage to the nerve is usually reversible if the injurious mechanism is discontinued within 8 h of initial injury. Early investigation of these nerves showed most of the early damage was occurring in the periphery of the nerve as opposed to the central segment were the greatest ischaemia occurred^[4]. When these compressed tissues were viewed under electron microscopy the typical morphological features seen included: dislocations of the nodes of ranvier, as well as paranodal penetration and and subsequent invagination^[7]. Figure 1 shows the common pathophysiological response to the damaging compression forces associated with nerve crush injuries.

Other less common causes of PNI can be attributed to thermal, electrocution, radiation, percussion and vibration injuries^[3,8]. Irrespective of the cause of injury "the functional outcome of PNI depends on the severity of the injury"^[9].

Classification of PNI

The severity of PNI was initially classified by Seddon and subsequently reclassified by Sunderland^[10,11] as shown in Figure 2. Neurapraxia is the least severe form of nerve injury in the Seddon classification equating to grade I in

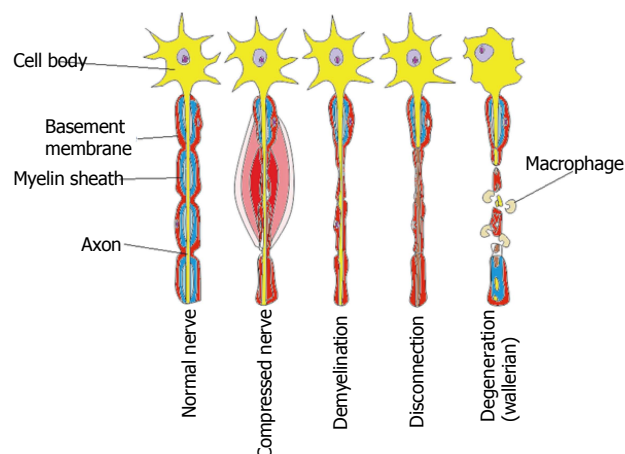


Figure 1 Changes occurring in a peripheral nerve following compression injuries adopted and modified from Ref^[9].

Sunderland. Although the overall anatomy of the nerve remains the same, areas of segmental demyelination lead to reductions in the frequency of transmitted electrical signals^[12]. Recovery from the primary injury is highly variable but most motor neuron lesions should resolve within 3 mo^[13]. Axonotmesis and neurotmesis lie along a continuum and again are subcategorised by Sunderland. Sunderland grade II is equivalent to axonotmesis with an intact endoneurium, grade III occurs where the endoneurium is compromised but the perineurium remains and grade IV is where only the epineurium remains as demonstrated in Table 1. Sunderland grade V or neurotmesis is associated with a complete transection of the peripheral nerve. Neurotmesis is an indication for surgical exploration to perform: debridement to healthy tissue, tensionless nerve approximation, accurate fascicle alignment and atraumatic mechanical coaptation of the nerve ends^[6]. The simplest nerve repair involves the approximation of the outermost layer of nerve tissue the epineurium. When 90% of the axonal tissue is lost, the remaining tissue heals by regeneration which is highly dependent upon the schwann cell (SC)^[12].

Wallerian degeneration

Wallerian degeneration is a process that results following nerve injury, where the axon separated from the neuron's cell body degenerates distal to the injury. Wallerian degeneration occurs in a proximo-distal direction in the distal nerve stump associated with loss of integrity of the granular structures of the axonal cytoskeleton^[14]. The cytoskeletal changes occurring were described in terms of a "cell body reaction"^[15]. Multiple cellular changes occur within the injured nerve tissue including chromatolytic changes in neuronal cell body, eccentric positioning and change in nerve function to a nerve capable of growth and sprouting^[9]. There is an influx of extracellular ions which cause macrophage influx and eventually apoptosis of the axon^[1]. During the wallerian degeneration process SCs replicate whilst altering their morphology to produce mRNA to produce substances such as p75, nerve

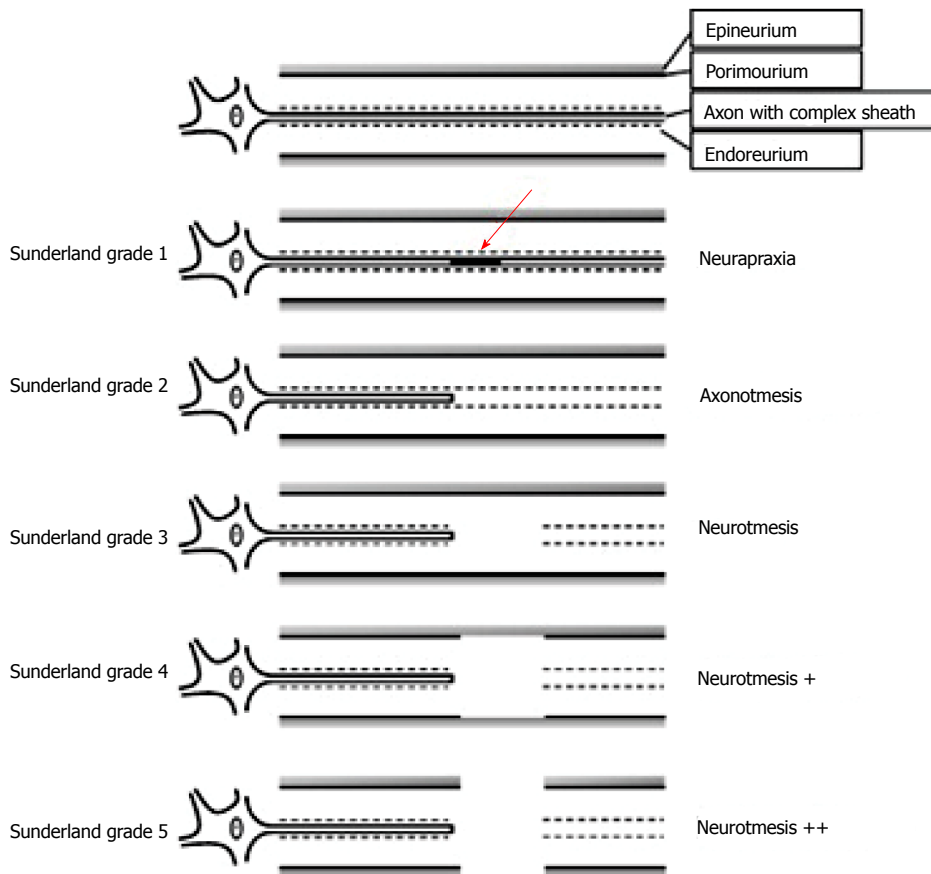


Figure 2 Sunderland classification of nerve injury adapted from Deumens *et al*^[9], 2010.

growth factor and monocyte chemoattractant protein-1^[16]. Reciprocal changes occur within the proximal nerve stump as SCs try to bridge the gap between the two nerve endings. Distal to proximal degeneration occurs within the proximal nerve ending. The degree of nerve tissue loss is dependent on the proximity of the nerve to the cell body in the spinal cord^[1]. It has been shown that the survival rate of motor neurones close to the cell body following spinal nerve avulsion in the context of brachial plexus injuries is 10%-20%^[17]. Lesions distal to the cell body cause axon death to occur up to the nearest node of ranvier in comparison to proximal lesions which may lead to apoptosis of the whole axon.

ADIPOSE DERIVED STEM CELL AND PERIPHERAL NERVE REPAIR

A key part of current peripheral nerve reconstruction involves the harbouring of SCs from autologous nerves. However, this technique is limited by the associated morbidity of sacrificing viable neurological tissue as well as the slow growth of SCs *in vitro*^[18]. Adipose derived stem cells (ADSC) still remain a therapeutic option since they are multipotent and thus have an inherent ability to differentiate into mature adipocytes, chondrocytes, osteoblasts and muscle cells (Figure 3). Similar to the neural stem cell they are able to differentiate into specialised neurospheres under

the action of growth factors^[19-21]. This is combined with the benefit of being “easily obtained, rapidly expanded, showing low immunogenicity and can be differentiated into SCs *in vitro*”^[22], thus making them potentially ideal for future peripheral nerve regenerative processes. The exact mechanism of how ADSCs interact with the regenerating nervous system is not known and theories are discussed in this review.

ADSC characterisation and harvesting

ADSC reside around pericytes structures within the mesodermally derived adipose tissue and are active during the first 50 d of the porcine embryo development and the 2nd trimester in humans^[23]. They are multipotent cells, which are able to differentiate into multiple tissues of mesodermal origin including bone or skeletal muscle endodermal and ectodermal tissues such as neurone like tissue^[20,24]. The ADSC express similar proteins and differentiating capabilities as cord and bone marrow mesenchymal stem cells (BMMSCs)^[25,26]. They are the best characterised multipotent stem cell population^[20] and form the basis of many of the early studies investigating the role of mesenchymal stem cells in nerve regeneration^[27,28]. However, the ADSC have the advantages of minimally invasive harvesting and high cellular yield of between $0.25-0.375 \times 10^6$ cells per millilitre of liquid fat^[29]. Since the early work of Rodbell *et al*^[30] on adipocyte extraction; type I collagenase extraction has been a popular

Table 1 Summary of seddon and sunderland classifications following peripheral nerve injury adapted from^[98]

Sunderland	Seddon	Injury	Neurosensory impairment	Recovery potential
I	Neuropraxia	Intrafascicular oedema, conduction block	Neuritis, paraesthesia	Ful (1-7 d)
		Segmental demyelination	Neuritis, paraesthesia	Full (1-2 mo)
II	Axonotmesis	Axons severed, endoneurial tube intact	Paraesthesia, episodic dysesthesia	Full (2-4 mo)
III		Endoneurial tube torn	Paraesthesia	Slow, Incomplete (12 mo)
IV		Only epineurium intact	Hypoaesthesia, dysaesthesia neuroma	Neuroma
V	Neurotmesis	Loss of continuity	Anaesthesia, extreme pain and neuroma formation	None
VI		As per V	As per V	Nil

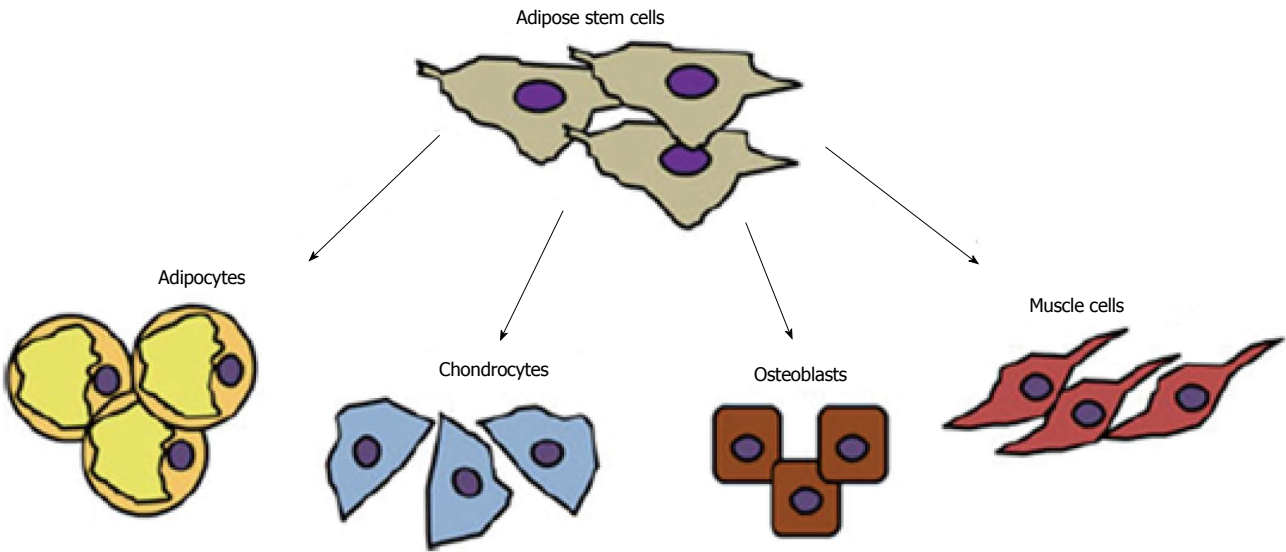


Figure 3 Differentiation of adipose derived stem cells into adipocytes, chondrocytes, osteoblasts and muscle cells adapted from Minteer *et al*^[97].

method of adipocyte isolation. Following the discovery of ADSCs by Zuk's team in 2001^[31], multiple techniques of ADSC isolation have been described^[32]. The collagenase ADSC technique of isolation involves the gradual separation of the adipose tissue layers followed by numerous washes with PBS removing the contaminating RBCs, and incubation with type I A collagenase solution^[33]. The infranatant is removed, followed by centrifugation to form the stromal vascular fraction (SVF) pellet. The SVF is acquired which contains the ADSCs which will be used for cell culturing and experimentation. The final processing of the SVF before culture is the repeated washing with PBS to remove the final adherent RBCs and adipocytes. Alternative, more economical methods of ADSC isolation including the use of trypsin and red blood cell lysis buffer solution have been described as an alternative to type I collagenase usage. Despite the relative expense and large number of steps required of the collagenase isolation method, it has remained the most popular method of ADSC isolation and results in up to 5 × more ADSCs available for experimental use^[19,31-33].

The vast majority of studies in the literature for ADSC culture, involve the use of fetal bovine serum (FBS) as a standard ADSC culture condition^[34]. FBS has multiple functions in tissue culture including a source of: growth factors, protein transport, fatty acid lipids as well as protease inhibitors^[35]. Due to the approximately 1000 different

chemicals within FBS, a serum free alternative has been difficult to manufacture with similar cellular efficacy to FBS. Serum free alternatives to FBS are limited in their availability and usefulness in the culturing of these and other types of MSCs^[35]. Xenogenic and serum free growth factor media better comply with the good manufacturing practice (GMP) which is essential for any cell therapies which are to be considered in trials of animals or humans. Xenogenic and serum free alternatives have shown much promise thus far and the treated mesenchymal stem cells (MSCs) retain similar morphology, genetic expression, differentiation and proliferative capacities as those treated with xenogenic serum culture media^[34,36]. Xenogenic serum free alternatives include human serum in the form of allogenic and autologous serum, allogeneic umbilical cord blood serum as well as human platelet derivatives^[35].

Neurological differentiation of the ADSC

Early studies by Woodbury and colleagues provided evidence for the ability of MSCs to transdifferentiate into a neural phenotype^[28]. They used human and rat BMMSCs under the stimulation of 1 mmol/L β-mercaptoethanol (BME). Following stimulation, these cells expressed neuron specific enolase (NSE); they were morphologically transformed so that they developed contracted cell bodies and terminal bulbs appearing similar to mature neurons.

Table 2 Table of various neuron markers their distributions and function adapted from^[99]

Marker	Distribution	Protein function
GFAP	Mature cells of glial origin	Specifically found in astroglial cells
MAP 2	Mature marker of neurones	Thought to be involved in microtubule assembly during neurogenesis
Nestin	Developing nervous system, myogenic, endothelial and hepatic cells	Intermediate filament protein expressed in multiple tissues
NF-70	Most PNS neurones	Structural protein supporting large axons and synapses
NSE	Early neuronal marker	Three variants: α , β , γ with only the γ isoenzyme expressed in neuronal tissue
S100	Cytoplasm of SCs, astrocytes and astrogliosis	Non-specific cell marker involved in multiple cell processes including cell cycle progression and differentiation
Synaptophysin	Brain synaptic vesicles	Directs vesicle associated membrane protein-2 to intracellular compartments
TAU	Axons	β Neuronal microtubule associated protein stabilising microtubules and link to signalling pathways to cytoskeleton
β III-tubulin	Central and peripheral nervous system axons	β tubulins heterodimerize to form microtubules involved in axon guidance

GFAP: Glial fibrillary acid protein; MAP2: Microtubule associated protein-2; NSE: Neuron specific enolase; PNS: Peripheral nervous system; SCs: Schwann cells.

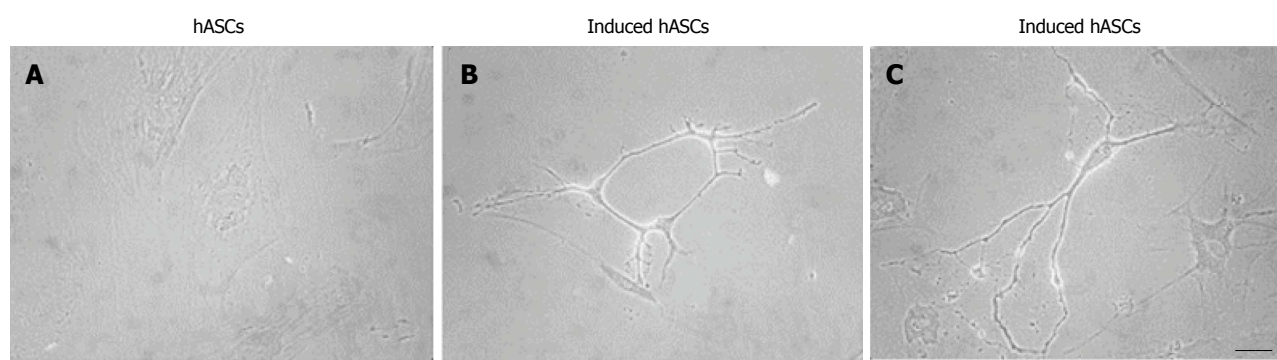


Figure 4 Morphological differences in stimulated vs unstimulated adipose derived stem cell. A: Unstimulated adipose derived stem cells (ADSCs) grown under normal conditions with fibroblast like morphology; B and C: Induced ADSC with a neuronal like phenotype reproduced from Cardozo *et al*^[40] 2012. hASCs: Human adipose-derived stem cells.

Since ADSC share many characteristics with BMMSCs and have easier harvesting, many groups pursued ADSCs for the same reason^[37,38]. Other groups followed on from this early work using a similar neurogenesis protocol to Woodbury, whilst Ashijan *et al* substituted BME for insulin, indomethacin and isobutylmethylxanthine (IBMX)^[37,38]. Despite the modifications of previous work, their differentiated cells lacked the mature markers of neural development and were not tested for their ability to respond following electrophysiological stimulation^[26]. Further concern was raised by other early reviewers Safford's team addressed both of these inherent problems in their modified protocols of Zuk and Woodbury^[39]. They used the combinations of butylated hydroxyanisole/KCL/valproic acid/forskolin/hydrocortisone/insulin. They also successfully demonstrated the expression of mature SC markers such as: glial fibrillary acid protein (GFAP), microtubule associated protein-2 (MAP2), β -III tubulin; the presence of voltage-gated calcium channels and the ability to upregulate the glutamate receptor. A summary of neural markers and tissue they originate from is shown in Table 2.

Following the varying successes of early models to induce MSCs to a neural lineage some authors believe both genetic and immunocytochemical staining are necessary to confirm this transdifferentiation^[26]. Cardozo

et al^[40] recently showed the cell morphological, genetic and immunocytochemical changes associated with the previous methods of chemical differentiation of ADSCs. A comparison between differentiated and undifferentiated ADSCs over a 14 d period was performed by real time polymerase chain reaction (RT-PCR) showed a reduction in the levels of GFAP and nestin in the differentiated cells whilst there was an increase in β -III-tubulin, NSE, TAU, MAP2 synaptophysin^[40]. Figure 4 shows the classic morphological changes seen in ADSCs undergoing chemical differentiation under the action of BME.

Alternative methods of inducing chemical differentiation to a neural lineage involves the addition of growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) along with BME to ADSCs which again results in the changes in cellular morphology and expression of SC markers such as S100, GFAP, p75 and β -III tubulin^[41,42]. Using these protocols the differentiated ADSCs promoted significantly greater neurite outgrowth from the NG108-15 motor neurones than uncultured ADSCs suggesting a local stimulatory effect between the stem cell and the neuron. Further modifications of techniques have shown the potential of IBMX alone to differentiate ADSC into cells expressing ectodermal markers such as neurofilament 70 and cyclic nucleotide phosphodiesterase

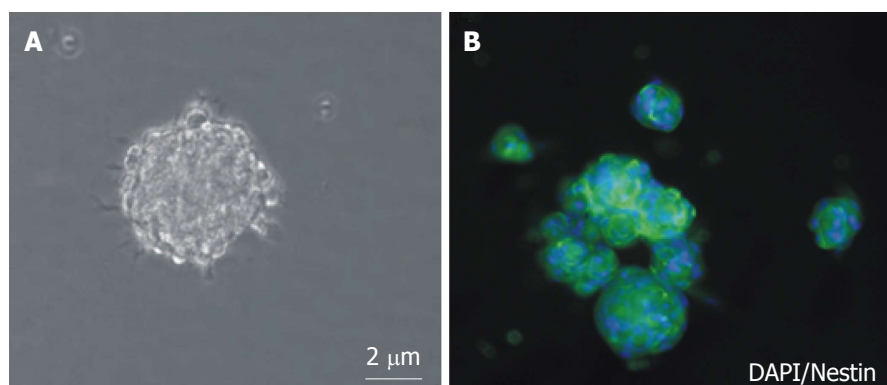


Figure 5 Appearance of neurospheres (A) Phase contrast image of free floating neurospheres; B: Fluorescence staining of nestin (Green) and nuclear stain (Blue) of neurosphere adapted from Radtke *et al.*^[43], 2009.

Table 3 Comparison the different methods of chemical differentiation of adipose derived stem cells

Nerve differentiation chemicals	Histochemical markers positive	Ref.
B27/bFGF/EGF	MAP2, β -III tubulin, S-100, GFAP, Nestin,	[19,21,42-45,47]
IBMX/dexamethasone/insulin/indomethacin	S100, GFAP	[94]
β -mercaptoethanol/butylated hydroxyanisole/retinoic acid/EGF/bFGF	GFAP, Nestin, β -III tubulin, NSE, TAU, MAP2 and Synaptophysin	[40]
β -mercaptoethanol/retinoic acid/PDGF/bFGF/forskolin/GGF-2	GFAP, S100	[41,58,59,95]

GFAP: Glial fibrillary acid protein; MAP2: Microtubule associated protein-2; bFGF: Basic fibroblast growth factor; NSE: Neuron specific enolase; EGF: Epidermal growth factor; IBMX: Isobutylmethylxanthine; PDGF: Platelet derived growth factor; GGF-2 glial growth factor-2.

(CNPase); which are associated with oligodendrocytes from the CNS as well as SCs.

Alternatively, similar immunocytochemical markers are seen when ADSCs are cultured exclusively with bFGF and epidermal growth factor (EGF) to form neurospheres. Figure 5 demonstrates the morphological appearances of neurospheres staining positive for nestin as well as the non-specific nuclear stains. Neurospheres were originally found to develop from neural stem cells. However, these confluent groups of differentiated cells can be obtained from ADSCs and express the SC markers such as S100, P75 and GFAP^[43]. A recent study compared the differentiation protocols of BME *vs* bFGF and EGF together^[28,44,45]. The neurospheres produced from the latter protocol had higher cell viability and improved expression of neural markers when tested at weeks 1 and 3^[45]. The higher cell viability of ADSCs in the absence of BME suggests an increased toxicity in the cells exposed to BME. This observation has been suggested in the literature previously using BMSCs^[27] and similar findings are reported by authors using dimethyl sulfoxide (DMSO) as the principle differentiation chemical^[46]. Razavi *et al.*^[47] 2013 demonstrated greater neuron generation relative to glial cells when ADSCs were co-cultured with neurotrophic factor secreting ADSCs compared to control neurogenic and conditioned media. They showed the expression of MAP2 to be two times greater than GFAP in the control group. Neurotrophic ADSC containing media contained much higher levels of both brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF)^[48].

Neuronal markers of differentiation

As shown in Table 3, there are a variety of markers which are expressed during the various neurological differentiation

protocols of ADSCs within the literature. The molecular code model of ADSC differentiation has been mentioned elsewhere in the literature thus far^[40]. In this model each marker is representative of neural tissues at different stages of neural differentiation and maturity. Each different neurological differentiation protocol results in the expression of a range of different cell surface proteins or markers. The markers expressed are dependent on various factors including cell passage, culturing media, as well as the differing types of induction media^[49]. When the process of neurogenesis is compared *in vitro* ADSC differentiation model, the gradual changes in cell surface markers have been documented previous studies depending on the maturity of the tissue and are found to be time dependent^[40,50].

THERAPEUTIC STRATEGIES AND EXPERIMENTAL MODELS FOR PNI REPAIR USING NERVE CONDUITS AND ADSCS

As discussed earlier, multiple *in vitro* studies have shown the transformation in the morphology of ADSCs under a range of stimuli to form tissues similar to neural tissue. Multiple animal studies have been described using ADSC to treat a range of canine and murine specific neurological conditions^[51,52]. To bridge the gap in the damaged peripheral nerves some authors used conduits combined with ADSCs whilst others used ADSC therapy with nerve allografts. Since axons only grow a short distance beyond their own reparative matrix and an intact endoneurium is associated with better outcomes, there has been a strong research focus on bridging the gap *via* conduits as well as

reconstruction of the extracellular matrix^[1,53].

Nerve conduits

Nerve conduits are used in peripheral nerve gaps of up to 3 cm in length^[54]. Natural conduits include the autogenous vein nerve conduits (AVNCs), allografts and autogenous nerve harvesting^[14]. A variety of synthetic conduits are also available for nerve repair, including Polyglycolic Acid (PGA), Poly-carprolactone (PCL), Collagen, Polyvinyl alcohol which have passed FDA testing and thus permitted for use in humans^[55].

The rat sciatic nerve model remains a classic clinical model for investigating the effects of cells on peripheral nerve regeneration. PCL conduits have been tested over a 6mm gap model to compare the nerve regenerative effects of autografts as well as PCL conduits containing ADSCs with and without nerve guides and a negative control of an unrepaired nerve^[56]. At 3 wk the cells containing the guides and ADSCs were associated with a significant improvement in the sciatic nerve index (SNI) compared to other groups ($P = 0.010$). However, this effect only lasted for 5-6 wk before the sciatic nerve function became similar to the other groups. The re-harvested sciatic nerves which were observed after cellular implantation of ADSCs were also thicker than those with the conduit alone. This suggests a possible interaction between the implanted ADSC and the conduit material, thus aiding the regeneration of the peripheral nerve. Similar results were found by other authors who used sciatic nerve defects of 13 mm^[57]. In their study there were several experimental groups, which included rats receiving allografts from similar breeds as a positive control. The other experimental groups included rats receiving silastic conduits containing either differentiated or undifferentiated ADSCs; these both of these groups also either had or didn't have exchange of ADSC media on days 14 and 28. The groups of rats, which received allografts as well as those receiving the silastic conduit containing differentiated ADSCs and media exchange, had the highest Sciatic Functional Index (SFI) when measured at 3 and 4 mo. In this paper the authors also reviewed another motor function and found that the extensor postural thrust was highest in the allograft group as well as the differentiation and undifferentiated ADSCs within silastic conduits with media exchange. The findings from this study suggest a role for ADSC in peripheral nerve recovery and also stress the importance of maintaining the stem cell *via* supplementation with fresh nutrients. Despite the multifaceted improvements in motor function, the presence of ADSCs leads to a slower improvement than compared to sensory function. Similar to a previously mentioned^[56] study, the ADSCs did not differentiate into SCs within the injury model.

When fibrin conduits have been used for 10 mm rat sciatic nerve models; it was associated with decreased growth associated protein (GAP-43) expression in the dorsal root ganglion relative to the spinal cord and could account for the difference in sensory and motor nerve

regeneration seen in previous studies^[58]. These findings may suggest ADSCs are better at regenerating motor neurones specifically^[56]. Fibrin conduits have also been used in rat 10 mm sciatic nerve model and compared to SCs alone, ADSCs and BMMSCs^[42] (Figure 6). SCs were able to regenerate over significantly greater distances than ADSCs and BMMSCs. However, there was no statistical significant difference between the ADSC and BMMSC. Poly-3-hydroxybutyrate conduits have also been used within a fibrin matrix conjugated with PDGF, bFGF and glial growth factor-2 for 10 mm sciatic nerve gap models^[59]. The variables in this study included ADSCs with and without growth factors. Axonal regeneration was significantly improved in all of the models which contained ADSCs with P values < 0.05 . The addition of these growth factors didn't result in a significant improvement in nerve regeneration. In this study ADSCs survived for a short period, which may be explained by immune consumption or an inability to form a niche in the requisite environment^[18].

Alternatively, microporous conduits over a 15 mm nerve gap have been used to compare the effects of ADSCs, ADSCs co-cultured with SCs, SCs alone or dental pulp stem cell co-cultured with SCs^[60]. ADSC and SC co-culture was associated with better motor recovery and nerve conduction velocity. ADSC and SC co-culture resulted in 50% increase of NGF release which is thought to be an important factor in SC development^[61]. Table 4 provides a summary of the animal studies using ADSCs in peripheral nerve recovery.

Cell based therapy and xenogeneic acellular nerve matrices

Adipose cell transplantation without the use of a conduit is an option for reconstructing peripheral nerves. The cells may be administered systemically *via* intravenous, subcuticular or intramuscular routes as used for traditional drug therapy. When ADSCs have been introduced intravenously, they spread throughout the body and locate to damage tissue. In the context of traumatic brain injury, these cells spread *via* the reticuloendothelial system directly into the diseased brain tissue^[62]. In this study, the observed benefits of human ADSC injection were largely dependent on the recipient rat's age. In the older rats, fewer cells transited through the spleen. This subsequently led to differences in cell distribution within injured parts of the rat's brains. In other studies when ADSCs were administered *via* the intravenous route they were also shown to improve neuropathic pain in rats which had the ill effects of chronic pain simulated^[63].

In other studies when ADSCs were administered *via* the subcutaneous route, the cells were found to stay within the area of infiltration and did not translocate to distal sites such as the spleen or brain^[64]. For up to 1 year the transplanted cells were found to slowly migrate from the upper to lower subcutaneous adipose layers. Many of the cells were found to differentiate into adipocytes following injection. Therefore, this route of administration is unlikely to be the adequate to allow a sufficient yield of stem cells to engraft within the

Table 4 Animal studies modelling adipose derived stem cells and conduits for peripheral regeneration

Animal models	Details	Measured outcomes	Ref.
Rat	Polycaprolactone conduit for 6 mm nerve gap	SNI, nerve thickness	[56]
Rat	Fibrin conduit 10 mm nerve gap	Axon regeneration	[42]
Rat	Polyhydroxybutyrate conduit 10 mm nerve gap	Axon regeneration	[59]
Mouse	Nerve crush model and local administration ADSC ± matrigel	Axon regeneration	[48]
Rat	Decellularised vascular conduit v autogenous nerve and vascular conduits containing ADSC	Axon regeneration, nerve conduction velocity	[90]
Rat	Silastic conduit for 13 mm nerve gap	SNI, EPT, sensory nerve function	[57]
Rat	Silicon conduit ± collagen or ADSCs	Axon regeneration, nerve conduction velocity	[100]
Rat	Nerve crush model with systemic injection of ADSC	Axon regeneration, motor recovery	[68]
Rat	Genipin-Gelatin-Tricalcium phosphate conduit 10 mm nerve gap	SNI	[92]
Rat	ADSC injected into repaired chronic denervated nerve	Axon regeneration, PFI	[89]
Rat	Microporous conduit for 15 mm nerve gap	Motor recovery	[60]
Rat	Fibrin conduit for 10 mm nerve gap	Motor recovery, GAP-43 expression in dorsal root ganglion	[58]
Dog	Polytetrafluoroethylene tube and alginate hydrogel 17 mm nerve gap	Axon regeneration	[51]

ADSC: Adipose derived stem cell; SNI: Sciatic nerve index; GAP-43: Growth associated protein.

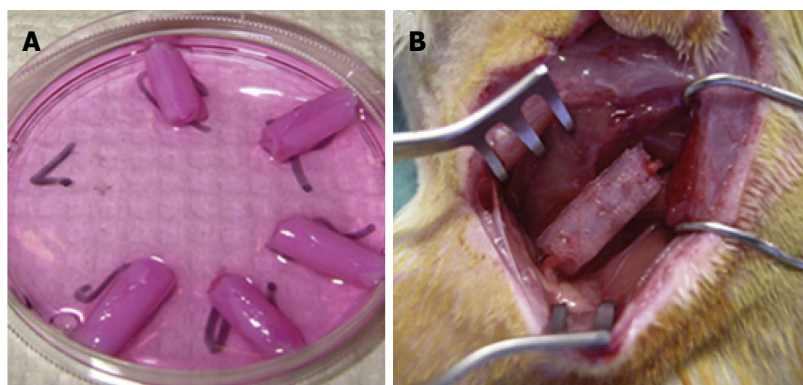


Figure 6 Fibrin nerve conduits. A: Fibrin conduits placed in DMEM prior to seeding with ADSCs; B: Fibrin conduit being inserted surgically to bridge a rat sciatic nerve injury^[42]. ADSCs: Adipose derived stem cells; DMEM: Dulbecco's modified eagle medium.

injured neural tissue.

Conduits are currently not used in humans for nerve gaps greater than 3 cm nor are they used on large nerves^[65]. Combinations of bridging acellular nerve matrices and rate ADSCs have been used in 15 mm sciatic nerve gaps in rats^[66]. Similar to conduits studies motor response and nerve conduction velocities are regularly measured. The implanted ADSCs and nerve graft were associated with improved walking track analysis, nerve conduction and increased number of myelinated fibres within the grafted tissue. When human ADSCs were injected into an acellular nerve graft as therapeutic cells, similar improvements in rat motor peripheral nerve function were obtained^[67].

In a sciatic crush nerve model, infiltration of ADSCs was associated with significant improvements in SFI score and axonal regeneration which was marked by increased GAP-43 expression in the recovering nerves^[68]. The allogenic ADSCs also reduced the immune response to the nerve injury and were able to migrate through the reticuloendothelial system to home to the zone of injury. Using ADSC therapy the authors were able to maintain the levels of glial cell line derived neurotrophic factor (GDNF) which has been proven to be important in increasing the number of myelinated axons in previous sciatic nerve injury models^[69]. The effects of transforming growth

factor beta-1 (TGFβ-1) on the survival of ADSCs injected into xenogeneic acellular nerve matrices (XANM) has been reviewed in multiple animals^[52]. In a 50 mm nerve defect, the combination of XANM-ADSC-TGFβ-1 led to significantly greater areas of axonal regeneration compared to XANM-ADSC or XANM- TGFβ-1; but the level was similar to that seen when an autograft was implanted. Significantly greater proportions of cells from the XANM-ADSC-TGFβ-1 compared to the XANM-ADSC survived over a 28 d time period (0.46 ± 0.16) *vs* (0.2 ± 0.13). TGFβ-1 lead to increased expression of genes for collagen I, collagen III and Laminin which are key structures in developing neurones^[2,70]. TGFβ-1 also led to a 7x increase in the release of vascular endothelial growth factor (VEGF) which was thought to be neuroprotective. When the ranges of growth factors secreted by ADSCs was tested, IGF-1 was found to be the most important in the prevention of caspase 3 and akt mediated apoptosis in rat cerebellar granule neurone^[71]. The theoretical interactions between ADSCs and the regenerating nerves are shown in Figure 7.

Electrical stimulation techniques

Electrical stimulation has been shown experimentally to improve neurite outgrowth particularly towards the negatively charged cathode electrode and axonal

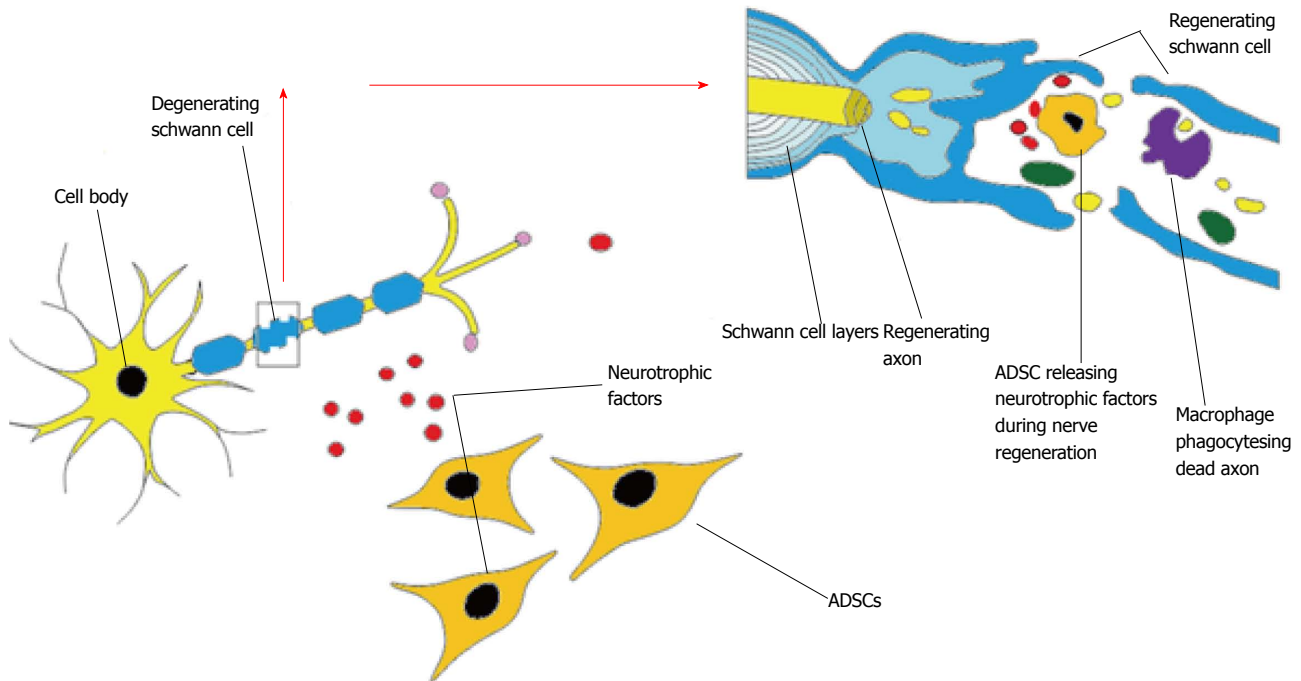


Figure 7 Potential interaction of adipose derived stem cells and the effects on peripheral nerve regeneration. ADSCs: Adipose derived stem cells.

regeneration^[72,73]. Electrical stimulation of ADSCs has been used in a number of studies for various potential clinical applications^[74-78]. BMMSC have been shown to respond in different ways to various form of electrical stimulation including capacitive coupling (CC), pulsed electromagnetic field, degenerate wave (DW) as well as the direct current (DC)^[79]. When these transformed cells were tested in assays those which had CC and DW stimulation showed greater invasive propensity to invade a collagen type I matrix *vs* DC treated cells which showed the greatest galvanotaxis effects. These differences in the behaviour of these cells could be used in an engineered approach for regenerating nerves. Adipose stem cells have also been shown to have improved galvanotaxis following DC stimulation were they preferentially migrate to the cathode electrode; assemble perpendicular to the applied electric field, disassemble gap junctions and modify the expression of multiple genes^[74,75]. The ability of adipose stem cells to respond to electrical stimulus is explained by findings of multiple ion channels found within this heterogeneous group of cells^[80]. The galvanotaxis effect has been found to be dose dependent and involves a number of cellular pathways including is mediated through MAPK, PI3K and its downstream target Akt as well as ROCK pathway and transient increases of intracellular calcium^[74]. More recently electrical stimulation of mesenchymal stem cells (MSC) has been combined with surfaces which have high conductivity to improve the cues for necessary to obtain neural differentiation^[81]. A range of different conductivities of Polyaniline (PANI) was combined with standardised electrical stimulation of MSCs. The higher conducting polymers of PANI were associated with greater expression of neural phenotypes Nestin and β -III-tubulin confirmed by RTPCR and

immunocytochemistry. These cells also underwent morphological changes to appear as neuron like cells although their functionality was not confirmed. On the surface of highest conductivity the cellular processes extended further than those surfaces which were less conductive.

DISCUSSION

ADSC based therapy remains an area of strong scientific research; they have been shown to improve the neurological recovery in murine nervous system ischaemic models^[71,82]. ADSCs can differentiate into a range of supporting structures such as Schwann like cells which carry many of the markers of mature PNS cells using chemical or neurosphere differentiation^[37,38,83]. These cells also have a morphological phenotype similar to mature SCs. Scepticism exists within the literature as to the functionality and safety of these differentiated cells with many believing the *in vitro* morphological and cytological changes to be secondary to the toxicity of the chemical differentiating protocols^[27]. The regeneration of diseased tissue occurs through the 3 processes of cell engraftment and differentiation, release of neurotrophic factors and immunosuppression^[84]. The cell engraftment is necessary for the ADSCs to form a cell niche, whilst differentiation is required for these cells to transdifferentiate from mesodermal to ectodermal lineages. Cell engraftment occurs following administration of these cells into a host environment. The ADSCs release a range of neurotrophic factors such as EGF, TGF β -1, VEGF, bFGF, HGF, IGF-I, and BDNF secreted at different phases of tissue regeneration which is also thought to aid the healing of the damaged peripheral nerve^[48,84-86]. In particular TGF β -1/3

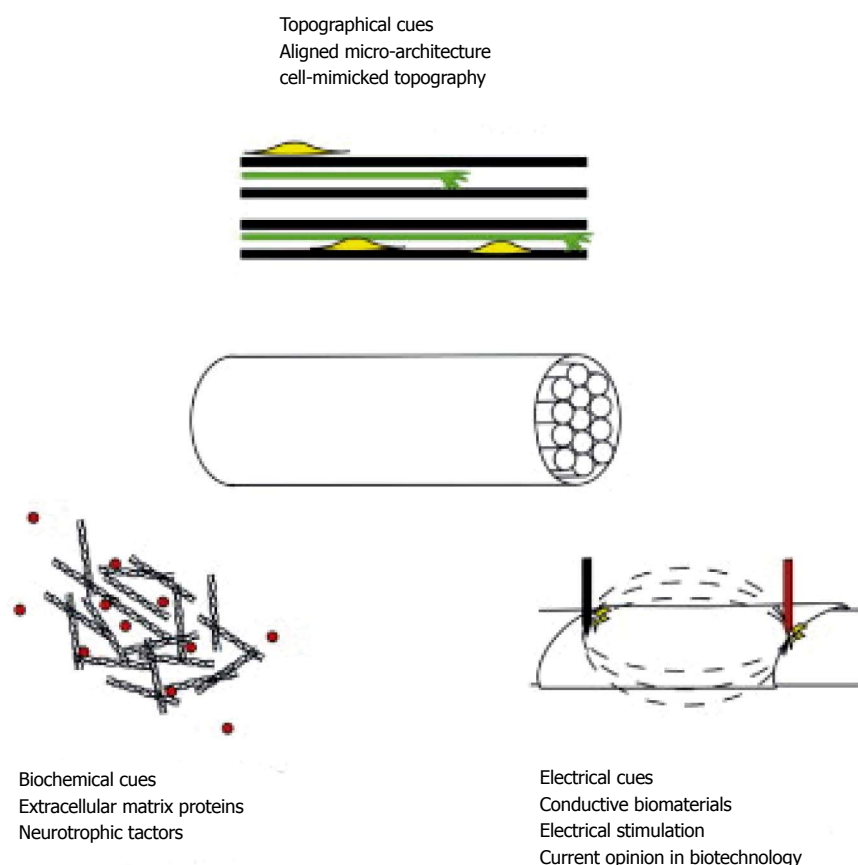


Figure 8 Interactions of topography, biochemical and electrical cues in engineering nerve tissues taken from^[72].

are found in high concentrations amongst undifferentiated ADSC may have future immunomodulatory roles which are greater than that of BMMSC^[87,88]. The combination of greater immunomodulatory effects and easier harvesting further justifies the greater emphasis of ADSC as opposed to BMMSCs. The question of whether ADSC can regenerate PNS neurons remains contentious. In smaller murine sciatic nerve models, ADSC neurotrophism has been shown to benefit both motor and sensory functions^[57,60]. The sensory recovery appears to be faster than that of motor recovery^[57]. The reason for this is currently unknown. Despite the numerous improvements in motor and sensory indices discussed in multiple studies following ADSC placement; there is yet to be a unified opinion regarding the precise mechanism of ADSC engraftment and differentiation at the nerve injury site^[56,57,89,90]. This uncertainty is confounded by a lack of convincing evidence of ADSC transformation to neural tissue at the repair site^[56,57]. It could be the improvements in the neurological recovery of injured animals was secondary to ADSCs providing a surge of growth factors which stimulate the remaining SCs still active within the nerve^[60]. The authors believe the improvements in the multiple facets of neuron recovery in murine models may be related to their ability to stimulate the remaining surviving SCs through angiogenesis and the release of the numerous growth factors mentioned previously^[52,91]. This would therefore suggest that ADSCs were partially able to regenerate a damaged neuron by stimulating the remaining functional cells within the neuron.

The murine model for nerve defects ranging from 1-2 cm in size remains the standard model for investigating ADSCs and nerve regeneration^[42,57-60,92]. Studies have since been scaled up to a canine model to observe ADSC roles in larger animals more comparable to human sized defects^[51,52]. Similar results were found to murine models where the ADSCs regenerated nerves to higher functionality in terms of faster nerve conduction velocities than those without ADSC application. Future research should be translated to larger animals in the longer term since multiple murine models have been trialled with variable successes. ADSCs may be placed *in vivo* in pre-differentiated or in undifferentiated states. Studies have shown the cells have neurotrophic effects in both states; however differentiated cells retain their regenerative capacity for longer^[57]. Future challenges will involve the supplementation of ADSCs to enable them to maintain their regenerative properties for the time required *in vivo*.

Conduits remain an option for nerve reconstruction but are limited to defects less than 3 cm in size^[93]. The conduits which have been used are a combination of natural and synthetic materials produced by a range of manufacturing processes. As discussed by Marquadt and Sakiyama Elber 2013 the ideal conduit would contain a combination of biochemical, topographical and electric cues to help determine the fate of the regenerative cells within the construct^[72] (Figure 8). The combination of all 3 of these factors would possibly yield an improvement on the results seen with the current conduit engineering techniques. Concern exists with the stability of cells

treated with high concentrations of glial growth factors. There are many studies in the literature where ADSCs are given large doses of glial growth factors and are forced to become either neurone or schwann like cells^[40-42,58,59,94,95]. The long term stability of these cells is largely unknown which may limit their clinical translatability into a peripheral nerve lesion human model. Alternative and potentially safer techniques using combinations of electrical stimulation and highly conductive topographical materials with MSCs have been used to obtain neural lineage absence of augmenting glial growth factors^[81]. This growth factor negative technique of producing neural like cells may be useful and a safer option whilst becoming a platform regenerative medical technology using ADSC for peripheral nerve regeneration. These transformed cells could be co-cultured with SCs taken at the time of surgery and these schwann cells used to maintain the neural phenotype of these cells over a long time period.

Cell therapy with Xenogenic Acellular Nerve Matrices (XANMs) is a viable option where the natural extracellular matrix of the PNS is replaced. When injected systemically ADSCs had a homing response to the injured neurological tissues and passed through multiple cell systems to get to their target organs^[68]. Porcine ANM have been used in rat models and were found to be well tolerated by the rat immune system despite the infiltration and interactions with host cell fibroblasts^[70]. Therefore, it appears that if ANM are sufficiently decellularised and/or immunosuppressant therapy is used ADSC and XANM may be a therapeutic alternative to autografts alone. ANM may be suitable for long nerve gaps where a like for like tissue is required. This could potentially be supplemented with ADSC which could help regenerate the nerve as well as provide immunosuppressive cues to limit the amount of immunosuppressant required to prevent rejection of the xenogenic material.

CONCLUSION

Peripheral nerve injury recovery remains a problem despite advances in the surgical and medical management of this debilitating illness. There are multiple studies using ADSCs in a variety of animal models to aid the recovery. At present the role of the ADSC in PN injury is still being investigated and the optimum timings and methods of insertion of ADSC has yet to be determined. For translation into humans; ADSC therapy will need to build upon the knowledge already acquired from *in vitro* and *in vivo* small animal studies. The preliminary results so far suggest a future role in peripheral nerve regeneration, but future research is need towards further optimisation and safety. The literature is saturated with the use of growth factors and other inducing chemicals such as BME and future studies should use less toxic methods of differentiating the stem cells. Electrical stimulus could provide a useful alternative method of reducing the number and concentrations of growth factors required to obtaining a neural lineage from the ADSCs.

The authors also feel greater focus should be given to surface modifications of nanomaterials to aid stem cell differentiation. Modifications of surface chemistry and nanotopography can be further adapted to support the expression of neural proteins within ADSCs. Material surface modifications along with electrical stimulation and essential glial growth factors could provide the stimulus required to obtain the neural lineages shown in previous studies, whilst reducing the risks of long term malignant transformation. The transplanted cells also need to be observed into larger animals than murine, as well as monitored for longer periods of time. This long term active surveillance would help to determine the malignant transformation potential of the ADSCs exposed to supraphysiologic levels of growth factors.

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Mesenchymal stem cell tracking in the intervertebral disc

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including analgesia and physiotherapy often fails and surgical treatment, such as spinal fusion, is required. Stem cells offer an exciting possible regenerative approach to IVD disease. Preclinical research has demonstrated promising biochemical, histological and radiological results in restoring degenerate IVDs. Cell tracking provides an opportunity to develop an in-depth understanding of stem cell survival, differentiation and migration, enabling optimization of stem cell treatment. Magnetic Resonance Imaging (MRI) is a non-invasive, non-ionizing imaging modality with high spatial resolution, ideally suited for stem cell tracking. Furthermore, novel MRI sequences have the potential to quantitatively assess IVD disease, providing an improved method to review response to biological treatment. Superparamagnetic iron oxide nanoparticles have been extensively researched for the purpose of cell tracking. These particles are biocompatible, non-toxic and act as excellent MRI contrast agents. This review will explore recent advances and issues in stem cell tracking and molecular imaging in relation to the IVD.

Key words: Intervertebral disc; Stem cells; Cell tracking; Magnetic resonance imaging; Intervertebral disc degeneration

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Core tip: Mesenchymal stem cell (MSC) transplantation shows exciting promise for the future regenerative approach to intervertebral disc (IVD) disease. Extensive preclinical research has demonstrated benefits from MSC treatment in disc degeneration. Cell tracking, with iron oxide nanoparticles and MRI, provides an opportunity to develop an in-depth understanding of stem cell survival, differentiation and migration, enabling optimization of stem cell treatment. This review summarizes the current literature relating to MSC tracking in the IVD, which is limited to short term monitoring. Medium to long-term cell tracking is required to accelerate translation of MSC treatment in the IVD to clinical practice.

Abstract

Low back pain is a common clinical problem, which leads to significant social, economic and public health costs. Intervertebral disc (IVD) degeneration is accepted as a common cause of low back pain. Initially, this is characterized by a loss of proteoglycans from the nucleus pulposus resulting in loss of tissue hydration and hydrostatic pressure. Conservative management,

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INTRODUCTION

Low back pain is the leading cause of disability in the developed world^[1]. Lifetime prevalence is 75%-80%^[2], with the annual cost in the United States alone estimated to be as high as \$500 billion^[3]. Low back pain is strongly linked to disc degeneration, with a two-fold increase in chronic lower back pain in patients with radiological evidence of degeneration^[4,5].

Current treatments, including analgesia, physiotherapy and spinal fusion only address symptoms, not the underlying disease. Regenerative strategies, such as stem cell therapy, provide an exciting future in the treatment of intervertebral disc (IVD) disease. Tracking and long term monitoring of these cells is essential to develop an understanding of their survival, migration, proliferation and differentiation *in vivo*, which will enable optimization of this promising therapy. Magnetic resonance imaging (MRI) combined with contrast agents is the modality of choice for cell tracking. This review will summarize recent advances in stem cell tracking, current problems and their application to the treatment of IVD disease.

PATHOLOGY OF BACK PAIN

The intervertebral disc is composed of 3 main regions: the tough annulus fibrosis (AF), peripherally, the amorphous nucleus pulposus (NP), centrally, and cartilaginous endplates which bind the disc to the adjacent superior and inferior vertebral bodies^[6]. With ageing and degeneration, significant cellular and matrix changes occur within the IVD. An early hallmark of disc degeneration is the loss of proteoglycans (PG), and associated water molecules, from the NP and AF, accompanied by structural changes to the lamellae of the AF^[7-11]. With the loss of the water binding PGs from the disc, its functional capacity as a hydroelastic cushion is diminished, leading to additional mechanical stresses acting on the fibrocartilaginous AF. With time, these events can result in the presence of concentric and radiating tears in the lamellae of the AF that may eventually extend into the NP^[7,8,11-13]. In addition, there is emerging evidence that damage to the cartilaginous endplate plays a role in the pathophysiology of degenerative disc disease, up-regulating matrix degrading enzymes and inflammatory cytokines in the NP^[14]. Structural deficiencies in the NP are considered to provoke neovascularisation and growth of nerve fibers, normally confined to the periphery of the AF, to the deeper regions of the disc^[13,15]. The establishment of these extended nerve fibers has been cited as a major cause of chronic lower back pain in degenerate discs^[15-17].

CURRENT TREATMENT OF BACK PAIN

Current strategies to treat low back pain fail to regenerate

the intervertebral disc or even reverse the degenerative process. Analgesics, non-steroidal anti-inflammatory drugs, physical therapies and other multimodal palliative modalities represent the mainstay of conservative therapy for low back pain^[18-20]. There is no evidence, however, that any of these therapies provide long-term benefit by improving the underlying pathobiology of disc degeneration. In fact, recent research has demonstrated simple analgesia does not improve recovery time from acute low back pain^[21]. When non-operative treatments fail, surgical interventions such as spinal fusion or total disc arthroplasty are commonly undertaken. These interventions aim to remove the pain generator, however, many patients remain with chronic pain and disability. Furthermore, spinal fusion has biomechanical consequences, which accelerates degeneration at adjacent levels^[22-24].

REGENERATIVE STRATEGIES FOR THE INTERVERTEBRAL DISC

Recent research suggests novel biological therapies can provide restorative treatment of disc degeneration. Cell types investigated include NP cells^[25-29], chondrocytes^[30-33] and mesenchymal stem cells (MSC)^[29,30,34-43]. MSCs, initially isolated from bone marrow, can also be derived from a range of tissues, including adipose and synovial^[44,45]. In addition, MSCs are reported to be non-immunogenic and, in contrast to embryonic stem cells, cannot undergo malignant transformation^[46,47]. Moreover, MSCs have the capacity for self-renewal, enabling maintenance of an undifferentiated phenotype in multiple subcultures^[48]. However, in the appropriate environment, MSCs are capable of differentiating into multiple cell types including chondrocytes, osteocytes, tenocytes and adipose cells^[44,45].

There are numerous preclinical studies investigating the use of MSCs in rat, rabbit, goat and porcine models^[29,30,34-43]. MSC implantation in the rabbit model has resulted in an increase in PG content, partial restoration of disc height and disc hydration^[42,43]. In the ovine model, intra-discal MSCs treatment has been shown to restore disc extracellular matrix, increase disc height and reduce radiological and histological grading scores 6 mo following injection^[49,50]. How stem cells produce these effects remains unclear from current preclinical studies.

Early clinical research has been promising. Yoshikawa *et al*^[51] reported two cases of autologous MSC implantation in markedly degenerate intervertebral discs. Symptomatic and radiological improvement was demonstrated without significant adverse effects^[51]. A larger series of 10 patients treated with autologous MSCs demonstrated improvement in pain, disability and disc hydration^[52]. Prior to translation to clinical practice, greater understanding of the mechanism of action of these cells is required.

NEED FOR CELL TRACKING

Longitudinal tracking of MSCs is necessary to ensure survival, evaluate distribution and assess if cells migrate to affected pathological sites in the IVD. Previous methods,

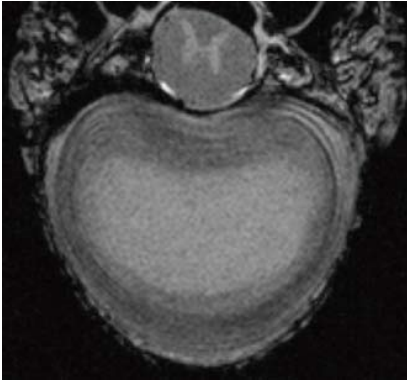


Figure 1 9.4 Tesla magnetic resonance imaging-Gradient Echo 2* weighted image of ovine lumbar intervertebral disc (normal control). Image obtained from our group 2014/02/18, not previously published.

based on histological analysis provide a snapshot view of the cells at a set time point. This is useful at the end of a scientific study but these methods cannot be translated to the clinical setting. Cellular labeling provides a non-invasive technique to assess the cells *in vivo* at any given time point. Thus, it can assess cell viability, track cell migration patterns and provide some information on efficacy. It may provide an understanding on mechanism of action, for example, potentially being able to determine whether cells differentiate into chondrocytic cells or act to modulate the resident native cell population through paracrine actions. In addition, cell tracking is required to ensure MSCs retention, as leakage of transplanted cells outside the disc has been reported to induce osteophyte formation^[53].

CURRENT IMAGING TECHNIQUES

In vivo, non-invasive techniques are required for cell tracking in the research and clinical setting. Multiple imaging modalities have been utilized, including MRI^[54-58], positron emission tomography (PET)^[59-62], single photon emission computed tomography^[63], bioluminescence imaging^[64,65], fluorescence imaging^[66-72] and computed tomography (CT)^[73]. MRI has been accepted as the best modality given its high spatial resolution (< 100 microns), prolonged effective imaging window, lack of ionizing radiation and ability to provide detailed anatomical information^[74,75]. There are now very high resolution MRI machines available such as 9.4 Tesla which provides high quality images (Figure 1).

CELL LABELING FOR MRI

The ideal contrast agent would be highly sensitive, biocompatible, non-toxic, easily taken up by the targeted cells and provide clear contrast between the labeled cells and surrounding tissue. Due to their superior sensitivity and excellent biocompatibility, iron oxide nanoparticles are the preferred contrast agent for cell labeling^[76]. Superparamagnetic iron oxide nanoparticles (SPIONs) consist of an iron oxide core, usually magnetite (Fe_3O_4) or

maghemite ($\gamma\text{Fe}_2\text{O}_3$) and a biocompatible coating. Coating substrates include dextran, carboxydextran, polyethylene glycol (PEG), polystyrene and silica^[77]. Iron oxide nanoparticles can be subdivided into standard SPION, 60 to 150 nm in size, and ultrasmall superparamagnetic iron oxide nanoparticles (USPION) which measure 10 to 20 nm^[78,79]. The type of coating, size and method of synthesis affect the SPIONs biocompatibility and magnetic properties^[80].

MRI generates images by utilizing the differences in proton density and the local magnetic environment of hydrogen atoms^[75]. There are two MR relaxation time constants, T1 and T2. Commonly used contrast agents, paramagnetic gadolinium analogues, alter longitudinal (T1) relaxation time of hydrogen protons and appear hyperintense. The abovementioned, superparamagnetic nanoparticles, affect the transverse (T2) relaxation time of hydrogen protons and appear hypointense. Due to their size, USPION demonstrate additional T1 effects^[75]. Iron oxide nanoparticles can be detected at micromolar concentrations and offer sufficient sensitivity to be identified on T2* weighted imaging^[81].

MR cell labeling requires the transfer of particles from extracellular to intracellular. The simplest method is spontaneous uptake of particles by phagocytic cells such as macrophages. While enhanced with an appropriate biocompatible coating, iron oxide nanoparticles are not efficiently taken up by stem cells^[82,83]. However, labeling can be facilitated by incubation with cationic transfection agents, including poly-L-lysine and protamine sulfate^[82,84-86]. If extended incubation time is not appropriate, other rapid techniques, magnetoelectroporation or magnetosonoporation, can be employed^[87,88].

A number of contrast agents have been approved for clinical use in medical imaging. Previously, the most commonly used iron oxide nanoparticle for cell labeling was Feridex[®], which contains an iron oxide core and dextran coating^[77]. Dextran coated SPIONs have been shown to be biocompatible and biodegradable *via* iron metabolism through Kupffer cells, located in the liver^[89]. Another widely used SPION, Resovist[®], has a carboxydextran coating^[90,91]. Both these products have been discontinued from production by the pharmaceutical companies^[77,92]. Other commercial products continue to be utilized, such as SiMAG[®], an SPIO with an unmodified silica surface. For example, Markides *et al*^[93] labeled MSCs with SiMAG[®] in a rheumatoid arthritis mouse model.

Extensive research has been devoted to designing novel iron oxide nanoparticles for the purpose of stem cell labeling^[92]. van Buul *et al*^[94] demonstrated ferumoxides (Endorem[®]) complexed with protamine sulfate are superior to ferucarbotran particles for cell labeling. Subsequently, this group demonstrated safety and efficacy of the ferumoxide-protamine sulfate complex for MSC labeling in articular cartilage repair^[95]. USPION have also been investigated recently. Coated with dextran and PEG and combined with protamine sulfate, USPIONs have been cultured with human Adipose Derived Stem Cells (hADSCs) within a three dimensional scaffold^[96]. *In vitro*, no effect on cell viability or osteogenic differentiation was seen from cell

labeling. The USPIOs were effectively internalized by the hADSC and demonstrated T2* signal change. Hypointense regions, representing labeled cells, were seen *in vivo* 28 d following implantation^[96]. Further research is required to optimize SPIONs for cell tracking.

ISSUES WITH CELL LABELING

Transfection agents are potentially toxic and, furthermore, there is capacity for iron oxide nanoparticles *in vivo* to cause toxicity to other organs, including liver and spleen^[97,98]. Small polyhedral SPIONs with a silica coating have shown efficient MSC labeling without the need for a transfection agent and may offer a solution^[99].

T2 signal change is due to the overall effect of magnetic nanoparticles rather than total number of cells^[100]. Typically, a few hundred cells are required for detection with conventional MRI sequences^[77]. Stem cells are known to proliferate following transplantation, leading to dilution of the iron oxide label and loss of MR signal over time^[77]. If cells divide asymmetrically, with one daughter cell receiving the majority of nanoparticles, rapid dilution of signal can occur to an undetectable level^[101]. Labeled cells could also become undetectable if they migrate in small rather than large groups. Sensitivity may be improved with post acquisition software analysis or a higher magnetic field strength.

A number of endogenous substances produce negative (or hypointense) MR signal, such as blood products containing haemosiderin or methaemoglobin. This leads to challenges differentiating blood product from labeled cells in an injured IVD. Novel MRI methodology has been adopted to help differentiate the labeled cells from endogenous substances, such as Inversion-Recovery With ON-Resonant Water Suppression, which delineates SPION labeled cells as positive contrast^[102]. Further novel sequences are being developed to provide an exciting possibility to enhance non-invasive cell tracking.

Iron oxide nanoparticles fail to differentiate between live and dead cells. SPION signal has been demonstrated in the CNS long after cell death^[103]. Multimodal imaging may be required to ensure cellular function, such as combining MRI with PET imaging. A study investigating iron oxide labeled stem cells in hemi-Parkinsonian rats used this multi modal technique. MRI visualized stem cells in the striatum and PET confirmed cellular viability^[104].

CELL LABELING IN THE INTERVERTEBRAL DISC

To date, there is limited published research tracking MSCs in the IVD and this is summarized in Table 1. Saldanha *et al.*^[105] demonstrated feasibility by imaging MSCs labeled with SPION (Feridex®) *in vitro* to quantitatively characterize signal intensity loss using T1, T2 and T2* relaxation parameters. T2* weighted gradient echo (GRE) images demonstrated the most significant loss of signal intensity from labeled cells. Conversely, SPION labeled cells were indistinguishable

from unlabeled cells on T1 weighted imaging^[105]. This group also demonstrated SPION labeled cells, loaded in a fibrin gel and injected *via* fluoroscopic guidance, could be identified *ex vivo* within the IVD of excised rat tails^[105]. Further research by Prologo *et al.*^[62] imaged MSCs labeled with a radioactive marker (iodine-124 2'fluoro-2'-deoxy-1β-D-arabinofuranosyl-5-iodouracil) using CT and PET. Four female pigs had approximately 100000 labeled MSCs injected under fluoroscopic guidance to the NP of discs which had degeneration induced 10 d prior. CT and PET were performed immediately following and three days after cell delivery. One animal was inaccurately injected. Results from the other three demonstrated accurate delivery and maintenance of labeled cells at three days^[62]. While demonstrating useful preclinical results, using radioactive cell labeling in conjunction with CT and PET requires a significant amount of ionizing radiation, a consideration if used in clinical practice.

Recently, Barczewska *et al.*^[106] developed a technique for real time non-invasive monitoring of minimally invasive MSC delivery. IVD degeneration was induced in porcine discs of 3 animals *via* a fluoroscopic guided IVD vaporization procedure. Subsequently, the animals were placed in a 3T MRI scanner and 3×10^6 SPIO labeled MSCs were injected into the IVD through a plastic catheter in two divided injections. T2 weighted imaging was performed prior to, and following, each injection. Results demonstrated a statistically significant difference in signal intensity between both SPIO labeled MSCs compared with unlabeled MSCs and SPIO compared with control discs^[106].

Although limited, current research has demonstrated MRI is a suitable modality for detecting SPION labeled MSCs in the IVD. Current data, utilizing both MRI and CT/PET, provides only short term tracking of labeled cells, demonstrating adequate initial placement of transplanted cells. Further research is required to monitor long-term retention of MSCs in the IVD. Moreover, longitudinal tracking of injected cells is required to improve understanding of the therapeutic mechanism of action of MSCs in IVD disease.

ADDITIONAL BENEFITS OF MRI IN CELL TRACKING

In addition to monitoring labeled MSCs, MRI has the potential to non-invasively measure response to biological therapies in IVD disease. Several techniques have been developed to classify disc degeneration by MR image criteria^[107-110]. All are based on conventional T2 weighted sagittal imaging characteristics, which correspond to later morphological changes in IVD degeneration. The Pfirrmann classification system is the most widely used in the literature, utilizing an algorithm to generate five morphological grades^[107]. Research has demonstrated acceptable intra and inter-observer variability for grading systems^[107,109]. Ultimately, however, each is based on qualitative assessment and prone to inter-observer

Table 1 Review of stem cell tracking in the intervertebral disc in animal models reported in the literature

Ref.	Animal model	Cell type	Label	Imaging	Results
Saldanha <i>et al.</i> ^[105] , 2008	Rat tail (<i>ex vivo</i>)	Human MSCs (Loaded in fibrin gel-Tisseel)	FE-Pro (Feridex and Proatmine Sulphate)	3T MRI	No significant effect of labeling on MSC viability Hypointensity (signal loss) seen in discs injected with labeled cell
Prologo <i>et al.</i> ^[62] , 2012	Porcine <i>n</i> = 4	Human MSCs	Iodine-124 2'-fluoro-2'-deoxy-1b-D-arabinofuranosyl-5-iodouracil	PET/CT	Inaccurate delivery in 1 animal Three animals showed persistence and containment of labeled cells 3 d following injection
Barczewska <i>et al.</i> ^[106] , 2013	Porcine <i>n</i> = 1 (<i>ex vivo</i>) <i>n</i> = 3 (<i>in vivo</i>)	Porcine MSCs	Molday ION USPION	3T MRI	<i>Ex vivo</i> -Injected labeled cells clearly visualized <i>In vivo</i> -hypointense regions identified immediately following injection of labeled cells. Histopathology performed 2 wk later confirmed the presence of MSCs in the disc

MSC: Mesenchymal stem cell; MRI: Magnetic resonance imaging; USPION: Ultrasmall superparamagnetic iron oxide nanoparticle; PET/CT: Positron emission tomography/computed tomography.

variability. In addition, conventional MRI sequences lack sufficient sensitivity to identify early disc biochemical changes^[111].

Novel MRI sequences have the potential to quantitatively assess early IVD degeneration. T1 ρ MRI uses long duration, low power radiofrequency, or spin-lock (SL) pulses, applied on-resonance to lock magnetization in the transverse plane. Spin-locked magnetization relaxes with the time constant T1 ρ , spin lattice relaxation in the rotating frame^[112,113]. Post acquisition computer software analysis can be utilized to generate a quantitative T1 ρ measurement for a region of interest, such as the NP. T1 ρ has been shown to be sensitive to PG content in articular cartilage and the IVD^[114,115]. Furthermore, Borthakur *et al.*^[112] demonstrated T1 ρ values in IVDs correlate with low back pain. Another quantitative method recently developed is the NP voxel count, which has been shown *in vivo* to quantitatively assess disc degeneration. This assesses nuclear size and hydration with T2-relaxation time measurements^[116]. These imaging techniques have the potential to non-invasively, quantitatively, monitor response to biological treatments, as well as diagnose early IVD degeneration.

SUMMARY AND EXPERT OPINION

MSC transplantation shows exciting promise for the future regenerative approach to IVD disease. Cell labeling techniques are integral to ongoing research, to ensure accurate delivery and long-term retention of cells. Current research is limited to only short term monitoring. A greater understanding of the mechanism of action is required, with longitudinal tracking of implanted MSCs. Further research is required to discover if cells remain *in situ* long term, if they remain viable and if they track to sites of pathology in the IVD. MRI, with SPIONs as contrast agents, provides an excellent imaging modality for cell tracking. Some issues remain, such as dilution of signal through cell division or migration and differentiating labeled cells from intrinsic negative signal. These may be addressed with stronger magnetic fields, post-processing analysis and novel MRI techniques. Furthermore, novel MRI sequences can non-invasively quantify early biochemical changes in disc

degeneration. In conjunction with conventional MRI, this provides a mechanism for measuring response to biological therapy, in both the research and clinical setting. Ongoing effective research will facilitate the translation of MSC treatment for IVD disease to clinical practice.

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***In vivo* imaging of endogenous neural stem cells in the adult brain**

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eNSC mobilization non-invasively in the live brain remain scarce, but considerable progress has been made in this field in recent years. This review summarizes and discusses the current imaging modalities suitable to monitor eNSCs in individual experimental animals over time, including optical imaging, magnetic resonance tomography and-spectroscopy, as well as positron emission tomography (PET). Special emphasis is put on the potential of each imaging method for a possible clinical translation, and on the specificity of the signal obtained. PET-imaging with the radiotracer 3'-deoxy-3'-[¹⁸F]fluoro-L-thymidine in particular constitutes a modality with excellent potential for clinical translation but low specificity; however, concomitant imaging of neuroinflammation is feasible and increases its specificity. The non-invasive imaging strategies presented here allow for the exploitation of novel treatment strategies based upon the regenerative potential of eNSCs, and will help to facilitate a translation into the clinical setting.

Key words: Neural stem cells; Positron emission tomography; Magnetic resonance imaging; 3'-deoxy-3'-[¹⁸F]fluoro-L-thymidine; [¹¹C]PK11195

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Abstract

The discovery of endogenous neural stem cells (eNSCs) in the adult mammalian brain with their ability to self-renew and differentiate into functional neurons, astrocytes and oligodendrocytes has raised the hope for novel therapies of neurological diseases. Experimentally, those eNSCs can be mobilized *in vivo*, enhancing regeneration and accelerating functional recovery after, *e.g.*, focal cerebral ischemia, thus constituting a most promising approach in stem cell research. In order to translate those current experimental approaches into a clinical setting in the future, non-invasive imaging methods are required to monitor eNSC activation in a longitudinal and intra-individual manner. As yet, imaging protocols to assess

Core tip: Endogenous neural stem cells (eNSCs) in the adult mammalian brain can be mobilized by, *e.g.*, pharmacological methods to facilitate regeneration and enhance functional recovery in neurological disease. In order to translate experimental approaches into the clinical setting, non-invasive imaging of eNSCs is required to monitor their fate *in vivo*. This review summarizes current imaging modalities suitable to monitor eNSCs in individual experimental animals over time, including optical imaging, magnetic resonance tomography and-spectroscopy, as well as Positron-Emission-Tomography, placing emphasis on the specificity of the signal obtained, as well as on their potential for clinical translation.

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NEURAL STEM CELLS IN THE ADULT MAMMALIAN BRAIN

The discovery of neural stem cells (NSCs) in the adult brain and their ability to self-renew and differentiate into tissue-appropriate, functional cell types has raised intense scientific interest and the hope for radical new therapies of neurological diseases. Altman *et al*^[1] first detected the ability of the adult mammalian brain to generate new neurons, and their work was followed up by Kaplan *et al*^[2]. Using radioactive thymidine to label all dividing cells and histological examination of postmortem brains, they found labeled neurons. This proved that new neurons in the adult brain can be generated following cell division. After those pioneer studies focusing on neurogenesis, later studies aimed to detect the immature precursor cells capable of differentiating into all three cell fates of the central nervous system (CNS): neurons, astrocytes and oligodendrocytes. Several biomarkers have been suggested to identify those undifferentiated stem cells in the brain, the first one being the intermediate filament nestin (neuroepithelial stem cell intermediate filament^[3]). Subsequently, NSCs were characterized in the developing and in the postnatal/adult mammalian brain^[4-11]. More biomarkers were consecutively identified including *Sax2*, *Shh* pathway components, PDGF, EGFR, GFAP, Hes3, Hes5, Musashi, and CD133^[5,12-22]. However, since no single marker has yet been identified to unambiguously distinguish NSCs from somatic cells, co-stainings are usually required to comprehensively characterize them. It is now well accepted that endogenous NSCs (eNSCs) persist in the adult mammalian brain in at least two distinct regions, the subventricular zone, lining the lateral ventricles, and the dentate gyrus of the hippocampus^[23,24].

Insults to the CNS such as cerebral ischemia or neurodegenerative disease result in a mobilization of eNSCs and their migration towards the compromised areas^[25-29], constituting a physiological regenerative response of the brain. However, in most cases the intrinsic regenerative response of eNSCs is obviously not sufficient to lead to functional recovery. Experimentally, it has been shown that eNSCs can be mobilized pharmacologically for therapeutic purposes. Early studies showed that activating the tyrosine kinase receptors for fibroblast growth factor 2 and epithelial growth factor on eNSCs by introducing those growth factors into the lateral cerebral ventricle of experimental animals stimulates the proliferation of eNSCs *in vivo*^[30,31]. Ligands for these receptors also show benefit in animal models of cerebral ischemia, where growth factor-induced eNSC mobilization is associated with enhanced functional recovery from motor deficits^[32,33]. Recently,

several other drugs have been identified to mobilize eNSCs in the naïve and in the injured rodent brain, including Notch-ligands^[13], angiopoietin 2^[14], the neural cell adhesion molecule mimetic peptide FG Loop^[34], and aromatic-turmerone^[35]. Enhancing this (physiological) mobilization of eNSCs by pharmacological intervention leads to an improvement of neurological function after, *e.g.*, cerebral ischemia^[13,32,33]. To convey these regenerative effects, it seems that the differentiation of eNSCs into mature neurons that functionally integrate into the damaged circuitry-previously thought to be required for regeneration-only plays a minor role^[36]. Rather, eNSCs secrete trophic factors supporting neuroprotection such as glial-derived neurotrophic factor, vascular endothelial growth factor, or *Shh*^[13,37]. Other regenerative processes induced by NSCs include remyelination, angiogenesis, remodeling, and immunomodulation^[38,39]. Since treatments based on the transplantation of stem cells harbor several disadvantages including poor long-term cell survival, a lack of integration into the host circuitry, immune reactions against the transplants, and limited availability of appropriate cells^[40], mobilizing the endogenous neural stem cell niche for therapeutic purposes constitutes a most promising approach in stem cell research.

IMAGING ENDOGENOUS NEURAL STEM CELLS *IN VIVO*

Developing strategies to mobilize the endogenous NSC niche *in vivo*, with the aim to later translate them into clinical applications, creates the need for *in vivo* imaging technology to monitor those interventions. Imaging techniques should be non-invasive, so they can be applied repetitively in the same individual in a longitudinal fashion, and thus track quantity and localization of endogenous NSC over any period of time. While considerable progress has been made in recent years to track transplanted, pre-labeled cells^[41-47], the detection of endogenous NSCs in the living brain remains elusive. Current approaches to image eNSCs *in vivo* include (1) the use of transgenic animals whose eNSCs exhibit certain imaging properties; (2) labeling eNSCs *in vivo* by injecting a labeling substance into the brain; or (3) imaging some intrinsic and putative unique property of eNSCs with a tailored imaging assay.

Transgenic animals expressing a fluorescent or bioluminescent protein under the control of a stem cell-characteristic promoter such as nestin or doublecortin render their eNSCs detectable by optical imaging techniques^[48-52]. Under ideal conditions, optical imaging can specifically detect clusters of about 10³ cells *in vivo*, but its sensitivity is limited by the relatively poor spatial resolution with shallow tissue penetration^[53]. Moreover, the need for transgenic mice limits the potential applications of optical imaging and prohibits its translation into a clinical setting.

In the attempt to (specifically) label eNSCs *in vivo*, various methods have been suggested. Labels can be micro-injected directly into (or close to) the neurogenic

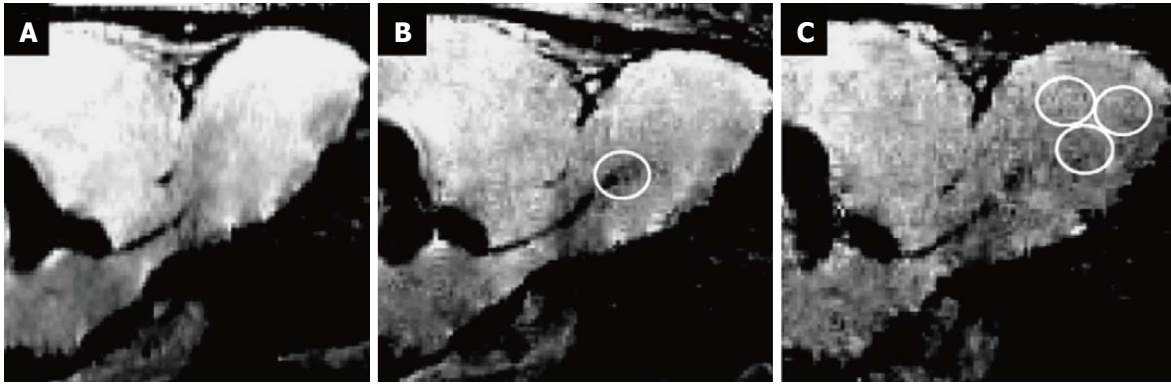


Figure 1 Monitoring the migration of endogenous neural stem cell labeled with iron oxide particles in the rat brain *in vivo*, using magnetic resonance imaging. Migrating cells were imaged 1 d (A), 3 d (B), and 8 d (C) after labeling, migrating from the subventricular zone (left in the sagittal images) to the olfactory bulb (on the right). White circles surround migrating cells. Granot *et al.*^[54], with permission. MRI: Magnetic resonance imaging.

niches, or they can be coupled to a vector in order to specifically target certain cells. Direct injection of a paramagnetic label such as small particles of iron oxide allows for cell detection using magnetic resonance imaging (MRI) with excellent spatial resolution on the single cell level (Figure 1)^[54-59]. However, this type of labeling is neither specific for a certain cell type, nor does it reflect cell viability, as only the particles themselves are visualized^[53]. Moreover, directly injecting iron oxide in *e.g.*, the lateral ventricles of the brain results in image distortion in the region of injection, allowing only for the detection of cells migrating away from the injection site^[54,57,58]. A more specific *in vivo* labeling approach is achieved by attaching an imaging label to a retro- or lentiviral vector, thus targeting proliferating cells in particular. This has been shown to be effective for optical imaging techniques after introduction of firefly luciferase^[60,61] or channelrhodopsin-2^[62]. Alternatively, ferritin can be introduced into proliferating (stem) cells using viral vectors, rendering them detectable for MR-imaging^[63,64]. Recent progress in this field has also been made by the development of a monoclonal antibody binding to neural precursor cells, coupled to magnetic glyconanoparticles allowing for their detection by MRI^[65]. While these approaches are quite promising to track eNSCs in individual experimental animals, a major disadvantage of those *in vivo* labeling approaches is the lack of applicability in human beings due to the invasiveness of the labeling procedure.

Truly non-invasive imaging techniques, utilizing methods that could be translated from the bench to the bedside, are rare. Manganas and colleagues investigated the spectrum of electromagnetic energy from established neurogenic niches using proton magnetic resonance spectroscopy (1H-MRS). They observed a prominent peak in the spectrum at the frequency of 1.28 parts per million (ppm) in the hippocampus of the adult mammalian brain *in vivo*, which was not observed in other regions of the brain^[66]. Later studies investigating this phenomenon have shown that it is not specific to eNSCs or neurogenesis, but closely related to apoptosis and quiescence^[67,68]. Since apoptosis is a major selection

process during neurogenesis, 1H-MRS may be used as an indirect method to detect neurogenesis within the known neurogenic niches and under physiological conditions. Disorders of the CNS associated with increased apoptosis of neural- or other non-neural cells, however, cannot be studied with this approach. Besides, MRS offers quite a low spatial resolution, failing to notice small clusters of cells and leading to the low sensitivity of this method.

We took advantage of the proliferative activity of eNSCs to develop an imaging assay using positron emission tomography (PET). Extensive studies in neurooncology have established the radiotracer 3'-deoxy-3'-[¹⁸F]fluoro-L-thymidine ([¹⁸F]FLT) to label proliferating cells in the adult rodent and human brain, allowing for non-invasive imaging of tumor cell proliferation with PET^[69-72]. [¹⁸F]FLT is a thymidine analogue that is incorporated into the DNA of dividing cells-similar to bromodeoxyuridine (BrdU) well established for their immunohistochemical detection- where it is irreversibly trapped, its positron-emitting properties allowing for its non-invasive detection^[73]. No evidence of toxicity or other complications have been reported following intravenous injection of [¹⁸F]FLT^[74]. [¹⁸F]FLT-PET detects ceasing glioma cell proliferation as soon as 3 d after initiation of an anti-proliferative treatment, as we have previously shown in mice and humans^[69,72]. Besides tumor cells, proliferating NSCs incorporate [¹⁸F]FLT both *in vitro* as well as *in vivo* after its systemic (intravenous) injection into adult rats^[75]. Thus, [¹⁸F]FLT labels proliferating eNSCs in the neurogenic niches of the healthy rodent brain with high sensitivity (Figure 2A), corresponding to BrdU-accumulation (Figure 2B). Moreover, [¹⁸F]FLT-PET quantifies eNSC mobilization mediated by pharmacological stimulation (Figure 2C). The resulting PET-signal can be quantified to reflect the extent of eNSC mobilization^[75]. Using a high-resolution PET-scanner and optimizing image reconstruction, the detection level can be as low as 10^4 cells. However, the PET-signal is not specific to endogenous NSC, since other proliferating cells are labeled as well. To increase specificity of this imaging assay, multi-modal imaging protocols can be applied as detailed below.

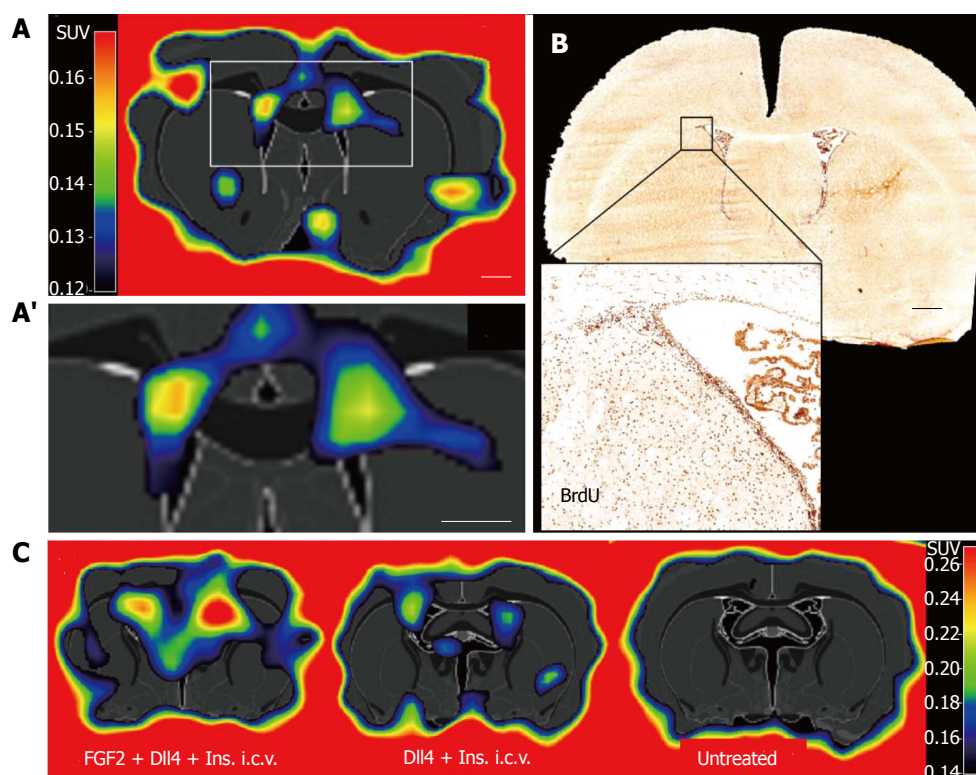


Figure 2 [^{18}F]FLT labels proliferating endogenous neural stem cell in the neurogenic niches of the healthy rodent brain. A, A': eNSC proliferation in the subventricular zone of adult rats as assessed by [^{18}F]FLT-PET; B: The signal corresponds to BrdU-positive cells in the region; C: Activation of eNSC by pharmacological stimulation with fibroblast growth factor 2, delta-like 4, and insulin is visualized with [^{18}F]FLT-PET. eNSC: Endogenous neural stem cell; [^{18}F]FLT-PET: 3'-deoxy-3'-[^{18}F]fluoro-L-thymidine-positron emission tomography; i.c.v.: Intracerebroventricular application. Adapted from Rueger *et al.*^[75], with permission.

STEM CELL-MEDIATED REGENERATION AFTER FOCAL CEREBRAL ISCHEMIA

Stroke is the third leading cause of death and the leading cause of adult disability in the Western world^[76]. Since rescue of affected neurons can only be achieved by re-perfusion within a very narrow time window, treatment is mainly confined to the amelioration of neurological deficits and the prevention of further events. Especially in the subacute and chronic phase, *i.e.* days to months after stroke, therapeutic options are limited to physiotherapy, ergotherapy and logopedia to rehabilitate impaired neurological functions. However, from the pathophysiological point of view, evolution of ischemic damage is not limited to the minutes after vessel occlusion. After the disruption of blood flow below a threshold has led to rapid necrotic cell death within a localized region, the surrounding tissue that has been spared in this initial phase consecutively also undergoes relevant, but less rapid changes which aim at encapsulating the necrotic tissue, clearing of debris, and facilitating regeneration. These processes - often referred to as neuroinflammation - involve the rapid activation of glial cells (microglia, astrocytes) as well as recruitment of hematogenous cells (granulocytes, T-cells, monocytes/macrophages) from the blood stream^[77-82].

While neuroinflammation on the one hand contributes to the evolution of secondary damage to the surrounding

tissue by the excessive production of reactive oxygen species and pro-inflammatory cytokines secreted by the immune cells, it also has beneficial effects on the prevention of secondary tissue damage^[83]. Besides the containment of necrotic tissue, another most relevant beneficial aspect of stroke-induced neuroinflammation is the induction of a strong regenerative response, leading to a robust expansion of eNSCs^[84]. Quality, extent and timing of neuroinflammatory processes determine whether manipulating that particular response after stroke will be deleterious or therapeutically beneficial. The activation of resident microglia can under some circumstances be neurotoxic^[85], under others neuroprotective^[86], depending on the specific activating conditions^[87]. Interestingly, differentially activated microglia also have opposing effects on NSC^[88]. eNSC are attracted to the site of the lesion by various inflammation-associated cytokines such as stromal cell-derived factor-1^[89-91], tumor necrosis factor- α , and interferon- γ ^[84,92]. This mobilization of eNSCs has been shown for various ischemia models in experimental animals, including transient global ischemia^[28], transient focal ischemia^[26,36], or permanent focal ischemia^[93]. However, without any further pharmacological mobilization, the vast majority of newly generated neuroblasts in ischemic stroke models die by the time they have reached the peri-infarct area^[36]. Moreover, neurogenesis after stroke seems to play even less a role in humans than it does in rodent models^[94]. Those recent findings highlight the

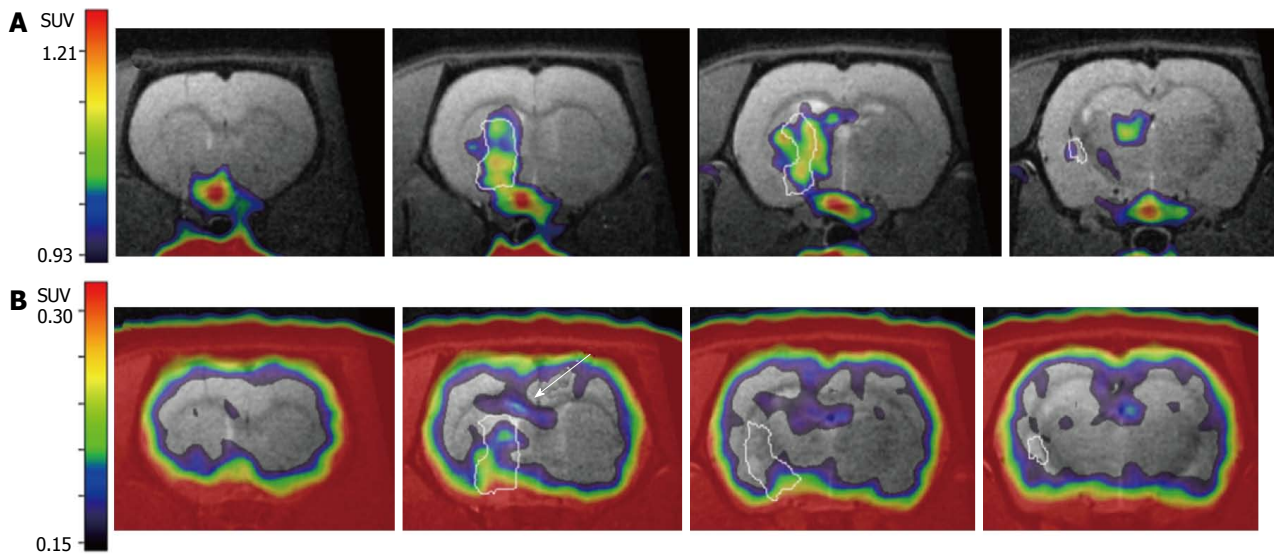


Figure 3 Conclusive differentiation between cell proliferation from endogenous neural stem cell and immune cells. After transient focal ischemia (lesion outlined in white), A: Neuroinflammatory processes are visualized with [^{11}C]PK11195-PET, depicting inflammation in the infarct core as well as in the ischemic border zone; B: [^{18}F]FLT-PET data acquired in the same imaging session without moving the animals in the scanner demonstrates additional cell proliferation in the subventricular zone (white arrow), originating from endogenous neural stem cells. Adapted from Rueger *et al*^[97], with permission. [^{18}F]FLT-PET: 3'-deoxy-3'-[^{18}F]fluoro-L-thymidine-positron emission tomography.

importance of developing novel therapeutic approaches to additionally mobilize and invigorate eNSCs after stroke, either by pharmacological^[34,35] or by non-pharmacological treatments^[95].

In order to advance those experimental therapeutic approaches into the clinical setting, non-invasive imaging assays have to be established that reliably monitor eNSC activation in the rodent and human brain under the pathophysiological condition of stroke. In this context, the post-ischemic neuroinflammatory processes introduced above resemble a major impediment, since immune cells proliferate in the ischemic brain just as eNSCs do, and [^{18}F]FLT-PET does not differentiate between stem cell- and immune cell-derived proliferation. An elegant way to circumvent this problem consists of an imaging assay specific to (neuro-)inflammatory cells that does not visualize eNSCs. The radiotracer [^{11}C]PK11195 fulfils this requirement by selectively binding to the translocator protein-18 kDa expressed on inflammatory cells, specifically visualizing post-ischemic cellular neuroinflammatory processes^[96]. Since [^{11}C]PK11195 is radiolabeled with the isotope 11-C with a half-life of ~20 min, sequential PET-imaging with [^{18}F]FLT is possible by waiting for ~5 half-lives or 100 min between scans. During this time, the animal remains anesthetized within the PET-scanner, avoiding any movement, and enabling an exact co-registration of the imaging data^[97]. This co-registered imaging data on [^{11}C]PK11195- and [^{18}F]FLT-accumulation allows for a conclusive differentiation between cell proliferation from eNSCs and immune cells (Figure 3).

Therefore, PET is a promising tool to image eNSCs in a non-invasive fashion in neurological disorders that can be translated from bench to bedside. However, to further advance the imaging technology in this direction, complementary verification of imaging parameters

with immunohistochemical analyses are still needed, and the actual translation of such strategies to a clinical environment has yet a long way to go. However, the non-invasive imaging strategies presented here will help to facilitate translation into the clinical setting, and allow for the exploitation of novel treatment strategies based upon the regenerative potential of eNSCs.

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Identify multiple myeloma stem cells: Utopia?

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and hematologic tumors, so the idea of CSCs has been proposed for MM, even if MM CSCs have not been defined yet. The existence of myeloma CSCs with clonotypic B and clonotypic non B cells was postulated by many groups. This review aims to focus on these distinct clonotypic subpopulations and on their ability to develop and sustain MM. The bone marrow microenvironment provides to MM CSCs self-renewal, survival and drug resistance thanks to the presence of normal and cancer stem cell niches. The niches and CSCs interact each other through adhesion molecules and the interplay between ligands and receptors activate stemness signaling (Hedgehog, Wnt and Notch pathways). MM CSCs are also supposed to be responsible for drug resistance that happens in three steps from the initial cancer cell homing microenvironment-mediated to development of microenvironment-independent drug resistance. In this review, we will underline all these aspects of MM CSCs.

Key words: Bone marrow microenvironment; Cancer stem cells; Multiple myeloma; Stem cells niche; Stemness

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Core tip: Multiple myeloma is a still incurable malignancy. Several study about multiple myeloma cancer stem cells showed their ability of self-renewal, survival and drug resistance. Besides, these cells are able to initiate and develop tumor when transferred into mice recipients. So understanding multiple myeloma cancer stem cells mechanisms become important to design new efficient targeting strategies for multiple myeloma. The aim of this review is to elucidate the state of art about multiple myeloma cancer stem cells and their critical role in maintenance of disease.

Abstract

Multiple myeloma (MM) is a hematologic malignancy of monoclonal plasma cells which remains incurable despite recent advances in therapies. The presence of cancer stem cells (CSCs) has been demonstrated in many solid

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MULTIPLE MYELOMA

Multiple myeloma (MM) is the second most common hematologic malignancy after non-Hodgkin lymphoma. It is characterized by uncontrolled proliferation of malignant plasma cells (PCs) that infiltrate in the bone marrow (BM), although small numbers of MM cells can be encountered in the peripheral blood circulation.

The first effect of MM is on the bone, but the blood and the kidneys are also involved. In bone marrow, malignant PCs induce damages in two ways: (1) proliferating cells form clusters disrupting the physiological structure of bone and causing osteolytic lesions; and (2) MM cells secrete not only cytokines and growth factors (GFs) that promote tumor progression and survival aging on MM cells themselves and on BM stromal cells (BMSCs) but also PCs secrete high amounts of monoclonal paraprotein (M protein) an abnormal immunoglobulins (Igs). Tumor cells interfere with hematopoiesis inducing a reduction in the number of white blood cells, condition known as leukopenia and increase the risk of infection. Moreover the decrease in red blood cells results in anemia, and low platelets level (thrombocytopenia) reduces normal blood clotting. Finally, the Bence Jones proteinuria, free light chain κ and, more important, λ , induces interstitial nephropathy and kidney failure because of their precipitation in distal tubules and collecting ducts^[1].

MM is usually preceded by a pre-malignant stage termed monoclonal gammopathy of undetermined clinical significance (MGUS) which progresses to overt MM at a rate of 0.5% to 3% per year^[2].

MGUS is characterized by a low number of PCs in BM and it isn't related to organ damages. MGUS has an increasing prevalence with age, affecting early 6% of over 60 years, but no treatment is indicated in these patients. Many studies evidence that patients with MGUS have a risk approximately 1% per year to develop to myeloma or to other related diseases^[3].

Another stage is represented by smouldering multiple myeloma (SMM). In these patients, the tumour burden is higher than in MGUS; they have a higher risk of progression to symptomatic myeloma and they require therapy. The current care for SMM patients is the monitoring disease and in case of progression, the treatments are recommended^[4].

MM it's still incurable despite the implementation of novel therapies and the great part of patients relapse even if initially they response to therapy. This is due to the presence of clonogenic cells inducing the so-called undetectable minimal residual disease (MRD)^[5].

BONE MARROW MICROENVIRONMENT AND THEIR PRECURSOR CELLS

MM progression is strongly supported by bone marrow microenvironment. A complex and mutual interactions between PCs and BMSCs support tumor cells growth, migration, survival, differentiation, drug resistance and angiogenesis^[6,7].

MM microenvironment is composed by extracellular matrix (ECM) proteins such as laminin, vitronectin, fibronectin, collagen, and by a large number of different stromal cells: fibroblasts, osteoblasts/osteoclasts, endothelial cells (ECs) and endothelial progenitor cells (EPCs), cells of immune system, hematopoietic and mesenchymal stem cells (HSCs and MSCs). During cancer progression, tumor cells are able to modify the surrounding stroma to build a promoting microenvironment from whose take advantages. The interplay between PCs and BMSCs is mediated by several cytokines, receptors and adhesion molecules^[8].

Plasma cells

The majority of PCs is identified in the bone marrow and a critical role in B cells development is played by stromal cells. Indeed they get in touch and secrete cytokines and growth factors needed to B cells maturation. The early stages of B cells proliferation depend on the interplay of PCs with stromal cells through vascular cell adhesion molecule-1 (VCAM-1) and thanks to growth factors such as interleukin-7 (IL-7), stem cell factor (SCF) and CXC chemokine ligand 12 (CXCL12)^[9].

During the development of B cells, within BM, the B cell precursors became independent from interaction with microenvironment and from cytokines secreted by stromal cells, and in the final stage, immature B cells, located near to the central sinus, lose expression of CXC chemokine receptor-4 (CXCR4), CXCL12 receptor, and they are released from marrow. Therefore, entering in the central sinus, immature B cells migrate to the spleen, where they complete their development becoming naïve mature B cell. Then, they may pick up antigen within the tissue, alternatively, B cells may return to bloodstream and they continue to look for antigen activating themselves. The activation of B cells induces the differentiation into memory B cells, PCs or plasmablasts that will move to the BM.

First, plasmablasts and PCs to stromal cell and derived factors (*e.g.*, IL-6), for their survival, besides chemokines appear responsible for plasmablast entry into BM as well as PCs retention. PCs express CXCR4 on their surface and migrate towards CXCL12, produced by stromal cells and sinusoidal ECs in the BM, showing that PCs express membrane antigen required for their localization in the BM.

The most specific PCs surface marker is Syndecan-1 (CD138), that has been shown to bind fibronectin, collagen and basic fibroblast growth factor (b-FGF); PCs express the adhesion molecules CD44 and very late antigen-4 (VLA-4) the presence of several interplays between PCs and stroma.

Myeloma PCs express on their surface antigens such as $\alpha_v\beta_3$, VLA-4, LFA-1 MPC-1, CD54, CD56 which allow interaction with BMSCs and stimulate the production of IL-6, RANK ligand (RANKL), insulin-like growth factor, tumor necrosis factor alpha (TNF α), vascular endothelial growth factor (VEGF), stromal cell-derived factor (SDF-1) promoting their proliferation^[10-12].

During maturation, PCs acquire specific lineage antigens and lose early markers, thus, BM PCs exhibit CD138, the survival factor Bcl-2, adhesion molecules such as VLA-4 and the chemokine receptor CXCR4, while they lose the B cell phenotype [CD19, CD20, CD22, human leukocyte antigen-DR (HLA-DR), Pax-5] and the death receptor CD95^[13].

Cancer associated fibroblasts

Fibroblasts represent the main component of BM stroma. Several fibroblasts, activated by cytokines and growth factors circulating in the microenvironment such as FGF and transforming growth factor-beta (TGF- β), are called cancer associated fibroblasts (CAFs). Markers identifying CAFs are alpha smooth muscle actin (alpha SMA), fibroblast activation protein, fibroblast-specific protein-1, Thy-1, desmin, and S100A4 protein^[14]. CAFs are able to promote cancer cell growth and to increase the invasiveness of cancer and stromal cells through cell-cell interactions, the production of pro-invasive cytokines, chemokines and inflammatory factors. This interplay between PCs and CAFs may serve as direction for cancer migration, breaking of the adjacent ECM and basement membrane, which represents the first step for cancer cells escape into the blood system^[15,16]. Furthermore, CAFs act also in immune responses producing pro-inflammatory cytokines and chemokines which attract immune cells such as macrophages, neutrophils, and lymphocytes to cancer region^[17].

Tumor-associated macrophages

Macrophages moved towards cancer bulk attracted by chemotactic factors and here they differentiate into tumor-associated macrophages (TAMs) favoring tumor progression^[18]. Macrophages allow tumor cells to escape immune-surveillance creating a particular microenvironment characterized by chronic inflammation and immune tolerance allowing cancer to escape immune-surveillance^[19]. Besides TAMs release a number of factors such as VEGF, hepatocyte growth factor (HGF), matrix metalloproteinase-2 (MMP-2), IL-8 which influence ECs behavior. Our group showed that BM macrophages in patients with active MM contribute to build neovessels through vasculogenic mimicry^[20,21]. TAMs preserve their own CD14 and CD68 lineage markers, indicating that they do not trans-differentiate into ECs, but only adapt themselves functionally, phenotypically and morphologically to ECs, under VEGF and FGF-2 stimulation produced by PCs^[22]; moreover macrophages secrete themselves VEGF and FGF-2, inducing BM angiogenesis^[8]. Finally, BM monocytes and macrophages from patients with MM are able to form capillary-like structures through vasculogenesis *in vitro* and contribute to vasculogenic mimicry *in vivo*^[23].

Osteoblasts and osteoclasts

In patients with MM the physiological balance between bone resorption and bone formation is often altered as results of a dysregulation of osteoclast and osteoblast activity, resulting in the formation of osteolytic lesions,

accompanied by bone pain^[24]. Many factors are involved in osteoclast activation, including macrophage inflammatory protein-1 α (MIP-1 α), IL-3, and IL-6 and receptor activator of NF- κ B ligand (RANKL)^[25]. RANK is a transmembrane receptor expressed by osteoclast cells; the interaction of PCs with BMSCs induce an increase of RANK ligand (RANKL) expression which binds its receptor expressed by osteoclast precursor cells, promoting their differentiation by NF- κ B and Jun-N-terminal kinase pathways^[26]. A great contribution to bone destruction is also due to the inhibition of osteoclast apoptosis by RANKL and to the suppression of osteoblast activity. Besides osteoblasts are important in this mechanisms cause they are able to supports MM cells growth and survival thanks to the secretion of IL-6^[27,28].

Endothelial cells and angiogenesis

Tumor ECs are more different from that of healthy vessels. They have an higher proliferation rate, in according to the enhanced angiogenesis that typically contributes to tumor progression. MM ECs highly express antigens such as VEGFR-2 and Tie/Tek, FVIII-RA, CD31 and VE-cadherin and their activity depends on growth factors circulating in BM microenvironment. They have an unusual shape and they are very permeable thanks to the presence of fenestrae, vesicles and a discontinuous basement membrane and they participate to the formation of new vessels with tumor cells capable to form mimic vessels. Angiogenesis plays a critical role in tumor progression important for tumor growth, invasion and metastasis not only in solid malignances but also in haematological malignances, such as MM. Angiogenesis is a multistep process which occurs in the switch from the avascular to the vascular phase in MGUS patients and leads to the transition to MM. Our group demonstrated an increase of angiogenesis in BM biopsies from patients with active MM compared with MGUS patients which suggest the passage from an avascular phase in MGUS or SMM to a vascular phase in active MM. The angiogenic switch is accompanied by mutations in PCs which acquire an angiogenic phenotype secreting growth factors (VEGF, FGF, HGF and others), and stimulating chemotaxis and proliferation of stromal cells^[21].

EPCs and BM vasculogenesis

Vasculogenesis was first described as a phenomenon occurring in early embryogenesis, and was believed to not occur in adult tissues. In 1997, Moschetta *et al*^[29] purified a population of circulating cells that showed characteristics typical of ECs as well as progenitor cells, and identified these cells as “endothelial progenitor cells” (EPCs). In humans, EPCs are identified by the expression of VEGFR-2, CD34, VE-cadherin, CXCR4, CD31, CD133, CD105, CD144, CD106, and CD117 (c-Kit). Mobilization of EPCs may occur in response to factors secreted by ischemic tissues and by inflammatory and tumor cells and it results in the generation of new vessels. When EPCs are recruited to tumor sites, they differentiate in mature ECs, sustain neovessel formation *via* paracrine secretion of

proangiogenic growth factors and they integrate into the nascent vessels which are a mosaic of ECs, EPCs, tumor cells and macrophages^[29].

Many studies indicate that BM-derived circulating EPCs can take part to tumor angiogenesis and to sustain tumor cells proliferation. A great amount of EPCs have been found in the BM of patients with active MM compared with treated MM, MGUS, or healthy people, highlighting the increased angiogenic activity in MM patients. Besides, Ria *et al.*^[30] showed for the first time that EPCs had phenotypic and functional characteristics of the mature endothelium. In the MM BM microenvironment, they postulated that PCs and inflammatory cells recruit EPCs into tumor site, they induce their differentiation into ECs and they contribute directly in the formation of new vessels thus contributing to tumor vasculature.

STEM CELLS IN MULTIPLE MYELOMA

The implication of B cells in the pathogenesis of MM has been investigated by many groups because normal and myeloma PCs arise from their differentiation. The rearrangement of immunoglobulin gene and their resulting antibody allows to understand the different relationships between different clones in B cell tumors. Sequencing of immunoglobulin genes of MM PCs has underlined the presence of somatic hypermutation without intraclonal variation suggesting that MM arises from a post germinal center B cell compartment^[31]. Already in many tumors it has been shown the existence of cancer stem cells (CSCs) or cancer-initiating cells^[32-34]. While CSCs markers differ from one to another, their peculiar characteristics are common, such as self-renewal, tumorigenesis and drug resistance. Therefore, these stemness abilities are useful for identifying the MM stem cells. The idea of CSCs model bases on the concept that cancers are similar to hematopoietic system with an asymmetric division where CSCs should maintain cancer cells population.

The possible existence of MM CSCs was first postulated by Drewinko *et al.*^[35] that demonstrated the presence of a small population of MM cells with the capability of self-renewal in experiments with MM cell lines and primary cell lines from patients with MM. Then, Hamburger *et al.*^[36] and Pilarski *et al.*^[37] showed, respectively *in vitro* and *in vivo*, the capacity for self-renewal of MM primary lines. Finally, the onset of relapse in some patients after treatment gives the idea that, maybe, MM CSCs really exist.

Many studies underline the presence of different subpopulations able to give rise to MM: clonotypic B cell, clonotypic non B cell and side population (SP) cell.

Clonotypic B cells

MM cells are more functionally and phenotypically heterogeneous population. Within the tumor it has been identified a mature normal cell population and a minor one which is able to form tumor after transplantation into susceptible recipients in contrast to the first one. Matsui *et al.*^[38] demonstrated that clonotypic B cells are

capable to give rise to monoclonal immunoglobulin-secreting PCs *in vitro*. In particular, CD19⁺ B cells, isolated from MM patients, showed the capacity to form a new tumor in xenograft models implying the existence of cells with self-renewal ability. Moreover they found that CD138⁺CD34⁺ cells were able to form colonies *in vitro*, like CD138⁺ PCs *in vivo*, which present the same intracellular immunoglobulin light-chain restriction as MM patients.

Normal B cells maintain long-term immunologic memory thanks to the ability for self-renewal as well as the clonotypic B cells of MM lead to belief that myeloma PCs may arise from this compartment. Indeed Matsui *et al.*^[39] studying the CD138⁺ clonogenic myeloma cells, found that only cells which co-express CD19 and CD27 cell membrane antigen, typical of memory B cells, were able to form colony *in vitro*. They also reported that CD19⁺CD27⁺CD138⁺ cells isolated by peripheral blood of MM patients engrafted NOD/SCID (Non-obese diabetic/severe combined immunodeficiency) mice and gave rise to mature CD138⁺ MM PCs secreting M protein. CD19⁺ B cells isolated by these engrafted mice were able to induce MM in other recipient mice, underlining their self-renewal potential. These results mark that MM-initiating cells with tumorigenic ability, localized in clonotypic post-germinal B center, may differentiate and build up again the bulk of MM cells. Moreover, Boucher *et al.*^[40] showed that CD34⁺CD19⁺ immature B cells and CD34⁺CD19⁺ mature B cells, but not CD34⁺CD19⁺ cells, harvested from MM patients form colonies, suggesting that undifferentiated clonotypic B cell may present MM-initiating ability.

More recently, Kirshner *et al.*^[41] evaluated a novel *in vitro* 3D stromal culture system to study typical properties of BM microenvironment, in which results that tumor growth derived from clonotypic B cells. Pilarski *et al.*^[37] demonstrated that cells from the peripheral blood of patients with late-stage of MM or from patients with minimal residual disease, or cells mobilized through G-CSF, engrafted NOD/SCID mice. Engrafted mice presented high levels of circulating M protein and bone lesions as in patients with myeloma; besides these tumor cells could be transplanted successfully into secondary recipients indicating self-renewal ability. Indeed, Chaidos *et al.*^[42] found that the amount of circulating clonotypic B cells correlates with disease progression.

Clonotypic B cells play an important role in MM disease because they are also detected in MM patients with complete remission, becoming potential source for MM-initiating cells which could relapse.

Clonotypic non B cells plasma cells

Although all the evidences that clonotypic B cells could be MM CSCs, many studies demonstrate the clonogenic potential of non-B cell plasma cell population in MM.

First experiments demonstrating clonogenic ability of non-B cells was realized by Yaccoby *et al.*^[43]. They successfully induced human MM disease by intraosseous transplantation of CD38⁺CD45⁺ human cells in SCID mice implanted with rabbit femurs (SCID-rab mice)

or with human fetal bone fragments in SCID-hu mice creating a humanized microenvironment^[44]. In these models, the rabbit or human implanted bone fragments promote MM growth within the bone with several clinical aspects of MM including lytic bone lesions, hypercalcemia and circulating M protein. While, in the same work, Yaccoby *et al.*^[43] demonstrated that CD38⁺CD45⁺ peripheral blood B cells weren't able to engraft into SCID-hu mice as well as CD19⁺ B cells did not allow the xenograft in SCID-rab mice. But PCs regained from SCID-hu models were successfully transferred to secondary and tertiary recipients to produce MM disease with the clinical symptoms. In contrast, plasma cell-depleted BM cells did not induce MM disease in these models.

Hosen *et al.*^[45] studied the clonogenic MM plasma cells in terms of CD138 negativity in SCID-rab mice. Phenotypic CD138⁺CD19⁺CD38⁺⁺ PCs isolated from MM patients were engrafted in SCID-rab mice developing MM disease; moreover CD138⁺ PCs from patients were also able to induce MM in mice, although more slowly than CD138⁺ cells.

Recent studies carried out by Kim *et al.*^[46] tested the clonogenic potential of plasma cells in relation with BM microenvironment using NOD/SCID/common cytokine receptor γ chain-deficient (NSG) and recombinase-activating gene 2/common cytokine receptor γ chain-deficient (RAG2-/-) mice. The results of this work underlined the ability of only differentiated CD138⁺CD38⁺ cells, risen from patients, to repopulate of B lineage cells in human bone-bearing mice but no engraftments were detected in human bone-free mice. Besides, serial transfer of the disease to secondary recipients were possible. In these models, completely differentiated MM PCs enriched MM-initiating cells contrary to B cells. All these data showed that MM PCs can induce MM *in vivo* even in the absence of CD19⁺ B cells.

Finally, Paino *et al.*^[47] evaluated the presence and function of CD20⁺ putative MM stem cells population in several MM cell lines. In the RPMI8226 cell line they found a small subpopulation of CD20^{dim+} that was not essential for CD17-SCID mice engraftment. Moreover, CD20^{dim+} cells didn't differentiate into CD20⁻ cells, even if CD20⁻ cells can differentiate into CD20^{dim+} cells, suggesting a sequential differentiation order. All these outcomes showed that CD20 isn't a marker related to MM CSCs.

SP population

Moreover, the SP phenotype is characteristic of stem cells in various normal tissues as described by Challen *et al.*^[48] SP cells show a strong ABC (ATP-binding cassette) transporter activity resulting in high ability to efflux dyes as Hoechst 33342, a substrate for the ABC transporter ABCG2, also known as breast cancer resistance protein 1. SP cells have characteristics of stem cells such as the ability of self-renewal, expression of stem cell-like genes and resistance to chemo- and radio-therapy.

Loh *et al.*^[49] detected SP cells in both MM cell lines

and primary MM cells. Also Jakubikova *et al.*^[50] studied SP fraction in the same lines too. Their research showed that SP population is mainly presented in both CD138⁺ and CD138^{low} without any correlation between the lack of CD138 expression and amount of this fraction. Indeed they demonstrated that SP is composed of cells highly proliferating and with tumorigenic ability. Besides, they also showed that SP cells were susceptible to lenalidomide treatment in a dose- and time-dependent manner, in contrast, other authors displayed that lenalidomide results ineffective against other possible MM CSCs subpopulations (clonotypic CD19⁺ cells)^[39].

Interestingly, IMiDs reduced SP cells rate in co-culture with BMSCs, invalidating interplay between CSCs and the BM microenvironment; even if they did not affect ABC transporter function^[51].

Bone marrow stem cells

Bone marrow is a source of different tissue-specific stem cells, in fact BM hosts Bone marrow hematopoietic stem cells (BM-HSCs) and Bone marrow mesenchymal stem cells (BM-MSCs). Probably in the BM a universal adult progenitor exists and its phenotype could be modified by the local environment. Stem cells may move from BM to another tissue through the blood circulation and can differentiate into various types of stromal cells^[52]. A large part of BMSCs is composed by HSCs, important for the homeostasis of blood system, giving rise to all blood cells (lymphocytes, erythrocytes, monocytes, granulocytes and platelets). The distribution of HSCs in the BM is well-organized: the majority of HSC are located within the endosteal region, while progenitors and mature cells are principally sited in the central marrow area in proximity to the central marrow vessels^[53,54]. Most HSC express the CD34 antigen, an integral membrane glycoprotein that functions as a regulator of hematopoietic cell adhesion to BMSCs^[55]. Antigens such as CD90, CD117, and CD133 are also expressed by HSCs^[56]; moreover they express CD90, CD117, and CD133^[56] but not CD38, CD45RA, CD71, HLA-DR, or any other lineage-specific antigen according to their immaturity^[57]. Following injury HSCs are mobilized from the BM niche and they start proliferating to supply new mature cells.

The BM mesenchymal stroma is essential for the normal functioning of HSCs, ensuring their renewal and differentiation, creating an ideal microenvironment and contributing to the formation of the HSC niche^[58]. The first evidence of MSCs was reported by Cohnheim on "mesenchymal precursor cells" defining those cells as fibroblastoid, adherent, and extravasated cells at sites of tissue injury. In the early 1990s, these cells began to be known as "mesenchymal stem cells", cause they exhibit multipotential differentiation and self-renewal capacity^[59]. MSCs isolated from the adult BM have the ability to differentiate into osteoblasts, adipocytes, chondrocytes *in vitro* and to heterotopic osseous tissue when transplanted *in vivo*. MSCs express a large variety of surface markers including CD29, CD44, CD49a-f, CD51,

CD73, CD105, CD106, CD166 and they don't express typical hematopoietic lineage receptors such as CD11b, CD14, CD45^[60]. Wallace *et al.*^[61] compared BM-MSCs from patients with myeloma at diagnosis and normal donors to further examine the role of the bone marrow stroma in myeloma. They established that BM-MSCs from myeloma showed the same expression of adhesion molecules and of integrin, such as VLA-2, VLA-4, VLA-5, β 1, L-selectin, and CD44 and were negative for VLA-2, β 2, and β 3; however they had a weaker expression of VCAM-1 and fibronectin compared with normal BMSCs. Adhesion and migration cell abilities depend on the expression of adhesion receptors and on the type and concentration of ECM proteins. BM-MSCs low expression of cellular fibronectin, may cause localization and retention of malignant PCs in the BM because PCs lines show to move on fibronectin. Besides BM-MSCs express an intracellular RHAMM, a receptor for hyaluronan-mediated motility which induces the migration of several kinds of cells such as smooth muscle cells, fibroblasts, neuronal cells, and leukocytes. RHAMM activation results in a transduction signal which leads to cellular mitosis. Finally this study showed an over-expression of cytokines and growth factors by BM-MSCs, including IL-6 critical for PCs proliferation, survival and resistance. The analysis of mesenchymal cells in myeloma underlines the importance of BMSCs in myeloma cell growth and progression.

STEM CELL NICHE

Stem cells are located in a specific microenvironment defined niche. The niche and stem cells interact each other through adhesion molecules that activate molecular signals able to ensure stemness. For the first time, the idea of a stem cell niche was suggested by R. Schofield in 1978 for the HSC in BM^[62]. Schofield called "stem cell niche" the cellular environment, retaining stem cells. Until the stem cell restrains in the niche, its differentiation is avoided and so they replicate indefinitely as stem cell. Many groups investigate several types of stem cells and respective niches.

Osteoblastic niche

In human, hematopoiesis occurs in BM, where microenvironment creates optimal conditions for the HSCs maintenance and differentiation. Quiescent HSCs have been recognized near to the endosteal surface of the BM, in the trabecular bone, forming a source of HSC that can induce hematopoiesis after tissue damage. Lo Celso *et al.*^[63] showed that after transplantation, HSCs were localized closest to the endosteum and that as differentiation progressed, they moved to different locations^[63]. There are many pathways and molecules involved in the maintenance of stemness and stem niche. HSCs and osteoblasts interact through adhesion molecules, as N-cadherin (N-cdh) that is considered the most important cell-cell adhesion molecule expressed by many cancer cells. Vandyke *et al.*^[64] showed that expression of N-cadherin in primary MM cell lines correlates with

poor prognosis; in fact it has been demonstrated that N-cdh regulates HSCs proliferation^[64]. Sadler *et al.*^[65] proved that, inhibiting N-cadherin through a neutralizing antibody, the interaction between MM cells and stroma is prevented and it is induced the proliferation of HSCs compartment. Thus, stem cells are quiescent (G_0 stage) and they are not going to enter into the cell cycle until they received stimulations. Arai *et al.*^[66] found that osteoblasts express angiopoietin-1 (Ang-1) that binds Tie2 (tyrosine kinase receptor) expressed by BM-HSCs. This bond induces the activation of β 1-integrin and N-cadherin contributing to the close interaction between osteoblasts and stem cells necessary to ensure the maintenance of the stem cell and self-renewal capability.

The niche size is regulated by bone morphogenetic protein (BMP) signaling through its binding to osteoblasts receptor type IA (BMPRIA)^[67]; besides the stimulation by PTH (parathyroid hormone)/PTHrP (parathyroid hormone-related peptide) receptors produces high levels of Jagged 1, a Notch ligand, which recruit more HSCs in the niche. A study highlight Osteopontin (Opn) as another important osteoblasts receptor; in fact in these work knock-out mice showed that transplanted HSCs were randomly distributed within BM, whereas HSCs should be usually located at the endosteal region. This data suggests that Opn is involved in the interplay between HSCs and osteoblastic niche and it decreases HSCs proliferation^[68]. Furthermore, c-Myc is another regulator of HSCs fate: c-Myc deficient mice have a higher amount of HSCs in BM and their differentiation was maintained by interactions mediated by adhesion molecules between HSC and the niche, such as N-cadherin and integrins. Contrary, overexpression of c-Myc induces HSCs differentiation^[69]. Also ECM and its components play an important role in osteoblastic niche. Glycosaminoglycan hyaluronic acid (HA), is the major component of the ECM and its receptor, CD44, is a pleiotropic transmembrane protein presented by several cell types, including the HSCs and progenitors. HA is important in supporting and regulating hematopoiesis.

Osteoblasts are sited in endosteal area where there is a high calcium concentration, so Adams *et al.*^[70] supposed that on HSCs a sensor for calcium exists and it contributes to their homing and localization in the BM. They demonstrated this in CaR-deficient mice, showing that even if they had a healthy amount of primitive HSCs in the circulation and in spleen, they had very few of these cells in the BM^[70].

Vascular niche

Recently, vascular niche, was found within the sinusoidal vessels in BM or spleen. The presence of a vascular niche is confirmed by HSCs migration to the vascular region of the BM after injury, restoring hematopoiesis. So the vascular niche encourages proliferation, differentiation and the release in the bloodstream of HSCs^[71]. HSCs were identified as CD150⁺CD244⁺CD48⁻ cells, and many of them were in sinusoidal endothelium^[72], near to cells that express high level of CXCL12^[73]. Vascular niche is

also important not only in maintain HSCs but also in the regulation of the various phases of hematopoietic processes within BM, such as mobilization, migration, and differentiation of HSCs. So the discover of the vascular niche make the attention on its crucial role in blood system and hematopoiesis.

Kopp *et al.*^[74] showed that the only translocation of megakaryocyte progenitors near vascular sinusoids of BM was able to induce megakaryocyte maturation and platelet production. This process depends on chemokines such as SDF-1 and bFGF that enhance expression of adhesion molecules, as VLA-4 on megakaryocytes and VCAM-1 on BMECs. Besides, the breakdown of VE-cadherin-mediated adhesion BMEC makes in vascular niche incapable to induce megakaryocyte differentiation and to release platelet in peripheral blood. This focuses on the importance of ECs structural integrity for HSCs differentiation and homing. BM-ECs of sinusoidal vessels represent a barrier between BM stroma and the peripheral blood circulation, suggesting that they are implied not only in hematopoiesis but also in stem cells mobilization and homing^[74].

The different functions of osteoblastic and vascular niches aren't yet known. A difference between the two niches is the oxygen level: vascular niche presents a higher oxygen level than osteoblastic niche. In this conditions, cells resume their cell cycle, undergoing mitosis^[75].

Furthermore, a new model of hematopoiesis is proposed: in osteoblastic niche, in hypoxic environment, the HSCs are in G₀ stage. Under stimulation of cytokine such as G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-7, IL-3, IL-12, SCF, and flt-3 ligand and chemokines like IL-8, Mip-1 and SDF-1, HSCs could move to the vascular niche, undergoing differentiation and supplying cells of bloodstreams^[76]. When no longer HSCs needed, they could return from the vascular niche to the osteoblastic one, where they revert to the G₀ stage again. The movement of the HSCs between osteoblastic and vascular niches appears to be necessary for well-balanced hematopoiesis^[77].

Cancer stem cell niche

It has been proposed that as well normal stem cells niche exists, as CSCs niche could exist and interplay with the cancer niche may have a similar role in differentiation, proliferation and self-renewal capability of tumor cells. The interaction between the niche and CSCs prevents their differentiation in response to chemokines and growth factors as happens for HSCs in the osteoblastic niche. Increasing evidence proved that factors secreted by the cancer microenvironment regulate cancer cells. The niche is able to anchor stem cells in their appropriate microenvironment through adhesion molecules, such as cadherin and β -catenin. In addition, recent data support the role of the vascular niche in initiating metastasis; MMPs family molecules are involved in the process of cancer migration and also integrins have been reported to be associated with tumor cell homing and mobilization

in which SDF1 and CXCR4 play an essential roles. For metastatic process, tumor cells must reduce cell-cell contacts and migrate to distant sites. Thus, these molecules, involved in stem cells mobilization from the niche, represent possible targets to block tumorigenesis, cancer progression and metastasis^[78]. Evidences showed that factors derived from tumor stroma niche are able to regulate cancer cells and to direct their diffusion, in fact genetic studies have shown that stromal cells are altered in many tumors and supports cancer progression. Specialized microenvironments of BM-ECs are needed for the homing and engraftment of both normal HSCs and cancer cells^[79]. There are several possible hypothesis for CSCs niche: (1) CSCs may not require specific niche for survival and are able to survive in the healthy stem cell niche; (2) a distinct CSCs niche may be necessary for their activation and CSCs may dependent on this tumor-niche for expansion; or (3) an inhibitory niche for CSCs could exists which provides factors that induce differentiation or death; (4) CSCs may provide signals that activate an otherwise quiescent niche, thus signals from the CSCs could result in amplification of an activated niche that already exists; and (5) CSCs may be niche independent and they may have acquired the ability to provide themselves with the necessary factors for expansion and self-renewal^[80].

PATHWAYS IN MM STEM CELLS

Many signaling that preserve physiological stem cells, are also involved in CSCs maintaince and self-renew. Three pathways results most activated in cancer stem cells: Hedgehog (Hh), Wnt and Notch.

Hedgehog signaling plays a crucial role during embryonic development, and it regulates cell proliferation, migration and differentiation. Hh signal transduction involves three ligands *i.e.*, Sonic (SHh), Indian (IHh) and Desert (DHh) which bind the cell surface antigen Patched (PTCH). Hh pathway normally inhibits the Protein Smoothed SMO, a trans-membrane receptor with a high homology with coupled G protein. When ligands bind PTCH, SMO is de-repressed and regulates the activity of 3 GLI proteins which act as transcriptional regulators. In particular, GLI1 induces the expression of cell cycle regulator cyclin D1, inducing mitosis; GLI2 can act as positive or negative transcriptional regulator depending on post-transcriptional and translational modification; whereas GLI3 is a negative downstream effector of Hh and down-regulates genes transcription. Altered Hh signaling has been found in many human tumors. In myeloma, *PTCH*, *SMO* and *GLI1* were over-expressed in both human cell lines and primary MM PCs compared to normal PCs and B cells^[81].

Wnt pathway is an ancient and conserved signaling which regulates cell fate determination, migration and differentiation. In MM Wnt pathway is activated by the interplay of BM microenvironment. Wnt stimulation by bond with Frizzled (Fz) activates several intra-cellular pathways, including the canonical Wnt/ β -catenin way and the non-canonical Wnt/ Ca^{2+} one. Komiya *et al.*^[82]

showed that genetic modifications of Wnt signaling induce alterations in development and function of several organs, in particular, down-regulation of Wnt pathway supports proliferation of both MM cell lines and primary patient samples. Finally, the inhibition of Wnt pathway by small molecule inhibitor disrupts the maintenance of MM cells both *in vitro* and *in vivo*. This result encourages the development of Wnt-targeted inhibitors for MM therapy^[82,83].

Notch signaling is an evolutionary pathway highly conserved both in vertebrates and in invertebrates. Notch pathway is involved in various neoplastic processes such as tumor angiogenesis, EMT, metastasis.

Mammals express four Notch transmembrane receptors (Notch-1, -2, -3, -4) and five ligands named Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3, DLL4 which mediate Notch activation (cell to cell) in trans and inhibition (on the same cell) in cis. Activation of Notch receptors consists in three proteolytic cleavage which result in the formation of "notch transcriptional complex" (NTC) inducing the expression of many genes related to differentiation and survival, including hairy/enhancer of split (HES), the family of helix-loop-helix transcription factors, cyclin D, c-Myc^[84]. In MM, Notch activation advances cell proliferation and MM progression. Agarwal *et al.*^[81] searched the presence of Notch on BM clonotypic B cells from MM patients and found great expression of Notch that underlines Notch signaling implication in MMSCs.

DRUG RESISTANCE

Drug resistance remains an important complication to the cure of most cancers. During therapies cancer bulk initially response, but, over time, cancer stem cells may become drug resistant, thus therapies fail to eradicate them. So CSCs have not only the classic capacities of self-renewal and proliferation, but also are more resistant to chemo- and radiotherapy^[85]. But, in MM patients, drug resistance mechanisms that could arise during treatments, not explain completely MRD onset, though the BM microenvironment present components that can induce drug resistance and could reduce drug activity such as cytokines, stromal cells and ECM compounds.

Drug resistance development consists of three phases: (1) Cancer cells homing to the protective microenvironment represented by BM; (2) Initial microenvironment-mediated drug resistance; and (3) Development of microenvironment-independent resistance and acquired drug resistance.

Cancer cells homing to the protective microenvironment represented by BM

Cytokines and chemokines are more important in hematopoietic cells homing within BM. The major axis is the CXCR4/SDF-1 one, that promotes myeloma cells migration and homing to BM *in vivo* and *in vitro*^[86]. SDF-1 or CXCL12 is constitutively expressed by BMSCs and it is the main source of chemokines in adult. It retain hematopoietic cells and progenitors for growth and differentiation and impounds mature B cells to BM.

CXCR4 (CD184) is a G-coupled cell surface receptor and can be expressed by normal and malignant cells. The bond CXCR4/SDF-1 mediates cell survival, adhesion and migration contributing to tumor progression in several cancers. Moreover VLA-4-mediated adhesion to ECM (in particular to fibronectin and collagen) enhances the drug resistance in MM^[87]. The inhibition of this pathway not only blocks tumor homing and engraftment, but also avoids cell adhesion-mediated drug resistance and cells retain in BM decreasing VLA-4 expression.

Initial microenvironment-mediated drug resistance

In this phase, tumor cells acquire a soluble factor- and/or cell adhesion-mediated drug resistance (CAM-DR) *i.e.*, *de novo* drug resistance. The BM collects GFs and cytokines necessary to maintain cellular homeostasis. Among these GFs, IL-6 is essential to MM. The binding to its receptor on target cells induces STAT3 signaling that inactivates Fas-mediated apoptosis by up-regulation of Bcl-Xl with anti-apoptotic effect. Besides Frassanito *et al.*^[88] supported that myeloma clones secreting IL-6 were more resistant to both spontaneous and drug-induced apoptosis than non-IL-6 secreting clones that were sensible. Furthermore Voorhees *et al.*^[89] showed that blockage of IL-6 signaling in MM cell lines increases their sensibility to bortezomib.

Interaction between tumor cells and BMSCs is more complex than adhesion of integrin to ECM compounds alone because it involves other pathways and signaling events activated by adhesion molecules. In drug resistance development, cell-cell and cell-ECM interacts are more important and integrin-mediated adhesion to ECM compounds and stromal cells induces pathways that regulate proliferation, migration, and survival of normal hematopoietic cells. But integrins are also important in the tumorigenesis and integrin expression patterns are altered in tumor cells.

The role of VLA-4 has been investigated by several groups in mediating *de novo* drug resistance in hematopoietic malignancies. In these cases, CAM-DR is leaded by the interaction of cancer cells to ECM components and/or BMSCs *via* integrin $\alpha 4\beta 1$. Damiano *et al.*^[90] demonstrated that drug resistant MM cells up-regulate some integrins as $\alpha 4$, $\beta 1$ and $\beta 7$ while, when drug-sensible myeloma cells are seeded on fibronectin, a reversible *de novo* drug resistance phenotype was observed.

Thus, CAM-DR is characterized by non-transcriptional mechanisms into drug resistant cells. Adhesion-mediated survival and drug resistance pathways induced by soluble factors contribute to MRD allowing the development of more complex drug resistance mechanisms caused by the selective pressure of chemotherapy. Also the study of *de novo* drug resistance mechanisms from CAM-DR explains how tumor microenvironment promotes drug resistance onset.

Development of microenvironment-independent resistance and acquired drug resistance

Into BM microenvironment, cancer cells can survive to chemotherapies activity, resulting in MRD. In particular,

under continuous pressure of chemo-treatments, tumor cells acquire intrinsic genetic and epigenetic changes that lead to drug resistance phenotype without extracellular stimuli as soluble factor- and/or cell adhesion-mediated drug resistance. Furthermore, acquired drug resistance is mediated by intrinsic changes at the transcriptional level while *de novo* drug resistance is mediated by post-transcriptional mechanisms^[90]. A possible solution to destroy MM CSCs is targeting self-renewal and drug resistance signals specifically activated in CSCs: Hedgehog (HH), Wnt, and Notch pathways^[91,92].

For example, Nefedova *et al.*^[93] showed Notch1 receptors expression on MM cell lines that stimulates their adhesion to BMSCs, which express the membrane Notch ligand Jagged. The binding induces up-regulation of p21^{Cip1/WAF1} that encourages growth inhibition and protection from drug-induced apoptosis.

Indeed, the existence of a drug resistant sub-clone, which may be composted by CSCs and may expand during therapy, may represent the reason of tumor treatments fail^[94]. Most important, Peacock *et al.*^[95] affirmed that overexpression of RAR α 2 provided MM CSCs (CD138⁺ MM cells) drug resistance by activation ABCC3 gene through stem cell related pathways Hh (hedgehog) and Wnt. In effect, MM CSCs express also some functional markers such as drug efflux pumps (ABCC3), ALDH1 and RAR α 2 which have been associated with clonogenic potential and resistance to chemotherapy contributing to drug resistance and relapse in MM patients^[96].

Moreover, MM CSCs also express a telomerase which has a fundamental role in controlling normal stem cell functions and cancer drug resistance. Inhibitors of telomerase activity block MM CSCs clonogenic potential *in vitro* and *in vivo*, triggering differentiation of CD138⁺ cells into CD138⁺ cells and decreasing the number of ALDH1⁺ cells^[96].

CONCLUSION

Taken all together, these above studies provide strong evidence for existence of well-defined stem/progenitor cells possessing the three prominent features common to CSCs in all cancers: self-renewal, proliferation and drug resistance^[97].

Because MM remains still incurable, the idea of the MM CSCs has been suggested to explain the ability of self-renewal, survival and drug resistance of some myeloma cells. To evaluate this hypothesis, several groups use clonotypic B and non B cells and SP cells and transfer them into mice models with opposite results due to the different ability of BM microenvironment to sustain their survival inducing MM. Interestingly, there are many resemblances between the HSCs and MM CSCs about extracellular and intracellular receptors and signalings which can be employed to improve targeted therapy. So stemness pathways such as Hedgehog, Wnt and Notch, constitutively activated by the interaction between MM CSCs and their niche could represent new potential targets for MM treatment. The comprehension of interactions between

MM CSCs and the BM microenvironment will enable us to establish the necessary reasons for MM CSCs maintenance and avoidance from therapies inducing drug resistance^[98].

However, despite the introduction of novel therapy against MM, it still remains incurable. This shows the necessity to further elucidate causes of MM onset and implement innovative strategies in order to improve patient outcomes and reduce the rate of relapse.

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Stem cells for the treatment of musculoskeletal pain

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health conditions affecting more than one third of the adult population worldwide. Pain from various mechanisms and origins is currently underdiagnosed and undertreated. The complexity of molecular mechanisms correlating pain and the progression of musculoskeletal diseases is not yet fully understood. Molecular biomarkers for objective evaluation and treatment follow-up are needed as a step towards targeted treatment of pain as a symptom or as a disease. Stem cell therapy is already under investigation for the treatment of different types of musculoskeletal-related pain. Mesenchymal stem cell-based therapies are already being tested in various clinical trials that use musculoskeletal system-related pain as the primary or secondary endpoint. Genetically engineered stem cells, as well as induced pluripotent stem cells, offer promising novel perspectives for pain treatment. It is possible that a more focused approach and reassessment of therapeutic goals will contribute to the overall efficacy, as well as to the clinical acceptance of regenerative medicine therapies. This article briefly describes the principal types of musculoskeletal-related pain and reviews the stem cell-based therapies that have been specifically designed for its treatment.

Key words: Molecular biomarkers; Musculoskeletal pain; Stem cells

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Core tip: This minireview discusses how biomarker discovery and stem cell therapies for musculoskeletal pain can advance knowledge and contribute to improvement of quality of life of individuals suffering from this condition.

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Abstract

Musculoskeletal-related pain is one of the most disabling

INTRODUCTION

The musculoskeletal (MSK) system is comprised of the bones, joints, tendons, ligaments and other connective tissues within the body of an organism to provide form, support, stability and movement. The structural and functional integrity of these distinctive parts is crucial for organ functioning, as well as for its integration with other systems. The functioning of the MSK system influences complex inter-system connectivity, including from interactions between physical activity and the environment to interpersonal relationships and social integration. The status of the MSK impacts metabolic activities and overall health in individuals of all ages, as well as functional independence and cognitive preservation in the elderly^[1].

MSK diseases are a group of various acute and chronic traumatic, degenerative, tumoral or congenital conditions affecting one or several components of the MSK system. More than 150 MSK diseases or symptoms have been identified, comprising the most frequent cause of physical disability in the developed world. The global burden of MSK disease is dramatically rising, mainly due to the ageing of the population and incidence of musculoskeletal trauma^[2]. Pain and loss of function are major symptoms associated with MSK disease (Figure 1), which impact quality of life and result in increasing health expenditures that are a major concern at individual, familial and societal levels.

Although current therapies only partially address MSK diseases, regenerative medicine (RM) provides the potential for efficient prevention, early detection, and treatment^[3]. The use of stem cell therapies and stem cell-based tissue engineering offer clinically relevant solutions for the treatment of bone and cartilage defects, osteoarthritis (OA), inflammatory arthritis and tendon injuries^[4]. Moreover, RM provides an opportunity to design therapies that are noninvasive, avoid the use of inert implants, and shorten rehabilitation time^[5]. Although the primary goal of RM is complete structural and functional restoration of MSK tissues and organs, it may not be a realistic one due to the heterogeneous nature of MSK diseases, which include a wide diversity of associated pathologies and co-morbidities. As an example, different stages of OA and the complex situations generated by associated metabolic conditions present as distinct clinical entities. Such cases can be regarded as different forms of the same disease, which should be considered when designing RM therapies rather than aiming for a “one-size-fits-all” solution.

A clearly defined therapeutic goal backed by realistic assessment of individual necessities could help increase clinical applicability of RM interventions. It is known that MSK-related pain ranges from merely a symptom to a disease that solicits specific therapeutic intervention, such as with neuropathic pain in advanced stages of OA, or in OA associated with diabetes mellitus, complex regional pain or entrapment syndromes. Pain treatment should be regarded as a distinct therapeutic goal for functional and

structural rehabilitation of the MSK system. This article briefly describes principal types of MSK-related pain and reviews stem cell-based therapies that are specifically designed for its treatment.

PRINCIPAL TYPES OF MSK-RELATED PAIN

Acute pain

Two of the most common causes of acute pain are MSK trauma or inflammation. Trauma can be isolated or encountered during the onset or flare-up of chronic conditions (such as inflammatory arthritis, OA, or tendonitis). The intensity and duration of acute pain varies, and usually correlates with the intensity and duration of the producing agent. Acute pain has been defined by the International Association for the Study of Pain as “the normal, predicted physiologic response to an adverse chemical, thermal, or mechanical stimulus[...]associated with surgery, trauma, or acute illness^[5].” However, in its severe forms, acute pain needs to be regarded as potentially life threatening^[6,7].

Acute pain is the result of tissue-specific nociceptor activation, sensitization and hyperalgesia. Nociceptors are the sensory receptors activated by injury that have different reactivity levels, and are tissue- and stimuli-dependent. Primary hyperalgesia from trauma or acute inflammation is produced by nociceptor sensitization. Secondary hyperalgesia refers to the increased responsiveness of central nervous system neurons to otherwise normal or subliminal peripheral afferent input, termed central sensitization, resulting in increased pain responses to stimuli that are outside the area of the initial injury^[8]. Acute pain is normally self-limited, and can resolve completely or transform into chronic pain and/or generate immediate or delayed complications ranging from circulation and infection to complex post-traumatic syndromes.

Chronic pain

Chronic pain in the MSK system can occur due to unresolved acute pain, or arise as a distinct phenomenon. Degenerative diseases, tumors of articular cartilage, tendon, ligament or intervertebral discs, typically produce chronic pain of progressive intensity that alternates between periods of spontaneous relief and activation. MSK-related chronic pain particularly impacts the quality of life of the individual and family, as it is commonly associated with loss of mobility, which is the primary impediment to living independently^[8]. Moreover, chronic pain is involved in functional decline, muscle weakness and increased propensity to fall in the elderly^[9]. Individuals of all ages experiencing severe disabling chronic pain report poorer self-rated health, mental well-being and social functioning, and have high levels of depression and work loss^[10].

Chronic pain results from the persistent activation of nociceptors that produce increased release of neurotransmitters in the central neurons located in the dorsal horn of the spinal cord. At the same time, neuroinflammatory

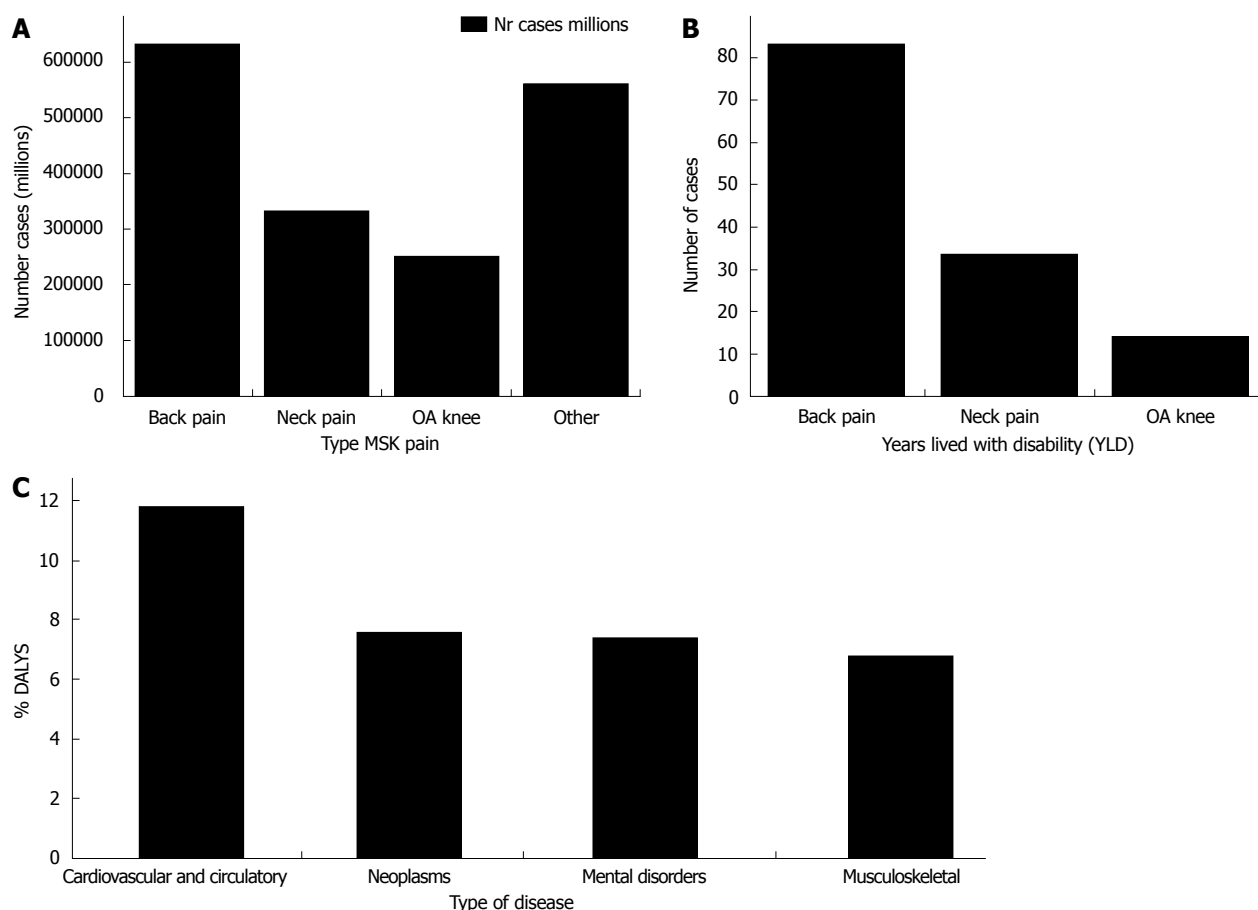


Figure 1 Prevalence of musculoskeletal pain. A: Number of persons affected by musculoskeletal pain (millions); B: Global number of years lived with disability (YLD; millions); C: Causes of disability-adjusted life years (DALY). Data are as of 2010, updated from the Source Global Burden of Disease 2010 Study^[75]. OA: Osteoarthritis; MSK: Musculoskeletal.

peptides, such as substance P and calcitonin gene-related peptide, are released from the nerve endings at the site of injury and/or tissue inflammation and potentiate the inflammatory response^[11].

Neuropathic pain

Both mechanisms of peripheral and central sensitization have been involved in the appearance of a distinct form of chronic pain associated with MSK diseases, neuropathic pain. Neuropathic pain is the direct consequence of a lesion in the somatosensory nervous system^[12], involving tissue-specific nerve endings, medullary dorsal horn neurons, and the peripheral or central neurotransmitter balance. Neuropathic pain is a consequence of disproportionately enhanced intensity from painful stimuli (hyperalgesia) or of altered modality, such as hyperpathia or allodynia (pain produced by otherwise non painful stimulation). MSK-related neuropathic pain can also result from nerve entrapment, complex regional pain syndrome, joint pain associated with diabetes mellitus, and sensory nerve injury in advanced stages of OA^[13].

The assessment and discrimination of neuropathic pain remains difficult and subjective, as it is currently diagnosed from the description of pain characteristics (e.g., “burning”, “aching”, “lancinating”). However, new methods have been described that are designed to measure

peripheral and central pain in subjects with OA, which could allow for objective MSK pain evaluation in clinical settings in the near future. Quantitative sensory testing^[14] and assessment of pain threshold using algometers have been used to demonstrate pain sensitization in OA patients, which is likely due to the increased firing of peripheral nociceptors^[15]. In addition, brain neuroimaging tools, such as functional magnetic resonance imaging or fluorodeoxyglucose positron emission tomography, have been used to observe the activation of brain pain centers in hip and knee OA patients^[16,17].

Pain in the MSK system has distinct characteristics depending on the tissue of origin, such as the various modalities of tissue-specific pain receptor activation. For example, muscle-, tendon- or joint-specific pain receptor activation generates pain of different intensities, duration and therapeutic responsiveness. Improved knowledge about the molecular pathways involved in the generation of different tissue-specific types of pain is important for the design of targeted therapies^[18].

MOLECULAR MECHANISMS OF MSK PAIN

Inflammation plays an important role in the occurrence

of acute and chronic MSK pain. Persistent aberrant inflammation is related on a molecular level to MSK pain produced by peripheral or central sensitization. The proinflammatory cytokines interleukin (IL)-1, IL-2 and IL-6^[19] are associated with different intensities of MSK pain^[20]. Tumor necrosis factor (TNF)- α , a key molecule involved in inflammatory processes in various acute and chronic MSK diseases^[21], is also a pain mediator^[22] that maybe involved in the development of neuropathic pain of MSK origin^[23]. IL-17 has been demonstrated as involved in the development of neuropathic pain in rodent models^[24], and may contribute to neuropathic pain from rheumatoid arthritis as it is present in the synovial fluid of patients and IL-17 knockout mice do not develop collagen-induced arthritis^[25]. Proinflammatory cytokines are also released by microglia (the immune cells of the central nervous system) in response to trauma, infection, inflammation and ischemia, thereby contributing to the immune response as well as to alteration of neuronal function. Neuropathic pain has been shown to result from microglial activation *via* adenosine 5'-triphosphate receptors or persistent stimulation of p38 mitogen-activated protein kinase^[26].

OA pain can effectively be interrupted by local anesthetic treatment or after total joint replacement, suggesting that structures responsible for pain are in contact with the intra-articular milieu^[27]. Synovial tissue is one of the joint components that generate pain *via* several mechanisms. The extensive network of free nerve endings in synovial tissue could be implicated in both inflammatory and neuropathic pain. Increased synovial mononuclear infiltration and overexpression of inflammatory mediators, such as TNF- α and IL-1 β , are implicated in pain production during early stages of OA^[28]. During late stages of OA, the local nerve endings in fibrotic tissue^[29] or within lymphoid aggregates^[30], as well as the increased expression of neuromediators such as substance P and calcitonin gene-related peptide, could contribute to generation of neuropathic pain^[31]. Subchondral bone marrow and marrow cavities of osteophytes contain perivascular and free nerve fibers as well as nerve trunks that could be involved in generating neuropathic pain in advanced stages of OA^[32,33].

Indeed, OA progression has been shown to correlate with pain. A community-based population study found that pain was a predictor of structural and radiographic changes in hip OA; patients with hip pain were more likely to exhibit minimum joint space narrowing and undergo total replacement^[34]. In addition, a lesser degree of knee pain was weakly associated with loss of knee cartilage volume as assessed by quantitative magnetic resonance imaging, but not with Western Ontario and McMaster Osteoarthritis Index scores, joint space width, or levels of urinary biomarkers of cartilage degradation^[35]. However, pain intensity did not correlate with the severity of rotator cuff tear in terms of size, retraction, or superior humeral head migration^[36,37]. In our own experience, severe pain in patients with knee OA and diabetes mellitus is not correlated with radiologic and magnetic resonance

imaging findings (unpublished data) even in the absence of diabetic polyneuropathy or peripheral vascular disease, and advanced stage OA (Kellgren-Lawrence 3) can be detected by radiologic examination in some asymptomatic patients (Figure 2). These findings may be explained by individual differences in pain thresholds, psychological and emotional states, or the presence of comorbidities.

STEM CELLS FOR PAIN TREATMENT

Advancements in stem cell research may provide more effective on-pharmacologic treatment methods for various types of MSK-related pain. Such therapies could reduce or eliminate the need for repetitive drug administration and have longer-lasting effects, thus improving patient quality of life and reducing healthcare costs. To this end, several stem cell types are envisaged as having a potential for immediate clinical application: mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), and genetically modified stem cells.

MSCs for the treatment of MSK diseases

MSCs or mesenchymal stromal cells are phenotypically heterogeneous populations of adult progenitors of mesodermal origin^[38]. Initially thought to reside only within bone marrow stroma^[39], MSC populations are found in a large variety of mesenchymal (fat^[40], muscle^[41], trabecular bone^[42]) or non-mesenchymal (dental pulp^[43], umbilical cord blood^[44]) tissues. MSCs of various origins have been shown to proliferate into clinically relevant amounts of cells and to differentiate into several specific mesenchymal lineages such as osteoblasts, chondrocytes, tenocytes, and adipose tissue. MSCs are currently being tested in several RM strategies of cell therapy and tissue engineering for the repair/regeneration of MSK tissues. Based on their capability of engraftment, *in vivo* differentiation and integration within the host, MSCs were initially sought for structural repair of damaged or diseased tissue^[45,46]. More recently, MSCs have been shown to exert cytokine-mediated paracrine effects contributing to tissue repair not only by direct participation, but also by means of recruiting local cells to induce repair^[47].

The immunomodulatory properties of MSCs contribute to the restoration of tissue homeostasis when used as a cell therapy^[38]. Moreover, MSCs are considered immune privileged as they express low major compatibility class I molecules but not class II, making them suitable as a source for allogeneic cells^[48]. However, recent reports describe the generation of antibodies against, and immune rejection of, allogeneic-infused MSCs. Although the clinical manifestations are benign, it is not known whether the immunogenicity influences their therapeutic effect^[49]. MSCs are known to modulate allogeneic immune responses by inhibiting maturation of dendritic cells and the proliferation and differentiation of B and T lymphocytes^[50]. MSCs act by decreasing inflammatory cytokines, such as IL-1 β and TNF- α , and by enhancing the amount of anti-inflammatory cytokines released by T cells^[51]. Autologous or allogeneic

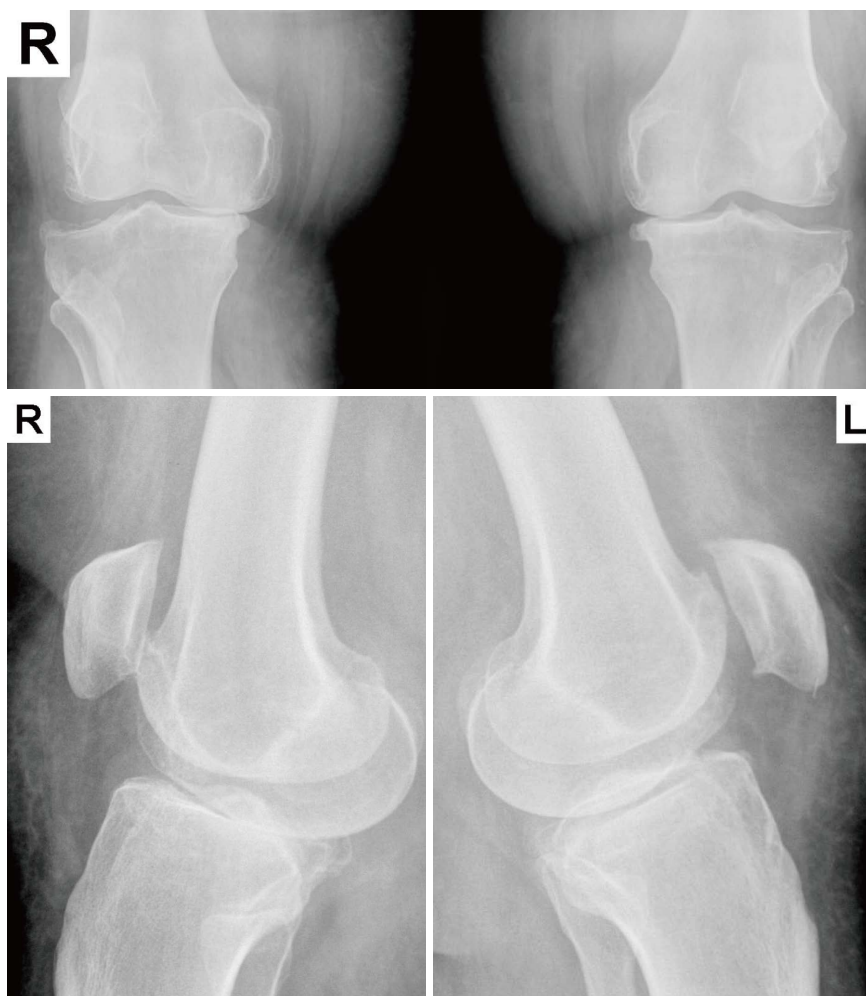


Figure 2 Radiologic evidence of asymptomatic Kellgren–Lawrence grade 3 knee osteoarthritis. Bilateral knee anteroposterior (top) and profile (bottom) plain X-rays from a 57-year-old asymptomatic female patient taken during emergency care after a traffic injury.

MSCs have already been used in several clinical trials for the treatment of various MSK diseases, such as bone defects and non-unions, cartilage defects, osteoarthritis, and tendon suture augmentation^[52,53].

MSCs for the treatment of MSK pain

The immunomodulatory properties of MSCs together with their natural capability of homing to the site of injury and inflammation make them attractive for the treatment of post-traumatic or inflammatory pain, and they are currently being used in preclinical and clinical trials for the treatment of various types of MSK-related pain. Proinflammatory cytokines in the serum or synovial fluid has been associated with OA-related pain in animal models^[54], IL-6 and TNF- α in synovial fluid^[55], and TNF- α and IL-1 β in vastus lateralis muscle of late stage OA subjects correlate with the degree of experienced pain or muscle atrophy^[56]. It was suggested that the increased intensity of pain in those with degenerative disc disease compared to those with herniated discs is due to increased levels of TNF- α and IL-8 in the nucleus pulposus^[57].

The use of MSCs as an anti-inflammatory agent is considered an attractive and convenient method for pain relief. In a pilot study, low back pain and disability produced by degenerative disc disease was successfully treated by local MSC infusion, to a degree comparable with surgery

for spinal fusion^[58]. Encouraging interim results have emerged from an ongoing phase 2 trial testing injections of allogeneic MSCs into degenerated lumbar vertebral discs, indicating significant pain reduction and functional improvement when compared to patients treated with hyaluronic acid^[59]. Successful use of MSCs has been demonstrated for OA treatment in animal models^[60], and for prevention of post-traumatic arthritis^[61]. The alteration of intra-articular and serum cytokine levels reported by these studies may provide a basis for a method of pain treatment in both degenerating and traumatized joints.

A systematic review including eight completed studies and a total of 844 procedures of local autologous MSC delivery for the treatment of OA in human indicates that the procedure is safe, with no reported major adverse effects related to implanted cells^[62]. Pain assessed by visual analogue score and functional outcome in 50 knee OA patients were significantly improved by treatment with MSC concentrate (buffy coat) compared with arthroscopic debridement only^[63]. Functional improvement after 24 mo as measured by the American Orthopaedic Foot and Ankle Society's ankle-hindfoot scale was reported after surgical debridement of talar dome defects and local delivery of concentrated bone marrow nucleated cells supported by collagen powder or hyaluronic acid membrane as scaffolds^[64]. Core decompression and local delivery of

bone marrow auto grafts improved Harris Hip scores and significantly reduced the need for arthroplasty when the procedure was performed in patients before the collapse of the joint surface^[65]. Percutaneous delivery of culture-expanded autologous MSCs into OA knee joints resulted in improvement of visual analogue scores, physical therapy assessments, as well as increased cartilage volume^[66]. The same group conducted a safety study on 227 clinical cases receiving intra-articular delivery of cultured MSCs for the symptomatic treatment of various OA joints, and reported three cases of self-limiting cell-related complications as the only safety issues^[67]. No malignant transformations were detected during the two years of follow-up.

A large variability remains among reported methods for MSC isolation, purification and culture expansion. The development of MSC-based therapies requires concerted efforts for the establishment of optimized and standardized protocols for these methods, as well as for cryopreservation and storage of MSCs, which will significantly improve their clinical translation and provide new opportunities for cell-based pain treatment for MSK diseases^[68].

MSCs for the treatment of MSK-related neuropathic pain

Although neuropathic pain is a potentially disabling disease that dramatically decreases quality of life, the complexity of the involved molecular pathways makes it difficult to treat. Neuropathic pain from MSK conditions is often underdiagnosed and undertreated^[69], and when diagnosed, is treated with pharmacologic agents such as antidepressants, opioid analgesics and tramadol, which have a risk for addiction as well as of inducing other side-effects that can interfere with a normal, active life^[70].

Neuropathic pain has been associated with neuro-inflammation and IL-17 signaling in a mouse model of peripheral nerve injury^[24]. Inhibition of TNF- α -induced astrocyte and microglia activation can attenuate neuropathic pain, and IL-6 inhibition was shown to prevent vincristine-induced allodynia in rodents^[71]. In humans, increased biochemical markers of inflammation were found in patients with painful diabetic neuropathy^[72], and neuropathic pain correlates with joint inflammation in knee OA^[13]. In addition, increased levels of IL-1 β and IL-6 were found in cerebrospinal fluid of patients with complex regional pain syndrome^[73].

MSCs have been investigated as anti-inflammatory agents for the treatment of MSK-related neuropathic pain. In a mouse model of streptozotocin-induced diabetes, toll-like receptor 3-primed, immunosuppressive human MSCs^[74] attenuated neuropathic pain behaviors^[75]. Systemically delivered human MSCs also exhibit long-lasting improvement of neuropathic pain in a spared-nerve injury mouse model, observed as decreases in IL-1 β and IL-17, and increases in anti-inflammatory IL-10 and a marker of alternatively activated macrophages in the spinal cord^[76]. These increases in anti-inflammatory cytokines were detected for up to 90 d, demonstrating the lasting effect from a single injection of MSCs.

MSCs have also been examined as neuroprotective

agents in neuropathic treatment. Intravenous infusion of bone marrow mononucleated cells reversed neuropathic pain in rats produced by chronic constriction of the sciatic nerve, though the underlying mechanism of action could not be clarified^[77]. Bone marrow MSCs injected into lumbar dorsal root ganglia of mice with sciatic nerve injury migrated to ganglia on the ipsilateral side and prevented mechanical and thermal allodynia by attenuating injury-induced neuropeptide expression^[78]. In preclinical studies, MSCs were found to have a role in neuroprotection and axonal growth stimulation^[79]. There are several clinical trials underway assessing the safety and efficacy of allogeneic MSCs for the treatment of spinal cord injury^[80]. The potential for MSCs to reverse somatosensory system dysfunction in MSK-related neuropathic pain is still a matter of further investigation.

iPSCs for the treatment of MSK pain

The generation of iPSC populations from adult somatic cells by forced expression of several transcription factors represents a major scientific breakthrough and provides a new tool for RM. Patient-specific iPSCs could be used as autologous cell sources for any RM application by avoiding immune rejection and improving therapeutic specificity^[81]. iPSC technology is being developed for RM application, with the first clinical trial underway in Japan for the treatment of age-related macular degeneration^[82]. However, several important problems still need to be addressed, such as the method of delivery, the efficiency of reprogramming factors, and the genomic stability and epigenetic memory of reprogrammed cells. iPSCs will provide a major contribution to understanding molecular disturbances involved in the production of persistent, recurrent and MSK-related neuropathic pain through the generation of patient-specific disease models. Moreover, patient-specific iPSC-based models of complex neurosensory mechanisms that culminate in the generation of pain may enable the development of new drugs for patient and disease-targeted therapies.

Genetically modified stem cells

Stem cell-mediated gene therapy is regarded as a promising method of gene delivery that could provide long-term therapeutic impact^[83]. Due to their lifespan and homing capabilities, MSCs are appealing tools for sustained gene delivery in various therapeutic scenarios. MSCs can be engineered to express a variety of proteins both *in vivo* and *in vitro* for use in a variety of genetic or acquired diseases. The expression of these therapeutic genes could last the lifetime of the patient, thus dramatically improving quality of life and reducing healthcare costs. MSC-mediated gene therapy for chronic pain management is therefore an appealing avenue for research. *In vitro*, expression of the human preproenkephalin gene by MSCs increased the production of the opioid peptide, met-enkephalin, which could serve as a safe supply for the treatment of opioid-sensitive pain^[84]. Furthermore, hybrids produced by the fusion of opioid-producing chromaffin cells and

MSCs express a catecholamine-producing phenotype, which could be used to generate a clinically significant population of therapeutic cells^[85].

ESTABLISHING ENDPOINTS FOR THE TREATMENT OF MSKDISEASES

MSK-related pain is a widespread, potentially disabling condition for which effective treatments are lacking^[86]. MSK pain can evolve from a symptom to a difficult-to-treat condition. Mechanisms of central sensitization are known to contribute to the maintenance of a local inflammatory microenvironment that aggravates pain and affects regional structure and function. Its roles in the abolishment of biomechanical stimuli necessary for normal tissue maintenance and repair have not yet been fully evaluated. A correlation between muscular atrophy and/or sarcopenia and MSK degeneration has been demonstrated^[87], and MSK pain-related stiffness and muscular atrophy consistently decrease quality of life and interact with reparatory mechanisms. MSK system loss-of-function could be at least partially prevented if pain as a symptom or a disease could be more effectively treated. However, the molecular mechanisms implicated in pain-mediated MSK disease progression remain to be elucidated^[88]. The identification of reliable biomarkers for diagnostic and targeted therapy is a stringent clinical necessity in MSK pain treatment.

RM and the use of stem cells show promise for several chronic non-life-threatening yet disabling conditions such as MSK-related pain. Several ongoing clinical trials are testing various forms of autologous or allogeneic, freshly isolated or cultured MSCs for the treatment of OA^[89], tendon^[90] and intervertebral disc degeneration^[91]. It is therefore possible that therapeutic goals for MSK diseases can be re-established with the availability of more tangible results. Possible strategies such as elimination of local nociceptive stimuli, reversing the mechanism of central sensitization or somatosensory neuroprotection using paracrine effects from local, regional or systemic MSC delivery could prevail over the attempts of recomposing MSK structure.

The large majority of current clinical trials focusing on tissue repair/regeneration have pain reduction as secondary endpoint. From a clinical perspective, it is important to evaluate the long-term safety and efficacy of pain control in disabling MSK disorders. Not only will this allow for accelerated acceptance of such therapies among clinicians and patients, but it will also likely contribute to structural rehabilitation generated by intrinsic mechanisms that resume in the absence of pain. The discovery of pain-related biomarkers will offer a tool for quantitative evaluation of pain and its correlation with degree of structural impairment in various MSK conditions. Furthermore, validation of these biomarkers will provide important therapeutic targets as well as methods for disease evaluation and treatment follow-up.

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Advances in cellular technology in the hematology field: What have we learned so far?

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Abstract

Despite the advances in the hematology field, blood transfusion-related iatrogenesis is still a major issue to be considered during such procedures due to blood antigenic incompatibility. This places pluripotent stem cells as a possible ally in the production of more suitable blood products. The present review article aims to provide a comprehensive summary of the state-of-the-art concerning the differentiation of both embryonic stem cells and induced pluripotent stem cells to hematopoietic cell lines. Here, we review the most recently published protocols to achieve the production of blood cells for future application in hemotherapy, cancer therapy and basic research.

Key words: Hematopoietic stem cells; Progenitor; Induced pluripotent stem cells; Reprogramming; Embryonic stem cells

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Core tip: In the past few years, considerable advance has been made possible in the field of cellular therapy, both in its basic and clinically applied form. Stem cells may have important future applications in hematology, with the possibility of supplying the blood needed for transfusion with the use of blood products produced *in vitro*. Thus, in this paper, we summarize the recently established protocols for differentiating both embryonic and induced pluripotent stem cells into blood cells for possible future use for hemotherapy.

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INTRODUCTION

Hemotherapy is considered to be the collection of techniques in which whole blood or its fractions are transfused into a patient in order to re-establish hemostasis and to treat certain conditions. It may be carried out using stocked whole blood or by using processed concentrates, red blood cells (RBCs), platelets or plasma, according to the needs of the patient. The blood transfusion, however, is closely related to causing iatrogenic events as a consequence of predictable or unpredictable reasons. Transfusion-related acute lung injury and hemolytic transfusion reaction (HTR) are the more commonly related conditions following blood transfusions which hinder the execution of this procedure with the life-threatening nature of these consequences^[1].

In order to minimize the inherent risks, blood transfusion requires immunological compatibility between the blood of the donor and recipient to avoid HTR and to ensure the therapeutic efficacy of the RBC concentrates. Identifying the blood group-related antigens in the surface of blood cells to be infused and the determination of hematological and immune status of the recipient serum are mandatory procedures before the blood transfusion. Many scientific works have made efforts towards the production of universal RBCs which may be transfused regardless of the antigenic phenotype of the recipient. These efforts tend to focus on management of cells by treating the AB antigens with polyethylene glycol or by erasing the AB epitopes by enzymatic digestion with glycosidase^[2,3]. However, these strategies have limited benefits and do not apply to silencing phenotypes other than the AB antigens or to impose the expression of a specific antigen in the cells.

The previously cited immunological compatibility between donor and receiver is due to the presence of membrane antigens on the donor cells that are bound to elicit an immune response in the receiver. More commonly discussed are the AB antigens that define the ABO group to which the individual belongs, so as to be used as guidance in blood transfusions, and are also aimed at when attempting to diminish eliciting an immune response of donor cells^[2,3]. However, transfusion-related iatrogenesis still happens despite using ABO compatible blood. Considerations about other possible antigens are necessary due to the occurrence of immune response that is not connected to the AB antigens. Thus, the phenotype of antigens of individuals may be classified into two clinically relevant groups, defined according to the frequency in which those antigens appear: public

antigens, the collection of antigens present in the great majority of the population; private antigens, those found in very few individuals^[4]. In order to prevent immune response, it is intuitively necessary to ensure compatibility of the greatest number of antigens possible. Matching donor and recipient bloods correctly would be highly unlikely due to the numerous phenotypes of blood cell antigens, rendering it necessary to perform immunological testing on the serum of the recipient in order to screen for the presence of antibodies against the most common antigens, namely public antigens. However, attainment of RBCs expressing a negative phenotype for a public antigen or a positive phenotype for a private antigen is difficult. Circumvention of this problem may only be done reliably by the installation of worldwide facilities for banking rare bloods^[4], which is logistically complicated and would add high costs to blood transfusions^[5].

It is necessary to consider the context of where the production of blood cells that induce no or less immune response is in order to achieve safety of the blood transfusion processes. The development of *in vitro* protocols capable of generating functional antigen-controlled cells is mandatory, as well as the compilation of those protocols. The use of stem cells characterizes an attempt to engineer blood cells which could serve the purpose of supplying the need of health systems for considerably voluminous amounts of safer blood products for transfusion, as well as for other applications in research that may culminate in future therapeutic protocols, including the production of cancer-targeted lymphocytes. Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been reported to be successfully differentiated *in vitro* to cells constituting blood products^[6-8].

DIFFERENTIATION OF ESCS TOWARDS BLOOD CELL PRODUCTION

ESCs may provide an inexhaustible and donorless source of cells for human hemotherapy, with the possibility of being indefinitely propagated in appropriate culture conditions. In addition to the proliferation competence of ESCs, these cells also display potentiality to differentiate into all tissues found in an individual, including hematopoietic differentiation. The possibility of manipulating the expression of antigen genes by homologous recombination is another feature that makes ESCs a suitable tool to generate blood cells of interest^[9]. Thus arises great interest in using human ESCs in order to supply the need for blood products. *In vitro* hematopoietic differentiation of ESCs has already been well documented along with the hematopoietic precursors involved, erythroid, myeloid, macrophage, megakaryocytic and lymphoid^[10-17]. Nevertheless, large-scale production of functioning blood cells is still in development. Substantial technological advances have been made in engineering mature hematopoietic tissue from murine ESCs; publications by Kitajima *et al.*^[18] (2003), Kennedy *et al.*^[19] (2003) and Fraser *et al.*^[20] (2003) display the practical aspects of murine cell differentiation^[18-21].

Here we have chosen to review the protocols being established in order to differentiate human ESCs into the various cell lineages of mature blood cells, including the differentiation to megakaryocytes through which platelets may be acquired, as well as to analyze the results obtained by the most recent advances.

Production of erythrocytes

The *in vitro* generation of RBCs is of particular interest as an alternative to classic transfusion in the sense that it could provide cells of a particular phenotype circumventing the problems related to immune response upon transfusion and, in addition, it would diminish the risk of infection by blood-borne pathogens^[22-24]. However, the viability of using the produced cells depends on their functionality and the capability of the method of producing enough quantity of blood product, factors still being developed by ongoing research.

Various protocols intended to achieve satisfactory erythrocytic differentiation of ESCs have been developed. As a consensus, the protocols rely on appropriate culture conditions and the use of cytokines that will be discussed later. Erythropoietin (Epo), responsible for activating anti-apoptotic pathways and stimulating hemoglobin synthesis, and stem cell factor (SCF) act mainly to promote proliferation of the erythroid progenitor cells and seem to be the two central factors in this differentiation^[25]; nevertheless, more recent research has been able to perform erythrocytic differentiation independently of Epo^[26], as detailed later.

The underlying regulatory molecular mechanism involved in the differentiation discussed in this topic requires alteration in expression of transcription factors of the GATA family. GATA1 is closely related to hematopoietic differentiation, including the erythroid lineages, and is mostly expressed during the final steps of the pathway by which RBCs are formed. GATA2, however, is responsible for maintaining the less differentiated status of the cells and proliferating^[27].

Production of erythrocytes via hemangioblasts:

Although extensively investigated, *in vitro* hematopoietic differentiated RBCs were not reported to successfully carry oxygen until the studies by Lu *et al.*^[28] in 2008, in which hemangioblasts were used as an intermediate for differentiation. Despite the success, the RBCs derived from *in vitro* differentiation still displayed structural differences concerning the globin chains expressed in the cells^[28].

Hemangioblasts are considered to be bipotential cells which differentiate into both hematopoietic cells and endothelial cells, placing them as an alternative for generating functional blood cells. Several research groups have already attempted to produce a significant amount of hemangioblasts which could be differentiated to erythrocytes as a final aim with clinical applications^[28-30]. However, the production of hemangioblasts is still considered to be insufficient due to its high costs and low

quantity of cells of interest produced.

In 2007, Lu *et al.*^[31] issued two publications in which a cheaper and significantly more efficient previously established protocol to produce hemangioblasts was detailed and tested. Also, the oxygen-carrying capability of the erythroid cells later produced was tested^[28,32]. The protocol established by Lu *et al.*^[31] is summarized in Tables 1 and 2.

Characterization of the cells produced after the steps in Table 1 displayed that the cells were nucleated and significantly larger than erythrocytes but showed an abundance of hemoglobin, whereas the cells obtained after the protocol in Table 2 were more similar to RBCs when morphologically compared as this protocol promoted enucleation of the cells.

The number of cells obtained with the delineated protocol was 800% higher than in previous studies^[31]. In addition, most of the cells produced expressed the RBC marker membrane proteins and did not express myelomonocytic or megakaryocytic antigens, 75% were CD 71 positive and 30% were CD 253a positive. Moreover, the erythroid cells successfully functioned as an oxygen carrier. Thus, the protocol employed by Lu *et al.*^[31] and summarized in Tables 1 and 2 is an advance in the attempts to produce RBCs.

Production of erythrocytes independently of erythropoietin:

More recently, Kim *et al.*^[26] proposed a method by which a substantially higher RBC production from ESC was possible without the use of Epo as a differentiation inducer^[26].

In this study, erythrocytic differentiation was achieved by transfecting the gene for the F36V-MPL protein with lentiviral vector which consists of the intracellular domain MPL and the drug binding extracellular domain F36V^[33]. The normal MPL protein is activated by thrombopoietin, while a small molecule, AP20187, may also activate the F36V-MPL protein, homodimerizing the MPL domain that activates erythropoiesis. MPL domain activation was found to up-regulate the expression of GATA1, commonly related to differentiation to erythroid cells. Additionally, the protein transduced in the cells does not suffer internalization as a negative feedback mechanism when the ligand binds to the external domain. These cited features show that the protocol used by the group has the capability of producing apparently superior results compared to previous studies^[26].

In the study by Kim *et al.*^[26], differentiation occurred satisfactorily and more efficiently. Globin chain expression and enucleation was higher than in previous studies using Epo, with the conclusion that the homodimerization of the transfected protein intracellular domain is more potent at promoting erythropoiesis than induction with Epo^[26]. Although it signifies a great step forward in the understanding of the underlying mechanisms of this differentiation, the use of lentiviral vectors poses a severe hindrance in its utilization for therapy due to the augmented possibility of malignant transformation of the

Table 1 Steps taken to obtain erythroid cells from human embryonic stem cells by the formation of hemangioblasts

Step-Days	Procedure
1-3.5 prior to step 2	EB formation is stimulated by plating hESCs on ultra-low attachment plates with serum-free medium supplemented with BMP-4, VEGF ₁₆₅ and basic FGF; 48 h later, half of the medium renewed and accreted with SCF, thrombopoietin and FLT3 ligand; EBs are formed and induced to differentiate to hemangioblasts
2-d 0 to 10	EBs formed are dissociated with trypsin and a single cell suspension is obtained; The cells are re-suspended in appropriate medium and further put in contact with BGM with added FGF and t-PTD-HoxB4 fusion protein, where they are expanded for 10 d; The hemangioblasts are expanded enough after this period of 10 d
3- d 11 to 20	Differentiation to erythroid cells may be achieved by culturing the hemangioblasts obtained in step 2 for 5 d with the addition of BGM supplemented with Epo to the medium used in step 2; The cells are then expanded for up to seven more days on Stemline II-based medium supplemented with SCF, Epo and methylcellulose
4-d 21	Erythroid cells obtained after step 3 were suspended in IMDM with 0.5% BSA in order to be collected by centrifugation The cells are washed with IMDM and BSA and normally plated so that non-erythroid cells adhere, allowing the non-adherent to be separated by collecting the medium and centrifugation

hESCs: Human embryonic stem cells; FGF: Fibroblast growth factor; BMP-4: Bone morphogenetic protein-4; SCF: Stem cell factor; BGM: Blast-colony growth media; Epo: Erythropoietin; IMDM: Iscove modified Dulbecco medium; BSA: Bovine serum albumin.

Table 2 Enucleation of Hemangioblasts. The technique here summarized starts by taking the hemangioblasts at the 7th day of step 2, clarified in Table 1

Step	Procedure
1	Hemangioblasts obtained on day 7 of the procedures described in Table 1 are filtered and plated with Stemline II medium supplemented with inositol, folic acid, monothioglycerol, transferrin, insulin, ferrous nitrate, ferrous sulphate and BSA, along with penicillin-streptomycin solution
2	For 7 d, the medium described in step 1 had hydrocortisone, SCF, interleukin-3 and Epo added; Cell density was kept at 10 ⁶ cells/mL
3	After the 7th day, SCF and interleukin-3 were no longer added to the medium
4	The cells were co-cultured with human mesenchymal stem cells or OP9 mouse stromal cells at different days between days 19 and 36; Medium was composed as described in Step 1 with the addition of Epo

SCF: Stem cell factor; Epo: Erythropoietin; BSA: Bovine serum albumin.

transfected cells.

Production of platelets

The genesis of platelets, both *in vivo* and *in vitro*, depends on the formation of the megakaryocytes as an intermediate. Megakaryocytes are polyploidy cells that underwent successive DNA replications without mitosis. Platelets are the result of the maturation of megakaryocytes in which they are fragmented^[34]. Efforts in understanding the dynamics of megakaryocytic differentiation as well as the final maturation to platelets are important in order to make advances in the *in vitro* production of this blood product that may be used for research about platelets and coagulation and eventually for thrombocytopenia treatment.

In 2002, Eto *et al*^[35] described the production of large quantities of megakaryocytes from murine ESCs. The ESCs were co-cultured with OP9 stromal cells and the differentiation was induced by thrombopoietin, IL-6 and IL-11 in order to obtain polyploid megakaryocytes and subsequently pro-platelets. The results were confirmed by the expression of α IIb β 3 and platelet glycoprotein Ib α as the cells obtained could successfully bind to fibrinogen by the α IIb β 3 integrin responding to platelet

agonists. No hematopoietic stem cell, erythrocyte and leucocyte markers were found to be expressed after differentiation^[35].

Years later, further research by Gaur *et al*^[36] managed the production of megakaryocytes starting with human ESCs, which also displayed properties of mature cells, responding to platelet agonists and binding to fibrinogen. Cell culture was also performed with OP9 cells and differentiation was induced by human thrombopoietin^[36].

Although Mks have been successfully produced by successful differentiation of both mouse^[35,37,38] and human ESCs^[12,39-42], a great part of the protocols employed required co-culture with stromal cells, imposing difficulties in determining the exact requirements for direct differentiation of ESC to megakaryocytes.

Production of mast cells

Mast cell differentiation using ESCs has been well documented by Kovarova *et al*^[43,44] since 2001. The process follows three steps: firstly, Ebs are formed after placing the ESCs in ultra-low attachment plates; secondly, the EBs are then transferred to Matrigel-treated plates where induction to differentiation starts by supplementation with SCF, IL-6, IL-3 and Fms-like tyrosine kinase 3 ligand (Flt-

3L) which yields non-adherent hematopoietic progenitors; and thirdly, the progenitors collected in the second step are further differentiated by adding fetal bovine serum (FBS), IL-6 and SCF^[43,44]. Although mast cells are not documented in the literature as prone to transfection, the *in vitro* differentiation of mast cells is relevant in the studies on inflammatory diseases^[44].

Production of macrophages: Mature macrophages can be classified into two groups, M1, which mainly mediate defence against pathogens and inflammatory response, and M2, more connected to wound healing and tissue remodelling. Fetal macrophages present with a M2-like phenotype that accounts for the scar-free wound healing in embryos. Thus, studies have been performed in order to study the properties of ESC-derived macrophages and their applications^[45]. Evidence was found that in spite of the M2 properties *in vivo* in embryos, the macrophages obtained from ESC differentiation do not promote better cutaneous tissue healing^[45]. In spite of these findings, the *in vitro* production of macrophages is still important for providing a cellular model for infections as these cells are the specific hosts for certain pathogens, namely, HIV-1, dengue virus, *Leishmania* and *Mycobacterium tuberculosis*^[46].

van Wilgenburg *et al.*^[46] provided the most recent detailed protocol for macrophage *in vitro* production, rendering the macrophage differentiation protocol more efficient and predictable^[47]. Two protocols were developed by the group: a “quick protocol”, in which EBs were formed by culturing the ESCs on a layer of mouse feeder cells before mechanical dissociation of the bodies that were then cultured on ultra-low attachment plates where differentiation was stimulated by IL-3 and macrophage colony-stimulating factor (M-CSF); and the “defined protocol”, where no feeder layer or serum was applied, EBs were formed by the spinning method^[48] and the medium used was the XTVIVO™ serum-free medium^[45].

Production of T lymphocytes

de Pooter *et al.*^[49] successfully differentiated mouse ESCs into T lymphocyte lineages following the culture conditions previously established by Nakano *et al.*^[50] where the stem cells were co-cultured with OP9 feeder cells and differentiation was induced by the flt-3 ligand and IL-7.

In vitro T-cell differentiation was reported to happen after ESCs were differentiated to hematopoietic stem cells (HSC), as described by Vodyanik *et al.*^[51]. When culturing the ESCs on a feeder layer of stromal cells and supplemented with Flt-3L, IL-7 and SCF, it was possible to attain a substantial number of hematopoietic progenitor cells (HPCs) which were later committed to the differentiated T lymphocytes^[52]. However, a detailed protocol to achieve efficient human T lymphocyte differentiation from ESCs has not yet been devised.

Production of B lymphocytes

In 2003, the production of functional B lymphocytes was described by Cho *et al.*^[53,54]. Flt-3L and IL-7 were

added to the medium on defined days; Flt-3L was added on the fifth day and IL-7 on the eighth day, when pre-B cells were obtained. Fully differentiated and functional B lymphocytes were produced after stimulation with lipopolysaccharide (LPS). The B cells produced expressed the markers that indicate commitment to this lineage, including IgM which had its expression enhanced by LPS stimulation^[54].

In the most recent publication on the subject, the induction of lymphopoietic differentiation of ESCs was found to yield two HPC phenotypes: CD34+CD45RA+CD7- and CD34+CD45RA+CD7+. CD7- had its potential turned more to the production of B cells but could differentiate into natural killer (NK) and T cells, while CD7+ was more likely to generate NK and T cells^[55].

Production of eosinophils

Hamaguchi-Tsuru *et al.*^[56] published a detailed protocol to differentiate eosinophils from murine ESCs and evidence of their functionality in 2004. The differentiation was achieved by cultivating the cells on OP9 stromal cells with stimulation of IL-5 and either IL-3 or granulocyte-macrophage colony stimulating factor (GM-CSF). Eotaxin was also employed in an attempt to differentiate the cells which was only achieved when combined with either IL-3 or IL-5. During the differentiation process, the level of expression of GATA1 transcription factor was found to increase the relationship between GATA1 and hematopoiesis, briefly explained earlier in this review. In addition, the expression of CCAAT binding proteins, IL-3 receptor, GM-CSF receptor and major basic proteins was also augmented^[56].

So far, no definite and detailed protocol has been established for the differentiation of eosinophils from human ESCs.

Production of neutrophils

A neutrophil differentiation protocol was documented and investigated by Lieber *et al.*^[57,58] in which mouse ESCs were used. The differentiation protocol followed was performed in three steps: firstly, ESCs were cultured on an OP9 feeder layer under conditions that elicited EB formation until the 8th day; secondly, the day 8 EBs were dissociated and placed in a new medium with added basic fibroblast growth factor (FGF), IL-6, kit ligand (KL), IL-11 and leukemia inhibitory factor (LIF); and the third step promoted the final differentiation into mature neutrophils by transferring the cells to a new medium supplemented with G-CSF, GM-CSF and IL-6^[57,58].

A neutrophil production protocol for human ESCs was more recently published. EB formation was stimulated by bone morphogenetic protein-4 (BMP-4), SCF, FTL-3L, IL-6, IL-6 receptor fusion protein and thrombopoietin. Further differentiation required G-CSF in order to attain mature neutrophils which expressed the lineage markers^[59].

Production of dendritic cells

Standard well-defined protocols for producing dendritic

Table 3 Summary and comparison of protocols employed for differentiation of embryonic stem cells and induced pluripotent stem cells

Hematopoietic lineage	Culture conditions for ESCs	Culture conditions for iPSCs	Cytokines used in ESC differentiation	Cytokines used in iPSC differentiation	Ref.
Erythrocytes	Cells grown initially on mitomycin-treated Mouse Embryonic Fibroblasts; BGM and Stemline II media are used; Final step was in co-culture with OP9 cells or human mesenchymal stem cells	Cells are grown in co-culture with human fetal liver-derived feeder layer	BMP-4, VEGF ₁₆₅ , FGF, thrombopoietin, FLT-3L, t-PTD-HoxB4 fusion protein, Epo, IL-3	IL-3, SCF, Epo, BMP-4, Insulin-like growth factor-1	[28,31,32,74]
Megakaryocytes	Cells are grown in co-culture with OP9 stromal cells	Feeder-free culture in serum-free Stemline medium or in OP9 or C3H10T1/2	IL-6, IL11 and thrombopoietin	BMP-4, VEGF, FGF, SCF, thrombopoietin, FLT-3L, IL-11	[35,75,76]
Mast cells	Cells are grown in co-culture with OP9 stromal cells	Mice iPSCs were initially co-cultured in on mitomycin-treated Mouse Embryonic Fibroblasts with LIF added. In the final step, OP9 cells were used as feeder layer	SCF, IL-6, IL-3 and FLT-3L	IL-3, IL-6 and SCF	[43,44,77]
Macrophages	Cells are grown in co-culture with OP9 stromal cells, but in an alternative method, no feeder layer was used, EBs were produced by the spinning method	Cells are grown in co-culture with OP9 stromal cells	IL-3 and M-CSF	FGF, BMP-4, VEGF, IL-3, IL-6, IL-11, SCF	[45,78]
T lymphocytes	Cells are grown in co-culture with OP9 stromal cells	Initial culture was done on irradiated SNL76/7 cells and further steps on OP9-DL1 cells	FLT-3L, IL-7 and SCF	FLT-3L, IL-5	[49-52,79]
B lymphocytes	Cells are grown in co-culture with OP9 stromal cells	Cells are grown in co-culture with OP9 stromal cells	FLT-3L, IL-7	FLT-3L, IL-3, IL-7 and SCF	[53-55,80]
Eosinophils	Cells are grown in co-culture with OP9 stromal cells	No established protocol	IL-5, IL-3, GM-CSF and Eotaxin	No established protocol	[56]
Neutrophils	Cells are grown in co-culture with OP9 stromal cells	Cells are grown in co-culture with OP9 stromal cells	BMP-4, SCF, FLT-3L, IL-6, IL-6 receptor fusion protein, thrombopoietin and G-CSF	VEGF, IL-3, SCF, thrombopoietin and G-CSF	[59,81]
Dendritic cells	Cells are grown in co-culture with OP9 stromal cells	Cell culture was feeder free, initially on mTeSR1 medium and posteriorly using X-VIVO 15 medium	GM-CSF, IL-4, TNF- α	BMP-4, VEGF, SCF, GM-CSF, IL-4	[60,61,82]
Natural killer Cells	Cells are grown in co-culture with M210-B4 cells initially and AFT024 cells in the final step	Cells are grown in co-culture with M210-B4 cells initially and AFT024 cells in the final step	IL-3, IL-7, IL-15, SCF and FLT-3L	IL-3, IL-7, IL-15, SCF and FLT-3L	[63,83]

ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; FGF: Fibroblast growth factor; BMP-4: Bone morphogenetic protein-4; SCF: Stem cell factor; BGM: Blast-colony growth media; Epo: Erythropoietin; BSA: Bovine serum albumin.

cells (DC) from human ESCs are available. The procedure involves formation of EBs on an OP9 cell monolayer and requires the use of GM-CSF, IL-4 and tumor necrosis factor- α (TNF- α) as cytokines to induce differentiation. The process requires the formation of HPC before a final step that gives rise to a mature DC due to the presence of GM-CSF and IL-4^[60,61].

Production of natural killer cells

Natural killer (NK) cells have particular importance in the cytotoxic response of effective immunity. The modulation of these cells has been investigated as they may be useful tools in the treatment of neoplasms, which has been recently been explored^[62].

NK cell differentiation was done by culturing the ESCs initially on a monolayer of M210-B4, a murine bone marrow lineage, as feeder cells. CD34+ and CD45+ were isolated from the first step and differentiated to NK when co-cultured with another feeder cell, AFT024, on medium

supplemented with β -mercaptoethanol, ascorbic acid and the cytokines IL-3, IL-7, IL-15, SCF and FLT-3L^[63].

DIFFERENTIATION OF INDUCED PLURIPOTENT STEM CELLS TOWARDS BLOOD CELL PRODUCTION

iPSCs was the term coined to describe the first cell lineage produced by reverting the differentiated status of somatic cells by using defined factors and four transcription factors were found to be necessary to reprogram fibroblasts, oct-4, Sox-2, Klf-4 and c-Myc^[64]. The cells acquired could successfully differentiate into cells of the three germ lines and resembled the ESCs in several other morphological aspects as well as in the gene expression signature^[64]. The understanding of the underlying mechanisms of cellular reprogramming provided by Takahashi *et al.*^[64] allowed further studies to develop protocols to reprogram other

somatic cell lines and produce iPSCs^[8,65,66]. The advances in the iPSC subsidized studies focused on their use for tissue production through cellular differentiation. Several following works managed to develop differentiation protocols to be applied to iPSCs, including hematopoietic differentiation^[67-69]. Additionally, cellular reprogramming readily encouraged considerable advances in tissue engineering since the development and use of iPSCs is not bound by the same hindrances imposed by the ethical constraints inherent to ESCs^[64].

The differentiation of iPSCs may also circumvent the problems related to immunological incompatibility upon engraftments and transfusion as iPSCs can be produced in a patient-specific manner^[64,70,71]. However, the production of these cells still is highly costly and widely inefficient with the proportion of cells which are successfully reprogrammed. Safety issues also have to be taken into consideration since lentiviral vectors were employed by Raya *et al.*^[68] and most of the following attempts to produce iPSCs. Nevertheless, several steps have been recently taken towards a safer and more efficient production of iPSCs^[64-66].

Considering the lack of ethical hindrances, the possibility of patient-specific cellular reprogramming and the great differentiation potential of iPSCs, they are a great candidate for use in obtaining donor-free blood products^[67-69].

In 2009, Lengerke *et al.*^[72] cultured fibroblast-derived iPSCs under conditions similar to those used for ESC procedures, enabling expansion of a long period. In addition, when differentiation was induced by BMP-4, EB formation was evidenced from the first day and flow cytometry provided evidence of the hematopoietic profile of the cells obtained after day 17^[72].

Protocols for hematopoietic differentiation of iPSCs are currently being devised and tested for functionality and resemble the protocols used in ESCs. The already established protocols for differentiating iPSCs into hematopoietic cell lineages were recently reviewed by Lim *et al.*^[73]. In order to summarize the state-of-the-art on the matter, Table 3 displays the culture conditions and cytokines employed in order to achieve differentiation as well as to compare the protocols used for ESCs.

Apart from the previously stated advantages of employing iPSCs for the production of blood products, the cells obtained with cellular reprogramming technology have been reported as useful tools for progression in the field of immunotherapy.

In 2013, Themeli *et al.*^[84] successfully produced tumor-targeting T lymphocytes by differentiating iPSCs. Differentiated NK cells were also investigated for cancer immunotherapy^[85]. Although little has yet been produced, the available data regarding iPSC aspects of differentiation potential as well as the possibility of using patient-specific cells make iPSCs a promising alternative to generate immunotherapeutic blood products^[86,87].

Besides the applications cited above, the knowledge of cellular reprogramming may also be applied to cancer cells in order to produce iPSCs that can potentially predict the progression of the disease and serve as an *in vitro*

model^[88,89]. Miyoshi *et al.*^[89] published the attainment of cancer stem cells by transfecting defined transcription factors to gastrointestinal neoplastic cells in 2009. More recently, iPSCs were produced starting from pancreatic carcinoma cells^[90]. Considering that pluripotency has also been achieved from hematopoietic somatic cells^[91-93], reprogramming is a promising technology for studying the development of hematological cancers which are of great importance in the population^[94].

CONCLUSION

From what could be perceived from data in the literature, differentiation of blood cells from either ESCs or iPSCs is an on-going field of studies from which various applications can be investigated. Most hematopoietic cells are already being successfully produced from pluripotent stem cells; however, there is an ever-progressing search for more efficient protocols in order to achieve sufficient production in the number of cells for transfusion through a safe protocol that also yields functioning blood cells.

Apart from the enterprise towards acquiring blood products through ESC and iPSC differentiation, the hematopoietic differentiation of these cells can also be applied for other purposes. T and NK cell-based immunotherapy seems to be an alternative to cancer treatment with future applications, as well as the reprogramming of cancer cells that may be of great use in determining the pathways through which carcinogenesis occurs.

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Methods of induced pluripotent stem cells for clinical application

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diseases. In particular, applying iPSCs clinically holds the promise of addressing the problems of immune rejection and ethics that have hampered the clinical applications of embryonic stem cells. However, as iPSC research has progressed, new problems have emerged that need to be solved before the routine clinical application of iPSCs can become established. In this review, we discuss the current technologies and future problems of human iPSC generation methods for clinical use.

Key words: Induced pluripotent stem cells; Cell reprogramming

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Core tip: Each induced pluripotent stem cells methodology has advantages and disadvantages, as in the case of autologous vs allogenic transplantation, and the choice of appropriate strategy may vary depending on the intended use. Additionally, to avoid tumorigenesis and to establish effective differentiation into the intended cells, further investigation is needed to identify the most suitable iPSC line and how these lines should be selected.

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Abstract

Reprogramming somatic cells using exogenous gene expression represents a groundbreaking step in regenerative medicine. Induced pluripotent stem cells (iPSCs) are expected to yield novel therapies with the potential to solve many issues involving incurable

INTRODUCTION

In 2006, Takahashi *et al*^[1] established a novel method of reprogramming mouse somatic cells using exogenous expression of genes related to pluripotency. The cell lines established by this group were named induced pluripotent stem cells (iPSCs) and demonstrated the same pluripotency and self-renewal properties that are characteristic of embryonic stem cells (ESCs). The following year, the

same group also succeeded in generating iPSCs from human somatic cells^[2]. These groundbreaking steps have been expected to lead to novel regenerative cell therapies with the potential to solve many problems surrounding incurable diseases. In particular, the clinical application of iPSCs is expected to solve the problems of immune rejection and ethics that are currently key obstacles in the clinical use of ESCs. However, as research into iPSCs has progressed, new problems to solve have emerged before iPSCs can be established as cell sources for patients. In this review, we discuss the current technology and future problems surrounding human iPSC generation methods for clinical applications.

BENEFITS AND PROBLEMS OF ESCS AND IPSCS IN CLINICAL APPLICATION

For treating diseases that lack self-repairing cells, the transplantation of artificially generated cells is one attractive means for curing the diseases. In fact, regenerative cell transplantation therapies have been expected to treat incurable diseases, such as spinal cord injury^[3], neurodegenerative disease^[4], heart failure^[5,6], diabetes^[7], and retinal disease^[8].

ESCs have the capacity to self-renew and differentiate into cells of the three germ layers. The development of suitable cultivation systems for maintaining the pluripotency of ESCs marked their promise as a cell source for regenerative medicine since human ESCs were first generated in 1998^[9]. Numerous efforts have been made since then to realize the promise of making specific differentiated cells from ESCs. However, the ethical problem of needing human zygotes to generate human ESCs has remained unsolved. Additionally, immune rejection remains an issue because of the limited number of ESC cell lines and the ability of the cell lines to match the huge number of human leukocyte antigen (HLA) type combinations found in patients.

These problems with ESCs and the ongoing need for regenerative therapies drove further research efforts, such as Dr. Yamanaka's 2006 success in generating iPSCs from somatic mouse cells^[1] and reports in 2007 by Takahashi *et al.*^[2] and Yu *et al.*^[10] of the successful generation of human iPSCs. In the original mouse work, 24 transcription factors showing high expression in ESCs were chosen as candidate reprogramming triggers^[1]. Finally, the forced expression of *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC* together with a retrovirus evoked mouse somatic cell reprogramming into the pluripotent state, and the same combination of four factors forced human fibroblasts into iPSCs^[2]. At the same time, Yu *et al.*^[10] successfully reprogrammed human fibroblasts using the combination of *OCT4*, *SOX2*, *NANOG*, and *LIN28*. These methods attracted much attention because these iPSC lines could potentially overcome the immune rejection and ethical issues hampering the development of ESCs for clinical use. Therefore, iPSCs showed promise as the breakthrough

technology in regenerative medicine.

COMBINATION OF REPROGRAMMING FACTORS FOR IPSC GENERATION

Since iPSC generation methods were first reported, numerous efforts have been made to adapt them to clinical applications^[5,11]. The reported generation methods vary in the combinations of reprogramming factors, vehicles for exogenous genes, and cell types to generate the iPSCs. Therefore, current discussions in the literature focus on selecting the most appropriate iPSC generation method for clinical use.

In relation to the combination of reprogramming factors for iPSC generation, the first reported combination of *OCT3/4*, *SOX2*, *KLF4*, and *C-MYC*, known as the Yamanaka factors, are generally used for iPSC generation. Combining only three of these factors and omitting *C-MYC* was also reported to achieve successful iPSC generation, although with reduced reprogramming efficiency^[12,13]. These alternative methods arose following concerns about using *C-MYC*, which is a known oncogene in human cells. Another group also reported the generation of mouse iPSCs, which showed more efficient germline transmission in chimeric formation experiments using the combination of *OCT3/4*, *SOX2*, *KLF4*, and *TBX3* as reprogramming factors^[14]. Subsequently, iPSC generation using *L-MYC* instead of *C-MYC* was reported with improved efficiency in both reprogramming somatic cells and germline transmission of generated iPSCs^[15]. Furthermore, *GLIS1* was also reported as a candidate alternate factor for *C-MYC* that showed high reprogramming efficiency, less incomplete reprogramming, and reduced tumor formation in iPSC-derived mice^[16]. Recently, improved iPSC quality was also achieved by introducing *Zscan4*, which is highly expressed at the zygotic genome activation stage. Forced expression of *Zscan4* in combination with the Yamanaka factors improved iPSC quality as demonstrated by tetraploid complementation^[17].

Recently, lineage-specific genes were substituted for *OCT3/4* or *SOX2*^[18]. In this report, *OCT3/4* was replaced with an early mesendodermal lineage marker, such as *GATA3*, and *SOX2* was replaced with an early ectodermal lineage marker, such as *ZNF521*, in reprogramming using the Yamanaka factors. This finding raised the possibility that *OCT3/4* and *SOX2* might act as lineage specifiers for cell reprogramming and showed that reprogramming factors are not limited to genes associated with pluripotency^[18].

Although methods for checking the quality of iPSCs such as germline transmission experiments are not applicable to human iPSCs, such studies can be useful for seeking the best combination of reprogramming factors to generate human iPSCs that are of sufficient quality for clinical use.

GENE-DELIVERY VEHICLES FOR IPSC GENERATION

Gene-delivery vehicles are also an important for selecting a suitable method of iPSC generation for clinical use, and to date, many gene delivery vehicles have proven to be applicable to iPSC generation. Initially, retrovirus vectors, such as pMXs^[2,19,20], pLib^[21] or pMSCV^[22,23], were used for the delivery of reprogramming factors into somatic cells. Importantly, transgenes introduced with retrovirus vectors have been known to be silenced in pluripotent states^[22,24], and therefore, silencing of transgene expression in iPSCs has been thought to be an important result of successful reprogramming^[25]. Lentiviral vectors have also been used for successful iPSC generation because lentiviral vectors achieve a higher efficiency of infection than retrovirus vectors^[10,26]. Importantly, transgenes introduced by lentiviral vectors are more resistant to being silenced in pluripotent states than those transgenes introduced by retrovirus vectors^[27].

The genomic integration of transgenes that occurs as a result of these virus vectors was thought to be a problem for the clinical use of iPSCs because the delivered transgenes have the potential to be reactivated after cell reprogramming and thus drive oncogenesis in the iPSC-derived cells^[28]. Furthermore, these transgenes have the potential to disrupt functional genes, even if they are silenced and not expressed. Therefore, many efforts have been made to generate iPSCs without the genomic insertions. For example adenovirus vectors are routinely used to introduce transient gene expression in target cells. Furthermore, a replication-defective pHIHG-Ad2 vector, was used to successfully reprogram somatic cells into iPSCs^[29]. Therefore, although adenoviral vectors still integrate into the genome of target cells at extremely low frequencies^[30] and the reprogramming efficiency is significantly lower than that with retrovirus, this method generates iPSCs that do not transfer residual transgenes into the host genomes.

More recently, a Cre-deletable lentivirus system was used for the successful generation of iPSCs^[31]. However, although these systems can avoid transgene reactivation, the risk of gene breaks being introduced near the insertion site is present because the LoxP sequence remains in the host genome after removing the insert sequence by Cre recombinase^[32].

In addition to virus vectors, the Sendai virus was also successfully used to generate iPSCs^[33,34]. The Sendai virus genome is negative-sense single-stranded RNA. Because replication occurs in the cytoplasm, this virus vector does not pose a danger of genome insertion. Therefore, this method solves both problems of gene disruption near the insertion site and reactivation of transgenes. Additionally, the residual Sendai virus RNA can be removed from the infected cells using siRNA^[35], and temperature-sensitive mutations^[36] were also reported, improving the potential clinical suitability of this iPSC generation method.

As another approach for safe iPSC generation, a virus-

free reprogramming method has received attention. For example, an iPSC generation method using episomal vectors has also been developed^[37-40]. The early attempts using this method yielded lower efficiencies of successful reprogramming than those achieved using retrovirus, and only a low percentage of iPSC lines generated using this method had no plasmid integration. However, later modifications of the episomal vector method yielded a higher reprogramming efficiency using a combination of plasmids encoding *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and shRNA for *TP53*^[41,42].

Generating iPSCs using a piggyBac transposon was reported as another method to avoid the reactivation of residual transgenes and gene breaks in the host genome^[43-45]. The piggyBac transposon is a moth-derived DNA transposon^[46] that is highly active in mammalian cells and able to be completely eliminated from the host genome using the piggyBac transposase^[47]. Despite generating integration-free iPSCs by this method, the reported reprogramming efficiency was lower than that with retrovirus, and thus further improvements are needed^[43-45].

As another way to avoid introducing genetic material, introducing reprogramming factors such as RNAs or proteins has attracted much attention. Indeed, the direct delivery of synthetic mRNAs has been shown to successfully reprogram somatic cells to a pluripotent state^[48]. In this study, *in vitro* transcribed RNAs were modified to avoid the endogenous antiviral cell defense. As a result, this method achieved a higher iPSC generation efficiency than the original retrovirus system^[48]. Successful reprogramming of somatic cells has also been achieved using microRNAs^[49], whereby expression of the *miR302/367* cluster containing five different miRNAs, *miR302a/b/c/d* and *miR367*, reprogrammed human fibroblasts more efficiently than previous retrovirus systems^[49]. Such RNA-based reprogramming avoids both breaks in existing genes and the reactivation of transgenes. Therefore, these methods hold much promise as novel iPSC generation methods that could be applicable for clinical use.

Similarly, recombinant proteins were also reported as a successful means of gene introduction for generating iPSCs^[50,51]. These protein-based methods are also attractive for clinical application because of the absence of breaks in existing host genes and the reactivation of transgenes; however, the generation efficiencies remain lower compared to those in the existing retrovirus systems^[50,51].

Finally, small-molecule drugs have been investigated for establishing safe methods of iPSC generation for clinical application because they are nonimmunogenic, cost-effective, and easy to handle^[52]. Recently, successful reprogramming of mouse somatic cells without transgene introduction was achieved with small-molecule drug combinations^[53]. This strategy has many merits for applying the iPSC generation method for clinical use, and therefore, further research into applying this method to human somatic cells is expected in the near future.

TYPES OF DONOR CELLS FOR IPSC GENERATION

Generating iPSCs in clinical practice also requires the consideration of the most appropriate type of donor cells. At first, iPSCs were generated from mouse fibroblasts^[1], followed by successful reprogramming of mouse hepatocytes and gastric epithelial cells^[54]. Subsequently, terminally differentiated somatic cells have also been reprogrammed, including mouse B lymphocytes^[55] and pancreatic beta cells^[56]. With respect to human cells, iPSCs have been generated from human dermal fibroblasts^[2,10] and many types of human somatic cells^[5].

As a matter of course, clinical applications of cell therapies require that tissue collection from patients be as minimally invasive as possible, and harvesting human dermal fibroblasts by biopsy leaves a small scar on the patient's body. Recently, iPSCs were generated from human keratinocytes induced from plucked hair, a process that is much less invasive than biopsy^[23,57]. However, several hairs are needed to obtain the successful cell outgrowth of keratinocytes in some cases, and therefore, a more stable protocol for primary culture is needed for routine clinical practice.

Dental tissue has also been proposed as a unique cell source for iPSC generation. Dental stem cells^[58,59] and mesenchymal stromal cells derived from human third molars^[60] were successfully reprogrammed and thought to be potentially useful material for clinical iPSC generation. Oral gingival^[61] and oral mucosa fibroblasts^[62], which can be obtained less invasively, were also investigated for iPSC generation. These methods are advantageous for clinical application because they involve a minimally invasive approach for the patients.

Cord blood was also reported as another cell source for generating iPSCs^[63,64]. Early studies with cord blood yielded lower reprogramming efficiencies than those achieved with lentivirus or retrovirus systems. However, a modified method involving the knockout of p53, which was previously shown to increase the efficiency of reprogramming^[65-70] for iPSCs, increased the efficiency of generating iPSCs from CD34-positive cells which were sorted from cord blood cells^[71]. Additionally, gene introduction using Sendai virus vectors successfully reprogrammed CD34-positive cord blood cells more efficiently than lentiviral or retroviral vector used to reprogram cord blood cells^[36]. Cord blood cells have attracted much attention because cord blood-derived cells do not require laborious mobilization or an invasive biopsy before introducing reprogramming factors. Using cord blood cells for iPSC generation also avoids the risk of transferring over accumulated genetic mutations into iPSC. Additionally, banked cord blood cells are relatively uncomplicated for use in iPSC generation because their immunological information is already available in cord blood banks^[72].

Peripheral blood cells are also an attractive cell source because the method for cell sampling from patients is less

invasive. Peripheral blood cells are more easily accessible as a cell source than the dermal fibroblasts obtained by skin biopsy. In the first study to generate iPSCs from human blood cells, the donor needed to be injected for 3 days with G-CSF to mobilize the CD34-positive cells, and the reprogramming efficiency was not higher than that of previous studies^[73]; however, since then there have been many efforts to effectively generate iPSCs from peripheral blood cells. For example, less invasive methods have since been reported for generating iPSCs from peripheral mononuclear blood cells^[34,74-77], whereby mononuclear blood cells from donors or frozen samples were induced using the Yamanaka factors with a retrovirus^[74,77], lentivirus^[75,76], or Sendai virus^[34]. In these experiments, the majority of iPSCs generated from mononuclear cells had TCR gene rearrangements, indicating that these cell lines were derived from T lymphocytes, and the reprogramming efficiencies with the Sendai virus were similar to those with the previous retrovirus system^[34]. Additionally, to avoid generating iPSCs containing genome rearrangements, methods were developed in which iPSCs were generated from peripheral mononuclear cells cultured under conditions that inhibit the proliferation of lymphocytes^[78,79] and from CD34-positive cells that were mobilized without additional drug administration to the donors^[80]. Sampling of peripheral blood is one of the least invasive procedures available, and therefore, generating iPSCs from peripheral blood could be one of the most appropriate methods for the clinical applications of iPSCs.

REMOVING ANIMAL PRODUCTS FROM CULTURE SYSTEMS

One of the most important issues to address in applying iPSCs to clinical therapy is that the culture systems for generating iPSCs contain animal-derived products with potential and unpredictable risks to patients^[81]. The initial culture system for ESC generation contained fetal calf serum in the culture medium and mouse embryonic fibroblasts as a feeder layer. These animal-derived products conferred a risk of transferring exogenous antigens, unknown viruses, or zoonotic pathogens to the generated cell populations^[9]. Thus, many investigations have been conducted to reduce such risks by establishing animal product-free culture systems for human iPSCs.

First, human-derived feeder cells have proven to be a useful alternative to mouse cells for human iPSC generation^[82]. However, these feeder cell preparations need significant time and effort, and in the case of clinical therapies requiring the mass culture of human iPSCs in some situations, using human-derived feeder cells for culturing human iPSCs is not an optimal strategy. As an option for culturing human iPSCs without feeder cells, Matrigel has proven to be a useful alternative that enables the stable culture of human pluripotent stem cells^[83-85]. Although Matrigel allows the generation of human iPSCs without animal-derived feeder cells^[86,87], Matrigel itself was derived from Engelbreth-Holm-Swarm mouse

sarcoma cells^[88]. Therefore, other types of matrices, such as CellStart^[89,90], recombinant proteins^[91-93], and synthetic polymers^[94,95], which do not contain animal-derived agents, have been tested and used as feeder-cell substitutes for the successful maintenance and generation of human pluripotent cells.

Developing animal product-free medium for iPSC generation is also an important practice for achieving safe therapy using iPSCs. The culture media used in the early generation of human ESCs contained fetal bovine serum^[9]. To remove unpredictable agents that might cause the differentiation of human ESCs, knockout serum replacement (KSR) has now been established as a defined material for maintaining human ESCs^[96] and is also used for human iPSC generation^[2]. Additionally, mTeSR1 medium was developed as a chemically defined medium for maintaining human pluripotent cells and is used for defined condition cultures of human pluripotent stem cells^[97]. However, because KSR and mTeSR1 also contain animal-derived products, new media have now been commercially developed as xeno-free media for maintaining human pluripotent stem cells and have already been used successfully for iPSC generation; these media include TeSR2^[98], NutriStem^[99], Essential E8^[91], and StemFit^[100].

SELECTING THE MOST APPROPRIATE IPSC LINE FOR CLINICAL USE

One of the most intractable problems for applying iPSCs to clinical therapy is the variety of iPSC lines with respect to differentiation tendency and tumorigenic risk. Additionally, the laboratory in which the iPSCs are generated could influence global gene expression patterns of those iPSCs due to small possible differences in the culture conditions^[101]. Therefore, how to select iPSC lines that are appropriate for a specific clinical use in terms of safety and differentiation ability remains a topic of intense discussion.

As described above, in contrast to generating iPSCs with Yamanaka factors, introducing *TBX3*^[14], *L-MYC*^[15], or *GLI1*^[16] instead of *C-MYC* has yielded high-quality iPSCs with respect to the efficiency of germ line transmission and prognosis of iPSC-derived mouse cells. These reports implied that selecting suitable combinations of reprogramming factors was an important consideration for clinical therapy using iPSCs. However, these reports were derived from mouse experiments, and how these findings translate to human iPSCs remains unknown. Of course, the quality index of iPSCs using chimeric formation is not applicable to human iPSCs. Therefore, another index of iPSC quality that is applicable to human iPSCs is required.

With respect to assessing the effects of the type of donor cell on the quality of generated iPSCs, the teratoma-forming propensity of neural stem and progenitor cells derived from mouse iPSCs was shown to differ depending on the donor cell type^[102]. This finding suggested that the quality of the iPSCs should be considered when the type of donor cell is selected. Since that early report, an epigenetic

memory of tissue of origin donor cell type that affected the differentiation tendency of iPSCs was reported^[103,104]. Importantly, the effect of epigenetic memory was not demonstrated when pluripotent stem cells generated by nuclear transfer were used. Additionally, another recent study showed that human iPSCs contain more CpG sites that retain the DNA methylation pattern of the parental donor cells than human ESCs generated by nuclear transfer^[105]. Although the fact that the differentiation tendency derived from epigenetic memory will not remain after long-term culture^[103], these reports also suggested the importance of selecting the donor cell type when applying iPSCs clinically.

The effect of donor cell type on differentiation tendency was also reported in human iPSCs^[106], but experiments that compared donor cell type, gene vehicle type, and volunteers on hepatic differentiation tendencies of iPSCs showed that the differentiation tendency of iPSCs depended on the volunteer from which the iPSCs were generated^[107]. Interestingly, in this latter study, hepatic differentiation tendencies that were derived from donor cells were not observed, implying that such differences derive from the volunteer cell donor when epigenetic memory does not remain after long-term culture.

With respect to the tumorigenic tendencies of human iPSCs, a study of gene expression and DNA methylation in 21 human iPSC lines and 2 human ESC lines showed activated expression of genes containing specific LTR7 sequences in some human iPSC clones that showed a neural differentiation-defective phenotype and formation of teratomas when they were differentiated into dopaminergic neurons and transplanted in mouse brains^[108]. Another report on 21 human iPSC lines and 6 human ESC lines indicated that certain human iPSC clones were in a pro-oncogenic state, as shown by the ectopic presence of secretory tumor tissue during *in vitro* cartilage differentiation^[109]. These reports together implied that a marker foreseeing pro-oncogenic differences in iPSC lines would be required for establishing safe therapy using iPSCs.

AUTO-TRANSPLANTATION AND ALLO-TRANSPLANTATION OF IPSCS

One of the most important advantages expected of iPSCs for transplantation therapy is avoiding immune rejection and therefore avoiding combination immunosuppressive treatment. Indeed, autologous iPSCs were used in the first human clinical trial of iPSCs started by Dr. Masayo Takahashi at the RIKEN Center for Developmental Biology in Kobe in 2013 for treating age-related macular degeneration with iPSC-derived sheets of retinal pigment epithelium. With respect to the immunogenicity of autologous iPSCs, in the first report that aroused discussion on its credibility, immunogenicity showed in the case of iPSCs was not observed in the case of ESCs^[110,111]. Subsequently, experiments with isogenic transplantation of mouse iPSCs revealed the successful engraftment of iPSC-derived tissue without immunosuppressive treatment and

verified the advantages of using auto-transplantation of iPSCs for avoiding immunosuppressive treatment^[112,113]. However, a recent study demonstrated immune rejection upon transplanting autologous undifferentiated mouse iPSCs *in vivo*, and rejection was imperceptible upon transplanting autologous terminally differentiated mouse iPSCs^[114]. Although this report supports the presence of immunogenicity in undifferentiated iPSCs^[91] and conflicts with the successful autologous engraftment of undifferentiated iPSCs in other reports^[93,94], it supports the advantages of using auto-transplantation of iPSC-derived terminally differentiated cells for avoiding immunosuppressive treatment. On another front, whether the immunogenicity in undifferentiated iPSCs contributes to removing contaminated undifferentiated cells from iPSC-derived cell populations and avoiding tumorigenesis after transplantation in the same manner as removing undifferentiated cells from cell populations before transplantation^[115-117] remains to be clarified.

Allo-transplantation of iPSC-derived cells was also expected to provide a useful strategy for transplantation therapy using iPSCs due to saving cost and time in generating autologous iPSC lines for transplantation. Furthermore, as described above, selecting appropriate iPSC lines on a patient-by-patient basis will require significant numbers of studies for verification. Therefore, although the transplantation of iPSC-derived cells from an allogenic donor with foreign HLA requires lifelong immunosuppressive treatment of the recipient, the concept of homozygous HLA-typed iPSC banks may be feasible for achieving generalized therapy using iPSCs^[41,118,119] and, indeed, this type of approach is already progressing^[120].

CONCLUSION

The invention of iPSCs was groundbreaking for novel regenerative medicine and has been expected to lead to regenerative therapies with the potential to advance the treatment and management of incurable diseases. Importantly, iPSCs could overcome the problems of immune rejection and ethics that remain with ESCs. However, each strategy of using autologous or allogenic iPSCs has advantages and disadvantages, and the choice of appropriate strategy may vary depending on the intended use. Additionally, there remain many factors that affect establishing transplantation therapy using iPSCs. To avoid tumorigenesis and establish effective differentiation into the intended cells, further investigation is needed to clarify which iPSC line is the most suitable and how these lines can be best selected.

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Adult human neural stem cell therapeutics: Current developmental status and prospect

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recover neurological deficits, it is still under development so as not to show significant treatment effects in clinical settings. In this review, we discuss the scientific and clinical basics of adult neural stem cells (aNSCs), and their current developmental status as cell therapeutics for neurological disease. Compared with other types of stem cells, aNSCs have clinical advantages, such as limited proliferation, inborn differentiation potential into functional neural cells, and no ethical issues. In spite of the merits of aNSCs, difficulties in the isolation from the normal brain, and in the *in vitro* expansion, have blocked preclinical and clinical study using aNSCs. However, several groups have recently developed novel techniques to isolate and expand aNSCs from normal adult brains, and showed successful applications of aNSCs to neurological diseases. With new technologies for aNSCs and their clinical strengths, previous hurdles in stem cell therapies for neurological diseases could be overcome, to realize clinically efficacious regenerative stem cell therapeutics.

Key words: Adult neural stem cell; Neurological diseases; Stem cell therapy; Preclinical trial; Clinical trial

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Core tip: In this review, we compare advantages and disadvantages of various types of stem cells for regenerative therapy in neurological disease, and discuss the preclinical and clinical developmental hurdles of stem cell technologies. While at present, adult neural stem cells (aNSCs) have clinical advantages, technical issues in the isolation and expansion of aNSCs prevent active preclinical and clinical applications of aNSCs. However, several papers have recently reported scientific breakthroughs, on the basis of which broad application trials using aNSCs could be performed. In this review, we also summarize the current status of preclinical and clinical applications of aNSCs for various neurological diseases.

Abstract

Over the past two decades, regenerative therapies using stem cell technologies have been developed for various neurological diseases. Although stem cell therapy is an attractive option to reverse neural tissue damage and to

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STEM CELL THERAPY FOR NEUROLOGICAL DISEASES

Neurological diseases are derived from the loss of functional neurons in the central nervous system (CNS). Although acute localized neurodegeneration could result from a temporal localized injury, such as stroke and trauma, chronic neurodegeneration usually develops over a long period of time, and has unclear multifactorial causes. Functional neurological deficits in chronic neurological diseases originate from either loss of a specific neuronal subtype, or universal brain damage. Alzheimer's and Huntington's diseases result in non-specific death of neurons in the brain, whereas Parkinson's disease is characterized by the specific and localized damage of dopaminergic neurons located in the substantia nigra. In the brain and spinal cord, amyotrophic lateral sclerosis (ALS) and traumatic spinal cord injury induce diffuse motor neuronal loss and localized nonspecific neural tissue damage, respectively. Although these neurodegenerative conditions have unique morphological pathologies, the molecular mechanisms for the neuronal death are complicated and ambiguous, making the development of mechanism-based therapeutic modalities elusive. Since functional loss of neural cells is the common final pathway of various neurological diseases, regardless of specific etiologies, regenerative treatment using stem cells that could repair damaged neural tissue is a viable and non-specific therapeutic option.

TYPES OF STEM CELLS AND THEIR APPLICATIONS TO NEUROLOGICAL DISEASES

Stem cells have two important characteristics: proliferation capacity, and differentiation potential into multiple cellular lineages. According to the source, stem cells can be classified into embryonic, fetal, and adult stem cells (ESCs, FSCs, and ASCs, respectively). Pluripotent ESCs are obtained from the blastocyst of fertilized egg^[1]. ESCs proliferate robustly and have multi-potent differentiation potentials into three germ layer cells, which consist of the whole body^[2]. However, ethical concerns^[3-5], and risks of adverse effects, such as immune rejection and tumor formation^[6] prevent their clinical applications. Recently, it was reported that somatic cells could be reprogrammed into pluripotent state, by overexpression of Oct4, Sox2, Klf4, and c-Myc^[7,8]. Although these induced pluripotent stem cells (iPSCs) maintain the merits of ESCs, iPSCs still

have limitations, such as low efficient generation, and the formation of teratomas or tumors *in vivo*. These critical limitations provoke hesitation in the use of ESCs and iPSCs as clinical therapies. As other sources of stem cells, fetal organs that contain FSCs have been suggested. In spite of the advantages of FSCs, including proliferation capacity, limited differentiation potential, and lack of teratoma formation^[9], ethical problems of using fetal tissues still remain.

ASCs are classically defined as multi-potent cells that originate from various tissues within the adult body, including the bone marrow, skeletal muscle, central nervous system, and adipose tissue^[10,11]. The important benefit of ASCs is possible autologous transplantation, in which stem cells can be primarily cultured from and applied to the same patient. This benefit bypasses the ethical problems that ESCs and FSCs harbour. However, in spite of these advantages of ASCs, their limited differentiation and proliferation ability interrupts their widespread use.

Therefore, in the current status, technical and ethical considerations indicate that compared with other stem cells, ASCs are the most clinically applicable.

ADULT NEURAL STEM CELLS FOR NEUROLOGICAL DISEASES

Among various ASCs, mesenchymal stem cells (MSCs) are the most widely used, and furthest progressed in preclinical and clinical trials^[12]. The strengths of MSCs are relatively simple isolation, and *in vitro* expansion techniques. However, there are concerns about the clinical applications of MSCs^[13]. First of all, in the culture methods of MSCs, bovine serum should be used. Because the dangers of bovine serum have not been well characterized, potential risks in clinical applications still exist^[14]. Although xeno-free culture methods for MSCs have been developed, their quality needs to further study. Moreover, many previous studies suggested that the beneficial effects of MSCs for neurological diseases might originate from their paracrine effects involving immune modulation and/or secretory growth factors, and not from direct neuroregenerative effects producing functional neural cells^[15-17].

Compared to MSCs, NSCs are cultivated and expanded in media containing low, or no bovine serum^[18-22]. Many preclinical studies using NSCs suggest that NSCs not only have beneficial paracrine effects in the regeneration and repair of neural tissue, but also direct differentiation potential into diverse neuronal lineages, to form networks with surrounding neuronal cells^[23-25]. Since the ultimate goal of regenerative treatment for neurodegenerative diseases is the functional repair of damaged neural tissues, NSCs seem to be a more optimal choice for neurological diseases.

Adult NSCs are tissue-resident multi-potent neural progenitor cells that have self-renewal capacity, so long as they

can be maintained undifferentiated. NSCs have the potential, under appropriate culture conditions, to differentiate into multiple neural cells, such as neurons, astrocytes, and oligodendrocytes. NSCs are observed in the developmental stage and mature CNS of mammalian species^[26-29], specifically in the subventricular (SVZ) and subgranular zones (SGZ)^[30-32]. The neurogenic niche surrounding SVZ and SGZ represents a unique microenvironment that regulates the survival and differentiation of NSCs^[28,33].

TECHNICAL HURDLES AND RECENT BREAKTHROUGHS IN THE USE OF ADULT NEURAL STEM CELLS FOR NEUROLOGICAL DISEASES

Depending on the types of neurological diseases, undifferentiated NSCs themselves, or differentiated neural cells, have been applied to verify their efficacy in preclinical animal models. However, *in vitro* expansion of differentiated neural cells to acquire the necessary amount of cells for transplantation is very difficult, because differentiated cells cannot proliferate well. Therefore, regardless of transplantation cell types, aNSCs first need to be properly isolated, and effectively expanded *in vitro*. Compared with other stem cells, such as ESCs, fetal NSCs, and MSCs, aNSCs reside in restricted areas of the adult CNS^[31,32], and have limited capacity to proliferate^[34,35]. Therefore, difficulties in the primary isolation and stable *in vitro* expansion of aNSCs are major technical obstacles to be resolved, for the utilization of aNSCs.

Up to now, several research teams have addressed these difficulties, using various scientific and technical approaches. Surgical samples from the adult CNS are usually very small (1-2 mL). As the number of resident aNSCs within the tissue is also very small, isolation techniques have been optimized to increase the success rate of the primary isolation of aNSCs. To acquire aNSCs, CNS tissues are physically minced, and enzymatically digested into single cells. Among them, the enzymatic digestion is a critical step, because it directly affects the survival of aNSCs. The compositions of dissociating enzymes and incubation times are various among investigators. Papain, trypsin, and collagenase have usually been used, and in some reports, papain dissociation was suggested to be most optimal for the primary isolation of aNSCs^[36,37].

After the mechanical and enzymatic dissociation of CNS tissues, the resulting single cells have been cultured by two alternative methods: the neurosphere, and adherent culture methods. Conventionally, the neurosphere culture method has been used for *in vitro* culture of NSCs^[38-47]. This method was first used in the primary isolation of NSCs from murine brains. The neurosphere culture method was also applied to maintain aNSCs from human brains. However, difficulties in the stable *in vitro* expansion of aNSCs using suspension culture methods resulted in the need for another culture method to be developed.

Moreover, a single neurosphere may not be derived from a single NSC^[48]. The possible heterogenic origin of neurospheres could not guarantee the homogeneity of *in vitro* expanded aNSCs in the suspension culture conditions^[49-51].

To overcome the weak points of the neurosphere culture method, others, as well as ourselves, developed alternative adherent culture methods for NSCs^[18-21,44,52-54]. Each group used their own coating plates to attach NSCs to the plates, and various culture medium compositions. Laminin and poly-L-ornithine (PLO) have frequently been used to coat plates, which increase the adherent efficiency of NSCs. To maintain stemness and proliferation of NSCs, the amount of EGF and basic FGF have been optimized^[55]. For example, we expanded aNSCs from temporal lobectomy samples of epilepsy patients without any neoplastic diseases, on PLO-coated plates in a DMEM/F12 media supplemented with 1% B27, 1% penicillin/streptomycin, EGF (50 ng/mL), bFGF (50 ng/mL), and 0.5% fetal bovine serum (Table 1)^[18]. Using the adherent culture method, aNSCs were expanded *in vitro* from 10⁴ to 10¹² cells within 8 subcultures for 2 mo. Moreover, the expression of Nestin and Sox2 as NSC markers was stably maintained^[18]. If the number of aNSCs required for transplantation is 10⁷ per patient, at least one hundred thousand patients could be treated with a primary culture of aNSCs.

Table 1 summarizes various primary culture and *in vitro* expansion techniques for aNSCs. As indicated in Table 1, major obstacles in the primary isolation and stable *in vitro* expansion of aNSCs have been, or are being resolved, which would increase their clinical applicability.

CURRENT THERAPEUTIC STATUS OF ADULT NEURAL STEM CELLS FOR NEUROLOGICAL DISEASES

Since technical breakthroughs for the preclinical and clinical utilization of aNSCs were introduced relatively recently, scientific results showing treatment effects of aNSCs against neurological diseases are, at present, limited^[18,56-59]. However, many previous reports have indicated that NSCs, compared with other stem cell types, are optimal for neurological diseases, since neural functional recovery requires direct neural cell supplementation, besides indirect paracrine effects. With brief presentation of the current developmental status of stem cell therapeutics for individual neurological disease, Tables 2 and 3 summarize preclinical and clinical results, respectively, of aNSCs against various neurological diseases.

Ischemic stroke

Various human stem cells and their derivatives can differentiate into neurons restoring functional losses in the rodent stroke model^[60,61]. In particular, human ESC-derived NSCs, injected into the ischemic penumbra region in rat brains with ischemic stroke, have been reported to

Table 1 Isolation and *in vitro* culture methods for adult neural stem cells

Culture methods	Cell source	Dissociating method	Media composition	Plate coating	Maximal <i>in vitro</i> culture	Ref.
Adherent culture method	Temporal lobe	Physical Mincing and enzymatic digestion with papain	DMEM/F12 supplemented with 10 ng/mL bFGF, 20 ng/mL TGF α , 2.5 μ g/mL heparin, 2% B27 (without retinoic acid), 10 mmol/L hepes, and 1% FBS			[54]
	Temporal lobe	Mechanical trituration and enzymatic dissociation using papain and DNase I	DMEM/F12 supplemented with 1% B27, 50 ng/mL EGF, 50 ng/mL bFGF, and 0.5% FBS	Poly-L-ornithine	18 passages	[18]
Neurosphere culture method	Hippocampal and lateral ventricle wall tissue	Mechanical dissociation and enzyme digestion using hyaluronic acid, kynurenic acid, and trypsin	DMEM/F12 supplemented with 10 ng/mL EGF, 20 ng/mL EGF, B27, and 2 mmol/L glutamine			[39]
	Temporal lobe	Enzymic digestion with trypsin	N2 medium supplemented with 5% FBS	Poly-2-hydroxyethyl methacrylate		[40]
	Hippocampus Amygdala Frontal cortex Temporal cortex	Enzymic digestion with hyaluronidase, kynurenic acid, and trypsin	DMEM/F12 supplemented with 0.6% glucose, 2 mmol/L glutamine, 3 mmol/L sodium bicarbonate, 5 mmol/L HEPES buffer, 25 mg/mL insulin, 10 mg/mL heparan sulfate, 100 mg/mL transferrin, 20 nmol/L progesterone, 60 mmol/L putrescine, 30 nmol/L selenium chloride, 20 ng/mL EGF, and 20 ng/mL bFGF-2			[41]
	Temporal lobe from 11-wk-old postnatal male Hippocampus, ventricular zone, motor cortex and corpus callosum from and a 27-year-old male	Enzymic digestion with papain, DNase I, and neutral protease	Initially, DMEM/F-12 supplemented with glutamine and 10% FBS After 24 h, DMEM/F12 supplemented with BIT-9500 (bovine serum albumin, transferrin, insulin, 20 ng/mL bFGF, 20 ng/mL EGF, and 20 ng/mL PDGF-AB) and 25% conditioned medium from rat stem cells that produces secretory bFGF and glycosylated form of cystatin C	Fibronectin	More than 70 population doublings in the 11-wk-old postnatal male More than 30 population doublings in the 27-year-old male	[42]
	Temporal lobe	Mechanical dissociation and enzymic digestion with DNase I and trypsin	DMEM/F12 supplemented with 1 mol/L HEPES, 2% B27, 0.1% EGF, and 0.1% bFGF		11 mo	[43]
	Temporal lobe	Enzymic digestion with Papain and DNase I	DMEM/F12 supplemented with bFGF and EGF		3–6 wk	[44,114]
	Lateral ventricular roof	Mechanical dissociation and enzyme digestion with DNase I and trypsin	DMEM/F12 supplemented with bFGF, EGF, and B27		3–7 wk	[45]
	Hippocampus containing hilus, temporal cortex, and subventricular zone including anterior horn and segmented lateral ventricle	Physical mincing and enzyme digestion with trypsin	DMEM/F12 supplemented with N2, 35 μ g/mL bovine pituitary extract, 5% fetal calf serum, 40 ng/mL EGF, and 20 ng/mL bFGF		> 60 population doublings	[46]
	Biopsies from filum terminale below conus medullaris	Physical mincing and enzyme digestion with trypsin	DMEM/F12 supplemented with B27, 10 ng/mL LIF, 10 ng/mL bFGF, and 20 ng/mL EGF	Ultra-low attachment dish		[47]

TGF α : Transforming growth factor alpha; DMEM/F12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; FBS: Fetal bovine serum; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor.

move to the lesions, and improve motor performances^[62]. Moreover, human adult temporal lobe-derived NSCs, grafted into the contralateral ventricle of the rat brains with focal cerebral stroke, significantly reduced the

infarction area, and showed recovery of motor function^[18]. When human fetal NSCs were transplanted into ischemic lesions of rodent brains, they migrated toward the injured regions and differentiated into neurons^[63,64].

Table 2 Preclinical results of adult neural stem cells against neurodegenerative diseases

Targeted disease animal model	Cell source	Injection method	Result	Animal species	Ref.
Demyelinated spinal cord injury	Frontal cortex, temporal cortex, hippocampus, and subventricular/subependymal zone of frontal lobe	The midline of the dorsal columns of the spinal cord at three longitudinal sites	The cells elicited extensive remyelination with a peripheral myelin pattern similar to Schwanncell myelination The remyelinated axons conducted impulses at near normal conduction velocities	Rat	[56]
Multiple sclerosis (lyssolecithin-demyelinated brain)	Temporal lobe	Local injection to demyelinated brain regions	Transplanted cells migrated to lesions without extending into normal white matter. Implanted progenitors matured as oligodendrocytes, and developed myelin-associated antigens	Rat	[57]
Global brain ischemia	Temporal lobe	The posterior periventricular region above the hippocampus	Adult human NPCs survived, migrated into ischemic regions, and differentiated into functional neural cells No information about therapeutic effects	Rat	[58]
Global brain ischemia	Temporal lobe	Left hippocampus After <i>in vitro</i> differentiation	Injected cells migrated preferentially into an ischemic lesion, which was mediated by SDF-1 α and CXCR4 signaling pathways No information about therapeutic effects	Rat	[59]
Focal ischemic stroke	Temporal lobe	Contralateral lateral ventricle	Transplanted cells reduced infarction volumes and enhanced motor activity	Rat	[18]

Initial clinical trials with stem cells have been completed in stroke^[61]. Unfortunately, no significant clinical outcomes were observed when autologous MSCs were injected intravenously into ischemic patients^[65]. Although other clinical studies adopting intravenous or intra-arterial administration of autologous bone marrow-derived stem cells in stroke patients are in progress or planning^[23], NSC-based regenerative treatment with both paracrine and neuronal supplementation effects would be more effective. Recently, a clinical trial for stroke with immortalized NSCs generated from human fetal cortex was planned in the United Kingdom^[23], which would yield scientific data that might possibly demonstrate the superior regenerative and treatment activities of NSCs.

Although there are scientific data demonstrating the therapeutic effects of aNSCs on ischemic stroke, aNSCs have not applied to clinical trials for ischemic stroke yet. In contrast, clinical trials using MSCs for ischemic stroke are continuously planned and performed world widely. Compared with MSC clinical trials, the most different feature of NSC trials is the injection route; while MSCs are usually injected intravenously, NSCs are stereotactically transplanted in the brain. Since the penetration of MSCs across brain-blood barrier is still controversial, direct implantation of NSCs into the brain would potentiate the therapeutic effects against ischemic stroke.

Spinal cord injury

Human NSCs transplanted into a mouse model of spinal cord injury were observed to differentiate into neurons and oligodendrocytes to lead the recovery of locomotion^[66]. Treatment mechanism study indicated that neurons derived from transplanted stem cells integrated into the host neuronal circuitry and mediated functional recovery^[19,67]. On the other hand, the functional recovery after NSC transplantation into spinal cord injury models was proportional to the number

of transplanted stem cell-derived oligodendrocytes and the amount of regenerated myelin^[68]. Those preclinical results indicate that the supplementation of mature neural cells by implanted stem cells would also be important in clinical settings, for the functional recovery of spinal cord injury patients.

Highly refined oligodendrocyte progenitor cells (OPCs) generated *in vitro* from human ESCs differentiated into oligodendrocytes, and induced remyelination of the demyelinated spinal cord of mouse^[69]. Based on these observations, a first phase I clinical trial using human ESC-derived OPCs is under planning by the United States company, Geron^[23]. This first clinical trial has raised worries about the risk for tumorigenicity, which is difficult to determine in preclinical situations^[70]. Since the results from animal models could not be directly translated into human, the possible risks need to be further validated. Moreover, utilization of aNSCs, instead of fetal origin stem cells, would reduce the possible tumorigenicity, due to their limited proliferation potential.

Parkinson's disease

Human embryonic mesencephalic tissue which contains many post-mitotic dopaminergic neuroblasts was tried clinically, which have showed proof of concept that regenerative approach could have therapeutic effects in Parkinson's disease (PD) patients^[71]. Dopaminergic neuroblasts for preclinical animal models have been cultured from various different stem cell sources, including ESCs^[72-79], fetal NSCs and precursors of embryonic ventral mesencephalon^[80-83], adult NSCs from the SVZ^[84], bone marrow stem cells^[85], and fibroblast-derived iPSC cells^[86].

Although a small portion of dopaminergic neurons derived from transplanted cells contain disease-specific Lewy bodies 11 to 16 years after transplantation^[87,88], implanted cells remained viable^[23,89]. However, definitive

Table 3 Current clinical trials of neural stem cells against neurodegenerative diseases

Disease	NSC source	Brief title	Trial ID	Condition	Location	Period	Cell source	Route	Phase
ALS	Fetal NSCs	Human neural stem cell transplantation in amyotrophic lateral sclerosis	NCT01640067	Recruiting	Italy	2012-07-09 -2016-09	Fetal neural stem cells	Spinal cord	I
	Adult NSCs	Dose escalation and safety study of human spinal cord derived neural stem cell transplantation for the Treatment of amyotrophic lateral sclerosis	NCT01730716	Enrolling participants by invitation only	United States	2012-11-06 -2014-09	Spinal cord of a single fetus 8 wk of gestation	Spinal cord after laminectomy	II
		Human spinal cord derived neural stem cell transplantation for the treatment of amyotrophic lateral sclerosis	NCT01348451	ongoing	United States	2011-04-28 -2015-12	Spinal cord of a single fetus 8 wk of gestation	Spinal cord after laminectomy	I
Stoke	Commercial NSC	Pilot investigation of stem cells in stroke	NCT01151124	ongoing	United Kingdom	2010-06-09 -2015-03	CTX0E03 DP allogeneic neural stem cells	Putamen region of the brain	I
	Adult NSCs	Pilot Investigation of stem cells in stroke phase II efficacy	NCT02117635	Recruiting	United Kingdom	2014-04-16 -2015-12	CTX0E03 DP allogeneic neural stem cells	Intracranially via stereotaxic	II
SCI	Adult NSC	Safety study of human spinal cord-derived neural stem cell transplantation for the treatment of chronic SCI	NCT01772810	Recruiting	United States	2014-08 -2014-05	Human spinal cord-derived neural stem cell	Direct injections into spinal parenchyma	I
	Adult NSCs	Study of human central nervous system stem cells in Patients with thoracic spinal cord Injury	NCT01321333	Ongoing	Canada, Switzerland	2011-03-21 -2015-05	Human central nervous system stem cells	Thoracic spinal cord	I , II
		study of human central nervous system stem cell transplantation in cervical spinal cord injury	NCT02163876	Recruiting	United States	2014-10 -2017-05	Human central nervous system stem cells	Cervical spine	II

NSCs: Neural stem cells; ALS: Amyotrophic lateral sclerosis; iPSCs: Induced pluripotent stem cells.

successful clinical trials have not yet been reported in the case of human stem cell-derived dopaminergic neurons. In contrast, a group of patients who had embryonic mesencephalic graft showed dyskinesia^[90-92]. Those reports have provoked major concern about the possible side effects of transplanted ESC cell-derived dopaminergic neuroblasts^[77], and the need for safer stem cell sources, such as aNSCs.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease with premature degeneration of motor neurons in the CNS^[23,93]. For the regeneration and/or supplementation of motor neurons, motor neurons were generated *in vitro* from ESCs^[94-97], fetal NSCs^[98-100], and iPSCs^[101,102].

Recently, transplantation of fetal spinal cord- or iPSC-derived NSCs was reported to be effective in slowing down the disease progression of ALS animal models^[103,104]. Based on the preclinical results, a phase I clinical trial of intra-spinal cord injection of fetal NSCs into ALS patients was attempted in the United States. Clinical outcomes from 6 to 18 mo after the transplantation showed that the intervention did not accelerate disease progress^[105]. In contrast, their

efficacy could not be determined, although higher dose of injection showed better results in some evaluating factors. On-going and/or planned phase II and III clinical trials would further determine the optimal therapeutic dose, and their therapeutic efficacy against ALS^[106].

Until now, there have been few preclinical and clinical trials using aNSCs for ALS. However, ALS could be a good treatment target of aNSCs, having regard to its fatality, and lack of proven therapeutic options for it.

Alzheimer's disease

Alzheimer's disease (AD) is the most frequent neurological disease, which is characterized by the increased amyloid plaques and neurofibrillary tangles in the brain^[107]. Amyloid plaques are extracellular aggregations consisting of amyloid-peptides. Neurofibrillary tangles are intracellular aggregations of hyperphosphorylated tau, a microtubule-associated protein within neuron^[108]. The causative relationship between amyloid plaques/neurofibrillary tangles and AD is still under investigation^[109]. Widespread non-specific neuronal death in the AD brain makes stem cell-based regeneration challenging. For effective cell therapy for AD, NSCs need to migrate to multiple regions

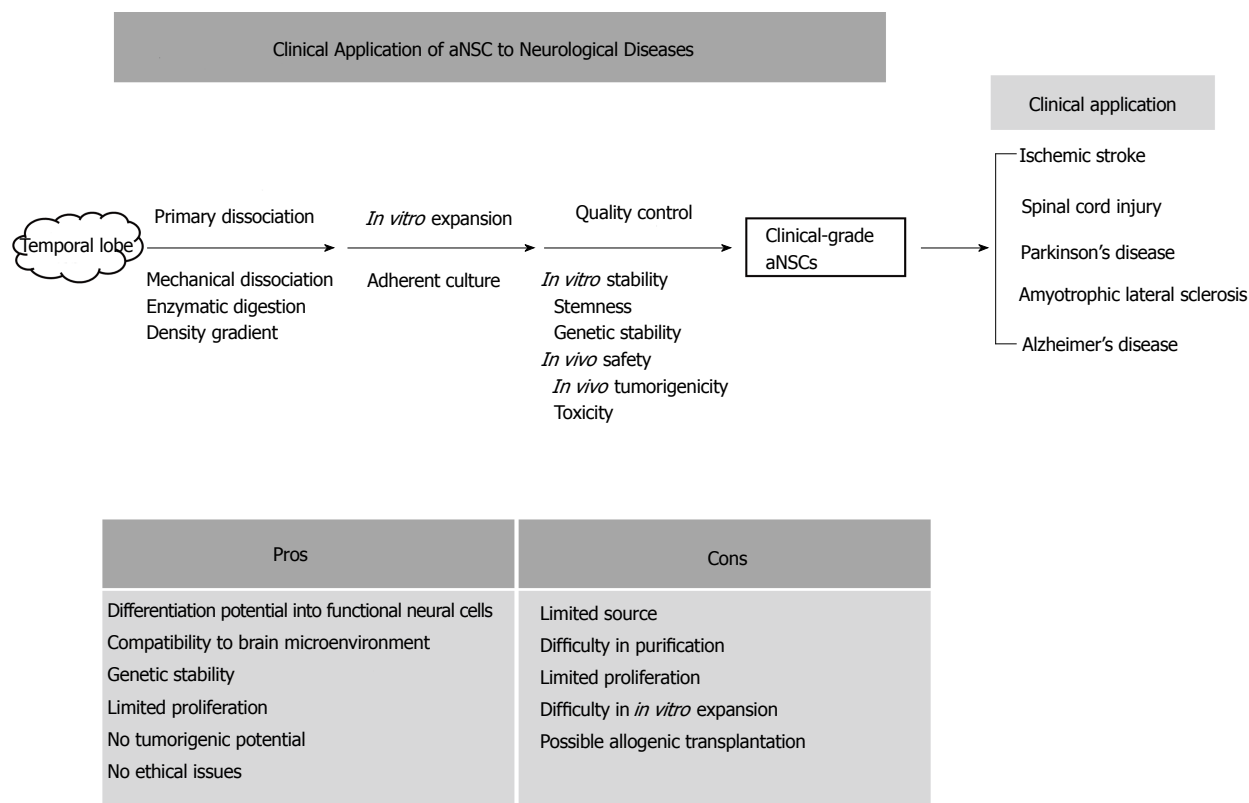


Figure 1 A diagrammatic summary of the approaches/strategies of adult neural stem cells and their pros/cons. aNSCs: Adult neural stem cells.

of the brain and then differentiate into numerous multiple subtype neural cells^[110]. Moreover, the effect of amyloid plaques on the survival, migration, and differentiation of injected stem cells should be taken into consideration^[111].

Human NSCs transplanted into the brains of AD animal models showed little neurogenesis, but unwanted gliosis around the plaque-like structures^[112]. Therefore, stem cell-based regenerative therapies need to be further developed preclinically, before clinical applications to AD. The disappointing preclinical data have resulted in few clinical trials using NSCs against AD. However, MSC is in relative advanced clinical trial stages. For example, human umbilical cord blood-derived MSCs are currently in a phase I clinical trial. Most trials using MSCs hire one-time direct injection of MSCs into the patient's brain. As AD is a progressive disease, long term investigations are necessary, to examine the lasting effects, as well as the safety of transplanted stem cells^[113].

PERSPECTIVES

Based on few scientifically proven treatment modalities for neurological diseases, and the regenerative potentials of stem cells, cell therapies using various stem cells have been preclinically and clinically applied to neurological diseases. There are many controversies about the therapeutic effects of stem cell treatments and their treatment mechanisms. The controversies could be derived from the diverse types of stem cells, and from their unique pros and cons.

Compared with other stem cell sources, aNSCs have several advantages, such as differentiation potential into functional neural cells, limited proliferation capacity, and few ethical problems (Figure 1). Although difficulties in the isolation and *in vitro* expansion of aNSCs prevent the active applications of aNSCs to neurological diseases, the technical obstacles have been continuously resolved. Therefore, at the current status, aNSCs can be attractive stem cell sources, to be introduced into the preclinical and clinical trials targeting various neurological diseases.

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Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries

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Abstract

Recent advances in stem cell biology have shed light on how normal stem and progenitor cells can evolve to acquire malignant characteristics during tumorigenesis. The cancer counterparts of normal stem and progenitor cells might be occurred through alterations of stem cell fates including an increase in self-renewal capability and a decrease in differentiation and/or apoptosis. This oncogenic evolution of cancer stem and progenitor cells, which often associates with aggressive phenotypes of the tumorigenic cells, is controlled in part by dysregulated epigenetic mechanisms including aberrant DNA methylation leading to abnormal epigenetic memory. Epigenetic therapy by

targeting DNA methyltransferases (DNMT) 1, DNMT3A and DNMT3B *via* 5-Azacytidine (Aza) and 5-Aza-2'-deoxycytidine (Aza-dC) has proved to be successful toward treatment of hematologic neoplasms especially for patients with myelodysplastic syndrome. In this review, I summarize the current knowledge of mechanisms underlying the inhibition of DNA methylation by Aza and Aza-dC, and of their apoptotic- and differentiation-inducing effects on cancer stem and progenitor cells in leukemia, medulloblastoma, glioblastoma, neuroblastoma, prostate cancer, pancreatic cancer and testicular germ cell tumors. Since cancer stem and progenitor cells are implicated in cancer aggressiveness such as tumor formation, progression, metastasis and recurrence, I propose that effective therapeutic strategies might be achieved through eradication of cancer stem and progenitor cells by targeting the DNA methylation machineries to interfere their "malignant memory".

Key words: Cancer stem and progenitor cells; DNA methylation; Epigenetic therapy; Aza-cytidine; Aza-deoxycytidine

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Core tip: Several types of cancers can be developed from genetic and/or epigenetic instabilities of stem and progenitor cells. Epigenetic abnormality involving dysregulation of DNA methylation has been reported to implicate in cancer aggressiveness. Inhibition of DNA methyltransferase activity by DNA methylation inhibitors has shown promising results toward treatment of myelodysplastic syndrome, a disease associated with leukemic stem cells. This review summarizes evidences which are pertinent to the antitumorigenic potential of DNA methylation inhibitors 5-Azacytidine and 5-Aza-2'-deoxycytidine on cancer stem and progenitor cells including those of leukemia and other solid tumors.

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INTRODUCTION

The study of stem cell biology, especially that of hematopoiesis, has provided an insight into the biology of cancers^[1,2]. In particular, normal stem cells and cancer cells share one of their characteristics, which is an ability to proliferate without differentiation, the property known as self-renewal. Three concepts of cancer biology are proposed based on the study of hematopoietic stem cells and their tumorigenic counterparts as the paradigm: (1) normal stem cells and cancer cells might possess similar mechanisms to control their proliferation or self-renewal; (2) cancer cells can be derived by differentiation of normal stem cells *via* mutation of transit-amplifying cells or progenitors; and (3) there might be cancer stem cells inside tumors where the tumorigenesis occurred with normal stem cells^[3]. Leukemia, which is the cancers of hematopoietic system, has provided the best evidence that normal stem cells are the target of oncogenic evolution, and that leukemogenesis is driven by cancer stem cells. For example, the tumor initiating cells of myelodysplastic syndrome (MDS), which can develop into acute myeloid leukemia (AML), are cancer stem cells, at least those with genetic deletion of chromosome 5q^[4]. Cancer stem and progenitor cells have been suggested to involve with relapse in many types of cancers^[5-13]. This tumorigenic recurrence of cancer stem and progenitor cells, which often associates with more aggressive phenotypes compared with differentiated cancer cells, can occur *via* accumulation of genetic mutations and/or epigenetic defects leading to selective advantage and hence the oncogenic evolution of cancer stem and progenitor cells.

DEFECTS OF DNA METHYLATION IN CANCERS

Gene expression must be tightly regulated by both genetic and epigenetic mechanisms in non-cancerous normal cells, thereby avoiding malignant transformation. Both genetic- and epigenetic-dependent mechanisms ensure that expression of genes, whose function is involved in cell proliferation, apoptosis, self-renewal and differentiation, is normally controlled in temporal- and spatial-dependent manners. Deregulated epigenetic mechanisms have been shown to implicate in tumorigenesis. One epigenetic mechanism, which has been prominently focused in cancer research, is DNA methylation which also represents one of the most active fields of epigenetic research.

DNA methylation is one of the most common defects in epigenetic regulation observed in tumorigenesis. Earlier studies have suggested that cancer cells possess global DNA hypomethylation, which results in chromosomal

instability^[14]. However, in the past decade most cancer epigenetics studies have paid attention to regulatory region-associated DNA hypermethylation^[15]. DNA hypermethylation of CpG dinucleotides has been well-documented in many types of cancer such as leukemia and colon cancer, and has been proposed as a hallmark of tumorigenesis^[16]. Specifically, DNA hypermethylation at promoters of tumor suppressor- and differentiation-associated genes has been frequently reported. The DNA hypermethylation of those genes then leads to a reduced gene expression, which in turn provides a selective advantage to cancer cells. Thus, down-regulation of the genes by DNA hypermethylation might result in the emergence of cancer stem and progenitor cells^[17,18].

DNA methylation has been widely accepted to mediate malignant memory of cancer cells due to its stable inheritance during cell division. Gene regulatory network specific to cancer stem and progenitor cells might play a direct role in dysregulation of DNA methylation. For example, the pluripotency-associated transcription factors OCT4 and NANOG have been shown to directly up-regulate expression of the maintenance DNA methyltransferase (DNMT) DNMT1 in mesenchymal stem cells^[19]. In fact, maintenance of DNA methylation has been reported to be critical for cancer stem cell properties in leukemic, colon and lung cancer stem cells^[20-22], which might be required to suppress expression of differentiation- and apoptosis-related genes. For instance, *p16* and *MLH1* are frequently hypermethylated in certain types of cancer. Of interesting is *MLH1*, which is a member of DNA mismatch repair pathway. Thus the epigenetic instability of *MLH1* might be a cause of genetic instability of cancer stem and progenitor cells. The epigenetic defects of other genes involved in different pathways related to cell migration, hormone receptors, and cell cycle control, have also been described in tumorigenesis^[18]. Different patterns of DNA hypermethylation have also been found in different types of cancers, which might be explained by cellular function of the cells in respective tissues^[16]. In addition, the pattern of DNA hypermethylation might be influenced by an interaction between DNMTs and polycomb repressive complex 2 (PRC2) protein subunits^[23], since different tumors also have different patterns of PRC2 binding^[17,24,25]. Epigenetic therapy based on DNA methylation inhibitors including 5-Azacytidine (Aza) and 5-Aza-2'-deoxycytidine (Aza-dC) has promised a therapeutic regimen to patients with DNA hypermethylation-induced cancer diseases^[15]. Therefore, understanding how DNMTs transfer a methyl group to genomic DNA, how they function in cancer cells, and how the drugs inhibit DNMT activity is crucial for development of superior anti-cancer therapy.

MOLECULAR MECHANISM UNDERLYING DNA METHYLTRANSFERASE ACTIVITY

DNA methylation is responsible for multiple chromatin-related processes including regulation gene expression by adding a methyl group from S-adenosyl methionine

(SAM) to the C5 position of a cytosine base mostly within CpG dinucleotide context at gene regulatory regions such as promoters and CpG islands. The CpG methylation is catalyzed by DNMTs. Five DNMT family proteins, *i.e.*, DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L, have been identified in mammalian genomes^[26]. Among these, DNMT2 does not function as a methyltransferase for DNA but as a RNA methyltransferase^[27,28]. In addition, DNMT3L does not possess the methyltransferase catalytic domain^[29], leaving only DNMT1, DNMT3A and DNMT3B that are functional DNA methyltransferases^[30]. Two mechanisms underlying DNA methylation have been reported. The first mechanism is maintenance DNA methylation pattern, of which all three DNMTs have the ability to copy DNA methylation pattern from DNA template to daughter DNA strand^[31-37]. In one study, Sharma *et al*^[34] have proposed a homeostatic inheritance system for maintenance of DNA methylation, whereby high levels of 5-methylcytosine stabilize DNMT3A and DNMT3B and prevent them from proteolysis^[34]. The second mechanism is *de novo* DNA methylation which can only be performed by DNMT3A and DNMT3B. Apart from repressing gene expression *via* methylation at gene promoters, DNA methylation also plays important roles in many epigenetic phenomena involved in development for example genomic imprinting, X chromosome inactivation and higher-order chromatin organization^[38,39]. Hence, apart from cancers, dysregulation of DNA methylation are also implicated in pathogenesis of many diseases.

Several DNMT domains play important role in the methylation catalysis. Region IV harbors a prolyl-cysteinyl dipeptide that functions as the active site (Figure 1). Regions I and X share amino acid residues, which are critical for binding of SAM. Region V contains glutamyl/asparaginyl residue, which functions to protonate nitrogen 3 (N3) of targeted cytosine during the methyltransferase reaction. The enzymatic mechanism of DNA methylation begins with flipping cytosine out of the DNA helix upon binding with a DNMT. The conformational change of the cytosine nitrogenous base ensures that carbon 5 (C5) of the base aligns at the catalytic pocket with a bound SAM, and that C6 is at close proximity to a cysteine residue at the active site of the enzymes. The base flipping leads to formation of a covalent bond between C6 of the cytosine in DNA and the cysteine residue mediated by nucleophilic attack of sulfhydryl group of the cysteine to the C6 resulting in a change from C5 = C6 double bond into C5-C6 single bond (Figure 2A). The chemical bonding between C6 and cysteine then leads to an increase in electron flow toward C5 position of cytosine. At the same time, a methyl group from SAM is transferred to C5 generating C5-CH₃ of the cytosine and S-adenosyl-homocysteine as the products. The proton at C5 position is then abstracted due to its weak bonding with C5 resulting in β -elimination at the C5-C6 single bond and in the reformation of the C5 = C6 double bond, which return a single electron to the cysteine of DNMT and discharge to the enzyme^[30]. The C5-cytosine is now become methylated giving rise to methylcytosine.

INHIBITION OF DNMTS BY AZA-DC

The ability of Aza and Aza-dC to induce not only cell death but also differentiation had encouraged the exploitation of the compounds for treatment of cancers associated with stem/progenitor cells such as leukemia^[40,41]. Unlike Aza which can be incorporated into both DNA and RNA, Aza-dC is only incorporated into DNA and is therefore a specific inhibitor for DNA but not RNA methyltransferases^[42,43]. Consequently, the difference between the two epigenetic drugs results in differential cellular responses and causes distinct changes in the gene expression patterns of cells treated with Aza-dC and Aza^[44,45].

Intracellular uptake of Aza-dC is mediated by the human concentrative nucleoside transporter 1 HCNT1, also known as SLC28A1^[46]. Once inside cells, Aza-dC is then phosphorylated by deoxycytidine kinase yielding Aza-dCMP. Aza-dCMP is further phosphorylated to Aza-dCDP and subsequent Aza-dCTP, the active substrate form for DNA replication, by dCMP kinase and diphosphokinase, respectively. Hence the irreversible inhibition of Aza-dC on DNA methylation takes place only if Aza-dCTP is used as a substrate by DNA polymerases during DNA replication at S phase of the cell cycle, but not from free Aza-dC itself. Within DNA and with the presence of DNMTs, which bind to genomic DNA, the Aza-dC base can make a covalent bond with a DNMT enzyme (Figure 2B), disrupting the function of the enzyme. Mechanistically, because the nitrogen atom at position 5 (N5) of Aza-dC has a higher electronegativity than C5 of cytosine, the incorporated Aza-dC can covalently trap the DNMT enzyme. A single bond between N5-C6 is formed upon the nucleophilic attack of sulfhydryl group to the C6. However, unlike C5 in cytosine, the N5 can abstract a proton, and hence stably form a single bond to the proton with a strong affinity due to its high electronegativity. The DNMT is therefore trapped with the incorporated Aza-dC through a covalent bond between C6 and the cysteine residue leading to a DNA-DNMT adduct. Thus only in the presence of DNMTs can Aza-dC function to inhibit DNA methylation activity^[47]. At chromatin level, gene reactivation activity of Aza-dC has been mechanistically demonstrated by Jones *et al*^[31]. The authors devised a novel technique called *Accesible/ble*^[48]. They find that approximately 2% of chromatin regions, which are demethylated after Aza-dC treatment in a colon cancer cell line, govern a feature of open chromatin^[48] possibly associated with deposition of the histone variant H2A.Z^[49]. Importantly, they show that of all genes reactivated by Aza-dC treatment, 90% of them possess DNA demethylation and chromatin accessibility supporting the role of the DNA methylation inhibitor as a gene reactivator. Interestingly, many reactivated genes are also tumor suppressor genes^[48].

Several reports have demonstrated that depletion of DNMT1, DNMT3A or DNMT3B can counteract the cytotoxic and apoptotic effect of Aza-dC on treated cancer cells, confirming the essential role of DNMTs in mediating the Aza-dC response^[47,50-52]. In contrast to

	I II III	
DNMT1	LRLTDVTSCHGLSEGFHQAGIS-DTLWAIEMWDPAAQAFRLNNPGSTVFTEDCNILKL	1213
DNMT3A	IRVLSLFDGIIITGLLVKLDLGIQVDRIYASEVCEDSITVGMVRHQG-----KI	681
DNMT3B	IRVLSLFDGIIITGLVLKELGKIKVGYVASEVCEESIAVGTVKHEG-----NI	622
	IV	
DNMT1	VMAGETTSNRGQRLPQKGDVEMLCGGPQGFSGMNRFNSTYSKFKNSLVVSFLSYCDY	1273
DNMT3A	MYVGDVRSVTQKHIEWGPFDLVIGGSNDLSIVNPARKGLYEGTG-RLFFEFYRLLDH	740
DNMT3B	KYVNDVRNITKKNIEEWGPFDLVIGGSNDLSNVNPARKGLYEGTG-RLFFEFYHLLNY	681
	V	
DNMT1	YR-----PRFFLEENVNFVSFKRSMVLKLTLRCLVRMGYQCTFGVLQAGQYGAQTR	1326
DNMT3A	ARPKEGDDRPFFWLFENVVAMGVSDKR-----DISRFLESN-----PVMIDAKEVS-----	786
DNMT3B	SRPKEGDDRPFFWLFENVVAMKVGDKR-----DISRFLECN-----PVMIDAIKVS-----	727
DNMT1	RRAILAAAPGEKLPFLPEPLHVFAPRACQLSVVVDKKFVSNITRLSSGPFRTITVRDT	1386
DNMT3A	-----	
DNMT3B	-----	
	VI VII VIII	
DNMT1	MSDLPEVRNGASALEISYNGEPQSWFQRQLRGAQYQPILRDHICKDMSALVAMHIPL	1446
DNMT3A	-----AAHAAAYF--	794
DNMT3B	-----AAHAAAYF--	735
DNMT1	APGSDWRDLPNIEVRLSDGTMARKLRYTHHDKRNGRSSSGALRGVCSCVEAGKACDPAAR	1506
DNMT3A	-----WGNLP-----GMNRPASTVNDK-----LELQECLEHGRIAK--FS	828
DNMT3B	-----WGNLP-----GMNRPVIASKNDK-----LELQDCLEYNRIAK--LK	769
DNMT1	QFNTLIPWCLPHTGNRRHHWAGLYGRLEWDGFFSTVTNPEPMGKQGRVLHFEQHRVSV	1566
DNMT3A	KVRTITTRSNSIKQKGDQHF-----VFMEKEDILWC	861
DNMT3B	KVQTITTKSNSIKQGNQLFP-----VVMNGKEDVLWC	802
	X	
DNMT1	RECARSQGFDPDYRLFGNIL--DKHR-----QVGNVAPPPLAKAIGLEIKL	1610
DNMT3A	TEMERVFGFPVHYTDVSNMSRLARQ-----LLGRSWSVPVIRHLFAPLKE	907
DNMT3B	TELERIFGFPVHYTDVSNMGRGARQK-----LLGRSWSVPVIRHLFAPLKD	848

Figure 1 High similarity of critical amino acid residues at the catalytic domain of DNA methyltransferases. Multiple alignment of amino acids within the catalytic domain of three catalytically active human DNA methyltransferases DNMT1, DNMT3A and DNMT3B is shown. Highlighted amino acids with roman letters shown above indicate critical residues of the catalytic domain. DNMT: DNA methyltransferases.

the apoptotic response mediated by DNMTs, whether DNMTs also mediate stem cell differentiation induced by Aza-dC is not well understood. Epigenetic therapy by induced differentiation offers an option for treating malignancies, in which cancer tissues contain both cancer stem/progenitor cells and differentiated cancer cells, such as chronic leukemia, teratocarcinoma, and other solid tumors. Thus elucidating how cell differentiation can be triggered by Aza-dC or Aza could have an enormous impact on epigenetic therapy targeting cancer stem and progenitor cells.

INHIBITION OF EPIGENETIC PATHWAYS IN CANCER STEM AND PROGENITOR CELLS

Leukemia

Leukemia is considered to be the prototypical model for development of epigenetic therapy. MDS together with AML have been identified for being diseases of cancer stem and progenitor cells^[4,53], which might contribute to relapse and resistance of chemotherapies. In fact, the DNMT inhibitor Aza is the first epigenetic drug which is approved by the Food and Drug Administration for treatment of MDS. Aza and Aza-dC have initially been shown to induce differentiation of Friend erythroleukemia cells^[54]. In this study, the authors show that Aza-dC has a greater effect on reduction of cell proliferation compared

with Aza when used at the same concentration, which might be associated with an increase in iron uptake and heme biosynthesis^[55]. Later on, Pinto *et al*^[56] have reported the first evidence of Aza-dC as a differentiation inducing agent of primary cells derived from patients with AML^[56]. The differentiation inducing effect of Aza and Aza-dC has proven them to be promising therapeutic approaches aiming at induction of leukemic stem and progenitor cells toward non-malignant differentiated cells^[57,58].

The induction ability of myeloid differentiation by Aza-dC in myeloid leukemic cells correlates with an up-regulation of CD15, myeloperoxidase, lysozyme and the tumor suppressor p15^[59]. Several mechanisms have been proposed to underline Aza- and Aza-dC-induced differentiation of myeloid leukemia. For example, Aza-dC facilitates tumor necrosis factor alpha-induced monocytic differentiation of two AML cell lines NB4 and U937 by demethylation of *DIF2* (also known as *IER3*) promoter and thus up-regulation of the gene^[60]. Similarly, an upstream regulatory element within PU.1 promoter has been shown to be demethylated by Aza leading to an up-regulation of PU.1 and its target genes^[61]. Co-treatment of CD34⁺ cells derived from MDS patients by Aza and granulocyte colony-stimulating factor led to myeloid differentiation^[61]. Another study has shown that Aza-dC induces expression of olfactomedin 4 (*OLFM4*), of which an over-expression led to apoptosis and differentiation HL60 cell line^[62]. Treatment of myeloid leukemia cells with Aza-dC also led to an induction of

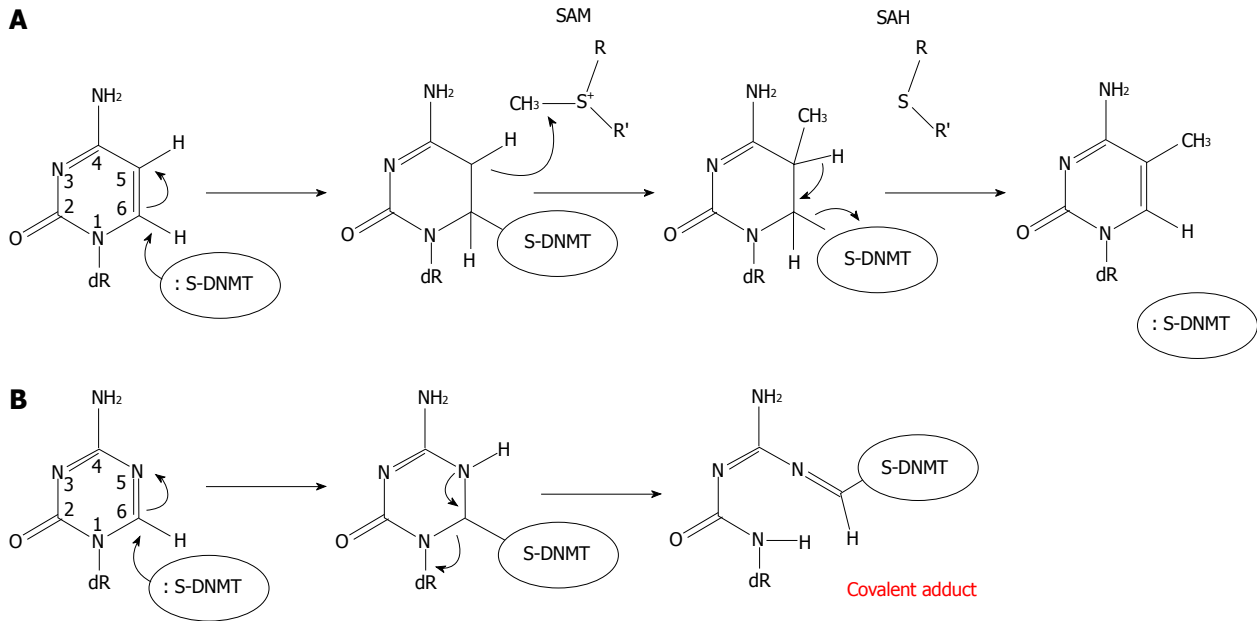


Figure 2 Mechanism of DNA methylation and its inhibition by 5-Aza-2'-deoxycytidine via covalent trapping. A: Cytosine methylation by a DNMT is initiated by the formation of a covalent bond between the cytosine base and a cysteine residue at the active site of the methyltransferase. SAM then transfers a methyl group to the cytosine generating a methylcytosine. B: A DNMT is covalently trapped by the DNA-incorporated 5-Aza-2'-deoxycytidine (Aza-dC) adduct. The sulfhydryl group of the cysteine residue at the active site of DNMT forms a covalent bond with C6 of the Aza-dC in DNA. However, the active methyl group from SAM cannot be transferred to Aza-dC by DNMT. This covalent trapping mechanism then leads to a gradual loss of free DNMTs, because they are covalently linked with Aza-dC within DNA. Therefore, the loss of free DNMTs then precedes passive DNA demethylation. Thus cells expressing high levels of DNMTs will respond to Aza-dC at a higher extent compared with cells expressing low levels of DNMTs (see Figure 3). R: Methionine/cysteine; R': Adenosine; Dr: Deoxyribose-5-phosphate; DNMT: DNA methyltransferases.

differentiation-associated genes including the erythroid-lineage transcription factor GATA1, which inhibits proliferation of the cancer cells^[20]. A link between DNA hypermethylation and AML1/ETO-mediated repression of microRNA expression has been suggested as a means to inhibit apoptosis and differentiation of leukemic stem and progenitor cells^[63,64]. In AML and chronic myeloid leukemia cell lines, Aza treatment reactivated expression of microRNA-193a, which suppresses translation of oncogenes including c-kit. Furthermore, an ectopic over-expression of microRNA-193a in primary cells of AML blasts led to apoptosis and differentiation of the cells^[63]. Remarkably, Tsai *et al*^[65] have recently shown that application of Aza or Aza-dC at low and non-acute toxic doses has successfully reduced stem cell characteristics of leukemia and also other cancer stem and progenitor cell lines and of primary tumors, without adverse effects on normal human bone marrow cells^[65]. The low concentrations of the epigenetic drugs can demethylate both CpG island- and non-CpG island-containing promoters, which up-regulate expression of tumor suppressor- and differentiation-associated genes.

Recently, a failure of Aza to eradicate cancer stem and progenitor cells in patients with MDS and AML has been reported^[66]. However, it has yet to be determined the mechanisms underlying this failure in order to improve new strategies of Aza treatment, and to design new drugs and clinical regimes that can completely diminish the cancer stem and progenitor cells of MDS and AML, either through an induction of cell death or

differentiation. Interestingly, tranylcypromine, an inhibitor targeting histone H3 lysine 4 demethylase LSD1 (also known as KDM1A) which is pivotal for maintaining the stem cell state of AML cells^[67], shows a promising result when combined with retinoic acid to induce myeloid differentiation of AML cells^[68]. Therefore, combination therapies between DNA methylation inhibitors, preferentially with low doses^[65], and other molecules which induce apoptosis and/or differentiation of cancer stem and progenitor cells of MDS and AML cells might offer an alternative direction to target the malignant cells in MDS and AML patients.

Although the effect of DNMT inhibitors on cytotoxicity and differentiation of lymphoblastic leukemia is less studied, a recent finding has suggested the role of Aza-dC in induction of apoptosis and differentiation of B-cell acute lymphoblastic leukemia (B-ALL) cell line NALM-6 and T-cell acute lymphoblastic leukemia (T-ALL) cell line CCRF-CEM^[69]. The apoptotic induction by Aza-dC in B-ALL cells might be due to reactivation of *HES5* gene, which has been shown to be hypermethylated in primary B-ALL cells^[70]. Further investigations are needed to ascertain whether Aza and Aza-dC induces differentiation of primary B-ALL and T-ALL cells, and to elucidate mechanisms responsible for the eradication of cancer stem and progenitor cells of B-ALL and T-ALL by DNMT inhibitors.

Medulloblastoma

Medulloblastoma is the most common malignant

brain tumor found in children, and is a disease of cancer stem cells which possess activity of cell signaling pathways resemble to that of embryonic stages of neural progenitors^[71]. Recent genome-wide DNA methylation analyses have suggested that DNA methylation plays a major role in the pathogenesis of medulloblastoma by repression of genes involved in developmental regulation thus avoiding cellular differentiation^[72,73]. In addition, DNA methylation might also be important for medulloblastoma to avoid cell death, since an inhibition of DNA methylation sensitized medulloblastoma cell lines to undergo apoptosis induced by interferon gamma and TNF-related apoptosis-inducing ligand (TRAIL)^[74]. Treatment of medulloblastoma cell lines by Aza-dC has been shown to induce expression of *SPINT2*, which is an inhibitor of hepatocyte growth factor signaling and is hypermethylated in a number of patients with medulloblastoma^[75]. Interestingly, an ectopic expression of *SPINT2* led to a decrease in proliferation, colony survival and cell migration of medulloblastoma cell lines. Moreover, mice transplanted with *SPINT2* over-expressing medulloblastoma cells show a prolonged survival compared with controls suggesting that *SPINT2* functions as a tumor suppressor *in vivo*^[75]. Similar to *SPINT2*, expression of *CRABP2*, which encodes a retinoic acid binding protein, is silenced by DNA methylation in medulloblastoma. Treatment of the cancer cells by Aza-dC restored expression of *CRABP2*, which in turn allowed neuronal differentiation of medulloblastoma cells induced by retinoic acid^[76]. Co-treatment of Aza-dC and the histone deacetylase (HDAC) inhibitor valproic acid also resulted in a reduction of medulloblastoma formation in *Patched* mutant mouse models, and extended survival of the mice^[77]. Thus further preclinical studies on combined therapies using DNA methylation inhibitors and HDAC inhibitors might be worthwhile for treatment of patients with medulloblastoma.

Glioblastoma

While medulloblastoma is the most prevalent brain tumor in children, glioblastoma is the most common and aggressive brain tumor found in adults. The role of DNA methylation in repression of genes involved in differentiation seems to be similar between glioblastoma and medulloblastoma. For example, DNA hypermethylation has been reported at CpG island upstream of *CRABP2*. Inhibition of DNA methylation by Aza-dC treatment in primary glioblastoma resulted in activation of *CRABP2* expression and differentiation of the cells^[76,78]. However, in contrast to medulloblastoma^[74], treatment of glioblastoma stem-like cells by Aza-dC failed to sensitize the cells toward TRAIL-induced apoptosis, although expression of caspase-8 was induced by Aza-dC^[79]. A connection of DNA methylation with p53 pathway has been described, in which Aza-dC treatment led to demethylation at p53 promoter. As the result, p53 then directly binds to the promoter region of the G protein-coupled formylpeptide receptor, and represses its expression leading to differentiation of glioblastoma

cells^[80]. Aza-dC has also been reported to induce expression of microRNA-137, which is involved in anti-proliferation and differentiation of glioblastoma stem cells^[81].

Neuroblastoma

Neuroblastoma has been proposed to develop from undifferentiated neural crest progenitor cells, which possess the potency to differentiate into peripheral neural and mesenchymal lineages^[82]. It is a pediatric cancer, which might be explained by the embryonic origin of neural crest progenitor cells. DNA methylation has been reported to epigenetically silence expression of caspase-8, which is a mediator of apoptosis in neuroblastoma^[83]. Co-treatment of Aza-dC and either chemotherapeutic drugs cisplatin or etoposide induced cell death possibly through caspase-8 reactivation^[84]. In addition, as mentioned above for medulloblastoma^[74], the same study also shows that a combined treatment of Aza-dC and interferon gamma led to apoptotic induction *via* TRAIL pathway in neuroblastoma cell lines^[74]. It would be of interest to further investigate whether Aza-dC and interferon gamma might synergistically and extensively induce differentiation of the cancer progenitor cells as neuroblastoma have been shown to differentiate by Aza-dC treatment^[85,86].

Prostate cancer

Androgen receptor (AR) is an important survival factor in prostate cancer. However, in some cases of prostate cancer, AR can function as a tumor suppressor gene^[87]. The inhibitory role of AR in aggressiveness of prostate cancer is supported by loss of AR expression *via* promoter DNA methylation^[88-91]. Interestingly, expression of AR is also reduced in prostate cancer stem cells particularly with high CD133 expression level^[92]. Treatment of prostate cancer stem cells with Aza-dC led to an up-regulation of AR and a decrease in proliferation and tumor formation. Moreover, Aza-dC treatment also induced differentiation of the cancer stem cells, which is correlated with a reduced expression of stem cell-associated genes *OCT4* and *NANOG*^[92]. Thus, this study suggests that the inhibition of DNA methylation might be clinically translated for patients having prostate cancers with androgen-independent feature.

Pancreatic cancer

Pancreatic cancer stem cells have been shown to be sensitive to Aza-dC, which reduces tumor sphere formation and induces apoptosis of the cancer stem cells through activation of caspase-3 pathway^[93]. The tumor suppressor activity of Aza-dC on pancreatic cancer stem cells is due to its ability to induce expression of microRNA-34, which in turn suppresses expression of the growth factor VEGF-B^[93]. In addition to the apoptotic induction effect, Aza-dC has also been demonstrated to induce differentiation of the pancreatic cancer progenitor cell lines MIA PaCa-2^[94] and PANC1^[95]. Further investigations on how Aza-dC induces differentiation of pancreatic cancer stem and progenitor cells at molecular level are important toward its clinical translation.

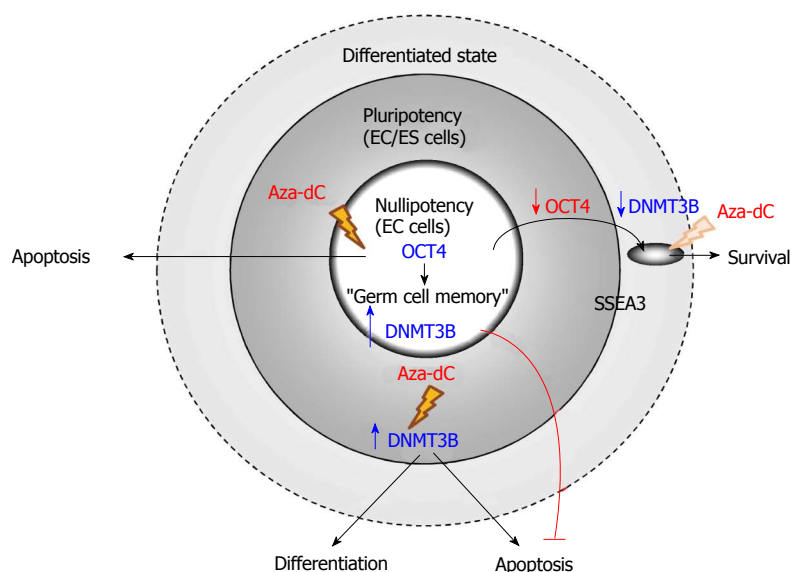


Figure 3 5-Aza-2'-deoxycytidine induces apoptosis of human teratocarcinoma stem cells but not their differentiated counterparts. Human nullipotent embryonal carcinoma (EC) cells, the stem cells of teratocarcinoma, are highly sensitive to 5-Aza-2'-deoxycytidine (Aza-dC), which induces apoptosis of the cancer stem cells. On the other hand, OCT4-knockdown differentiated nullipotent EC cells are resistant toward Aza-dC treatment. In human pluripotent EC cells NTERA2, DNMT3B mediates apoptosis induced by Aza-dC, whereas the enzyme mediates differentiation of human ES cells induced by the inhibitor. DNMT: DNA methyltransferases; ES: Embryonicstem; OCT4: Octamer-binding transcription factor 4.

Testicular germ cell tumors

Germ cell tumors (GCTs) with stem cell characteristics comprise of seminoma and non-seminoma, which belong to type II testicular GCTs. Seminoma and non-seminoma have been proposed to develop from carcinoma *in situ*, also known as intratubular germ-cell neoplasia unclassified lesion or testicular intratubular neoplasia, which is the malignant counterpart of gonocytes^[96,97]. Although the human seminoma-like cell line TCam-2 is resistant to Aza-dC even applied at 10 $\mu\text{mol/L}$ ^[98], human embryonal carcinoma (EC) cells, the stem cells of teratocarcinoma, are extremely sensitive to the epigenetic drug of which only 10 nmol/L is sufficient to reduce viability of EC cells. This cytotoxicity of Aza-dC on human EC cells is mediated by DNMT3B, which is a marker of human pluripotent stem cells^[99] and is highly expressed in human EC cells^[51]. Subsequently, DNMT3B has been reported to play a significant role in activation of DNA damage response and expression of p53 target genes induced by Aza-dC in human pluripotent EC cell line NTERA2^[100].

Albeit having the cytotoxic effect on the cancer stem cells, Aza-dC fails to induce apoptosis of differentiated cells derived from human nullipotent EC cells, which can be isolated from teratocarcinoma more frequently than their pluripotent counterparts^[52]. Although human nullipotent EC cells undergo apoptosis induced by Aza-dC, they do not differentiate. On the other hand, Aza-dC induces both differentiation and apoptosis of human pluripotent EC cells and also human embryonic stem (ES) cells. The ability to induce apoptosis of Aza-dC depends on DNMT3B in human pluripotent EC cells but not in their nullipotent counterparts (Figure 3). Similarly, the ability to induce differentiation of Aza-dC in human ES cells also depends on DNMT3B, supporting the role of DNMT3B in facilitating differentiation of human ES cells^[101]. The failure of Aza-dC to induce differentiation of human nullipotent EC cells suggests that mechanism(s) other than DNA methylation is responsible for maintaining the stem cell state of nullipotent EC cells^[102], which

might still possess a remnant of germ cell memory^[103]. Therefore, to effectively eradicate teratocarcinoma comprising of EC cells and their associated teratoma tissues, combined therapies between Aza-dC and agents that target the differentiated cancer cells should be considered.

CONCLUSION

Progresses in an understanding of normal stem cell biology have revolutionized the concept of cancer stem and progenitor cells, which might be the key toward success in treatment of a number of cancer diseases by targeting at cancer stem and progenitor cells. Aza and Aza-dC, although being the drugs currently used for treatment of hematologic malignancy, have held a great promise for treatment of solid tumors^[65]. Future studies on mechanisms underlying differentiation and/or apoptosis induced by Aza and Aza-dC on cancer stem and progenitor cells are required for effective treatment of the epigenetic drugs. Several questions emerge including; (1) Do gene regulatory networks, which are specifically associated with cancer counterparts of stem and progenitor cells, up-regulate expression of DNMTs? (2) Which signaling pathways and their downstream target genes are functionally perturbed by Aza and Aza-dC treatment? (3) Are cell death pathways other than apoptosis involved in the anti-proliferation effect? (4) Are Aza and Aza-dC effective to eradicate cancer stem and progenitor cells in xenograft tumors? (5) In case of relapse, do recurrent cancer stem and progenitor cells evolve a distinct "malignant memory" at epigenomic levels compared with the cancer cells isolated before admission? and, if so, (6) Does the oncogenic evolution lead to more aggressive phenotypes such as a reduced differentiation potential of cancer stem and progenitor cells? Treatments with Aza and Aza-dC can lead to certain toxicity and side effects to hematopoietic, nervous as well as metabolic systems^[104-107]. Nevertheless, they have been recognized as having lower toxicity compared

to traditional chemotherapy. In fact, low-dose treatment of Aza-dC in patients with MDS^[108] and solid tumors^[109] resulted in complete response of the patients. Thus using low-dose DNA methylation inhibitors may provide a safe therapeutic choice^[65] especially for elderly patients^[107,110]. Whether long-termed treatments of low-dose Aza and Aza-dC would perturb fates of stem and progenitor cells without side effects and toxicity in patients have yet to be elucidated. Addressing these questions will not only provide an understanding of cancer stem cell biology but also shed light on development of novel clinical regimes.

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Perinatal stem cells: A promising cell resource for tissue engineering of craniofacial bone

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Abstract

In facing the mounting clinical challenge and suboptimal techniques of craniofacial bone defects resulting from various conditions, such as congenital malformations, osteomyelitis, trauma and tumor resection, the ongoing research of regenerative medicine using stem cells and concurrent advancement in biotechnology have shifted the focus from surgical reconstruction to a novel stem

cell-based tissue engineering strategy for customized and functional craniofacial bone regeneration. Given the unique ontogenetical and cell biological properties of perinatal stem cells, emerging evidence has suggested these extraembryonic tissue-derived stem cells to be a promising cell source for extensive use in regenerative medicine and tissue engineering. In this review, we summarize the current achievements and obstacles in stem cell-based craniofacial bone regeneration and subsequently we address the characteristics of various types of perinatal stem cells and their novel application in tissue engineering of craniofacial bone. We propose the promising feasibility and scope of perinatal stem cell-based craniofacial bone tissue engineering for future clinical application.

Key words: Craniofacial bone regeneration; Bone tissue engineering; Extraembryonic tissue; Perinatal stem cells

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Core tip: Given the unique ontogenetical and cell biological properties of perinatal stem cells, emerging evidence has suggested these extraembryonic tissue-derived stem cells to be a promising cell source for extensive use in regenerative medicine and tissue engineering. In this review, we summarize the current achievements and obstacles in stem cell-based craniofacial bone regeneration and subsequently we address the characteristics of various types of perinatal stem cells and their novel application in tissue engineering of craniofacial bone. We propose the promising feasibility and scope of perinatal stem cell-based craniofacial bone tissue engineering for future clinical application.

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INTRODUCTION

The craniofacial bone has an essential role in supporting the adjacent soft tissues, providing anchorage for dental structures, maintaining structural stability for many physical functions and composing the esthetics of the human body. Craniofacial bone defects resulting from various conditions, such as congenital malformation, trauma, osteomyelitis and tumor resection, often lead to large psychomedical burdens as well as difficult reconstructive challenges for both patients and craniofacial surgeons^[1-5]. Current treatments for such skeletal defects often require invasive and technically challenging operations such as alloplastic reconstruction, bone grafting from various anatomic areas like the iliac crest and fibula and microvascular free-flap techniques^[3,4,6-8]. While these procedures have been proven to be reliable and effective, they also result in secondary donor site defects, associated intra- and post-operative complications and extended hospitalization. Furthermore, these techniques were largely limited by the scarcity of donor bone graft volume and patient physical conditions and struggled to restore pre-operative form and function. Suggested by recent clinical studies, stem cell-based bone tissue engineering has been recognized as a promising strategy for both aesthetic and functional reconstruction of craniofacial bone defects^[9-14].

To date, several stem cell sources, such as adult mesenchymal stem cells or mesenchymal stromal cells (MSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have displayed promising osteogenic potential *in vivo* and *in vitro* and thus have been proposed as a potential cell source for bone tissue engineering^[9,11-13,15,16]. However, the limitations associated with these stem cell sources are also significant^[13,17-20]. In the last decade, the list of putative human stem cell sources was amended to include human perinatal extraembryonic tissues, such as amniotic fluid, fetal membranes (amnion and chorion) and umbilical cord^[21-23]. Due to their unique ontogenetic relationship to fetal development, the extraembryonic perinatal stem cells represent an intermediate cell type which has recently been described to combine qualities of both their adult stem cell counterparts and ESCs and possess immunoprivileged characteristics, as well as a broad multipotent plasticity^[24-29]. Most importantly, these cells, simply isolated from extraembryonic tissues which are normally discarded after birth, effectively avoid ethical issue involvement. All these attractive characteristics make perinatal stem cells a promising and noncontroversial source of stem cells for extensive use in craniofacial bone tissue engineering (CBTE).

In the present review, we summarize the current research progress on stem cell-based craniofacial bone regeneration and subsequently we address the characteristics of various perinatal stem cells and their novel application in CBTE.

CRANIOFACIAL BONE TISSUE ENGINEERING AND STEM CELLS

Craniofacial bone tissue engineering

As an alternative to surgical reconstruction, multidisciplinary developments in cell and molecular biology, developmental biology, materials science and bioengineering promoted the tissue engineering approach, which was composed of stem/progenitor cells, biocompatible scaffolds and biochemical signals, to regenerate large tissue defects^[30]. The ongoing tissue engineering strategy offers several potential benefits, including the avoidance of secondary donor site defect, reduction of hospitalization and medical burdens and most importantly, the ability to closely restore the normal anatomic structure and function. Among the variety of current tissue regenerative indications based on the tissue engineering strategy, craniofacial bone defect is particularly suited to be tissue engineered. In fact, stem cell-based bone tissue engineering has already entered many preclinical or clinical applications in the craniofacial region.

Adult MSC-based craniofacial bone tissue engineering

Currently, one of the most well-defined and utilized stem cell types in CBTE is the adult MSCs. These stem cells have been isolated and identified from various tissues such as bone marrow, adipose, muscle, dental pulp and periodontal ligament^[9,11,12,14,31,32]. In 2001, Shang *et al.*^[33] showed augmented healing of sheep cranial defects with calcium alginate gel containing autologous bone marrow-derived MSCs (BMSCs). In 2004, Cowan *et al.*^[34] first demonstrated that hydroxyapatite-coated polylactic-co-glycolic acid scaffolds seeded with adipose-derive MSCs (AMSCs) promoted bone regeneration of critical-size calvarial defects using a rodent model^[34]. More recently, a stem cell-based temporomandibular joint (TMJ) condylar bone graft using a tissue engineering strategy was reported, which suggested the possibility of generating an entire articular condyle in the same shape and dimensions of a human TMJ *in vivo*, with both cartilage and bone layers from a single population of MSCs^[35]. These observations were constantly supported in various craniofacial defect models of mouse, rabbit and canine, with the utilization of different MSC and biomaterial scaffolds^[9,11,12,14,36-42]. In clinical experiments, autologous BMSCs were seeded onto bioscaffolds and transplanted to repair the mandible or alveolar defect in patients and showed satisfied ossification at the regenerated site in terms of both esthetic and functional outcome^[10,43-45]. In 2009, Mesimäki *et al.*^[46] reported the first use of a microvascular custom-made ectopic bone flap developed from a preformed titanium cage filled with autologous AMSCs, β -tricalcium phosphate (β -TCP) granules to reconstruct a maxillary defect. Most recently, Thesleff *et al.*^[47] described a novel method to reconstruct critical-sized calvarial defects of adult patients using autologous AMSC and β -TCP granules, providing evidence that AMSCs combined with β -TCP can be used

to regenerate calvarial bone, with favorable clinical results. Although there have been many reports of preliminary success using adult MSCs both in the laboratory and clinic for craniofacial bone regeneration, these stem cell sources are still significantly limited by their scarcity, invasive cell collection and cell aging^[15,16,19,48].

Pluripotent stem cell-based craniofacial bone tissue engineering

After the revolutionary discovery and characterization of ESCs and iPSCs, further advances have been made towards applying bone tissue engineering methods with these promising pluripotent stem cells^[15,16,19,20,49]. Harkness *et al.*^[50] reported a subpopulation of fibroblast-like ESCs which showed up-regulation of osteogenic specific markers and production of extracellular mineralization when cultured in osteogenic induction medium. The implantation of these cells in a critical-sized calvarial defect of immune deficient mice resulted in promoted new bone formation and partial repair of the calvarial defect^[50]. More recently, Ye *et al.*^[13] demonstrated de novo osteogenesis of iPSCs within a critical-sized calvarial bone defect^[13]. However, the application of ESCs and iPSCs in craniofacial regeneration are still at a preliminary stage and significantly limited by controversial political and ethical concerns, as well as genomic instability, tumorigenesis and immune rejection^[16-18]. The drawbacks of these stem cell types may restrict their utility and performance in further clinical practice, prompting increasing interest in alternative stem cell sources for bone tissue engineering.

PERINATAL STEM CELLS

Perinatal stem cell research has been attracting mounting interest worldwide in recent years. Various populations of stem cells, most of which are comprised within the category of mesenchymal stem cells, have been isolated from different extraembryonic-associated tissues.

Amniotic fluid-derived stem cells

Amniotic fluid (AF) is the nourishing and protective liquid located within the fetal sac throughout the gestational stage which contains heterogeneous cells originating from embryonic and extraembryonic tissues^[51,52]. Previously, cells obtained from AF were mostly used for diagnostic purposes, such as sex determination, detection of fetal infections and genetic diseases. During the last decades, different groups of researchers have provided evidence that human amniotic fluid could be a pool of stem cells. In 2003, Prusa *et al.* demonstrated that a distinct subpopulation of cells expressing OCT4, a key transcript factor for pluripotent human stem cells and preventing differentiation of stem cells, can be found in human amniotic fluid. In the same year, In't Anker *et al.*^[53] demonstrated that human amniotic fluid contained a fibroblast-shaped cell population positive for mesenchymal markers such as CD90, CD105, CD73 and CD166 but negative for the hematopoietic markers such as CD45, CD34 and CD14^[53]. Notably,

De Coppi *et al.*^[54] successfully isolated a multipotential subpopulation of stem cells in the amniotic fluid by fluorescence-activated cell sorting for c-kit positive cells. These amniotic fluid-derived stem cells (AFSCs) maintain a round shape for 1 wk after isolation and begin to change to a fibroblastoid morphology from the second week. Long term culture shows a high self-renewal capacity of these cells with > 300 population doublings, far exceeding Hayflick's limit. The doubling time of the undifferentiated cells is noted to be 36 h, with little variation during passaging. Analysis of stem cell markers shows that the AFSCs express pluripotent markers SSEA4 and OCT4, as well as typical mesenchymal markers, but they did not express the full complement of pluripotent markers, such as SSEA1, SSEA3, TRA1-60 or TRA1-81, indicating that AFSCs are not as primitive as ESCs and yet maintain greater potential than most adult MSCs^[54-56]. Later, the existence and multi-differentiation capabilities of AFSCs into cells and tissues from all three embryonic germ layers was widely confirmed by various independent reports^[54,57-63]. Promising results in terms of structural and functional outcomes highlight the true clinical potential of AFSCs in cell-based therapies and tissue engineering perspectives.

Amnion-derived stem cells

The amniotic membrane is the innermost fetal layer of the placenta which lines the amniotic cavity and assures the normal growth and development of the fetus during gestation. Normally, two types of stem cells can be isolated from the amniotic membrane through mechanical separation and the subsequent enzymatic digestion, namely amniotic epithelial cells (AECs) and the amniotic mesenchymal stem cells (AMMSCs)^[64]. What makes these cells especially attractive is that large amounts can be isolated from an uncontroversial material that is usually discarded after birth. AECs, developing from the central region of the epiblast, build a single layer adjacent to the amniotic fluid and display a cobblestone-like epithelial morphology^[64,65]. While AECs reside in the innermost layer of the amnion, AMMSCs differentiated from somatopleuric mesodermal cells reside in the stromal matrix of the amniotic membrane and exhibit a fibroblastoid morphology^[66-68]. Given their different ontological and cell biological characteristics, subpopulations from both cell types have been confirmed to express a wide range of pluripotency markers, such as OCT4, SOX2, SSEA4, SSEA3, as well as typical mesenchymal markers^[65-70]. Further *in vitro* and *in vivo* studies confirmed their multilineage differentiation potential into cells derived from all three germ layers, such as neuron-like cells, cardiomyocytes, chondrocytes, osteocytes and hepatocytes^[69,71-81]. Most importantly, the immunomodulatory capacity and immunoprivileged status of amnion-derived stem cells make them a promising candidate for allogeneic transplantation and stem cell based therapies^[2,25-27,82-91]. However, standardized collection, quality control and further preclinical and clinical research are still needed before safe amnion-derived stem cell banking products can be achieved^[92].

Chorion-derived mesenchymal stem cells

The chorionic membrane of human term placenta is a rich source of stem cells from which mesenchymal stem cells from chorionic villi and chorionic plate can be isolated^[24,93]. Notwithstanding the potential contamination of decidual maternal stem cells suggested by many studies of term chorionic cells with both fetal and maternal origin, or even pure maternal origin cells only, previous studies of these chorion-derived mesenchymal stem cells (CMSCs) have been focused on primary isolation and limited cell characterization^[24,66]. Like their stem cell counterparts from the placenta, CMSCs participate in placental tissue generation, maintenance, repair and possess a intermediate phenotype of adult MSCs and pluripotent stem cells^[93-95]. *In vitro*, the CMSCs have been reported to be more primitive than adult MSCs with evidence of greater self-renewal and potential to differentiate beyond mesenchymal cell lineages to other lineages such as hepatocyte-like cells and neuron-like cells^[24,93,95-98]. *In vivo*, the CMSCs have recently been reported to have a therapeutic effect on liver injury and osteogenesis imperfecta through not just direct hepatocyte and osteocyte differentiation, but also secretion of cytokines and inhibition of donor cell apoptosis^[97,98]. Very recently, Jones *et al.*^[99] reported that CMSCs isolated from first trimester but not term human placenta accelerated tissue repair in dermal excision skin wounds and improved bone quality and plasticity in osteogenesis imperfecta of mice. Interestingly, the first trimester CMSCs showed an earlier state of stemness, such as smaller size, faster kinetics, unique formation of embryoid bodies and higher expression levels of NANOG, SOX2, c-MYC and KLF4, than its term isolated counterparts. However, collection of CMSCs in the first trimester is technically challenging and usually requires early termination of pregnancy, an ethical obstacle to clinical applications.

Umbilical cord-derived mesenchymal stem cells

The umbilical cord (UC) is an elastic cord that connects the fetus to the placenta during pregnancy. Anatomically, the UC consists of two umbilical arteries (UA) and one umbilical vein (UV) embedded in a mucous connective proteoglycan-rich matrix called Wharton's jelly (WJ). Since more than a decade ago when two independent teams reported their successful isolation of mesenchymal like cells from porcine and human umbilical cord respectively^[100,101], there has been increasing interest in studying the potential of umbilical cord-derived mesenchymal stem cells (UCMSCs) for regenerative medicine. Generally, UCMSCs have been reported to be isolated from different parts of the UC such as UA, UV, WJ, umbilical-lining membrane and umbilical cord blood (UCB)^[102]. Based on the optimized methods to isolate, culture and cryopreserve human UCMSCs, a human UC-MSC bank complying with good manufacturing practices has been established^[103]. Given the controversy that exists on whether UCB or umbilical vessel-derived MSCs can be isolated, MSCs isolated from these various parts of UC have been shown

to resemble adult BMSCs while retaining some specific characteristics. *In vitro*, UCMSCs appear with a fibroblastoid morphology and display a greater expansion capacity and faster doubling time than adult BMSCs. Their morphology and proliferative characteristics did not change even after 30 passages^[100,101]. These cells consistently express typical mesenchymal markers as well as α -smooth muscle actin and low levels of pluripotency markers, such as Oct4, Sox2 and Nanog^[100,104,105]. Moreover, UCMSCs express a lower level of HLA-ABC than BMSCs and were negative for CD31, CD34, CD45, HLA-DR, CD80 and CD86^[106]. *In vitro* and *in vivo* studies confirmed their multi-differentiation potential of mesodermal lineages, such as osteocytes, chondrocytes and cardiomyocytes, as well as other lineages such as neuron-like cell and hepatocytes^[100,107-111]. Most importantly, UCMSCs possess immunomodulatory effects *in vitro* and *in vivo* and have been shown to be well tolerated in allogeneic transplantation experiments^[28,106,112]. Promising results of preclinical and clinical studies using UCMSCs for treatment of liver fibrosis, systemic lupus erythematosus, Sjogren's syndrome and GVHD have been reported which will further promote the therapeutic application of UCMSCs in cell-based regenerative medicine^[112-115].

APPLICATION OF PERINATAL STEM CELLS IN CRANIOFACIAL BONE TISSUE ENGINEERING

Due to their same ontogenetic origin, perinatal stem cells isolated from different extraembryonic tissues possess similar phenotypic and functional properties. In fact, AFSCs, AECs, AMMSCs, CMSCs and UCMSCs have been shown to exhibit osteogenic differentiation capacity both *in vitro* and *in vivo*^[77,93,97,116-119]. In limited comparative studies, perinatal stem cells such as AFSCs and UCMSCs have been shown to display lower basal levels of bone-related genes, higher proliferation rate and much later senescence than BMSCs, resulting in a delayed but robust osteogenic differentiation capacity, which render them a promising candidate for cell banking and stem cell-based bone tissue engineering^[120-122].

In face of the mounting clinical demand and suboptimal techniques for craniofacial skeleton reconstruction, perinatal stem cells may be particularly useful for autologous or allogeneic CBTE for newborns and children and may also be used for adult patients after banking at later stages of life (Figure 1). Although preclinical applications of perinatal stem cells in craniofacial bone regeneration are still limited, recent research progress of these cells revealed multiple possibilities for their potential applications in CBTE.

One of the most advanced applications of perinatal stem cells in CBTE was demonstrated by the use of UCMSCs in restoring rat cranial defects. Chen and colleagues investigated the *in vitro* and *in vivo* bone regenerative performance of human UCMSCs and BMSCs with RGD-modified macroporous calcium phosphate cements (CPC), using a critical-sized athymic rat parietal bone defect (8 mm in

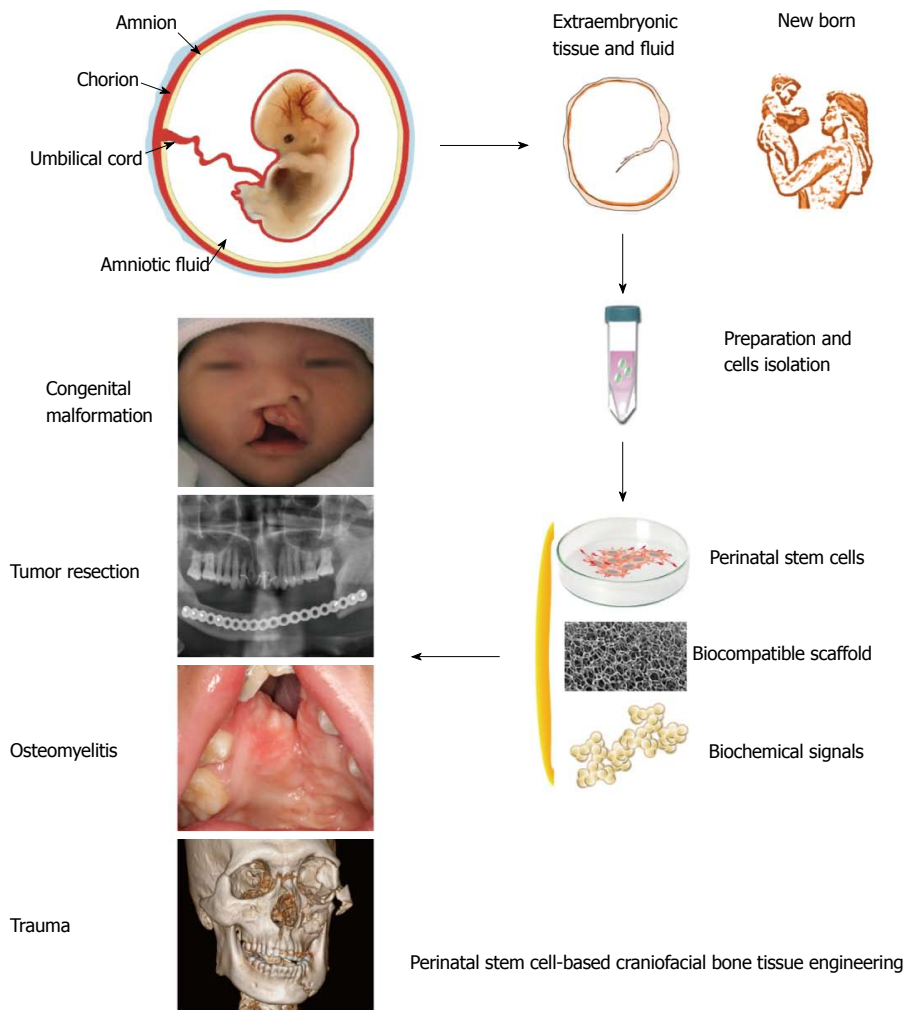


Figure 1 Schematic illustration of the perinatal stem cell-based craniofacial bone tissue engineering. Generally, perinatal stem cells are easily harvested with no harm to the baby or mother and hold a most promising perspective for extensive application in tissue engineering. In the most visionary view, through sophisticated control of perinatal stem cells, biocompatible scaffolds and a signaling system, we will finally be able to bring the perinatal stem cell-based bone tissue engineering strategy to the customized and functional clinical reconstruction of craniofacial bone defects resulting from congenital malformations, osteomyelitis, trauma and tumor resection.

diameter) model^[123]. They observed similarly high expressions of osteogenic specific genes and high percentages of live cells and cell density on CPC for UCMSCs and BMSCs. After implantation for 24 wk, the new bone tissue of UCMSC-CPC and BMSC-CPC groups *in vivo* exhibited similarly higher bone mineral density, new bone amount and vessel density compared to the CPC control group. Notably, this observation was in agreement with a former study by Liu *et al.*^[124] using human UCMSCs and partially demineralized bone matrix for reconstruction of critical-sized parietal bone defects (5 mm in diameter) in athymic rats. Interestingly, Barboni *et al.*^[125] reported AEC-enhanced bone regeneration using an ovine maxillary sinus augmentation model. Calcium-phosphate synthetic bone substitutes, alone or engineered with ovine AECs, were grafted bilaterally into maxillary sinuses of six adult sheep. After implantation, the scaffold integration and bone deposition are positively influenced by allotransplanted AECs. Sinus explants derived from an AEC-scaffold group displayed a reduced fibrotic reaction, a limited inflammatory response and an accelerated process of angiogenesis compared with the control group. In addition, a direct osteogenic differentiation of ovine AECs *in vivo* was observed at 45 d post operation, suggested by the presence of AECs-derived osteocalcin-expressing osteocytes entrapped within the newly deposited bone matrix. The general concept of perinatal stem cell-based bone tissue engineering has also

been validated using ASCs in several craniofacial bone defect models. A commercial magnesium-enriched hydroxyapatite (MgHA)/collagen-based scaffold seeded with or without ovine AFMCs was implanted in the bilateral maxillary sinus of sheep models for up to 90 d. Results showed that application of AFMCs increased new bone deposition and stimulated a more rapid angiogenic reaction^[126]. In another study, researchers developed two combined constructs by seeding dental pulp stem cells (DPSCs) and AFSCs onto silk fibroin scaffolds respectively. The AFSCs-scaffold construct as well as DPSC-scaffold construct promoted early bone regeneration in the critical-sized rat parietal bone defects compared with scaffold alone^[127]. However, the relative long-term outcome of AFSCs in craniofacial bone repair was still in question and the optimal scaffold composition for this particular application remains to be defined^[128,129]. In addition, suggested by most of the discussed studies, perinatal stem cells may play an essential role in new vessel formation in the regeneration site, which indirectly promotes the restoration of craniofacial bone defects^[123,125,126,128].

CONCLUSION

The ontological and anatomical origins of stem cells have profound influences on their biological properties and performance in regenerative medicine. Promoted

by the developments in cell and molecular biology, developmental biology and tissue engineering, the unique and advanced properties of perinatal stem cells have been largely unveiled within the last decade: (1) the collection, preparation and application of perinatal stem cells are scarcely involved with ethical issues; (2) most perinatal stem cells are easily harvested and manipulated with no harm to the baby or mother; (3) compared to adult MSCs which decreased in cell number, proliferation ability and differentiation capacities with age, perinatal stem cells are initially available in substantial numbers and exhibit greater proliferative activity than BMSCs, indicating the advantage for rapid expansion and consequent downstream application; (4) with an intermediate multipotency between pluripotent stem cells and adult MSCs, perinatal stem cells do not form a teratoma while maintaining a broad multi-differentiation capacity *in vitro* and *in vivo*; and (5) in terms of their involvement in materno-fetal immunotolerance, perinatal stem cells exhibit low immunogenicity and potentially high immunomodulatory capacity, indicating a clinical prospective of allogeneic cell transplantation. However, the mechanisms underlying the immunoprivileged and immunosuppressive effects of perinatal stem cells have not yet been clearly defined.

Despite the problems researchers still face in translating scientific concepts from the bench to the bedside, these exciting experimental and preclinical achievements from intense research efforts suggest perinatal stem cells may offer a most promising scope and will continue to produce new and exciting progress for the novel cell-based CBTE in the future.

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Stem cell therapy for retinal diseases

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brain, as well as the differentiation from pluripotent quiescent SC to adult retina, and its relationship with a primary retinal injury, different techniques of transplantation, management of immune rejection and tumorigenicity, its potential application in improving patients' vision, and, finally, approaching future directions and challenges for the treatment of several conditions.

Key words: Macular degeneration; Human embryonic stem cell; Induced pluripotent stem cell; Retinal pigment epithelium; Stargardt's disease

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Core tip: The stem cell (SC) retinal therapy has turned into an potentially useful way of treating blinding disorders, such as, Stargardt's dystrophy, and geographic atrophy. The Food and Drug Administration's approval for clinical trials using SC retinal therapy, and its good results, may appoint to future promising outcomes, providing an anatomical restoration of the retina, and a functional improval of visual function of several patients.

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Abstract

In this review, we discuss about current knowledge about stem cell (SC) therapy in the treatment of retinal degeneration. Both human embryonic stem cell and induced pluripotent stem cell has been growth in culture for a long time, and started to be explored in the treatment of blinding conditions. The Food and Drug Administration, recently, has granted clinical trials using SC retinal therapy to treat complex disorders, as Stargardt's dystrophy, and patients with geographic atrophy, providing good outcomes. This study's intent is to overview the critical regeneration of the subretinal anatomy through retinal pigment epithelium transplantation, with the goal of reestablish important pathways from the retina to the occipital cortex of the

INTRODUCTION

The retina is the light sensing, innermost part, in a multilayered structure that relays information to the occipital cortex of the brain *via* the optic nerve. The photoreceptors layer is located in the outer retina, and plays a major part in light perception and the phototransduction^[1,2]. The retinal pigment epithelium (RPE) is placed between the adjacent photoreceptors, and the underlying pentalaminar Bruch's membrane^[3]. It provides both metabolic and

functional support of photoreceptors, replenishing bleached photopigments that, after a photon is absorbed, initiates the phototransduction, through an active participation from the RPE and the photoreceptors^[1,2]. The RPE forms with the photoreceptors a functional unit, and its optimal functioning is critical to sight^[1,4]. Loss of the integrity of this unit, promotes apoptosis or degeneration of the photoreceptors^[1,2].

Retinal degeneration occurs in several forms, such as age related macular degeneration (AMD), Stargardt's macular dystrophy and retinitis pigmentosa (RP)^[2,4,5]. The AMD has a multifactorial pathophysiology, which results in photoreceptors degeneration in the macula. However, the inner retina remains intact, becoming suitable to the possibility of photoreceptors replacement as a potential therapy^[2]. Therapeutic options that could substitute affected retinal layers, aim to replace lost or injured retinal components, and reestablish the interaction concerning RPE and photoreceptors^[2,5,6]. Autologous full thickness grafts have been used most commonly in humans, but the pursuit for the best method to replace RPE is still ongoing^[7].

STEM CELLS AND CELL REPLACEMENT

After recent advances in regenerative medical treatment, the stem cell (SC) therapy started to be actively explored for eventually blinding retinal conditions. SC could be defined as pluripotent, with ability of self renewal, and able of differentiate or just be maintained quiescent, in order of a damage, following injury or stress^[8,9]. The introduction of the concept of SC therapy, after bombings in Hiroshima and Nagasaki, discovered that bone marrow (BM) transplanted into irradiated animals produced hematopoiesis. Hematopoietic SC (HSC) were identified in 1961, and their ability to migrate and differentiate into multiple cell types was documented^[8-10].

SC transplants may be xenogenic (from another species), allogenic (from another individual) or autograft (from the same individual)^[2,4]. The immunological ocular privilege provides, both allogenic and xenogenic intraocular grafts, a long term survival, when compared with similar grafts^[11]. The human embryonic (hESC) and the induced pluripotent (iPSC) consist, at this moment, as the major forms of retinal SC therapy^[4]. The hESC are derived from blastocysts, left from procedures of "*in vitro*" fertilization. They can remain undifferentiated, or develop into a settled differentiated population. Takahashi and Yamanaka, reprogrammed peripheral normal adult fibroblasts and lymphoblasts, into human iPSC^[11,12]. Both hESC and iPSC have similar characteristics and potential for retinal differentiation, with morphological similarities to the RPE, bringing a total new horizon for retinal degenerations, producing RPE in possibly unlimited amounts^[1,4,7].

Human iPSC are normal fibroblasts or lymphoblasts, and can be obtained from any adult individual, including the patient itself^[1,4]. The first findings about the hESC suggest that there was no sign of abnormal proliferation or

growth, such as no immune mediated transplant rejection in the patients. However, the possibility of immune rejection, teratogenicity and ethical restrictions need to be firmly established before bringing hESC into clinical application^[5].

The most common techniques of delivering SC are the intravitreal and the subretinal injections. The first approach resulted in a higher survival of SC, according prior reports. The subretinal pathway directs the material to targeted retinal regions with indicated treatment, promoting maturation of hESC RPC to photoreceptors^[2]. Unfortunately, lack of integration is a problem when using a suspension as orientation, resulting in unsuccessful outcomes^[2,7].

TARGET DISEASES

Loss of highly differentiated photoreceptors and RPE, is a common end point in retinal degenerations^[12,13]. First described in medical literature in 1874, AMD still has no permanent solution. The dry form of AMD refers to the condition in which the retina accumulates amorphous deposits, called drusen, with gradual degeneration of the RPE^[2,5]. In the wet form, CNV grow progressively, disrupting visual function. It may result in hemorrhagic alterations and fibrosis, affecting the macula^[13]. In both types of AMD, loss of photoreceptors has been documented^[5]. Current therapeutic options to the wet form include, photodynamic therapy (PDT) and, mainly, antivascular endothelial growth factor (anti VEGF) therapy^[5]. The dry form remains an unmet medical need with no available treatment^[5,12]. Hereditary retinal degenerations, including RP and Stargardt's macular dystrophy are important blindness related disorders^[8,13]. RP is an inherited disorder in which rod photoreceptors degenerate initially, but all photoreceptors frequently become involved as well, leading to total blindness^[2]. Stargardt's macular dystrophy was firstly described by Karl Stargardt, in 1905, and is the most common pediatric macular degeneration^[12,14]. Patients with this condition usually present with macular atrophy^[14].

RPE CELLS REPLACEMENT

The RPE is a hexagonal, monolayer of polarized pigmented cells, densely adherent to one another, as well as to the underlying Bruch's membrane. Among its many functions, it plays a major part in photoreceptor renewal^[2,4,12,13]. The AMD is frequently pictured as a local disruption of the RPE photoreceptor unit. Moving photoreceptors to intact underlying regions of the retina had demonstrated good results^[14,15]. Transplant rejection was not so manifested in dry form, as in other forms of this disorder^[4]. The RPE replacement, prior the development of advanced stages, may signify a crucial alternative, requiring refined surgical methods^[5]. The autologous RPE replacement is usually applied from RPE suspension, or autologous full thickness RPE and choroid transplantation. A clinical trial concluded that both had similar results^[4,5]. RPE suspensions may product underprivileged orientation and survival, which is an important factor to be considered^[2,4,5].

The transplantation of RPE polarized monolayer on a scaffold may provide higher integration and function^[4,5]. So far, the parylene membrane has been the only scaffold experienced for subretinal delivery, resulting in a good biocompatibility in RCS rats after implantation^[4,12,13]. The RCS rat is a model used in disorders involving the outer retina^[11,13]. Genetic studies have demonstrated that Mer is essential for RPE function, and its absence results in inherited abnormal phagocytosis of the photoreceptor outer segment, photoreceptor degeneration and retinal atrophy within 2 mo of birth^[1,5,16,17]. Prasad *et al*^[17], in 2006, demonstrated the action of Mer, suggesting that the Protein S is a biologically relevant ligand.

The natural formation of hESC into RPE is the most commonly used process to generate this material^[18]. Reestablishment of the normal interface had demonstrated good results^[14,15]. The hESC initially overgrows in a supplemented hESC medium, until formal contact of its borders. At this point, it is performed the removal of bFGF, and changed until they start to show RPE as a truly small, hexagonal and pigmented morphology^[11,13]. In 2004, Haruta demonstrated the generation of RPE derived from hESC, indicating functional recovery and biological function of phagocytosis^[1,2,16]. The hESC RPE not only display consistent structure with adult RPE, but also exhibit resemblances, as the ability to phagocytose photoreceptor outer segments. The hESC RPE in animals improved functional performance, after comparing with controls, and significant similarity compared to what is found in adult humans^[6,8,9]. The United States FDA recently approved a I / II clinical trial, currently ongoing with hESC, for the treatment of GA and Stargardt's macular dystrophy^[7,19]. Pan *et al*^[12], reported the first subretinal replacement of hESC RPE in subjects with Stargardt's and GA^[2,5]. A clinical study reported absence of immune rejection 4 mo after transplantation in a group of patients^[1,16]. Still, it is hard to find clinical trials showing the results of RPE suspension injection therapy. Ribeiro *et al*^[20] conducted a study to determine whether transplantation of a polarized monolayer of hESC RPE, supported on a nondegradable parylene membrane, into the subretinal space of 69 rats, would lead to RPE survival and tumorigenic effects, compared to suspension injection^[20]. Polarized monolayers of hESC RPE showed 12 mo survival of 50% and 25% in the hESC RPE suspensions, with no signs of tumoral formation. This survival was confirmed by robust antiTRA185, and antiRPE65 staining^[11,20]. Ribeiro *et al*^[20] showed that the confocal nearinfrared (NIR) 830 nm imaging modality, capable of visualizing subretinal pathology, was, also capable to identify the “*in vivo*” hESC RPE in the parylene membrane, with subsequent histological confirmation^[20].

Possibility of teratomas, management of immune rejection and histocompatibility with the hESC, turns it into a difficult clinical application^[11-13]. The iPSC is an interesting alternative to avoid immune rejection, but it is important to point out that iPSC may become immunogenic after reprogramming^[14,6,8,21]. It is predictable that the “autogenous” iPSC RPE transplantation could improve results in patients with retinal degeneration^[16]. The iPSC therapy is a possibility

for retinal replacement, though remaining problems to be solved, until applying it to daily practice. Thus, both hESC and iPSC, under certain biological conditions, may distinguish into actual RPE^[4].

PHOTORRECEPTORS REPLACEMENT

The degeneration of RPE always promotes the induction of photoreceptor damage. But when the loss of photoreceptors is settled, the resulting deficit is permanent. As such, replacing the injured RPE through SC therapy may help to reestablish the integrity of this functional unit^[2,11,13]. The generation of photoreceptors has garnered particular interest, probably related with the growing number of patients affected with macular degeneration and inherited disorders in the United States^[21]. Several groups have demonstrated successful photoreceptors' transplantations in animals, performing synaptic connections between grafts and host retina, and significant functional improvements^[2,22].

Retinal SC (RSC) has been cultured from the peripheral mammalian retina^[6]. RSC transplanted subretinally into animal models develop into photoreceptors after integration, with long term survival^[2,6]. The hESC may, also develop the RSC^[2,8]. In 1998, Reynolds *et al*^[1] cultured hESC from human blastocysts. In 2009, with the permission of the FDA, the first hESC clinical trial was approved for spinal cord injury.

The RPC exist in a more advanced ontogenetic stage than hESC, and constitutes the active component of fetal retinal transplants^[2,8]. Aftab *et al*^[23] showed that human RPC (hRPC), depending of the gestational period, exhibit optimal proliferative dynamics under certain conditions^[23]. After the transplantation of human fetal retina, opsin and recoverin could be also developed by the photoreceptors, improving patients' vision transiently^[6,8]. The use of hRPC from fetal retina was limit by the low capacity to expand “*in vitro*”. Baranov *et al*^[24] showed that hRPC expanded in culture conditions submitted to hypoxia, maintaining its multipotency and self renewal characteristics^[24,25].

Photoreceptors transplantation requires integration into both outer and inner retina, reforming functional synapses, and acting as an anatomical replacement therapy. This direct contact provides the differentiation of photoreceptors “*in vivo*”, which is hard to replicate “*in vitro*”. The inner retina of the transplant, including the outer limit membrane, act as a barrier to donor photoreceptors, and its removal may improve the success involving full thickness retinal grafts^[2]. In addition, the induced differentiated cells are not similar to photoreceptors in structure and morphology. A recent study successfully molded structures similar to outer segment from hESC^[16]. Although the production of phototransduction initiating protein, rhodopsin, it has been difficult to induce electrophysiological responses under light stimuli using the patch clamp technique, indicating a functional defect^[2,16]. Researchers treated ESC with Wnt and Nodal antagonists to differentiate SC^[2,16]. The inhibition of Notch signaling, also significantly promoted photoreceptor differentiation^[16]. In 2006,

Table 1 Clinical trials using stem cell for retinal therapy currently registered on ClinicalTrials.gov

Injury or disorder	Sponsor	Type or source of cells	Delivery	Identification
Stargadt's, AMD (GA)	Advanced cell technology, recruiting	hESC RPE	Subretinal transplantation	NCT01469832
AMD (GA), RP, ischaemic retinopathy	University of Sao Paulo recruiting	BMSC	Intravitreal injection	NCT01518127 NCT01560715 NCT01518842
AMD	Pfizer, ongoing	hESC RPE	Subretinal transplantation	NCT01691261
AMD (GA), RP, RVO, DR	UC Davis recruiting	BMSC	Intravitreal injection	NCT01736059
Stargadt's, Fundus Flavimaculatus, Juvenile Macular Dystrophy	Advanced cell technology, recruiting	hESC RPE	Subretinal transplantation	NCT01469832
Retinal and optic nerve damage	Retinal associates of South Florida, recruiting	BMSC	Retrobulbar, subtenon and intravenous injection	NCT01920867
AMD	Advanced cell technology, recruiting	hESC RPE	Subretinal injection	NCT01344993
AMD	Al-Azhar University, recruiting	BMSC	Intravitreal injection	NCT02016508
RP	Chaitanya Hospital, recruiting	MSC	Not informed	NCT01914913
RP	Mahidol University, recruiting	MSC	Intravitreal injection	NCT01531348
Myopic Macular Degeneration	UC Los Angeles, ongoing	hESC RPE	Subretinal transplantation	NCT02122159
Best disease	Mayo Clinic, recruiting	iPSC RPE	Not informed	NCT02162953
AMD	CHA Bio and Diotech, recruiting	hESC RPE	Subretinal transplantation	NCT01674829
Stargadt's	Advanced cell technology, recruiting	hESC RPE	Subretinal transplantation	NCT01345006
Stargadt's	CHA Bio and Diotech, recruiting	hESC RPE	Subretinal transplantation	NCT01625559

Legends^[29]. AMD: Age related macular degeneration; GA: Geographic atrophy; RVO: Retinal vein occlusion; DR: Diabetic retinopathy; SC: Stem cells; hESC RPE: Human embryonic stem cells derived RPE; iPSC: Induced pluripotent stem cells; BMSC: Bone marrow stem cells; RP: Retinitis pigmentosa; MSC: Mesenchymal stem cells.

Lamba *et al*^[1] promoted the formation of RPC, and generated photoreceptors from hESC in a culture with supplementation of IGF1, noggin (inhibitor of BMP pathway) and Wnt inhibition^[1,10,16]. Reynolds *et al*^[11], in 2009, implanted photoreceptor derived from hESC, with the objective of testing hESC to photoreceptor replacement in an animal model. They demonstrated the migration following intraocular injection of hESC derived material, settling into the appropriate layers, and expressing markers, included related to photoreceptors. After transplantation, there is differentiation into functional photoreceptors and restore light responses to the animals^[2].

The injection of RPC was promising, but, although it demonstrated robust integration, they did not produce photoreceptor appropriately. Fernandes *et al*^[2] demonstrated the subretinal delivery method promote maturation of hESC RPC to photoreceptors. Concluding, alternative approaches and techniques need to be explored to direct RPC transplantation as a therapeutic option, aiming significant survival after transplantation, integration into host retinas and photoreceptor specific differentiation^[2]. Scaffold delivery strategy has been shown to enhance the cell survival and differentiation in a variety of retinal degeneration models^[24].

RETINAL GANGLION CELL REPLACEMENT

The retinal ganglion cell (RGC) is the primarily part of the retina damaged by glaucoma^[26]. In general, the most successful transplantations mentioned before, aiming to

repair damaged retinas, restored photoreceptor cells, but not RGC^[2]. Nevertheless, RGC production from ESC cultures has been reported^[12,13]. Reconstructing the optic nerve through the regeneration of RGC may reestablish sight in glaucoma or traumatic optic nerve injury^[2].

NON NEURAL SC REPLACEMENT

The transplantation of nonneural SC, such as BM derived SC (BMSC), UCSC, HSC or MSC has been documented^[2,14]. BM transplants still consists in adjunct therapy in cancer patients^[8]. The injection of BMSC improves retinal circulation, enhancing survival of outer retina, bringing benefits in retinal degeneration^[2]. The BMSC is an important tool in regenerative therapy, directly related to HSC production^[6]. HSC is an accepted therapy today. It is a group of multipotent SC, obtained by directly removal^[8,9]. BM is supplemented as SC by peripheral blood or cord blood^[27]. The HSC therapy performed globally, such as the demand for donors, has progressing annually^[28]. Nevertheless, HSC transplantation is still related to significant morbidity and mortality, has a high cost and requires multidisciplinary infrastructure. The identification of small ESC in BM supports the notion that, if transplanted together with HSC, it would be able to participate in regeneration^[8,9,27].

CONCLUSION

Several ocular SC clinical trials ongoing have the objective of establish the safety and efficacy of this therapy in humans (Table 1). Additional sources of funding are

therefore imperative to maintain the research programs and integrate these basic and preclinical discoveries and launch early phase clinical trials within the coming years. Although the recent controversy involving changes in strategy planning and funding of the Center of Regenerative Medicine, from the United States NIH, studies of SC therapy for macular degeneration will not be affected.

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Obesity and weight loss could alter the properties of adipose stem cells?

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interesting source of multipotent stem cells has led to many studies exploring the clinical potential of these cells in cell-based therapies. Recent advances in understanding the secretory capacity of adipose tissue and the role of adipokines in the development of obesity and associated disorders have added a new dimension to the study of adipose tissue biology in normal and diseased states. Subcutaneous adipose tissue forms the interface between the clinical application of regenerative medicine and the establishment of the pathological condition of obesity. These two facets of adipose tissue should be understood as potentially related phenomena. Because of the functional characteristics of adipose stem cells, these cells represent a fundamental tool for understanding how these two facets are interconnected and could be important for therapeutic applications. In fact, adipose tissue stem cells have multiple functions in obesity related to adipogenic, angiogenic and secretory capacities. In addition, we have also previously described a predominance of larger blood vessels and an adipogenic memory in the subcutaneous adipose tissue after massive weight loss subsequent to bariatric surgery (ex-obese patients). Understanding the reversibility of the behavior of adipose stem cells in obese and in weight loss is relevant to both physiological studies and the potential use of these cells in regenerative medicine.

Key words: Adipose stem cells; Subcutaneous adipose tissue; Obesity; Weight loss; Regenerative medicine

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Core tip: In this mini-review, we summarize recent aspects regarding obese subcutaneous adipose tissue with a focus in adipogenic and secretory capacities of adipose stem cells. In particular, we discuss how the occurrence of obesity and weight loss could alter the properties of stem cells and the consequences of using adipose-derived stem cells in regenerative medicine.

Abstract

The discovery that adipose tissue represents an

Our previous results reveal that, after massive weight loss subsequent to bariatric surgery, stem cells retain an adipogenic memory besides a predominance of larger blood vessels in subcutaneous adipose tissue. Inflammatory subcutaneous adipose tissue microenvironment found in obese patients and even the massive weight loss could alter adipose tissue-derived stem cells phenotype to a non-healthy state.

Baptista LS, Silva KR, Borojevic R. Obesity and weight loss could alter the properties of adipose stem cells? *World J Stem Cells* 2015; 7(1): 165-173 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i1/165.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i1.165>

INTRODUCTION

The discovery that adipose tissue represents an interesting source of multipotent stem cells has led to many studies exploring their clinical potential in cell-based therapies. Recent advances in understanding the secretory capacity of adipose tissue and the role of adipokines in the development of obesity and associated disorders have added a new dimension to adipose tissue biology under normal and diseased states. In the present mini-review, we summarize different aspects of adipose tissue stem cells and the role of these cells in obese subcutaneous adipose tissue. In addition, we discuss how the occurrence of obesity and weight loss after bariatric surgery could alter the properties adipose-derived stem cells and the consequences of using these cells in regenerative medicine.

OBESE SUBCUTANEOUS ADIPOSE TISSUE

White adipose tissue is highly complex and is composed of various cell types that interact dynamically with each other through the secretion of adipokines, including hormones, cytokines and chemokines. This tissue is now recognized as being equivalent to an endocrine organ^[1]. White adipose tissue is mainly divided into two fat depots: internal or visceral and subcutaneous. In humans, a major contribution of visceral adipose tissue to the development of insulin resistance and to the cardio-metabolic complications in obesity was proposed^[2]. The differences observed between visceral and subcutaneous adipose tissues are supported by studies showing that visceral and subcutaneous fat depots exhibit differential gene expression profiles^[3]. A recent study has suggested a common embryonic origin from mesothelium and mesothelium-derived cells for up to six internal fat depots, but not for the subcutaneous fat depot^[4].

Visceral and subcutaneous adipose tissues can significantly alter their mass according to the amount of triglycerides stored in adipocytes, however is more evident in subcutaneous fat tissues. Some authors assume that the

visceral fat depot is associated with metabolic syndromes, while subcutaneous fat has a metabolic buffer function, which means that an increase in the subcutaneous fat depot in the presence of a positive caloric balance is protective^[3,5]. However, during the development of obesity, the enormous increase in the subcutaneous fat mass results in a dysfunctional tissue. Tissue homeostasis is disrupted due to adipocyte hypertrophy, decreased adipogenesis and angiogenesis^[6].

The cellular content of subcutaneous adipose tissue includes a major population of specialized cells, the adipocytes, and a stromal vascular fraction (SVF) composed of preadipocytes, pericytes or multipotent stem cells, vascular wall and endothelial cells, macrophages^[7], lymphocytes^[8], eosinophils^[9], neutrophils^[10], mast cells^[11] and hematopoietic progenitor cells^[12]. It was initially proposed that adipocyte hypertrophy is the major mechanism by which subcutaneous adipose tissue expands. However, adipocyte hyperplasia also contributes to tissue expansion through the activation of multipotent stem cells, leading to the generation of new cells to sustain the demand for fat storage^[13,14]. Therefore, the capacity to dynamically expand or shrink throughout adult life is mediated by adipocytes, preadipocytes and multipotent cells with a regenerative capacity.

Lipid accumulation is not the only trigger for the development of obesity. One of the most important discoveries regarding the development of obesity was the description of the establishment of an inflammatory state characterized by an increase in the levels of local and systemic inflammatory cytokines and macrophage infiltration into subcutaneous adipose tissue^[15-18]. Besides, endoplasmic reticulum dysfunction is being given as evidence of adipocyte stress^[19], playing an upregulation role in inflammatory scenario of adipose tissue. The accumulated macrophages contribute to the production of inflammatory mediators, thus amplifying the inflammatory state of the adipose tissue and thereby representing an important factor in promoting insulin resistance^[17,18]. The macrophage population within inflamed obese adipose tissue can switch phenotypes between inflammatory and non-inflammatory states^[20]. Adipose tissues also secrete osteopontin, which also plays a role in the migration and differentiation of cells of the immune system^[21-25].

It has been suggested that preadipocytes of mouse adipose tissue could be responsible for most of the secretion of MCP-1^[22,24,25], which has been classically described regarding the role of this cytokine in monocyte mobilization from the bone marrow and the migration of monocytes through the peripheral circulation. Therefore, it was proposed that the inflammatory process that is established during the development of obesity could be initiated by preadipocytes that were affected by adipose tissue growth. In this case, both adipocytes and the preadipocytes would be responsible for the migration and, possibly, the activation of macrophages in adipose tissues.

The cellular events that lead to the establishment of this scenario have not been fully elucidated, and

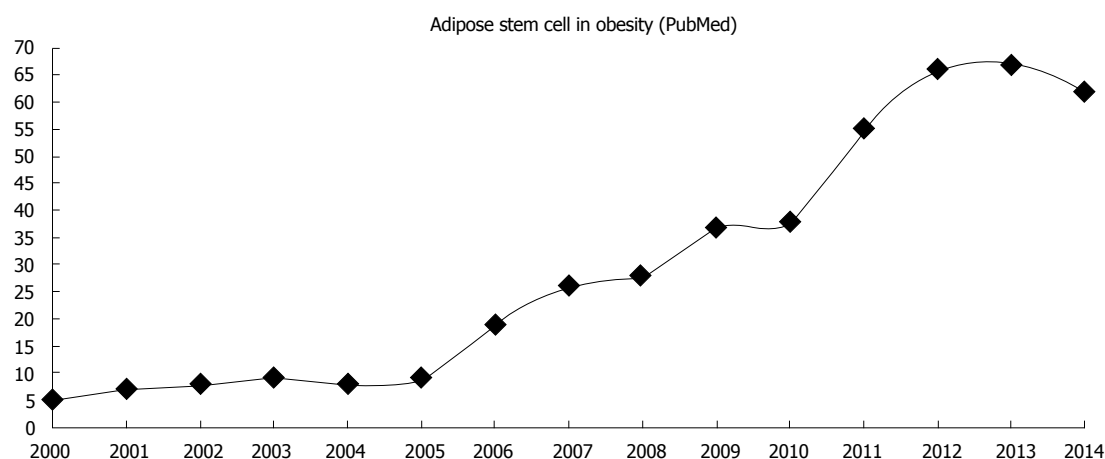


Figure 1 Annual number of publications referenced in the PubMed database between 2000 and 2014 with the sentence “adipose stem cell in obesity”.

the mechanisms by which obesity is associated with other metabolic disorders are not well understood. Because multipotent stem cells are directly involved in subcutaneous adipose tissue homeostasis, it is reasonable to postulate a pleiotropic role for stem cells in the vicious cycle of inflammation. In fact, since 2000, the number of scientific articles in the adipose stem cell field within the context of obesity has been increasing (Figure 1). However, there is only a few analyzing subcutaneous adipose tissue samples after massive weight loss^[26-30]; two of them with focus on multipotent stem cells^[26,30].

HEALTHY AND NON-HEALTHY ADIPOSE STEM CELLS

The term mesenchymal stem cells (MSC) was first used for a multipotent cell population that dwells in adult bone marrow stroma. Based on the concept of bone marrow MSC, this population of perivascular cells has been described in a variety of other adult tissues^[31-36], including adipose tissue^[37]. MSC contribute to tissue homeostasis, repair and regeneration^[38,39]. Data collected during the last 10 years suggest that MSC represent a specific adult tissue stem cell population, the plasticity of which is essential. It is not clear whether all the MSC populations, derived from different tissues throughout the body, represent a substantially similar cell category. Among the different sources of MSC already described, the adipose stem cells have attracted the attention of many scientists and physicians due to the frequently large amounts of subcutaneous adipose tissue that can be easily harvested using liposuction. In addition to being an important tool for tissue regeneration, due to their secretory capacity, adipose stem cells also represent possible regulators in the development of obesity. While many studies have reported on the participation of inflammatory and non-inflammatory macrophages, little is known about the events in obesity that involve resident stem cells.

The *in vitro*-amplified stem cell population derived from adipose tissue is now termed adipose tissue-derived stem cells (ASC)^[40]. ASC share common features with

MSC derived from other tissues, which are primarily based on the paracrine activity of these cells. ASC are similar to MSC derived from bone marrow, including the capacity to sustain hematopoiesis^[12] and immunomodulatory activities, but most importantly, ASC display specific features, among which are higher angiogenic and adipogenic potentials^[41]. Regarding the surface marker profile, ASC are distinguished by CD34 expression^[42].

In fact, results from *in vitro* assays and animal models have supported a beneficial role for ASC in tissue regeneration and immunomodulation *via* paracrine activity^[43]. However, ASC are responsive to inflammatory stimulus. Hoogduijn *et al.*^[44] showed in C57BL/6 mice that intravenously infused ASC home to the lung where they first induce an inflammatory response that in turn leads to immunomodulatory effects. Furthermore, after lipopolysaccharide stimulation ASC increases their inflammatory cytokines secretion^[45] with clones exhibiting varying degrees of paracrine activity^[46]. Stressful conditions on tissue homeostasis can lead to extreme cell behavior changes^[47], and specifically in subcutaneous adipose tissue it has been reported that some diseases can alter ASC function. For example, autologous ASC therapy treatment in a mouse model of multiple sclerosis showed no positive results on the disease progression^[48]. The angiogenic potential^[49,50] and wound healing^[51] capacity of ASC is altered by Diabetes.

There is a positive correlation between high body-mass index (BMI) and some types of malignant tumors^[52]. MSC may favor tumor development through the release of soluble factors and by direct cell contact, promoting proliferation, survival, and drug resistance. It is postulated that the inflammatory tumor microenvironment could change the phenotype of infiltrating MSC to a pro-inflammatory state^[53]. We could speculate that the tumor-derived MSC is in fact a non-healthy state, since they promote tumor progression.

In addition to producing new adipocytes and to participation in the growth of the vascular tree^[41], ASC could contribute in other ways to the development of obesity. First, these cells could generate macrophages by direct differentiation or indirectly, by supporting their generation from resident hematopoietic progenitor

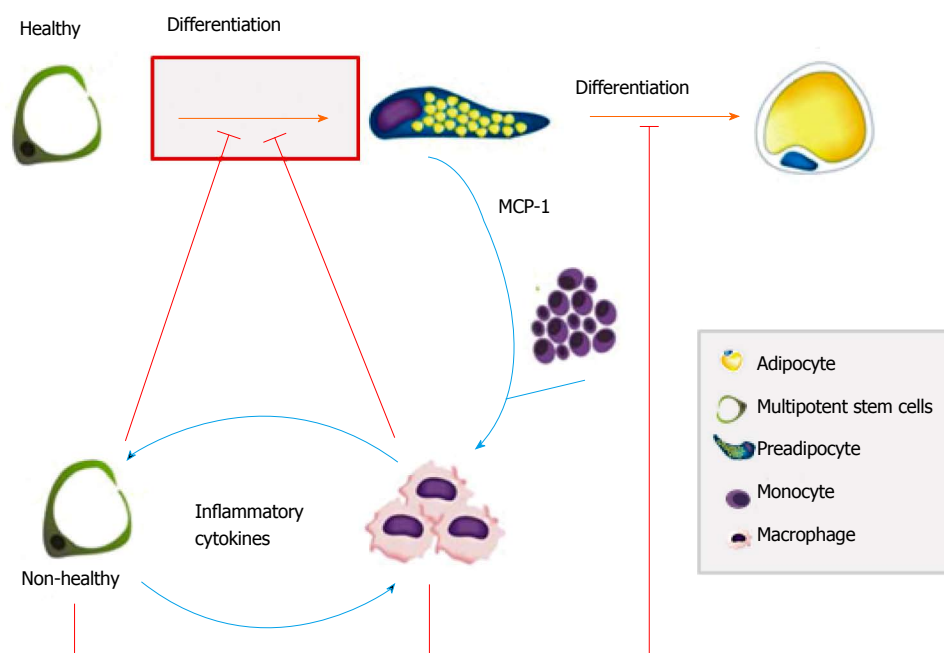


Figure 2 Multipotent stem cells as key players in tissue homeostasis disruption in obesity. The enormous increase in subcutaneous fat mass leads to an increase in secreted molecules such as monocyte chemoattractant protein 1 (MCP-1), which causes monocyte infiltration and subsequent macrophage differentiation (blue lines). Inflammatory cytokines may change multipotent stem cells to a non-healthy phenotype (blue lines) that in turn impairs (red lines) the normal adipogenesis of subcutaneous adipose tissue.

cells^[12]. Murine ASC have a phagocytic activity, both *in vitro*^[54] and *in vivo*^[55]. Human ASC also have this capacity^[56]. Recently, a macrophage population that expresses CD34 and is plastic-adherent and multipotent, similar to ASC, was identified in human white adipose tissue^[7]. Therefore, the idea that a significant proportion of the inflammatory macrophages present in the adipose tissue of obese patients may be generated *in situ* cannot be ignored^[57]. Second, ASC could also participate in obesity *via* cytokine secretion function. The endocrine function of adipose tissue depends upon the activity of the adipocytes and the cells from the SVF. Adipocytes are the main source of leptin and adiponectin, while inflammatory cytokines such as IL-6 and TNF- α , are mostly secreted by the stromal-vascular cells^[13,17,58], among which are ASC and preadipocytes. Consequently, ASC can be key players in obese subcutaneous adipose tissue as secretory cells.

Oñate *et al.*^[59] showed a low capacity for adipogenic and angiogenic differentiation and an upregulation on inflammatory genes^[60] in the ASC from obese patients. Once ASC and preadipocytes are exposed to inflammatory adipose tissue microenvironment, they may react in several possible ways including cytokines secretion, dedifferentiation and migration, aiming to reduce tissue damage^[47]. In this microenvironment, inflammatory cytokines secretion by non-healthy ASC could represent a failure to evade stress. Indeed, the cellular and molecular events in obesity may alter ASC producing a non-healthy and even inflammatory phenotype that in turn impairs the normal cellular physiology of the subcutaneous adipose tissue (Figure 2). Once a disruption in adipose tissue homeostasis begins, the metabolic buffer function

of subcutaneous adipose tissue^[3,5] can be lost, thereby worsening the systemic chronic inflammatory condition of obese patients.

This opens a new and fascinating pathway for modulating obesity through the possibility of manipulating the functional capacity of ASC^[61]. Massive weight loss after bariatric surgery reduces the inflammation as measured by C-reactive protein and IL-6 plasma levels^[62] and even the stress in endoplasmic reticulum of adipocytes^[63], however if ASC isolated from subcutaneous adipose tissue could return their healthy state is still unknown.

ADIPOSE STEM CELLS AFTER MASSIVE WEIGHT LOSS: IMPLICATIONS FOR REGENERATIVE MEDICINE

ASC are now considered to be regenerative cells that promote tissue repair and regeneration *in vivo*, which may occur *via* different mechanisms, including indirectly by the production of soluble factors or directly by the differentiation of the ASC themselves. The first clinical trial that used expanded ASC involved the treatment of fistulas in Crohn's auto-immune disease^[64-66]. More than a decade's worth of animal model studies and clinical trials using ASC^[67] suggest that cell therapy approaches lead to the reestablishment of function and the remodeling of tissue after injury, mostly *via* a paracrine mechanism. Paracrine factors include a set of growth factors that once released by the transplanted stem cells may abrogate local apoptosis and stimulate the proliferation and activation of resident cell progenitors^[45,46].

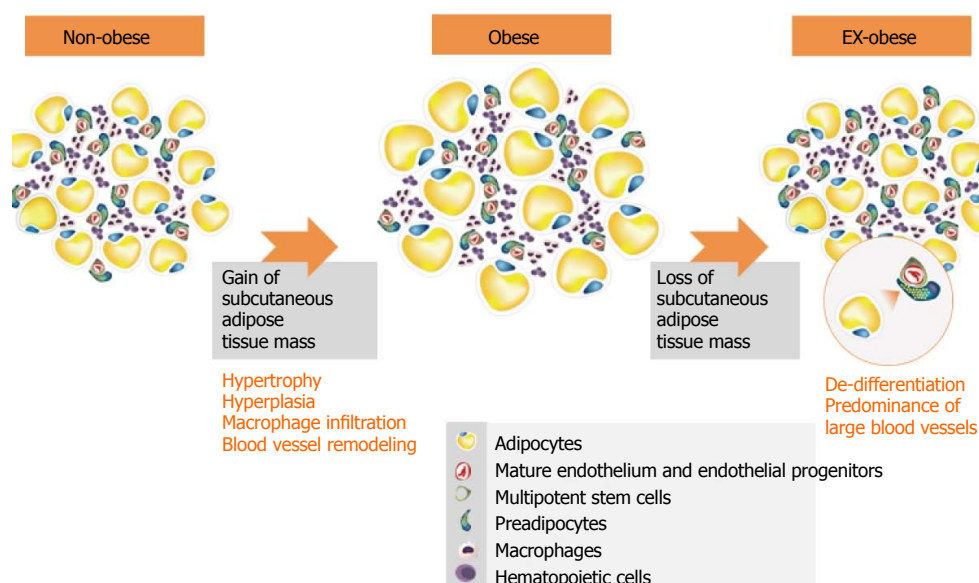


Figure 3 The enrichment of preadipocytes and the density of blood vessels in the adipose tissues of ex-obese patients may play roles in accelerated weight regain. Even after massive weight loss, ex-obese adipose tissue does not return to a normal cellular state. This adipose tissue is characterized by a higher percentage of CD34 cells and a greater number of small and large blood vessels^[26].

Obesity is an inflammatory disease, and relevant changes in the cytokine release profile are expected when comparing obese and non-obese patients and even after massive weight loss (ex-obese patients). In fact, it is known that epigenetic modifications could play a role in chronic diseases^[68,69]. The alterations in cellular behavior caused by the inflammatory microenvironment in obese subcutaneous adipose tissue could be partially maintained even after the decrease of inflammation because of the accumulation of epigenetic modifications. Therefore, it is critical to investigate the biology of ASC to understand their potential use in particular therapeutic approaches. If ASC are isolated from ex-obese patients, the regenerative potential of these cells may be modified, and distinct from that observed in the cell populations present in and derived from non-obese patients. Although massive weight loss has been associated with reduced inflammatory markers in plasma^[62], bariatric procedures could produce metabolic abnormalities, including the increase of bone resorption^[29]. It has been reported that after massive weight loss, subcutaneous adipose tissue returns to a non-inflammatory state with a significant decrease in inflammatory cytokines^[28,58] and immune cells infiltration^[27,29]. These cytokines are produced mainly by SVF and their decrease was observed during weight stabilization period^[58].

Mitterberger *et al.*^[30] have showed no alterations in ASC after massive weight loss; however we analyzed few days of adipogenic induction in contrast to previous studies^[26,59,60]. We have described a predominance of larger blood vessels in the subcutaneous adipose tissues derived from ex-obese patients (BMI 25-29.9 kg/m²), who had lost weight after treatment with bariatric surgery, compared to that from control patients (BMI 25-29.9

kg/m²), who had never developed morbid obesity. In the same study, ASC isolated from ex-obese patients attained a mature adipocyte phenotype faster than those obtained from non-obese patients^[26], suggesting an enrichment of the preadipocyte population.

We currently propose to find a potential biomarker to predict the adipogenic commitment of ASC and, therefore, a biomarker to predict the potential regaining of subcutaneous adipose tissue mass. Recently, Joe *et al.*^[70] have described two major subpopulations, based on CD34 surface marker expression, that dwell in murine skeletal muscle tissue. Using cell sorting techniques, the authors have shown that Lin^{neg}Sca-1^{pos}CD34^{pos} cells contain adipogenic progenitors, while Lin^{neg}Sca-1^{neg}CD34^{neg} cells contain osteogenic and chondrogenic activity. In a mouse model of acute lung injury, freshly isolated human umbilical cord blood CD34-positive cells are protective against the inflammation induced by a lipopolysaccharide challenge^[71].

The role of the CD34 surface molecule in ASC physiology has been recently discussed^[72]. In subcutaneous adipose tissue, the population of preadipocytes can be identified by flow cytometry using the following combination of cell surface markers: CD45^{neg}, CD146^{neg}, CD31^{neg}, CD34^{pos}^[42]. Our preliminary results have shown an accumulation of CD34-positive cells in subcutaneous adipose tissue of ex-obese patients. Furthermore, a novel adipose tissue macrophage subpopulation (CD45^{pos}/CD14^{pos}/CD34^{pos}/CD206^{pos}) had characteristics similar to ASC, including adherence, localization, morphology, and mesenchymal multipotency. This subpopulation may have migrated from the bone marrow and, once in the adipose tissue, may play an important role in tissue maintenance^[7] and also in the development of obesity.

The mechanisms by which the ASC from ex-obese patients are committed to the adipogenic lineage are unknown, but some results point to the existence of circulating preadipocytes that can be recruited and participate in the enlargement of the adipose tissue mass^[73]. Another possible mechanism for the preadipocyte accumulation in ex-obese adipose tissue relies on the fact that adipocytes can dedifferentiate *in vitro*^[41,74]. Considering that patients treated with bariatric surgery massively decrease food intake and increase energy expenditure through physical exercise, hypertrophied adipocytes tend to mobilize lipids through lipolysis and could dedifferentiate into preadipocytes (Figure 3). The enrichment of preadipocytes in the adipose tissues of ex-obese patients and the fact that the blood vessels in these tissues do not return to a normal diameter^[26] may play roles in accelerated weight regain.

Due to the high demand for breast augmentation, replacing devices with autologous fat and ASC is a fair option^[75]. However, this approach cannot be followed if previous breast cancer has occurred. Moreover, if the ASC from ex-obese patients have an adipogenic memory of obesity development, we could also postulate an inflammatory memory or even altered inflammatory properties. It is thus reasonable to consider that the ASC from ex-obese patients may worsen the evolution of a tumor.

Due to ASC plasticity, studies of the function and contribution of ASC during the development of obesity are fundamental to developing new therapeutic approaches to prevent or treat obesity and its complications. Evidence of the alteration of ASC function during the development of obesity is beginning to be evaluated^[59,60], but information is still scarce^[61], specially related to patients who lost weight after bariatric surgery. In fact, inflammatory subcutaneous adipose tissue microenvironment could change ASC phenotype to a non-healthy state, possibly maintained by epigenetic alterations even after massive weight loss. We believe that the use of cell sorting approaches is imperative to validate the use of ASC from obese and ex-obese patients for autologous cellular therapy.

CONCLUSION

In conclusion, subcutaneous adipose tissue forms the interface between the clinical application of regenerative medicine and the establishment of the pathological condition of obesity. These two facets of adipose tissue should be understood as potentially related phenomena. Due to their functional characteristics, ASC represent a fundamental tool for understanding how these two facets are interconnected and could be important for therapeutic applications. Efforts to increase the understanding of the biology of ASC isolated from adipose tissues with normal physiology and in disease states may thus lead to the appropriate use of these cells in regenerative medicine and will provide novel therapeutic insights regarding obesity and weight regain.

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Advances and challenges in the differentiation of pluripotent stem cells into pancreatic β cells

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Abstract

Pluripotent stem cells (PSCs) are able to differentiate into several cell types, including pancreatic β cells. Differentiation of pancreatic β cells depends on certain transcription factors, which function in a coordinated way during pancreas development. The existing protocols for *in vitro* differentiation produce pancreatic β cells, which are not highly responsive to glucose stimulation except after their transplantation into immune-compromised mice and allowing several weeks for further differentiation to ensure the maturation of these cells *in vivo*. Thus, although the substantial improvement that has been made for the dif-

ferentiation of induced PSCs and embryonic stem cells toward pancreatic β cells, several challenges still hindering their full generation. Here, we summarize recent advances in the differentiation of PSCs into pancreatic β cells and discuss the challenges facing their differentiation as well as the different applications of these potential PSC-derived β cells.

Key words: Embryonic stem cells; Induced pluripotent stem cells; Insulin-secreting cells; *In vitro*; *In vivo*; Differentiation; Maturation

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Core tip: Pluripotent stem cells (PSCs) including induced PSCs and embryonic stem cells are valuable sources for cell replacement therapies and disease modeling of diabetes. Although several studies reported the differentiation of PSCs into pancreatic β cells *in vivo*, still their response to glucose is very limited *in vitro* due to their immature nature. In this review, we summarize the current knowledge about the differentiation of PSCs into pancreatic β cells and discuss the challenges facing the differentiation and application of PSC-derived β cells.

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INTRODUCTION

Diabetes mellitus (DM) is a common disease, characterized by hyperglycemia caused by insufficient insulin production and/or resistance to insulin. Two different kinds of DM are

well characterized, type 1 (T1D) and type 2 (T2D). T1D is an autoimmune disease in which insulin-secreting β cells in pancreatic islets are permanently damaged by autoimmune attack, resulting in a lack of insulin production^[1]. T2D occurs when the pancreas produces insufficient amounts of insulin and/or the tissues of the body become resistant to normal or even high levels of insulin^[2]. Thus, T1D and T2D patients suffer from insulin insufficiency and damage of insulin-secreting β cells by distinct mechanisms^[3,4].

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced PSC (iPSCs) have the ability to differentiate into all cell types of the body^[5,6], which make them valuable sources for cell replacement therapies and other applications. Several reports have showed the generation of iPSCs from patients with different forms of diabetes^[7-12]. Also, the generation of patient-specific ESCs has been recently generated from somatic cells of diabetic patients^[13]. Thus, generation of functional β cells from human PSCs represents the most promising approach to treat diabetes. In addition to their high potential in cell therapy, β cells derived from patient-specific PSCs can be used as a disease model, where they provide a new approach to culture cells with a disease genotype and re-establishing pathogenesis *in vitro*. Disease modeling with such cells has the potential to give insights into the molecular mechanisms underlying diabetes and enable new cell-based drug discovery as well as autologous transplantation^[13,14].

Several studies reported the generation of pancreatic β cells from ESCs^[15-18] and iPSCs^[7,8,12,15,19]. However, a number of obstacles have arisen that render the generation of fully functional pancreatic β cells from PSCs^[14]. Therefore, in this review we summarize the current knowledge about the differentiation of ESCs and iPSCs into pancreatic β cells and discuss the challenges facing the differentiation and application of PSC-derived β cells.

DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO INSULIN-SECRETING β CELLS

It has been well documented that both human ESCs (hESCs) and human iPSCs (hiPSCs) share the same characteristics and properties and are able to differentiate into various types of cells under certain cultural conditions^[6,20,21]. Among these cells, insulin-secreting β cells differentiation from both hESCs and hiPSCs has been studied using similar differentiation protocols (Figure 1)^[8,15-17,19]. However, variation in the efficiencies of differentiation has been reported between different hPSC lines^[22,23]. These variations might occur due to the use of different protocols and different cell lines. The process of β cells differentiation is controlled by a complex network that depends on transcriptional regulation of genes involved in the development of the pancreas, the type and number of the differentiation factors, and the type and conditions of stem cell culture. Although many factors are important for successful generation of pancreatic insulin-secreting β cells

from PSCs, successful manipulation of culture conditions stands out as one of the more critical factors. Several initial studies have used the step-wise approach to differentiate insulin-secreting cells from hESCs. Under these cultural conditions, hESCs generate up to 12% pancreatic β cells; however, the ability of these differentiated cells to produce insulin in response to glucose was very limited^[16,18,24]. Therefore, a number of modifications in culture conditions have been done to improve the efficiency of β cell differentiation^[16,18,25,26]. These modifications include, but are not limited to, monolayer cultures^[16-18,25,27,28] or embryoid body (EB) technique^[29,30]. Also, several studies have used xeno-free culture system to generate pancreatic β cells from PSCs *in vitro*^[31-34]. Furthermore, *in vitro* modifications of cultural conditions to regulate signaling pathways demonstrated the vital role of specific pathways in the enhancement of β cell differentiation (Nostro *et al.*^[29], 2011). In hESCs, the regulation of transforming growth factor β (TGF β) signaling produces high yields of insulin-secreting cells that reaches up to 25%^[29]. Importantly, it has been proven that such critical refining in pancreatic differentiation protocols efficiently enhances the differentiation and maturation of hESC-insulin-secreting cells *in vivo*. These generated insulin-secreting cells showed several properties of functional β cells and were capable to respond efficiently to glucose stimulation in engrafted mice^[25].

Differentiation of pluripotent stem cells into definitive endoderm

Similar to embryonic pancreatic development, PSCs differentiation into pancreatic lineage is excised in several steps that start with the differentiation into definitive endoderm (DE) (Figure 1), which is recognized by the expression of specific markers. Previous studies showed a high percentage (60%-80%) of hESC-differentiated cells express a panel of specific DE endodermal markers such as SOX17, FOXA2, CXCR4, and GSC, but not the visceral endodermal marker, SOX7^[15,16,25,35-37]. The initiation of DE differentiation is properly induced in hESCs and hiPSCs by NODAL and WNT signals^[15,18,35,38]. NODAL signals have been previously reported to be the main inducer of endogenous endoderm^[39] and is activated by one of the members of TGF β family, activin A. Notably, the dose of activin A appears to be crucial for Nodal signaling activation and in-turn DE differentiation. A previous study showed that the use of activin A in a concentration of (50-100 ng/mL) leads to an efficient DE differentiation as compared to lower concentrations^[30,40]. In association with activin A, other factors have been shown to play an important role in DE differentiation. A recent study showed that treatment of hESCs with a combination of activin A, wortmannin (PI3K inhibitor), and CHIR99021 improves the percentage (90%) of the generated SOX17-positive cells^[41]. Furthermore, it has been previously shown that the combination of activin A with sodium butyrate^[16], PI3K pathway antagonists^[15,38], or Wnt signaling activators (WNT3A or CHIR9902) enhances the efficiency of DE differentiation in PSCs. It is worth to note that CHIR99021 has been found to be more potent

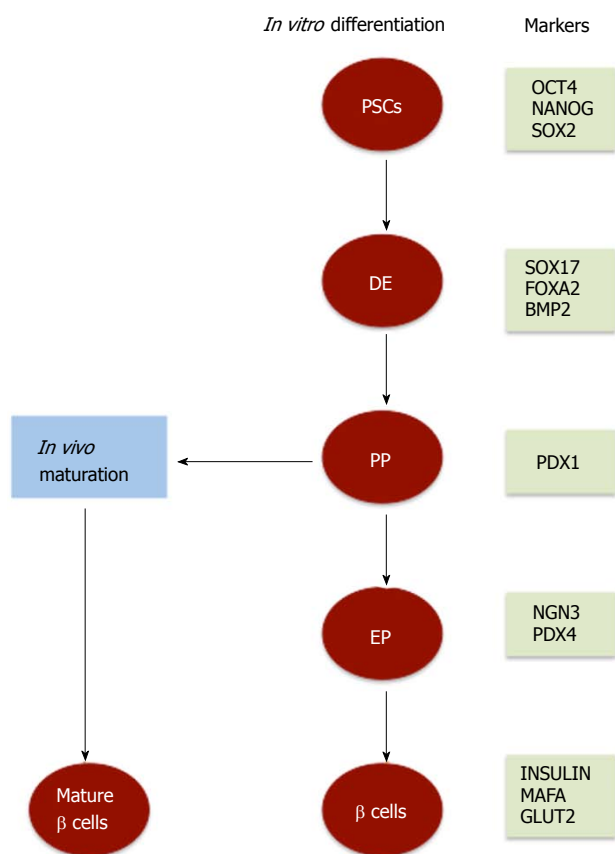


Figure 1 Schematic representation of the differentiation of pluripotent stem cells into pancreatic β cells. Pluripotent stem cells (PSCs) can be differentiated *in vitro* into pancreatic β cells after a step-wise differentiation protocol into definitive endoderm (DE), pancreatic progenitors (PP), pancreatic endocrine (PE) progenitors, and β cells. Transplantation of pancreatic progenitors derived from PSCs into immune-compromised mice induces their differentiation into glucose responsive pancreatic β cells (mature β cells). During pancreatic β cell differentiation, specific transcription factors are expressed at different stages. PDX1: Pancreatic and duodenal homeobox 1 gene; NGN3: Neurogenin 3; OCT4: Octamer-binding transcription factor 4; SOX: SRY (sex determining region Y)-box 2; FOXA2: Forkhead box protein A2; BMP: Bone morphogenetic protein; GLUT2: Glucose transporter 2.

in promoting SOX17- and FOXA2-positive endodermal cells than Wnt3A^[28]. Like wise, treating hESCs with GSK3 β inhibitor instead of WNT3A increases DE generation^[42]. Also, another TGF β family member, GDF8 (myostatin), has been found to be effective for stimulating DE^[43]. However, the efficiency of DE differentiation not only depends on GDF8, or activin A and its associated factors but also is enhanced by small molecules such as IDE1 and IDE2, which has been found to significantly induce the differentiation of approximately 80% of ESCs into SOX17-expressing DE cells^[44].

It is well known that DE eventually generates both pancreatic and hepatic tissues. To direct DE cells towards pancreatic differentiation *in vitro*, the alternative hepatic lineage differentiation is inhibited by treating the cells with different types of growth factors inhibitors, such as SU5402 (FGF receptor antagonist) and Noggin (BMP antagonist)^[36]. It has been shown that BMP signaling exerts two opposite

effects during pancreatic differentiation^[45]. In the dorsal endoderm, BMP signaling inhibition has been found to be required for specific differentiation into pancreatic lineage, whereas it is presence after the formation of pancreatic cells is essential to maintain pancreatic and duodenal homeobox 1 gene (*PDX1*) expression^[45].

Differentiation into pancreatic progenitors and endocrine cells

In the dorsal endoderm, BMP signaling inhibition has been found to be required for specific differentiation into pancreatic lineage, whereas its presence after the formation of pancreatic cells is essential to maintain pancreatic and *PDX1* expression^[45]. *PDX1* is a transcription factor that is expressed on all pancreatic precursor cells and has been shown to be essential for early pancreatic development^[46]. It has been found that the expression of *PDX1* is correlated with the pancreas developmental stages. During the early stages of endocrine specification, *PDX1* expression becomes restricted, whereas at later stages during β cells development its expression is upregulated as the protein enhances β cell function and is involved in insulin secretion^[46]. The differentiation of *PDX1*-expressing cells *in vitro* is regulated by several factors that range from signaling pathways inhibitors to protein kinase activators. For example, in hESCs, the differentiation of pancreatic progenitors expressing *PDX1* is induced by a small molecule, Indolactam V, that activates protein kinase C^[17], and enhanced by retinoic acid and dorsomorphin (a BMP type 1 receptor inhibitor) treatments^[28], whereas its proliferation is increased by inducing epidermal growth factor signaling^[15]. Two other signaling pathways, NOTCH and HEDGEHOG, have been shown to be involved in the DE differentiation into pancreatic endocrine cells^[16] (Figure 1). The inhibition of HEDGEHOG-signaling by Cyclopamine or KAAD cyclopamine induces the generation of *PDX1*-expressing cells^[17,18,25,27,29], whereas Fibroblast growth factor 10 activates NOTCH signaling, which is involved in the proliferation of *PDX1*-expressing pancreatic progenitors.

Another transcription factor to consider as a marker for late pancreatic cell development is neurogenin 3 (NGN3). NGN3 expression peaks during endocrine differentiation stage, which is subsequent to the generation of *PDX1* pancreatic progenitors. It has been previously shown that the capacity of PSCs to differentiate into pancreatic endocrine cells is highly affected by the seeding density of initial cultures. For example, Gage *et al.*^[47] demonstrated that high density hESCs led to remarkable increase in that rate of cell differentiation into *PDX1*- and NGN3-positive cells as compared to low density cultured cells. In contrast to *PDX1*, NGN3 expression is stimulated by a reduction in NOTCH signaling. It has been previously shown that the blockage of NOTCH signaling at this stage may be driven by DAPT (the gamma secretase inhibitor) treatment and thus drive NGN3-expressing cells formation^[17,18,27]. Interestingly, these cells have been found to be differentiated from *PDX1*-expressing pancreatic progenitors after specific treatments. A recent study used a xeno-free culture system

showed that a high NOGGIN concentration is crucial for inducing the differentiation of iPSCs into pancreatic progenitors (PDX1-positive) and then pancreatic endocrine progenitors (NGN3-positive cells)^[34]. A previous study showed that TGF β type 1 receptor inhibitor (SB431542) stimulates the differentiation of hESC-derived PDX1-positive pancreatic cells into NGN3-positive pancreatic endocrine progenitors^[28,48]. Another recent study reported the generation of NGN3-positive endocrine precursors after the inhibition of vesicular monoamine transporter 2 by reserpine and tetrabenazine in PDX1-positive cells^[49].

Differentiation into pancreatic β cells

The generation of mature pancreatic β cells from hESCs and hiPSCs is characterized by the expression of a panel of different factors, such as PDX1, MAFA, NKX6.1, NEUROD, ISL-1, and GLUT2, C-peptide, and INS (insulin)^[15]. Of these factors, NKX6.1 expression has been shown to be essential for the production of functional mature β cells. This significant role of NKX6.1 was remarkably clear in the *in vivo* studies done by Rezanian *et al.*^[50], where they showed that hyperglycemia significantly reduced in diabetic mice engrafted with cells expressing substantial amounts of NKX6.1. On the other hand, diabetic mice transplanted with cells expressing low levels of NKX6.1 remains hyperglycemic. In another study, it has been demonstrated that mice lacking NKX6.1 does not develop pancreatic β cells. However, re-expression of Nkx6.1 in pancreatic progenitors (PDX1-positive) restores the development of pancreatic β cells^[51]. It has been also reported that overexpression of some pancreatic development-transcription factors enhance pancreatic β cell differentiation^[52-54]. In human ESCs, enforced expression of the transcription factor PAX4 significantly promotes cell differentiation into pancreatic β cells^[55]. Also, overexpression of human PAX4 in hESC-derived pancreatic progenitors increases the number of insulin-secreting β cells, which produce only one hormone (insulin) by suppressing the expression of ARX and glucagon^[56]. In contrast, overexpression of PDX1 induces pancreatic endocrine cell differentiation, but not *in vitro* β -cell formation during the differentiation of hESCs into EBs^[57].

Several treatments, forskolin (an adenylate cyclase activator) and dexamethasone (a synthetic adrenocortical steroid)^[28], hepatocyte growth factor, insulin growth factor 1 and glucagon-like peptide 1^[9], have been used to enhance pancreatic β cell maturation *in vitro*. Several studies have previously reported the successful production of insulin-secreting cells *in vitro* from either hESCs or hiPSCs^[15,16,18,19,24,25,29,30,36,58-60]. However these differentiated β cells showed little ability to respond to glucose, which is considered one of the well-known and important parameters that recognize functional and mature β cells. These differentiated cells also lack the expression of specific mature pancreatic β cell markers such as NKX6.1 and MAFA^[19], that categorize them as immature non-functional β cells. Moreover, hPSC-derived β cells co-express multiple hormones such as INS, GCG (glucagon), and C-peptide

instead of secreting only insulin hormone, which is secreted only by mature β cells. Therefore, controversy exists as to the ability of PSCs to differentiate into fully functional pancreatic β cells^[61]. A previous study showed that endocrine precursors could be differentiated into glucose responsive β cells^[49], which is considered one of the major challenges during β cell differentiation process.

In vivo maturation of pluripotent stem cell-derived pancreatic β cells

Despite these contentious *in vitro* evidences, mentioned above, *in vivo* microenvironment is likely to be important for pancreatic β cells maturation. Several transplantation studies have demonstrated the differentiation of immature pancreatic β cells or pancreatic progenitors into mature β cells *in vivo*. In either healthy^[25,30] or streptozotocin (STZ)-induced diabetes mice^[62], functional and mature pancreatic β cells are successfully developed from hESC-derived pancreatic progenitors transplanted either under the mouse kidney capsule or fat pad. Interestingly, another study showed that pancreatic progenitor cells are still capable to differentiate into mature pancreatic β cells that efficiently secrete insulin within macroencapsulation devices that have been transplanted into diabetic mice^[63]. Like wise, in two different mouse models that mimic human T1D and T2D^[64,65] *in vivo*, transplanted iPSC-derived β cells efficiently differentiate into glucose-responsive pancreatic β cells. In these studies as well as another study that has used monkey iPSC-derived β cells^[48], the generated functional pancreatic β cells were found to improve hyperglycemia in the transplanted mouse models. Other studies reported that transplantation of pancreatic progenitors derived from PSCs into mice induces the differentiation of polyhormonal cells into glucose responsive pancreatic β cells (Figure 1). Interestingly, it has been found that such transition into mature β cells is associated with dynamic chromatin remodeling^[58,66-68].

Moreover, it has been found that urocortin 3 (Ucn3), which is a corticotropin-releasing factor, is highly expressed in pancreatic β cells and regulates glucose-stimulated insulin secretion^[69]. Although Ucn3 expression is detected in pancreatic β cells after *in vivo* maturation, it has not been detected after *in vitro* differentiation, suggesting the difference between *in vitro* and *in vivo* differentiation^[70]. Interestingly, the level of Ucn3 expression increases more than sevenfold between immature and mature pancreatic β cells^[70]. Thus *in vivo* maturation is critical for both the functionality and maturity of PSC-derived pancreatic β cells, indicating that there may be specific signals at the transplantation sites that induce and enhance β cell differentiation and maturation.

CHALLENGES AND FUTURE PERSPECTIVES

Several challenges need to be addressed before using PSCs in clinical applications and disease modeling. Differentiation of PSCs into mature, glucose responsive β cells is required for proper cellular therapies as well as for re-establishing the

disease phenotypes *in vitro* to understand the mechanisms underlying different forms of diabetes. Pancreatic β cell maturation is defined based on glucose-stimulated insulin secretion (GSIS). To date, although several reports showed an improvement in generating glucose-responsive insulin-secreting β cells *in vivo*, still their response to glucose is very limited *in vitro* due to their immature nature^[8,15-17,19]. A recent study has reported that insulin-secreting β cells differentiated from hPSCs are highly similar to human fetal pancreatic β cells than adult β cells^[71]. Also, it has been suggested that the inability of pancreatic β cells to produce adequate insulin is due to the gradual loss of a specific group of transcription factors, which is important for insulin secretion and glucose responsiveness. Previous studies reported that dysfunctional pancreatic β cells derived from hESCs fail to secrete PDX1, NKX6.1, and MAFA^[18,25,72], indicating that restoring the transcription factors is essential to recover β cell functions. Another study found that some genes related to early embryonic development (DPPA4, LIN28A, and LIN28B) continue to be expressed in the cells differentiated from PSCs, suggesting that the cells differentiated from PSCs are similar to the cells found in the very early stage of human development^[73]. Notwithstanding the above findings, transplantation of pancreatic progenitors derived from PSCs into immune-compromised mice induces their differentiation into glucose responsive mature β cells^[7,50,58,66-68]. Thus, future studies should focus on the signaling pathways regulating the maturation of pancreatic β cells *in vitro*.

Another problem hindering cell therapies of PSCs in diabetes is the low efficiency in producing insulin-secreting β cells. The current protocols are still lacking critical signals required for efficient generation of insulin-secreting β cells. Therefore, future studies should focus on studying the molecular mechanisms, guiding normal pancreatic development and to develop highly efficient differentiation protocols to obtain large number of insulin-secreting β cells.

Furthermore, one of the problems facing the stem cell therapies is the potential for unwanted cell growth. Undifferentiated PSCs are characterized by their ability to produce teratoma *in vivo* due to their high pluripotential capabilities. Previous studies suggested that encapsulation of the transplanted cells prevents overgrowth^[74,75]. In addition, encapsulation was found to be crucial to differentiate PSCs into pure culture of insulin-secreting β cells to avoid their contamination with undifferentiated cells. To use the functional pancreatic β cells to cure T1D, the repair of self-tolerance or eliminate the influences of autoimmunity must be considered, thus the immune system cannot destroy the newly transplanted insulin-secreting β cells. Therefore, several studies reported the use of encapsulated devices to protect pancreatic β cells from immunological attack. Glucose responsive insulin-secreting grafts have been generated from encapsulated CyT49-luc hESC-derived pancreatic epithelium and found that maturation occurs without an increase in cell biomass^[75]. Thus, encapsulation is considered as a possible tool to overcome some of the grand challenges in diabetes treatment by stem cell therapy.

Another challenge is related to the use of ESCs in

cell therapy due to ethical concerns and immune rejection problem. The use of patient-specific ESCs, which are immunologically compatible to the patient, would overcome the immune rejection issue since ESCs can be generated from the somatic cells of the patients using SCNT^[13,76].

In conclusion, there is a lot of work need to be done to obtain fully functional pancreatic β cells *in vitro*. For example, a genome-wide transcriptional analysis should be performed in each stage during pancreatic β cell differentiation to recognize the defects in the transcription factors. Also, comparing *in vivo* pancreatic development with *in vitro* pancreatic differentiation is important to identify the difference in gene expression, which may account for the functional differences. Finally, different differentiation protocols should be applied to understand the signaling pathways controlling the differentiation process *in vitro*.

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How do resident stem cells repair the damaged myocardium?

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Abstract

It has been a decade since the monumental discovery of resident stem cells in the mammalian heart, and the following studies witnessed the continuous turnover of cardiomyocytes and vascular cells, maintaining the homeostasis of the organ. Recently, the autologous administration of c-kit-positive cardiac stem cells in patients with ischemic heart failure has led to an incredible outcome; the left ventricular ejection fraction of the cell-treated group improved from 30% at the baseline to 38% after one year and to 42% after two years of cell injection. The potential underlying mechanisms, before and after

cell infusion, are explored and discussed in this article. Some of them are related to the intrinsic property of the resident stem cells, such as direct differentiation, paracrine action, and immunomodulatory function, whereas others involve environmental factors, leading to cellular reverse remodeling and to the natural selection of "juvenile" cells. It has now been demonstrated that cardiac stem cells for therapeutic purposes can be prepared from tiny biopsied specimens of the failing heart as well as from frozen tissues, which may remarkably expand the repertoire of the strategy against various cardiovascular disorders, including non-ischemic cardiomyopathy and congenital heart diseases. Further translational investigations are needed to explore these possibilities.

Key words: Regeneration; Heart failure; Cell therapy; C-kit; Cardiac stem cells; Cellular reverse remodeling; Miracine

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Core tip: The autologous transplantation of cardiac stem cells appeared to be safe and surprisingly effective for a small group of patients with chronic ischemic cardiomyopathy. Their specific feature as resident stem cells, the interaction with the surrounding tissue, and the natural selection during the cell culture process potentially contributed together to the outstanding consequences.

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INTRODUCTION

The advancement of emergency medicine enabled us to

rescue many patients with acute myocardial infarction, but they may eventually develop and suffer from chronic heart failure later in life. Accordingly, the demand of alternative approaches to chronic ventricular dysfunction is increasing dramatically. For decades, the mammalian heart was recognized as a postmitotic terminally differentiated organ, in which the number of cardiomyocytes was believed to be constant throughout the life span of organisms. Early this century, however, the existence of resident cardiac stem cells (CSCs) was unveiled^[1], questioning the validity of the long-lasting theory. The uninterrupted renewal of human myocardium in physiological and pathological circumstances has been depicted^[2], promoting our understanding of the self-renewing characteristic of the heart. This paradigm shift not only shook the biological dogma but revealed the possibility of an unprecedented therapeutic strategy for devastating diseases.

CLINICAL APPLICATION OF CARDIAC STEM CELLS

By virtue of the explorations following the discovery of resident CSCs, the human heart has been characterized by the persistent renewal of its components, muscles and vessels. Moreover, by utilizing animal models, c-kit-positive CSCs are shown to regenerate the diseased myocardium of ischemic and non-ischemic origins^[3-5]. Subsequently, in 2009 the first clinical use of autologous CSCs began; in this SCIPIO trial, the subjects with severe ischemic heart failure received an elective coronary artery bypass graft surgery, during which a tiny piece of the right atrial appendage was resected as the source of stem cells. Four months after the operation, only those with the left ventricular ejection fraction (LVEF) worse than 40% were included in the trial and randomly assigned to the control or cell-treated groups. While the cardiac function did not change in the control group, the LVEF dramatically improved in the treated patients, from 30% at the baseline to 38% after one year and to 42% after two years of cell administration^[6,7]. The infarct size evaluated by an MRI remarkably reduced following the cell therapy, and the symptom improved as well. It should be noted that the major adverse cardiac event rate was unchanged with or without this therapeutic intervention^[8].

DIRECT DIFFERENTIATION VS PARACRINE ACTION

A single intracoronary injection of one million CSCs, as described above, appeared to be safe and effective for 2 years or longer. How does this work? There are accumulated evidences suggesting that most cell types applied for heart failure, including skeletal myoblasts, bone marrow-derived cells, and cardiosphere-derived cells^[9,10], function through paracrine mechanisms^[11-13]. In this regard, CSCs are found to work through the sequential processes of engraftment, proliferation, and direct differentiation into various cardiac cell types^[14], in addition to the paracrine action^[15,16]. Actually,

a recent study showed that the injection of 200 million human mesenchymal stem/stromal cells (MSCs) and that of one million c-kit-positive CSCs into a two-week-old infarcted myocardium were similarly effective, revealing the great regenerative potential of resident stem cells^[17]. Interestingly, the combined usage of these distinct cells resulted in a synergistic effect, formulating an attractive regimen for myocardial infarction.

REGULATION OF IMMUNE RESPONSE

The environment of the host tissue is not always favorable for the transplanted cells to engraft, especially when allogeneic ones are used. Historically, MSCs were found to be immune privileged, due to their lack of major histocompatibility complex class II antigen. In fact, the endomyocardial injections of autologous and allogeneic bone marrow MSCs were similarly effective as a treatment for ischemic heart failure patients^[18]. More recently, Di Trapani *et al.*^[19] have demonstrated that certain stem cells of various sources, including CSCs, are able to regulate the immune response of the recipient^[19]. Such properties may have a role *in vivo* in enhancing their regenerative ability, especially in inflammatory circumstances. These reports would support the enthusiasm for the “off-the-shelf” usage of allogeneic CSCs. The obvious advantages include minimizing the lot-to-lot variation of cell therapy and its applicability to the acute phase of the disease. Following the differentiation of engrafted CSCs *in vivo*, however, the immune system of the host may reject them, just like in the case with rat MSCs^[20]. Although this particular study was carried out with rodents, stem cells of any given species would become immunogenic upon the acquisition of myogenic phenotypes. In the setting of allogeneic transplantation, therefore, the long-term benefit depends essentially on the paracrine action of the administered cells. Further evaluations would be needed to compare the advantages and disadvantages of respective therapeutic strategies.

REVERSE REMODELING AT CELLULAR LEVEL

As stated above, while the administered cells may affect the circumstance, the microenvironment within the host would also influence the destiny of the exogenous cells. Based on the studies using immunosuppressed animals, human CSCs can create more cardiomyocytes than the lost myocytes of the recipient, within one month after injection^[21]. Assuming a comparable growth behavior of the autologous CSCs, it might be unlikely that these cells divided continuously and frequently for more than 2 years. Instead, it would be reasonable to speculate that each differentiated progeny gradually matured to improve the global function of the organ. Lately, it was shown that microRNAs (miRs) can traverse gap junctions and influence the fate of the cells receiving these miRs. Specifically, miR-499, which is abundant in cardiomyocytes

and essentially absent in CSCs, can be transferred from myocytes to resident stem cells *via* gap junction channels, resulting in the enhanced differentiation of the primitive cells toward myocytic lineage^[22]. Therefore, it can be presumed that the presence or absence of matured myocytes in a microenvironment determines the destiny of stem cells *in situ*. Actually, this theory is consistent with the previous observation in which CSCs engrafted in remote myocardium, full of surviving mature cells, progressively differentiate to become indistinguishable from its surrounding myocytes within five weeks^[23]. On the contrary, at the infarcted/regenerated area, cardiomyocytes derived from injected CSCs tend to hold fetal/neonatal characteristics for the same period of time. Once committed to the myocytic lineage, CSCs themselves start to express miR-499, and the quantity increases as the differentiation process advances, which in turn may have an influence on neighboring immature cells through the mircrine mechanism. This cascade of progressive maturation may be called “cellular reverse remodeling”.

CONSEQUENCE OF CELL EXPANSION

Another essential factor can be pointed out in the cell preparation procedure. The necessity of CSC cultivation prior to injection basically excludes the autologous implantation at the acute stage, and this is generally considered to be a major drawback of somatic stem cell treatments. This inevitable culturing step, however, may hold an advantageous aspect as well. Because rapidly multiplying “juvenile” cells grow faster than “senile” ones on the dish, this maneuver practically functions as a natural selection process. Conversely, when cells harvested from a body are used directly to treat an acute illness, the cell population may be heterogeneous by nature within the patients as well as among the preparations for various patients. Needless to say, it is unlikely that a longer culturing period gives a better outcome; there should be a certain threshold. Accordingly, CSCs should be characterized better, in order to pick the right timing and method for the cell preparation step.

PERSPECTIVE

The clinical applications of resident CSCs began only five years ago. Due to the limited number of subjects and the relatively short observational period, we still do not yet know whether this therapy will improve hard endpoints such as the survival of patients. Also, the treatment was mainly applied to patients who required cardiac surgery. However, a recent research indicated that tiny pieces of the failing heart, approximately 5 mg in weight, are sufficient for CSC preparation^[24]. Moreover, our group has lately succeeded in culturing CSCs from small surgical specimens kept frozen for longer than a year; the isolated cells possessed properties comparable to that of those derived from fresh tissues (unpublished observation). This is clinically relevant and important, in light of the

potential repetitive treatment of a single patient and/or applications to adult congenital heart diseases. We have to investigate further to see whether such repeated CSC injections and its usage for pediatric patients are safe and effective as expected. This endeavor will be able to salvage a large number of patients who do not have adequate therapeutic options at the present time.

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Stem cells for spine surgery

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in countless animal models and select clinical trials. Unfortunately, the bench to bedside translation of this research has been slow. Nonetheless, stem cell therapy has received the attention of spinal surgeons due to its potential benefits in the treatment of neural damage, muscle trauma, disk degeneration and its potential contribution to bone fusion.

Key words: Stem cell; Spine surgery; Spinal cord injury; Peripheral nerve damage; Intervertebral disk regeneration; Fusion; Skeletal muscle regeneration

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Core tip: Stem cells have become an increasingly feasible option for the future treatment of spinal disorders. Recent scientific advances have allowed researchers and spinal surgeons alike to investigate the potential of stem cells in the regeneration of degenerated disks, healing spinal cord injury and helping bone growth in spinal fusion.

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Abstract

In the past few years, stem cells have become the focus of research by regenerative medicine professionals and tissue engineers. Embryonic stem cells, although capable of differentiating into cell lineages of all three germ layers, are limited in their utilization due to ethical issues. In contrast, the autologous harvest and subsequent transplantation of adult stem cells from bone marrow, adipose tissue or blood have been experimentally utilized in the treatment of a wide variety of diseases ranging from myocardial infarction to Alzheimer's disease. The physiologic consequences of stem cell transplantation and its impact on functional recovery have been studied

INTRODUCTION

In recent years, stem cells have become a focus of regenerative medicine. Adult stem cells, harvested directly from bone marrow, adipose tissue or blood have the ability to undergo mitosis as well as multipotent differentiation into a variety of cell lineages. The goal of stem cell therapy is to replace or replenish diseased tissue through the localized differentiation of transplanted stem cells into cells which advance the healing process or directly restore the tissue physically. Despite the years of research elucidating the physiology and the processes of stem cell differentiation, both the survival as well as the physical

and biochemical control over the stem cells when implanted into a body remains a challenge. Advances in material sciences have aided tremendously in providing a three-dimensional environment for the cells within a scaffold which allows for both the local retention of cells where they are intended to operate and simultaneously allowing the diffusion of nutrients to enable cell survival. Advances in genetic engineering on the other hand have allowed the modification of stem cells to induce the expression of selective growth factors to further aid in tissue reconstruction. Several challenges in spine surgery have been addressed by experimental ventures into stem cell therapy. Degenerative spinal disorders such as Degenerative Disk Disease have been sought to be addressed through the biological reconstruction of the disk by a variety of stem cells and growth factors, thereby potentially circumventing the need for surgery. The potentially devastating consequences of Spinal Cord Injury have been moderated through the implantation of stem cells to aid in the recovery of nerve cells. Spine surgery itself has been the focus of tissue engineers primarily to achieve bony fusion in the spinal fusion of vertebrae to attain stability. The iatrogenic injury of peripheral nerves and skeletal muscle surrounding the spine, which inevitably occurs during spine surgery whilst access to the spine is being prepared, although not as dramatic in its effect on the disability of the patient in the long-term has been addressed by many scientists. Overall, stem cell therapy, despite being in the experimental phase in most sub-disciplines, promises exciting opportunities to improve spine care and decrease the morbidity due to spine surgery in the future.

APPLICATION OF STEM CELLS IN SPINE SURGERY

Spine fusion

Spine fusion is performed to address the pain, deformity or neurologic deficit caused by degenerative conditions, spinal tumor, vertebral fractures and spinal deformities such as scoliosis and kyphosis amongst other indications. The bony fusion between two or more vertebrae eliminates the pain caused by aberrant motion of the vertebrae through immobilization. Lumbar fusion has been reported to have increased at a rate of 220% from 1990, more than the increases for knee and hip arthroplasty combined^[1]. Ambulatory lumbar spine surgery has been demonstrated to increase at a larger rate relative to inpatient surgery^[2]. Cervical and thoracolumbar fusions have also reportedly increased at a rate of 89% and 31%, respectively, mirroring the rapid increase in the utilization of the procedure^[3]. The introduction of new surgical technology has not proven to reduce reoperation rates^[4]. The vital elements in bony fusion are an adequate quantity of bone-forming cells (osteogenesis), an appropriate microenvironment directing bone synthesis through a variety of growth factors (osteinduction), and a scaffold or cage in which the growth of bone is well positioned (osteoconduction).

Despite the recent advances in cage design and bone fusion extender materials, pseudoarthrosis remains a pressing issue occurring in 13%-41.4% of patients^[5-8]. Risk factors for pseudoarthrosis have been reported to be older age, thoracolumbar kyphosis, smoking, diabetes mellitus, metabolic bone disease and female gender^[6,8-11]. As patients more than 60 years old represent the demographic with the largest increase in the rate of fusion surgery, the medical community has begun investigating alternatives to support the process of bone growth and fusion, for example with the implantation of stem cells^[1]. The gold standard for creating a bony fusion is the use of autograft bone from the iliac crest; however, this has been associated with increased morbidity. Allograft or synthetic bone graft extenders carry the osteoconductive, and to a different extent, the osteoinductive properties, but no cells that will bring the fusion together. Mesenchymal stem cells (MSCs) harvested from the bone marrow, adipose tissue, periosteum or skeletal muscle have been confirmed to differentiate into osteoblasts both *in vitro* and *in vivo*^[12-17]. Adipose derived stem cells (ADSCs) harvested from fat pads, although less commonly utilized in experimental models, are multipotent cells that can differentiate into adipocytes, osteoblasts, chondrocytes, or myocytes when cultivated in the correct microenvironment^[17-21]. Both types of cells have been demonstrated to have a significant effect on spinal fusion in a multitude of settings including a variety of culturing mechanisms, scaffolds and added growth factors. Bone morphogenetic protein-2 (BMP-2) is a growth factor which is increasingly used in spinal fusion, mostly on an off-label basis, which may be the reason for the increased incidence of complications associated with its utilization^[22]. Genetically modified MSCs which were induced to express BMP-2 were reported to induce spinal fusion in mice after injection into the paraspinal musculature comparable, in terms of rigidity, to the fusion achieved with instrumentation^[23,24]. Fu *et al*^[25] addressed the concern of complications associated with BMP-2 by examining if a reduced amount combined with MSCs would still yield acceptable fusion rates. They found that the group with MSCs seeded on alginate with a low dose of BMP-2 achieved equal fusion rates to the group treated with an iliac crest autograft in a rabbit model^[25]. Additional evidence that MSCs may potentially serve as a substitute for autograft or BMP-2 has been presented, however, slightly lower fusion rates were reported for the group treated with MSCs *vs* the group treated with BMP-2 in a rabbit model^[26]. Seo *et al*^[27] attempted to induce higher fusion rates in a rat model by transplanting MSCs seeded on hydroxyapatite in addition to fibroblast growth factor-4, but found that the group treated without the addition of the growth factor achieved the highest fusion rate^[27]. Other than selection of the appropriate growth factor, the level of osteogenic differentiation of the cells may also play a role. One study reported that 80% of rabbit spines treated with MSCs cultured in osteogenic differentiation medium fused *vs* only 33.3% of spines treated with cells that had been cultured without

the addition of differentiation medium^[28]. The efficacy of MSCs transplanted without amendments to culturing protocols, the addition of genetic engineering or growth factors has been less encouraging in a variety of animal models utilizing beta-tricalcium phosphate graft or porous ceramics^[29-31]. Recently, stem cells derived from adipose tissue have become popular in the tissue engineering community, in part due to the ease of cell harvesting from fat pads through liposuction. ADSCs expressing bone morphogenic proteins have proven effective for spinal fusion in animal models of metabolic bone disease^[32,33]. In a study comparing MSCs and ADSCs expressing BMP-2 seeded on collagen sponges, fusion rates were encouraging and not significantly different in the two groups of rat models^[34]. Due to the relatively easier clinical access to ADSCs in the patient, greater attention to their potential role in spinal fusion is warranted. Overall, the use of stem cells in clinical spine fusion has been restricted due to the limited number of cells which may be harvested through liposuction or bone marrow puncture. Cellular *in vitro* expansion is necessary to increase the number of viable pluripotent cells. This represents the greatest burden in the bench to bedside translation of stem cells in spine fusion, as two separate procedures, the availability of sophisticated instrumentation and educated personnel decrease the cost-effectiveness of the intervention^[35-37].

Disc regeneration

Degenerative Disk Disease results from a complex process regulated by biomechanical forces and molecular changes within the disk. A healthy disk consists of the nucleus pulposus rich in collagen type II fibers with a high content of proteoglycan and aggrecan to aid in the resistance to compression^[38,39]. It is surrounded by the annulus fibrosus, rich in collagen type I fibers which are arranged in a parallel fashion to withstand bending and twisting forces. A healthy disk is aneural and avascular due to the high proteoglycan content of the nucleus pulposus, receiving most of its nutrients by diffusion through the vertebral endplate^[40]. Starting at the second decade of life, the progressive calcification of the endplate results in a decrease in the nutrient supply to the disk^[41]. This has been hypothesized to result in phenotypic changes leading to decreased synthesis of proteoglycan and collagen type II, and increased synthesis of collagen type I and III as well as an increase in matrix metalloproteinase activity^[41-44]. Overall, the change in the biochemical composition of the disk results in gross morphologic changes and decreased disk height which contribute to the impingement of nerves^[45-47]. The clinical manifestation of Degenerative Disk Disease in the form of lower back pain is usually focused on conservative management including lifestyle- or work modifications, physical therapy, pain medication, acupuncture and epidural injections. If the symptoms are persistent, cause progressive deformity or neurologic compromise, surgery in the form of disc replacement or spinal fusion is considered^[48]. Growth factors, inflammatory cytokine antagonists and intracellular regulatory proteins are among the factors which have

been demonstrated to result in encouraging regeneration of nucleus pulposus cells *in vitro* and *in vivo*^[49-53]. The utility of these therapies in humans may be limited due to the rapid *in vivo* degeneration of the molecules used for the treatment. Gene therapy, although successfully utilized in animal studies, has significant risks concerning the vectors used for gene transduction. Stem cell therapy for Degenerative Disk Disease is based on the premise of reconstruction of the nucleus pulposus matrix. Nishimura and Mochida were the first to reimplant autologous nucleus pulposus cells in a disk herniation rat model and reported decreased degeneration of the annulus fibrosus, the endplate and the remaining nucleus pulposus when compared to the control group^[54]. As with bony fusion, most scientists have focused on MSCs for Degenerative Disk regeneration. MSCs can differentiate into cell lineages populating bone, cartilage, skeletal muscle and ligamentous tissue^[15]. As the exact phenotype of nucleus pulposus cells has yet to be determined, confirmation of the possibility of MSCs to differentiate into nucleus pulposus cells capable of proteoglycan production does not exist. Nonetheless, researchers have demonstrated that various environmental stimuli and genetic manipulations may result in an MSC differentiating into a nucleus pulposus-like cell. Richardson *et al*^[55] transfected MSCs using the transcription factor, SOX-9, and found that they differentiated into chondrocyte-like cells with the deposition of nucleus pulposus matrix markers collagen type II and aggrecan^[55]. Risbud *et al*^[56,57] experimentally cultured immobilized MSCs under hypoxic conditions with transforming growth factor-beta and found that these conditions prompted MSC differentiation towards nucleus pulposus-like cells^[56,57]. Similar differentiation of stem cells into cells which expressed nucleus pulposus-like phenotypic markers has been observed in rabbit studies. Sakai *et al*^[58,59] studied the effect of the transplantation of MSCs into both healthy and degenerated disks. They found that the implanted cells differentiated into nucleus pulposus-like cells, producing collagen type II and proteoglycan without harm to the rabbit^[58,59]. The degenerated disks showed significant improvement in height and hydration^[60]. Allogenic MSCs were transplanted into the intervertebral disk in a rat model, and demonstrated viability and proliferation^[61]. However, concerns regarding an immune reaction to allogenic stem cells in humans have limited the utilization of such cells in clinical trials. Orozco *et al*^[62] transplanted autologous MSCs into ten patients diagnosed with Degenerative Disk Disease^[62]. They found improvements in pain and disability within three months of treatment. Their study had severe limitations regarding the average age of the patients (35 years) and the number of patients (10). Nonetheless, these results exemplify the importance of arranging larger clinical trials to ease the translation of stem cell therapy from bench to bedside for patients suffering from Degenerative Disk Disease.

Spinal cord injury

Spinal cord injury (SCI) results from traumatic damage to the spinal cord which may have devastating consequences

or result in death^[63]. The most common causes of traumatic SCI are motor vehicle accidents, sports injuries, falls at home, and traumatic injury in the workplace^[64]. A total of 15-40 cases per million people are estimated to suffer a SCI every year, with most cases occurring in males 16-30 years of age^[65-67]. SCI consists of several complex phases which are yet to be elucidated fully on a molecular level. The primary or acute phase consists of the physical disruption and contusion of the nerves and the tissues surrounding the spinal cord^[68]. The force of the traumatic disturbance correlates directly with the amount of cell death^[69]. As a consequence, the spinal cord swells and concomitantly with the commonly associated hemorrhage impedes blood flow, causing hypoxia^[70-74]. The second or sub-acute phase of SCI is characterized by overlapping phases of sustained inflammation, oxidative and immune events. Excessive glutamate levels, the formation of reactive oxygen species and lipid peroxidation cause widespread neuronal and glial death, and axonal degeneration^[75-79]. The scar tissue which is generated during the third or chronic phase presents a physical and biochemical barrier for axonal regeneration, complicating recovery^[80,81]. Scientists have experimented with stem cell transplantation in the hope of promoting functional recovery after SCI. The intervention may be targeted at different phases, but should ideally enhance neuron and axon regeneration and remyelination through the creation of a favorable microenvironment or the direct physical replacement of cells^[82]. This may best be achieved through suppression of the inflammatory cascade resulting in cell apoptosis and necrosis^[83]. Embryonic stem cells, pluripotent cells derived from the inner cell mass of an embryo, have been considered as a treatment option for SCI^[84]. Although these cells can divide infinitely and have greater differentiation potential than adult stem cells, their use is highly controversial^[85-89]. A Chinese surgeon who claimed to have cured SCI in hundreds of patients without complications by injecting them with olfactory ensheathing cells isolated from aborted fetuses was received with great skepticism and sparked fierce debates about the ethicality of such research^[90-93]. The Gevron Corporation is the first company to have received approval to initiate a clinical trial assessing a human embryonic stem cell-derived candidate therapy for severe spinal cord injuries in the United States^[94]. Adult stem cells have more commonly been used in both *in vitro* and *in vivo* experimentation due to the ethical concerns regarding embryonic stem cells. MSCs are favored by many scientists due to the ease of cell harvest, isolation, expansion, and preservation^[35,95-97]. To date, no reports of immunologic reactions to allogeneic *vs* autologous cell transplants have been observed, making MSCs a very practical solution for cellular therapy^[98,99]. MSCs have been demonstrated to promote axonal regeneration and suppress demyelination^[100]. Several different studies in rat models found that MSCs induce nerve regeneration, modulate the production of inflammatory cytokines such as TNF- α and IL-6 and reduce myeloperoxidase activity^[101-105]. Menezes *et al*^[106] hypothesized that laminin may play a pivotal role in neuron and axon preservation and regeneration after finding deposits of the glycoprotein on the lesion site

in a rat SCI model^[106]. All of these studies reported that transplanted MSCs operate mainly through the creation of a favorable microenvironment by means of the secretion of a variety of neurotrophic factors^[107-111]. However, the *in vivo* differentiation of MSCs into neuron-like cells has been documented to be inefficient^[108-110]. Therefore, MSCs are as of now, not capable of directly repopulating and physically restoring the damaged tissue in SCI. Neural stem cells (NSCs) were sought as an option for stem cell therapy, specifically for their ability to overcome this deficit. NSCs are harvested from the subventricular zone and are capable of differentiation into neurons, oligodendrocytes and most commonly astrocytes^[112,113]. Nemat *et al*^[114] reported that the transplantation of NSCs into a contusion SCI in a monkey model facilitated hind limb performance recovery^[114]. Lee *et al*^[115] documented similar functional recovery in terms of hind limb recovery paired with reduced lesions and an increased density of axons and dendritic spines surrounding the transplanted NSCs in a rat model^[115]. Piltti *et al*^[116] examined the survival rates, migration and sensory fiber sprouting of transplanted NSCs in a rat model in the secondary or subacute phase *vs* the tertiary or chronic phase of SCI. They found that the number of surviving transplanted cells was lower in the group treated during the tertiary phase, but that these cells had a stronger effect by increasing the number of mature oligodendrocytes^[116]. The experimental utilization of stem cell therapy in SCI has been very limited to date. Several studies have reported sensory and motor improvements after 1-3 mo of stem cell transplantations combined with various other cells and growth factors^[117-121]. In contrast, Karamouzian *et al*^[122] stated that despite the feasibility and safety of cellular transplantations, the improvements in terms of functional recovery were not statistically significant in their study^[122]. The low numbers of patients in these studies make it difficult to provide a definitive statement on the clinical potential of stem cell transplantation for SCI.

CONCLUSION

Additional areas of interest which have not been clinically addressed with stem cell therapy are iatrogenic nerve and muscle injury caused by spinal surgery. Additional considerations are warranted with respect to the ethics and the cancerogenous risk of embryonic stem cell therapy, the potential immune reaction to autologous cell transplantation as well as the clinical morbidity of adult stem cell harvest. Overall, greater standardization of *in vitro* experimentation and animal models may aid the speed of translation of stem cell therapy in spinal surgery from bench to bedside.

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Basic Study

Early gestation chorionic villi-derived stromal cells for fetal tissue engineering

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Abstract

AIM: To investigate the potential for early gestation placenta-derived mesenchymal stromal cells (PMSCs) for fetal tissue engineering.

METHODS: PMSCs were isolated from early gestation chorionic villus tissue by explant culture. Chorionic villus sampling (CVS)-size tissue samples (mean = 35.93 mg)

were used to test the feasibility of obtaining large cell numbers from CVS within a clinically relevant timeframe. We characterized PMSCs isolated from 6 donor placentas by flow cytometry immunophenotyping, multipotency assays, and through immunofluorescent staining. Protein secretion from PMSCs was examined using two cytokine array assays capable of probing for over 70 factors in total. Delivery vehicle compatibility of PMSCs was determined using three common scaffold systems: fibrin glue, collagen hydrogel, and biodegradable nanofibrous scaffolds made from a combination of polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA). Viral transduction of PMSCs was performed using a Luciferase-GFP-containing lentiviral vector and efficiency of transduction was tested by fluorescent microscopy and flow cytometry analysis.

RESULTS: We determined that an average of 2.09×10^6 (SD $\pm 8.59 \times 10^5$) PMSCs could be obtained from CVS-size tissue samples within 30 d (mean = 27 d, SD ± 2.28), indicating that therapeutic numbers of cells can be rapidly expanded from very limited masses of tissue. Immunophenotyping by flow cytometry demonstrated that PMSCs were positive for MSC markers CD105, CD90, CD73, CD44, and CD29, and were negative for hematopoietic and endothelial markers CD45, CD34, and CD31. PMSCs displayed trilineage differentiation capability, and were found to express developmental transcription factors Sox10 and Sox17 as well as neural-related structural proteins NFM, Nestin, and S100 β . Cytokine arrays revealed a robust and extensive profile of PMSC-secreted cytokines and growth factors, and detected 34 factors with spot density values exceeding 10^3 . Detected factors had widely diverse functions that include modulation of angiogenesis and immune response, cell chemotaxis, cell proliferation, blood vessel maturation and homeostasis, modulation of insulin-like growth factor activity, neuroprotection, extracellular matrix degradation and even blood coagulation. Importantly, PMSCs were also determined to be compatible with both

biological and synthetic material-based delivery vehicles such as collagen and fibrin hydrogels, and biodegradable nanofiber scaffolds made from a combination of PLA and PLGA. Finally, we demonstrated that PMSCs can be efficiently transduced (> 95%) with a Luciferase-GFP-containing lentiviral vector for future *in vivo* cell tracking after transplantation.

CONCLUSION: Our findings indicate that PMSCs represent a unique source of cells that can be effectively utilized for in utero cell therapy and tissue engineering.

Key words: Placenta; Mesenchymal stromal cells; Chorionic villus; Fetal surgery; Tissue engineering

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Core tip: In this study we characterize mesenchymal stromal cells derived from early gestation human placenta chorionic villi (PMSCs) for the purpose of fetal tissue engineering. We examine cell expansion in early passages from chorionic villus sampling-size tissue samples, as well as PMSC surface marker expression, multipotency, intracellular protein expression, protein secretion, and compatibility with delivery vehicles and tracking methods often used for *in vivo* experimentation. We show that early gestation PMSCs are excellent candidates for future tissue engineering studies, particularly as it applies to in utero therapy for congenital anomalies.

Lankford L, Selby T, Becker J, Ryzhuk V, Long C, Farmer D, Wang A. Early gestation chorionic villi-derived stromal cells for fetal tissue engineering. *World J Stem Cells* 2015; 7(1): 195-207 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i1/195.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i1.195>

INTRODUCTION

Over the past three decades, fetal surgery has emerged as an effective treatment option for congenital diseases, including spina bifida, congenital diaphragmatic hernia, sacrococcygeal teratoma and cardiac malformations^[1,2]. *In utero* intervention allows clinicians to treat disease quickly after its onset and prevent debilitating symptoms before they ever occur^[3,4]. Mesenchymal stromal cell (MSC) therapy can augment existing *in utero* surgical techniques, and treatment with MSCs has been shown to promote wound healing^[5,6], protect damaged tissues^[7-11], and modulate the immune system^[12-15]. Cells obtained *via* methods such as chorionic villus sampling (CVS) allow for the potential development of cell therapies that are autologous (derived from the patient's cells) to the fetus^[16-18]. Autologous therapies are potentially preferable to allogeneic (not patient specific) therapies, as using the patient's own cells in a therapy should not elicit an immune response^[19]. However, where autologous therapies are not required, allogeneic

early gestation chorionic villus tissue can be an outstanding cell source for cell therapy as well.

The chorionic villi of early gestation placenta present an ideal source of autologous and allogeneic stromal cells. Placenta-derived mesenchymal stromal cells (PMSCs) are analogous to the MSCs routinely obtained from bone marrow in both surface marker expression and multipotency^[20,21]. Investigators interested in utilizing PMSCs or other fetal-derived mesenchymal stromal cells have demonstrated their potential for engineering bone^[22-24], cartilage^[25-27], and muscle^[28-30], as well as pancreatic^[31], neural^[17,17] and cardiac tissues^[18,32-34]. The gestational age of the source tissue may affect the therapeutic capabilities of the cells. One study comparing cells obtained from term chorionic villus tissue *vs* those from first trimester tissue noted that the cells from earlier gestation tissue had more stem-like properties, faster growth kinetics, and improved wound-healing capability *in vivo*^[35]. These data indicate that cells obtained from earlier gestation chorionic villus tissue may be preferential to term cells for tissue engineering purposes.

In this study we isolate PMSCs from chorionic villus tissue of early gestation placenta (GA 12-18 wk). We detail the initial growth phase of cells isolated from CVS-sized tissue samples using an explant culture method. PMSCs were characterized by flow cytometry immunophenotyping, multipotency assays and immunofluorescent staining for stem cell related transcription factors and intracellular markers, as well as by examining secreted proteins by membrane array. We demonstrate that these cells are compatible with multiple delivery matrices critical to their local distribution upon surgical transplantation. Lastly, PMSCs are shown to be efficiently transduced with a Luciferase-GFP-containing lentiviral vector which allows for cell tracking data to be obtained from *in vivo* transplantation experiments. These data provide further support for the use of early gestation PMSCs for *in vivo* tissue engineering purposes, and sets the stage for further investigation of the therapeutic profile of transplantable cell-seeded matrices.

MATERIALS AND METHODS

Isolation and culture of PMSCs from human early gestation placenta

Donated placental tissues were collected at the UC Davis Medical Center with approval from the Institutional Review Board. PMSCs were isolated from placentas of varying ages early in gestation (GA 12-18 wk) using an explant culture method. Chorionic villus tissue was dissected into small pieces (roughly 5 mm) and washed in sterile 1X phosphate-buffered saline (PBS) containing 100 UI/mL of penicillin, 100 µg/mL of streptomycin. Tissue was then dissected into even smaller pieces and evenly spread across tissue culture-treated dishes. Cells were allowed to migrate from tissue onto the dish before being harvested for subculture. Cells were cryopreserved in a freeze media containing fetal bovine serum (FBS) and 10% dimethyl sulfoxide beginning at passages 3-5 and

thawed for later characterization. Our standard culture media for all experiments was DMEM high glucose with 5% FBS, 100 UI/mL of penicillin, 100 µg/mL of streptomycin, 20 ng/mL recombinant human bFGF (Peprotech) and 20 ng/mL recombinant human EGF (Peprotech).

Early passage growth from CVS-size samples

Cell growth curves were generated from PMSCs obtained from CVS-size tissue samples in order to test the feasibility of obtaining significant cell numbers from a limited tissue mass. Early gestation chorionic villus tissue was carefully dissected and washed in sterile 1X PBS containing 100 UI/mL of penicillin and 100 µg/mL of streptomycin. Dissected tissue was then further cut into smaller pieces (about 20-50 mg) analogous to the amount of tissue obtained in routine chorionic villus sampling (CVS). The CVS-size samples were weighed before being further dissected and spread evenly across an individual well of a 6-well cell culture plate previously coated with CELLStart xeno-free substrate for 1 h at 37 °C. When cells reached 70%-80% confluence, individual wells were passaged and villus tissue removed. Cells were counted at each passage up to the third passage.

Flow cytometry immunophenotyping of PMSCs

Cells were detached for flow cytometry using Accutase (Invitrogen) and viable cell counts obtained using Trypan Blue staining. Cells were then fractioned into tubes containing approximately 1×10^6 cells per sample before staining with the following antibodies: FITC-CD44 (560977), PECy5-CD90 (555597), PE-CD73 (561014), Alexa Fluor 647-CD105 (561439), PE-CD29 (561795), PE-CD34 (560941), Alexa Fluor 647-CD31 (561654), FITC-CD45 (560976) or appropriate isotype controls (all from B.D. Biosciences). Prior to antibody staining, viability staining was completed using LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Molecular Probes) to detect dead cells. After viability and antibody staining, cells were fixed in 10% formalin for 30 min prior to analysis. Cell samples were analyzed on a BD Fortessa LSR Cell Analyzer and further data analysis and gating was performed using FlowJo software (Treestar, Inc).

Multipotency assays

Trilineage differentiation capability of PMSCs was assessed using the assays described below.

For osteogenic differentiation, PMSCs were grown in media consisting of DMEM containing 10% FBS, 10 mmol/L β-glycerol phosphate (Sigma Aldrich), 0.1 µmol/L dexamethasone (Sigma Aldrich) and 200 µmol/L ascorbic acid (Sigma Aldrich) for a minimum of two weeks. Upon differentiation cells were fixed in formalin and osteogenesis confirmed using Alizarin Red staining (Sigma-Aldrich).

For chondrogenic differentiation, PMSCs were grown as cell pellets in suspension in media consisting of DMEM containing 10% FBS, 10 ng/mL transforming growth factor

beta-3 (Peprotech), and 200 µmol/L ascorbic acid (Sigma Aldrich) for a minimum of 3 wk. Chondrogenic pellets were then fixed in formalin before subsequently being embedded in Optimal Cutting Temperature compound (Fisher Scientific) and frozen prior to sectioning. Pellet cross sections were then stained with Alcian Blue (Sigma-Aldrich) and Nuclear Fast Red (Sigma Aldrich) to confirm presence of glycosaminoglycans.

For adipogenic differentiation, PMSCs were grown in media consisting of DMEM containing 10% FBS, 1 µmol/L dexamethasone (Sigma Aldrich), 10 µg/mL insulin (Sigma Aldrich), 5 µmol/L isobutylxanthine (AdipoGen), and 200 µmol/L indomethacin (MP Biomedicals) for a minimum of 3 wk. Upon differentiation cells were fixed in formalin and stained with Oil Red O (Sigma-Aldrich) to identify lipid formation followed by hematoxylin to stain cell nuclei.

Immunofluorescent staining of PMSCs

Cells were fixed in formalin and permeabilized using 0.5% Triton X-100 in PBS prior to immunofluorescent staining. Non-specific binding of primary antibodies was blocked with 1% bovine serum albumin (BSA) in 1X PBS for one hour at room temperature. Primary antibody staining consisted of an overnight incubation with each of following primary antibodies at 4 °C in a solution of 1% BSA in PBS: Sox10 (R and D Systems, MAB2864), Sox17 (R and D Systems, MAB1924), Nestin (Millipore, MAB5326), S100β (Sigma-Aldrich, S2532), or NFM (Santa Cruz Biotech, SC-16143). Following primary antibody incubation, cells were then incubated with AlexaFluor 546-conjugated secondary antibodies (Molecular Probes, A10040) for 1 h at room temperature. Lastly, cell nuclei were counterstained using DAPI (Biotium, 40011). Stained samples were imaged using a Carl Zeiss Axio Observer D1 inverted microscope and post-processed with ImageJ software.

Cytokine array analysis of PMSC secreted proteins

Secreted factors were analyzed from the culture supernatant of PMSCs from a single donor (14 wk GA) using two cytokine array kits (Human Cytokine Array Panel A and Human Angiogenesis Array, both from RD Systems). PMSCs were seeded at a density of 7.5×10^5 per 100 mm culture dish. Culture supernatant was collected at 96 h, centrifuged to remove particulates and then the assays performed according to the manufacturer's instructions. Stained membrane blots were imaged on a Bio-Rad ChemiDoc MP, and images were analyzed using ImageJ software with the Dot Blot Analysis plugin. Integrated density values obtained from membrane images were then plotted using Microsoft Excel.

Delivery vehicle compatibility testing

A single PMSC line (15 wk GA) was tested for compatibility in three delivery vehicles: collagen and fibrin hydrogels, as well as aligned nanofiber scaffold made from synthetic polymers. Fibrin gels were formed using EVICEL® Fibrin Sealant kit. PMSCs were resuspended in DMEM and mixed

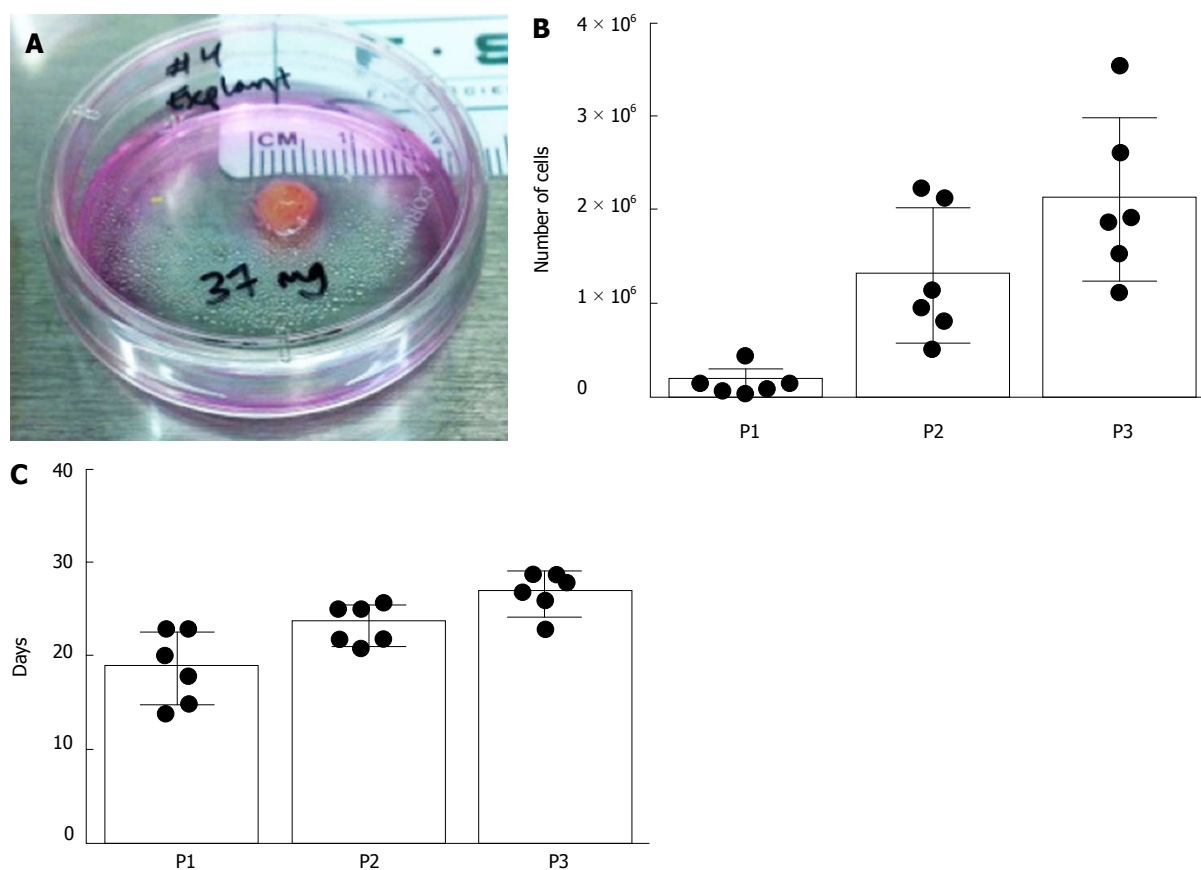


Figure 1 Early passage growth of placenta-derived mesenchymal stromal cells from chorionic villus sampling-size tissues. Placenta-derived mesenchymal stromal cells (PMSCs) were isolated and expanded from chorionic villus sampling (CVS)-size tissue samples ($n = 6$). A: Photograph of a representative CVS-size mass of human early gestation chorionic villus tissue. The average tissue mass used in this experiment was 35.9 mg; B: Cell expansion data from the first three passages show that an average of 2.09×10^6 cells were obtained by the third passage; C: Average days *in vitro* until passages 1, 2, and 3 were 18.83, 23.5, and 27 d, respectively.

with 70 mg/mL fibrinogen solution and 1000 U/mL thrombin. The final composition of fibrin hydrogel was 5 mg/mL fibrinogen, 5 U/mL thrombin and 8.3×10^4 cells/mL. Fibrin gel with embedded cells were seeded in 96-well plates at a volume of 100 μ L per well and incubated at 37 °C for 1 h to ensure complete gelation. After gelation was complete, 100 μ L of culture media was added to each well to cover the hydrogel layer. Collagen hydrogel was prepared by mixing stock 5 mg/mL bovine collagen I solution (Advanced BioMatrix) in DMEM containing PMSCs, yielding a final concentration of 2 mg/mL collagen I and 1.5×10^5 cells/mL. The collagen/cell solution was then seeded in 24-well plates at a volume of 500 μ L per well and allowed to gel at 37 °C for 1 h before the addition of 500 μ L culture media to cover the hydrogel. Aligned nanofiber scaffold has a two-layer structure and was prepared using electrospinning technology. The scaffold consists of polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA) polymers each of them making up 15% weight/volume of the final product. A rectangular piece of nanofiber scaffold (150 mm² surface area) was attached to the bottom of a 35 mm culture dish using double sided bonding tape 5 × 10⁵ cells in 400 μ L of DMEM were carefully pipetted on the membrane and incubated at 37 °C for 1 h to allow cells to adhere. After 1 h, 1 mL of culture medium was added to the 35 mm dish to cover the scaffold

with cells. Cell viability in each system was assessed using LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes) after 24–72 h. Stained constructs were imaged using a Carl Zeiss Axio Observer D1 inverted microscope, and images further processed with ImageJ software.

Lentiviral vector transduction

To allow for long term labeling for future *in vivo* transplantation applications, PMSCs from a single donor (17 wk GA) were transduced using a Luciferase-GFP-containing lentiviral vector. The viral vector used was a pCCLc-MNDU3-LUC-PGK-EGFP-WPRE construct obtained from the UC Davis/CIRM Institute for Regenerative Cures, Sacramento, CA. Cells were seeded overnight at a density of 7.5×10^5 cells per 100 mm dish, and the next morning were treated with transduction media containing 8 μ g/mL protamine sulfate and 10 μ L/mL viral vector for 6 hours. After incubation with the viral vector, cells were washed twice and normal culture media reintroduced. Cells were imaged with fluorescent microscopy to detect successful GFP expression using a Carl Zeiss Axio Observer D1 inverted microscope. At 5 d post-transduction the GFP expression was quantified by flow cytometry using a BD Fortessa LSR Cell Analyzer and collected data further analyzed using FlowJo software (Treestar, Inc).

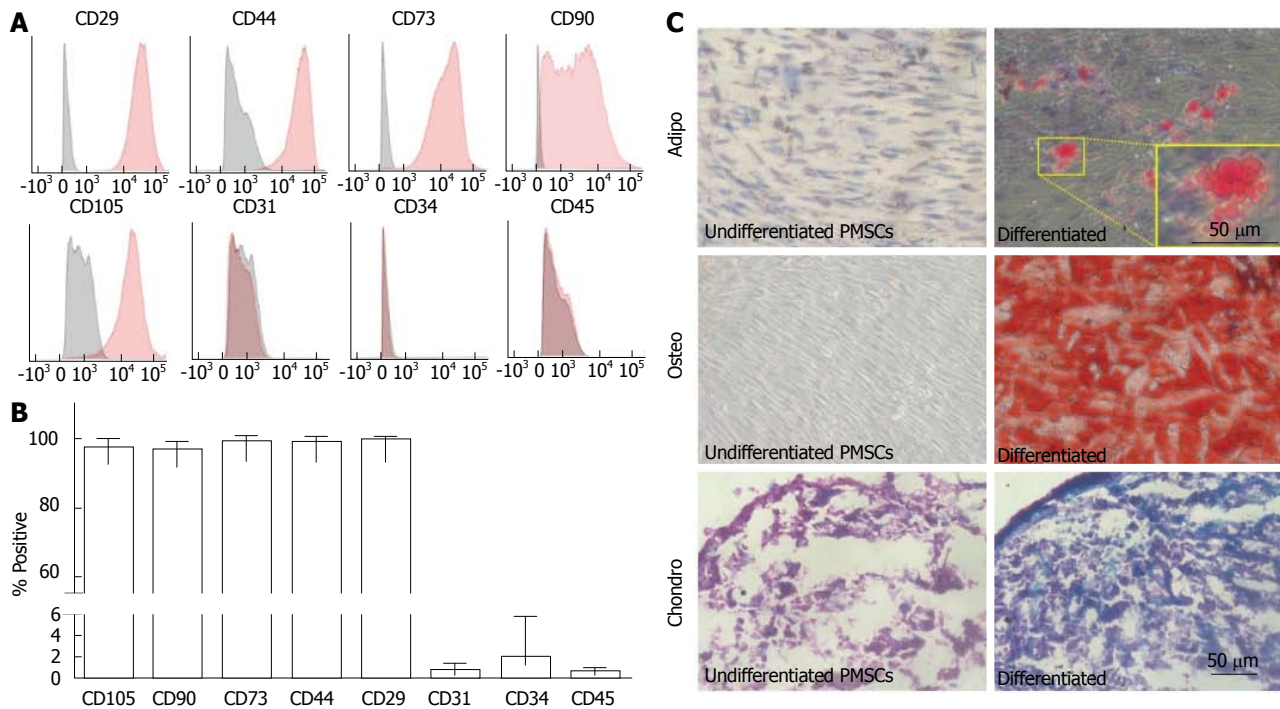


Figure 2 Placenta-derived mesenchymal stromal cells express typical mesenchymal stromal cells surface markers and are multipotent. A, B: Placenta-derived mesenchymal stromal cells (PMSCs) were found to be mostly positive for well-established MSC markers CD29, CD44, CD73, CD90, and CD105, and mostly negative for hematopoietic and endothelial cell markers CD31, CD34, and CD45 ($n = 6$); C: Cells were multipotent and were capable of differentiating into adipocytes, osteocytes and chondrocytes *in vitro*. Images in the left column show negative control samples (normal media only) while those on the right display cultures grown in induction media. Yellow boxes provide a magnified view of the selected area. Scale bar = 50 μ m.

RESULTS

Early passage growth from CVS-size Samples

Cell growth from CVS-size tissue samples demonstrated the feasibility of obtaining cells from biopsied early gestation chorionic villus tissue within a timeframe meaningful for autologous *in utero* cellular therapy, as shown in Figure 1. Expansion of PMSCs from CVS-size tissue samples (mean = 35.93 mg) was found to yield over 2×10^6 cells on average by the third passage ($n = 6$ samples). The first passage of cells to a monolayer occurred at an average of 18.8 d with a minimum and maximum time of 14 and 23 d until passage one, respectively. The cultures reached passage three by day 27 on average, and the longest culture time until passage three was observed to be 29 d.

PMSC display typical MSC surface marker profile and multipotency

Flow cytometry immunophenotyping displayed a profile for PMSCs similar to that typically assigned to mesenchymal stem cells from various sources. As shown in Figure 2A and 2B, cells were observed positive for well-established MSC surface markers CD105 (97.13%), CD90 (96.98%), CD73 (99.28%), CD44 (98.93%), and CD29 (99.58%). Additionally, they were mostly negative for hematopoietic and endothelial-related surface markers CD31 (0.62%), CD34 (1.96%), and CD45 (0.53%) ($n = 6$). Trilineage differentiation potential was observed for all cell lines assayed and results displayed in Figure 2C. PMSCs

exposed to induction media for 2 wk were capable of differentiating into osteocytes and adipocytes, confirmed by Alizarin Red staining to observe calcium deposition and Oil Red staining for lipid accumulation, respectively. In addition, PMSCs were capable of forming chondrocytes when cultured as pellets in induction-specific media for 3 wk, as confirmed by Alcian Blue staining. These results indicate that these cells are multipotent, which is another widely accepted hallmark of traditionally characterized MSCs.

Immunofluorescence staining of PMSC reveals stem cell phenotype

PMSCs were probed for developmentally significant transcription factors and stem cell-related intracellular proteins in order to describe their phenotype in a developmental context. All cell lines examined displayed positivity for transcription factors Sox10 and Sox17, as well as for intracellular stem cell-related proteins Nestin, S100 β , and neurofilament medium (NFM) (Figure 3).

Robust cytokine and angiogenic protein secretion by PMSCs

Supernatant from one PMSC line was collected and secreted cytokines were detected using two cytokine array kits together capable of probing for over 70 cytokines. A total of 34 factors with spot density values above 10^3 were detected in the culture supernatant in both assays combined (Figure 4). Density values for each array showed

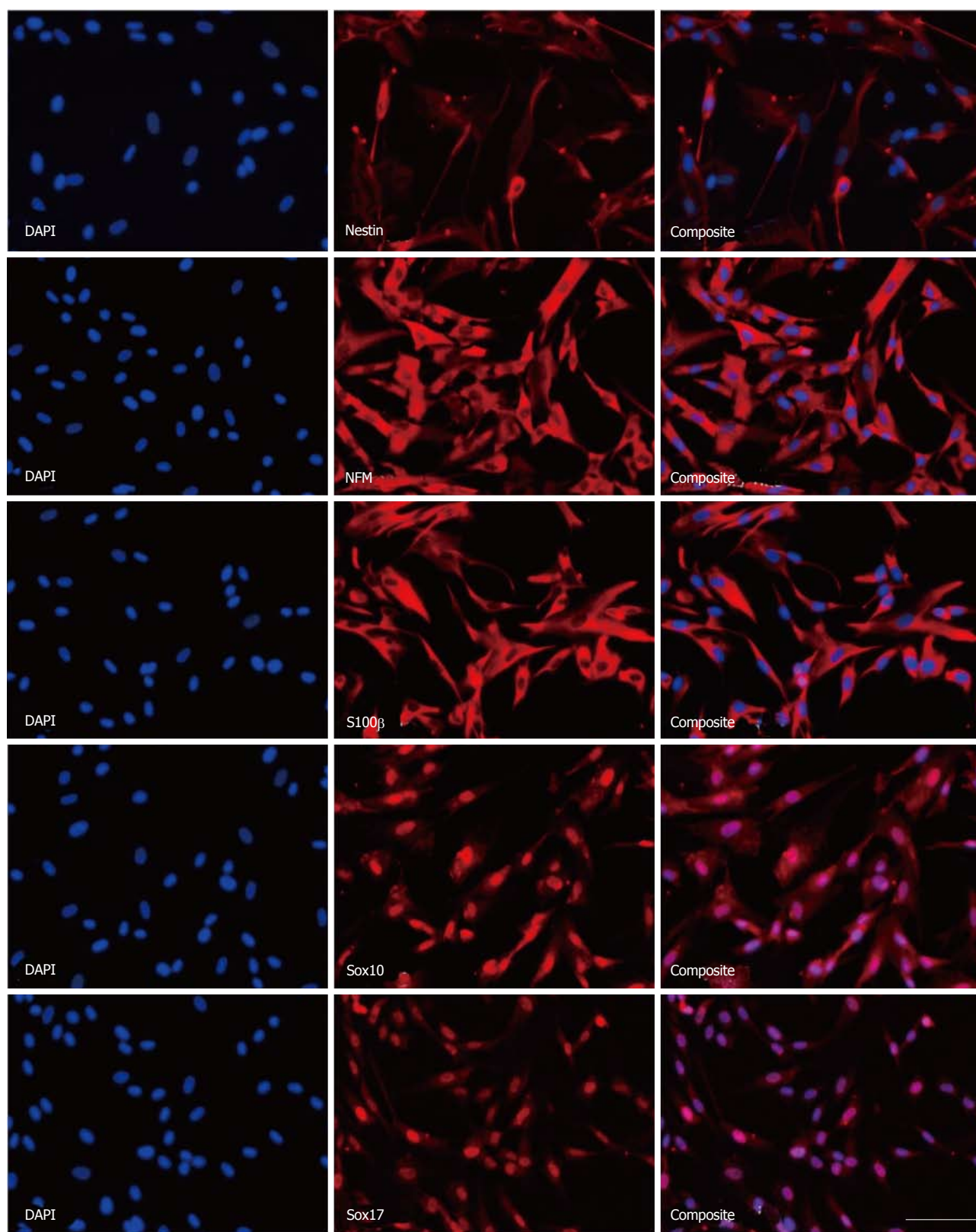


Figure 3 Immunofluorescence staining of placenta-derived mesenchymal stromal cells reveals stem cell phenotype. Placenta-derived mesenchymal stromal cells (PMSCs) were probed using immunofluorescence and found to express intracellular structural proteins Nestin, NFM, and S100 β that are often associated with neural-lineage phenotypes. Additionally, PMSCs were positive for developmental transcription factors Sox10 and Sox17. Scale bar = 100 μ m. NFM: Neurofilament medium.

variable range, with negative control spots having values of around 500. Density maximums were near 57000 in both kits (IL-8 for the angiogenesis array and MCP-1 for

the Panel A array). With the angiogenesis array we found 13 of the 30 detected factors (43.3%) to have densities above 10000, compared to 6 of 7 detected factors

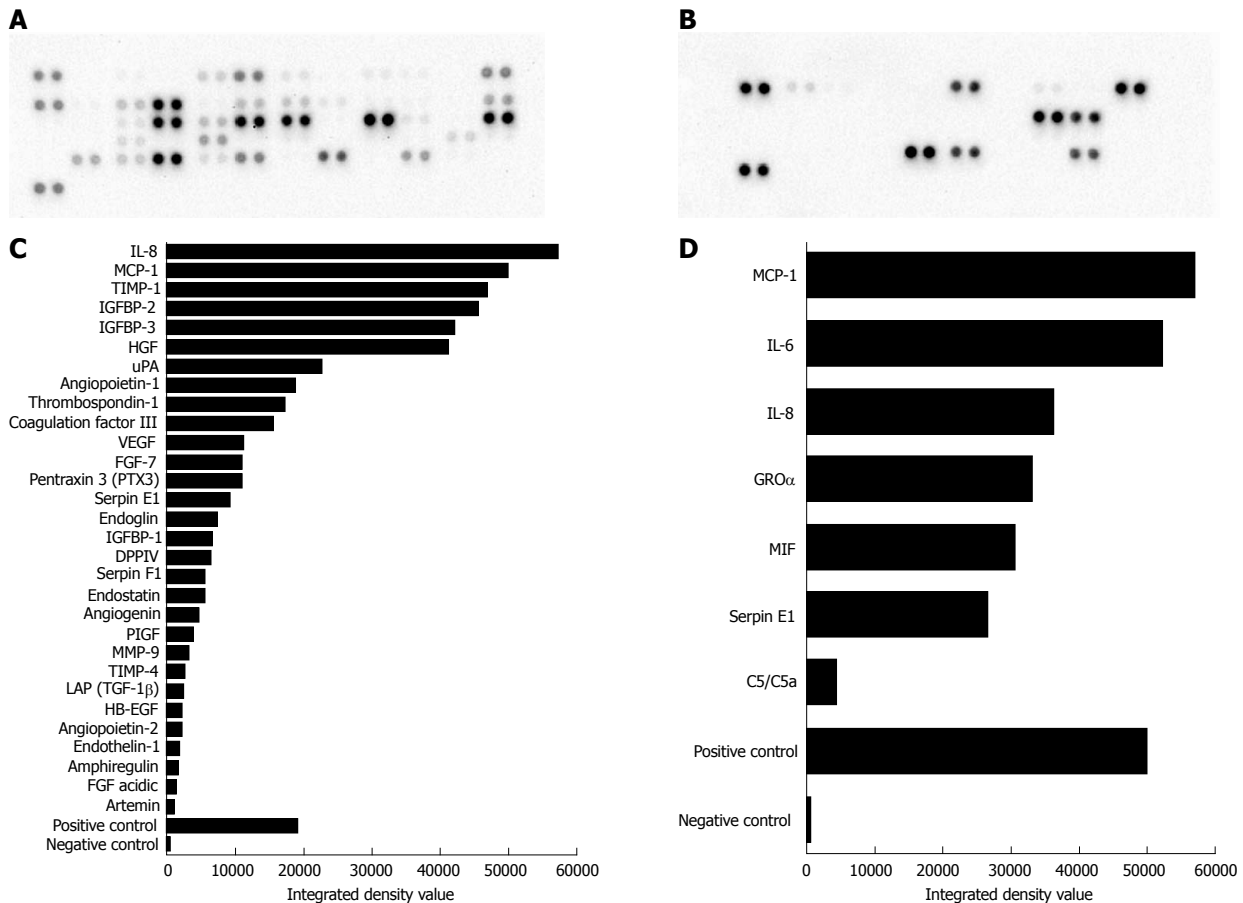


Figure 4 Robust paracrine secretion by placenta-derived mesenchymal stromal cells. A, C: Results from the human angiogenesis array kit detected 30 secreted factors with integrated density values above 10^3 . A: Original membrane image from the Angiogenesis array, and C: corresponding graph showing measured integrated density values of detected spots. Functions of proteins identified here include modulation of angiogenesis, chemotaxis, stimulation of cell proliferation, blood vessel maturation, blood coagulation, and extracellular matrix remodeling. B, D: Results from the panel A array kit detected 7 secreted factors with integrated density values above 10^3 . B: Original membrane image from the Panel A array; D: Corresponding graph showing measured integrated density values of detected spots. Many of these proteins have distinct functions related to the cell chemotaxis and immune response. TGF: Transforming growth factor; IL: Interleukin; VEGF: Vascular endothelial growth factor; GRO α : Growth regulated oncogene alpha; MCP-1: Monocyte chemoattractant protein 1; uPA: Urokinase-type plasminogen activator.

(85.7%) detected in the Panel A array. The functions of these factors are quite expansive and are detailed in Table 1. Among the most highly detected factors for both kits were cytokines and angiogenic factors such as MCP-1, IL-8, IL-6, GRO α , MIF, HGF, Angiopoietin-1, FGF-7, coagulation factor III and VEGF. Inhibitors of angiogenesis such as TIMP-1, Thrombospondin-1, and Pentraxin-3 were notably present as well, indicating the cells can modulate angiogenesis. IGF-binding proteins 2 and 3 were also highly detected, as was extracellular matrix degradation protease μ PA and its inhibitor Serpin E1. The presence of an additional 18 factors was observed at lower levels (integrated density < 10000). While most function in some way to modulate angiogenesis, many also have specific function beyond this process. Some of the more unique functions include vessel homeostasis and maturation (Angiopoietin 1 and 2), ribonuclease activity (Angiogenin), and initiating coagulation (Coagulation Factor III). Additional capabilities such as chemotaxis, stimulation of cell growth, and immunomodulation are shared among many of the detected factors.

PMSCs are compatible with delivery vehicle matrices and transducible for in vivo tracking

Cell loading experiments demonstrated that PMSCs are viable on both biological and synthetic delivery vehicle systems. Viable non-transduced cells were observed with green fluorescence in all vehicles tested as well as the two-dimensional control culture (Figure 5A). PMSCs seeded on the scaffolds had normal morphology indicating that cells were well adhered. Little to no red fluorescent staining was observed in each condition, indicating that cells seeded on each matrix remained viable with few, if any, dead cells. Additionally, PMSCs were readily transduced with the lentiviral vector (97.3% of total cell population) as confirmed by microscopy images for GFP-expression as well as by flow cytometry analysis of transduced and non-transduced cells (Figure 5B).

DISCUSSION

Early gestation chorionic villus tissue is a unique cell source, yielding robust mesenchymal stromal cells well-

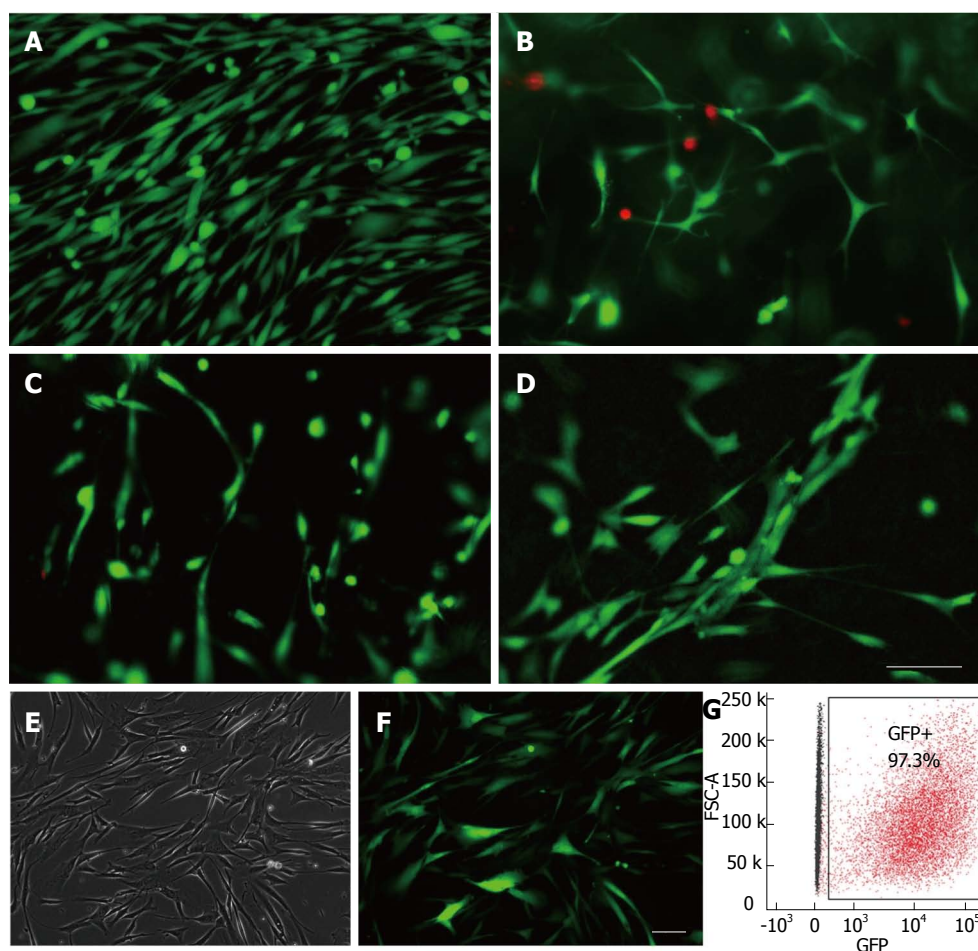


Figure 5 Placenta-derived mesenchymal stromal cells are compatible with biological and synthetic delivery vehicle matrices. A-D: Cell viability was assessed using Molecular Probes Live/Dead fluorescent assay kit for PMSCs cultured in various delivery vehicles. Results show that PMSCs were viable when culture in A: two-dimensional culture (control); B: 2 mg/mL collagen hydrogel; C: 5 mg/mL fibrin glue; D: an aligned PLLA/PLGA nanofiber scaffold. E-G: PMSCs can be efficiently transduced with Luciferase-GFP-containing lentiviral vector; E: phase contrast; F: green fluorescence images from the same field of view of transduced PMSCs; G: Flow cytometry analysis of transduced PMSCs indicate that they were transduced with an efficiency of 97.3%. Scale bars = 100 μ m. PMSCs: Placenta-derived mesenchymal stromal cell.

suites for autologous and allogeneic *in utero* cell therapy and tissue engineering. PMSCs isolated from CVS-size samples were consistently able to generate populations on the order of 10^6 cells in less than four weeks, indicating that simple explant culture provides feasible means of obtaining cells for an autologous fetal cell therapy or tissue engineering. For example, *in utero* repair of spina bifida typically occurs before 26 wk gestational age^[36], and our data show that CVS tissues obtained early in the second-trimester can produce sufficient cell numbers well before they are needed for the repair. Additionally, other structural and congenital defects such as cardiac malformations and congenital diaphragmatic hernia (CDH) may also be potential targets for autologous, tissue engineered constructs populated with PMSCs^[18,32,37].

The *in vitro* characteristics of early second-trimester PMSCs presented in this study are analogous to those of MSCs isolated from various source tissues^[38] in terms of surface marker expression and multipotency. The data also corroborate previous studies aimed at characterizing chorionic villus stromal cells from first and third trimester placentas^[20,39,40], showing that PMSCs described here

contain similarities to cells typically obtained from placentas younger and older in gestation.

Immunofluorescent staining revealed that PMSCs expressed developmentally significant transcription factors Sox10 and Sox17. Sox10 is critical for neurogenesis and maintenance of multipotency^[41-43], while Sox17 functions in the development of definitive endoderm and vasculogenesis^[44,45]. The uniformity of expression of these transcription factors in PMSC cultures may reflect the developmental origins of these cells and could serve as predictors of some related functional properties. All examined PMSC lines also expressed structural proteins Nestin, NFM, and S100 β . These proteins are often associated with a neural phenotype, but their appearance in cultures of non-fetal MSCs has been previously reported elsewhere^[46,47]. Taken in total, these results provide a characteristic phenotypic profile of these cells similar to that of traditionally studied bone marrow MSCs. Still, more work is necessary to better understand how the expression of proteins described above may impact the cell function after *in vivo* transplantation.

It is widely understood that MSCs derive many of

Table 1 Functions of placenta-derived mesenchymal stromal cells secreted factors

Factor	Array	Function	Ref.
Amphiregulin	Angiogenesis	Related to EGF, autocrine mitogen, cancer cell inhibition	[51,52]
Angiogenin	Angiogenesis	Angiogenesis, ribonuclease, mitogen	[53]
Angiopoietin-1	Angiogenesis	Angiogenesis, TIE2 agonist, vessel homeostasis/maturation	[54]
Angiopoietin-2	Angiogenesis	Angiogenesis, TIE2 antagonist, vessel homeostasis/maturation	[54]
Artemin	Angiogenesis	Neurotrophin, endothelial cell migration and proliferation	[55,56]
Tissue Factor	Angiogenesis	Angiogenesis, activation of coagulation cascade	[57,58]
DPPIV	Angiogenesis	Angiogenesis <i>via</i> ECM degradation and EC invasion	[59]
Endoglin	Angiogenesis	Angiogenesis, modulation of effects of TGF- β 1	[60]
Endostatin	Angiogenesis	Angiogenesis inhibition by inhibition of EC proliferation	[61]
Endothelin-1	Angiogenesis	Angiogenesis by stimulating EC proliferation and invasion	[62]
FGF acidic	Angiogenesis	Angiogenesis, EC mitogen	[63-65]
FGF-7	Angiogenesis	Angiogenesis, neuroprotection, wound repair	[66,67]
HB-EGF	Angiogenesis	Chemotaxis, cell growth, angiogenesis	[68]
HGF	Angiogenesis	Angiogenesis through EC motility and growth, neuroprotection	[65,69]
IGFBP-1	Angiogenesis	Modulation of IGF activity, cell chemotaxis and adhesion	[70,71]
IGFBP-2	Angiogenesis	Modulation of IGF activity, mitogen, osteogenesis	[70-72]
IGFBP-3	Angiogenesis	Modulation of IGF activity, inhibition of cell proliferation	[70,71]
LAP (TGF-1 β)	Angiogenesis	Angiogenesis, abundant and varied cell functions	[73,74]
MMP-9	Angiogenesis	Angiogenesis, ECM proteolysis aiding EC migration	[75]
Pentraxin 3 (PTX3)	Angiogenesis	Angiogenesis inhibition by binding FGF2, innate immunity	[76,77]
PIGF	Angiogenesis	Angiogenesis, related to VEGF family	[78,79]
Serpin F1	Angiogenesis	Angiogenesis inhibition, cell survival, immunomodulation	[80]
Thrombospondin-1	Angiogenesis	Angiogenesis inhibition	[81,82]
TIMP-1	Angiogenesis	Angiogenesis inhibition, neuroprotection	[83-85]
TIMP-4	Angiogenesis	Modulation of angiogenesis, EC migration	[86]
uPA	Angiogenesis	ECM proteolysis by converting plasminogen to plasmin	[87]
VEGF	Angiogenesis	Angiogenesis, EC migration and proliferation	[88,89]
IL-8	Both	Angiogenesis, leukocyte chemotaxis	[90-92]
MCP-1	Both	Monocyte chemotaxis, macrophage infiltration, angiogenesis	[93,94]
Serpin E1	Both	mPA inhibitor, wound healing, atherosclerosis	[95,96]
C5/C5a	Panel A	Anaphylatoxin, chemotaxis, immunomodulation	[97]
GRO α	Panel A	Chemotaxis, cell growth, thrombin-induced angiogenesis	[98]
IL-6	Panel A	Both pro- and anti-inflammation, myokine, cell metabolism	[99,100]
MIF	Panel A	Adaptive/innate immune response, inflammation modulation	[101]

TGF: Transforming growth factor; IL: Interleukin; VEGF: Vascular endothelial growth factor; GRO α : Growth regulated oncogene alpha; MCP-1: Monocyte chemotactic protein 1; uPA: Urokinase-type plasminogen activator.

their therapeutic properties from paracrine signaling^[48,49]; thus we employed cytokine array assays in order to examine PMSC cytokine secretion. This approach allowed us to identify an abundance of secreted factors with a wide array of functions. Many of the factors function directly or indirectly in the process of angiogenesis, either to stimulate or inhibit its onset. This seems to indicate that PMSCs are able to modulate the angiogenic process, possibly in response to other environmental cues. Indeed, the enormous variety of function in the factors detected seems to indicate that these cells are capable of exerting an extensive paracrine effect on their environment. In the context of the field of tissue engineering where it is necessary to “restore, maintain, or improve tissue function”^[50], PMSCs delivered locally to damaged tissue may be capable of enhancing the endogenous wound response through their paracrine function.

Cell fate and a suitable delivery vehicle must be determined in the course of developing a new cell therapy. Of crucial importance in this study is the examination of cell compatibility with several delivery systems capable of carrying and supporting PMSCs for transplantation. We have found that PMSCs are exceptionally well-suited

for seeding in several systems capable of delivering cells such as collagen hydrogel and fibrin glue, as well as nanofiber scaffolds made of synthetic polymers. These materials have distinct physical properties in terms of rigidity and biodegradability, making them ideal for tailored approaches to tissue engineering with cell-seeded matrices. These data demonstrate cell attachment and survivability on each of these matrices, which is critical to their effective use for *in utero* tissue engineering. Support for further investigation into the use of PMSCs for tissue engineering purposes is given by the efficiency at which they can be transduced with viral vectors. We show here that PMSCs are readily transduced (97.3% of total cell population) with a luciferase-GFP-containing lentiviral vector that can aid in tracking the cells after *in vivo* transplantation. PMSCs transduced with this viral vector can be tracked in two ways: by detection of luciferase expression in live animals injected with the luciferin substrate, and by GFP expression in histological sections after the transplanted animal is sacrificed. Future investigations into the therapeutic function of PMSCs *in vivo* are enhanced by this technique in that it may allow researchers to gain insight into the ultimate fate of

transplanted cells.

In this report, the characterization of early gestation PMSC populations establishes some basic properties of the cells in their surface marker expression and multipotency. Early gestation PMSCs appear to be an excellent candidate cell source for an autologous *in utero* cellular therapy, especially given their compatibility with several delivery vehicles. However, much work still needs to be completed to determine their potential therapeutic applications and to optimize relevant parameters for transplantation, such as delivery vehicle and cell dosage. In addition, more detailed characterization of cytokine secretion across various cell lines and gestational ages is needed in order to determine the optimal time frame for tissue collection and *ex vivo* cell expansion. It will also be necessary to characterize changes in cell secretion patterns for cells seeded on delivery vehicles in comparison to normal two-dimensional culture. Still, there is abundant potential for PMSCs to be utilized as a therapeutic cell source for transplantation, especially in the form of an autologous *in utero* cell therapy.

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COMMENTS

Background

In utero surgery has emerged in recent years as an effective option for early repair of many congenital anomalies and malformations. Additionally, many diseases for which *in utero* surgery is available may be amenable to augmentation with tissue engineered repair strategies using mesenchymal stromal cells (MSCs). MSCs have been shown to promote wound healing, protect damaged tissues, and modulate the immune system via paracrine secretion. The placenta has recently been described as an ethically unobjectionable and minimally invasive source of fetal MSCs.

Research frontiers

As the field of *in utero* surgery continues to expand, it is likely that future advances in regenerative medicine such as tissue engineering, stem cell and gene therapy will in turn be incorporated into clinical practice. The placenta represents a unique cell source for both autologous and allogeneic fetal stem cell therapies. Due to the developmental nature of many congenital malformations, fetal-derived cells may confer greater benefit to the fetus than traditional adult multipotent cells such as bone marrow or adipose-derived MSCs.

Innovations and breakthroughs

Early gestation placenta-derived mesenchymal stromal cells (PMSCs) were shown to be expandable to clinically relevant populations from chorionic villus sampling (CVS)-sized samples on a timeline applicable to autologous *in utero* therapy for disorders treated mid-gestation. The sheer variety of factors secreted by PMSCs reported here underscores their ability to affect a broad number of physiologic and cellular processes. Additionally, their compatibility with numerous delivery vehicle systems demonstrate that PMSCs can be surgically applied locally to damaged or injured tissues.

Applications

The results obtained in this study suggest that PMSCs have the potential to be highly therapeutic when used to augment current fetal surgical procedures.

Terminology

The chorionic villi are a functionally and anatomically distinct portion of the placenta. They consist of small, tree-like projections that facilitate nutrient transportation between the maternal and fetal blood supplies. CVS is a technique

by which a small portion of chorionic villus tissue can be obtained in a minimally invasive procedure as early as 8 wk of gestation.

Peer reviews

This report from Lankford et al demonstrates the extraction of stromal cells from chorionic-villi suitable for manipulation, expansion, and reimplantation with the ultimate goal of tissue engineering and repair of multiple congenital disease, namely those of the heart and spinal cord. These findings are particularly relevant to the translational field of medicine, as heart conditions and spinal bifida have recently been described as promising targets of *in utero* repair; thus the cells described here would be an excellent source for repair of these congenital anomalies. The authors have detailed an exciting area of research with solid findings that will be of significant interest to multiple investigators and clinicians in the field.

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Basic Study

Regulated genes in mesenchymal stem cells and gastric cancer

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gene expression was analyzed.

METHODS: Gene expression of MSCs and diffuse-type GC cells were analyzed by microarray. Genes related to stem cells, cancer and the epithelial-mesenchymal transition (EMT) were extracted from human gene lists using Gene Ontology and reference information. Gene panels were generated, and messenger RNA gene expression in MSCs and diffuse-type GC cells was analyzed. Cluster analysis was performed using the NCSS software.

RESULTS: The gene expression of regulator of G-protein signaling 1 (RGS1) was up-regulated in diffuse-type GC cells compared with MSCs. A panel of stem-cell related genes and genes involved in cancer or the EMT were examined. Stem-cell related genes, such as growth arrest-specific 6, musashi RNA-binding protein 2 and hairy and enhancer of split 1 (Drosophila), NOTCH family genes and Notch ligands, such as delta-like 1 (Drosophila) and Jagged 2, were regulated.

CONCLUSION: Expression of RGS1 is up-regulated, and genes related to stem cells and NOTCH signaling are altered in diffuse-type GC compared with MSCs.

Key words: Mesenchymal stem cells; Gastric cancer; Stem cells; Gene; Epithelial-mesenchymal transition

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Core tip: Recent studies have revealed that epithelial-mesenchymal transition (EMT) regulators play important roles in cellular phenotypes. This study has shown that EMT-related genes are regulated in diffuse-type gastric cancer and mesenchymal stem cells (MSCs). Regulator of G-protein signaling 1 (RGS1) was significantly up-regulated in diffuse-type GC compared with MSCs. These results are interesting and

Abstract

AIM: To investigate the genes regulated in mesenchymal stem cells (MSCs) and diffuse-type gastric cancer (GC),

provide insights about the mechanisms of the stem cell phenotype transition and gastric cancer progression. These insights will examine the novel role of EMT-related genes and RGS1.

Tanabe S, Aoyagi K, Yokozaki H, Sasaki H. Regulated genes in mesenchymal stem cells and gastric cancer. *World J Stem Cells* 2015; 7(1): 208-222 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i1/208.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i1.208>

INTRODUCTION

It is a mystery how the epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs) are correlated^[1,2]. Although it is known that the EMT is related to cancer migration and shares similar molecular characteristics with CSCs, whether the EMT occurs in CSCs or if there is any threshold for this phenotype between stem and cancer cells is largely unknown. The EMT is the cellular process involving the cell phenotype transitioning from an epithelial phenotype to a mesenchymal phenotype. Cancer cells experience the EMT when adhesion is less important, and the cells can freely migrate throughout the body. This mechanism is thought to be important in cancer cell invasion and migration and CSC development. The stem cell phenotype transforms from a differentiated stage to an undifferentiated stage and vice versa during the development of tissues and organs and during different disease states. Investigating stem cell-regulated genes is a highly important issue to elucidate the mechanisms of CSCs.

It has been shown that mesenchymal stem cells (MSCs) can promote the expression of an EMT phenotype in cancer cells *via* CD44, lysyl oxidase and TWIST^[3]. The relationship between MSCs and cancer cells, including the mechanism of stemness exhibited by cancer cells, remains unclear. Moreover, the self-renewal of colorectal cancer initiating cells is considered to be a new therapeutic approach^[4]. It is important to uncover the gene signature of stem and cancer cells to further understand the stemness of these cells. Studies to reveal the stemness phenotype have already been performed. A marker of the putative stem cell region of human intestinal crypts has been identified using a Fourier transform infrared microspectroscopy approach^[5-7]. Genes, such as necdin homolog (mouse) (*NDN*), EPH receptor A5 (*EPHA5*), nephroblastoma overexpressed gene (*NOV*) and runt-related transcription factor 2 (*RUNX2*), have been correlated with MSC passage numbers^[8]. Molecular signatures have also been investigated, and a prediction model of bone marrow stromal cell senescence has been discovered^[9]. Even gene expression at the single cell level can be determined using a recent technique^[10]. These new approaches, including a physical theory, may be important for the advancement of stem cell therapeutics.

Gastric cancer is classified into diffuse and intestinal types^[11,12]. Diffuse-type gastric carcinomas supposedly originate from nonmetaplastic gastric epithelium, and the intestinal type originates from the regenerating epithelium in chronic atrophic gastritis^[12]. Diffuse-type gastric cancer (GC) are known to correlate with the EMT^[13]. We have previously shown that diffuse-type GC can be distinguished with combination of cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*) and cadherin 2, type 1, N-cadherin (neuronal) (*CDH2*) gene expression in MSC and diffuse-type GC associated with the EMT^[14]. Considering that hepatoma cells are converted into human induced pluripotent stem cell-like cells using microRNAs, the difference between stem cells and cancer cells should be deeply investigated^[15]. Breast CSCs show both mesenchymal-like and epithelial-like phenotypes and transition between EMT- and mesenchymal-epithelial transition-like states^[16]. Diffuse-type GC has been reported to be involved in the remodeling of the pulmonary artery, which suggests cancer niche formation by diffuse-type GC^[17]. Considering the finding that MSCs provide microenvironment for CSCs, the features of MSC and cancer cells should be investigated^[18].

In this article, MSC gene expression has been investigated and compared with diffuse-type GC cells that exhibit a mesenchymal phenotype more than intestinal-type GC cells do, and panels of regulated genes in MSCs and diffuse-type GC have been generated to clarify the important molecular patterns that distinguish stem and cancer cells.

MATERIALS AND METHODS

Cell culture

MSCs from human bone marrow were cultured according to the manufacturer's protocol (Lonza Walkersville, Inc., Walkersville, MD, United States). Detailed methods have been previously described^[8]. Briefly, MSCs were cultured *in vitro* in mesenchymal stem cell growth medium (Lonza Catalog #PT-3001; mesenchymal stem cell basal medium plus SingleQuots™ of growth Supplements) at 37 °C in a CO₂ (5%) incubator. Each number in the MSC sample name indicates the passage number.

Total RNA extraction

Diffuse-type GC samples were surgically resected and obtained from the National Cancer Center Research Institute. Detailed information on total RNA extraction has been previously described^[14,19]. Briefly, total RNA was obtained by suspending the cells in ISOGEN lysis buffer from surgically resected samples, which were provided by the Central Hospital or East Hospital at the National Cancer Center^[14,19]. Total RNA of MSCs were purified using RNeasy kit (QIAGEN, Düsseldorf, Germany) as previously described^[8].

Microarray analysis

MSC gene expression data are available to the public *via* the NCBI Gene Expression Omnibus (GEO)^[8]. Diffuse-

type GC gene expression data are also available *via* the NCBI GEO^[14]. The accession numbers of data used for gene expression analysis are GSE7888 and GSE42252 for MSCs and diffuse-type GC, respectively. Briefly, total RNA from MSCs and diffuse-type GC was analyzed using a GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA, United States). Probe set and Gene Ontology information is based on annotation version na34. Cluster analysis was performed using NCSS 2007 (NCSS, LLC., Kaysville, UT, United States).

Statistical analysis

All data are presented as the mean $\pm$ SE. Statistical significance was calculated with GraphPad Prism® 6 or Microsoft® Excel® using the unpaired Student's t test or one-way ANOVA with Bonferroni's multiple comparisons test.

RESULTS

Regulated genes in diffuse-type GC and MSCs

Genes regulated in diffuse-type GC and MSCs were investigated and analyzed using cluster analysis (NCSS) (Figure 1). Probe sets that have a more than a 50-fold significant difference between MSCs and diffuse-type GC ($P < 10^{-9}$ in the equal variance hypothesis and $P < 0.01$ in the non-equal variance hypothesis) are shown in Figure 1. Each panel shows the gene expression in 30 probe sets. Panels A, B and C show the up-regulated probe sets in diffuse-type GC compared with MSCs, whereas panel D shows 29 up-regulated probe sets and one down-regulated gene, pentraxin 3, long (*PTX3*). In this analysis, regulator of G-protein signaling 1 (*RGS1*) was the most up-regulated gene in diffuse-type GC compared with MSCs (Figure 2A). *RGS4* gene expression was down-regulated in diffuse-type GC compared with MSCs (Figure 2B). The regulated RGS family genes are shown in Table 1. Additional up-regulated genes in diffuse-type GC compared to MSCs included spleen tyrosine kinase (*SYK*) and *CDH1*, which have been suggested to be mutated in cancer, based on an assessment of the public database Cancer Gene Census (<http://cancer.sanger.ac.uk/cancergenome/projects/census/>).

Stem cell gene panel

The expression of genes related to stem cells was investigated (Table 2). Considering that gene combination is important to understand stem cell phenotype, several genes involving to stem cells have been analyzed^[20]. *NANOG* and *SUZ12*, which have been found by gene expression analysis to be involved in MSC invasion was up-regulated in diffuse-type GC compared to MSCs^[21]. Gene expression of tumor necrosis factor receptor superfamily, member 19 (*TNFRSF19*), tended to decrease in diffuse-type GC compared with MSCs, but it was not significantly altered in MSCs and diffuse-type GC (Figure 3). The gene expression of *TNFRSF8*, also known as

CD30, was slightly up-regulated in diffuse-type GC compared with MSCs. The gene expression of growth arrest-specific 6 (*GAS6*), musashi RNA-binding protein 2 (*MSI2*) and hairy and enhancer of split 1, (*Drosophila*) (*HES1*), which have stem cell terms in the Gene Ontology biological process, were regulated in diffuse-type GC and MSCs (Figure 4).

Investigation of EMT markers

To examine the cellular phenotype of cancer and stem cells, the expression levels of EMT-related genes were investigated (Table 3). The gene expression of the *TWIST*, *ZEB* and *SNAI* families were examined because these genes have been reported to be associated with the EMT and cancer regulation^[22]. The gene expression of twist family bHLH transcription factor 1 (*TWIST1*) and paired related homeobox 1 (*PRRX1*) were down-regulated in diffuse-type GC compared with MSCs. The gene expression of zinc finger E-box binding homeobox 2 (*ZEB2*) was up-regulated in diffuse-type GC compared with MSCs. Previously, high mobility group AT-hook 2 (*HMGAT2*) has been shown to be involved in the EMT, *WNT*/ β -catenin signaling and malignant cancer^[23,24]. *HMGAT2* gene expression was down-regulated in diffuse-type GC compared with MSCs. The gene expression of one of the probe sets for catenin (cadherin-associated protein), beta 1, 88 kDa (*CTNNB1*) was up-regulated in diffuse-type GC compared with MSCs. The gene expression of the other two probe sets for *CTNNB1* was stable. Sp1 transcription factor (*SP1*) and lymphoid enhancer-binding factor 1 (*LEF1*) were up-regulated in diffuse-type GC compared with MSCs. *SP1* is known to be an important factor for cell cycle regulation and is up-regulated in various cancers^[25]. The *AKT* gene family is known to be important in cancer and has been suggested to regulate the EMT and stem cells in cancer cells^[26]. Three *AKT* isoforms were examined, and the gene expression of v-akt murine thymoma viral oncogene homolog 2 (*AKT2*) was up-regulated. However, some of the probe sets recognizing *AKT3* showed a down-regulation of *AKT3* gene expression. The gene expression of stratifin (*SFN*); F11 receptor (*F11R*); gap junction protein, beta 2, 26 kDa (*GJB2*); occluding (*OCN*); coxsackie virus and adenovirus receptor (*CXADR*); and keratin 8 (*KRT8*) were up-regulated in diffuse-type GC compared with MSCs.

Messenger RNA expression of NOTCH signaling-related genes

NOTCH signaling-related genes are involved in cancer, and *NOTCH* signaling is one of the target signaling pathways of CSCs^[27-30]. Next, *NOTCH* messenger RNA (mRNA) expression was examined (Table 4). *NOTCH1*, 3 and 4 gene expression was up-regulated, whereas *NOTCH2* gene expression was down-regulated in diffuse-type GC compared with MSCs (Figure 5A). The gene expression of delta-like 1 (*Drosophila*) (*DLL1*) and Jagged 2 (*JAG2*) were up-regulated in diffuse-type GC compared with MSCs

To confirm that the up-regulation of *RGS1* was not stomach specific, *RGS1* gene expression in normal tissues was investigated using various data in the database, such

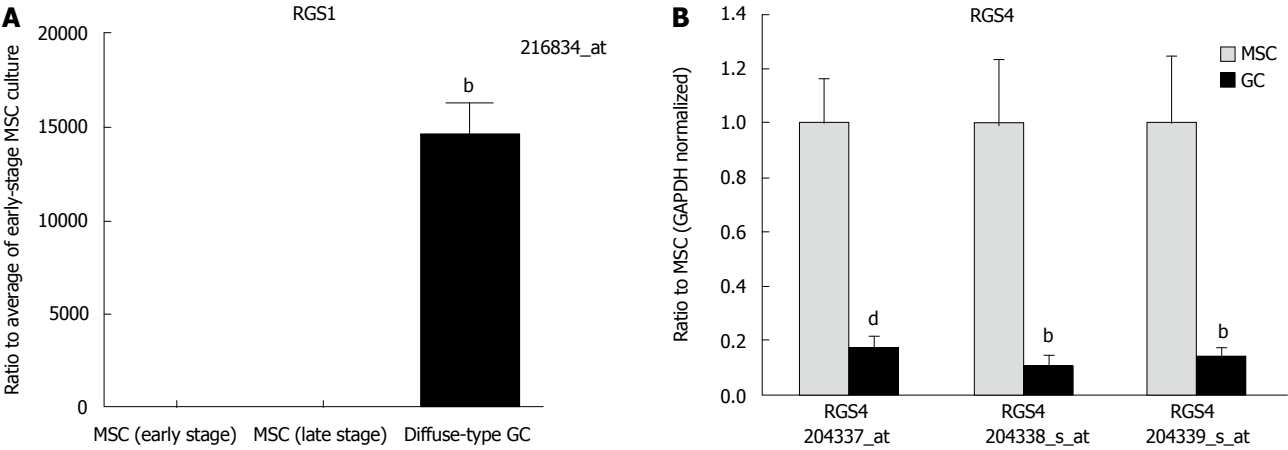


Figure 2 Regulator of G-protein signaling 1 and regulator of G-protein signaling 4 gene expression was up-regulated and down-regulated, respectively, in diffuse-type gastric cancer. A: regulator of G-protein signaling 1 (*RGS1*) gene expression was up-regulated in diffuse-type gastric cancer (GC) compared with early-stage mesenchymal stem cell (MSC) cultures [$n = 5$ in early-stage MSC cultures (B#4, C#4, F#5, G#4, and H#4), $n = 5$ in late-stage MSC cultures (B#28, C#24, E#28, G#28, and H#28), and $n = 5$ in diffuse-type GC (GC16, GC40, GC54b, GC60, and GC23s)]. Results represent the mean $\pm$ SE. $^bP < 0.01$ using a one-way analysis of variance with Bonferroni's multiple comparisons test; B: *RGS4* gene expression was down-regulated in diffuse-type GC compared with MSCs. Results represent the mean $\pm$ SE ($n = 12$ in MSC cultures and $n = 5$ in diffuse-type GC). $^bP < 0.01$ and $^cP < 0.001$ using Student's *t* test with the non-equal variance hypothesis.

Table 1 Gene expression alterations of regulator of G-protein signaling family genes in mesenchymal stem cells and diffuse-type gastric cancer

Gene symbol	Detailed description	<i>P</i> value	Ratio of diffuse-type GC to MSCs	Probe set ID
<i>RGS1</i>	Immune response / signal transduction / adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway / negative regulation of signal transduction / termination of G-protein coupled receptor signaling pathway / positive regulation of GTPase activity	0.013 0.001	1004 13808	202988_s_at 216834_at
<i>RGS2</i>	Regulation of translation / cell cycle / spermatogenesis / regulation of G-protein coupled receptor protein signaling pathway / negative regulation of signal transduction / negative regulation of phospholipase activity / negative regulation of cardiac muscle hypertrophy / termination of G-protein coupled receptor signaling pathway / negative regulation of mitogen-activated protein (MAP) kinase activity / positive regulation of GTPase activity / negative regulation of G-protein coupled receptor protein signaling pathway / brown fat cell differentiation / relaxation of cardiac muscle / relaxation of vascular smooth muscle / positive regulation of cardiac muscle contraction / regulation of adrenergic receptor signaling pathway	0.007	14.51	202388_at
<i>RGS3</i>	Inactivation of mitogen-activated protein kinase (MAPK) activity / signal transduction / regulation of G-protein coupled receptor protein signaling pathway / negative regulation of signal transduction / termination of G-protein coupled receptor signaling pathway / positive regulation of GTPase activity	1.00E-05	0.39	203823_at
<i>RGS4</i>	Inactivation of MAPK activity / regulation of G-protein coupled receptor protein signaling pathway / negative regulation of signal transduction / termination of G-protein coupled receptor signaling pathway / positive regulation of GTPase activity	3.00E-04 0.003 0.004	0.17 0.11 0.14	204337_at 204338_s_at 204339_s_at
<i>RGS5</i>	Signal transduction / regulation of G-protein coupled receptor protein signaling pathway / negative regulation of signal transduction / termination of G-protein coupled receptor signaling pathway / positive regulation of GTPase activity	0.01 0.008 0.009	47.79 81.82 95.52	209070_s_at 209071_s_at 218353_at
<i>RGS10</i>	Negative regulation of signal transduction / termination of G-protein coupled receptor signaling pathway / positive regulation of GTPase activity	0.003	3.92	204319_s_at
<i>RGS19</i>	Autophagy / G-protein coupled receptor signaling pathway / small GTPase mediated signal transduction / negative regulation of signal transduction / termination of G-protein coupled receptor signaling pathway / positive regulation of GTPase activity	9.00E-04	2.45	204336_s_at

RGS family genes that have significant alterations in MSCs and diffuse-type GC are shown. *RGS1*, *RGS2*, *RGS5*, *RGS10* and *RGS19* were up-regulated in diffuse-type GC compared with MSCs. *RGS3* and *RGS4* were down-regulated in diffuse-type GC compared with MSCs. RGS: Regulator of G-protein signaling; MSCs: Mesenchymal stem cells; GC: Gastric cancer.

as the GEO database. From data analysis of various types of normal human tissues (GSE2361 and GSE2193)^[31,32], *RGS1* has been shown to be expressed in normal stomach,

bladder, colon, corpus, liver, lung, placenta, prostate, skin, spinal cord, spleen, thymus, thyroid, and trachea, whereas *RGS1* gene expression is relatively high in the thymus and

Table 2 Gene expression alterations of stem cell-related genes in mesenchymal stem cells and diffuse-type gastric cancer

Gene symbol	Gene name	P value	Ratio of diffuse-type GC to MSCs	Probe set ID
NANOG	Nanog homeobox	0.0037	14.9	220184_at
SUZ12	SUZ12 polycomb repressive complex 2 subunit	0.0022	35.4	1566191_at
TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	0.0126	1.98	206729_at
CD9	CD9 molecule	0.0012	3.44	201005_at
ICAM3	Intercellular adhesion molecule-3	0.0143	2.69	204949_at
CD200	CD200 molecule	0.0013	4.79	209583_s_at
		0.0002	4.35	209582_s_at
THY1	Thy-1 cell surface antigen	0.0081	0.61	208850_s_at
		0.0301	0.79	208851_s_at
		0.0024	0.67	213869_x_at

Genes related to stem cells in the literature are shown with their gene expression alterations in MSCs and diffuse-type GC. *NANOG* and *SUZ12*, which have been found by gene expression analysis to be involved in MSC invasion was up-regulated in diffuse-type GC compared to MSCs. RGS: Regulator of G-protein signaling; MSCs: Mesenchymal stem cells; GC: Gastric cancer.

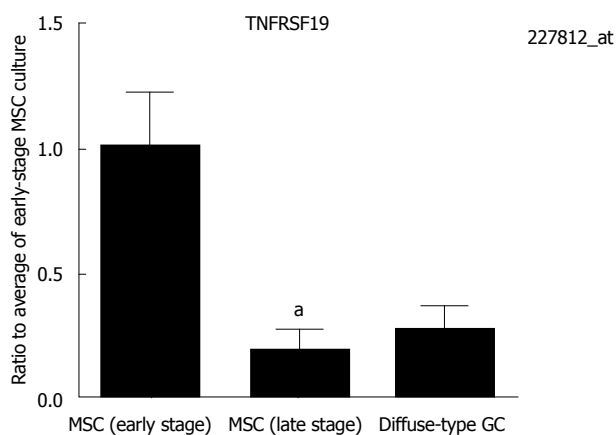


Figure 3 Tumor necrosis factor receptor superfamily, member 19 gene expression. Tumor necrosis factor receptor superfamily, member 19 (*TNFRSF19*) gene expression tended to decrease in diffuse-type gastric cancer (GC) but was not significantly altered compared with early-stage mesenchymal stem cell (MSC) cultures. *TNFRSF19* gene expression in late-stage MSC cultures was significantly down-regulated compared with early-stage MSC cultures. Results represent the mean $\pm$ SE [$n = 5$ in early-stage MSC cultures (B#4, C#4, F#5, G#4, and H#4), $n = 5$ in late-stage MSC cultures (B#28, C#24, E#28, G#28, and H#28), and $n = 5$ in diffuse-type GC (GC16, GC40, GC54b, GC60, and GC23s)]. * $P < 0.05$ using a one-way ANOVA with Bonferroni's multiple comparisons test.

spinal cord but not in the stomach.

DISCUSSION

Gastric cancer progression is associated with *Helicobacter pylori* and gastric stem cells are suggested to involve in the progression from chronic atrophic gastritis to gastric cancer^[33]. It may be assumed that *RGS1* plays a role in regulating G protein signaling and mitogen-activated protein (MAP) kinase signaling during gastric cancer progression, which may provide some hypotheses in which *RGS1* promotes stem cell transition into CSCs, CSCs induce *RGS1* expression in gastric cancer, or *RGS1* orchestrates with other molecules in EMT. In gastric cancer cells, *CDH1* isoform is found to increase gastric cancer cell invasion and angiogenesis, and some inflammatory mechanism is suggested to be

involved in *CDH1* regulation^[34]. Analysis of *Rgs1*(-/-) has shown that B cell responses to chemokines, plasma cell localization, and immune tissue architecture are disturbed when *Rgs1* is deleted^[35]. In our studies, *RGS1* was up-regulated in diffuse-type GC compared to MSC, which may suggest that *RGS1* plays a role to response cancer cells and indicate the possibility to involve in cancer immunity. It may be possible that *RGS1* protects the cells from cancer progression by enhancing anti-cancer immunity, or promote cell phenotype transition to cancer. The possibility that diffuse-type GC sample contains a variety of cells in cancer microenvironment should be considered. It may also be possible that *RGS1* is up-regulated in some cancer microenvironment cells. Because the dedifferentiation of epithelial cells into stem cells may play a role in regenerative medicine or diseases, including cancer, investigating the genes regulated in stem and cancer cells is important^[36]. Changes in the gene expression profiles of early- and late-stage MSC cultures have provided evidence regarding the importance of studying gene combinations to understand cellular phenotypes^[8]. Cell surface antigens, such as SSEA4 or TRA-1-81, should also be included for future quality control in stem cells^[37-39]. Furthermore, cell type-specific transcriptional profiles have been suggested for neural lineage cells, hepatic lineage cells, embryonic stem cells and induced pluripotent stem cells (iPSCs)^[40]. Interestingly, insulin-like growth factor-1 receptor signaling and EMT crosstalk is sufficient to down-regulate erlotinib function in cells, while exon 19 of the erlotinib-sensitive epidermal growth factor receptor is mutated in non-small cell lung carcinomas^[41]. Many cell-based iPSC models that are derived from patient cells have been investigated^[42], and successful genetic or epigenetic reprogramming of cellular identity is crucial for the development of these stem-cell based disease models^[42]. The investigation of the molecular combinations associated with different cell types is important for determining cellular phenotypes.

RGS1 has been shown to be related to cancer in some studies^[43-45]. In late-stage non-small cell lung cancer, single nucleotide polymorphisms in *RGS1* have been shown to

Table 3 Gene expression alterations of epithelial-mesenchymal transition-related genes in mesenchymal stem cells and diffuse-type gastric cancer

Gene symbol	Gene name	P value	Ratio of diffuse-type GC to MSCs	Probe set ID
<i>TWIST1</i>	Twist family bHLH transcription factor 1	0.0004	0.2	213943_at
<i>PRRX1</i>	Paired related homeobox 1	1.00E-05	0.3	226695_at
<i>ZEB2</i>	Zinc finger E-box binding homeobox 2	0.027	2.1	203603_s_at
		0.03	1.7	228333_at
		6.00E-05	3.9	235593_at
<i>TJP1</i>	Tight junction protein 1	0.184	1.2	202011_at
		0.795	1	214168_s_at
<i>CTNNB1</i>	Catenin (cadherin-associated protein), beta 1, 88 kDa	0.001	9.6	223679_at
<i>VIM</i>	Vimentin	0.031	4.7	1555938_x_at
		0.016	0.7	201426_s_at
<i>FN1</i>	Fibronectin 1	0.204	0.6	1558199_at
		0.015	0.7	210495_x_at
		0.004	0.7	211719_x_at
		0.001	0.6	212464_s_at
		4.00E-08	0.04	214701_s_at
		1.00E-10	0.02	214702_at
		0.008	0.7	216442_x_at
<i>AKT1</i>	v-akt murine thymoma viral oncogene homolog 1	0.187	1.2	207163_s_at
<i>AKT2</i>	v-akt murine thymoma viral oncogene homolog 2	8.00E-09	3.1	225471_s_at
		0.002	4.4	226156_at
		0.0006	5.6	236664_at
		0.0006	7.6	1560689_s_at
<i>AKT3</i>	v-akt murine thymoma viral oncogene homolog 3	0.0006	6.2	242876_at
		0.008	0.6	212607_at
		0.549	1.2	212609_s_at
		0.385	0.8	219393_s_at
		0.772	1.1	222880_at
		0.0003	0.5	242879_x_at
<i>SFN</i>	Stratifin	0.003	66	33322_i_at
		0.008	111	33323_r_at
<i>EPCAM</i>	Epithelial cell adhesion molecule	0.0009	725	201839_s_at
<i>F11R</i>	F11 receptor	0.0004	35	221664_s_at
		0.002	21	222354_at
		0.0009	201	223000_s_at
		0.012	25	224097_s_at
<i>GJB2</i>	Gap junction protein, beta 2, 26 kDa	0.023	69	223278_at
<i>LEF1</i>	Lymphoid enhancer-binding factor 1	0.002	26	221558_s_at
<i>JUN</i>	Jun proto-oncogene	0.042	4.8	201464_x_at
		0.022	5.1	201466_s_at
		0.079	3.8	201465_s_at
		0.019	4.1	213281_at
<i>TGFB1</i>	Transforming growth factor, beta 1	0.002	2	203085_s_at
<i>TGFB2</i>	Transforming growth factor, beta 2	0.07	0.4	209909_s_at
		0.055	0.6	220407_s_at
		0.588	0.8	228121_at
<i>TGFB3</i>	Transforming growth factor, beta 3	0.015	6.8	209747_at
<i>SP1</i>	Sp1 transcription factor	0.0002	3.4	224754_at
		2.00E-05	2.7	224760_at
<i>HMGA2</i>	High mobility group AT-hook 2	0.003	0.3	208025_s_at
<i>ITGA5</i>	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	0.007	0.5	201389_at
<i>OCLN</i>	Occludin	0.0008	8	231022_at
		0.012	9.4	235937_at
		0.027	11	209925_at
		0.0045	11	227492_at
<i>CXADR</i>	Coxsackie virus and adenovirus receptor	0.013	265	203917_at
		0.014	297	226374_at
		0.021	75	239155_at
<i>KRT8</i>	Keratin 8	0.012	128	209008_x_at

RGS: Regulator of G-protein signaling; MSCs: Mesenchymal stem cells; GC: Gastric cancer.

be associated with overall survival in patients who received chemotherapy^[43]. Silencing of *RGS1* and *RGS13* mRNAs resulted in enhanced responsiveness to chemoattractants

in a human B lymphoma line^[44]. *RGS1* has been suggested to be involved in regulatory T cell trafficking^[45]. Furthermore, *RGS1* is up-regulated in T cells from the

Table 4 Gene expression alterations of NOTCH signaling-related genes in mesenchymal stem cells and diffuse-type gastric cancer

Gene symbol	Gene name	P value	Ratio of diffuse-type GC to MSCs	Probe set ID
<i>NOTCH1</i>	Notch 1	0.004	6.9	218902_at
<i>NOTCH2</i>	Notch 2	0.001	0.7	202445_s_at
		1.00E-05	0.5	202443_x_at
		1.00E-05	0.5	212377_s_at
<i>DLL1</i>	Delta-like 1 (Drosophila)	0.007	31	224215_s_at
<i>JAG2</i>	Jagged 2	0.003	11	32137_at
<i>JAG1</i>	Jagged 1	0.003	1.5	209099_x_at
		0.022	1.4	216268_s_at
		0.49	1.1	209098_s_at
<i>WNT9A</i>	Wingless-type MMTV integration site family, member 9A	0.027	5.4	230643_at
<i>WNT2B</i>	Wingless-type MMTV integration site family, member 2B	0.026	4.4	206458_s_at
<i>WNT5A</i>	Wingless-type MMTV integration site family, member 5A	8.00E-05	0.3	213425_at
		3.00E-05	0.2	205990_s_at
<i>WNT5B</i>	Wingless-type MMTV integration site family, member 5B	0.0002	0.2	221029_s_at

RGS: Regulator of G-protein signaling; MSCs: Mesenchymal stem cells; GC: Gastric cancer.

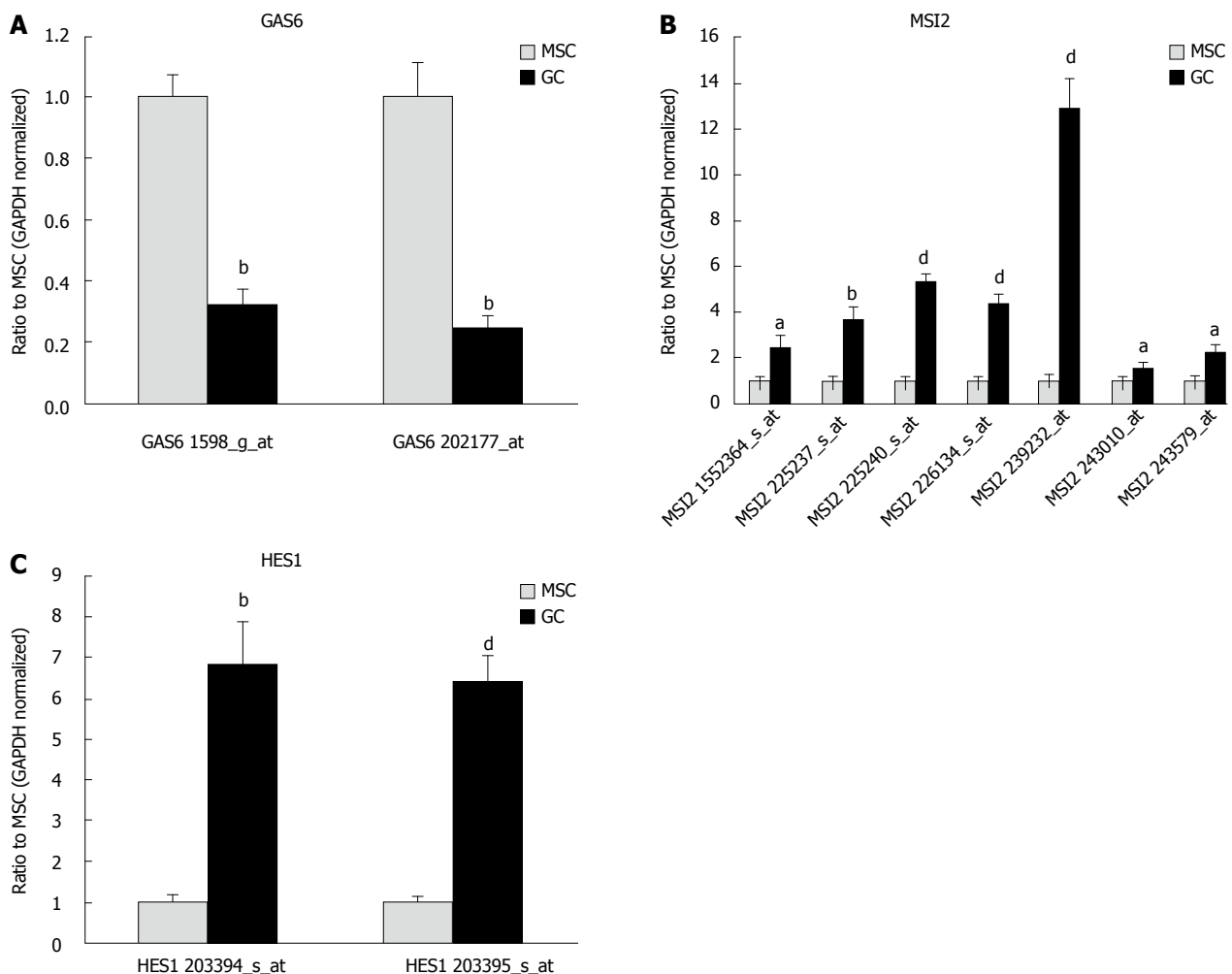


Figure 4 Gene expression of stem cell-related genes. A: The gene expression of growth arrest-specific 6 (*GAS6*) was down-regulated in diffuse-type gastric cancer (GC) compared with mesenchymal stem cell (MSCs). Results represent the mean $\pm$ SE ($n = 12$ in MSC and $n = 5$ in diffuse-type GC). ^b $P < 0.001$ using Student's *t* test; B: The gene expression of musashi RNA-binding protein 2 (*MSI2*) was up-regulated in diffuse-type GC compared with MSCs. Results represent the mean $\pm$ SE ($n = 12$ in MSC and $n = 5$ in diffuse-type GC). ^a $P < 0.05$, ^b $P < 0.01$, and ^d $P < 0.001$ using Student's *t* test; C: The gene expression of hairy and enhancer of split 1 (Drosophila) (*HES1*) was up-regulated in diffuse-type GC compared with MSCs. Results represent the mean $\pm$ SE ($n = 12$ in MSC and $n = 5$ in diffuse-type GC). ^b $P < 0.01$ and ^d $P < 0.001$ using Student's *t* test. All three genes have stem cell terms in the Gene Ontology biological process.

human gut compared with T cells from peripheral blood, and *RGS1* regulates gut T cell trafficking^[46]. Cancer cells

Table 5 Gene ontology of NOTCH family genes

Gene symbol Gene ontology biological process

NOTCH1
 0000122 // negative regulation of transcription from RNA polymerase II promoter // inferred from sequence or structural similarity /// 0001525 // angiogenesis // inferred from electronic annotation /// 0001701 // in utero embryonic development // inferred from electronic annotation /// 0001708 // cell fate specification // inferred from electronic annotation /// 0001837 // epithelial to mesenchymal transition // inferred from sequence or structural similarity /// 0001889 // liver development // inferred from electronic annotation /// 0001947 // heart looping // inferred from sequence or structural similarity /// 0002040 // sprouting angiogenesis // inferred from electronic annotation /// 0002052 // positive regulation of neuroblast proliferation // inferred from electronic annotation /// 0002437 // inflammatory response to antigenic stimulus // inferred from electronic annotation /// 0003157 // endocardium development // inferred from sequence or structural similarity /// 0003160 // endocardium morphogenesis // inferred from sequence or structural similarity /// 0003162 // atrioventricular node development // inferred from electronic annotation /// 0003169 // coronary vein morphogenesis // inferred from sequence or structural similarity /// 0003180 // aortic valve morphogenesis // inferred from mutant phenotype /// 0003181 // atrioventricular valve morphogenesis // inferred from sequence or structural similarity /// 0003184 // pulmonary valve morphogenesis // inferred from mutant phenotype /// 0003192 // mitral valve formation // inferred from mutant phenotype /// 0003198 // epithelial to mesenchymal transition involved in endocardial cushion formation // inferred from sequence or structural similarity /// 0003203 // endocardial cushion morphogenesis // inferred from sequence or structural similarity /// 0003207 // cardiac chamber formation // inferred from sequence or structural similarity /// 0003208 // cardiac ventricle morphogenesis // inferred from sequence or structural similarity /// 0003209 // cardiac atrium morphogenesis // inferred from sequence or structural similarity /// 0003213 // cardiac right atrium morphogenesis // inferred from sequence or structural similarity /// 0003214 // cardiac left ventricle morphogenesis // inferred from sequence or structural similarity /// 0003219 // cardiac right ventricle formation // inferred from electronic annotation /// 0003222 // ventricular trabecula myocardium morphogenesis // inferred from sequence or structural similarity /// 0003241 // growth involved in heart morphogenesis // inferred from sequence or structural similarity /// 0003256 // regulation of transcription from RNA polymerase II promoter involved in myocardial precursor cell differentiation // inferred from sequence or structural similarity /// 0003264 // regulation of cardioblast proliferation // inferred from electronic annotation /// 0003270 // Notch signaling pathway involved in regulation of secondary heart field cardioblast proliferation // inferred from electronic annotation /// 0003273 // cell migration involved in endocardial cushion formation // inferred from sequence or structural similarity /// 0003344 // pericardium morphogenesis // inferred from sequence or structural similarity /// 0006351 // transcription, DNA-dependent // inferred from electronic annotation /// 0006355 // regulation of transcription, DNA-dependent // traceable author statement /// 0006357 // regulation of transcription from RNA polymerase II promoter // inferred from electronic annotation /// 0006367 // transcription initiation from RNA polymerase II promoter // traceable author statement /// 0006955 // immune response // non-traceable author statement /// 0006959 // humoral immune response // inferred from electronic annotation /// 0007219 // Notch signaling pathway // inferred from mutant phenotype /// 0007219 // Notch signaling pathway // traceable author statement /// 0007220 // Notch receptor processing // traceable author statement /// 0007221 // positive regulation of transcription of Notch receptor target // inferred from sequence or structural similarity /// 0007275 // multicellular organismal development // inferred from electronic annotation /// 0007283 // spermatogenesis // inferred from electronic annotation /// 0007368 // determination of left/right symmetry // inferred from sequence or structural similarity /// 0007386 // compartment pattern specification // inferred from electronic annotation /// 0007409 // axonogenesis // inferred from electronic annotation /// 0007440 // foregut morphogenesis // inferred from electronic annotation /// 0007492 // endoderm development // inferred from electronic annotation /// 0007507 // heart development // inferred from mutant phenotype /// 0008284 // positive regulation of cell proliferation // inferred from direct assay /// 0008284 // positive regulation of cell proliferation // inferred from mutant phenotype /// 0008285 // negative regulation of cell proliferation // inferred from direct assay /// 0008544 // epidermis development // inferred from electronic annotation /// 0009790 // embryo development // inferred from electronic annotation /// 0009912 // auditory receptor cell fate commitment // inferred from electronic annotation /// 0010001 // glial cell differentiation // inferred from electronic annotation /// 0010467 // gene expression // traceable author statement /// 0010468 // regulation of gene expression // inferred from electronic annotation /// 0010718 // positive regulation of epithelial to mesenchymal transition // inferred from mutant phenotype /// 0010812 // negative regulation of cell-substrate adhesion // inferred from direct assay /// 0010832 // negative regulation of myotube differentiation // inferred from sequence or structural similarity /// 0014031 // mesenchymal cell development // inferred from sequence or structural similarity /// 0014807 // regulation of somitogenesis // inferred from electronic annotation /// 0021515 // cell differentiation in spinal cord // inferred from electronic annotation /// 0021915 // neural tube development // inferred from electronic annotation /// 0030154 // cell differentiation // inferred from electronic annotation /// 0030182 // neuron differentiation // inferred from electronic annotation /// 0030216 // keratinocyte differentiation // inferred from electronic annotation /// 0030279 // negative regulation of ossification // inferred from sequence or structural similarity /// 0030324 // lung development // inferred from electronic annotation /// 0030326 // embryonic limb morphogenesis // inferred from electronic annotation /// 0030334 // regulation of cell migration // inferred from electronic annotation /// 0030335 // positive regulation of cell migration // inferred from sequence or structural similarity /// 0030513 // positive regulation of BMP signaling pathway // inferred from sequence or structural similarity /// 0030514 // negative regulation of BMP signaling pathway // inferred from sequence or structural similarity /// 0030900 // forebrain development // inferred from electronic annotation /// 0031069 // hair follicle morphogenesis // inferred from electronic annotation /// 0031100 // organ regeneration // inferred from electronic annotation /// 0031960 // response to corticosteroid stimulus // inferred from electronic annotation /// 0032495 // response to muramyl dipeptide // inferred from electronic annotation /// 0032496 // response to lipopolysaccharide // inferred from electronic annotation /// 0035116 // embryonic hindlimb morphogenesis // inferred from electronic annotation /// 0035148 // tube formation // inferred from mutant phenotype /// 0035914 // skeletal muscle cell differentiation // inferred from electronic annotation /// 0035924 // cellular response to vascular endothelial growth factor stimulus // inferred from direct assay /// 0042127 // regulation of cell proliferation // inferred from electronic annotation /// 0042246 // tissue regeneration // inferred from electronic annotation /// 0042640 // anagen // inferred from electronic annotation /// 0043065 // positive regulation of apoptotic process // inferred from electronic annotation /// 0043066 // negative regulation of apoptotic process // inferred from electronic annotation /// 0043086 // negative regulation of catalytic activity // inferred from sequence or structural similarity /// 0045165 // cell fate commitment // inferred from electronic annotation /// 0045596 // negative regulation of cell differentiation // inferred from electronic annotation /// 0045603 // positive regulation of endothelial cell differentiation // inferred from electronic annotation /// 0045618 // positive regulation of keratinocyte differentiation // inferred from electronic annotation /// 0045662 // negative regulation of myoblast differentiation // inferred from mutant phenotype /// 0045665 // negative regulation of glial neuron differentiation // inferred from electronic annotation /// 0045668 // negative regulation of osteoblast differentiation // inferred from sequence or structural similarity /// 0045687 // positive regulation of glial cell differentiation // inferred from electronic annotation /// 0045892 // negative regulation of transcription, DNA-dependent // inferred from sequence or structural similarity /// 0045893 // positive regulation of

transcription, DNA-dependent // inferred from sequence or structural similarity /// 0045944 // positive regulation of transcription from RNA polymerase II promoter // inferred from direct assay /// 0045944 // positive regulation of transcription from RNA polymerase II promoter // inferred from sequence or structural similarity /// 0045955 // negative regulation of calcium ion-dependent exocytosis // inferred from electronic annotation /// 0046427 // positive regulation of JAK-STAT cascade // inferred from sequence or structural similarity /// 0046533 // negative regulation of photoreceptor cell differentiation // inferred from electronic annotation /// 0048103 // somatic stem cell division // inferred from electronic annotation /// 0048663 // neuron fate commitment // inferred from electronic annotation /// 0048708 // astrocyte differentiation // inferred from electronic annotation /// 0048709 // oligodendrocyte differentiation // inferred from electronic annotation /// 0048711 // positive regulation of astrocyte differentiation // inferred from sequence or structural similarity /// 0048715 // negative regulation of oligodendrocyte differentiation // inferred from sequence or structural similarity /// 0048754 // branching morphogenesis of an epithelial tube // inferred from electronic annotation /// 0050678 // regulation of epithelial cell proliferation // inferred from electronic annotation /// 0050679 // positive regulation of epithelial cell proliferation // inferred from electronic annotation /// 0050767 // regulation of neurogenesis // inferred from electronic annotation /// 0050768 // negative regulation of neurogenesis // inferred from sequence or structural similarity /// 0050793 // regulation of developmental process // inferred from electronic annotation /// 0055008 // cardiac muscle tissue morphogenesis // inferred from sequence or structural similarity /// 0060038 // cardiac muscle cell proliferation // inferred from electronic annotation /// 0060045 // positive regulation of cardiac muscle cell proliferation // inferred from sequence or structural similarity /// 0060253 // negative regulation of glial cell proliferation // inferred from sequence or structural similarity /// 0060317 // cardiac epithelial to mesenchymal transition // inferred from sequence or structural similarity /// 0060411 // cardiac septum morphogenesis // inferred from sequence or structural similarity /// 0060412 // ventricular septum morphogenesis // inferred from mutant phenotype /// 0060528 // secretory columnar luminal epithelial cell differentiation involved in prostate glandular acinus development // inferred from electronic annotation /// 0060548 // negative regulation of cell death // inferred from electronic annotation /// 0060740 // prostate gland epithelium morphogenesis // inferred from electronic annotation /// 0060768 // regulation of epithelial cell proliferation involved in prostate gland development // inferred from sequence or structural similarity /// 0060842 // arterial endothelial cell differentiation // inferred from sequence or structural similarity /// 0060843 // venous endothelial cell differentiation // inferred from sequence or structural similarity /// 0060948 // cardiac vascular smooth muscle cell development // inferred from sequence or structural similarity /// 0060956 // endocardial cell differentiation // inferred from sequence or structural similarity /// 0060979 // vasculogenesis involved in coronary vascular morphogenesis // inferred from sequence or structural similarity /// 0060982 // coronary artery morphogenesis // inferred from sequence or structural similarity /// 0061314 // Notch signaling involved in heart development // inferred from mutant phenotype /// 0061384 // heart trabecula morphogenesis // inferred from sequence or structural similarity /// 0061419 // positive regulation of transcription from RNA polymerase II promoter in response to hypoxia // inferred from sequence or structural similarity /// 0070986 // left/right axis specification // inferred from electronic annotation /// 0071372 // cellular response to follicle-stimulating hormone stimulus // inferred from direct assay /// 0072017 // distal tubule development // inferred from electronic annotation /// 0072044 // collecting duct development // inferred from electronic annotation /// 0072144 // glomerular mesangial cell development // inferred from electronic annotation /// 0072602 // interleukin-4 secretion // inferred from electronic annotation /// 0090051 // negative regulation of cell migration involved in sprouting angiogenesis // inferred from direct assay /// 0090090 // negative regulation of canonical Wnt receptor signaling pathway // inferred from electronic annotation /// 0097150 // neuronal stem cell maintenance // inferred from expression pattern /// 1901201 // regulation of extracellular matrix assembly // inferred from sequence or structural similarity /// 2000737 // negative regulation of stem cell differentiation // inferred from mutant phenotype /// 2000811 // negative regulation of anolix // inferred from mutant phenotype /// 2000974 // negative regulation of pro-B cell differentiation // inferred from sequence or structural similarity /// 2001027 // negative regulation of endothelial cell chemotaxis // inferred from direct assay

NOTCH2

0001701 // in utero embryonic development // inferred from electronic annotation /// 0001709 // cell fate determination // traceable author statement /// 0001890 // placenta development // inferred from electronic annotation /// 0002011 // morphogenesis of an epithelial sheet // inferred from electronic annotation /// 0002437 // inflammatory response to antigenic stimulus // inferred from electronic annotation /// 0003184 // pulmonary valve morphogenesis // inferred from mutant phenotype /// 0006351 // transcription, DNA-dependent // inferred from electronic annotation /// 0006355 // regulation of transcription, DNA-dependent // traceable author statement /// 0006367 // transcription initiation from RNA polymerase II promoter // traceable author statement /// 0006915 // apoptotic process // traceable author statement /// 0006917 // induction of apoptosis // traceable author statement /// 0006959 // humoral immune response // inferred from electronic annotation /// 0007050 // cell cycle arrest // inferred from direct assay /// 0007219 // Notch signaling pathway // traceable author statement /// 0007220 // Notch receptor processing // traceable author statement /// 0007275 // multicellular organismal development // non-traceable author statement /// 0007368 // determination of left/right symmetry // inferred from electronic annotation /// 0007399 // nervous system development // non-traceable author statement /// 0008285 // negative regulation of cell proliferation // inferred from direct assay /// 0009887 // organ morphogenesis // inferred from expression pattern /// 0010467 // gene expression // traceable author statement /// 0016049 // cell growth // inferred from direct assay /// 0019827 // stem cell maintenance // traceable author statement /// 0030097 // hemopoiesis // traceable author statement /// 0030154 // cell differentiation // inferred from electronic annotation /// 0030326 // embryonic limb morphogenesis // inferred from electronic annotation /// 0042246 // tissue regeneration // inferred from electronic annotation /// 0043065 // positive regulation of apoptotic process // inferred from electronic annotation /// 0043066 // negative regulation of apoptotic process // traceable author statement /// 0046579 // positive regulation of Ras protein signal transduction // inferred from direct assay /// 0046849 // bone remodeling // inferred from mutant phenotype /// 0050793 // regulation of developmental process // inferred from electronic annotation /// 0060413 // atrial septum morphogenesis // inferred from mutant phenotype /// 0060674 // placenta blood vessel development // inferred from electronic annotation /// 0061314 // Notch signaling involved in heart development // inferred by curator /// 0072602 // interleukin-4 secretion // inferred from electronic annotation

NOTCH3

0006351 // transcription, DNA-dependent // inferred from electronic annotation /// 0006355 // regulation of transcription, DNA-dependent // inferred from electronic annotation /// 0006367 // transcription initiation from RNA polymerase II promoter // traceable author statement /// 0007219 // Notch signaling pathway // traceable author statement /// 0007220 // Notch receptor processing // traceable author statement /// 0007275 // multicellular organismal development // inferred from electronic annotation /// 0010467 // gene expression // traceable author statement /// 0030154 // cell differentiation // inferred from electronic annotation /// 0030900 // forebrain development // inferred from electronic annotation /// 0045596 // negative regulation of cell differentiation // inferred from electronic annotation /// 0045665 // negative regulation of neuron differentiation // inferred from electronic annotation /// 0048661 // positive regulation of smooth muscle cell proliferation // inferred from electronic annotation /// 0048663 // neuron fate commitment // inferred from electronic annotation /// 0050793 // regulation of developmental process // inferred from electronic annotation /// 0072104 // glomerular capillary formation // inferred from electronic annotation

NOTCH4 0001569 // patterning of blood vessels // inferred from sequence or structural similarity /// 0001709 // cell fate determination // traceable author statement /// 0001763 // morphogenesis of a branching structure // inferred from sequence or structural similarity /// 0001886 // endothelial cell morphogenesis // inferred from electronic annotation /// 0006351 // transcription, DNA-dependent // inferred from electronic annotation /// 0006355 // regulation of transcription, DNA-dependent // inferred from electronic annotation /// 0006367 // transcription initiation from RNA polymerase II promoter // traceable author statement /// 0007219 // Notch signaling pathway // traceable author statement /// 0007220 // Notch receptor processing // traceable author statement /// 0007221 // positive regulation of transcription of Notch receptor target // traceable author statement /// 0007275 // multicellular organismal development // inferred from electronic annotation /// 0009790 // embryo development // inferred from sequence or structural similarity /// 0010467 // gene expression // traceable author statement /// 0030097 // hemopoiesis // traceable author statement /// 0030154 // cell differentiation // non-traceable author statement /// 0030879 // mammary gland development // inferred from direct assay /// 0045446 // endothelial cell differentiation // inferred from electronic annotation /// 0045596 // negative regulation of cell differentiation // non-traceable author statement /// 0045602 // negative regulation of endothelial cell differentiation // inferred from sequence or structural similarity /// 0045893 // positive regulation of transcription, DNA-dependent // traceable author statement /// 0050793 // regulation of developmental process // inferred from electronic annotation

interacting with the immune system may be future targets for anti-cancer therapy^[47]. Considering that *RGS1* is up-regulated in diffuse-type GC compared with MSCs, *RGS1* may be a potential target for cancer treatment. *RGS1* is involved in an adenylate cyclase-inhibiting G-protein coupled receptor signaling pathway, immune response, positive regulation of GTPase activity, and termination of a G-protein coupled receptor signaling pathway in the Gene Ontology biological process [Traceable author statement (TSA)]; Inferred from electronic annotation (IEA) (NCBI). From the investigation of EMT-related genes and *RGS1*, this study may suggest the possibility of future gene applications, including *RGS1*, to evaluate cancer cells to their sensitivity to treatment. microRNAs have been reported to be involved in *RGS5* regulation in neuroblastoma^[48]. Further assessments are needed to examine the possibility that *RGS1* could be a marker for responsiveness to cancer treatment.

NOTCH gene expression was altered in diffuse-type GC and MSCs. Gene expression of *NOTCH1*, 3 and 4 was up-regulated in diffuse-type GC compared with MSCs, whereas *NOTCH2* was down-regulated in diffuse-type GC. Upon Notch ligand cellular stimulation, the intracellular domain of the Notch receptor (NICD) translocates into the nucleus and regulates gene transcription, which leads to cell communication in signaling^[49,50]. The regulation of *NOTCH* family gene expression may play an important role in the

transcriptional regulation of cell phenotype transitions. *NOTCH1* expression is increased in gastric cancer^[51]. It has been shown that *NOTCH1* is associated with gastric cancer with non-cardia location and diffuse type^[51]. The up-regulation of *NOTCH1* in diffuse-type GC compared to MSCs in our studies is consistent with the results in previous reports. *NOTCH2* has been reported to be a candidate marker for prognosis of neoadjuvant-treated gastric cancer^[52]. Combination of expression of *GSK3B*, *CTNNB1*, *NOTCH2* was suggested to be correlated with patient survival. The previous study indicated that *NOTCH2* may be involved in chemotherapy resistance in GC^[52]. Considering that CSCs may be involved in drug resistance, the role of *NOTCH2* should be investigated in CSCs or EMT. *NOTCH2* expression was increased in post-therapeutic tumors^[53]. Since *NOTCH2* was down-regulated in diffuse-type GC compared to MSCs, *NOTCH2* may mark some cancer phenotype in different stages. *NOTCH3* was found to be increased in diffuse-type GC^[51,53]. The previous reports are consistent with the results in this study indicating that *NOTCH3* is up-regulated in diffuse-type GC compared to MSCs. Interestingly, *NOTCH4* is suggested to be related to gastric carcinogenesis targeted by microRNA *miR-181*^[54]. Our data indicating *NOTCH4* up-regulation in diffuse-type GC compared to MSCs is consistent with the previous findings suggesting involvement of *NOTCH4* in gastric carcinogenesis. *NOTCH1* and *NOTCH2* exhibit opposite functions and play important roles in cancer as oncogenes or cancer suppressors depending on the cancer species. The relationship with *NOTCH* signaling and gastric cancer signaling should be investigated in the future (Figure 6A). In the EMT hypothesis, it is possible that epithelial-phenotype cells expressing *NOTCH1* transition to mesenchymal-phenotype cells that express *NOTCH2* (Figure 6B). Integrin beta 1 expression is important for the cancer cell population, and the insights into the mechanisms of cell phenotype conversion by gene regulation may provide fundamental understanding of cancer development^[55]. Several pathways, including the claudin family genes and MAP kinase signaling pathways, have been investigated by gene set enrichment analysis, which may be the next step for the precise investigation of cancer drug response signaling^[56].

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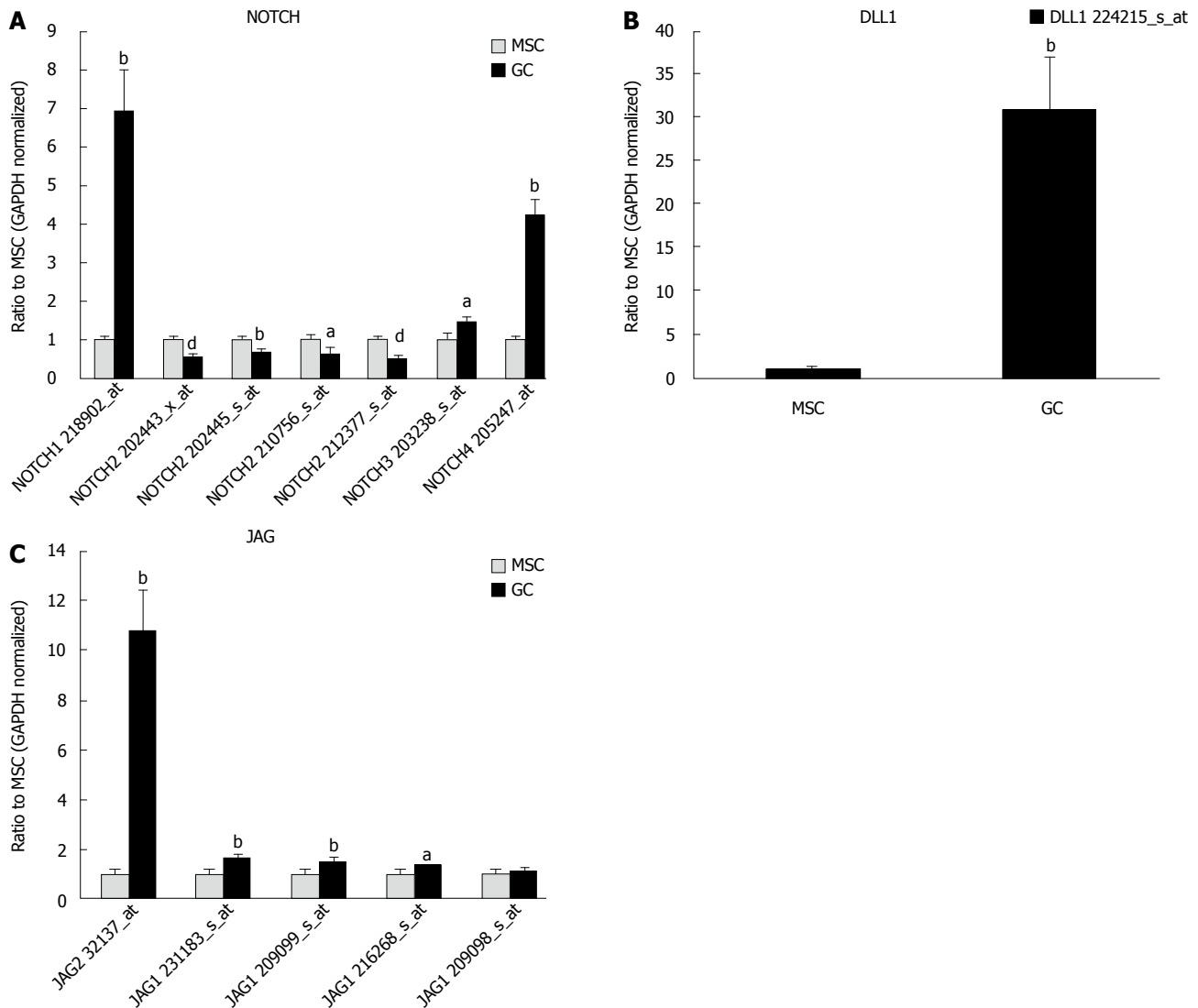


Figure 5 Gene expression of *NOTCH*, delta-like 1 (*Drosophila*) (*DLL1*) and *JAG*. A: The gene expression of *NOTCH1*, 3 and 4 was up-regulated in diffuse-type gastric cancer (GC) compared with mesenchymal stem cell (MSCs), whereas *NOTCH2* was down-regulated. Results represent the mean $\pm$ SE ($n = 12$ in MSC cultures and $n = 5$ in diffuse-type GC). ^a $P < 0.05$, ^b $P < 0.01$, and ^d $P < 0.001$ using Student's *t* test; B: *DLL1*, a *NOTCH* ligand, was up-regulated in diffuse-type GC compared with MSCs. Results represent the mean $\pm$ SE ($n = 12$ in MSC cultures and $n = 5$ in diffuse-type GC). ^b $P < 0.01$ using Student's *t* test; C: Jagged 2 (*JAG2*), a *NOTCH* ligand, was up-regulated in diffuse-type GC compared with MSCs. Jagged 1 (*JAG1*) was up-regulated in diffuse-type GC compared with MSCs. Results represent the mean $\pm$ SE ($n = 12$ in MSC cultures and $n = 5$ in diffuse-type GC). ^a $P < 0.05$ and ^b $P < 0.01$ using Student's *t* test.

COMMENTS

Background

Mesenchymal stem cells (MSCs) exhibit mesenchymal features and are useful in regenerative medicine. Gastric cancer (GC) can be classified into two types, including diffuse-type GC that has mesenchymal features and intestinal-type GC that has epithelial features. The epithelial-mesenchymal transition (EMT) is an important mechanism in cancer development. The EMT is known to be involved in cancer cell migration and malignancy. Cancer stem cells (CSCs) are known to be resistant to anti-cancer drugs and involved in cancer recurrence.

Research frontiers

Gene regulation in MSCs and diffuse-type GC may provide new insights into CSCs. In this study, regulator of G-protein signaling (*RGS*) has been identified to be up-regulated in diffuse-type GC compared with MSCs. It is a novel hypothesis where *RGS* signaling may be involved in the EMT pathway.

Innovations and breakthroughs

Recent progress in genome-wide analysis in cancer has revealed that the gene signature is important for cancer diagnosis and exploring the targets of cancer therapy. CSCs are one of the targets for cancer treatment because these cells

tend to be resistant to anti-cancer drugs and are involved in cancer recurrence. This study is the first to demonstrate that *RGS1* may distinguish diffuse-type GC and MSCs.

Applications

By understanding how *RGS1* is involved in the EMT and CSC signaling, this study represents potential future strategies for GC including diffuse-type GC treatment and prediction.

Terminology

RGS1 is protein involved in G-protein signaling and the regulation of GDP dissociation from the G alpha subunit and GTP binding to form trimetric G proteins. The EMT is the mechanism by which epithelial cells change into mesenchymal cells with a migratory capacity. Several adhesion molecules are involved in the EMT, and *RGS1* may contribute to the changes in signaling.

Peer review

The authors described a global gene expression analysis in comparison between MSCs and diffuse-type GC cells. The authors found that *RGS1* gene was upregulated in diffuse-type GC cells compared with MSCs, and that genes related to stem cells and *NOTCH* signaling were altered in diffuse-type GC cells compared with MSCs. The results may provide some insights in correlation

- between the EMT and CSCs. This paper summarizes important and actual data, it fulfils all criteria and demands put on articles published in World Journal of Stem Cells.
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Clinical Trials Study

Fetal *vs* adult mesenchymal stem cells achieve greater gene expression, but less osteoinduction

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mesenchymal stem cells (MSCs) and effects on stemness *in vitro* and function as a cell therapy *in vivo*.

METHODS: Bone marrow-derived adult and fetal MSC were isolated from an equine source and expanded in monolayer tissue culture. Polyethylenimine (PEI)-mediated transfection of pcDNA3-eGFP or adenoviral transduction of green fluorescent protein (GFP) was evaluated in fetal MSCs. Adenoviral-mediated transduction was chosen for subsequent experiments. All experiments were carried out at least in triplicate unless otherwise noted. Outcome assessment was obtained by flow cytometry or immunohistochemistry and included transduction efficiency, cell viability, stemness (*i.e.*, cell proliferation, osteogenic and chondrogenic cell differentiation), and quantification of GFP expression. Fetal and adult MSCs were then transduced with an adenoviral vector containing the gene for the bone morphogenic protein 2 (BMP2). *In vitro* BMP2 expression was assessed by enzyme linked immunosorbent assay. In addition, MSC-mediated gene delivery of BMP2 was evaluated *in vivo* in an osteoinduction nude mouse quadriceps model. New bone formation was evaluated by microradiography and histology.

RESULTS: PEI provided greater transfection and viability in fetal MSCs than other commercial chemical reagents. Adenoviral transduction efficiency was superior to PEI-mediated transfection of GFP in fetal MSCs ($81.3\% \pm 1.3\%$ *vs* $35.0\% \pm 1.6\%$, $P < 0.05$) and was similar in adult MSCs ($78.1\% \pm 1.9\%$). Adenoviral transduction provided significantly greater expression of GFP in fetal than adult MSCs (7.4 ± 0.1 *vs* 4.4 ± 0.3 millions of mean fluorescence intensity units, $P < 0.01$) as well as significantly greater *in vitro* BMP2 expression (0.16 pg/cell-day *vs* 0.10 pg/cell-day, $P < 0.01$). Fraction of fetal MSC GFP positive cells decreased significantly faster than adult MSCs ($1.15\% \pm 0.05\%$ *vs* $11.4\% \pm 2.1\%$ GFP positive at 2 wk post-transduction, $P < 0.05$). Cell proliferation and osteogenic differentiation *in vitro*

Abstract

AIM: To investigate adenoviral transduction in

were not affected by Ad transduction in both fetal and adult MSCs, but fetal MSCs had reduced chondrogenic differentiation *in vitro* when compared to adult ($P < 0.01$). Chondrogenic differentiation was also significantly reduced in Ad-GFP transduced cells ($P < 0.05$). Ad-BMP2 transduced adult MSCs induced new bone formation in more thighs than Ad-BMP2 transduced fetal MSCs (83% *vs* 17% of the six treated thighs per group, $P < 0.05$) and resulted in increased femur midshaft diameter due to greater extent of periosteal new bone (1.57 ± 0.35 mm *vs* 1.27 ± 0.08 mm, $P < 0.05$).

CONCLUSION: Fetal MSCs may be genetically manipulated *ex vivo* with adenoviral vectors. Nonetheless, the abbreviated expression of the exogenous gene may limit their applications *in vivo*.

Key words: Adenovirus; Bone morphogenic protein 2; Fetal; Green fluorescent protein; Mesenchymal stem cell; Gene transfer technique

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Core tip: Fetal mesenchymal stem cells (MSCs) can be genetically manipulated *ex vivo* with adenoviral vectors without major effects on stemness. Their greater expression of exogenous genes than adult MSCs is promising for MSC-mediated gene therapy. MSC-mediated bone morphogenic protein 2 gene delivery provides osteogenic induction *in vivo*, which could have clinical applications in bone regeneration. Nonetheless, the abbreviated expression of the exogenous gene provided *via* adenoviral transduction may limit their applications *in vivo*.

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INTRODUCTION

The best-characterized source of human mesenchymal stem cells (MSC) to date is bone marrow (BM)^[1]. Transplantation of BM-derived MSCs has been studied as a potential therapy for myocardial ischemic injury^[2,3], autoimmune diseases^[4], and musculoskeletal conditions such as osteoarthritis^[5]. MSCs could be useful in the active regeneration of joint and cartilage tissue because of their multipotency and fast proliferation, as a cell therapy acting to replace lost lineages and to modify existing cells. However, in certain degenerative diseases, such as osteoarthritis, MSCs are depleted and have reduced ability to differentiate and proliferate^[6]. As an alternative source of MSCs, young cells such as fetal (f) MSCs could be harvested from the umbilical cord, placental stroma, and

the amnion^[7-9]. Ethical concerns regarding the isolation of MSCs from fetal sources are minimized, as these tissues are normally discarded after birth^[1].

Zhang *et al*^[10] demonstrated that human fMSCs are a superior cellular candidate for bone tissue engineering applications. This related to their superior proliferation capacity, more robust osteogenic potential, and lower immunogenicity, as compared to MSC from postnatal sources^[10]. Nevertheless, the permissiveness to gene delivery and the effect of genetic engineering on stemness in these young cells has not been extensively studied. Gene delivery to fMSCs may differ from adult (a) MSC due to immaturity of gene expression cellular pathways and cell surface receptors, such as the coxsackievirus adenovirus receptor required for adenoviral transduction^[11]. To our knowledge, only two original research studies have evaluated gene delivery on fMSCs. Campagnoli *et al*^[12] demonstrated that fMSCs could be retrovirally transduced with high efficiency without selection. Also, Chan *et al*^[13] showed that human fMSCs retain their proliferative and differentiation capacities after stable lentiviral transduction. The paucity of studies addressing gene delivery in fMSCs, and the lack of studies on transient gene delivery in these young cells, highlights the need for more studies in these topics.

We chose to study the transient transduction characteristics of adenoviral vectors (Ad) in fMSC. Adenoviral vectors' lack of insertional mutagenesis result in a desirable safety profile, which added to their capacity to achieve high levels of transgene expression^[14], make them attractive for gene delivery particularly for bone regeneration. Our study addressed the effectiveness of Ad-mediated transduction of equine fetal bone marrow-derived MSCs. We hypothesized that Ad-mediated transduction will be efficient in fMSCs without affecting measures of cell stemness. In addition, Ad-transduced equine MSC-mediated bone morphogenic protein 2 (BMP2) gene delivery was studied to assess its ability to induce bone formation in a mouse model.

MATERIALS AND METHODS

Ethics

The use of animals in this study was carried out in accordance to regulations by The Ohio State University Institutional Animal Care and Use Committee (IACUC) in compliance with national guidelines. The IACUC reviewed and approved our Protocol 2009A0119.

Study design

Both bone marrow-derived aMSCs and fMSCs were isolated and expanded in monolayer tissue culture. Low passage MSCs were immunophenotyped by CD90 and CD34 surface expression by flow cytometry. Polyethylenimine (PEI)-mediated transfection of pcDNA3-egreen fluorescent protein (GFP) or Ad transduction of GFP were evaluated in fMSCs. Ad-mediated transduction was chosen for subsequent experiments. Outcome assessment included transduction efficiency, cell viability, stemness (*i.e.*,

cell proliferation, osteogenic and chondrogenic cell differentiation), and quantification and duration of *GFP* and *BMP2* gene expression. All experiments were carried out at least in triplicate unless otherwise noted. *In vitro* *BMP2* gene expression was assessed by ELISA. MSC-mediated *BMP2* gene delivery was then evaluated *in vivo* in an osteoinduction nude mouse quadriceps model.

Isolation and culture of MSCs from equine bone marrow

Adult MSCs were obtained from equine bone marrow of the sternum of adult horses (age range 2-5 years old) using methods described by Ishihara *et al.*^[15]. Fetal MSCs were isolated following a similar protocol from a 20-wk-old equine fetus. The mare was euthanized for terminal problems, while the fetus was apparently healthy. Isolated cells were then resuspended in 1 mL of 90% fetal bovine serum with 10% dimethyl sulfoxide for cryopreservation. Prior to each experiment, preserved cells were allowed to thaw at 37 °C, rinsed in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, United States), and were grown separately in T-75 flasks in DMEM supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, United States), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Cells were incubated at 37 °C and 5% CO₂. Medium was changed every 2-3 d. Cells were trypsinized when confluency was above 80% with Trypsin-EDTA (Gibco) after a washing step with Dulbecco's phosphate buffered saline (PBS, Gibco). MSCs were evaluated by microscopy for homogenous elongated spindle-shaped morphology. Cells were also immunophenotyped by flow cytometry for expression of CD90 after incubation with phycoerythrin (PE)-conjugated anti-hCD90 antibody (561970, BD Biosciences) detected with a 585/40 nm optical filter. Lack of expression of CD34 was assessed with allophycocyanin (APC)-conjugated anti-hCD34 antibody (555824, BD Biosciences) detected with a 675/25 nm optical filter.

Amplification and purification of plasmid DNA

Escherichia coli DH5α were transformed with a plasmid consisting of a pcDNA3 backbone containing a gene for the enhanced GFP. The transformed cells were amplified in lysogeny broth medium at 37 °C overnight at 225 rpm in a shaker incubator. Isolation and purification of the plasmid was carried out with a Midiprep kit (K2100, Life Technologies) according to the given protocol. The concentration and purity of the plasmid were determined by the 260/280 ultraviolet absorbance method with a Nanodrop 2000 (Thermo Scientific). The purified plasmid was stored at -20 °C.

PEI/DNA complex preparation

A 1 mg/mL transfection reagent solution was made from 50 mg of linear PEI (23966, Polysciences) in 50 mL of PBS adjusting the pH to 4.5 with HCl and dissolving at 70 °C, followed by filter sterilization and storage at 4 °C. On the day of transfection a stock solution was made with 100 µL of warm PBS, 5 µL of 1 mg/mL PEI, and 2

µL of the pcDNA3-eGFP plasmid, which is sufficient for transfection in one well in a 6-well plate. The transfection solution was upscaled as needed.

Adenoviral production and titering

Replication-deficient, E1-A-deleted adenoviral vector encoding either a 1547 base-pair open reading frame segment of human BMP2 (Ad-BMP2) or GFP (Ad-GFP) under the control of the cytomegalovirus promoter were previously generated^[16]. These were amplified in 80% confluent HEK 293A cells (R70507, Invitrogen) and titered by plaque assay following the methods described in the provided user guide by Life Technologies. Aliquots were stored at -80 °C.

Fluorescent microscopy and flow cytometry

A fluorescent Olympus 1X51 microscope was used to evaluate the PEI-mediated eGFP transfection and Ad-GFP transduction efficiency of MSCs in monolayers at various time points. Fluorescent and phase contrast images were merged using ImageJ.

Green fluorescence after gene delivery into aMSCs and fMSCs was assessed in an Accuri C6 BD Biosciences flow cytometer, excited by an argon 488 nm laser and detected by use of a 533/30 nm optical filter. The mean fluorescence intensity (MFI) data were collected from these cell populations. All data was analyzed with a minimum of 15000 events setting the respective negative control to less than 1% in BD CFlow software.

Transient gene delivery optimization

MSCs were seeded at a density of 6×10^4 cells/well in a 12-well plate to 75% confluency with 2 mL of DMEM per well. PEI, FuGENE 6 (Promega), and Lipofectamine (Invitrogen) were tested for *GFP* gene transfection in fMSCs. Transfection conditions were optimized for the three reagents. Transfection efficiency and viability were qualitatively assessed at 48 h post-transfection. In addition, transduction with Ad-GFP was tested at MOI of 0.5, 3, 15, 25, and 50 in fMSCs.

Transgene expression, proliferation, and viability of MSCs post-transduction

Adult MSCs or fMSCs were seeded at 1×10^5 cells in triplicate in T-25 flasks and transduced 24 h later or kept as control cells. Ad-GFP transduced cells (Ad-GFP-MSC) and control cells were evaluated by flow cytometry at 48 h, 1 wk, and 2 wk post-Ad-GFP transduction at MOI of 25. Outcome assessment included transduction efficiency, MFI, cell confluence to calculate cell proliferation, and cell viability by staining with 7-AAD (559925, BD Biosciences) detected with a 670 nm long pass filter. Cell morphology and GFP expression were documented with fluorescent microscopy.

Differentiation post-transduction

Chondrogenic and osteogenic differentiation was carried out following Lonza's protocol (AA-2501-16). The

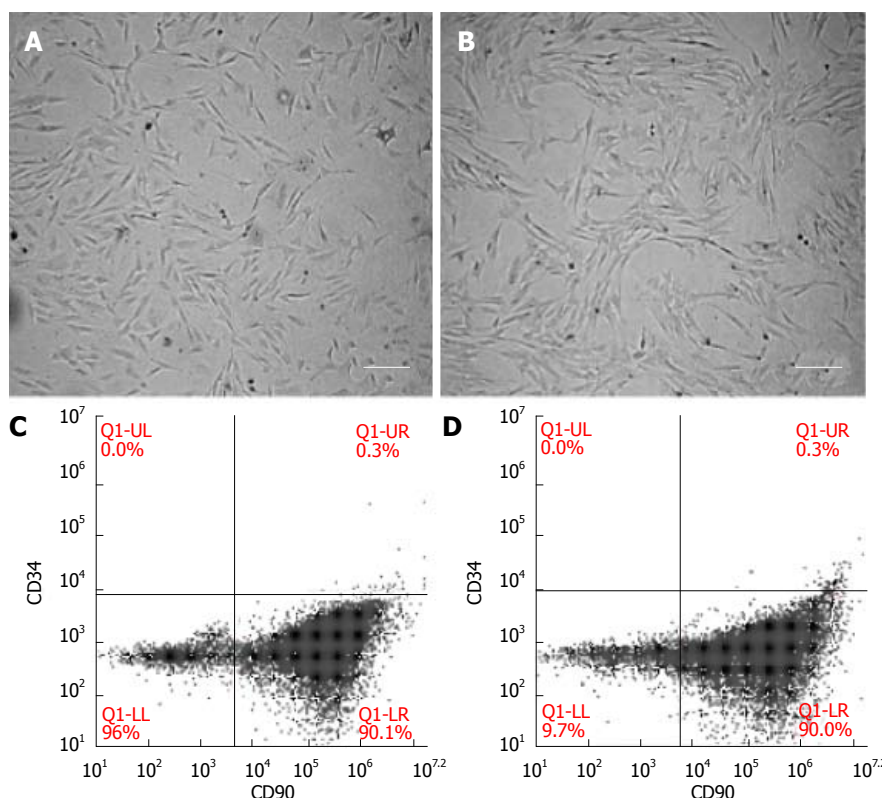


Figure 1 Morphology and immunophenotyping of fetal mesenchymal stem cells and adult mesenchymal stem cells. (A) fMSCs and (B) aMSCs were evaluated by microscopy for homogenous elongated spindle-shaped morphology. Scale bar = 100 µm; C and D: Corresponding positive expression of CD90 and absent CD34 as measured by flow cytometry. fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

cultures were stained with Von Kossa stain and toluidine blue for evaluation of osteogenesis and chondrogenesis, respectively, of Ad-GFP-MSCs compared to control MSCs. Images were evaluated in ImageJ software.

In vitro BMP2 expression and osteoinduction in vivo

MSCs at 80% confluency were transduced at MOI of 25 with Ad-BMP2 or used as control. On the following day, ascorbic acid was added to both, transduced and control cells, in the DMEM to a final concentration of 50 µg/mL. At day 3 after transduction, samples from the media were evaluated for BMP2 expression using methods described for the BMP2 Quantikine ELISA Kit (BD Biosciences, DBP200). BMP2 expression per cell was estimated through quantification of BMP2 standard provided with the kit and using media from untransduced cells as background.

Ad-BMP2 MSCs and control cells were trypsinized at day 3 post-transduction and trypan blue exclusion count was performed to confirm viability of at least 90%. The cells were washed twice with PBS and resuspended in 0.9% NaCl to a concentration of 2×10^6 cells/0.1 mL. Thighs from twelve 6-wk-old mice (BALB/c *Foxn1 nu/nu*, Taconic Farms, Hudson, NY, United States) were randomly assigned to five groups: aMSC, Ad-BMP2-aMSC, fMSC, Ad-BMP2-fMSC, or saline. Anesthesia was induced with 5% isoflurane (IsoFlo, Abbott Animal Health, North Chicago, IL, United States) in 100% oxygen and maintained with 0%-2% isoflurane delivered by a precision vaporizer. Cranial thigh injection sites were disinfected with three alternating scrubs of povidone iodine and 70% isopropyl alcohol. Mice received one intramuscular injection in each thigh of 0.1 mL cells

or saline using a 25 G needle. All mice recovered from anesthesia uneventfully. Three weeks after injection, mice were euthanized by CO₂ followed by cervical dislocation. The hind limbs were harvested, and the skin was removed.

Diagnostic imaging

Digital microradiography (Model LX-60, Faxitron Bioptics, Tucson, AZ, United States) was performed separately for each experimental group. Lateromedial views of the hind limbs were obtained at 30 kV for 10 s and analyzed with ImageJ at 40% contrast. Outcome measurements included: number of mineralized bodies (*i.e.*, ossicles), lateromedial area (mm²) of the ossicles, extent of periosteal new bone formation, and midshaft diameter (mm). Extent of periosteal new bone was scored as 0, 1, 2, 3, or 4 corresponding to no formation, new bone in less than 50% of the cranial aspect of the femur, more than 50% cranial, less than 50% involving both the cranial and the caudal aspects of the femur, or more than 50% cranial and caudal aspects involved, respectively.

Histological examination

Harvested limbs were fixed in 10% neutral buffered formalin for 4 mo. Samples were decalcified in 14% EDTA neutralized solution, overnight. The samples then underwent procedures for embedding, incising, and staining with toluidine blue or hematoxylin and eosin.

Statistical analysis

Data were reported as the mean $\pm$ SD. Group means were compared with two-tailed sample *t* test or One-way ANOVA. Relationships between categorical variables

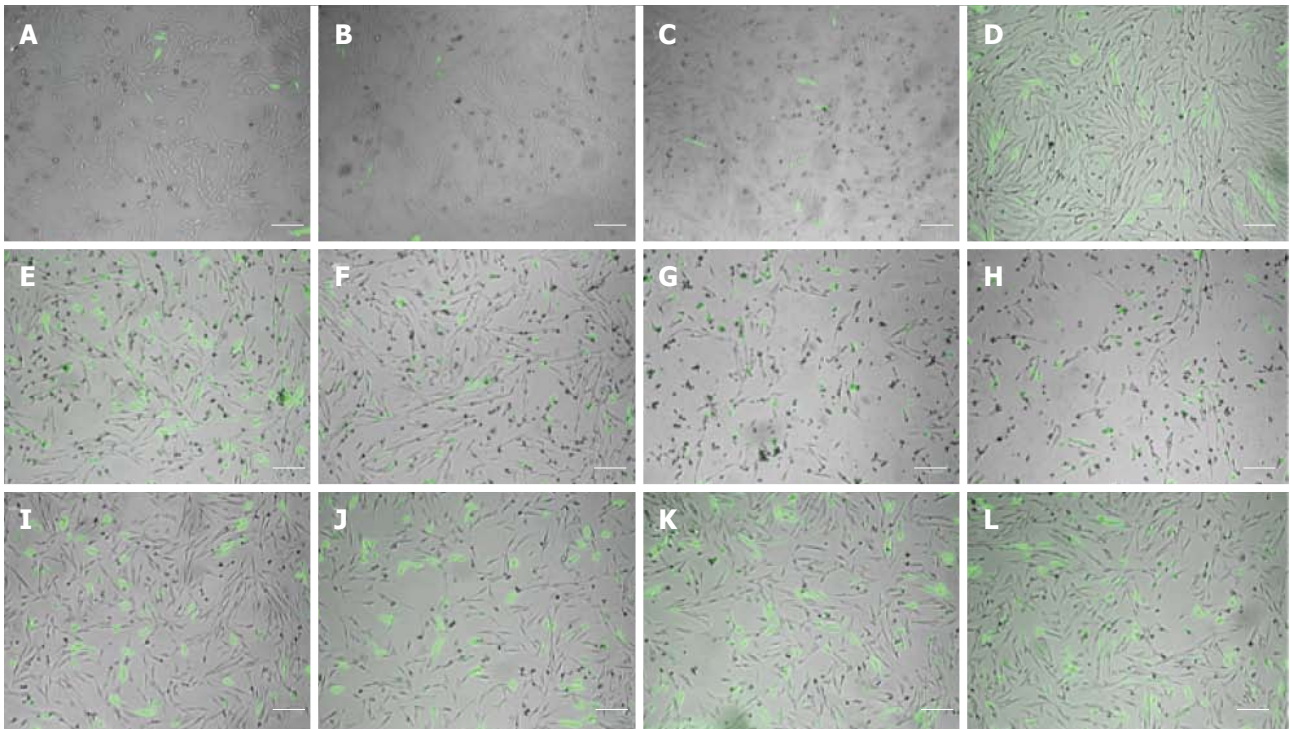


Figure 2 Chemical transfection of fetal mesenchymal stem cells. Representative merged photomicrographs for (A-D) FuGENE 6, (E-H) Lipofectamine and (I-L) PEI mediated transfection of pcDNA3-eGFP into fMSCs at 3:1, 3:2, 6:1, and 5:2 reagent (μL): DNA (μg) ratio, respectively. In combination, qualitative transfection efficiency and viability reveal that PEI was superior at all the tested conditions. fMSCs: Fetal mesenchymal stem cells; PEI: Polyethylenimine.

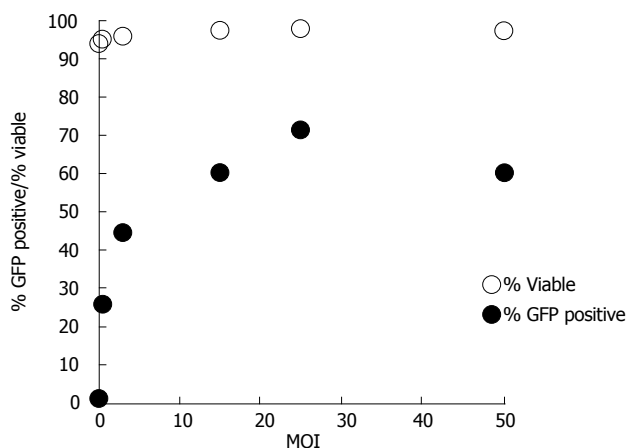


Figure 3 Optimization of E1-A-deleted-green fluorescent protein transduction and viability of fetal mesenchymal stem cells post-transduction. Transduction efficiency and viability as a function of MOI at 48 h post-transduction with Ad-GFP. Ad-GFP: E1-A-deleted-green fluorescent protein.

were tested by the chi-square method. Statistical analysis was performed using StatPlus. A value of $P \leq 0.05$ was deemed significant.

RESULTS

Morphology and phenotypic expression of MSCs in vitro

Cultured fMSCs and aMSCs displayed an elongated spindle-shaped population and maintained their morphology during subsequent passages (Figure 1A and

B). MSCs were more than 90% positive for CD90 and negative for the hematopoietic stem cell marker CD34 (Figure 1C and D).

Screening for a transient gene delivery reagent for fMSCs

A qualitative analysis of fluorescent photomicrographs 48 h after pcDNA3-eGFP transfection revealed that FuGENE 6 resulted in the lowest transfection efficiency (Figure 2A-D) and Lipofectamine resulted in the lowest viability (Figure 2E-H), while PEI provided superior transfection efficiency (Figure 2I-L) in fMSCs than FuGENE 6 or Lipofectamine transfection reagents at 3:1, 3:2, 6:1, and 5:2 μL of reagent to μg of DNA ratio.

Optimized PEI-mediated transfection of pcDNA3-eGFP (5:2 μL of reagent/μg of DNA ratio) resulted in a maximum efficiency of $35.0\% \pm 1.6\%$ as assessed by flow cytometry. No significant decrease in viability was observed at either of the tested MOIs when compared to control untreated cells (Figure 3). Ad transduction at MOI of 25 resulted in transduction efficiency above 70% with similar viability to control cells (Figure 3). Thus, Ad transduction at an MOI of 25 was selected for succeeding experiments.

GFP expression and cell proliferation of MSCs post-transduction

Ad-GFP transduction at MOI of 25 resulted in transfection efficiency of $81.3\% \pm 1.3\%$ in fMSC, which was not statistically different from the efficiency of $78.1\% \pm 1.9\%$

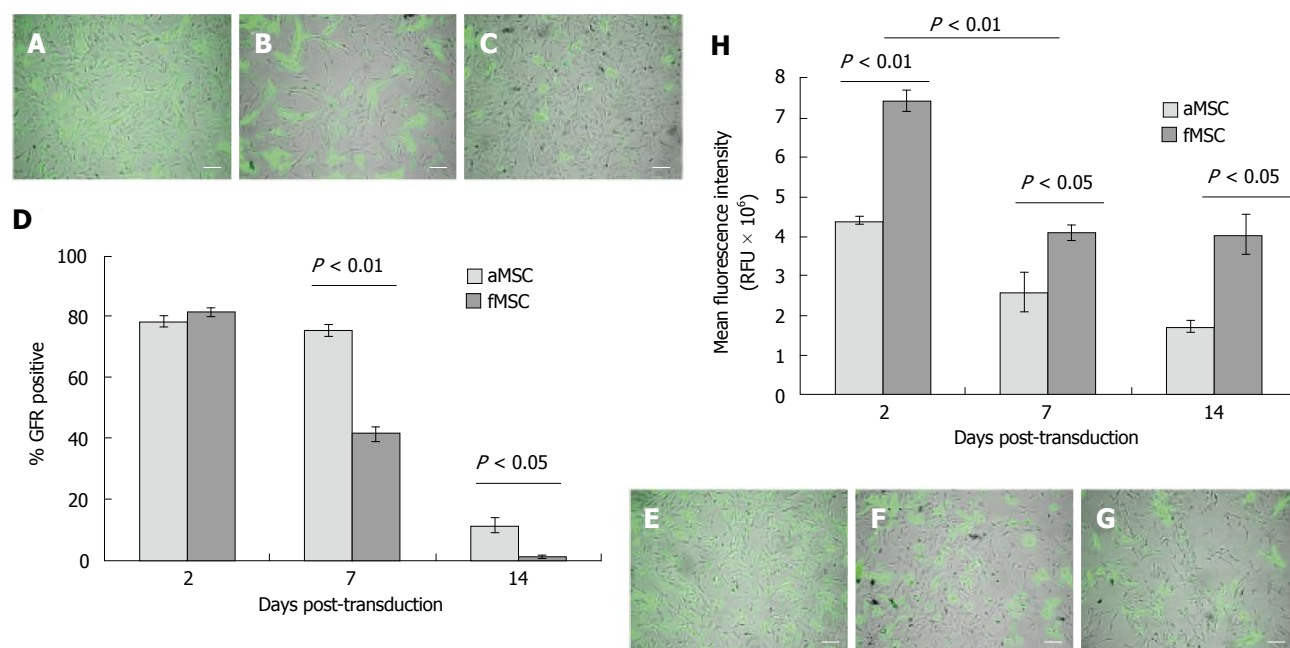


Figure 4 Percent green fluorescent protein positive MSCs and mean fluorescence intensity following E1-A-deleted-green fluorescent protein transduction. Representative merged photomicrographs for (A-C) fMSCs and (E-G) aMSCs following Ad-GFP transduction at 2, 4, and 10 d post-transduction, respectively. Scale bar = 100 μ m; (D) GFP positive cells over the period of 2 wk post-transduction and (H) mean $\pm$ SD fluorescence intensity. Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

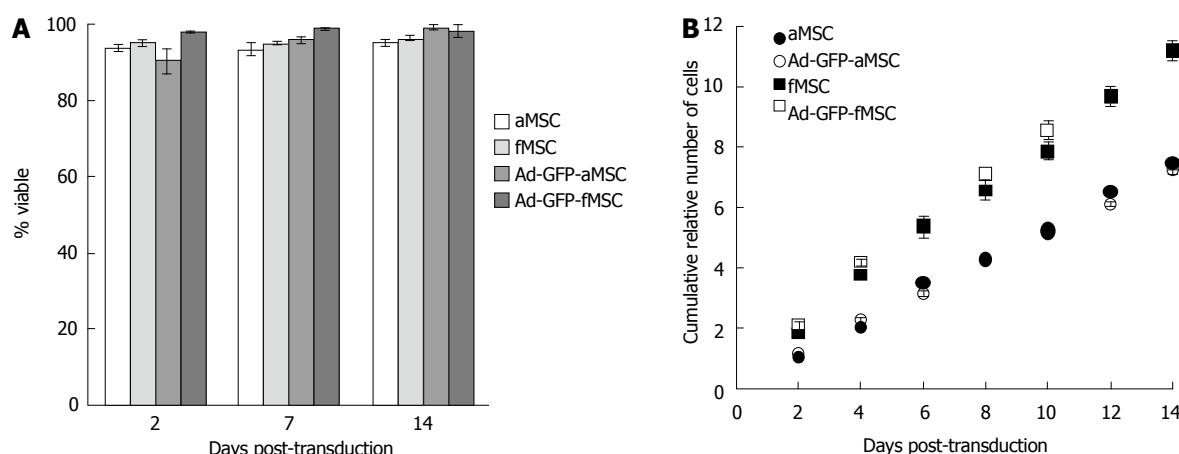


Figure 5 Mesenchymal stem cells viability and proliferation post-transduction. A: Mean $\pm$ SD percent of viable cells as determined by 7-AAD positivity in flow cytometry at the indicated days post-transduction with Ad-GFP; B: Cumulative relative number of cells of Ad-GFP-MSCs and control MSCs. An estimated cell proliferation rate of 45 h and 29 h for aMSC or fMSCs, respectively, was determined from the slope of the curves; not significantly different between treated and control cells. Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

in aMSCs. There was a greater decrease of GFP positive cells in fMSC than aMSC within 2 wk post-transduction (Figure 4A-G). Mean fluorescence intensity (MFI) was $7.43 \pm 0.08 \times 10^6$ and $4.40 \pm 0.25 \times 10^6$ channel numbers in fMSCs and aMSCs, respectively, at 48 h post transduction (Figure 4H). Ad-GFP-fMSCs retained superior gene expression intensity compared to Ad-GFP-aMSCs at one and two weeks post transduction.

Cell viability of both fMSCs and aMSCs in culture was not significantly affected by exogenous administration of GFP gene *via* Ad-transduction at MOI of 25 when compared to control untransduced cells (Figure 5A).

Number of cells was similar in both Ad-GFP-MSCs and control MSCs, with fMSCs showing a greater number of cells than aMSCs (Figure 5B), which in combination with results on viability reflect greater proliferation capacity of about 1.5 times faster proliferation than aMSCs (doubling time of 29 h *vs* 45 h). The latter, in combination with data on % GFP positive expression, suggests that Ad genome is gradually diluted out as cell division occurs.

Differentiation capacity post-transduction

Chondrogenic differentiation *in vitro* was significantly less for fMSCs than aMSCs and decreased post Ad-

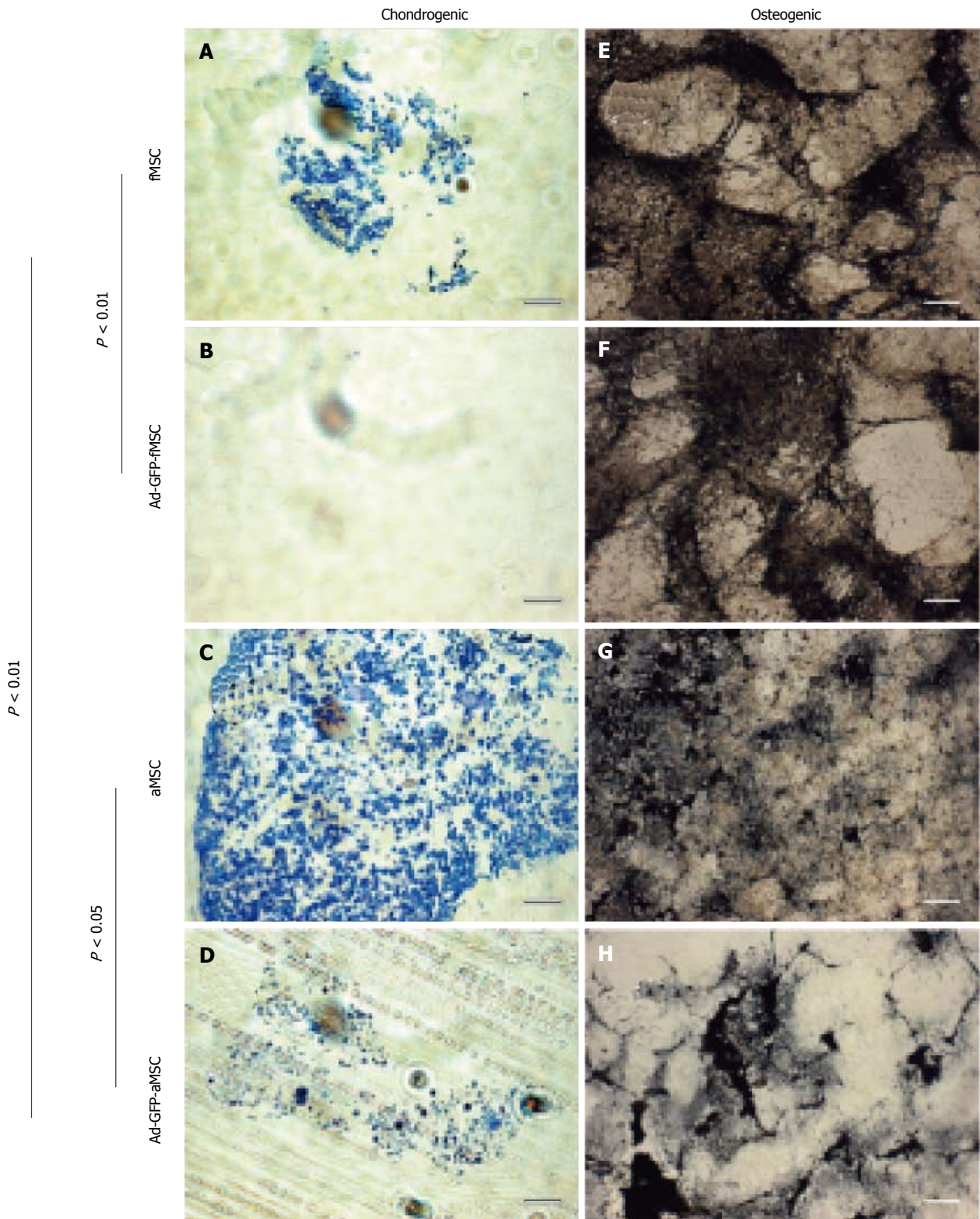


Figure 6 Chondrogenic and osteogenic differentiation of control mesenchymal stem cells and E1-A-deleted-green fluorescent protein-mesenchymal stem cells. A-D: Chondrogenic cell pellets, differentiation induced with chondrogenic medium and TGF β 3, scale bar = 250 μ m; E-H: Osteogenic differentiation induced in monolayer culture of MSCs, scale bar = 100 μ m. Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

GFP transduction as assessed by intensity and amount of toluidine blue staining in cell pellets (Figure 6A-D). Osteogenic differentiation was robust in transduced fMSC and aMSC, with no statistically significant difference

between groups (Figure 6E-H).

In vitro BMP2 expression by transduced MSCs

Supernatants collected 3 d post-transduction from

Table 1 Bone morphogenic protein 2 expression from adenoviral-transduced mesenchymal stem cells

Medium	O.D. (Mean $\pm$ SD)	Corrected O.D.	BMP2 (pg/cell-day)	Total BMP2 injected (ng/day)
fMSC	0.043 $\pm$ 0.004	-	-	-
aMSC	0.031 $\pm$ 0.002	-	-	-
Ad-BMP2-fMSC	2.99 $\pm$ 0.33	2.95	0.02	32.8 ^b
Ad-BMP2-aMSC	1.69 $\pm$ 0.27	1.66	0.01	18.5

^b*P* < 0.01 Ad-BMP2-fMSC *vs* Ad-BMP2-aMSC. BMP2: Bone morphogenic protein 2; MSCs: Mesenchymal stem cells; Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

Table 2 Summary data of the analysis of the microradiographs

Treatment group	Total thighs (n)	Thighs with new bone [n (% of total)]	Mean ossicle area (mm ²)	Mean extent of periosteal new bone	Mean midshaft diameter (mm $\pm$ SD)
Saline	4	0	0	0	1.24 $\pm$ 0.07
Ad-BMP2-aMSC	6	5 (83) ^a	0.78	1	1.57 $\pm$ 0.35 ^c
aMSC	4	0	0	0	1.26 $\pm$ 0.09
Ad-BMP2-fMSC	6	1 (17)	0.45	0	1.27 $\pm$ 0.08
fMSC	4	0	0	0	1.23 $\pm$ 0.07

^a*P* < 0.05 Ad-BMP2-aMSC *vs* Ad-BMP2-fMSC; ^c*P* < 0.05 intergroup comparison. BMP2: Bone morphogenic protein 2; MSCs: Mesenchymal stem cells; Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

Table 3 Association between ossicle formation and E1-A-deleted-bone morphogenic protein 2-mesenchymal stem cell treatment

Treatment group	No. of thighs (n)	Ossicles (n)	Mean ossicle area (mm ² $\pm$ SD; range)
Ad-BMP2-aMSC			
No ossicle	4		
Ossicle	2	6	0.78 $\pm$ 1.23 (0.03-3.20)
Ad-BMP2-fMSC			
No ossicle	5		
Ossicle	1	3	0.45 $\pm$ 0.12 (0.32-0.47)

BMP2: Bone morphogenic protein 2; MSCs: Mesenchymal stem cells; Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

monolayer cultures used for osteoinduction were evaluated for BMP2 expression. It was determined that Ad-BMP2-fMSCs transduced at MOI of 25 released significantly more soluble BMP2 than Ad-BMP2-aMSCs (0.16 pg/day-cell *vs* 0.10 pg/day-cell; Table 1). Also, BMP2 expression per injection of 2×10^6 transduced MSC was estimated at 3.28 and 1.85×10^4 pg/d with fMSCs and aMSCs, respectively (Table 1), consistent with the greater intensity of GFP expression in Ad-GFP-fMSCs than aMSCs.

***In vivo* MSC-mediated BMP2 delivery and osteoinduction**

Digital microradiography of the harvested thigh specimens revealed intramuscular bone ossicle formation, new periosteal bone, and femur cortical bone thickening in treated mice with Ad-BMP2-MSCs, but not in saline-injection control group (Figure 7A and B). Five of six thighs treated with Ad-BMP2-aMSC resulted in new bone formation, which was significantly greater than saline, untransduced, and Ad-BMP2-fMSC groups (Table

2). The Ad-BMP2-aMSC group also had significantly greater femur midshaft diameter due to cortical thickening and new periosteal bone when compared to saline, untransduced, and Ad-BMP2-fMSC groups (Table 2). Bone ossicles were identified in two of the six thighs treated with Ad-BMP2-aMSCs (Table 3 and Figure 7C) and of the other four thighs in the Ad-BMP2-aMSC group, 3 showed femur cortical thickening and new periosteal bone (Table 4 and Figure 7D), while no changes were found in the untransduced aMSC group (Figure 7E-F). One of the six thighs treated with Ad-BMP2-fMSCs showed ossicle formation (Figure 7G-H), none resulted in periosteal changes, and no changes were noted in the untransduced fMSC group (Figure 7I-J).

Histological examination confirmed new trabecular bone formation, normal in morphology, in the thighs of the Ad-BMP2-aMSC group (Figure 8A). This new trabecular bone contained highly cellular bone marrow (Figure 8B). A nodule of what appears to be our MSCs was identified in the untransduced aMSC group with no bone or cartilage formation (Figure 8C) as evaluated by H&E and toluidine blue stains, respectively (Figure 8D-E).

DISCUSSION

Our study confirmed that adenoviral vector-mediated transduction was efficient in fMSCs without affecting measures of MSC stemness. Chemical-mediated transfection was less efficient than Ad transduction, but PEI was superior and more effective in fMSCs than other commercial products. Fetal MSCs were not superior to aMSCs in osteoinduction *in vivo*. Both fMSCs and aMSCs were able to express BMP2 *in vitro* and to deliver BMP2 *in vivo*, which could have clinical applications in bone regeneration.

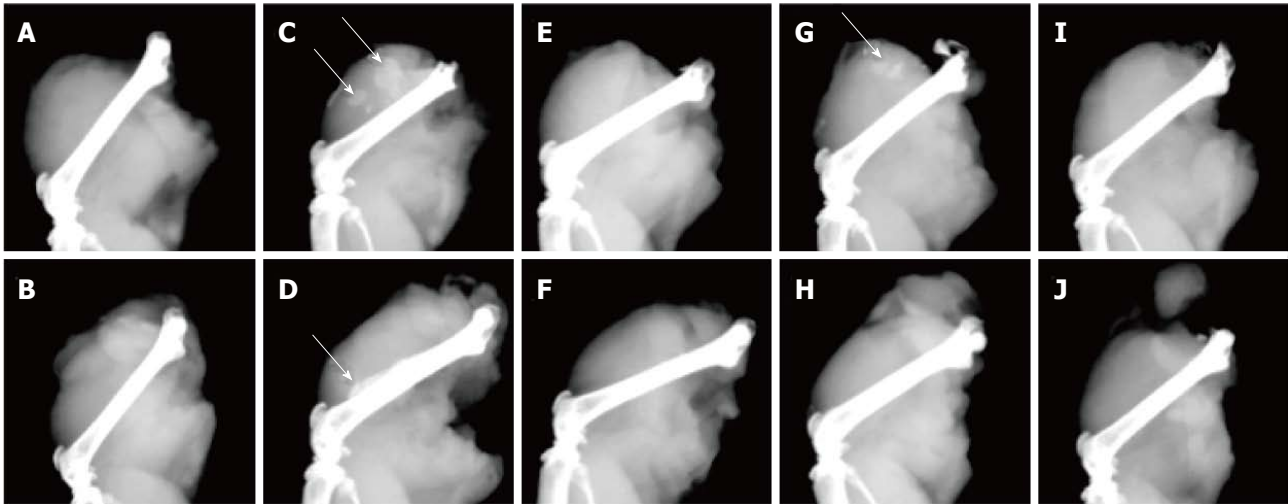


Figure 7 Osteoinduction *in vivo* by mesenchymal stem cell-mediated bone morphogenic protein 2 delivery. Twelve 6-wk-old SCID mice received a 100 μ L injection of 2×10^6 MSCs or saline into the quadriceps muscles. Muscle samples harvested from the mice were examined by microradiography, 21 d after treatment. Treatment groups consisted of (A-B) saline control; C-D: Ad-BMP2-aMSC injection; E-F: plain aMSCs; G-H: Ad-BMP2-fMSCs; I-J: injected with plain fMSCs. The white arrows point at ossicles. The green arrow points at cortical thickening and new periosteal bone. BMP2: Bone morphogenic protein 2; Ad-GFP: E1-A-deleted-green fluorescent protein; fMSCs: Fetal mesenchymal stem cells; aMSCs: Adult mesenchymal stem cells.

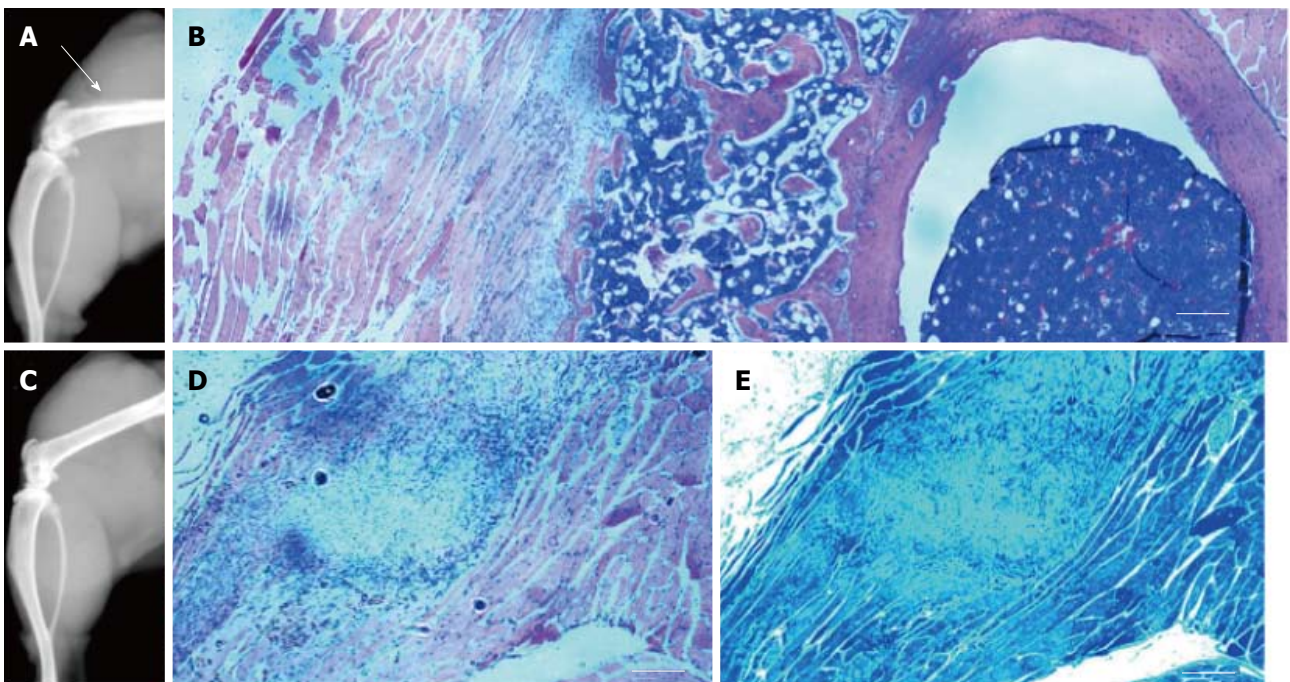


Figure 8 Histological examination of thighs after osteoinduction. A: Arrow points at new periosteal bone and cortical thickening of Ad-BMP2-aMSC treated mouse; B: Corresponding H and E photomicrograph showing normal bone formation with new bone marrow adjacent to the periosteum; C: Radiograph of control aMSC injected mouse and corresponding; D: H and E slide; E: toluidine blue-stained slide showing a nodule of MSCs and no bone or cartilage formation. Scale bar = 100 μ m. BMP2: Bone morphogenic protein 2; aMSCs: Adult mesenchymal stem cells.

In the evaluation of chemical-mediated transfection, we found that PEI provided a significant level of transfection efficiency in fMSCs. Our results are in accordance with a study by Wang *et al.*^[17], which showed that genetic modification of human MSCs by PEI with a relatively low efficiency of 4% achieved biologically significant effects *in vivo*, with transfection efficiency proportional to cells in S phase^[17]. Our analysis further supported the use of PEI as a transfection reagent in

MSCs over other reagents. While we achieved around 35% transfection efficiency of pcDNA3-eGFP ratio, we found PEI to be relatively nontoxic in contrast to Lipofectamine and FuGENE 6. In conclusion, PEI offers an alternative to viral vector transduction and advantages over other chemical transfection reagents, especially when funding to support the use of viral vectors is not available.

Furthermore, the current study showed that Ad transduction provided superior efficiency to chemical

Table 4 Extent of periosteal new bone in E1-A-deleted-bone morphogenic protein 2-adult mesenchymal stem cell treatment group

Extent of periosteal new bone	Thighs (n)	Midshaft diameter (mm)
0	3	1.27
1	2	1.76
4	1	2.10

transfection, including PEI. It has been shown that lentiviral and retroviral-mediated transduction are effective in fetal MSCs^[12,13], however these are non transient and oncogenic such that their confirmed risk may be even greater when used in a stem cell. Our study was the first to test transient adenoviral mediated transduction in fetal MSCs. The present study confirmed that Ad transduction of fMSCs did not significantly affect measures of stemness, consistent with the previous studies addressing lentiviral and retroviral-mediated transduction of fMSCs. Viability was maintained above 90% for both fetal and adult MSCs, consistent with Ishihara *et al.*^[18] who reported equine aMSCs to not be sensitive to cytotoxic effects associated with Ad transduction at MOI of 100 or less. Our study also confirmed that fMSCs maintained a higher proliferative capacity than aMSCs post-transduction. This is again consistent with previous studies on viral vector-mediated stable transduction^[12,13]. Also, our observed greater proliferative capacity of fMSCs than their adult counterpart is consistent with studies in human fMSCs^[19]. These advantages of fMSCs would suggest advantages *in vivo*.

Ad-mediated transduction provided a similar efficiency in both fMSCs and aMSCs, but significantly greater intensity with greater decrease of the fraction of GFP positive cells in fMSCs compared to aMSCs. The greater proliferation of fMSC is sufficient to explain the greater reduction in GFP positive cells resulting in a gradual dilution of the adenoviral genome as cell division occurs^[20-22]. This is due to the fact that Ad transduction does not result in DNA integration into the host genome; therefore the delivered gene will be expressed only as long as the viral genome is present^[11,18].

Osteogenic differentiation capacity was not affected by Ad transduction *in vitro*. Our results are consistent with those of Zhang *et al.*^[10], who suggested that human fMSCs are a superior cellular candidate for bone tissue engineering applications than their adult counterpart because of their higher proliferation capacity, lower immunogenicity, and robust osteogenic potential as assessed *via* mineralization *in vitro*. On the other hand, the chondrogenic capacity of aMSCs was superior to fMSCs when inducing chondrogenesis with TGFβ3, and both decreased post-transduction with Ad-GFP. In fact, TGFβ3 was recently shown to induce chondrogenesis in aMSCs by phosphorylating SMAD3 but not in fMSCs^[23], which explains in part the lower differentiation of fMSCs into chondrocytes found in the present study.

In the evaluation of osteogenesis *in vivo*, the present

study showed that Ad-BMP2-aMSCs are capable of inducing regenerative bone formation more effectively than Ad-BMP2-fMSCs. Naïve fetal or adult MSCs alone were not osteoinductive *in vivo*. In fact, previous work by Zhang *et al.*^[10] have demonstrated that predifferentiation and a scaffold were necessary for osteoinduction by naïve MSCs. Although fMSCs showed higher BMP2 expression post-transduction *in vitro*, fMSC resulted in minimal new bone formation *in vivo*. These findings may be related to the shorter expression of the transgene in fMSCs. Alternatively, initial high BMP2 expression and release from fMSCs could have caused a transient increase in number of osteoclasts derived from hematopoietic stem cells^[24] but there was no evidence of bone remodeling in our histology to suggest this mechanism was at work. Based on our *in vitro* findings with fMSC, and recent work from our laboratory demonstrating superior outcomes of neocartilage growth with a mosaic of 90% naïve and 10% Ad-BMP2-transduced juvenile chondrocytes^[25], it was anticipated that the dilution of BMP2 expressing cells in the rapidly proliferating fMSC population may be an advantage *in vivo*. In addition, Brady and colleagues recently demonstrated that recombinant BMP2 can induce chondrogenesis in pellet culture of fetal but not adult MSCs^[23]. However, we were unable to demonstrate this potential as either enhanced endochondral ossification or chondrogenesis *in vivo* by fMSC, which could be due to difference in delivery method (recombinant BMP2 protein *vs* BMP2 gene transduction) or the fact that our study was *in vivo*. It is also possible that chondrogenesis would not be identified by microradiography and chondral ossicles may have evaded histologic sectioning, but, regardless, we saw minimal evidence of activity of fMSC *in vivo*.

In conclusion, the present study demonstrated adenoviral vectors as an effective method to transiently deliver genes into fMSCs. Nonetheless, the relative faster decrease in the fraction of cells expressing the exogenous gene when compared to transduced aMSCs may limit their applications. Our study also suggested fMSC-mediated BMP2 gene delivery and osteogenic induction *in vivo* was less robust than aMSCs at least in their capacity to induce bone formation. Both Ad-BMP2-aMSCs and Ad-BMP2-fMSCs were capable of expressing BMP2 *in vitro* and can be employed to deliver BMP2 for osteoinduction *in vivo* for the purpose of bone regeneration. Cell delivery may offer advantages over direct vector delivery. In addition, Ad vectors lack insertional mutagenesis, resulting in transient expression of the exogenous gene, which may be desirable over stable expression in applications involving bone morphogenic proteins to induce bone or cartilage regeneration. Further studies are necessary to elucidate differences in osteoinductive mechanisms of aMSCs and fMSCs *in vivo*.

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COMMENTS

Background

In recent years, there have been hundreds of active clinical trials testing the use of mesenchymal stem cells (MSCs) in treating disorders such as myocardial infarction, osteoarthritis, spinal cord injury, and autoimmune diseases. Recent studies have demonstrated that fetal MSCs are a superior cellular candidate than adult MSCs for bone tissue engineering applications. While bone regeneration can be optimized with genetic engineering techniques, the permissiveness to gene delivery and the effect of genetic engineering on stemness in fetal MSCs has not been extensively studied.

Research frontiers

Bone regeneration is a complex process that requires interplay of molecular, cellular, and metabolic factors. In the area of bone tissue regeneration and MSCs, the current hotspot is genetic engineering, microenvironment, and homing of MSCs to optimize bone healing.

Innovations and breakthroughs

Previous studies have found that fetal MSCs can be retrovirally and lentivirally transduced with high efficiency and retain their proliferative and differentiation capacities. The present study demonstrated that adenoviral vectors are an effective method to transiently deliver genes into fetal MSCs and bone morphogenic protein 2 (BMP2)-transduced MSCs can be employed for osteoinduction *in vivo*.

Applications

The study results suggest that fetal MSCs can be genetically engineered with adenoviral vectors to deliver BMP2 and induce osteoinduction, with promising applications in bone tissue regeneration.

Terminology

MSCs are capable of self replicating and differentiating into mesenchymal tissues such as bone, cartilage, and adipose tissue. MSCs can be genetically engineered for applications in tissue regeneration. Adenoviral vectors can deliver genetic material into MSCs. These vectors lack insertional mutagenesis resulting in a desirable safety profile, which added to their capacity to achieve high levels of transgene expression make them attractive for gene delivery particularly for bone regeneration. BMP2 is a growth factor involved in signaling pathways for osteoinduction resulting in osteoblast differentiation of stem cells.

Peer review

The authors have asked some interesting questions and they have compared the fetal vs adult MSCs for their ability to express transgenes via viral and non-viral vectors, and their differentiation capability. It is a very good study. Conclusions are supported by their data.

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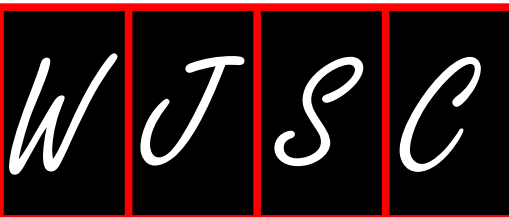
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EDITORIAL

- 235 SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer
Maiese K

REVIEW

- 243 Substrates for clinical applicability of stem cells
Enam S, Jin S
- 253 Hematopoietic stem cell-derived adipocytes and fibroblasts in the tumor microenvironment
Xiong Y, McDonald LT, Russell DL, Kelly RR, Wilson KR, Mehrotra M, Soloff AC, LaRue AC
- 266 Role of nanotopography in the development of tissue engineered 3D organs and tissues using mesenchymal stem cells
Salmasi S, Kalaskar DM, Yoon WW, Blunn GW, Seifalian AM
- 281 Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance
West JD, Dorà NJ, Collinson JM
- 300 Imprinted *Zac1* in neural stem cells
Daniel G, Schmidt-Edelkraut U, Spengler D, Hoffmann A
- 315 Induced pluripotent stem cells: Mechanisms, achievements and perspectives in farm animals
Kumar D, Talluri TR, Anand T, Kues WA
- 329 Cardiac disease modeling using induced pluripotent stem cell-derived human cardiomyocytes
Dell'Era P, Benzoni P, Crescini E, Valle M, Xia E, Consiglio A, Memo M
- 343 Stem cell therapy in inflammatory bowel disease: A promising therapeutic strategy?
Flores AI, Gómez-Gómez GJ, Masedo-González A, Martínez-Montiel MP
- 352 "Second-generation" stem cells for cardiac repair
Núñez García A, Sanz-Ruiz R, Fernández Santos ME, Fernández-Avilés F
- 368 Mesenchymal stem cells as a therapeutic tool to treat sepsis
Lombardo E, van der Poll T, DelaRosa O, Dalemans W

- 380** Transplantation of stem cell-derived astrocytes for the treatment of amyotrophic lateral sclerosis and spinal cord injury
Nicaise C, Mitrecic D, Fahnkar A, Lepore AC
- 399** Tooth-derived stem cells: Update and perspectives
Saito MT, Silvério KG, Casati MZ, Sallum EA, Nociti Jr FH
- 408** Could cancer and infection be adverse effects of mesenchymal stromal cell therapy?
Arango-Rodriguez ML, Ezquer F, Ezquer M, Conget P
- 418** Mitochondria as therapeutic targets for cancer stem cells
Song IS, Jeong JY, Jeong SH, Kim HK, Ko KS, Rhee BD, Kim N, Han J
- 428** Vital roles of stem cells and biomaterials in skin tissue engineering
Mohd Hilmi AB, Halim AS
- 437** Neural differentiation from embryonic stem cells *in vitro*: An overview of the signaling pathways
Chuang JH, Tung LC, Lin Y
- 448** Importance of the stem cell microenvironment for ophthalmological cell-based therapy
Wan PX, Wang BW, Wang ZC

MINIREVIEWS

- 461** Using induced pluripotent stem cells as a tool for modelling carcinogenesis
Curry EL, Moad M, Robson CN, Heer R
- 470** CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture
Álvarez-Viejo M, Menéndez-Menéndez Y, Otero-Hernández J
- 477** Adult stem cells in neural repair: Current options, limitations and perspectives
Mariano ED, Teixeira MJ, Marie SKN, Lepski G
- 483** Development of cancer-initiating cells and immortalized cells with genomic instability
Yoshioka K, Atsumi Y, Nakagama H, Teraoka H
- 490** Adult stem-like cells in kidney
Hishikawa K, Takase O, Yoshikawa M, Tsujimura T, Nangaku M, Takato T
- 495** Niche interactions in epidermal stem cells
Choi HR, Byun SY, Kwon SH, Park KC

- 502** Neural stem cells could serve as a therapeutic material for age-related neurodegenerative diseases

Suksuphew S, Noisa P

- 512** Molecular mechanism of extrinsic factors affecting anti-aging of stem cells

Wong TY, Solis MA, Chen YH, Huang LLH

ORIGINAL ARTICLE

Basic Study

- 521** Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair

Gantenbein B, Gadhari N, Chan SCW, Kohl S, Ahmad SS

Contents

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SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer

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proliferation for cardiac disease, vascular disorders, cancer, and neurodegenerative disorders is receiving great enthusiasm, especially those that focus upon SIRT1, a mammalian homologue of the yeast silent information regulator-2. Modulation of the cellular activity of SIRT1 can involve oversight by nicotinamide/nicotinic acid mononucleotide adenylyltransferase, mammalian forkhead transcription factors, mechanistic of rapamycin pathways, and cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene family members that can impact cytoprotective outcomes. Ultimately, the ability of SIRT1 to control the programmed cell death pathways of apoptosis and autophagy can determine not only cardiac, vascular, and neuronal stem cell development and longevity, but also the onset of tumorigenesis and the resistance against chemotherapy. SIRT1 therefore has a critical role and holds exciting prospects for new therapeutic strategies that can offer reparative processes for cardiac, vascular, and nervous system degenerative disorders as well as targeted control of aberrant cell growth during cancer.

Key words: FoxO; Mechanistic of rapamycin; Apoptosis; Autophagy; Cardiovascular; Cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene; Neurodegeneration; Progenitor stem cells; SIRT1; Cancer

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Core tip: SIRT1, a mammalian homologue of the yeast silent information regulator-2, holds exciting prospects for new therapeutic strategies that can offer reparative processes for cardiac, vascular, and nervous system degenerative disorders as well as targeted control of unchecked cell growth during cancer.

Maiese K. SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer. *World J*

Abstract

Cardiovascular disease, nervous system disorders, and cancer in association with other diseases such as diabetes mellitus result in greater than sixty percent of the global annual deaths. These noncommunicable diseases also affect at least one-third of the population in low and middle-income countries and lead to hypertension, elevated cholesterol, malignancy, and neurodegenerative disorders such as Alzheimer's disease and stroke. With the climbing lifespan of the world's population, increased prevalence of these disorders is expected requiring the development of new therapeutic strategies against these disabling disease entities. Targeting stem cell

THE IMPACT OF CARDIOVASCULAR DISEASE, CANCER, AND NEURODEGENERATIVE DISORDERS

Life expectancy is increasing in developed countries such as the United States and has been accompanied by a one percent decrease in the age-adjusted death rate from the years 2000 through 2011^[1]. Yet, a number of disorders on a global scale continue to plague the population with increased morbidity and mortality from cardiovascular disease, disorders of the nervous system, and cancer. The World Health Organization reports that in 2008, greater than 60% of 57 million global deaths were primarily due to cardiovascular diseases, diabetes, cancer, and respiratory disorders^[2]. Almost 80% of these noncommunicable diseases (NCDs) occur in low and middle-income countries. These NCDs affect approximately 30% of the population under 60 in low and middle-income countries. In contrast, in high-income countries, 13% of the population under 60 is affected. Hypertension and elevated cholesterol are significant risk factors for cardiovascular disease with hypertension alone contributing to approximately 13% of all deaths^[3]. Disorders such as hypertension and elevated cholesterol also contribute to acute neurodegenerative disease such as stroke, the fourth leading cause of death^[4,5]. With the increasing lifespan of the world's population and advancing age, it is expected that the incidence of neurodegenerative disorders also will grow. As an example, ten percent of the global population over the age of 65 is now affected with the sporadic form of Alzheimer's disease, but this is expected to increase significantly^[6-8]. Continued development of new therapeutic strategies directed against the NCDs of cardiovascular disease, neurodegeneration, and cancer are necessary to increase our armamentarium against the complexity of these disease entities.

THE SIRT1 PATHWAY

In this arsenal directed against cardiovascular disease, neurodegenerative disorders, and cancer, multiple therapeutic strategies are being advanced that involve novel stem cell applications. Targeting stem cell proliferation is being considered for cardiac disease^[9], vascular disorders^[10,11], cancer^[12,13], and neurodegenerative disease^[14-16]. However, it is the investigation of stem cells that focus upon sirtuins, mammalian homologues of the yeast silent information regulator-2 (Sir2), that are proving to be extremely

exciting.

Sirtuins are histone deacetylases that transfer acetyl groups from ϵ -N-acetyl lysine amino acids on the histones of DNA to regulate transcription^[17-19]. This family of histone deacetylases also mediates post-translational changes of proteins involved with cellular proliferation, survival, and senescence^[20-23]. There are seven identified mammalian homologues of Sir2 that include SIRT1 through SIRT7. Of these, SIRT1 has been tied to the modulation of multiple cellular functions that include protection against oxidative stress^[24-28], development of atherosclerosis^[29,30], modulation of vascular survival and senescence^[17,20,21,31,32], proliferation of cancer cells^[33-36], changes in diabetic cellular metabolism^[33-36], control of vascular tone through the transient receptor potential cation channel A1^[37], promotion of neuronal survival and cognitive function^[21,38-41], and the extension of lifespan^[25,42,43]. Furthermore, SIRT1 appears to be necessary for efficient post-reprogramming of telomere elongation, the maintenance of pluripotency, and the modulation of differentiation in induced pluripotent stem cells^[44]. In differentiated cells, SIRT1 also controls telomere length and maintenance^[45].

SIRT1 is dependent upon NAD⁺ as substrate^[17,38,46,47]. Through the salvage pathway of NAD⁺ synthesis, nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the conversion of nicotinamide to nicotinamide mononucleotide^[48]. Nicotinamide mononucleotide is subsequently converted to NAD⁺ by enzymes in the nicotinamide/nicotinic acid mononucleotide adenyltransferase (NMNAT) family. NAMPT is a rate-limiting enzyme in mammalian NAD⁺ biosynthesis pathway. Elevated levels of NAMPT activity increase cellular NAD levels as well as the activity of SIRT1 transcription.

The level of SIRT1 activity and its modulation in these cellular processes is considered to be an important factor in determining cell survival and protection against toxic insults. Insufficient SIRT1 activity can have a detrimental affect upon vascular cell survival^[22,23,49], protection against cardiovascular disease^[31], and prevention of neuronal injury^[28,50,51]. Yet, a reduction in SIRT1 activity also may be required to promote cellular survival in systems involving trophic factors such as insulin growth factor-1^[52].

Several biological systems can control the activity of SIRT1 (Figure 1). For example, NMNAT can modulate the deacetylating activity of SIRT1. In addition, mammalian forkhead transcription factors^[53] can bind to the SIRT1 promoter region that contains a cluster of five putative core binding repeat motifs (IRS-1) and a forkhead-like consensus-binding site (FKHD-L). As a result, forkhead transcription factors such as FoxO1 can govern SIRT1 transcription and increase SIRT1 expression^[54]. AMP activated protein kinase (AMPK) represents another pathway for the control of SIRT1 activity. AMPK is a member of the mechanistic

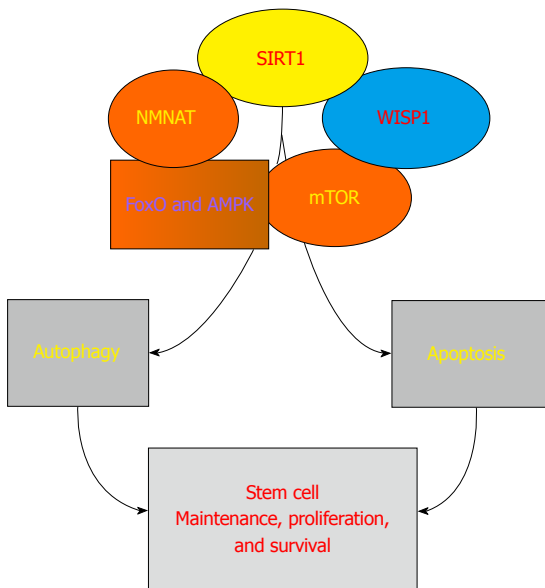


Figure 1 Schematic of SIRT1 pathways that can influence stem cell maintenance, proliferation, and survival. Several pathways can control SIRT1. For example, NMNAT can modulate the deacetylating activity of SIRT1, FoxO1 can govern SIRT1 transcription and increase SIRT1 expression, and AMPK can increase the cellular NAD⁺/NADH ratio leading to the deacetylation of downstream SIRT1 targets. SIRT1 subsequently can depress mTOR pathways and promote autophagy to preserve stem cell integrity during oxidant stress as well as promote neuronal growth. In addition, SIRT1 is necessary to initiate autophagy and transition cells from a quiescence state to an active state. WISP1 increases SIRT1 activity to protect cells from oxidative stress and apoptotic injury and blocks SIRT1 caspase degradation. NMNAT: Nicotinamide/nicotinic acid mononucleotide adenylyltransferase; mTOR: Mechanistic of rapamycin; AMPK: AMP activated protein kinase.

of rapamycin (mTOR) pathway that phosphorylates tuberous sclerosis protein 2 and inhibits the activity of mTORC1^[55,56]. AMPK can increase the cellular NAD⁺/NADH ratio leading to the deacetylation of downstream SIRT1 targets that include the peroxisome proliferator-activated receptor-gamma coactivator 1 α , FoxO1, and FoxO3a^[57]. AMPK also can increase NAMPT during glucose restriction that results in increased NAD⁺ and decreased levels of nicotinamide^[58], an inhibitor of SIRT1^[59]. Resveratrol, a SIRT1 activator, also has been shown to activate AMPK through SIRT1 dependent or independent mechanisms^[57,60].

STEM CELLS, SIRT1, APOPTOSIS, AND AUTOPHAGY

The impact of SIRT1 on cellular function is intimately associated with programmed cell death pathways that involve apoptosis and autophagy^[61-63]. Apoptosis leads to DNA degradation and caspase activation through an early phase that involves the loss of plasma membrane lipid phosphatidylserine (PS) asymmetry and a later phase that results in genomic DNA degradation^[64]. During the early phase of apoptosis, prevention of membrane PS externalization in injured cells is necessary to block the loss of functional cells that may

be removed by activated inflammatory cells^[56]. SIRT1 activation limits external membrane PS exposure during the early phases of apoptosis in mature cells^[22,23,65,66]. In endothelial progenitor cells, SIRT1 activity can counteract the “pro-apoptotic” effects of tumor necrosis factor- α (TNF- α)^[67]. During exposure to TNF- α , SIRT1 also has been shown to protect skeletal myoblast survival^[68]. Loss of SIRT1 activity in human mesenchymal stem cells yields a reduction of proliferation rate with increased apoptosis^[69]. During aging in the mouse auditory system, loss of SIRT1 in cochlear neurons and in the auditory cortex is associated with hearing loss^[70]. In addition, endothelial progenitor cell dysfunction with apoptotic cell death that can occur in smokers and chronic obstructive disease patients has been associated with the loss of SIRT1 expression^[71].

Stem cell survival with SIRT1 can be reliant upon forkhead transcription factors and mTOR (Figure 1). Although several studies involving differentiated cells support the premise that down-regulation of forkhead transcription factors by SIRT1 activation can protect against apoptotic cell death especially during oxidant stress^[22,23,65,72,73], other studies in embryonic stem cells suggest that SIRT1 down-regulation can lead to the acetylation/phosphorylation of forkhead transcription factor pathways such as FoxO1, and in association with phosphatase and tensin homolog (PTEN) and c-Jun N-terminal kinase (JNK), block oxidant stress induced apoptosis^[74]. However, in embryonic stem cells, the presence of SIRT1 also can be protective and appears to have an inverse relationship with mTOR^[35]. SIRT1 can depress mTOR mediated pathways as well as promote autophagy to preserve the integrity of embryonic stem cells during oxidant stress^[75]. SIRT1 can foster inhibition of mTOR signaling to promote neuronal growth^[76]. In addition, during high glucose exposure to mesangial cells, the loss of SIRT1 activity is necessary for mTOR to arrest mesangial cell senescence^[77].

It is important to note that during apoptotic cell injury with the induction of caspase activity, SIRT1 is susceptible to degradation by caspases. Although SIRT1 degradation also may be mediated by apoptotic pathways associated with p38^[78] and JNK1^[79], loss of SIRT1 activity can be the result of caspase degradation of the SIRT1 protein^[80] that can then accelerate further activation of caspases^[80,81]. In some systems that involve the cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene (CCN) family (defined by the first three members of the family that include cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene)^[12], the CCN member WISP1 increases SIRT1 activity to protect cells from oxidative stress and apoptotic injury^[28] (Figure 1). WISP1 also prevents SIRT1 degradation and oversees forkhead transcription activity with SIRT1 similar to other

cytoprotective pathways^[20,73,82] to block FoxO3a activity and prevent caspase activation that would otherwise lead to the degradation of SIRT1^[28,83-85].

In contrast to apoptosis, autophagy promotes tissue remodeling by recycling cytoplasmic components and eliminating no longer useful organelles^[62]. Macroautophagy is the classification of autophagy most commonly described^[86]. It involves the sequestration of cytoplasmic proteins into autophagosomes that fuse with lysosomes for degradation and are eventually recycled. In most cases, SIRT1 activation with the induction of autophagy appears to be vital to promote cell survival in mature cells. In differentiated chondrocytes during oxidant stress, knockdown of the forkhead transcription factors FoxO1 and FoxO3 result in cell death with decreased SIRT1 activity and reduced autophagic related proteins, suggesting that SIRT1 with the activation of autophagy is necessary for cellular protection^[24]. SIRT1 also plays a role in autophagic flux and promoting autophagy in mitochondria^[87] that may be required to maintain a healthy pool of mitochondria^[88]. In endothelial cells exposed to oxidized low density lipoproteins that can lead to atherosclerosis, SIRT1 up-regulation in conjunction with AMPK results in autophagy that is necessary for cellular protection^[89]. In models of cognitive loss with chronic intermittent hypoxia hypercapnia exposure, SIRT1 activation is able to block apoptotic cell injury, up-regulate autophagy, and improve cognitive performance^[90]. However, in pulmonary models of oxidant stress such as the exposure to cigarette smoke in bronchial epithelial cells, SIRT1 has been shown to prevent cell injury through the inhibition of autophagy^[91,92]. In regards to stem cells and the autophagic pathway, stem cells rely upon SIRT1 to modulate autophagic flux^[93]. In muscle stem cells, SIRT1 is necessary to initiate autophagy and transition muscle stem cells from a quiescence state to an active state^[94]. In endothelial progenitor cells, SIRT1 blocks apoptotic cell injury during oxidative stress through the induction of autophagy^[95].

STEM CELLS, SIRT1, AND THE CARDIOVASCULAR SYSTEM

In the cardiovascular system, SIRT1 expression can affect not only the survival of stem cells, but also the ability of stem cells to differentiate and the efficacy of these cells for therapeutic applications. Increased SIRT1 expression can improve the survival of cardiomyoblasts^[96] and prevent senescence and impaired differentiation in endothelial progenitor cells^[97]. In regards to treatment efficacy, mesenchymal stem cells that are subjected to SIRT1 over-expression exhibit increased blood vessel density in the area of cardiac infarcts, reduced cardiac remodeling, and improved cardiac performance in rodent models^[98], factors that may be associated with cardiac stem migration that is vital to tissue repair^[99]. SIRT1 also

can limit expression of aged mesenchymal stem cell phenotypes^[98]. Loss of SIRT1 in circulating endothelial progenitor cells that can occur during tobacco exposure or chronic obstructive pulmonary disease may lead to increased senescence and apoptotic cell death that presents increased risk for vascular disease or cardiac disease^[71]. SIRT1 also may improve the function of aged stem cells that are senescent. Aged mesenchymal stem cells that were exposed to pre-conditioning with glucose depletion exhibited increased expression of SIRT1 in addition to other proliferative entities such as growth factors and resulted in increased cardiac performance^[100].

STEM CELLS, SIRT1, AND NEURODEGENERATION

In the nervous system, SIRT1 has been tied to the differentiation, maturation, and maintenance of neurons. Loss of SIRT1 expression with the concurrent induction of heat shock protein-70 promotes neural differentiation, maturation of embryonic cortical neurons^[101], and the differentiation of human embryonic stem cells into motor neurons^[102]. SIRT1 also is considered a negative regulator of subventricular zone and hippocampal neural precursors in murine animal models. Knockdown of SIRT1 does not eliminate neural precursor numbers or proliferation but increases the production of neurons in the subventricular zone and the hippocampus^[103]. In the mouse cerebral cortex, repression of SIRT1 by the oncogene BCL6 leads to the conversion of neural stem cell/progenitor cells to become neurons^[104]. Neural stem cell differentiation also can be controlled through alternate pathways that involve SIRT1. In mouse neural stem cells, neuronal differentiation can be driven through the microRNA miR-34a that leads to decreased SIRT1 expression and DNA-binding of p53^[105]. Interestingly, in these studies, increased expression of SIRT1 enhanced the astrocytic subpopulation of cells^[105].

STEM CELLS, SIRT1, AND CANCER

The cellular proliferative effects of SIRT1 also play a critical role in tumorigenesis. For example, SIRT1 activity can maintain acute myeloid leukemia stem cells and confer resistance against chemotherapy^[106], stimulate endometrial cell tumor growth through lipogenesis^[107], maintain neural stem cells and promote oncogenic transformation^[108], and foster hepatocellular carcinoma^[109]. As a result, SIRT1 and agents that modulate SIRT1 activity may represent new therapeutic strategies against tumorigenesis. For example, down-regulation of endoglin, a protein over-expressed in tumor associated endothelial cells, leads to apoptotic cell death, DNA damage, inhibition of several DNA repair genes including SIRT1, and enhanced chemotherapy sensitivity^[110]. In addition, pathways linked to SIRT1

also may provide new strategies against cancer. Activation of p53 through SIRT1 inhibition can result in apoptotic cell death for quiescent leukemia stem cells in chronic myelogenous leukemia^[111]. In breast cancer, estrogen receptor- α can lead to SIRT1 expression that activates pro-survival genes in breast cancer cells, such as catalase and glutathione peroxidase, and inhibits tumor suppressor genes, such as cyclin G2 (CCNG2) and p53. In these breast cancer cells, if SIRT1 is inhibited, estrogen receptor-induced breast cell growth is blocked through apoptotic cell death^[112].

FUTURE CONSIDERATIONS

Cardiac disease, vascular disorders, neurodegenerative disease, and cancer lead to significant disability and death in the global population. Development of stem cell strategies for these disorders and the targeting of SIRT1 to drive stem cell viability and function holds great promise for the future. In the cardiovascular system, SIRT1 through stem cell proliferation can drive angiogenesis, improve cardiac performance following ischemic injury, limit cell senescence, and enhance the function of aged stem cells. In the nervous system, SIRT1 can be a negative modulator of neural precursors with the loss of SIRT1 leading to differentiation and maturation of embryonic stem cells in the nervous system. During tumorigenesis, SIRT1 foster the development of acute myeloid leukemia stem cells, promote oncogenic transformation of neural stem cells, and lead to hepatocellular cancer. Vital to the clinical outcomes controlled by SIRT1 is its level of activity overseen by pathways that include NMNAT, mammalian forkhead transcription factors, mTOR, and CCN family members such as WISP1 that determine cell survival through apoptosis and autophagy. Future work that can target SIRT1 and navigate stem cell proliferation under required conditions to either cellular proliferation or cellular death can open new avenues for the treatment of cardiovascular disorders, neurodegenerative disease, and cancer.

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Substrates for clinical applicability of stem cells

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as a type of substrate that can bring the benefits of regenerative medicine to clinical settings.

Key words: Human pluripotent stem cells; Extracellular matrix protein; Synthetic substrate; Peptide; Polymer; Scaffold; Hydrogel

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Core tip: This review article highlights numerous extracellular matrix proteins, peptide and polymer based substrates, scaffolds and hydrogels that have been pioneered for human pluripotent stem cell self-renewal for stem cell-based therapy. The benefits and shortcomings of these substrates as well as future direction that can bring the benefits of regenerative medicine to clinical settings are discussed.

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Abstract

The capability of human pluripotent stem cells (hPSCs) to differentiate into a variety of cells in the human body holds great promise for regenerative medicine. Many substrates exist on which hPSCs can be self-renewed, maintained and expanded to further the goal of clinical application of stem cells. In this review, we highlight numerous extracellular matrix proteins, peptide and polymer based substrates, scaffolds and hydrogels that have been pioneered. We discuss their benefits and shortcomings and offer future directions as well as emphasize commercially available synthetic peptides

INTRODUCTION

Ever since the derivation of human embryonic stem cells (hESCs) from the inner mass of the blastocyst^[1], great effort has been placed on extending the benefits of these pluripotent cells to regenerative medicine^[2,3]. However, human pluripotent stem cells (hPSCs) which include human induced pluripotent stem cells (hiPSCs) and hESCs, have traditionally been cultured on mouse fibroblast feeder layers in the presence of animal cell-conditioned media or on Matrigel that pose inherent risk of pathogenic contamination^[4] as well as the presence of non-human immunogenic epitopes such as N-glycolylneuraminic acid (Neu5Gc)^[5]. Consequently, research has focused on developing xeno-free, chemically defined media and substrates that are

compliant with current good manufacturing practice (cGMP), scalable, maintain high degree of hPSC purity, colony homogeneity and pluripotency. Here, we review substrates that have been developed for maintenance of hPSCs for clinical applications.

EXTRACELLULAR MATRIX PROTEINS

Feeder free cultures for hPSC expansion rely on Matrigel which constitutes the basement membrane components derived from mouse Engelbreth-Holm Swarm (EHS) tumor. The EHS tumor can easily yield a hundred grams of basement membrane components that are abundantly found in Matrigel such as collagen IV, laminin, heparin sulfate proteoglycans and nidogen/entactin^[6]. In addition, minor components can also be found in Matrigel which include proteases such as 72 kDa matrix metalloproteinase-2, 92 kDa matrix metalloproteinase-9, urokinase, tissue-type plasminogen activator as well as growth factors, like transforming growth factor beta, fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, insulin-like growth factors, and proteins (Amylase, Transferrin, Clusterin) in an undefined composition. Matrigel has been used as a substrate to study the long-term stability (40 passages) of hESCs^[7] as well as to derive and maintain new hESC lines for over 70 passages^[8]. Other studies have used Matrigel as an extracellular matrix (ECM) to induce hepatic differentiation of hESCs using hepatic growth hormone and activin A^[9], to expand hESCs on Matrigel-coated cellulose microcarriers in 3D suspension cultures^[10] and to study the effects of bone morphogenetic protein 4 on hESCs differentiation^[11]. As Table 1 highlights, the batch-to-batch variability and xenogeneic-origin makes Matrigel unsuitable for quality control or cGMP production of hPSCs. Consequently, various ECM protein components have been sought as alternatives to Matrigel.

Vitronectin

The N-terminal of the 75 kDa vitronectin protein consists of the binding sites for integrins *via* the Arg-Gly-Asp RGD sequence, which allows cell-vitronectin interaction^[12]. Braam *et al*^[13] have shown that recombinant vitronectin is as effective as plasma purified vitronectin for hESC expansion and mediates cell attachment *via* the α V β 5 integrins. In mTeSR1 medium, recombinant vitronectin supported several hESC lines for five passages while maintaining normal morphology, karyotype, differentiation potential and cell surface markers for stemness. However, other ECM proteins such as laminin, collagen I, collagen IV and fibronectin were only able to support cell growth in mouse embryonic fibroblast-conditioned media (MEF-CM)^[13]. The use of mouse myeloma cell line NS0 to obtain the recombinant vitronectin holds potential risk of sialic acid contamination but others have been

able to produce recombinant vitronectin in *Escherichia colivia* plasmid vectors^[14]. Prowse *et al*^[14] obtained recombinant somatomedin B (SMB) domain along with the RGD sequence of vitronectin (rVN SMB) and anchored the substrate to tissue culture plate through vitronectin's polyhistidine linker sequence.

Recently, a fully-defined, serum- and feeder-free medium called StemPro was developed for hPSC expansion and differentiation. The hESCs grown in an ascorbate (vitamin C) free StemPro media for over 10 passages showed similar binding affinity to another ECM protein purified from EHS tumor (called Geltrex) and pluripotency markers comparable to cultures grown on whole purified vitronectin and Geltrex^[14]. The surface density of human purified plasma vitronectin necessary for long term (> 30 passages) hESCs attachment, proliferation and differentiation was determined by Yap *et al*^[15] to be 250 ng/cm². However, the hESCs cultured on purified plasma vitronectin in mTeSR media showed slower (35 h) doubling time than Matrigel (25 h) but expressed normal pluripotency markers and karyotype^[15]. Another study examined the scale up potential of vitronectin and laminin-coated three dimensional (3D) substrates from traditional two dimensional (2D) tissue culture polystyrenes (TCPs) to 3D polystyrene microcarriers (MCs)^[16]. In this study, vitronectin was adsorbed onto TCPs and reached a surface density saturation of 510 ng/cm² compared with laminin at 850 ng/cm². Coating vitronectin and laminin to MCs allowed for a surface density saturation of 450 and 650 ng/cm², respectively. Even though, the two extracellular matrices showed similar adhesion and growth kinetics, the scale up to 3D culture resulted in a slower growth kinetics on the polystyrene MCs. This was explained by the hydrophobicity of MCs as well as the formation of large compact cell aggregates which deprive essential nourishments to the cells that are located in the center.

Laminins

Laminin provides adequate growth for hESCs *via* the integrin α 6 β 1 laminin specific receptor since it is the major type of integrin present on hESCs^[17,18]. The effect of various recombinant human laminin (rhLM) isoforms on hESC attachment and proliferation has been investigated. Miyazaki *et al*^[18] showed that hESC line KhES-1 cells adhere markedly to rhLM-332 as well as to rhLM-511 and rhLM-111 (least adherence) when compared to non-adherence on rhLM-211 and rhLM-411. In the study, KhES-3 cells were able to adhere to rhLM-211 and rhLM-411 but their growth rate was markedly lower on rhLM-332, rhLM-511 and rhLM-111^[18]. The primary reason for such a marked difference in adherence and growth is because rhLM-211 and rhLM-411 possess affinity for α 3 β 1 and α 7 β 1 which are laminin-binding integrin isoforms that are expressed on hESCs in a relatively low amount compared to α 6 β 1 integrins^[18]. This receptor specificity

Table 1 Benefits and shortcomings of Matrigel, extracellular matrix proteins, synthetic peptides, synthetic polymers and hydrogels

Substrate	Advantages	Disadvantages
Matrigel	Allows feeder-free cell culture Inexpensive Long-term hESCs culture ^[7,8]	Xenogeneic origin ^[6] Undefined components ^[6] Pathogenic contamination risk ^[4] Neu5Gc immunogenic epitope ^[5] Batch-to-batch variability ^[6]
ECM proteins	See subsections below	Batch-to-batch variability Degradation upon sterilization
Vitronectin		Pathogenic contamination risk
rhLM-332		Not-Scalable ^[30]
LM-E8		High production cost ^[30]
rh E-cadherins-Fc protein		Immunogenicity risk ^[17]
Fibronectin		Degradation upon sterilization
Vitronectin	Long-term hESC culture (> 30 passages) ^[15] α V β 5 integrin receptor mediated cell attachment ^[13]	Not-Scalable ^[16,30] High production cost ^[30]
rhLM-332	High α 6 β 1 integrin affinity ^[18]	
LM-E8	Smaller, easily purified, higher purity <i>vs</i> 780 ku laminins ^[20] Better stem cell adhesion than Matrigel and intact laminins ^[20] ROCK inhibitor Y-27632 not needed ^[20]	Not-Scalable ^[30] High production cost ^[30]
rh E-cadherins-Fc protein	hESC self-renewal, maintenance and pluripotency comparable to Matrigel TM ^[26]	Low cell adherence <i>vs</i> Matrigel ^[26]
Synthetic peptides	No batch-to-batch variation ^[36] Immunogenicity risk avoided ^[37] Since chemically synthesized Long-term hESCs culture ^[32,37,44]	High production costs ^[47,48] Sterilization difficulties ^[47] Easily degradable ^[47] Labor intensive cell passaging Limited scale-up potential of 2D platform ^[51]
Synthemax surface	Gamma irradiation sterilization ^[39] 2 yr shelf-life ^[39] hESCs cryopreserved and thawed on substrate ^[38] Scalable ^[38] Long-term hESCs culture ^[38-40]	
Synthetic polymers	Inexpensive ^[45,47]	Limited scale-up potential of 2D platform
PMVE-alt-MA	Easy and rapid fabrication ^[45,49]	
PMEDSAH	Highly manipulable ^[47]	
APMAAm	Long-term substrate stability ^[46]	
Polyacrylates		
Chitosan-alginate polymers		
(pDTEc) polymer scaffolds		
Hydrogels	<i>In-vivo</i> 3D type environment ^[58] Thermoresponsive and pH sensitive properties ^[54,55,58]	Difficult to analyze cells embedded in hydrogels
(AETMA-Cl)-DEAEA based		Enzymatic release of cells from hydrogel ^[57]
PDEAAm-based		
HA-based		
Alginate-collagen based		
PEG-based		
PPP-based		

hESC: Human embryonic stem cell; ECM: Extracellular matrix; LM: laminins; PMVE-alt-MA : Poly(methyl vinyl ether-alt-maleic anhydride); PMEDSAH: poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]; APMAAm: Aminopropyl methacrylamide; pDTEc: Poly(desaminotyrosyl tyrosine ethyl ester carbonate); AETMA-Cl: 2-(acryloyloxyethyl) trimethylammonium chloride; HA: Hyaluronic acid; PDEAAm: Poly(N,N-diethylacrylamide); PEG: Polyethylene glycol; PPP: Platelet poor plasma; 2D: Two dimensional.

is further established by the fact that although laminin-332 is not expressed on hESCs, rhLM-332 was an adequate substrate for hESCs adherence and proliferation due to its affinity for α 6 β 1 integrins^[18].

Since laminin isoform-511/-521 are the major laminins expressed on hESCs, another study investigated hESC self-renewal of cell lines HS420, HS207 and HS401 on a rhLM-511 substrate in the mTeSR1 variant (O3) and xeno-free TeSR1 variant (H3) media^[19]. The cells proliferated for 28 passages and normal karyotype was observed after 20 passages while exhibiting OCT4, SOX2 and Nanog pluripotency markers at a higher level than Matrigel and comparable

to those grown on feeder cells. Importantly, rhLM-511 affinity of the cells was higher than cell-cell adhesion as demonstrated by cell spreading when the cells were passaged as individual clumps and this highlights the role of rhLM-511 in cell motility.

Further refinement to laminin-coated substrates has been achieved by using laminin E8 fragments (LM-E8) that are composed of the α , β and γ chains' C-terminal regions^[20]. These truncated proteins are smaller, easily purified in a short time period and yield a higher purity compared with the 780 kDa LMs. Miyazaki *et al*^[20] used LM-511-E8 and LM 332-E8 to achieve greater hESC and hiPSC adhesion than MatrigelTM and intact LM-511

and 332 in a TeSR2 medium. The three hESC lines and two hiPSC lines cultured on LM-E8-coated substrates underwent proliferation for 30 and 10 passages, respectively, where disassociated cells attached strongly to LM-E8 substrates as compared to weak adhesion on Matrigel, fibronectin and vitronectin substrates^[20]. Importantly, LM-E8 substrates did not require the use of ROCK inhibitor γ -27632 which is needed for some recombinant protein-based substrates.

E-cadherin

E-cadherin, which is a transmembrane glycoprotein is involved in calcium-dependent cell-cell adhesion in epithelial as well as embryonic stem cells^[21]. It is essential for the maintenance of pluripotent state of stem cells due to their critical role in cell-cell adhesions^[22] as well as cell survival and renewal^[23]. This E-cadherin mediated pluripotency is quickly lost, as measured by the levels of Oct4, Nanog and Sox2, when siRNA is used to suppress E-cadherin expression on cells^[24]. This in turn causes a decrease in hESC proliferation^[25] and emphasizes the critical role that E-cadherins play in cell-cell adhesion, survival, renewal, proliferation and pluripotency. Nagaoka *et al*^[26] used recombinant E-cadherin-Fc-coated culture dishes (rhE-cad-Fc) where E-cadherins are fused with an IgG-Fc domain to coat tissue culture plates on which hESCs were examined for their pluripotency and propagation characteristics. The study demonstrated that both hESCs and hiPSCs that were cultured on rhE-cad-Fc protein-coated surface for > 13 passages in mTeSR1 medium were indistinguishable from those that were cultured on Matrigel as far as the rate of proliferation, cell morphology, maintenance of pluripotency and the ability to differentiate into multiple cell types were concerned. However, the ability of cells to adhere was decreased as a result of protease digestion cocktails using Accutase. This problem can be circumvented by using enzyme-free Cell Dissociation Buffer for passaging the cells. Therefore, rhE-cad-Fc can be considered superior to Matrigel as a substrate because of the use of more defined chemical conditions that yield similar results for hESC self-renewal, maintenance and pluripotency. E-cadherins have also been linked with matrix rigidity where hESCs on rigid substrates were shown to co-express E-cadherins and were OCT4⁺. In contrast, the softer substrates had large hESC aggregates that had an increased tendency to differentiate. Furthermore, hESCs on rigid substrates exhibit increased cytoskeleton contractility related to E-cadherin expression among cells^[27].

Fibronectin

The 220 kDa fibronectin dimer interacts with hESCs *via* the α 5 β 1 integrin receptor such that only 25 percent of adsorbed fibronectin is needed for hESC renewal^[28]. However, the 120 kDa central-binding domain of fibronectin is necessary for attachment

and maintenance of hESCs pluripotency. Using a high throughput screening of various ECM proteins, Brafman *et al*^[29] discovered that high concentrations of fibronectin and laminin (500 μ g/mL) supported hESC proliferation commensurate with Matrigel in a concentration dependent manner and fibronectin, collagen I and laminin were each able to support a high degree of pluripotency.

SYNTHETIC PEPTIDES

Even though, the protein based substrates such as recombinant vitronectin, laminin and rhE-cad-Fc coated tissue plates may be feeder free, they are not xeno-free and as a result pose significant immunogenic and pathogenic hazards^[4]. Additionally, such substrates are not scalable and have significant manufacturing costs associated with them^[30]. Being protein-based, they can also undergo degradation following common sterilization techniques. Consequently, peptide-based substrates offer better alternatives for maintaining hPSCs pluripotency, self-renewal, growth, scalability and clinical applicability.

Heparin binding peptides promote cell adhesion and spreading through their interaction with cell-surface glycosaminoglycans (GAGs) which are involved in cell-cell recognition and adhesion, cell-matrix interactions and receptor-signal complexes^[31]. In a study conducted to determine the optimal peptides for hESC self-renewal, Klim *et al*^[32] discovered that surfaces which display heparin binding peptide GKKQRFHRNRKG, derived from vitronectin, supported adhesion and self-renewal of hESCs at the lowest peptide surface density (0.5%) while other surface presenting various heparin binding peptides did not allow for cell attachment. These heparin binding peptides exhibit increased levels of Oct-4 and SSEA-4 expression, exhibit similar rates of growth, long term stem cell propagation (17 passages) and differentiation capabilities that are equal to Matrigel. The heparin binding peptide GKKQRFHRNRKG has been used in polyacrylamide hydrogels of varying stiffness where H9 hESCs were cultured for 12 passages in mTeSR1 medium supplemented with 10 μ mol/L ROCK inhibitor Y-27632^[33]. Not only was the hESC self-renewal confirmed but it was found that GAG binding on stiffer materials conveyed mechanosensing information that allowed for F-actin polymerization and enabled cell adhesion. Additionally, the nuclear localization of transcriptional co-activators Yes-associated protein and transcriptional coactivator with PDZ-binding motif (TAZ) on stiffer matrices allowed for hESCs pluripotency^[33].

Self-assembled monolayers (SAMs) with peptide sequence TVKHPDALHPQ and LTTAPKLPKVTR have also been shown to promote hESC adhesion and proliferation for 3 passages^[34]. However, non-clonal abnormalities were detected in cells cultured on TVKHPDALHPQ SAMs while LTTAPKLPKVTR SAMs

did not exhibit karyotypic abnormalities. Similarly, 18 different laminin-derived peptides were also screened and five (including RNIAEIIKDI) were capable of supporting hESCs proliferation and pluripotency comparable to MatrigelTM[35]. The presence of relatively low receptor concentration on cells requires the presentation of laminin-derived peptides at a higher concentration despite being presented in a uniform density and specific orientation.

More recently, Au-coated glasses have been modified with poly[oligo(ethylene glycol) methacrylate] polymer brushes through surface initiated polymerization and peptides that are derived from vitronectin^[36]. A 0.75 mmol/L vitronectin peptide concentration is deemed necessary for hiPSC expansion for up to 10 passages in mTeSR1 medium. Using micro-contact printed polydimethylsiloxane stamp, cell adhesion was easily regulated only on the vitronectin peptide and pluripotency markers and self-renewal capabilities were observed to be similar to Matrigel. A more potent version of the RGD sequence in the form of cyclic RGD peptide has been used to modify surfaces on which hESCs can adhere more prominently than on linear RGD peptides at a 4 fold higher rate^[37]. Normal pluripotency markers, karyotype and differentiation potential similar to Matrigel have been observed for cells cultured for 10 passages as well as in mTeSR1 medium.

Peptide acrylate surfaces

Peptide acrylate surfaces (PAS) are synthetic surfaces that are composed of acrylates which are conjugated to biologically active peptides^[38]. One such platform is the so called Synthemax Surface where the synthetic surface is manufactured by covalently linking the vitronectin-derived RGD sequence to the acrylate surface^[39]. Melkounian *et al*^[38] have used this technique to deposit various peptide sequences, as well as the vitronectin sequence, onto acrylate surfaces in a process that deposits carboxylic acid containing acrylate onto the surfaces of cultures and then conjugates amine-containing peptides to these surfaces using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide. Here, bone sialoprotein-PAS and vitronectin-PAS were used to culture hESC line H7 for more than 10 passages in a defined medium. The authors concluded that the hESCs grown on PAS system were not different to the cells that were grown on Matrigel when comparing cell viability, colony morphology, doubling time and hESC marker expression of OCT4, TRA-1-60 and SSEA-4. Furthermore, hESCs can be cryopreserved and thawed onto PAS in addition to their ability to undergo directed differentiation into functional cardiomyocytes. This research demonstrated the scalability of feeder free, xeno-free and chemically defined PAS substrate that are suitable for clinical applications. Jin *et al*^[40] further examined the expansion and differentiation capabilities of hiPSCs on synthemax surface. The authors concluded

that there is no significant difference between the doubling time and pluripotency marker expressions for hiPSCs cultured on Matrigel and Synthemax substrate. However, the cells cultured on the Synthemax substrate showed more compact morphology than Matrigel. The researchers were able to demonstrate definitive endoderm differentiation of hiPSCs and were able to decipher the specific binding of $\alpha V\beta 5$ receptors for Synthemax Surface while the Matrigel substrate bound to various integrin receptors. Lastly, cell-cell interfaces on Synthemax Surface showed broad and dense actin filaments as well as the up-regulation of zyxin which demonstrates the involvement of various cytoskeletal proteins in hiPSCs attachment and proliferation.

Synthemax Surface has also been used for retinal differentiation of hiPSCs^[41] and to show efficient single cell passaging of hPSCs, using ROCK inhibitor Y-27632, similar to the results obtained on LM-E8 substrates^[42]. It has been used to obtain oligodendrocyte progenitor cells (OPCs) from hESCs that express higher levels of OPC specific proteoglycan, NG2, as compared to OPCs grown on xenogeneic-derived ECM^[43]. The researchers concluded that Synthemax Surface can replace the xenogeneic-derived ECM for hESCs differentiation into OPCs. Recently, Synthemax Surface was used to expand hESCs for 10 passages and to obtain insulin-producing β cells within 21 d in a defined serum-free media^[44].

Nevertheless, the high production costs, degradation upon sterilization, labor intensive cell passaging and limited scale-up potential of 2D peptide surfaces have caused researchers to seek alternative substrates in the synthetic polymer arena.

SYNTHETIC POLYMERS

Poly(methyl vinyl ether-alt-maleic anhydride)

Brafman *et al*^[45] used a high throughput screening approach to identify supports for hPSCs self-renewal as well as optimal conditions for pluripotency and proliferation for hPSCs. The authors examined 90 polymers with different functional groups, chemical compositions and molecular weights. They discovered that poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) is most capable of supporting both short and long term maintenance of hESCs for HUES1 and HUES9, as well as one hiPSC line for over five passages. PMVE-alt-MA and poly(acrylic acid) cause greatest hPSC proliferation rates at discrete molecular weights of 1.25×10^6 Da and 4.5×10^5 Da, respectively. Of the 16 polymers, cells on PMVE-alt-MA did not exhibit detachment or differentiation. Furthermore, one hiPSC line and two HUES 1/9 lines were successfully cultured for over five passages with expression of normal pluripotency markers and differentiation capabilities and showed an increased expression of $\alpha 5$ and αv integrin receptors. Importantly, the authors showed that hPSCs can

further secrete endogenous ECM proteins in the absence of exogenous ECM proteins in defined medium thereby highlighting the role of ECM proteins in hPSC self-renewal.

Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]

Long term hESC growth has been demonstrated on poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH)-coated TCPs *via* surface initiated graft polymerization technique that grafts PMEDSAH onto ozone-activated surfaces of TCPs^[46]. For 25 passages, H9 and BG01 cells that were grown on PMEDSAH attached, proliferated, exhibited long term growth along with normal genetic and proteomic makeup as exhibited through Nanog, OCT3/4 and SOX2 expression and possessed differentiation potential. H9 cells exhibited higher cell-aggregate adhesions when grown in human cell conditioned medium as compared to BG01 cells. However, both these cell lines showed comparable cell doubling times, pluripotency and karyotype when compared with cells grown on Matrigel^[46]. Consequently, variability exists in the response of these cell lines to various media conditions. H9 hESCs grown on PMEDSAH were able to be cultured on chemically defined StemPro media for 10 passages. Additionally, hiPSCs grown on PMEDSAH have also been shown to proliferate in defined xeno-free conditions for 15 passages while retaining pluripotency markers, normal morphology and karyotype^[47]. However, the hiPSC-derived mesenchymal stem cell unequally preferred osteogenic and chondrogenic differentiation rather than adipogenic lineage.

Aminopropyl methacrylamide

Other synthetic polymer substrates such as the methacrylamide-based aminopropyl methacrylamide have been used to culture hESCs for over 20 passages in mTeSR1 media^[48]. The H9-hOct4-pGZs cells showed higher expression of pluripotency markers than Matrigel as well as higher proliferation rate at passage 1 and 22 but the proliferation rate slowed down at latter passages. Importantly, bovine serum albumin (BSA) remains crucial for hESC attachment, growth and proliferation since it was shown to be adsorbed onto the APMAA surfaces in an unfolded state^[48].

Polyacrylates

One study applied a high throughput screening approach to high-density polymer microarrays to obtain polymers with either 2-carboxyethyl acrylate or 2-(methylthio) ethyl methacrylate containing monomers that allowed cell adhesion at specific ratios^[49]. Furthermore, an equal ratio of 4-tert-butylcyclohexyl acrylate and n-butyl methacrylate was found in polymers supporting greatest adhesion. The study demonstrated short term (5 passages) pluripotency potential of hESCs cultured on these surfaces.

Chitosan-alginate polymers

As natural polymers that mimic GAG structure, chitosan-alginate (CA) 3D scaffolds have been used for hESC self-renewal^[50]. The cells grown on these scaffolds exhibit three times higher alkaline phosphatase activity and the expression of pluripotency markers were similar to hESCs grown on human fibroblast feeder layers. Additionally, hESCs which are recovered *via* EDTA and K₂HPO₄ solution express over 95% cell viability and the CA scaffolds allow for easy passaging of cells. Another study used alginate-chitin polymer based scaffold, which mimic the GAGs units of the ECM^[51]. Combined conditions of high cell seeding density, avoidance of fluid shear stresses *via* encapsulation as well as increased surface area to volume ratio of encapsulated cells, and cryopreservation with high cell viability (> 75%) allows for long term culture (10 passages) and quick non-cytotoxic harvesting of cells through enzymatic disassociation of microfibers with further scalability.

Poly(desaminotyrosyl tyrosine ethyl ester carbonate) polymer scaffolds

Synthetic scaffolds composed of poly(desaminotyrosyl tyrosine ethyl ester carbonate)^[52], a tyrosine derived polycarbonate polymer^[53], have been used to study the effects of geometries on hESCs self-renewal and proliferation. Carlson *et al.*^[52] discovered that microfibrillar architecture of poly-D-lysine pretreated scaffolds supports hESC survival and colony formation as this geometry allows cell-cell and cell-matrix contact and extensive ECM deposition of laminin and collagen IV with some collagen I deposition. The endogenous production of laminin was an essential factor for hESC adhesion and survival.

HYDROGELS

Although synthetic polymers represent a more economical approach with more quality control over the manufacturing process, the 2D topography does not mimic the *in vivo* cell-cell and cell-ECM interactions. Hydrogels, serve as viable alternatives to 2D cultures that hold the potential for clinical scale hESC production. Using acrylate and acrylamide monomers, one study used 2-(acryloyloxyethyl) trimethylammonium chloride (AETMA-Cl) and 2-(diethylamino) ethyl acrylate (DEAEA) in a 3:1 ratio to construct thermoresponsive hydrogels^[54]. The undifferentiated colonies can be passaged without enzymatic disassociation by reducing the temperature from 37 °C to 15 °C for 30 min, followed by gentle pipetting since hydrogel swelling alone was not sufficient for hESC removal. The hydrogel adsorbed BSA and cells that were grown on the hydrogel demonstrated slower growth and lower total expansion rate over 5 d compared to Matrigel. Additionally, microdeletions and duplications on some chromosomes were present in both Matrigel and hydrogel culture conditions. Nonetheless, hESCs were able to be cultured

for over 20 passages in mTeSR1 medium on glass coverslips coated with the hydrogel and expressed pluripotency markers.

Another acrylamide-based Poly(N,N-diethylacrylamide) (PDEAAm) thermoresponsive hydrogel uses pentapeptide YIGSR-NH₂ to mimic B1 chain amino acid sequence of laminin^[55]. Here, the hydrogel was further modified with (NH₄)₂SO₄ salt to yield highly porous interconnected (NH₄)₂SO₄-PDEAAm hydrogels that were able to support adhesion and growth of hESCs better than large NaCl generated random pores. The polyacrylamide hydrogels can also be used to direct the differentiation to specific lineages in microwells since microwells accumulate molecules above the hydrogel cut off range of 40 to 70 kDa^[56]. As a result, microwells direct stem cells to be differentiated into neural, ectodermal and endodermal lineages while relatively small mesodermal inducing factors diffuse away and large mesodermal inducing factors accumulate in the microwells.

Hyaluronic acid (HA) based hydrogels were used by Gerecht *et al.*^[57] to culture hESCs in MEF-CM. The encapsulated hESCs formed colonies of varying sizes and maintained doubling time similar to 2D cultures. After 20 d, undifferentiated colony morphology was observed but a high cell seeding density between 5-10 × 10⁶ is necessary to prevent apoptosis. The hydrogel allowed for enzymatic release (hyaluronidase) of cells that achieved cell viability of 76.5% ± 8%^[57]. Additionally, Ikonen *et al.*^[58] used HA with hydrophilic pH-sensitive hydrogels to demonstrate adhesion and expansion of hESC cardiomyocytes. Here, the collagen-mimicking hydrogel nanofibers allowed for cell adhesion and growth due to their hydrophilicity but HA further augmented the cell survival and provided a more structurally sound hydrogel. Furthermore, the thinnest nanofibers (4.2 nm) were supportive of hESC cardiomyocytes culture.

Clinical scale applications require microenvironments that not only self-renew hESCs but are also able to direct their differentiation. To this end, Dixon *et al.*^[59] have used alginate-collagen hydrogels such that self-renewal of hESCs is sustained on alginate dominated state but differentiation can be induced by "switching" to a collagen predominant microenvironment via EDTA/sodium citrate based treatment. This process further changes the elasticity of the matrix from approximately 21.37 ± 5.37 kPa to ~4.87 ± 1.64 kPa. Moreover, the early switching (day 3) correlates with ectodermal differentiation while day 5 switching results in mesodermal and endodermal commitment. This hydrogel configuration offers advantages, namely the preservation of hydrogel structure and the relatively high hESC cell density (approximately 2 × 10⁷) obtained before differentiation^[59].

Polyethylene glycol (PEG)-based hydrogel functionalized with vinyl sulfone (VS) macromers with multiarms have been shown to maintain hESC self-renewal where the 8 multiarm PEG-VS hydrogel (10% PEG) proved to be ideal for hESC self-renewal and stemness expression^[60]. However, the hydrogel needs to be optimized for specific

cell lines since some of the cells lines demonstrated weak stemness markers. Another PEG-based thermoresponsive substrate utilized recombinant protein factors as a poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) hydrogel^[61]. Here, 10⁷² fold expansion over 60 passages was achieved in hPSCs using single cell passaging and stem cells were able to be further differentiated into dopaminergic neurons at even higher numbers.

Another novel hydrogel is derived from human platelet poor plasma (PPP) gelled in the presence of DMEM media, which contains calcium ions^[62]. The resulting coagulation cascade forms a stable hydrogel. HESCs were able to be cultured for 25 passages and fibronectin was speculated to play a role in hESC adhesion via α5β1 integrins^[63]. Despite being relatively inexpensive, the PPP-based hydrogel was not xeno-free due to the N2 and B27 supplements in the media. Furthermore, scalability may be an issue being that the system is donor dependent.

CONCLUSION

Although many substrates exist for the self-renewal and expansion of hPSCs, most of the substrates possess limitations hinder their clinical applicability. As summarized in Table 1, Matrigel is xenogeneic in origin, contains undefined components and can be immunogenic. The relatively high production costs, immunogenicity risks and difficulties with sterilization^[16,17,30] of ECM proteins limit their scalability potential while synthetic polymers, although being inexpensive and easily fabricated, have shown limited 3D scale-up capabilities. Hydrogels address the need for 3D *in vivo*-like environment but are not easily scalable.

Lastly, synthetic peptides are easily degradable^[47] and difficulties with sterilization and high production costs exist^[47,48]. Nevertheless, Synthemax Surface has shown to be capable for clinical applications since it can be sterilized via gamma irradiation, has a long shelf life of two years and can be stored at room temperature^[39]. Furthermore, hESCs can be cryopreserved and thawed on substrate and studies have demonstrated its capability for long term hESCs self-renewal and maintenance^[38-40]. Its scalability has also been demonstrated in T75 flasks^[38]. Therefore, we conclude that Synthemax Surface is an ideal substrate for clinical applicability.

FUTURE DIRECTION

Although feeder free, xeno-free and chemically defined media has been developed, the clinical applicability of hESCs depends on synthetic substrates that can easily and economically be manufactured, can undergo common sterilization methods without degradation (*i.e.*, reusability) and yield large number of cells as required for transplantation dosage (2 × 10⁸ cells/kg per dose)^[64]. Microcarriers and suspension cultures have

been used to meet these demands but their limitations in controlling aggregate size, passaging challenges, shear forces in stirred cultures and difficult cell extraction from microcarrier have caused researchers to seek alternatives. As a result, significant need exists for developing scalable synthetic substrates like Synthemax that can work with multiple cell lines (without conferring epigenetic modifications to cells), easily cryopreserved, are non-labor intensive (*i.e.*, automation), adaptable to induce differentiation conditions and require fewer exogenous factors to maintain hPSCs self-renewal and expansion.

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Hematopoietic stem cell-derived adipocytes and fibroblasts in the tumor microenvironment

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Abstract

The tumor microenvironment (TME) is complex and constantly evolving. This is due, in part, to the crosstalk between tumor cells and the multiple cell types that comprise the TME, which results in a heterogeneous population of tumor cells and TME cells. This review will focus on two stromal cell types, the cancer-associated adipocyte (CAA) and the cancer-associated fibroblast (CAF). In the clinic, the presence of CAAs and CAFs in the TME translates to poor prognosis in multiple tumor types. CAAs and CAFs have an activated phenotype and produce growth factors, inflammatory factors, cytokines, chemokines, extracellular matrix components, and proteases in an accelerated and aberrant fashion. Through this activated state, CAAs and CAFs remodel the TME, thereby driving all aspects of tumor progression, including tumor growth and survival, chemoresistance, tumor vascularization, tumor invasion, and tumor cell metastasis. Similarities in the tumor-promoting functions of CAAs and CAFs suggest that a multipronged therapeutic approach may be necessary to achieve maximal impact on disease. While CAAs and CAFs are thought to arise from tissues adjacent to the tumor, multiple alternative origins for CAAs and CAFs have recently been identified. Recent studies from our lab and others suggest that the hematopoietic stem cell, through the myeloid lineage, may serve as a progenitor for CAAs and CAFs. We hypothesize that the multiple origins of CAAs and CAFs may contribute to the heterogeneity seen in the TME. Thus, a better understanding of the origin of CAAs and CAFs, how this origin impacts their functions in the TME, and the

temporal participation of uniquely originating TME cells may lead to novel or improved anti-tumor therapeutics.

Key words: Hematopoietic stem cell; Cancer associated adipocyte; Mesenchymal stromal cell; Tumor progression; Cancer associated fibroblast; Plasticity; Metastasis; Fibrocyte

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Core tip: This review examines the roles of cancer-associated adipocytes (CAAs) and cancer-associated fibroblasts (CAFs) in remodeling of the tumor microenvironment (TME), presents evidence for a unique hematopoietic stem cell origin for both CAAs and CAFs, and discusses potential therapeutic implications of this novel origin. Studies highlighted herein emphasize the necessity of developing an understanding of the origins of cells in the TME and the importance of multipronged therapeutic targets directed at preventing both the incorporation and effects of stromal remodeling by the cells of the TME.

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INTRODUCTION: THE TUMOR MICROENVIRONMENT

The "seed and soil" hypothesis suggests that an appropriate host microenvironment ("soil") must be present for the optimal growth of tumor cells ("seed")^[1-3]. Although this paradigm was initially proposed by Stephen Paget in 1889, research efforts have predominantly focused on the epithelial component of solid tumors and tumor cell-intrinsic factors leading to tumorigenicity. However, in the last decade, Paget's hypothesis has again come to focus and it has been recognized that the epithelial "seed" and stromal "soil" components co-evolve and interact during tumor progression^[4]. Seminal works from Weinberg's group have shown that this stromal compartment, often referred to as the reactive stroma or tumor microenvironment (TME), directly and indirectly supports tumor survival, growth, vascularization, escape from immune surveillance, drug resistance, and metastasis *via* extracellular matrix (ECM) remodeling and production of growth factors, cytokines, and chemokines (reviewed in^[5-7]). The TME is comprised of a variety of cell types including endothelial cells, perivascular cells, immune cells, adipocytes, and fibroblasts/myofibroblasts.

These cells interact with one another as well as with tumor cells to create an intricate network of cellular crosstalk and bidirectional regulation.

This crosstalk results in a heterogeneous population of tumor cells exhibiting varying degrees of differentiation, unregulated proliferation, the capacity to migrate and invade through surrounding tissue, and the ability to establish a dense irregular and leaky vascular network, all critical steps in metastatic tumor progression. Concomitantly, this crosstalk leads to changes in the local stromal populations, contributing to the heterogeneity of TME cells. The heterogeneity of the cells of the TME, the factors they contribute and their broad functional ability to promote all aspects of tumor progression make the "soil" a challenging and complex therapeutic target. Many factors contribute to the heterogeneity of these cell types, including exposure to the local tumor milieu, the plasticity between cells of the TME, and the multiple potential origins of each cellular population. Understanding the mechanisms behind this heterogeneity could lead to the identification of novel therapeutic targets for cancer. This review will focus on two stromal cell types, the cancer-associated adipocyte (CAA) and the cancer-associated fibroblast (CAF). The adipocyte is a stromal cell type that has recently been implicated in tumor initiation, growth, and metastasis (reviewed in^[8]). Several epidemiologic studies have linked obesity with multiple types of cancer^[9-11]. Recent clinical studies have reported a positive correlation between the presence of CAAs at the tumor margin and poor patient outcome, suggesting that CAAs contribute to the permissive pro-TME, particularly in adipocyte-rich tissues, such as the mammary gland^[12,13] (and reviewed in^[14]). CAFs, the most abundant cellular component of the TME in solid tumors, have a significant impact on tumor progression during multiple stages^[5-7]. While more extensively studied than CAAs, the numerous roles of CAFs in tumor progression and metastasis are still under investigation. Like CAAs, CAFs have clinically been correlated with tumorigenesis and poor prognosis in many cancer types^[15-18]. Similarities in the pro-tumorigenic functions of CAAs and CAFs suggest that these TME cell types may act in concert to promote tumor progression, indicating that therapeutic targeting of the TME may need to encompass both cell types. Herein, we will examine the phenotype and function of CAAs and CAFs in remodeling of the TME, present evidence for a unique hematopoietic stem cell origin for both CAAs and CAFs, and discuss potential therapeutic implications of this novel origin.

CONTRIBUTIONS OF CAAS AND CAFS TO TME REMODELING

Cancer has been likened to a perpetual wound

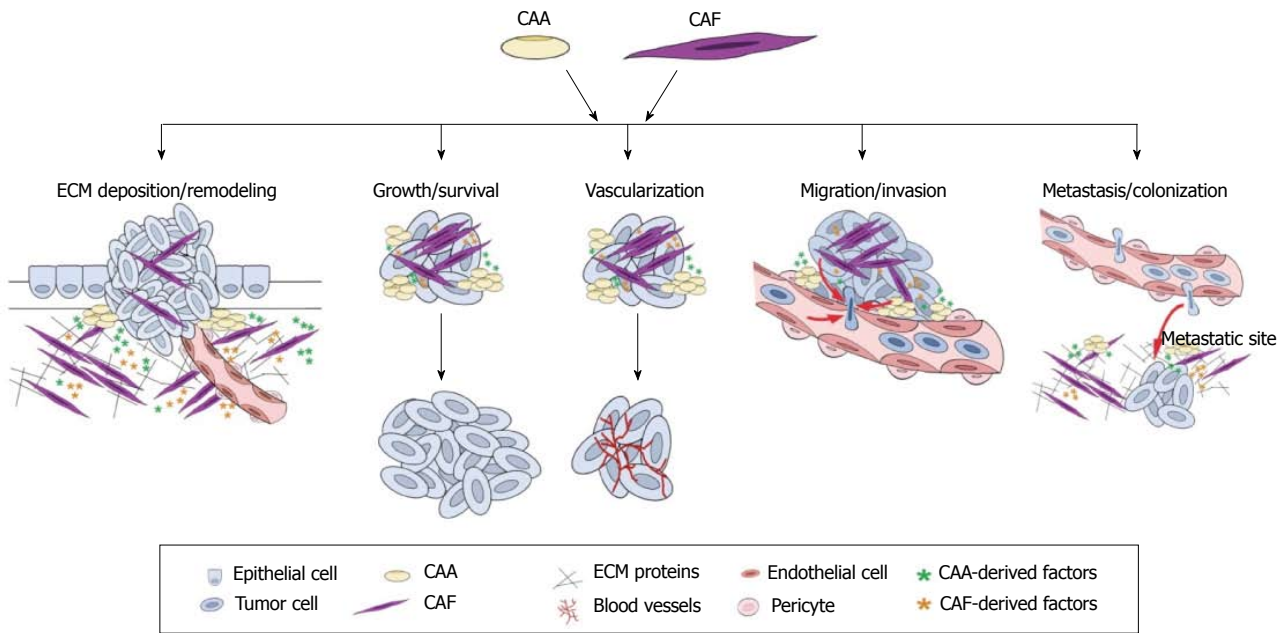


Figure 1 Multifactorial contributions of cancer-associated adipocytes and cancer-associated fibroblasts to tumor progression and metastasis. Research in the last decade has highlighted the importance of the tumor microenvironment in cancer progression. While there are numerous stromal cell types that contribute to the tumor microenvironment, this illustration depicts roles for cancer-associated adipocytes (CAAs) and cancer-associated fibroblasts (CAFs) in promoting the multiple stages of tumor progression and metastasis. ECM: Extracellular matrix.

healing process^[19] since both processes begin with the formation of a reactive stroma. During wound healing the reactive stroma resolves rapidly, but, during cancer progression, this actively remodeling, inflammatory state is perpetuated. CAAs and CAFs have been shown to play a role in a variety of tumor promoting processes including ECM deposition/degradation, inflammation and immune surveillance, tumor growth and survival, angiogenesis, invasion, and metastasis^[5,6,20-24], suggesting similarities in the pro-tumorigenic functions of these cells. As summarized in Figure 1, this section will discuss the CAA and CAF phenotypes and their roles in generating and maintaining the reactive stroma associated with cancer progression and metastasis.

The activated phenotype of CAAs and CAFs

Adipocytes, surrounded by fibroblasts, preadipocytes, pluripotent stem cells, endothelial cells, and immune cells, are the major components of the adipose tissue. Apart from their traditional function in energy storage, adipocytes are also considered endocrine cells, producing hormones, growth factors, cytokines and adipokines, including leptin, adiponectin, resistin, vascular endothelial growth factor (VEGF), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6)^[25]. During interaction with cancer cells, adipocytes acquire phenotypic changes and are reprogrammed to an activated state during which they are referred to as CAAs^[26]. CAAs are generally located at the invasive front of tumors^[21]. Several reports suggest that cancer cells can induce metabolic changes in adipocytes,

resulting in enhanced lipolytic activity and an inability to properly store triglyceride^[27]. Moreover, transforming growth factor- β (TGF- β), secreted by cancer cells or local stroma, is a potent inhibitor of adipocyte differentiation^[28]. Thus, the associated morphological changes upon activation include loss of lipid content (delipidation) and acquisition of a fibroblast-like/preadipocyte phenotype (de-differentiation)^[21]. Functional alterations in CAAs include loss of terminal adipocyte markers and products (adiponectin, resistin, fatty acid binding protein-4 (FABP4), hormone sensitive lipase (HSL), and CCAAT/enhancer binding protein- α (C/EBP α)) and an increased production of pro-inflammatory cytokines IL-6, IL-1 β , plasminogen activator inhibitor-1 (PAI-1)^[21]. As detailed below, in this activated state, CAAs produce adipokines and inflammatory factors that have been shown to promote tumor progression in adipocyte-rich environments.

In non-malignant tissues, fibroblasts provide structure and ECM scaffolding for tissues. In a wound environment, these fibroblasts become activated, produce increasing amounts of ECM proteins and migrate to wound interfaces to cause wound contraction and closure. In both wound healing and the TME, fibroblast activation is marked by increased α -smooth muscle actin (α SMA) protein expression, along with increased expression of vimentin, desmin, fibroblast specific protein, platelet-derived growth factor receptor α and β (PDGFR α and β), fibroblast activation protein (FAP), or a combination of these markers. TGF- β is present in both the wound and TMEs and has been shown to both induce and suppress differentiation and

tumorigenesis in a dose and context specific manner^[29]. With respect to fibroblasts, TGF- β has been shown to upregulate α SMA expression^[30,31], induce expression of FAP^[32], and promote collagen synthesis^[33], hallmarks of activated fibroblasts. Like CAAs, activated fibroblasts in the TME are also characterized by increased and altered production of inflammatory cytokines, chemokines, ECM components, and growth factors. The clinical importance of this activated phenotype is highlighted by molecular profiling studies of CAFs and matched normal fibroblasts. Studies in non-small cell lung cancer^[16] and breast cancer^[34] revealed that the cancer-associated gene signatures in CAFs correlated to disease outcome.

CAAs and CAFs in ECM remodeling and establishment of a reactive stroma

CAAs have been shown to play an important role in stromal remodeling during tumorigenesis. Type VI collagen, a soluble ECM protein, was reportedly up-regulated in peritumoral adipocytes during tumorigenesis^[22] and was shown to promote early mammary tumor progression *in vivo*^[23]. The $\alpha 3$ chain cleavage product of type VI collagen, endotrophin, augmented fibrosis, angiogenesis and inflammation through recruitment of macrophages and endothelial cells^[23]. Rio and colleagues found that the native $\alpha 3$ chain of type VI collagen constituted a specific substrate for matrix metalloproteinase (MMP)-11, whose collagenolytic activity was functional in fat tissue ontogenesis as well as during cancer invasive steps^[24]. Interestingly, they also reported that invasive breast cancer cells induced the expression of MMP-11 in the neighboring CAAs^[20], suggesting that exposure to tumor cells promotes stromal remodeling abilities of CAAs.

CAF function to generate and remodel ECM through production of collagens, fibronectin, and laminin^[30,35], and proteases such as MMPs^[36,37]. Collagens, fibronectin, and laminin contribute to the stiffness and density of the stroma, give structural support to the tumor cells, and provide important mechano-signals in the TME. Additionally, the expression of α SMA by activated fibroblasts was shown to promote matrix contraction^[38-40], suggesting a direct effect on matrix stiffness. Like CAAs, CAFs also produce MMPs that degrade matrix collagens, fibronectins, and proteoglycans, profoundly contributing to structural remodeling of the TME. It has also been demonstrated *in vitro* that fibroblast overexpression of FAP, a serine protease selectively produced by CAFs, remodeled the ECM by increasing expression levels of α SMA, fibronectin, and collagen I^[41]. These data indicate that, orchestrated by the tumor-stroma crosstalk, CAAs and CAFs actively remodel the ECM to favor local tumor progression.

In addition to elaboration and remodeling of matrix, CAAs and CAFs may also promote changes in the local stroma by contributing to the inflammatory

state of the TME. As described above, CAAs have been shown to produce a variety of inflammatory cytokines including IL-6, IL-8, IL-1 β and TNF- α ^[8,26]. Through production of factors such as IL-1 β , IL-23, TGF- β , IL-6, and IL-8^[42-44], CAFs exert considerable influence over the inflammatory state of the TME. CAF production of ECM components such as hyaluronic acid further drive the inflammatory state by recruiting tumor-associated macrophages^[45] that promote tumor vascularization and proliferation. While the interactions between CAAs, CAFs and immune cells are only beginning to be explored, it is clear that cellular cross-talk modulates the inflammatory state of the TME, potentially influencing tumor-specific immunity.

Tumor growth and survival

Both CAAs and CAFs aid the tumor in meeting requirements for rapid growth by providing structural matrix as described above as well as directly promoting tumor cell proliferation and survival. Rapid, unchecked proliferation is characteristic of tumor cells, and as the cells of the TME remodel the reactive stroma, tumor cell proliferation is further accelerated. In addition to proteases and ECM constituents, CAAs provide their high energy content lipids to cancer cells resulting in accelerated tumor progression^[46]. In support of this, morphologically, CAAs at the tumor invasive front are smaller than those observed at a distance, which implies lipolysis. In the case of ovarian cancer, the cancer cell-adipocyte interaction initiated HSL-mediated lipolysis in the adipocytes, releasing fatty acids, which were then taken up by the ovarian cancer cells for energy production through β -oxidation^[46]. In a PC-3 model of prostate cancer, the translocation of lipid from adipocytes to prostate cancer cells was visualized by Fourier transform infrared spectroscopy^[47]. Together these studies suggest that in multiple cancer types, CAAs supply the TME with energy rich lipids that may act to promote tumor growth by supplying tumor cells with essential metabolites.

Furthermore, adipocytes secrete adipokines into the TME, such as TNF α , IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1/CCL2), and leptin which have been shown to enhance tumor growth locally and systemically in a variety of cancer types (reviewed in^[8]) including prostate^[48,49] and breast^[14,21] cancers. Another aspect of adipocyte pro-tumorigenic activity is their contribution to chemo/radio-therapy resistance. The antitumor effect of vincristine, daunorubicin and dexamethasone to acute lymphoblastic leukemia (ALL) was impaired under the influence of adipocytes, which augmented ALL cell survival due to increased expressions of Bcl-2 and Pim-2^[50]. Likewise, breast tumor cells co-cultivated with adipocytes or recombinant IL-6 exhibited radioresistance, an increase in the effector

kinase Chk1, and a decrease in cell death^[51]. Taking into account that CAAs secrete elevated levels of IL-6 in the TME, CAAs could promote chemoresistance *via* multiple mechanisms. These studies shed light on a new role of CAAs in fostering a chemo/radio-resistant phenotype in cancers and suggest targeting CAAs may increase effectiveness of conventional chemotherapy treatments.

CAFs also promote tumor growth through the production of factors that have been shown to be involved in proliferation of tumor cells, including TGF- α ^[52], TGF- β ^[53], hepatocyte growth factor (HGF)^[54], and others (reviewed in^[44,55]). Through production of MMPs, CAFs act to release stored growth factors from within the matrix, further contributing to an enriched host microenvironment and promoting proliferation of tumor cells (reviewed in^[37]). Studies have identified a novel mechanism of cellular respiration, coined the “reverse Warburg effect” that involves an interplay and exchange between tumor cells and stromal cells whereby tumor cells take up energy-rich metabolites from CAAs^[8] and CAFs^[56] for use in the mitochondrial TCA cycle. This may contribute to the rapid proliferation of tumor cells by directing cellular energy towards cell division rather than cellular respiration. CAFs, like CAAs, have been implicated in promoting tumor chemoresistance. The expression and organization of collagen type I has been inversely correlated with intratumoral uptake of chemotherapeutic agents *in vivo* as it contributes to increased interstitial fluid pressure, forming a barrier to trans-capillary transport of agents^[57]. DNA vaccine targeting of FAP on CAFs led to decreased deposition of a collagen I rich matrix and improved chemotherapeutic drug uptake in pre-clinical animal studies^[58]. Direct targeting of CAF-derived FAP also suppressed primary tumor cell growth in a pre-clinical murine model of multi-drug resistant breast cancer^[58]; however, this finding has not held in Phase II clinical trials. *In vitro* studies revealed that adherence of melanoma cells to fibroblast monolayers allowed for reduction of the cytotoxic effects of cisplatin, supporting a role for the CAF-induced ECM^[59] in tumor cell survival. CAFs from melanoma and prostate cancer were found to be less sensitive to etoposide and vincristine due to expression of a non-mutated but functionally deficient form of p53^[60]. Together, these findings suggest CAFs promote tumor growth and survival through multiple mechanisms and these effects may contribute to both the primary tumor and metastatic site.

Tumor vascularization

Angiogenesis is a critical step in tumor progression, without which tumors cannot maintain growth beyond 1-2 mm³^[61]. Many of the key factors required to initiate the angiogenic switch in solid tumors are produced by CAAs and CAFs. Adipocytes are known to produce multiple angiogenic factors [VEGF, fibroblast growth

factor-2 (FGF2)], adipokines (leptin, adiponectin, resistin) and cytokines (IL-6), all of which stimulate angiogenesis and contribute to an overall pro-angiogenic microenvironment for tumor progression^[62]. CAFs have been shown to produce angiogenic factors including stromal derived factor-1 (SDF-1)^[63], TGF- β ^[64], IL-6^[65] and VEGF^[66,67], which support endothelial cell proliferation and tumor vascularization. In addition to their production of angiogenic cytokines, CAFs may play a more direct role in tumor vascularization through their ability to serve as vascular support cells. CAF and myofibroblast expression of α SMA closely links this cell type with the pericyte, a fibroblast-like cell, which plays a supportive role for endothelial cells in both normal and tumor systems. Thus, CAAs and CAFs are a source for critical angiogenic factors and may be involved in flipping the hypothetical switch to a vascularized tumor site, thereby acting to support an essential early step in tumorigenesis.

Invasion and metastasis

One of the initial steps in metastasis of solid tumor is the migration and invasion of the tumor cell through the ECM and through the basement membrane. This is followed by intravasation of tumor cells into a local blood vessel and the extravasation of the tumor cell to colonize and proliferate at a distant site. CAAs secrete similar levels of MMP-2 and MMP-9 compared to normal adipocytes^[21], whereas MMP-11 is highly expressed by CAAs in the proximity of invading cancer cells, but not in normal resting adipocytes^[24]. The role of MMP-11 (stomelysin-3) in tumor biology is still unclear (reviewed in^[37,68]). However, MMP-11 has been shown to promote tumorigenesis and function through its proteolytic activity^[69,70], but only weakly degrades matrix molecules. CAFs have been shown to produce a variety of matrix metalloproteinases (MMP), including MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13, and MMP-14 (reviewed in^[36,37]). Degradation of ECM allows tumor cells to cross the structural barrier of the basement membrane, a key step in tumor metastasis (reviewed in^[37]). In an elegant imaging study, Gaggioli^[35], demonstrated that CAFs are able to degrade matrix to form tracks through the ECM that allow invading tumor cells to efficiently follow behind.

CAAs and CAFs also directly affect the migratory and invasive abilities of tumor cells through production of adipokines, cytokines, and chemokines. Adipocyte/CAA-secreted IL-6 has been shown to play a key role in mediating adipocyte-dependent invasive activity of both breast cancer cells and melanoma cells^[21,71]. FAP production by fibroblasts was linked to the increased invasion of pancreatic cancer cells in a β_1 -integrin/FAK mediated fashion^[41]. In breast cancer, CAFs were shown to increase the invasive ability of DCIS epithelial cells and this was related to their production of MMP-9 and MMP-14^[72,73]. In addition, these factors secreted by CAAs and CAFs may flood

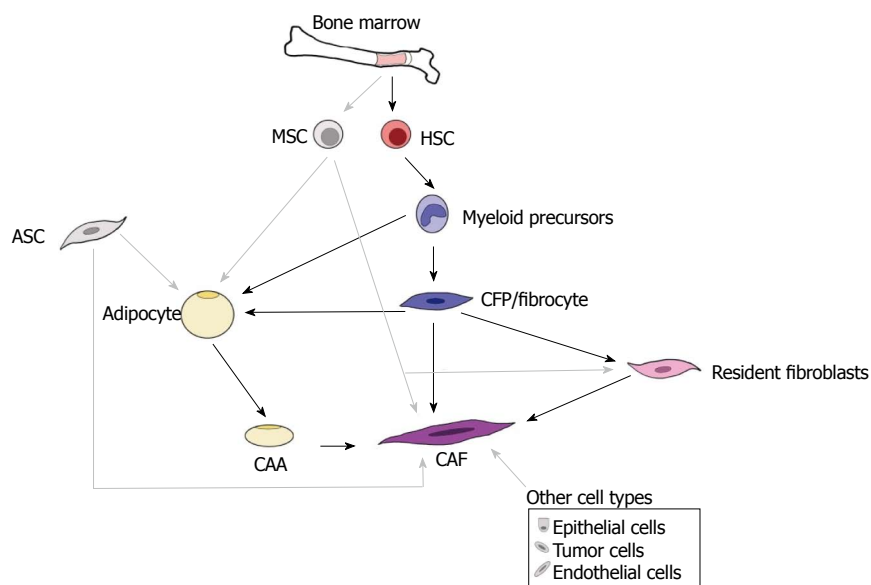


Figure 2 Origins of cancer-associated adipocytes and cancer-associated fibroblasts. This drawing illustrates the complex and ever-growing understanding of the origins for cancer-associated adipocytes (CAAs) and cancer-associated fibroblasts (CAFs). CAAs and CAFs are generally thought to arise from resident tissue cells, the adipose stem cell (ASC) and resident fibroblast, respectively. However, alternative sources for adipocytes and CAAs have been demonstrated including cells of the bone marrow, specifically those of the myeloid lineage (e.g., macrophages, CFPs, and fibrocytes). In addition to resident fibroblasts, CAFs have been shown to be derived from myeloid progenitors (CFPs, fibrocytes), mesenchymal stromal cells (MSCs), ASCs, CAAs, epithelial cells, tumor cells, and endothelial cells. It is possible that these multiple sources are reflected in the morphological, phenotypic, and functional heterogeneity described for adipocytes and for CAFs. HSC: Hematopoietic stem cell; CFPs: Circulating fibroblast precursors.

the circulation with signals for distant metastatic sites to initiate their own expression of chemokines that will aid the tumor cell in homing to the metastatic site and preparing the site for colonization once the tumor cell arrives^[74]. Research conducted on human omental adipocytes indicates they secrete IL-6 and IL-8 and that antibody-mediated inhibition of either factor resulted in reduced homing of ovarian cancer cells *in vitro* and *in vivo*, although inhibition of IL-8 was more efficient at reducing homing of cancer cells *in vivo*^[46]. Once ovarian tumors are established on the omentum, they may convert adjacent adipocytes into CAAs, which results in a positive feedback loop leading to increased IL-6 production and further recruitment of cancer cells to the omentum. It has also been suggested that tumor cells do not metastasize alone, rather, they “travel” with stromal cells. Studies from Duda *et al.*^[75] demonstrated by cannulating primary tumor bearing mice, that tumor cells are shed with stromal cells in heterotypic “clumps” from the primary tumor. The stromal component, which included fibroblasts, acted to support the viability of the tumor cells while traveling through the circulation to the metastatic site. Together, these studies suggest that CAAs and CAFs promote the migratory and invasive phenotype in a variety of solid tumors and highlight the importance of elucidating mechanisms to target both CAAs and CAFs.

ORIGINS OF CAAS AND CAFS IN THE TME

Both CAAs and CAFs are generally thought to arise from tissues adjacent to the tumor; however, recent studies have begun to demonstrate alternative sources, including other resident stromal cells, epithelial cells, and bone marrow. This complex and ever-growing understanding of the origins for CAAs and CAFs can be appreciated in Figure 2. It is possible that these

multiple sources are reflected in the morphological, phenotypic, and functional heterogeneity described for adipocytes from different fat depots^[76] and for CAFs^[77,78]. Given that this heterogeneity is a significant hurdle in therapeutically targeting the TME populations, it will be essential to elucidate the multiple origins for these cells as well as to examine the impact these origins may have on cellular function.

Origins of CAAs

The expansion of adipose tissue is achieved *via* increases in size (hypertrophy) and/or number (hyperplasia) of adipocytes. Mature adipocytes are postmitotic, therefore, adipocyte hyperplasia requires new adipocytes be produced from their adipogenic precursors. A long-standing paradigm of adipocyte generation is that all adipocytes are differentiated from mesenchymal progenitor cells resident in the vascular stroma, referred to as adipose stem cells (ASCs), where the regional fat depots eventually form^[79,80]. However, these progenitor cells are found associated with adipose vessels^[79], bringing up the possibility that circulating progenitors, such as those provided by bone marrow, home to adipose tissue through the bloodstream followed by extravasation across the endothelium of blood vessels, subsequently undergoing adipogenic conversion. To test this hypothesis, several groups transplanted GFP-labeled bone marrow into wild-type mice^[81-83]. Two of these groups detected GFP-expressing adipocytes in the major adipose depots^[81,82], while one failed to detect these cells^[83], perhaps due to low marker expression or limited engraftment. When engrafted mice were treated with rosiglitazone or a high fat diet that stimulated adipogenesis, the number of GFP-expressing adipocytes was elevated, and cells were often found in clusters, suggesting clonal growth from bone marrow-derived progenitors^[81]. While these studies support a bone marrow origin for CAAs, it is unclear which bone

marrow stem cell serves as the CAA progenitor.

It is commonly held that the bone marrow contains two types of stem cells, the mesenchymal stromal cell (MSC) and the hematopoietic stem cell (HSC). MSCs are defined by their adherence to plastic and potential to differentiate into mesenchymal tissue cells such as bone, fat, muscle, cartilage, and fibroblasts^[84-87]. HSCs are defined by their capability of hematopoietic reconstitution *in vivo* and have also been shown to give rise to other tissue cell types including mast cells and osteoclasts. Our laboratory has developed a method for transplantation of a clonal population from a single sorted HSC defined as an EGFP⁺Lin⁻Sca-1⁺c-Kit^{hi}CD34⁻ cell. Using this single cell transplantation model, we have demonstrated that HSCs give rise to a variety of mesenchymal cell types including adipocytes^[88], osteocytes and chondrocytes^[89], cardiac valve interstitial cells^[90], circulating fibroblast precursors^[91], and fibroblasts and myofibroblasts in multiple tissues^[92-94] (reviewed in^[95]). Further use of our unique clonal cell transplantation model revealed the generation of adipocytes *in vivo* from clonally derived bone marrow HSCs^[88]. Similar to findings from Crossno *et al.*^[81], we found that rosiglitazone stimulated adipogenesis from the HSC^[88]. *In vitro*, clones giving rise to monocytes/macrophages under hematopoietic conditions were also able to generate adipocytes under adipogenic conditions, suggesting a differentiation pathway from HSCs-myeloid precursors-adipocytes^[88]. Similar results were obtained from the Klemm group who confirmed the *de novo* generation of a subset of adipocytes from bone marrow myeloid progenitor cells using a non-transplant transgenic mouse model in which LacZ expression was restricted to the myeloid lineage^[96]. Moreover, Sterieter and colleagues reported that circulating fibrocytes were capable of adipogenic differentiation both *in vitro* and *in vivo*^[97]. Due to the dual hematopoietic/mesenchymal nature of fibrocytes, they may be considered as an intermediate for myeloid-derived adipocyte population. These studies demonstrate the ability of the HSC to give rise to adipocytes through the myeloid lineage. While studies have not yet directly examined the role of HSC-derived CAAs in tumor progression, findings from our laboratory suggest that they can enhance tumor growth and tumor cell motility in breast cancer and melanoma models (unpublished data).

Origins of CAFs

Traditionally, CAFs are thought to arise from resident tissue fibroblasts^[98]. However, recent studies have suggested alternative sources including other resident stromal cells, epithelial cells, epithelial-mesenchymal transition or endothelial-mesenchymal transdifferentiation (EndoMT), and adipose tissue. Several studies have also suggested a bone marrow origin for myofibroblasts and CAFs^[99] (and reviewed in^[100-102]). As described above, the bone marrow provides a rich source for both MSCs and HSCs. Using

our model for transplantation of a clonal population from a single sorted HSC in conjunction with a variety of solid tumor models, we have demonstrated the presence of HSC-derived fibroblasts in tumor sections from mice transplanted with a clonal population of cells derived from a single, sorted HSC^[91,103]. Analysis of sections from Lewis lung carcinoma (LLC) and melanoma (K1735-M2) tumors harvested from clonally engrafted animals showed the presence of HSC-derived CAFs^[91,103]. These EGFP-expressing cells had a fibroblastic morphology and constituted 8%-28% of the tumor stromal cells^[103]. Characterization of these HSC-derived cells indicated that they were activated fibroblasts, based on expression of α SMA and mRNA expression of collagen I^[103]. Also prevalent in the specimens were EGFP⁺ pericyte-like perivascular cells, suggesting that HSCs contribute to tumor vasculature^[103].

Work from our laboratory has also identified a population of circulating fibroblast precursors (CFPs) that express markers of both hematopoietic cells (CD34, CD45) and fibroblasts [collagen I (Col I), discoidin domain receptor-2 (DDR2; a collagen 1 receptor)]^[91] (and unpublished data). The CD45⁺DDR2⁺ population was shown to differentiate along the monocyte/macrophage lineage, contain the CD34⁺Col I⁺ fibrocyte and rapidly differentiate to collagen 1⁺, α SMA⁺ cells with fibroblastic morphology. Using our clonal hematopoietic stem cell transplantation model, we conducted an *in vitro* examination of CFPs/fibrocytes derived from peripheral blood cells of clonally engrafted mice^[92]. In these studies, nucleated blood cells were cultured and the appearance of EGFP⁺ (HSC-derived), spindle-shaped or polygonal cells was detected by the seventh day. Flow cytometric time course analysis of the cultured cells demonstrated decreasing CD45 expression and increasing DDR2 expression. Our studies have demonstrated that CFPs may be stimulated to express markers of activated fibroblasts including collagen, vimentin, and α SMA by exposure to tumor conditioned media. This demonstrates a possible differentiation pathway from the HSCs-myeloid precursors-CFP and with exposure to tumor, HSCs-myeloid precursors-CFP-CAF^[91]. These findings are supported by our *in vivo* data demonstrating the activated fibroblast phenotype of HSC-derived cells recruited from the bone marrow to the tumor stroma^[91].

CAA and CAF common origins and plasticity

As summarized in Figure 2, multiple origins for CAAs and CAFs have been proposed. Evidence also suggests that CAAs and CAFs may share a common origin. Data from our laboratory using clonal cell lineage tracing demonstrated a monocyte lineage origin for adipocytes, specifically the Mac1^{lo} fraction of bone marrow^[88]. Similarly, lineage and gene expression analyses demonstrated that adipocytes and adipocyte progenitors arise from the hematopoietic stem cell *via* the myeloid lineage^[96]. Our studies of CAFs

and their circulating precursors demonstrated that these cells originate in the Mac1^{hi} population of peripheral blood and that their participation in tumor may be regulated by MCP-1^[91]. Additional evidence suggests plasticity between preadipocytes and macrophages, with preadipocytes being a source for macrophages^[104] and tissue macrophages being a source for preadipocytes^[105]. Histological evidence of a high ratio of adipocytes to fibroblasts at the tumor invasive front and an extremely high fibroblast-like cell to adipocyte ratio observed at the tumor center, suggests that CAAs may transition and/or give rise to CAFs as tumor progresses^[14]. Breast cancer cells were also shown to induce de-differentiation of adipocytes to a more fibroblastic phenotype^[21]. Human adipose tissue derived stem cells (hASCs) were found to give rise to CAF-like cells when cultured with conditioned media from MDA-MB-231 or MCF-7 breast cancer cell lines^[106]. Under tumor conditions, the hASC-derived CAF-like cells were shown to have a myofibroblastic phenotype, with increased expression of α SMA and tenascin C. This change in phenotype was found to be dependent upon TGF β signaling in the hASCs. Studies have yet to directly demonstrate a CAF to CAA conversion, however, we have observed *in vitro* that non-adherent bone marrow cells, enriched for hematopoietic progenitors, cultured in the presence of M-CSF and mouse serum give rise to lipid laden cells with a fibroblast-like morphology (unpublished observation). Together, these studies support an HSC origin for both CAAs and CAFs, suggesting plasticity exists between adipocytes, CAAs, and CAFs. This plasticity may be one mechanism by which heterogeneity of the TME is generated.

CONCLUSION

Evidence suggests that CAAs and CAFs play a critical role in tumor progression as well as patient prognosis and survival. It is thought that the metabolic changes associated with obesity underlie the increased risk of cancer and cancer-related mortalities. It has been estimated that excess weight and obesity were responsible for 20% of all cancer deaths in women in the United States^[107], consistent with poor outcome of cancer in overweight/obese patients (reviewed in^[108]). While no direct comparisons of CAAs derived from distinct sources have been conducted, it is clear that HSC-derived adipocytes represent a subpopulation distinct from conventional white and brown adipocytes based on their low expression level of leptin, low mitochondrial/peroxisomal content and oxidative capacity, and elevated inflammatory cytokine production^[96]. HSC-derived adipocytes also share numerous features with CAAs including low expression of terminal adipocyte markers and high expression levels of inflammatory cytokines, indicating that HSC-derived adipocytes may be considered "activated" contributors to the TME. Like CAAs, HSC-

derived adipocytes were found to be smaller in size than "resident" adipocytes (^[81] and our unpublished observation), but whether, as with CAAs, this is related to a higher rate of lipolysis in these cells requires further exploration. It has been noted that HSC-derived adipocytes preferentially accumulate in visceral adipose tissue (VAT) rather than subcutaneous adipose tissue (SAT)^[96]. Excess adiposity in VAT is specifically linked to type 2 diabetes and certain forms of cancer^[76]. As compared to adipocytes from SAT, VAT adipocytes exhibited higher rates of fatty acid turnover and lipolysis^[109] and produced more IL-6 and less adiponectin and leptin^[110]. These data could indicate that VAT and SAT adipocytes are generated from different progenitors, or functional changes in different depots are due to differential accumulation of adipocytes arising from distinct progenitors. Furthermore, the accumulation of HSC-derived adipocytes was increased in female mice over males, which may have important inference in human biology^[96], as women generally possess a higher percentage of body fat and tend to disproportionately gain fat in VAT following menopause. Coupled with preferential accumulation of HSC-derived adipocytes in VAT, this pattern of adiposity represents a higher risk of adipose-related gynecological cancers for postmenopausal women and suggests HSC-derived CAAs may represent a novel target these patients.

Several studies suggest that CAFs and their unique phenotypes are associated with increased malignant potential. In the case of breast cancer, women with denser breast tissue have an increased tendency to develop cancer^[15]. The presence of a fibrous stroma was found to be associated with poor prognosis in squamous cell carcinoma^[18]. In non-small cell lung carcinoma, molecular analysis revealed a gene signature for CAFs that was associated with patient prognosis^[16]. Interestingly, recent pre-clinical studies in pancreatic ductal adenocarcinoma have demonstrated a protective role for CAFs^[111,112], suggesting that the role of these cells is tumor-type dependent. CAFs are a heterogeneous population of cells that can differ based on both location within the tumor and between tumor types and demonstrate different phenotypes, activation states, and/or functions throughout tumor progression. This diversity may, in part, be due to the multiple proposed origins of CAFs, which have led to CAFs being referred to as a "cell state" rather than a specific cell type^[113]. A more in depth understanding of the origins of CAFs may shed light on the array of markers expressed by these cells, help to better define the "CAF", and elucidate their roles based on origin and tumor type.

Given the essential roles of the TME in tumor development, progression and metastasis, it is clear that successful anti-tumor therapeutics should include those directed at the support cells of the TME. A key step towards this goal is the gaining knowledge of the role(s) of the different TME cell types (*e.g.*, CAAs

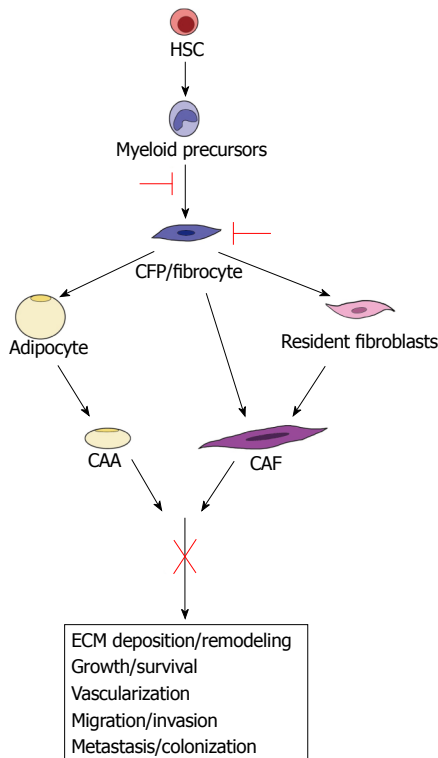


Figure 3 Therapeutic implications. We hypothesize that the identification of pathways from the hematopoietic stem cell (HSC) to the cancer-associated adipocyte (CAA) and cancer-associated fibroblast (CAF) provides a potential opportunity to target these pro-tumorigenic cells both early in their differentiation and at multiple points in their maturation, which may lead to a more encompassing downstream inhibition of their contributions to tumor progression and metastasis.

and CAFs), their origins, their temporal participation and mechanisms by which they influence tumor. However, the complexity of the crosstalk between the cells of the TME, the broad impact of CAAs/CAF on the reactive stroma and their contribution to the evolving TME has made therapeutically targeting individual stromal cell derived factors difficult. For example, targeting fibroblast activation protein alpha (FAP α) produced by activated CAFs, while showing promise in preclinical studies^[58,114,115], was demonstrated to have no beneficial response in Phase II clinical trials for metastatic colorectal cancer and soft-tissue sarcoma patients^[116] (and reviewed in^[117]). However, we propose that targeting CAAs, CAFs and their precursors based on their origin may lead to significant advances in treatment by directly targeting the cells before their incorporation into tumor rather than targeting their varied products and multiple effects (Figure 3). We and others have shown that HSCs give rise to adipocytes *via* the myeloid lineage^[88,96]. Likewise, our studies demonstrate an HSC origin for CAFs *via* the same myeloid lineage^[91]. Studies have also demonstrated a myeloid lineage origin for the fibrocyte^[118] that also gives rise to adipocytes^[97] and fibroblasts^[92,119]. These HSC-derived CAAs (unpublished observation) and CAFs^[91,103] (and unpublished observation) contribute

to the TME and have a significant impact on tumor progression in mouse models. Identification of pathways from the HSC to the CAA or CAF provides a potential opportunity to target CAAs and CAFs both early in their differentiation and at multiple points in their maturation. For example, early inhibition of CFP/fibrocyte differentiation from the myeloid lineage would lead to fewer CAA and CAF precursors available for incorporation in the TME. Likewise, directly targeting CFPs/fibrocytes in circulation may prevent their incorporation into the local and metastatic TME as both CAAs and CAFs, essentially hitting two arms of pro-tumorigenic stromal cells. Given their ability to invade, circulate and extravasate, therapeutically targeting HSC-derived CFPs/fibrocytes may also directly affect the population of CAFs demonstrated to chaperone cancer cells to metastatic sites. Finally, the ability to isolate HSC-derived CAA/CAF progenitors in circulation, combined with their intrinsic ability to home to tumor^[91], may provide a novel modality for drug delivery vehicles for chemotherapy. Thus, targeting the precursors of CAAs and CAFs may lead to a more inclusive and encompassing downstream inhibition of their multiple contributions to tumor progression and metastasis. Taken together, these studies highlight the necessity of developing an understanding of the differences and similarities between TME cell types of multiple origins as well as research directed at elucidating the differentiation pathway of these populations for the ultimate goal of TME-based anti-tumor therapy.

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Role of nanotopography in the development of tissue engineered 3D organs and tissues using mesenchymal stem cells

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Abstract

Recent regenerative medicine and tissue engineering strategies (using cells, scaffolds, medical devices and gene therapy) have led to fascinating progress of translation of basic research towards clinical applications. In the past decade, great deal of research has focused on developing various three dimensional (3D) organs, such as bone, skin, liver, kidney and ear, using such strategies in order to replace or regenerate damaged organs for the purpose of maintaining or restoring organs' functions that may have been lost due to aging, accident or disease. The surface properties of a material or a device are key aspects in determining the success of the implant in biomedicine, as the majority of biological reactions in human body occur on surfaces or interfaces. Furthermore, it has been established in the literature that cell adhesion and proliferation are, to a great extent, influenced by the micro- and nano-surface characteristics of biomaterials and devices. In addition, it has been shown that the functions of stem cells, mesenchymal stem cells in particular, could be regulated through physical interaction with specific nanotopographical cues. Therefore, guided stem cell proliferation, differentiation and function are of great importance in the regeneration of 3D tissues and organs using tissue engineering strategies. This review will provide an update on the impact of nanotopography on mesenchymal stem cells for the purpose of developing laboratory-based 3D organs and tissues, as well as the most recent research and case studies on this topic.

Key words: Nanotopography; Mesenchymal stem cells; Tissue engineering; Nanotechnology; Three dimensional organs/tissues; Scaffolds

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Core tip: Tissue engineering and nanotechnology are both exciting fields that have enormous potentials to revolutionise medicine as we know it today. Use of nanotechnology is an attractive and effective way to control and direct biological events at cellular levels. Nanoscale architecture plays a pivotal role directing cellular activities. Here, the use of nanotopography for the purpose of 3D organ/tissue regeneration using mesenchymal stem cells (*i.e.*, their proliferation, differentiation and function), is reviewed by investigating the most recent, innovative, and effective studies in this field.

Salmasi S, Kalaskar DM, Yoon WW, Blunn GW, Seifalian AM. Role of nanotopography in the development of tissue engineered 3D organs and tissues using mesenchymal stem cells. *World J Stem Cells* 2015; 7(2): 266-280 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i2/266.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i2.266>

INTRODUCTION

It is becoming progressively evident that with an ever increasingly older population and high costs associated with meeting the healthcare demands^[1] as well as the shortage of organs and effective therapeutic methods^[2], the field of medicine has to move towards cutting edge, laboratory engineered techniques and devices if, we are to avoid a drastic world-wide healthcare collapse in the near future. To this end, the fields of nanotechnology, regenerative medicine and tissue engineering (TE) are expanding at a rapid pace. Recent regenerative medicine and TE strategies (using cells, scaffolds, medical devices and gene therapy) have led to fascinating progress of translation of basic research towards clinical applications^[1,3]. In the past decade, great deal of research has focused on developing various three dimensional (3D) organs, such as bone^[4], skin^[5], liver^[6], kidney^[7], and ear^[8], using such strategies in order to replace or regenerate damaged organs for the purpose of maintaining or restoring organs' functions.

Human organs are responsible for various important functions of the body including, but not limited to, digesting food, serving as a barrier against infections, recognising and coordinating the body's response to its internal and external environmental changes, and providing oxygen (to be used for cellular respiration) as well as removing excess carbon dioxide. They are also responsible for maintaining homeostasis, transmission of information and generating force^[3]. In cases when one or a few of the organs are severely damaged, to an extent that they are no longer capable of reconstructing or regenerating themselves, tissue engineering and nanotechnology based strategies, using previously established knowledge on cellular behaviour^[9,10],

could be employed to develop and construct tailored therapies, devices, or even whole organs. To this end, nanostructured, bio-inspired, or biological materials have attracted a great deal of attention as they poses unique chemical, mechanical and surface characteristics that could prove useful for organ or tissue TE.

The surface properties of a material or a device are key aspects in determining the success of the implant in biomedicine, as the majority of biological reactions in human body occur on surfaces or interfaces^[11]. Furthermore, it has been established in the literature that cell adhesion and proliferation are, to a great extent, influenced by the micro- and nano-surface characteristics of biomaterials and devices^[12,13]. In addition, it has been shown that the stem cells', mesenchymal stem cells (MSCs) in particular, functions can be regulated through physical interaction with specific nanotopographical cues^[3,14], further indicating the importance of surface characteristics at nanometre length scale. Therefore, guided stem cell proliferation, differentiation and function are of great importance in the regeneration of 3D organs and tissues using TE strategies.

This review will provide an update on the impact of nanotopography on MSCs for the purpose of developing laboratory-based 3D organs, as well as the most recent research and case studies on this topic.

NANOFABRICATION OF 3D SCAFFOLDS WITH STEM CELLS

Previously, most research and investigations focused on growing cells in a petri dish (2D). However, in nature cells use 3D template of extracellular matrix (ECM) to shape functional tissues^[15]. Micro- and nano-scale chemical and physical cues from the ECM environment control and direct various key cell behaviours including their adhesion, proliferation, migration and differentiation^[16-18]. Therefore, the construction of a synthetic system that mimics the natural ECM and its component has become a field of topical interest^[19].

Recent investigations have shown rapid success in TE of sophisticated and complex nanoenvironments suitable for 3D growth of stem cells for the purpose of organ and tissue regeneration^[19-22]. So far, various biofabrication techniques have been developed and employed to design an ideal 3D synthetic ECM-mimetic system that resembles the architecture and mechanical properties of the natural ECM^[3]. Natural or synthetic polymers are used as scaffold materials and, depending on their nature, suitable biofabrication techniques are used to create a 3D environment with nanotopographical cues that can lead to controlled and directed growth and differentiation of stem cells toward a specific tissue or organ regeneration. Numerous studies have covered the currently available fabrication techniques for natural or synthetic polymers^[15,23-27]. In general, the available fabrication techniques can

Table 1 Classification of various types of nanotopography (nanofabrication) methods

Energy source	Method	Mechanism and final outcome	Processable polymers
Thermal	Replica modelling	Creating negative shape of the mold by thermal cross-linking of cavity-filled pre-polymer	Thermocurable polymers, <i>e.g.</i> , poly(dimethyl siloxane)
	Nanoimprint lithography	Creating negative shape of the mold by plastic deformation of polymer above T _g	Thermoplastic, <i>e.g.</i> , polystyrene, poly(lactic acid), and conductive polymers, <i>e.g.</i> , polyaniline and polypyrrole
Optical	Block copolymer lithography	Creating nanoscale hole, line and lamellar structures by microphase separation of two immiscible polymers	Block copolymer, <i>e.g.</i> , polystyrene-block-poly(methyl methacrylate), styrenebutadiene-styrene
	Photolithography	Depending on mask design and selective UV exposure, solubility is changed	Photo curable polymers, <i>e.g.</i> , photoresist, polyurethane-based
	E-beam lithography	Formation of arbitrary patterns using different electron beam pathways and selective irradiation of focused electron beams to change solubility	E-beam sensitive polymers, <i>e.g.</i> , polymethyl methacrylate
	Direct laser writing	Formation of arbitrary patterns by selective cross-linking of the polymer by laser irradiation	Photo-curable polymers
Chemical	Microcontact printing	Creating extruded patterns of elastomeric stamp using relative surface energy difference needed for transferring materials	Proteins and self-assembled monolayers
	Dip-pen lithography	Formation of arbitrary patterns by direct writing of molecules with a sharp tip	Self-assembled monolayers
	Salt leaching/gas foaming	Formation of a block of polymer with voids by dissolution of salt particles (salt leaching) and/or bubble formation in the polymer block (gas foaming)	Solvent soluble polymers, <i>e.g.</i> , thermoplastic and conductive ones
Electrical	Electrochemical deposition	Forming negatively shaped molds by electrochemical reduction of the polymer	Conductive polymers
	Electrospinning	Drawing a three dimensional nanofibrous mesh from the polymer solution using an electric field	Solvent soluble polymers
Physical	Capillary force lithography	Formation of partially filled negative shape of the mold by capillary rise of thermoplastic polymer above T _g	Thermoplastic and solvent soluble polymers
	Micromolding in capillaries	Creating a negative shape of the mold by capillary-driven microchannel filling	Solvent soluble polymers
	Wrinkle	Formation of random or aligned micro- or nanolines using mechanical buckling Mechanical buckling between elastic substrate and rigid film	Elastomeric polymers, <i>e.g.</i> , polydimethylsiloxane
	Crack	Formation of aligned or inter-crossing line patterns by mechanical fracturing of the stiff film adhered onto elastic substrate	Elastomeric polymers

Adapted from Kim *et al.*^[3].

be classified into different categories based on their energy source, *i.e.*, thermal, optical, physical, chemical or electrical (Table 1). It is beyond the scope of this paper to review the different available techniques in each of these categories, however, a review conducted by Kim *et al.*^[3] could be referred to for further and in detail information on this topic.

CHARACTERISTICS OF MSCs AND THEIR APPLICATION IN TE OF 3D ORGANS

An extensive number of studies have demonstrated the great potentials of using MSCs for TE approaches^[28-33]. Among many advantageous characteristics of MSCs the fact that these cells can be isolated from several tissues and that they have the potential to be expanded in culture and exhibit multilineage differentiation (Figure 1) make MSCs a highly interesting stem cell source for TE and regenerative medicine research^[31]. Other interesting properties of MSCs include; their ability to self-renew, modulate immune responses, and their availability (they can be obtained from a small scale aspirate of bone marrow or adipose tissues)^[34-36]. Furthermore,

MSCs can be isolated from adults, therefore, allogeneic transplant of these cells would eliminate raising ethical issues in regards to their use in TE and regenerative medicine^[36].

Among most potential characteristics of MSCs, it is probably their ability of multilineage differentiation that is mostly exploited for TE and regenerative medicine purposes. The differentiation of MSCs is controlled by some regulatory genes and induction chemicals that lead to the specific differentiation of these progenitor cells^[37,38]. In addition to growth factors and induction chemicals, various biomaterials (*i.e.*, natural and synthetic polymers) are used to provide appropriate scaffolding for the proliferation and differentiation of MSCs for the purpose of reconstruction of several hard and soft tissues and organs, such as bone, cartilage, tendons, and skin^[39,40] (Figure 2).

THE ROLE OF NANOTOPOGRAPHY ON THE GROWTH AND PROLIFERATION OF MSCS

As mentioned earlier, surface nanotopography of

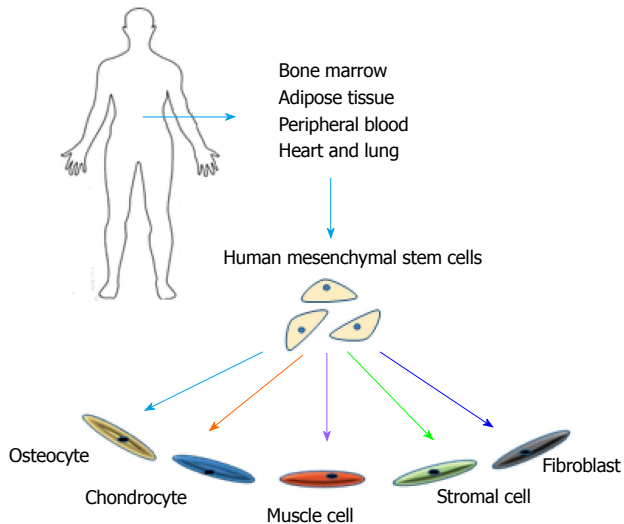


Figure 1 Potentials and sources of mesenchymal stem cells. Mesenchymal stem cells can be collected from various sources within human body and have the ability to differentiate into a variety of lineages.

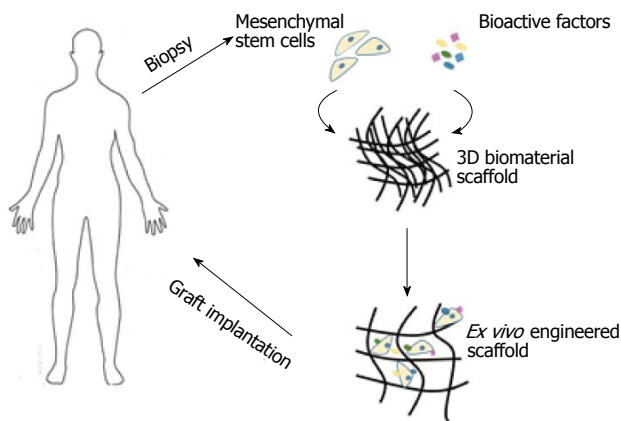


Figure 2 Overview of tissue engineering strategy of incorporating scaffolds with mesenchymal stem cells. Mesenchymal stem cells (derived directly from the patient) are expanded in the laboratory, whereby the necessary environment for their growth has been prepared. These cells are then seeded onto a scaffold and either allowed to differentiate *ex vivo* pre-implantation or the scaffold is immediately implanted.

biomaterials can evoke specific cellular responses. Materials with unique nanotopographical characteristics offer properties, similar to growth factors, which can be used to induce specific biological performances of safe and cost effective manners in the human body^[41]. Previous studies show that various nanotopographical cues can potentially impact the adhesion^[42,43], orientation^[44], and cytoskeletal organisation^[45] of MSCs as well as their self-renewal^[46], proliferation and differentiation^[41]. Furthermore, nanotopographical cues could influence morphology, migratory capacity, gene expression and subsequently the fate of MSCs^[47,48].

It has been shown that nanofeatures including nanopits, nanogratings and nanoprotusions have the potentials to influence the cell morphology, proliferation and differentiation of MSCs^[49,50] (Figure

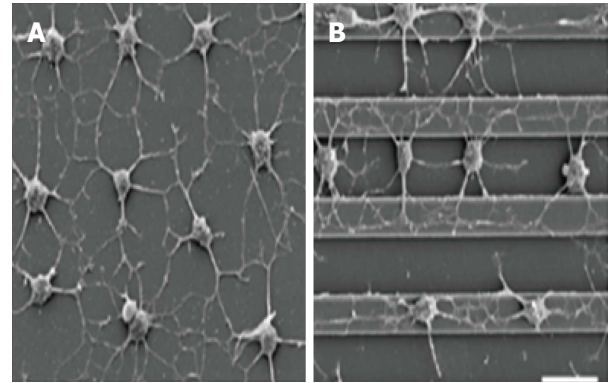


Figure 3 Comparison of different topography strategies employed to investigate the effects of anisotropic vs isotropic cytoskeletal tension on cultured mesenchymal stem cells. (A) nonpatterned substrates caused randomly oriented cell protrusions to be formed, while (B) alignment of elaborated processes in the direction of the grooves were induced by micropatterned surfaces, mimicking the native structure and orientation of the natural extracellular matrix proteins^[52].

3). For instance, it has been shown that homogeneously nanopatterned and chemically modified surfaces can have direct effect on cellular responses of MSCs, including their self-renewal abilities, control over their initial cell interactions and subsequently their cell phenotype, by creating arrays of nanodots using dip pen nanolithography^[51] (Figure 4). Furthermore, differentiation and proliferation of human MSCs (hMSCs) were investigated on nanogratings of 350 nm width combined with biochemical cues such as retinoic acid, and it was shown that synthetic nanostructures can induce hMSCs to differentiate into neuronal lineage^[52]. This study, conducted by Yim *et al.*^[52], also confirmed the significance of nanotopography as it revealed that retinoic acid alone on unpatterned surfaces did not lead to strong neuronal marker expression as it was shown on surfaces with nanogratings. Other nanopatterned structures, such as grooves, ridges, and pores as well as holes, nodes, or rods are of other commonly techniques currently employed to change unpatterned surfaces for MSCs to grow on and to direct their cellular responses^[53]. Such nanostructures have great applications to all areas of TE. For instance, Andersen *et al.*^[54] investigated adhering nanoparticles containing different small-interfering RNAs (siRNAs) into nanostructured scaffolds consisting of nanopores and reported of spatial retention of the RNAs within nanopores seeded with MSCs, which resulted in enhanced osteogenic and adipogenic differentiation of MSCs^[54]. This is an exciting finding as the ability of directing a single type of differentiation plays a crucial role in developing specific 3D organs. In another study, Watari *et al.*^[55] used topographically-patterned substrates containing anisotropically ordered ridges and grooves to modulate osteogenic differentiation in hMSCs^[55]. They reported that hMSCs cultured on 1400 or 4000 nm pitches, compared to those seeded on 400 nm pitch or planar control, exhibit better elongation

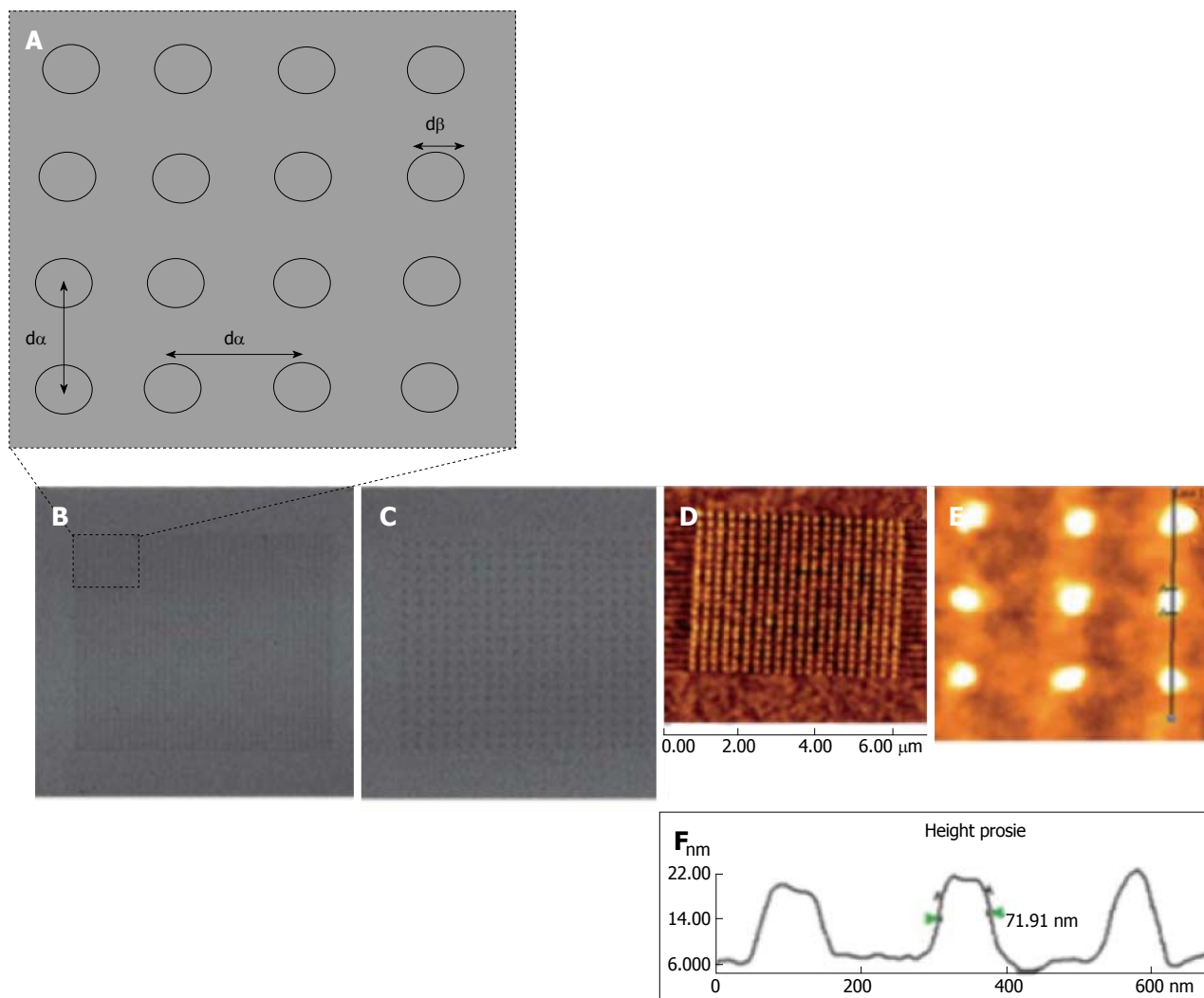


Figure 4 Nanopatterned gold surfaces examination for the effect of both the nanotopography and terminating chemical functionality. A: Nanopatterned surfaces used for mesenchymal stem cell control and differentiation exhibiting dot to dot pitch ($d\alpha$) and dot diameter ($d\beta$); B: Lateral Force Microscopy (LFM) image of small area 280 nm pitch array; C: LFM image of 140 nm pitch array; D-F: Atomic force microscopy topographical image of an alkanethiol resist array fabricated on gold surface following chemical etching. An average diameter feature ($d\beta$) of 70 nm was shown on the cursor profile^[53].

and alignment, while they showed a significant decrease in Runt-related transcription factor 2 (RUNX2) and bone gamma-carboxyglutamic acid-containing protein (BGLAP) expression. Their data also revealed that 400 nm pitch increased extracellular calcium deposition. Watari *et al.*^[55] concluded that specific size scale of topographic cues could directly influence the osteogenic differentiation of hMSCs both with and without osteogenic agents. This is another important finding that could enable one to manipulate and develop nanostructures that could lead to controlled and directed differentiation of stem cells for the purpose of TE of 3D organs. Very recently, the effect of topographical design, in the form of nano-pillar, nano-hole and nano-grill, on hMSCs were investigated by Wu *et al.*^[56] in which these nanotopographies were applied onto a polycaprolactone surface using thermal nanoimprinting. Their findings revealed that nanotopographical patterns trigger changes in the morphology and cytoskeletal structure of hMSCs. They

also found that, compared to non-patterned surfaces, nano-pillar and nano-hole topography determined MSCs chondrogenesis, resulting in specific cartilage formation. Furthermore, Kilian *et al.*^[57] showed that geometric nanotopography cues, that increase actomyosin contractility, could influence and direct the osteogenesis of bone marrow-derived hMSCs. Such geometric cues direct and control mechanochemical signals and paracrine/autocrine factors necessary for specific differentiation of MSCs, also observed during the *in vivo* investigation of the microenvironment of the differentiated cells.

CASE STUDIES ON THE APPLICATION OF NANOTOPOGRAPHY GUIDED TE OF 3D ORGANS/TISSUES USING MSCS

Bone

Reconstruction of large bone defects caused by

surgery, trauma or tumours are common deficiencies, which present a significant medical challenge^[58]. Autologous bone grafting is the gold standard for treating bone defects, but obstacles such as limited sources of tissue, and bone resorption before bone healing, have raised interests in synthetic materials as potential bone substitutes^[59]. Furthermore, bone grafting has proven challenging for large bone defects reconstruction^[60]. This is mainly due to difficulties in harvesting enough bone grafts from a healthy bone, potential postoperative pain, risk of infection, risk of hypersensitivity, risk of paresthesia, and time constraints^[61,62]. TE, using stem cells, provides the opportunity to avoid the established drawbacks of bone graft materials for the purpose of reconstructing or regenerating bone defects at variety of scales. MSCs, because of their interesting properties, have been demonstrated as an attractive cell source for bone TE applications^[63]. Controlled and directed differentiation of MSCs into osteoblasts (bone cells) is therefore a key aspect of this process. As mentioned above, nanotopographical cues could be used to influence MSCs cell behaviour and differentiation toward specific lineages. For instance, in a very recent study, McCafferty *et al.*^[64] demonstrated the use of nanotopography to induce osteogenic differentiation of human bone marrow derived MSCs. They sputter deposited thin films of bioactive calcium phosphate onto a polycrystalline titanium nanostructured surface. These sputter deposited surfaces supported high levels of bone marrow-derived hMSCs proliferation and adhesion, determined by DNA quantification. Moreover, they were also able to directly promote significant levels of osteogenic differentiation. In this study, gene expression, alkaline phosphatase activity and immunocytochemical localisation of key osteogenic markers showed that the nanostructured titanium surfaces and the bioactive calcium phosphate coatings could direct differentiation towards an osteogenic lineage. The addition of the calcium phosphate chemistry to the topographical profile of the titanium was found to induce increased bone marrow-derived hMSCs differentiation compared to that observed for either the titanium or calcium phosphate coating without an underlying nanostructure. Therefore, the results presented a clear benefit from a surface engineering strategy that combines a defined nanoscale surface topography with a conformal bioactive chemistry. In another study by de Peppo *et al.*^[65] osteogenic response of hMSCs to titanium-coated hemisphere-like topographic nanostructures of 50, 100, and 200 nm was assessed. Their aim was to look at the influence of different sizes of nanoscale topographies on the morphology, proliferation, and osteogenic differentiation of hMSCs *in vitro*. Here, the nanostructuring was fabricated using colloidal lithography and the desired structure sizes were achieved by etching the original 200 nm polymeric

particles (polystyrene particles) and further heat-treating them above the transition temperature of polystyrene (118 °C), to create semispheroidal nanoparticles to increase their surface attachment abilities. Their results showed that there is a direct relationship between the proliferation and osteogenic differentiation of hMSCs and the size of the underlying structures, demonstrating that by varying the scale of the nanotopographic features at nanoscale, one can control the osteogenic behaviour of hMSCs. de Peppo *et al.*^[65] concluded that colloidal lithography in combination with coating technologies can enable structuring well defined nanoscale topographies to control and direct hMSCs growth and differentiation. Dalby *et al.*^[66] also investigated the osteogenic response of hMSCs to semi-ordered and random nanotopographies performed by colloidal lithography and polymer demixing on silicon and showed that hMSCs react robustly to nanotopographic features down to 10 nm in size with a low aspect ratio^[66]. In this study, scanning electron microscopy of primary hMSCs on flat controls and scaffolds with nanotopographic structures showed that hMSCs exhibited strong reaction to nanofeatures as their filopodia extended and curled around these features (Figure 5). Dalby *et al.*^[66] concluded that their recorded osteogenic response of hMSCs to nanotopographies could be employed to construct and design scaffolds with an appropriate osteogenic “environment” instead of planar control structures in order to direct and control MSCs growth and differentiation.

Furthermore, Rosa *et al.*^[67] examined the osteoinductive potential of titanium (Ti) surfaces with nanotopographic features, yield by chemically treating polished Ti discs with H₂SO₄/H₂O₂, and cultured them with rat MSCs under osteogenic and non-osteogenic conditions. Untreated polished Ti surfaces were used as controls. Their findings revealed that Ti surfaces with nanotopography boosted cell proliferation and alkaline phosphatase (Alp) activity of rat MSCs under both osteogenic and non-osteogenic conditions (Figure 6). They also demonstrated that nanotopography upregulated the gene expression of major bone markers under both of the test conditions. Interestingly, they noticed that obtustatin, an α 1 β 1 integrin inhibitor, was able to reduce higher gene expression of key bone markers and Alkaline Phosphatase (ALP) activity on Ti Scaffolds with nanotopographic features. Therefore, suggesting that obtustatin signalling pathway plays a crucial role in determining the osteoinductive effect of nanotopography on MSCs, a finding that can be exploited as a novel mechanism of accelerating and/or enhancing MSCs osseointegration for the purpose of TE of complex organs or tissues in the future.

Cartilage

Cartilage defects, caused by osteoarthritis, trauma or sport, are considered serious clinical problems. So far,

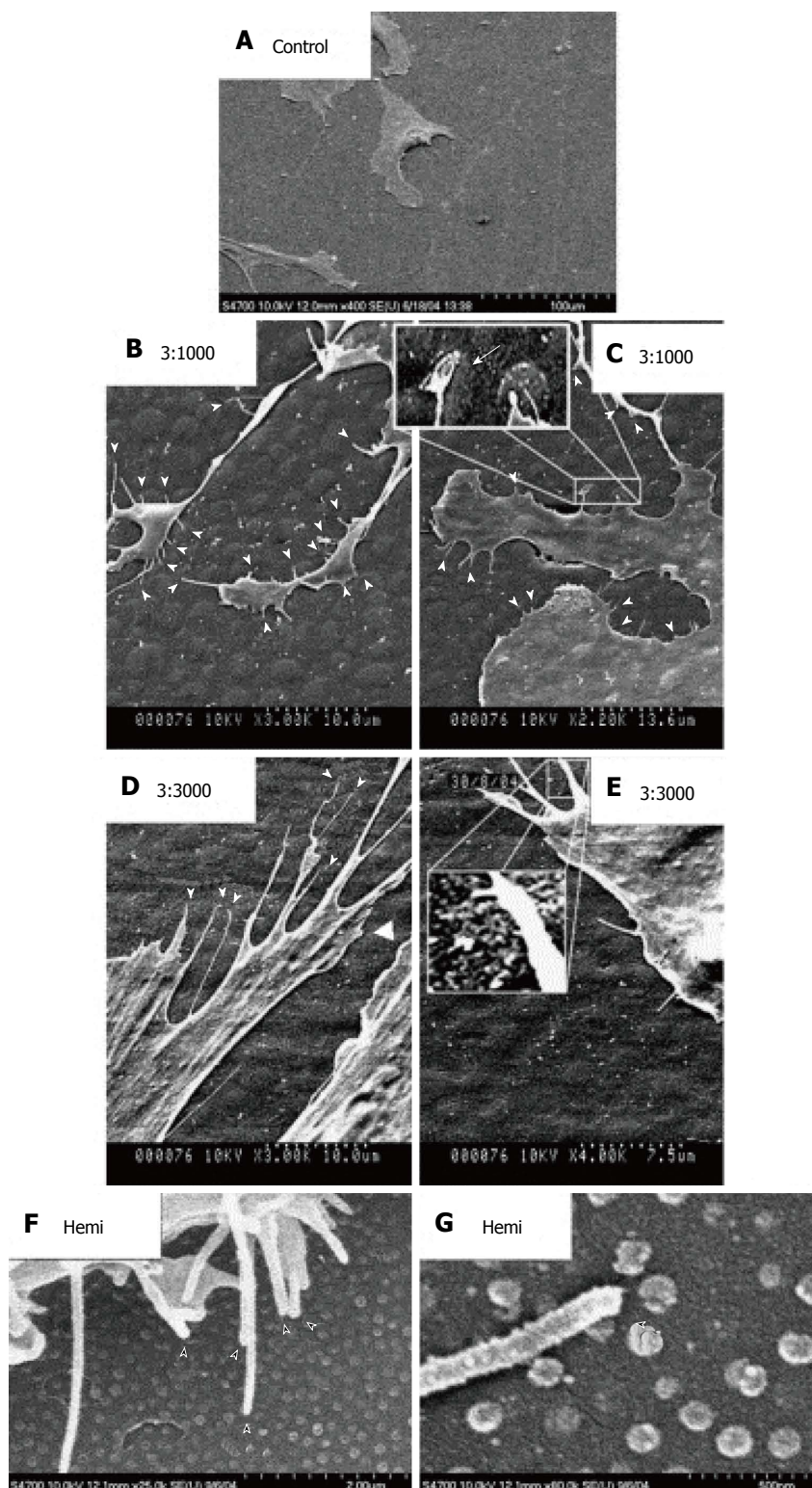


Figure 5 Scanning electron micrographs of human mesenchymal stem cells cultured on control and test materials. A: On planar control materials cells showed normal morphologies; B, C: Filopodial of the human mesenchymal stem cells (hMSCs) interacts with the 3:1000 substrates (arrowheads); C: hMSC filopodia are curving around an island; D, E: Filopodial interactions with the 3:3000 substrates (arrowheads); E: A filopodia curving around an island is clearly observed; F: filopodial interactions with the hemi substrates (arrowheads); G: Filopodia curving around a hemisphere (arrowhead)^[68].

TE of cartilage has proven to be much more difficult than many other organs or tissues, due to cartilage's inherently poor regenerative ability^[68]. Therefore, developing a functional TE system, capable of improving the regenerative ability of this tissue, would be of great interest. Most research on this field have been focused on using polymeric scaffolds with stem cells, in particular MSCs^[8,68-70]. MSCs are considered the "gold standard" of stem cell source for cartilage TE as their

differentiation to chondrocytes can be easily controlled and directed using various techniques, in particular nanotopography^[8,56].

Previous studies have shown that a more rounded, spheroidal cell shape can enhance the rate of chondrogenesis, through increasing the expression of chondrocyte-related genes, markers, and proteins^[71,72]. Based on this, Zhong *et al.*^[73] attempted to create a microenvironment suitable for MSCs fibrochondrogenesis

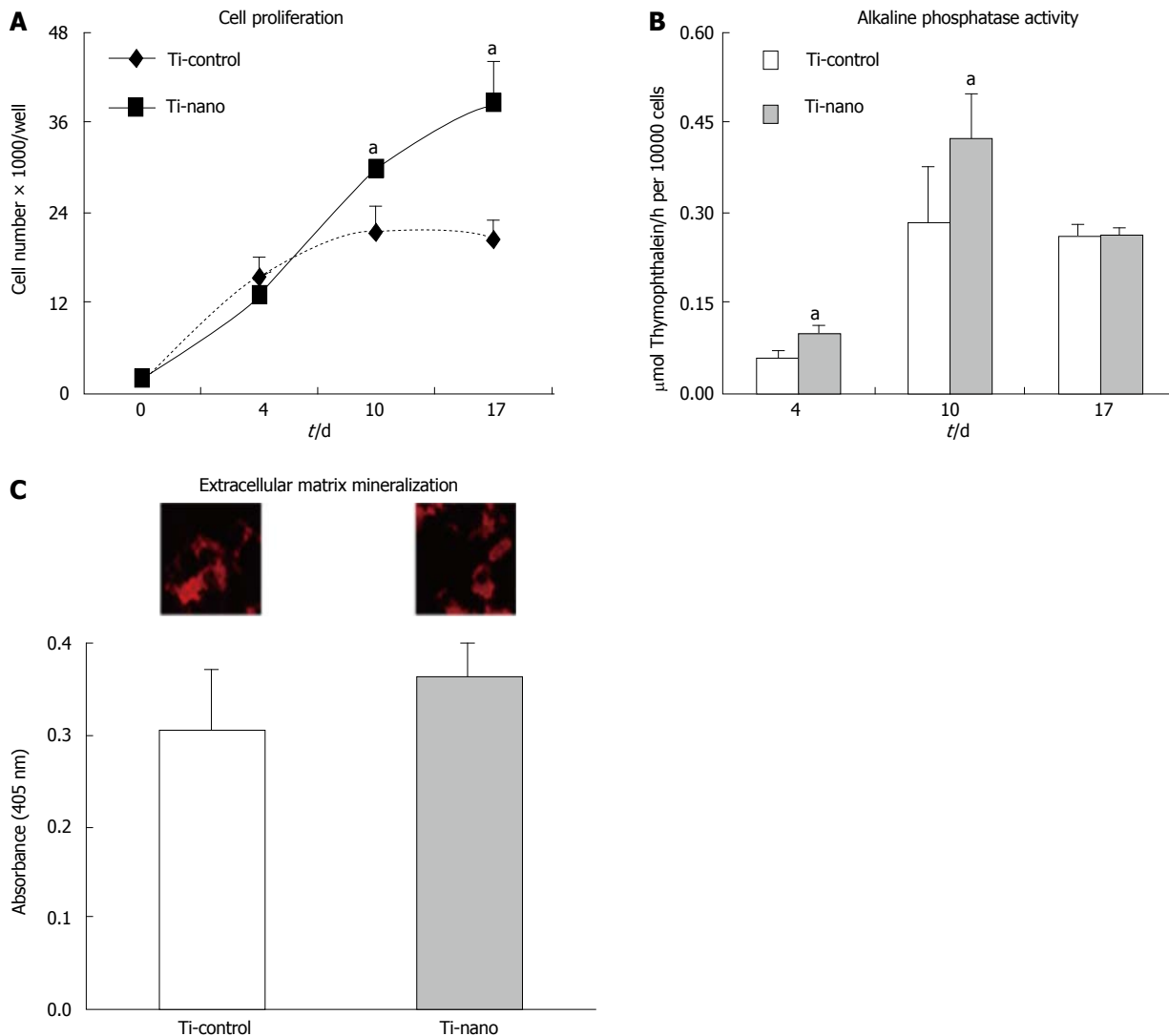


Figure 6 Investigation of the effects of nanotopography on proliferation. (A), alkaline phosphatase (Alp) activity (B), and extracellular matrix mineralization (C) of mesenchymal stem cells differentiated into osteoblasts and cultured on nanotopography in an osteogenic medium compared to control Ti surfaces. A: The number of cells was significantly increased on Ti with nanotopography on days 10 ($P = 0.07$) and 17 ($P = 0.03$); B: Higher Alp activity was supported by Ti surface with nanotopography supported on days 4 ($P = 0.01$) and 10 ($P = 0.04$); C: The difference in the level of calcium mineralisation in the matrix (insets) was not statistically significant ($P = 0.13$) by comparing both surfaces. The data are presented as mean $\pm$ standard deviation ($n = 4$). ^aIndicates statistically significant difference^[67].

using simultaneously integrated nanotopography and flow stimulus. They developed a biomimetic microfluidic device consisting of aligned nanofibers of poly lactic-co-glycolic acid (PLGA), and micorchambers of different angles. The micorchambers were used to enable flow direction to create different angles with PLGA nanofibers. Their findings showed that the combination of fluid flow, nanotopography-induced cues, and the direction of flow in relation to the aligned nanofibers contributed towards the round shape morphology of MSCs, associated with fibrochondrogenesis during chondrogenic differentiation of these cells.

Although, most studies have shown positive contributions of various nanotopographical cues on MSCs growth and differentiation, a few studies have concluded that certain types of nanotopography could have adverse effect on the differentiation of MSCs into chondrocytes for the purpose of repairing

or regenerating cartilage. The study by Wu *et al.*^[56] is a good example on this statement as their findings revealed that MSCs experienced delayed chondrogenesis on samples with nano-grill topography. They observed radically different morphological, proliferation and chondrogenesis changes as well as significantly higher upregulation of type II collagen on nano-pillar and nano-hole surfaces compared to nano-grill surfaces, where the expression of collagen I marker was drastically higher (Figure 7). Similarly, Trujillo *et al.*^[74] reported of decreased chondrogenic differentiation of adipose-derived MSCs on nanowire surfaces. They used sintering and solvent-free nanotemplating to fabricate polycaprolactone (PCL, as control) and nanowire (NW) samples. After 4 and 7 d of culturing the samples with adipose-derived MSCs, both groups demonstrated positive support for cell attachment and proliferation. Once chondrogenic differentiation

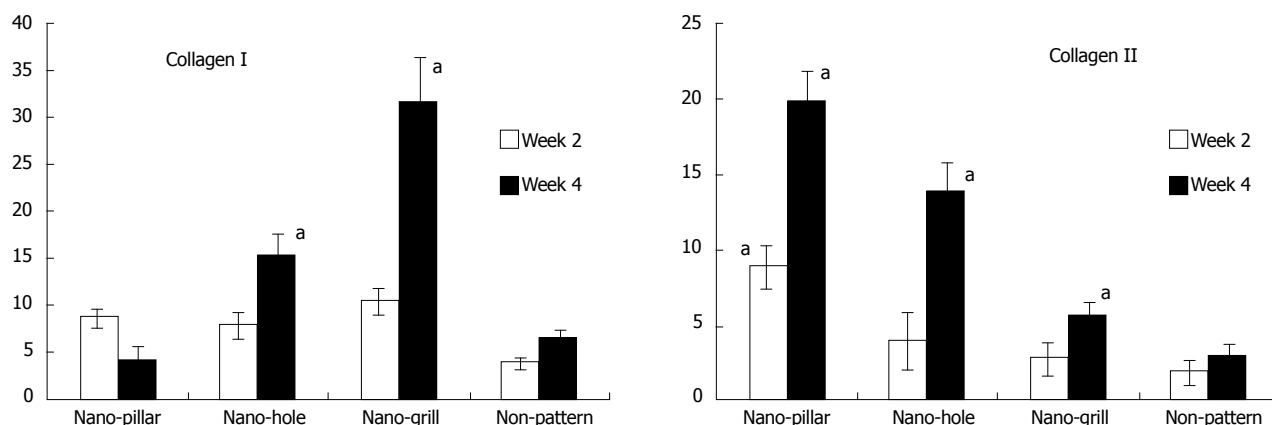


Figure 7 The effect of nano-patterned surfaces on the expression of cartilaginous genes. Real time polymerase chain reaction was used to analyse mRNA expression levels of cartilaginous genes at week 2, 4 or 6 of chondrogenic differentiation, which was normalised to their respective glyceraldehyde-3-phosphate dehydrogenase expression and expressed as fold changes relative to undifferentiated mesenchymal stem cells. $n = 3$ per group, mean $\pm$ SD. ^a $P < 0.05$ which was considered statistically significant compared to non-patterned surface^[56].

media was supplemented to the cultures, alcian blue staining was used to confirm the presence of acidic polysaccharides, such as sulphated glycosaminoglycans, normally found in articular and hyaline cartilage tissue. At 3 wk, it was evident that there had been significantly higher production of polysaccharides on PCL compared to NW. The authors also investigated PCL and NW samples under adipogenic differentiation conditions and found the results to be reversed, *i.e.*, there was increased adipogenic differentiation of adipose-derived MSCs on the NW samples. These findings indicate that nanotopography can have bias and in some cases unexpected effects on the differentiation of MSCs towards a particular lineage. However, further research is required on this particular topic in the future, in order to better understand the underlying mechanism of such adverse events.

Skin

Millions of burn injuries occur worldwide that cause serious harm to skin and subsequently to the general health of patients, as the first line of a patient's defence is compromised. In cases where the injuries are too severe for the natural repair process to take place, skin TE is considered. Skin, with a surface area of 1.8 m², is the largest organ in the body, which consists of two layers: the outer protective epidermis and inner corium or dermis. Currently the main obstacle in front of skin TE is *in vitro* culture time required to grow epithelial sheets large enough to be used for severe cases. This is particularly dangerous as the longer the wound takes to heal, the patient is at higher risks of acquiring infection^[75]. Another issue is that epithelial cells are very sensitive and adhering them to the burned surfaces is very difficult^[76].

Like in other diseases, stem cells can help improve the healing and regenerating process of skin. Previously, bone marrow-derived MSCs have been shown to differentiate into epithelial cells of skin^[77], and promising results have been achieved in treating skin wounds,

especially chronic ones^[78,79]. Wounds treated using bone marrow-derived MSCs have shown accelerated wound closure with rapid re-epithelisation, cellularity and angiogenesis^[80]. Incorporation of these cells into a suitable scaffold, which closely mimics the native micro- and nanotopographical characteristics of the ECM of the skin, may offer improved opportunity to repair or regenerate skin^[81]. For this to happen, two events should take place; (1) MSCs should be directed to the sites of injury; and (2) they should differentiate into cells of skin lineage. This is achievable by designing scaffolds with specific nanotopographical features^[82].

Based on the highly oriented nanogrooved structures of natural ECMs in human body, Kim *et al.*^[83] designed nanotopographically variable grooved matrices, using UV assisted capillary force lithography, with curable polyurethane acrylate (PUA) polymer. The PUA nanogrooved matrices were then gelatine coated prior to cell culturing with hMSCs. In this study, the effect of nanotopographical density was investigated on hMSCs migration and proliferation for wound healing purposes. It was shown that hMSCs migrate into the target area (the wounded, cell free area) and that the hMSCs on nanogrooved matrices exhibited a significantly higher speed of cell migration than those on the flat controls. They also investigated various densities of nanogrooves and found that as the density of the nanogrooved matrices increases, the speed of hMSCs migration increases proportionally. Their analysis of hMSC proliferation on nanogrooved matrices, compared to flat ones, revealed no significant differences, hence concluding that proliferation of hMSCs may not be influenced by nanogrooves.

Recently, great deal of attention has been focused on electrospun nanofibrous for skin regeneration^[75,81,84,85]. Using various nanotopographical designs, highly porous meshes of ultrafine fibers could be fabricated that closely resemble the nanotopography of the natural ECM of human skin^[84]. Jin *et al.*^[86] investigated the *in*

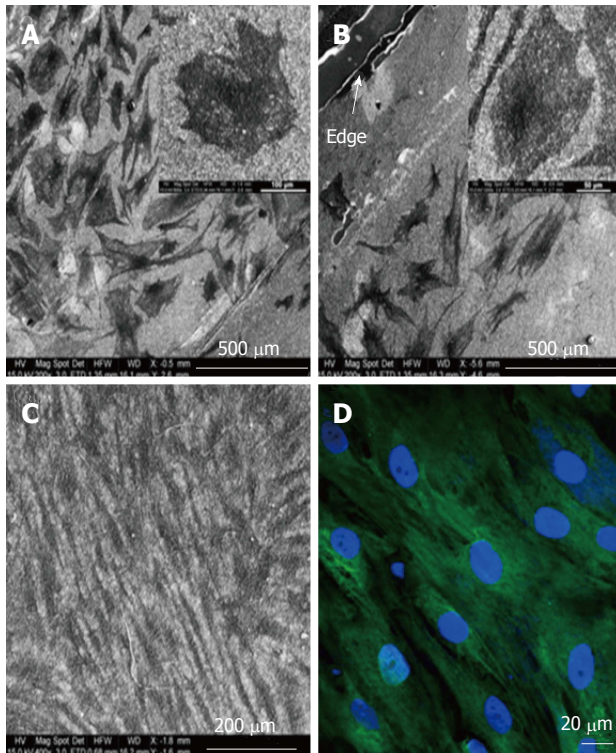


Figure 8 Electron microscopy images of differentiated and undifferentiated mesenchymal stem cells on Coll/PLLCL nanostructured nanofibers. A: Mesenchymal stem cell (MSC) directed to differentiate along the epidermal lineage when cultured in epidermal induction medium; B: Epidermally differentiated MSC on the edge of a Coll/PLLCL scaffold (as shown by arrow); C: Electrospun Coll/PLLCL nanofibers seeded with undifferentiated MSC cultured in normal growth medium; D: MSC grown in normal growth medium on Coll/PLLCL nanofibers stained with Ker 10, after 15 d cell culture, imaged using laser scanning confocal microscope^[86].

vitro differentiation potentials of bone marrow-derived MSCs to epidermal cells on electrospun collagen/poly (L-lactic acid)-copoly (3-caprolactone) (Coll/PLLCL) nanofibrous scaffolds^[86]. To further mimic the structure of the natural ECM of human skin, they incorporated Coll/PLLCL nanofibrous scaffolds with collagen, at a ratio of 30:70, respectively. Their findings demonstrated that electrospun Coll/PLLCL nanofibers enhanced the level of MSC and scaffold interaction and that the electrospun scaffolds could mimic the native skin ECM. Furthermore, their results showed controlled and directed differentiation of MSCs along the epidermal lineage on Coll/PLLCL nanofibrous scaffolds (Figure 8), suggesting their potential use in skin TE applications.

POTENTIAL ADVERS EFFECTS OF ERODED NANOPARTICLES ON MSCS

Based on the evidence presented in this review, it is clear that nanotopography of a surface can have a great influence on the various cellular behaviours of MSCs including; their attachment, proliferation and most importantly their differentiation towards a specific lineage. However, based on the literature,

there have been various reports of the adverse effects of nanoparticles on the cellular behaviour of MSCs as the result of implant erosion over-time^[87-89]. This is especially a concern of biodegradable materials as they have been specifically designed to be deteriorated once implanted in their host. In long-term clinical application, the physiochemical properties of implants are influenced by their constant chemical, mechanical, and biological interactions with their host tissue and its surrounding environment^[90]. These factors cause the degradation of the implant and subsequently to the release microscale and/or nanoscale wear particles in their immediate vicinity. These released particles no longer exhibit any of the nanotopographical characteristic of the implant surface, prior to implantation.

Released nanoparticles, once exposed to tissues and bodily fluids, tend to absorb macromolecules in their vicinity and depending on the surface characteristics of the nanoparticles (e.g., their surface chemistry and surface energy), these macromolecules become attached onto the surface of the nanoparticles, leading to potential modification or functionalization of the surface of the nanoparticles^[91,92]. Attachment of such macromolecules could change the affinity of a nanoparticle to bind with a specific protein, on the surface of a particular type of cells, which in return could have serious unaccounted for or adverse toxic effects on the proliferation and/or differentiation of cells^[93]. For instance, there has been a report on serious DNA damage caused to MSCs when cultured with metallic silver nanoparticles, even at lower concentrations^[88]. Calcium phosphate nanoparticles, very commonly used for bone tissue engineering applications, have also shown to affect MSC proliferation in a size-dependent manner, with larger particles causing more serious harm^[87]. Furthermore, studies conducted by Hu *et al.*^[94] and Liu *et al.*^[89] showed that calcium phosphate nanoparticles could also affect MSC differentiation depending on their concentration and form of appearance. They reported that increasing the concentration of calcium phosphate nanoparticles, especially in the form of amorphous particles, rather than crystals, could negatively affect the osteogenic cell differentiation and matrix mineralisation of MSCs.

Other types of nanoparticles such as metallic ones are also of great concerns, as their release could lead to serious nanotoxicity in biomaterials and different cell lines. Various groups have investigated adverse systematic effects of titanium nanoparticles on different cell lines such as endothelial^[95], lymphoblastoid^[96], and fibroblasts^[97] cells and reported of inflammatory reactions, DNA damage, and induction of apoptosis, respectively. However, until a year ago, there was no concise report on the adverse effect of titanium nanoparticles on the cellular behaviour of bone marrow-derived MSCs, despite titanium implants majorly being used for medical applications, such as in bone TE. As the result, very recently, Hou *et al.*^[98] investigated

Table 2 Significant studies on nanotopography and mesenchymal stem cells for developing 3D bone, cartilage and skin

Tissue/organ	Nanotopographical cues	Description	Outcome	Ref.
Bone	Nano-ridges, and nanogrooves nanotopography surfaces	The effect of nanotopographic ridges and grooves on MSCs morphology, proliferation and differentiation to osteoblast cells were investigated	Osteogenic differentiation can be controlled and directed using specific size scale of topographic cues with or without osteogenic agents	Watari <i>et al</i> ^[55]
	Implementing nanostructures of different sizes	The effect of titanium-coated hemisphere-like topographic nanostructures of various sizes (50, 100, and 200 nm) on hMSCs cellular behaviour towards osteoblast lineage was investigated	Osteogenic differentiation of hMSCs is dependent on the size of the underlying nanotopographical structures. Colloidal lithography combined with coating technologies can have great potentials for fabricating nanoscale topography on scaffolds	de Peppo <i>et al</i> ^[65]
	Bioactive calcium phosphate thin films sputter deposited onto a polycrystalline titanium nanostructured surface	Calcium phosphate thin films were used to study the cellular response of hMSCs to nanostructured titanium surfaces with the aim of directing them towards osteogenic differentiation	Various <i>in vitro</i> studies revealed that the use of nanostructured titanium surfaces and the bioactive calcium phosphate coatings could allow for directed and controlled differentiation of hMSCs towards osteogenic lineage. The combination of the two materials together showed higher rate of osteogenic differentiation compared to that of each of these materials on their own	McCafferty <i>et al</i> ^[64]
	Polished Ti surfaces chemically treated with H ₂ SO ₄ /H ₂ O ₂ to create nanotopography	Chemically treated Ti surfaces with nanotopography and seeded with rat MSCs were used to investigate their osteoinductive potentials compared to untreated surfaces. Signalling pathways responsible for osteoinductive effect of nanotopography on MSCs were also investigated	Ti surfaces with nanotopography exhibited increased cell proliferation and alkaline phosphate activity. Gene expression of key bone markers was upregulated on nanotopography surfaces, under non-osteogenic conditions, compared to control	Rosa <i>et al</i> ^[67]
Cartilage	A biomimetic microfluidic device embedded with aligned nanofibers consisting of microchambers of different angle	A device was developed to create a microenvironment that integrates nanotopography and flow stimulus of the ECM of natural cartilage for the purpose of investigating the effect of microfluidic and nanotopography on the cellular behaviour and fibrochondrogenesis of MSCs	The angle of flow direction, in relation to the aligned nanofibers, affects MSCs behaviour. Fibrochondrogenesis of MSCs was evident when the flow direction was perpendicular to the aligned nanofibers	Zhang <i>et al</i> ^[76]
	Nanowire nanotopographic surfaces	Polycaprolactone nanowires surfaces were fabricated using a solvent-free gravimetric template technique to investigate their nanotopographical effects on the adhesion, proliferation, differentiation and ECM synthesis of adipose-derived MSCs	The results demonstrated that adhesion and proliferation of adipose-derived MSCs were enhanced on nanowire surfaces compared to the control. Nanowires also effected the morphology of these cells Interestingly, it was shown that nanowires supported adipogenic differentiation of these cells rather than chondrogenic differentiation	Trujillo <i>et al</i> ^[74]
	Nano-pillar, nano-hole and nano-grill nanotopography surfaces	Nano-pillar, nano-hole and nano-grill structures were formed on polycaprolactone surface using thermal nanoimprinting to investigate their effect on chondrogenic differentiation of hMSCs	Nanotopographical patterns have the ability to induce changes in MSC morphology and cytoskeletal structure towards a specific lineage, in this case chondrocyte cells. Delayed chondrogenesis was observed on nanogrill topography compared to nano-pillar and nano-hole topography, which enhance MSC chondrogenesis	Wu <i>et al</i> ^[56]
Skin	Electrospun nanofibrous scaffolds	Electrospun Coll/PLLCL and PLLCL nanofibrous scaffolds were prepared to investigate the proliferation and differentiation of MSCs to epidermal lineages	Cell proliferation was significantly higher on Coll/PLLCL nanofibrous scaffolds compared to PLLCL scaffolds. MSC morphology was also different on Coll/PLLCL nanofibrous scaffolds compared to control. Electrospun Coll/PLLCL exhibited similar properties to the native skin ECM	Jin <i>et al</i> ^[86]
	Nanotopographically variable grooved matrices	Nanotopographically variable grooved matrices, using UV assisted capillary force lithography, with curable PUA polymer were fabricated and then coated with gelatine to investigate the effect of nanotopographical density on hMSC migration and proliferation for wound healing purposes	As the density of the nanogrooved matrices increased, the speed of hMSCs migration increased proportionally. It was shown that hMSC proliferation was not significantly different on nanogrooved matrices, compared to flat control. Therefore, suggesting that proliferation of hMSCs may not be influenced by the nanogrooves	Kim <i>et al</i> ^[83]

PUA: Polyurethane acrylate; Coll/PLLCL: Collagen/poly(lactic acid)-co-poly(3-caprolactone); Ti: Titanium; hMSCs: Human mesenchymal stem cells.

the effects of titanium nanoparticles on adhesion, migration, proliferation and differentiation of MSCs and

reported of serious negative effects of the nanoparticles on MSC migration as particle size increased. They

also demonstrated that exposure of MSCs to titanium nanoparticles negatively affected their osteogenic differentiation^[98].

DISCUSSION AND FUTURE PROSPECTIVE

In this review, the role of nanotopography on controlling and directing cellular behaviour of various types of MSCs, with respect to specific tissues and organs, has been described. Based on the studies presented here, it can be established that various nanofabrication methods can be employed to design and fabricate nanostructured scaffolds with distinct nanotopographical cues to control and direct various cellular behaviours of MSCs including; their attachment, proliferation and most importantly their differentiation towards a specific lineage (Table 2). Therefore, incorporating nanotopography on the design of scaffolds would open doors to new generation of TE strategies for the development of functional organs and tissues.

The review of the literature demonstrated that most studies on this topic have been focused on bone TE applications. In most of these studies, such strategy seemed to improve the proliferation and differentiation of MSCs for repair or regeneration of bone. In most cases, authors have reported of controlled and directed osteogenesis of these cells on various polymeric based composite or nanocomposite scaffolds. However, there seems to be a clear lack of investigation into the potentials of using nanotopography for the development of more complex organs or tissues such as heart, kidney, or bladder. This is despite of the fact that some studies have confirmed the abilities of MSCs to differentiate into various other cell types; including muscle cells, stromal cells and fibroblast cells. Therefore, in the future, TE and regenerative medicine could greatly benefit from research focused on developing more complex organs or tissues using nanotopography guided differentiation of MSCs, while at the same time, there needs to be a more comprehensive investigation on the potential adverse effect of various types of nanoparticles, released from eroded nanotopographical surfaces, on the cellular behaviour of MSCs.

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Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance

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hypothesis is most widely accepted. This proposes that stem cells in the basal layer of the limbal epithelium, at the periphery of the cornea, maintain themselves and also produce transient (or transit) amplifying cells (TACs). TACs then move centripetally to the centre of the cornea in the basal layer of the corneal epithelium and also replenish cells in the overlying suprabasal layers. The LESC hypothesis maintains the corneal epithelium during normal homeostasis and become more active to repair significant wounds. Second, the corneal epithelial stem cell (CESC) hypothesis postulates that, during normal homeostasis, stem cells distributed throughout the basal corneal epithelium, maintain the tissue. According to this hypothesis, LESC are present in the limbus but are only active during wound healing. We also consider a third possibility, that the corneal epithelium is maintained during normal homeostasis by proliferation of basal corneal epithelial cells without any input from stem cells. After reviewing the published evidence, we conclude that the LESC and CESC hypotheses are consistent with more of the evidence than the third hypothesis, so we do not consider this further. The LESC and CESC hypotheses each have difficulty accounting for one main type of evidence so we evaluate the two key lines of evidence that discriminate between them. Finally, we discuss how lineage-tracing experiments have begun to resolve the debate in favour of the LESC hypothesis. Nevertheless, it also seems likely that some basal corneal epithelial cells can act as long-term progenitors if limbal stem cell function is compromised. Thus, this aspect of the CESC hypothesis may have a lasting impact on our understanding of corneal epithelial maintenance, even if it is eventually shown that stem cells are restricted to the limbus as proposed by the LESC hypothesis.

Key words: Eye; Cornea; Corneal epithelium; Limbal epithelium; Stem cell; Lineage tracing

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Abstract

In this review we evaluate evidence for three different hypotheses that explain how the corneal epithelium is maintained. The limbal epithelial stem cell (LESC)

Core tip: This review article evaluates the evidence for different hypotheses that have been proposed to explain how the corneal epithelium is maintained. It identifies core observations in favour of the conventional limbal epithelial stem cell (LESC) hypothesis and an alternative corneal epithelial stem cell hypothesis and describes how lineage-tracing experiments are helping to reconcile the two sets of conflicting evidence in favour of the LES C hypothesis.

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INTRODUCTION

It is widely accepted that adult corneal epithelium is maintained by stem cells located in a region called the limbus, at the corneal periphery. However, this limbal epithelial stem cell (LESC) hypothesis has been challenged by an alternative corneal epithelial stem cell (CESC) hypothesis, based on experimental studies with mice^[1]. This accepts that LESCs exist but proposes that they only contribute to corneal epithelial repair in response to wounding and that, during normal homeostasis, the corneal epithelium is maintained solely by stem cells scattered throughout the corneal epithelium itself. It has also been proposed that, in the absence of a wound, the corneal epithelium is maintained entirely by proliferation of its own basal cells without any involvement of stem cells^[2]. The main purpose of this review is to compare the evidence for the alternative LES C and CESC hypotheses in order to identify where there is common ground and where differences need further experimental investigation. However, we also consider whether the experimental evidence is consistent with the possibility that the corneal epithelium is maintained without stem cells.

THE CORNEAL AND LIMBAL EPITHELIA

The cornea is the specialised, avascular, transparent, dome-shaped region of the anterior ocular surface, which refracts light through the pupil to the lens and provides a protective, impermeable barrier. It consists of three cellular layers: (1) the inner corneal endothelium, which, despite its name, is a type of epithelium; (2) the middle corneal stroma, comprising specialised fibroblasts, called keratocytes, embedded in a collagen and proteoglycan matrix; and (3) the outer, non-keratinised, stratified squamous epithelium, comprising 5-6 layers of keratinocytes, which is kept moist by the tear film. The corneal epithelium is a very dynamic tissue. Differentiated cells are continuously shed from the outer layer and replaced by cells

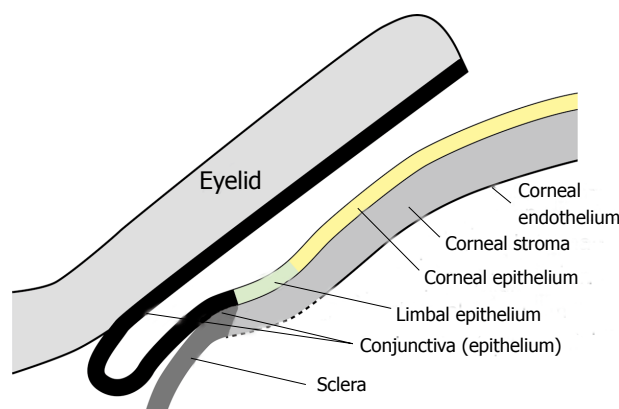


Figure 1 Diagrammatic representation of the tissues of the mouse ocular surface and eyelid.

produced in the proliferative basal layer. According to the conventional stem cell paradigm these proliferative basal cells are considered to be transient (or transit) amplifying cells (TACs) and are replenished by stem cells. The early generation TACs are sometimes referred to as progenitor cells.

The limbus is a narrow transition zone, which encircles the cornea (Figures 1 and 2). The stroma and epithelial layers of the cornea extend into the limbus where they become the limbal stroma and limbal epithelium. However, the corneal endothelium does not extend into the limbus and is replaced by the drainage channels of the trabecular meshwork. On the other side of the limbus, the stroma merges with the sclera, which forms most of the ocular surface (the white part of the human eye) and the limbal epithelium becomes the conjunctiva. The conjunctiva is an epithelium, which covers the anterior sclera, folds back to form the conjunctival sac and lines the inner surface of the eyelids (Figure 1). Thus, the conjunctiva attaches the eyeball to the eyelids and orbit and permits some rotation of the eyeball in the orbit. Unlike the transparent cornea, both the limbus and the conjunctiva are vascularised.

The human limbus contains radial fibrovascular ridges, called the palisades of Vogt, which project upwards from the stroma deep into the epithelium (Figure 2) but many species, including mice, do not have limbal palisade structures. Another anatomical difference between these species is that, in the mouse, the corneal epithelium is thickest in the centre of the cornea and has fewer cell layers in the peripheral cornea and limbus whereas, in humans, the limbal epithelium is thicker (about 8-10 cell layers) than the corneal epithelium (5-6 layers)^[3].

It is widely accepted that some basal limbal epithelial cells are stem cells^[4,5] and that the limbal stroma, vasculature and other associated cell types provides a suitable stem cell niche microenvironment, which is required to maintain the LESCs in a relatively undifferentiated state^[6,7]. The limbal palisades (Figure 2) increase the area of interface between the limbal

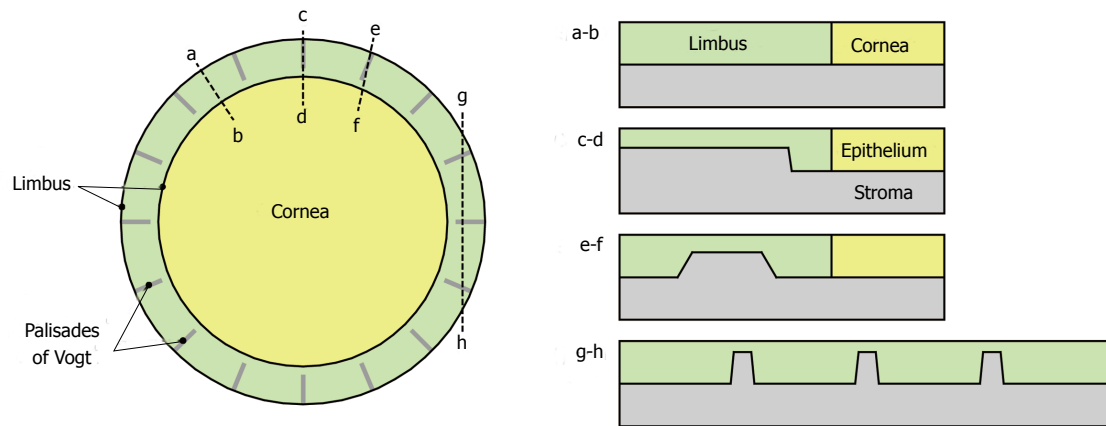


Figure 2 Palisades of Vogt. Diagram showing the arrangement of the palisades of Vogt (upward projections of limbal stroma into the limbal epithelium) in the human limbus in plan view (left) and how they might appear in differently orientated sections through the limbus (right). Only 16 palisades are shown but in reality there are many more. For anatomy see references^[114,115].

epithelium and stroma, so increasing the size of the region that is likely to harbour the LESC niches. Furthermore, it has been suggested that LESC niches may be particularly enriched in two types of epithelial crypts associated with the palisades. One type of crypt is formed by the regions of limbal epithelium between the upward-projecting stroma of the limbal palisades and these have been named "limbal crypts" (LCs)^[8,9]. The other type of crypt (named "limbal epithelial crypts"; LECs) are more sparsely distributed (only 6-7 per eye) and are formed from epithelial projections from the periphery of the limbal palisades, which extend either radially from the limbus into the conjunctival stroma or circumferentially within the limbus (perpendicular to the palisades)^[10,11]. However, many species do not have limbal palisades and associated crypts, which could, therefore, be considered to be species-specific adaptations, possibly related to eye size. Thus, if the limbus provides a niche microenvironment, presumably it is either not dependent on these structures or differs among species. Maintenance of the niche microenvironment is more likely to depend on the presence of the vasculature and other cell types that are present in the limbus in all species.

ALTERNATIVE HYPOTHESES OF CORNEAL EPITHELIAL MAINTENANCE

We consider two hypotheses, which propose alternative ways that stem cells may maintain the corneal epithelium, and a third hypothesis, which does not include stem cells. For the present purposes, we define a stem cell as an undifferentiated cell with high proliferative potential that is capable of renewing itself and also producing one or more differentiated cell types with lower proliferative potential. While most adult stem cells are multipotent, generating multiple cell types, stem cells that maintain the corneal epithelium are generally presumed to be unipotent,

only producing the corneal epithelial cells. Although one report shows that they may also produce the goblet cells that enter the corneal epithelium in response to large wounds^[12], we have not considered this possible additional role of the stem cells in this review.

Limbal epithelial stem cell hypothesis

According to the conventional LESC hypothesis (Figure 3A and D), LSCs act as a source of new basal corneal epithelial cells (the TACs) during normal corneal epithelial homeostasis and become more active during episodes of significant wound healing^[13], although small wounds may be healed without upregulating stem cells. In this scheme LSCs remain in the limbus where they maintain themselves and also generate the first generation of TACs. Some of these early TACs move to the overlying, non-mitotic suprabasal epithelial layers, and become terminally differentiated. Other early generation TACs continue to divide and move centripetally in the basal layer to maintain the corneal epithelium. Once cells leave the basal layer they differentiate and move rapidly through the suprabasal layers to the superficial layer from where they are shed. It seems that both daughter cells of a dividing basal cell usually share the same fate^[14] so that they either both remain in the proliferative basal layer or both move suprabasally. It is not known what determines whether cells leave the basal layer. For example, it could be a combination of declining suprabasal cell numbers, caused by cell loss, and overcrowding in the basal layer, caused by cell proliferation, as described for the *Drosophila notum*^[15].

Corneal epithelial stem cell hypothesis

The corneal epithelial stem cell (CESC) hypothesis accepts that there are stem cells in the limbus but proposes that these are only activated for repairing wounds and that during normal homeostasis the corneal epithelium is maintained by stem cells

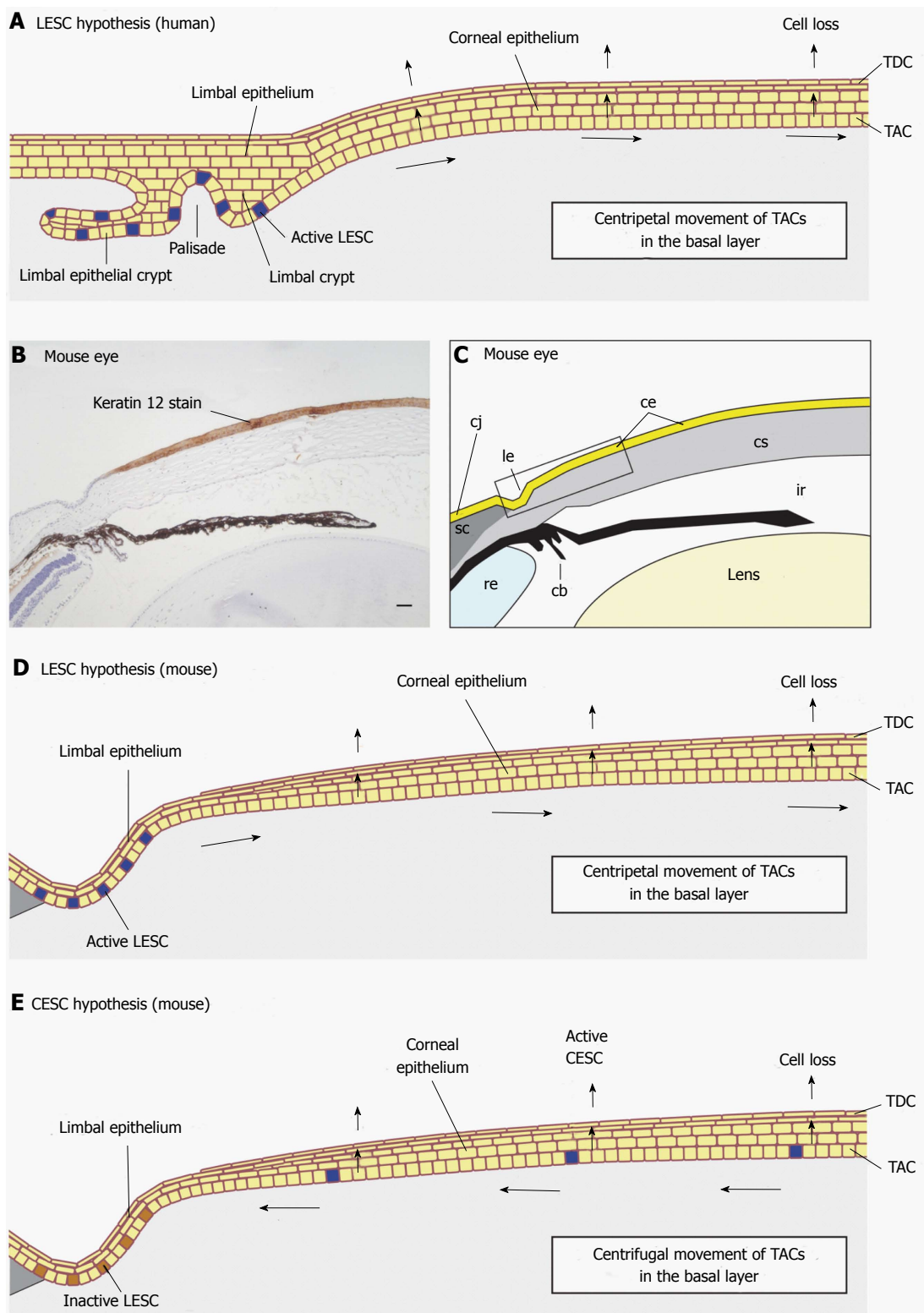


Figure 3 Limbal epithelial stem cell vs corneal epithelial stem cell hypotheses. A: Diagram of human corneal epithelial maintenance according to the limbal epithelial stem cell (LESC) hypothesis showing active LESCs in the limbal epithelium in both a limbal crypt and a limbal epithelial crypt. The LESCs divide slowly replacing themselves and producing daughter transient (or transit) amplifying cells (TACs), which divide more quickly and move centripetally from the basal layer of the limbal epithelium to the basal layer of the corneal epithelium. After a final cell division TACs leave the basal layer, move through the suprabasal layers and are shed from the surface as terminally differentiated cells (TDCs); B: Histological section showing mouse cornea, limbus and part of the conjunctiva immunohistochemically stained for keratin 12 (K12; dark brown staining) to show the border between the corneal epithelium (K12 positive) and limbal epithelium (K12 negative); C: Drawing of photograph shown in (B) with different tissues labelled. The boxed area shows part of the limbal and corneal epithelia, equivalent to that represented in (D) and (E); D: Diagram of mouse corneal epithelial maintenance according to the LESC hypothesis. The principles are the same as described for (A); E: Diagram of mouse corneal epithelial maintenance according to the corneal epithelial stem cell (CESC) hypothesis. The CESC divide slowly replacing themselves and producing daughter TACs, which divide more quickly and move centrifugally as originally proposed^[1]. After a final cell division TACs leave the basal layer, move through the suprabasal layers and are shed from the surface. cb: Ciliary body; ce: Corneal epithelium; cj: Conjunctiva; cs: Corneal stroma; ir: Iris; le: Limbal epithelium; re: Retina; sc: Sclera. Photograph (B) is reproduced from Mort *et al.*^[18] with kind permission of Springer Science + Business Media.

Table 1 Evidence discriminating between alternative hypotheses

Observations	Species	Ref.	Consistent with hypothesis?		
			LESC	CESC	GBL
Holocline-producing cells are present in limbus but not cornea	Human	[25]	++	±	±
Holocline-producing cells are present in limbus and cornea	Pig	[1]	±	++	±
Production of clonogenic spheres from limbus and central cornea	Human	[29]	±	++	±
Limbal epithelial cells are superior to corneal epithelial cells for corneal repair	Human	[32,33]	++	+	±
The central corneal epithelium can maintain itself when isolated from the limbus	Rabbit, mouse, human	[1,39-42]	+ ^a	++	++
Label-retaining cells are present in the limbus but not the corneal epithelium during normal homeostasis	Mouse, rabbit	[13,52-54,57]	++	+	0
After a 40-d chase ³ H-TdR-labelled cells are present in both the limbus and the corneal epithelium during normal homeostasis	Rabbit	[2]	+ ^b	+ ^b	+ ^b
Cells move centripetally from the periphery of the corneal epithelium during normal homeostasis	Human, mouse	[58,61-63]	++	± ^c	± ^c
Mosaic patterns change after birth and clones of labelled cells emerge from the limbus as radial stripes	Mouse	[27,72,77-79]	++	0	0
Distribution of rare stripes in corneas of <i>KRT5-LacZ</i> ^{+/+} transgenic mice	Mouse	[17]	++	±	±
Transplanted limbal tissue contributes to replacing experimentally debrided corneal epithelium	Rabbit, mouse	[1,76]	++	++	+
Transplanted limbal tissue does not contribute to the unwounded corneal epithelium	Mouse	[1]	0	++	++
More tumours arise from the limbal epithelium than corneal epithelium	Human	[80]	++ ^d	+	+
Diverse cell types and blood supply makes limbus a likely stem cell niche	All species	[6]	++ ^d	+	+
Distribution of markers associated with undifferentiated or stem cell phenotype	Human, mouse, rabbit	See Table 2	++	±	±
Lineage tracing studies show that limbal cells contribute to the unwounded corneal epithelium during normal homeostasis	Mouse	[64]	++	0	0

++: Expected for hypothesis; +: Consistent with hypothesis; ±: Not consistent with hypothesis unless specific assumptions are made or technical issues compromise the interpretation of the experiment; 0: Not consistent with hypothesis. ^aThis is compatible with the LES C hypothesis if self-maintenance of the central corneal epithelium is a back-up mechanism that is used when homeostasis is compromised and LESCs are unable to maintain the corneal epithelium. ^bThe chase period may not have been sufficient to identify label-retaining cells (see text). ^cThe CESC hypothesis, as originally stated^[1], proposed that corneal epithelial cell movement was centrifugal but this assumption is not necessary. However, it requires *ad hoc* assumptions to account for centripetal movement across the full radius. ^dEvidence is circumstantial. LES C: Limbal epithelial stem cell.

scattered throughout the corneal epithelium itself^[1]. This hypothesis is based largely on surgical transplantation experiments in mice. These experiments showed that labelled limbal epithelial tissue, transplanted to the limbus of immunologically compromised mice, only produced labelled clones in the cornea if the host corneal epithelium was subsequently removed. The authors reasoned that if LESCs were active during normal homeostasis, as the LES C proposes, the donor limbal tissue should colonise the cornea without being stimulated to do so by wounding. However, others have pointed out that the CESC hypothesis is not consistent with some of the earlier experimental evidence^[16-18].

Germinative basal layer hypothesis

A third possibility harks back to earlier explanations before the importance of tissue stem cells was recognised. Like the CESC hypothesis, this proposes that the corneal epithelium is normally maintained entirely from cells in the basal layer of the corneal epithelium but unlike the other two hypotheses it proposes there are no stem cells in either the limbal or corneal epithelia. Haddad *et al.*^[2] referred to the basal layer as the “germinative basal layer” and proposed this alternative mechanism for corneal epithelial maintenance to explain the results of their label-retaining cell experiment with rabbits. These

results are inconsistent with other label-retaining cell experiments, as discussed below. However, we have considered this hypothesis because there is evidence that some other adult tissues are maintained during normal homeostasis by proliferation of more differentiated cell types. In such cases, stem cells are either absent or only active during wound repair. For example, this type of tissue maintenance has been proposed for pancreas β -cells^[19], epidermis^[20], lung^[21] and liver hepatocytes^[22,23].

EXPERIMENTAL EVIDENCE AND EVALUATION OF ALTERNATIVE HYPOTHESES

The three alternative hypotheses are discussed below with respect to the available experimental evidence and evaluations are summarised in Table 1.

Cells with high proliferative potential

One of the hallmarks of stem cells is that they have a greater proliferative potential than TACs and this can be identified using *in vitro* colony-forming assays with cultured cells. The proliferation characteristics of cultures of explanted epithelial cells can be investigated in culture and clones derived from single cells can be classified as holoclones, mero clones and

paraclones. These are thought to represent *in vitro* descendants of stem cells, TACs and differentiated cells, respectively^[24]. (On indicator dishes in culture, holoclones form large, smooth-edged, fast-growing colonies with large numbers of small tightly packed cells. Meroclones form smaller colonies that are irregular in outline and include a mixture of small tightly packed cells and larger more loosely packed cells, which are predominantly at the edge. Paraclones form small, diffuse colonies and most cells are large, flattened and loosely packed). Clonal analysis of cells from the human ocular surface epithelia identified holoclone-producing cells in the limbal epithelium but not the corneal epithelium from a 54-year-old individual^[25], suggesting that stem cells are present in the limbal epithelium.

However, there is also evidence that some cells of the central cornea are self-sustaining and have high proliferative potential. Majo *et al.*^[1] showed that cultured corneal epithelial cells of many species produced colonies of cells *in vitro* although there were significant species differences. Pig corneal epithelial cells grew particularly well and clonal analysis of cultured pig cells identified holoclone-producing cells in the central corneal epithelium as well as the limbus. This suggests that stem cells are present in the central corneal epithelium of pigs as well as the limbus but this result cannot be evaluated fully as the age of pigs was not given and fetal cells with stem cell characteristics may persist in younger individuals^[26-28].

The production of clonogenic spheres of cells in culture has also been associated with the presence of stem cells and these have been isolated from the human limbus and central cornea, although isolation is most efficient from the limbus and from younger individuals^[29]. These culture experiments imply that both the limbal epithelium and the central corneal epithelium have cells that are able to behave like stem cells in clonogenic assays *in vitro* which argues against the LESC and germinative basal layer (GBL) hypotheses. However, if culture conditions unmasked proliferative potential of corneal epithelial cells, which is not expressed during normal homeostasis *in vivo*, this result would be compatible with all three hypotheses.

Despite their slow proliferation *in vivo* (see next section and reference^[30]), human limbal epithelial cells grow well in culture and have a higher mitotic rate than corneal epithelial cells^[31]. Furthermore, clinical observations indicate that human limbal tissue is superior to central corneal tissue for treating patients with severely wounded corneal epithelia, which is likely to reflect a greater proliferative potential. The corneal epithelium can be restored using grafts of human limbal epithelial tissue^[32,33] or cells cultured from explanted limbal tissue^[34-38]. Although the limbus is the preferred source of cells for clinical therapeutic use, this does not help determine whether LSCs are active during normal tissue homeostasis (LESC hypothesis)

or only during wound healing (CESC hypothesis).

There is also evidence that the central cornea of several species contains highly proliferative cells. For example, rabbit central cornea is able to survive for months after the limbus is removed or separated from the cornea^[39,40], although corneal integrity slowly degenerates and it does not heal properly after corneal wounding. Similarly, the mouse corneal epithelium was able to sustain itself for four months after the limbus was cauterised to destroy the limbal epithelium^[1]. It has also been reported that some patients with symptoms of total LESC deficiency retain central islands of normal corneal epithelium for several years^[41] and in one case this appeared to be sufficient to restore the corneal epithelium^[42]. These studies show that the central cornea can maintain itself to some extent when the limbus is eliminated or disconnected. This implies that the central corneal epithelium has cells that are able to act as progenitors, if LSCs are unable to maintain the corneal epithelium. However, again this does not show whether these cells act as progenitors during normal homeostasis so it does not provide conclusive evidence against the LESC hypothesis.

Cell division characteristics and identification of slow-cycling label-retaining cells

Stem cell populations maintain themselves and produce more differentiated cells throughout the lifetime of the organism. This is sometimes interpreted as requiring stem cells to divide asymmetrically (producing one stem cell and one TAC) but this does not mean that each division of every stem cell has to be asymmetric as long as the population average achieves this. There have been few attempts to identify asymmetrically dividing cells in the ocular surface^[43] and results are insufficient to discriminate among the three hypotheses.

A more widely studied characteristic of many stem cells is that they are relatively quiescent so divide infrequently. A slow cell division rate is not an obligatory phenotype of stem cells but it has been used to try to locate the stem cells that maintain the corneal epithelium. Slow-cycling cells (including putative stem cells) are usually identified as "label-retaining cells". These are cells that retain a DNA-label such as BrdU or ³H-TdR, or a chromatin label such as GFP-tagged histone-2B, after prolonged labelling and a chase period to dilute the label from more rapidly dividing cells. The prolonged period of labelling is to label as many cells as possible including relatively quiescent stem cells that divide infrequently. The chase period is calibrated to dilute label from most cells in the tissue but not any slow-cycling cells (which include putative quiescent stem cells). This method is useful for identifying the location of putative stem cells but is not specific and will also identify other slow-cycling cell types and cells that divide during the labelling

period and then stop dividing when they terminally differentiate.

The ocular surface is a suitable tissue for label-retaining cell experiments because the corneal epithelial TACs divide quite frequently so will readily dilute the label. (The average mitotic rate has been estimated for rats as 14.5% per day for the whole corneal epithelium^[44] and this equates to approximately 37% for just the mitotic basal layer, based on the relative basal and suprabasal cell numbers in mouse corneas^[45]). Similarly, BrdU experiments indicate that almost 50% of basal corneal epithelial cells are in S-phase of the cell cycle, during a 24-h labelling period^[46]. The effectiveness of the chase period is also helped by the constant loss of cells from the superficial layer, as stem cells will not be lost in this way. It has been estimated that once cells leave the basal corneal epithelial layer the time to cell loss (turnover time) is only about 7 d (range 3¹/₂ to 14 d) for mice, rats and humans^[45,47-50] but a longer turnover time of between 14 and 21 d has been estimated for rabbits^[51].

Most investigations have identified label-retaining cells in the basal limbal epithelium but not in the corneal epithelium either after wounding^[5,13] or during normal homeostasis in mice^[13,45,52-54], rats^[55,56] and rabbits^[57]. Two caveats about the exclusive location of label-retaining cells to the limbus in these experiments should be mentioned: (1) species differences in cell cycle kinetics and technical differences between studies may affect the number of cells that remain labelled so the chase period needs to be optimised for each species (for example, in these studies, chase periods for treatments without wounding varied from 4 to 11 wk); (2) using a relatively short chase period of 4 wk, Chen *et al.*^[56] showed that approximately 20% of the label-retaining cells were slow-cycling Langerhans cells rather than putative slow-cycling stem cells. (These Langerhans cells also shared two other characteristics of putative stem cells, discussed below, as they were positive for the marker ABCG2 and had a high nucleus to cytoplasm ratio). Nevertheless, the results of all these studies consistently identified label-retaining cells in the basal limbal epithelium but not in the basal corneal epithelium and it is likely that most of these will be stem cells. Thus, these studies favour the LESC hypothesis, unless there is an additional stem cell population in the corneal epithelium, which is not slow cycling. They also argue against the GBL hypothesis unless none of the limbal label-retaining cells are stem cells.

A completely different result was found for one study with rabbits^[2], which prompted the authors to conclude that the corneal epithelium is not maintained by LSCs but by virtually all the cells of the basal corneal epithelium (referred to here as the GBL hypothesis). Rabbits were given 3 intravitreal injections of ³H-TdR at intervals of 4 d. After a 41-d chase (49 d after the first injection) the labelling index was higher in the corneal epithelium (17.8%) than the limbal

epithelium (3.8%)^[2]. However, the high labelling index suggests that many of the labelled cells were TACs, rather than slow-cycling stem cells, and the chase period was insufficient to detect label-retaining cells. Paradoxically, the chase period was comparable to that used in an earlier experiment, which identified label-retaining cells in the limbus but not corneal epithelium of rabbits^[57]. In this experiment, BrdU was infused from an osmotic mini-pump for 14 d, the pump was removed at 17 d, and the rabbits were left for a further 38-d chase period (*i.e.*, until 55 d after the beginning of labelling). In the ³H-TdR study^[2], autoradiography was used to detect label in high quality semi-thin sections and it is possible that this is more sensitive than the BrdU immunofluorescence used earlier^[57]. If so, more cell divisions and a longer chase period would be required to dilute the ³H-TdR below detectable levels in the majority of cells in order to identify any label-retaining cells. It would, therefore, be worth repeating the ³H-TdR experiment with a longer chase period before drawing conclusions that contradict the other label-retaining cell studies.

Movement of corneal epithelial cells

Early experimental evidence showed that cells moved from the limbus to the cornea to repair a corneal wound in guinea pigs^[58]. The observation that donor corneal epithelial cells, transplanted to the centre of rabbit corneas, were replaced by host cells more quickly at the periphery of the transplant also suggested that new host cells were moving centripetally from the periphery of the cornea to replace the older donor cells^[59]. Other indirect evidence that cells move centripetally from the limbus during homeostasis of unwounded corneas is reviewed elsewhere^[60]. More importantly, direct observations of radial epithelial movement during normal corneal homeostasis have consistently demonstrated that cells move centripetally from the periphery to the centre of the cornea. This supports the LESC hypothesis, which proposes that LSCs remain in their limbal niche but TACs move centripetally to maintain the corneal epithelium.

These experiments also provided estimates of the rate of centripetal movement of corneal epithelial cells for the unwounded cornea. This was estimated to be 28 μ m/d from observations of one human subject over 24 h using *in vivo* confocal microscopy^[61]. For mouse corneas, the rate of centripetal movement has been estimated as 11-26 μ m/d using three different approaches involving direct observations of labelled cells. Corneal epithelial cells labelled with India ink moved 17 μ m/d over 7 d^[62], patches of brightly fluorescent cells moved 26 μ m/d over 7 wk in mosaic GFP transgenic mice^[63] and fluorescent clones of cells extended 11 μ m/d over 12 wk in K14-CreERT2;R26R-confetti transgenic mice (from 9 to 21 wk after tamoxifen-activation of the reporter transgene)^[64]. Furthermore, the evidence from the mosaic GFP

transgenic mice^[63] and tamoxifen-activated reporter transgenic mice^[64] demonstrated that the same clonal lineage of cells moved across the full radius from the periphery to the centre. This is in contrast to cells in the conjunctiva, which do not move significantly at all^[62,65].

When Majo *et al.*^[1] proposed the CESC cell hypothesis they also proposed that the corneal and conjunctival epithelia continuously expand towards the limbus, which they described as a zone of equilibrium, so any movement in the corneal epithelium was predicted to be centrifugal. This is inconsistent both with the absence of movement in the conjunctiva^[62,65] and the convincing, direct evidence that movement of corneal epithelial cells is centripetal not centrifugal^[62-64]. The evidence for centripetal cell movement in the corneal epithelium is inconsistent with the CESC hypothesis as originally proposed^[1] but there is no need to link the stem cell location and movement aspects of the original CESC hypothesis. In principle, it would be possible for the corneal epithelium to be maintained by stem cells, scattered throughout the tissue, without invoking centrifugal movement. It is likely that TACs produced by CESC would only move radially because evidence from various mosaics and chimaeras implies that lateral movement is constrained (discussed in the next section). In theory, radial movement of TACs could be either centripetal or centrifugal but, as noted above, evidence for centripetal movement is compelling.

The causes of centripetal movement are not known and suggestions include: (1) population pressure from the periphery due to production of new TACs by LESC^[66-68]; (2) preferential loss of epithelial cells from the central cornea^[30,60]; (3) differential stiffness of cornea and limbus^[69]; (4) chemotaxis^[62]; (5) stimulation by corneal nerves^[70]; and (6) response to endogenous electric currents^[71].

If the LESC hypothesis was incorrect, centripetal movement could still be explained by a mechanism other than population pressure from the limbus. However, if LESC were absent or only active during wound healing, a peripheral source of cells would be required to replace peripheral cells that move centripetally, during normal homeostasis. This might be provided by limbal TACs for the GBL hypothesis or CESC in or near the limbus for the CESC hypothesis. Thus, both the CESC and GBL hypotheses could account for centripetal movement of cells in separately maintained regions on the same radius. However, evidence from transgenic mice shows that a single clone of cells moves across the full radius^[63,64], implying that there is a single source of cells in the limbus or peripheral cornea, rather than multiple sources throughout the cornea. This is more difficult for the CESC and GBL hypotheses to explain unless it is argued that not all radial regions are maintained by a single CESC or progenitor TAC. Another problem for the CESC hypothesis is that the CESC would tend to

move centripetally with the TACs, and so accumulate in the centre, unless the CESC were somehow stabilised in unidentified niches and the TACs could move past them. Overall, centripetal movement strongly favours the LESC hypothesis and it is difficult to reconcile this with the other two hypotheses without *ad hoc* assumptions.

Change in mosaic patterns after birth

In addition to direct studies of cell movement in real time, changes in patterns in several types of mosaic mice have provided additional evidence that cells emerge from the limbus at the periphery of the corneal epithelium and continue to move centripetally across the cornea. Mosaic patterns in adult mouse and rat chimaeras and mouse X-inactivation mosaics (*XLacZ* mosaics) are arranged as radial stripes in the corneal epithelium^[27,72,73], which is consistent with either centripetal or centrifugal movement, without significant lateral dispersion. Similar radial stripes, have been observed with various endogenous markers in human corneas, including traces of pigment^[58] and various opacities, cell inclusions or drug-induced lipidosis associated with vortex keratopathy (cornea verticillata) or hurricane keratopathy^[60,66,74,75]. In many cases the stripes form a spiral-pattern in the centre, which fits well with the more direct evidence for centripetal movement, discussed above, because centripetal movement of labelled cells transplanted to the rabbit limbus sometimes formed a similar spiral^[76].

Before about 5 wk of age, the pattern in X-inactivation mosaics is completely different from the adult radial stripes and the β -gal-positive and β -gal-negative cell populations initially form randomly orientated patches^[27,72]. Groups of β -gal-positive and β -gal-negative cells emerge from the periphery by about 5 wk and extend as radial stripes across the cornea. The simplest interpretation is that the formation of stripes coincides with the onset of activation of stem cells in the limbus that generate new cells, which replace those produced during development^[27]. This is supported by similar observations with mosaic transgenic mice^[77,78] and lineage tracing with a GFP-tagged lentiviral marker^[79], as illustrated in Figure 4, and is consistent with the LESC hypothesis but not with the CESC or GBL hypotheses.

One problem with these mosaic systems is that similar proportions of labelled and unlabelled cells were present so many of the radial stripes may comprise more than one adjacent clone that are similarly marked. Observations on *KRT5-LacZ*^{+/-} transgenic mice showed that they had rare β -gal-positive stripes in a predominantly β -gal-negative corneal epithelium, so largely avoiding the problem of multiple adjacent clones^[17]. The distribution of β -gal-positive stripes was not consistent with predictions of centrifugal extension of clones of labelled cells from β -gal-positive CESC distributed randomly in the corneal epithelium and the simplest interpretation is that the stripes represent clonal lineages derived from LESC located

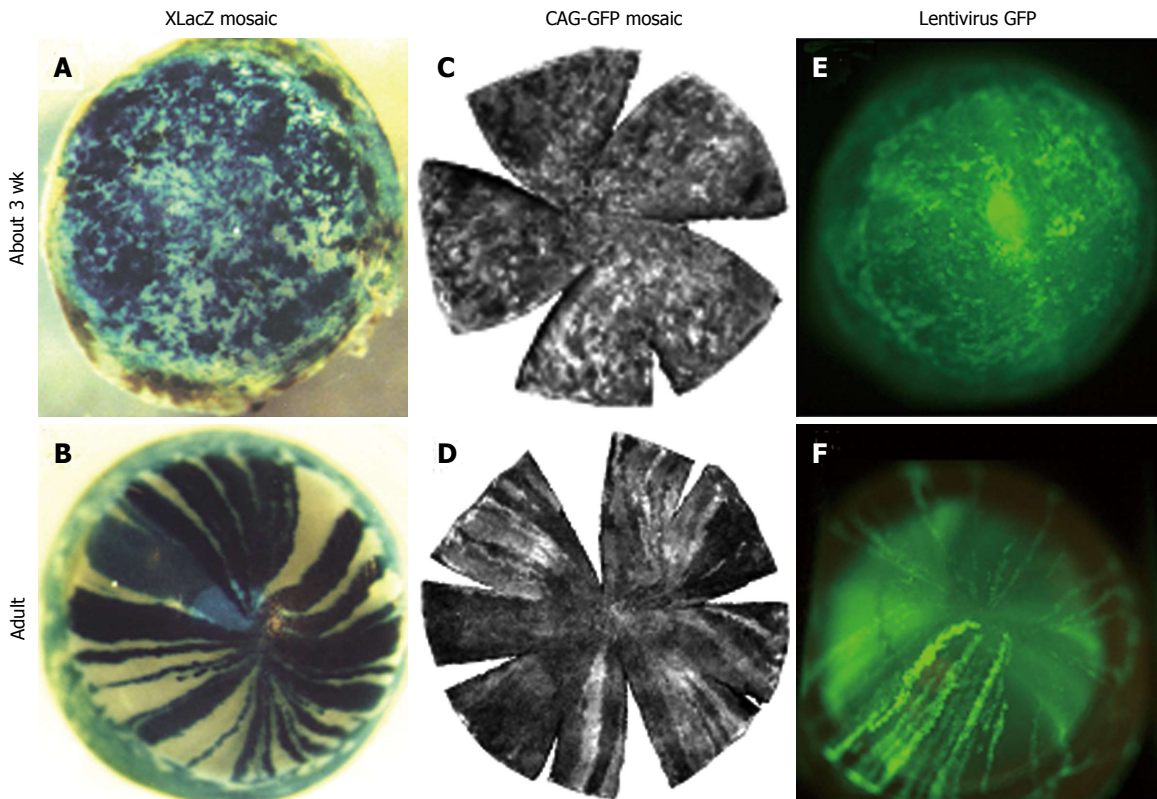


Figure 4 Transition from randomly orientated patches to radial stripes in corneal epithelia of different types of mosaic mice between 3 wk and adulthood. A and B: β -gal staining in XLacZ X-inactivation mosaics^[27]; C and D: Green fluorescent protein (GFP) fluorescence in CAG-GFP transgenic mosaics^[77]; E and F: GFP fluorescence in corneal epithelium after transfecting conceptuses with lentiviral vectors encoding GFP at embryonic day 9 or 10^[79]. Photographs (A and D) are reproduced from *Developmental Dynamics*^[27] with kind permission of John Wiley and Sons, (C and D) are reproduced from *Molecular Vision*^[77] with kind permission of the authors and editors, and photographs (E and F) are reproduced from *Molecular Therapy*^[79] with kind permission of the authors and the Nature Publishing Group. This combination of photographs was first published by Mort *et al*^[16].

in the limbus. However, analysis of striped patterns in *KRT5-LacZ*^{+/-} corneas is not unequivocal and similar analyses with inducible lineage markers are required, as discussed below.

Transplantation experiments

Bradshaw *et al*^[76] labelled rabbit limbal tissue *ex vivo* and transplanted it back to the limbus of the donor rabbits after first debriding the corneal epithelium across the full diameter. The labelled cells quickly colonised the corneal epithelium but, as the corneal epithelium was completely removed, this is equivalent to wound healing rather than normal corneal homeostasis. Majo *et al*^[11] transplanted either β -gal-positive limbal or central corneal tissue from transgenic mice into the limbus of β -gal-negative, immunocompromised mice and both sources of tissue produced similar results. Consistent with the earlier experiment with rabbits^[76], labelled clones of donor cells moved centripetally into the corneal epithelium if the host corneal epithelium was removed but it failed to contribute to the corneal epithelium if the host cornea was left intact. Thus, although the transplanted limbal tissue contributed to corneal repair, it did not contribute to steady state corneal maintenance during normal tissue homeostasis, as predicted by the LESC

hypothesis. This was a key result, which prompted Majo *et al*^[11] to propose the CESC hypothesis.

Circumstantial evidence

In addition to the specific investigations discussed so far, there are two circumstantial observations that favour the limbus as a site for stem cells. First, tumours of the ocular surface commonly involve the limbus^[80] and for other systems it has been suggested that tumour cells may preferentially arise from stem cells^[81,82]. This provides only weak, circumstantial evidence in favour of the LESC hypothesis.

Second, it is generally agreed that stem cells need a specialised niche environment to maintain the stem cell phenotype and this is likely to involve interactions with several cell types^[6,83]. For example, signalling from the microvasculature plays an important role in the mouse neural stem cell niche^[84]. Undeniably, the limbus provides a more diverse population of cell types than cornea and this is enriched further by its blood supply and for this reason it seems arguably a more likely location for a stem cell niche than the cornea. As already mentioned, an additional issue is that a stem cell niche in the basal corneal epithelium might be unstable because of the continuous centripetal movement of TACs. These considerations also make

Table 2 Examples of marker gene expression differences between the basal limbal and corneal epithelia during normal homeostasis identified by immunostaining

Positive markers			Negative markers		
Marker	Species	Ref.	Marker	Species	Ref.
Integrin $\alpha 9$	Mouse, human	[3,105,106]	Keratin 3	Rabbit	[4]
$\Delta Np63^1$	Human	[3,107,108]	Keratin 3/Keratin 12	Human	[3,108]
$\Delta Np63\alpha$	Human	[96]	NGF receptor (p75NTR)	Human	[3]
ABCG2	Human, rat, rabbit	[3,57,91-93,108]	Involucrin	Human	[3]
Vimentin	Human	[108]	Connexin 43	Human	[3,108]
Keratin 19	Human, mouse	[108,109]	E-cadherin	Human	[3]
Keratin 15	Human, mouse	[109]	Nestin	Human	[108]
N-cadherin	Human	[110]			
Bmi1	Human	[111]			
C/EBP δ	Human	[111]			
ABCB5	Human, mouse	[54]			

¹ $\Delta Np63$ is not expressed in the human basal corneal epithelium^[107] but it is expressed in mouse and rat corneal epithelia^[27,112,113]. Positive markers are expressed in basal limbal but not basal corneal epithelium. Negative markers are expressed in basal corneal but not basal limbal epithelium.

it more likely that stem cell niches would be located preferentially in the limbus rather than the cornea.

Although some tissues are maintained during normal homeostasis by stem cells in the main body of the tissue, the limbus is not the only putative stem cell niche with a more peripheral location. For example, there are two types of stem cells that maintain the epithelium that lines the intestinal crypts and villi: crypt base columnar cells and position +4 reserve stem cells. These are both located near the base of the intestinal crypts, from where they produce TACs, which move up the crypt and generate the different functional cell types of the villus epithelium^[85]. Maintenance of the corneal epithelium by stem cells located in the limbal epithelium, as proposed by the LESC hypothesis, is essentially analogous to the way the intestinal epithelium is maintained. The circumstantial evidence that the limbus is a likely location for a stem cell niche supports the LESC hypothesis. However, it does not provide strong evidence against the CESC hypothesis, which accepts that LSCs exist, or the GBL hypothesis, which predicts there are no stem cells and so no niches.

Stem cell markers and phenotype

The Holy Grail of stem cell research is to find a phenotype or cell marker that allows the stem cells to be unequivocally distinguished from all neighbouring cells, including early generation TACs, and isolated for further study. This has not yet proved possible for the putative stem cell population(s) that maintain the corneal epithelium. Early evidence that the basal limbal epithelium contained stem cells was produced by an immunohistochemical study of keratin 3 (K3), which is considered to be a corneal differentiation marker^[4]. K3 is expressed in the basal and suprabasal layers of the rabbit corneal epithelium but only the suprabasal layers of the limbal epithelium, leading to the conclusion the basal limbal epithelium was less differentiated than the other epithelial layers. The

mouse has no K3^[86] but K12, which normally pairs with K3, is present and expression is restricted to the cornea^[87], as shown in Figure 3B. Several authors have also noted that cell morphology of cells in the basal limbal epithelium was more characteristic of stem cells (smaller, euchromatin-rich, high nucleus to cytoplasm ratio) than the corneal epithelium^[3,88] but, as already noted, Langerhans cells in the limbus also share this phenotype^[56]. These observations are consistent with the hypothesis that the limbus contains stem cells but no more than that.

The discovery of the K3 difference between basal limbal and corneal epithelia, as a whole, was followed by a quest for a specific cell marker to identify the LSCs within the limbal epithelium. Many candidate markers have been proposed based on differential expression studies (reviewed in reference^[18]) or conventional immunostaining (Table 2) but no definitive marker has been found, that is known to be expressed in putative stem cells in the limbus but not in neighbouring early generation TACs.

Some of the markers expressed in the limbal but not the corneal epithelium have been identified as putative stem cell markers in other tissues. ATP-binding cassette transporters (ABC transporters) are a family of transmembrane proteins whose functions include the transport of (potentially harmful) metabolic products out of the cells^[89]. Conceptually, they may form a component of the molecular mechanisms by which long-lived stem cells reduce the potential for genomic damage over their extended lives, and their expression has been correlated with stem cell activity^[90]. ABCG2 expression in the limbus is one such example and cells expressing this marker can be isolated as a "side population" by fluorescence-activated cell sorting (FACS)^[3,57,91-95]. However, as noted above, some of the ABCG2-positive, label-retaining cells with a high nucleus to cytoplasm ratio cells in the rat limbus have been identified as Langerhans cells rather than epithelial stem cells^[56].

It has recently been shown that ABCB5 appears to be a promising new marker for LESC and early TACs in both mice and humans, which should also allow enrichment by FACS sorting^[54].

Despite the absence of a marker that is only expressed in the stem cells, Δ Np63 α has proved useful for identifying cultures of human limbal cells with sufficient LESC and early TACs for clinical transplantation^[37,96].

RESOLVING THE LESC VS CESC DEBATE

Some of the evidence discussed so far (summarised in rows 1-15 of Table 1) is inconclusive. Evidence from holoclone experiments with pig and human tissues is inconsistent and, in any case, the critical thing is to understand how the corneal epithelium is maintained *in vivo* during normal homeostasis. Various studies have shown that some cells in the central corneal epithelium are capable of acting as long-term progenitor cells. On the face of it, this favours the CESC and GBL hypotheses. However, these observations are also consistent with the LESC hypothesis if some basal corneal epithelial TACs have a latent proliferative potential that is only used if LESC function is compromised so homeostasis is disrupted. Drawing attention to this latent proliferative potential is an important outcome of Majo *et al.*'s^[1] investigations even if the CESC hypothesis ultimately proves to be incorrect.

Other evidence provides better discrimination. There are several strong arguments against the GBL hypothesis. It is inconsistent with the consensus of results from label-retaining cell experiments and the evidence that mosaic patterns in the mouse cornea change after birth, when clones of cells emerge from the peripheral cornea and form radial stripes. The GBL hypothesis also has difficulty accounting for the convincing evidence that corneal epithelial cells move centripetally across the full radius. In our view, this evidence (summarised in Table 1) is sufficient to exclude the GBL hypothesis and it is, therefore, not considered further.

These same observations also argue against the CESC hypothesis. However, results of the label-retaining cell experiments could be accommodated by the CESC hypothesis if CESC were not slow cycling. Nevertheless, the CESC hypothesis is inconsistent with the developmental switch from randomly orientated patches to stripes that emerge from the periphery in various types of genetic mosaics in mice and then extend across the full radius to the centre. By the same token, the CESC hypothesis requires *ad hoc* assumptions to account for centripetal movement, if the same clone of cells moves across the full radius.

Conversely, the LESC hypothesis is inconsistent with the observation that when genetically marked limbal tissue was surgically transplanted to the limbus of immunocompromised mice, donor cells failed to

move into the cornea unless the corneal epithelium was removed^[1]. Thus, the LESC and CESC hypotheses each have difficulty accounting for one type of evidence. In each case, critical evidence is based on experiments with mice so there are no grounds for suggesting that maintenance of the corneal epithelium differs between mice and humans or other species.

Clinically, it may not matter whether the LESC or CESC hypothesis is correct as both agree that the limbus is a suitable source of stem cells for therapeutic use. However, we need to know where the stem cells that maintain the corneal epithelium during normal homeostasis are located in order to understand the biology of this process. The key issue that needs to be resolved is why evidence from mouse mosaics and transfection with lentiviral markers conflict with results of surgical transplantation experiments. Analysis of mosaics show that, during normal homeostasis, clones of cells appear at the corneal periphery at around 5 wk after birth and extend centripetally across the corneal radius, consistent with activation of LESC. In contrast, surgical studies in mice show that transplantation of labelled cells to the limbus fail to colonise the cornea in a similar way.

One possible explanation is that the transplanted limbal tissue failed to colonise the corneal epithelium because the surgical manipulation or other aspects of the experimental procedure somehow perturbed normal homeostasis and affected the outcome. In principle, one way of testing this would be to label individual cells in the limbus of adult mice, using a genetic switch rather than surgical transplantation and test whether any of the labelled cells produce long-lived clones of cells that colonise the corneal epithelium. Similar genetic labelling of cells in the adult basal corneal epithelium would also allow investigation of whether these produce long-lived clones of cells in the corneal epithelium. This requires lineage tracing experiments and some possibilities are discussed below.

It should be borne in mind that some of the apparent contradictions between the different hypotheses may be more imagined than real, and arise as a result of different research groups attempting to subdivide and label what is, perhaps, a continuum of biological situations. It seems certain that limbal stem cells exist, that they can contribute to regeneration of the cornea, and yet that the basal corneal epithelial cells themselves also have massive regenerative potential. There is no reason why the balance between limbal-mediated and corneal-mediated corneal regeneration should not shift over the lifetime of the animal, with age, disease and injury, and no reason why the balance should necessarily be the same in different species.

Although we talk about wounded and unwounded corneas as if they are separate entities, in fact the corneal epithelium is, of necessity constantly regenerating, because of normal desquamation of cells. Desquamation rate is modulated by rate of blinking,

tear film composition, irritants and abrasive dust in the environment, chronic abrasion caused by *e.g.*, contact lens wear and diseases such as trachoma, acute minor scratches, ranging through to significant physical or chemical injuries and acute infections such as Herpes simplex keratitis. This continuum of insults to the corneal surface may require different levels of limbal response to support the regenerative potential of the corneal epithelial cells. It may be that the genuinely uninjured cornea does not require limbal input, but the genuinely uninjured corneal epithelium does not exist. Experimentally, factors such as the abrasive nature of bedding and dust from food that laboratory mice are exposed to, may modulate corneal regeneration and be a source of variation between research institutes. Furthermore, the large circular central corneal wounds that are so widely used as models of induced regeneration do not really recapitulate any of the most common injuries that happen in life.

There may also be problems with the practical definition of stem cells, particularly with regard to their property of "immortality". We accept that TACs do not renew indefinitely and therefore have a finite lifespan, whereas stem cells do not have this constraint. However, outside the laboratory, a stem cell cannot outlive the individual. We do not know the maximum lifespan of a TAC but this is likely to vary stochastically. If the longer-lived TACs survive for 6 mo or more, this will encompass the effective lifespan of most laboratory mice used experimentally. At that stage, the difference between a long-lived TAC and a stem cell becomes blurred. On the other hand, in larger animals such as humans, a lifespan of 6 mo or a year for a TAC is utterly insignificant in terms of the lifespan of the individual. Differences such as this may start to explain why stem-like regenerative ability may be assigned to the corneal epithelium on experimental small animals, while at the same time the data do not reflect clinical experience in humans.

Lineage tracing experiments

To test whether any cells in the limbal and/or corneal epithelia can generate long-lived clones of cells that colonise the corneal epithelium, an inducible lineage tracing method is required that can label some of the putative stem cells in the adult at a chosen time without surgical intervention or disturbing homeostasis. Sophisticated, inducible lineage tracing methods using Cre/*loxP* transgenic mice are now available that can be used to throw a genetic switch to label a chosen cell population with a fluorescent or histochemical marker that will identify them and all their mitotic progeny. Such methods have been used to trace stem cell lineages in other tissues, including the hair follicle^[97], intestinal epithelium^[98-100] and ovarian surface epithelium^[101], and this approach could be used to help resolve the LESC vs CESC debate. A similar approach has already been used, in conjunction with a multi-coloured reporter construct, to trace clonal lineages in

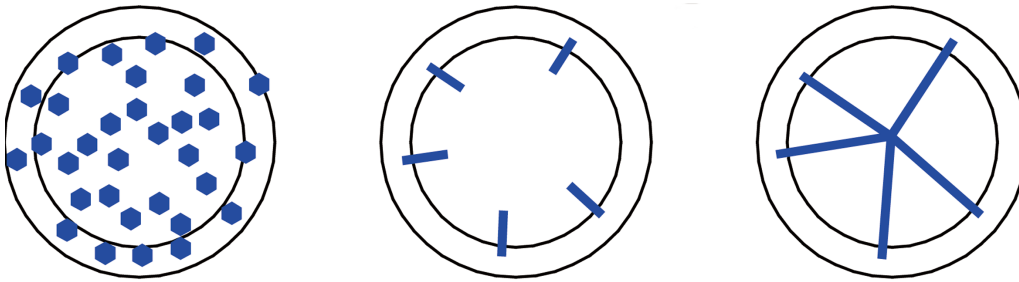
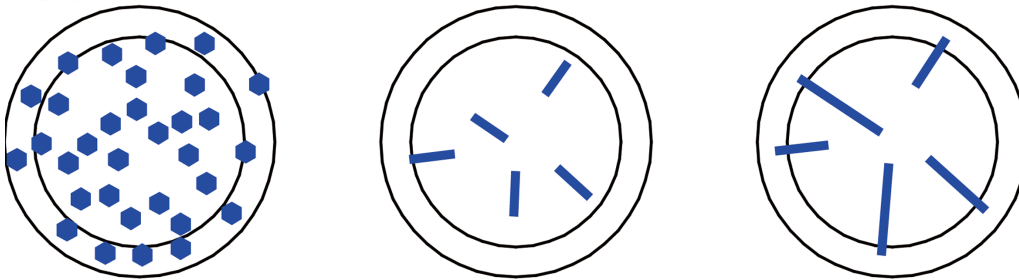
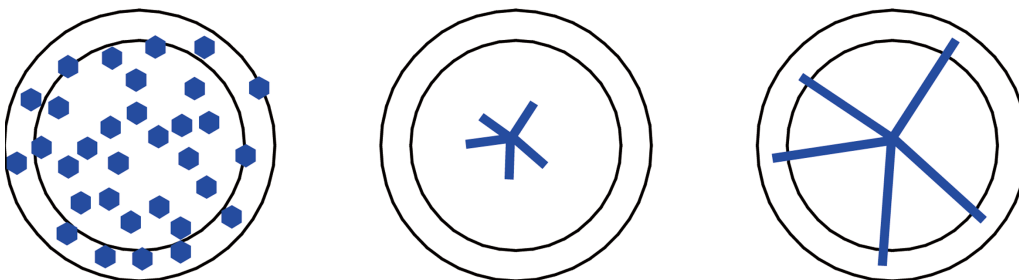
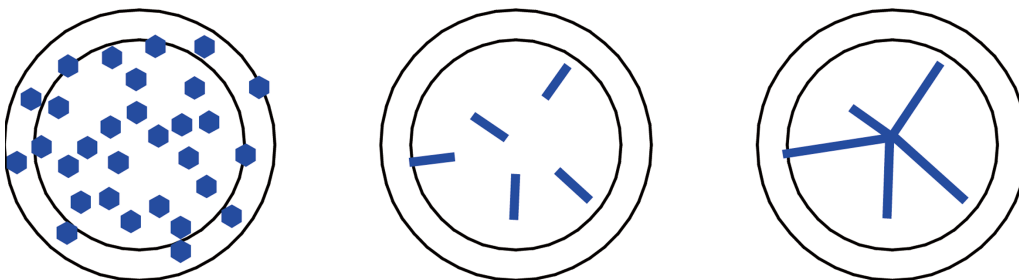
the ocular surface of zebrafish and demonstrate that, as in mice, the initial patchwork of cells established in the embryo is replaced by a radial pattern of clones that extend from the limbus^[102].

We have begun to explore this Cre/*loxP* lineage tracing approach using transgenic mice in which a reporter transgene is ubiquitously expressed once the flanking *loxP* sites are recombined by active Cre recombinase to remove an upstream stop sequence. Cre recombinase is provided in the form of a CreER fusion protein, which is produced by another transgene under the control of a ubiquitous promoter. The CreER fusion protein is normally sequestered in the cytoplasm unless the mouse is treated with tamoxifen. This binds to the modified oestrogen receptor (ER) and translocates CreER to the nucleus, where it can recombine the *loxP* sites, so removing the stop sequence and activating the reporter transgene. Use of a ubiquitous promoter to drive expression of CreER provides an unbiased approach, which allows putative stem cells in the limbus or cornea (and any other tissue) to be labelled. However it is not specific for stem cells so initially most of the labelled cells will not be stem cells but, by including a chase period, short-lived clones founded by labelled TACs will be shed, leaving long-lived clones founded by labelled stem cells.

The genetic switch is activated when the mouse is injected with tamoxifen. Delaying tamoxifen treatment, until well after the adult stem cells are activated, should ensure that the genetic switch is thrown to label individual adult stem cells, during normal homeostasis. This avoids labelling ancestral cells, which could each generate multiple labelled stem cells. Furthermore, by titrating the dose of tamoxifen it should be possible to label a relatively small proportion of cells so only a few stem cells will be labelled per eye. Together, this will ensure that most of the clones of labelled cells that remain as stripes or patches after the chase period are individual clones produced by single stem cells. The predicted results for such an experiment are shown, for the LESC hypothesis and three versions of the CESC hypothesis, in Figure 5.

Tamoxifen-inducible labelling of a low proportion of stem cells in adults is a significant advantage over analysis of other types of mosaics, where the cells are labelled early in development and many patches and stripes of labelled TACs are likely to be derived from multiple adjacent labelled stem cells. This is because labelling a cell in the embryo will produce a large clone of labelled cells, some of which will remain close together throughout development. Later, when adult stem cells are specified, some adjacent stem cells will probably be clonally related making it difficult to identify TACs descended from a single stem cell in these mosaic systems.

Examples of results of a preliminary experiment of this type are shown in Figure 6 and indicate that the strategy suggested in Figure 5 is feasible. The cornea

A LESC hypothesis (centripetal movement)**B** Original CESC hypothesis (centrifugal movement)**C** Central CESC and centrifugal movement**D** CESC throughout cornea and centripetal movement

Time 1

Time 2

Time 3

Figure 5 Hypothetical results from a lineage tracing experiment to distinguish between the limbal epithelial stem cell and corneal epithelial stem cell hypotheses. In each figure the inner disc represents the corneal epithelium and the outer ring represents the limbal epithelium. If a reporter transgene is driven by a tamoxifen-inducible, ubiquitous promoter, a proportion of all the cell types in the ocular surface (and other tissues) will be labelled shortly after tamoxifen treatment. The frequency of labelled cells will depend partly on the dose of tamoxifen, which could be titrated to ensure only a few stem cells are labelled per eye. Time 1 is shortly after tamoxifen-treatment and the labelled cells may have divided to produce a small clone of labelled cells. By Time 2, the short-lived labelled clones produced by labelling transient (or transit) amplifying cells should have been shed from the corneal epithelium but long-lived labelled clones produced by long-lived labelled stem cells will remain. Expectations for distributions of labelled cells at Times 2 and 3 vary for the different hypotheses. A: The limbal epithelial stem cell hypothesis predicts clones of labelled cells will extend radially from the limbus and, by Time 3, clones of labelled cells will span the full radius; B: The original corneal epithelial stem cell (CESC) hypothesis predicts clones of labelled cells produced by labelled stem cells may also extend radially but will extend centrifugally from stem cells located throughout the corneal epithelium itself. Clones of labelled cells that do not arise from the centre of the cornea will not span the full radius; C: If the CESC hypothesis is modified so that all the CESC are at the very centre of the cornea, centrifugal movement will produce clones of labelled cells that span the full radius by Time 3 but at Time 2 there should be no labelled peripheral cells produced by stem cells; D: If the CESC hypothesis is modified so that the CESC are located throughout the corneal epithelium but movement is centripetal, clones of labelled cells that do not arise from the periphery of the cornea will not span the full radius. To distinguish between the various hypotheses it will be necessary to compare patterns of labelled clones at different times after tamoxifen treatment.

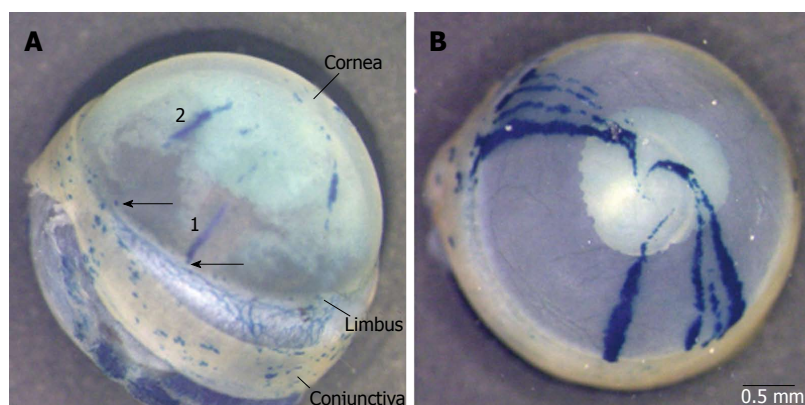


Figure 6 Preliminary results from a lineage tracing experiment to distinguish between the limbal epithelial stem cell and corneal epithelial stem cell hypotheses. Eyes from CAGG-CreER; R26R-LacZ mice that were injected with tamoxifen to induce LacZ reporter gene expression and stained for β -galactosidase (β -gal) activity after different chase periods. The pigmented iris is visible through the cornea and appears grey, whereas the β -gal staining is blue. A: Side view of a β -gal-stained eye, after a chase period of 9 wk and 4 d, with several radial β -gal-positive stripes and small patches in the cornea and numerous β -gal-positive patches in the conjunctiva. (The conjunctiva is torn near the limbus and hangs down at the bottom right of the photograph, so the sclera is visible between the limbus and conjunctiva.) Stripe 1 is a limbus-cornea (LC) stripe, with its more peripheral end in the limbus (arrow), consistent with expectations of the limbal epithelial stem cell (LESC) hypothesis (see Figure 5). It also appears to be aligned with other β -gal-positive patches towards the centre of the cornea so it may be part of a longer discontinuous stripe. Stripe 2 is a cornea-cornea stripe with both ends in the cornea, consistent with the corneal epithelial stem cell hypothesis. However, it is radially aligned with a small β -gal-positive patch in the limbus (arrow), which could be the location of a β -gal-positive LESC. If so, stripe 2 might be a discontinuous stripe, which extended from a LESC that was not continuously active (consistent with the LESC hypothesis); B: Anterior (frontal) view of a β -gal-stained eye, after a 14-wk chase period, with eight radial β -gal-positive stripes. All eight stripes are LC stripes with one end at the limbus and many extend the full radius and have a curved end, consistent with a central spiral pattern, as reported for other chimaeric and mosaic eyes^[27,72,73]. CAGG-CreER; R26R-LacZ mice were produced by crossing CAGG-CreER and R26R-LacZ mice [full names Tg(CAG-cre/Esr1*)5Amc and B6.129S4-Gt(ROSA)26Sortm1Sor/J; references^[116,117]]. LacZ reporter gene expression was induced at 12 wk by 3 injections of tamoxifen (100 μ g/g body weight per injection).

illustrated in Figure 6A was stained for β -gal activity after chase period of 9¹/₂ weeks. Stripe 1 has its peripheral end in the limbus and its more central end in the cornea so, using the stripe classification system described for mosaic *KRT5*^{LacZ/-} transgenic mice^[17], it is classified as a limbus-cornea (LC) stripe. This is consistent with expectations of the LESC hypothesis (Time 2 in Figure 5A), particularly as the stripe does seem to extend into the limbus itself (arrow in Figure 6A). A peripheral stripe could also be consistent with the original CESC hypothesis (Figure 5B) or the centripetal version shown in Figure 5D, although, in the latter case, peripheral stripes would be expected to be entirely within the cornea (CC stripes). This LC stripe fits less well with the hypothesis that all the CESC are located in the very centre of the cornea (Figure 5C) unless additional assumptions are made (e.g., stripes have already extended to the periphery by 9¹/₂ weeks).

Stripe 1 also appears to be radially aligned with other β -gal-stained tissue located more centrally so it may be a longer discontinuous stripe. Discontinuous stripes have been discussed elsewhere for *KRT5*^{LacZ/-} transgenic mosaics^[17] and also occur in mouse chimaeras and X-inactivation mosaics^[27,72,103]. The discontinuities in β -gal staining shown in Figure 6A could reflect: (1) separate clones of cells derived from β -gal-positive and β -gal-negative CESC or TACs that are radially aligned; (2) dispersal of a β -gal-positive clones by incursions from laterally adjacent β -gal-negative clones; or (3) alternating contributions of more than one stem cell to a single radial stripe if individual stem cells cycle through phases of activity

and quiescence.

Stripe 2 in Figure 6A is a cornea-cornea (CC) stripe with both ends in the cornea, consistent with the predictions of the original CESC hypothesis at Time 2 (Figure 5B) or the centripetal version shown in Figure 5D. However, it might also be consistent with the LESC hypothesis (Figure 5A) because this stripe is radially aligned with a small β -gal-stained patch in the limbus, which could mark the location of a β -gal-positive LESC. If so, stripe 2 might be a discontinuous stripe formed in two phases. During the first phase a β -gal-positive region might have extended from a β -gal-positive LESC, which was active during the early part of the chase period. This could have been followed by extension of a β -gal-negative region generated by an adjacent active β -gal-negative LESC, if the β -gal-positive LESC became inactive during the later part of the chase period.

The eye shown in Figure 6B was stained after a 14-wk chase period and has eight β -gal-positive stripes, all of which are LC stripes, consistent with the LESC hypothesis (Time 3 in Figure 5A) and both the original CESC hypothesis (Figure 5B) and the variant shown in Figure 5C. However, the variant CESC hypothesis shown in Figure 5D predicts that only a small proportion of stripes would extend right to the limbus. Some of these stripes span the full radius, consistent with the LESC hypothesis (Figure 5A) and the variant CESC hypothesis shown in Figure 5C. It could also be consistent with the original CESC hypothesis (Figure 5B) and the centripetal version, shown in Figure 5D, if full-radius stripes comprised several shorter stripes produced by β -gal-positive

CESCs aligned on the same radius. However, most of the cornea is β -gal-negative so it seems unlikely that the *LacZ* reporter would be activated in radially aligned CESCOs but not in many of the other CESCOs, located elsewhere in the corneal epithelium.

The preliminary results shown in Figure 6 and the alternative explanations of discontinuous stripes indicate that the interpretation of the stripe patterns may be more complicated than predicted in Figure 5, so detailed analysis of many more eyes and different chase periods will be required to resolve the LESC vs CESC debate. Nevertheless, this lineage-tracing approach appears to be a promising way of resolving the conflicting evidence from transplantation experiments and mosaics. Although important, the evidence from conventional mosaics is limited because the time of stem cell labelling cannot be controlled and occurs early in development. Even lineage tracing with a GFP-tagged lentiviral marker (Figure 4E and F) relied on marking one cell population before birth^[79]. In contrast, a tamoxifen-inducible transgenic reporter system enables cells to be labelled at a specific time in the adult without the risk of disturbing homeostasis with surgical intervention.

While this review was in preparation, a similar lineage tracing study was published online, in a preliminary form, and this has already been mentioned in the section on cell movement^[64]. This used K14-CreER^{T2}; R26R-confetti mice, where the keratin 14 (K14 or *Krt14*) promoter, rather than a ubiquitous promoter, was used to drive tamoxifen-inducible CreER^{T2}. The multi-colour "R26R-confetti" fluorescent reporter^[100] was used to identify labelled cells. This reporter is based on an earlier "brainbow" construct^[104] and is also similar to that used for the zebrafish cornea^[102]. According to the authors, immunofluorescence showed that K14 protein was present in basal epithelial cells in the mouse limbus but the central corneal epithelium had much lower levels. Thus, limbal epithelial cells will be preferentially targeted for labelling, making it difficult to test whether CESCOs exist. On the other hand, a chase period to remove short-lived clones formed by TACs rather than stem cells is less important than for the experimental design shown in Figure 5, where CreER is expressed ubiquitously.

Mice were treated with tamoxifen at 6 wk and corneas of the same mice were imaged repeatedly to allow changes in size and position of different clones to be tracked in real time. This elegant time-lapse study showed that labelled cells began to emerge from the limbus 5 wk after tamoxifen treatment and the same stripes were tracked at 9, 13, 17 and 21 wk, as they extended into the centre of the unlabelled cornea without overlapping. The frequency of labelled clones was higher than for the preliminary results illustrated for tamoxifen-treated CAGG-CreER; R26R-*LacZ* mice, shown in Figure 6. However, because the multi-coloured R26R-confetti reporter randomly labelled cells with one of up to ten different colours, individual

adjacent clones could be distinguished easily. In addition to the radial stripes, the authors reported the presence of some rare, small patches of labelled cells in the cornea, which they suggested might have arisen in the cornea itself.

This is an important study as it is the first lineage tracing investigation of the mouse cornea with an inducible marker that was activated in adults. In particular, the informative, real-time study with live mice proved that individual cells labelled in the adult limbal epithelium could form long-lived clones that extend centripetally across the complete radius of the cornea during normal homeostasis. This effectively provides a non-surgical equivalent of the limbal transplantation experiment described by Majo *et al.*^[11] and supports the interpretation that the outcome of the transplantation was adversely affected by the surgical procedures and homeostasis was perturbed. If so, this would undermine the evidence for the CESC hypothesis and reconcile the conflicting evidence from surgical transplantation experiments and conventional mosaics. In effect, all the available evidence would then be consistent with the LESC hypothesis. To further clarify the situation, additional investigations, driving CreER from a promoter expressed in the corneal epithelium, are now required to investigate whether long-lived clones can arise in the adult cornea as well as the limbus.

CONCLUSION

There is strong evidence that the corneal epithelium is maintained by stem cells rather than solely by proliferation of more differentiated cells in the basal corneal epithelium. Most evidence also favours the conventional LESC hypothesis, which proposes that limbal epithelial stem cells maintain the corneal epithelium during normal homeostasis. Although limbal transplantation experiments favour the alternative CESC hypothesis, this result could be reconciled with the LESC hypothesis if surgical transplantation perturbed normal homeostasis and affected the outcome. This possibility is supported by a recent non-surgical, lineage-tracing experiment, which demonstrated that clonal derivatives of cells in the limbal epithelium move into the corneal epithelium during normal homeostasis. Thus, the available evidence supports the conclusion that, during normal homeostasis, the corneal epithelium is maintained by stem cells in the limbus, which produce daughter TACs that migrate centripetally, rather than any stem cells in the corneal epithelium itself. However, if homeostasis is compromised, so the limbal epithelial stem cells are unable to maintain the corneal epithelium, it seems likely that TACs in the basal corneal epithelium can act as long-term progenitors and maintain the tissue for a considerable time in the absence of functional LESCOs.

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Imprinted *Zac1* in neural stem cells

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important role for NSC quiescence, proliferation and differentiation. Overall, transcriptional, epigenomic, and genomic mechanisms seem to coordinate the functional relationships of NSCs and imprinted genes from development to maturation, and possibly aging.

Key words: *Zac1*; Cell fate decisions; Neural stem cells; Genomic imprinting; *Igf2-H19*; *Dlk1*; *p57^{Kip2}*; *Necdin*; Differentiation; Imprinted gene networks

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Core tip: Both neural stem cells (NSCs) and imprinted genes participate in the same developmental processes. Here, we will explore the possibility that these two processes actually interact with each other. We will exemplarily consider the role of single imprinted genes in NSC biology based on their functional relationship to the imprinted gene *Zac1*, which is itself at the focus of this review due to its role in directing neuronal vs astroglial differentiation of NSCs and as a central hub of an imprinted gene network comprising genes important to NSC biology.

Abstract

Neural stem cells (NSCs) and imprinted genes play an important role in brain development. On historical grounds, these two determinants have been largely studied independently of each other. Recent evidence suggests, however, that NSCs can reset select genomic imprints to prevent precocious depletion of the stem cell reservoir. Moreover, imprinted genes like the transcriptional regulator *Zac1* can fine tune neuronal vs astroglial differentiation of NSCs. *Zac1* binds in a sequence-specific manner to pro-neuronal and imprinted genes to confer transcriptional regulation and furthermore coregulates members of the p53-family in NSCs. At the genome scale, *Zac1* is a central hub of an imprinted gene network comprising genes with an

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INTRODUCTION

Neural stem cells (NSC), also known as neural precursor cells (NPC), are the common source of all neuronal and glial cells, including astrocytes and oligodendrocytes, in the developing and adult CNS. They arise from the neuroepithelial layers which line the spinal canal and forebrain ventricles at early embryonic stages and reside in circumscribed regions in the postnatal

brain to produce in a spatio-temporal controlled manner a variety of cell-types. Following a series of symmetric proliferative divisions, NSCs progress to asymmetric neurogenic divisions. Hereby, the parent cell maintains the progenitor state while the daughter cell migrates to its final destination, exits from the cell cycle, differentiates and participates in the formation of complex neural networks^[1]. Although the last decade has witnessed major progress on the pathways and genes coordinating NSC behavior, a potential role of imprinted genes has been largely ignored. In this review, we will consider current evidences for the general impact of imprinted genes in NSC cell fate decisions and differentiation with a particular focus on their relationship with the imprinted gene *Zac1*, as well as the role of *Zac1* itself, which encodes a versatile transcriptional regulator.

GENOMIC IMPRINTING

Although equivalent complements of paternally and maternally expressed autosomes are transmitted from parent to offspring, select autosomal regions can be lastingly silenced as a result of their parental origin. This process takes place in the respective parental germ cells and is known as genomic imprinting. Germline-derived imprints are preserved during fertilization and somatic development with few remarkable exceptions (see below). Some 120 genes with a verified imprinting status have been identified in mouse, which are largely conserved in human and correspond to less than 1% of the genome^[2]. A considerably larger number of genes with a strong bias in allele-specific expression has been recently detected in mice brains although an authentic imprinting status has still to be proven^[3,4]. In this respect we note that the reported number of genes showing allele-specific expression differences in mice actually matches general estimates on tissue-specific and allele-specific differences in human gene expression (approximately 10%) based on common genetic variations (*i.e.*, single nucleotide and copy number polymorphisms)^[5]. This observation speaks against the hypothesis that most of these allelic differences originate from genomic imprinting.

Multiple molecular mechanisms govern imprinted gene expression, they include CpG methylation of specific DNA sequences, ncRNAs, alterations in chromatin structure, and posttranslational histone modifications (*e.g.*, lysine acetylation, lysine and arginine methylation, serine phosphorylation, and covalent binding of the small peptide ubiquitin)^[6,7]. Above all, DNA methylation is at the center of the imprinting process and is thought to catalyze the establishment and life-long maintenance of genomic imprints. Differentially methylated regions (DMR), which harbor CpG-rich regulatory sequences, play a critical role in determining parental allele-specific expression. With few exceptions (*i.e.*, *Zac1*) imprinted

genes cluster in huge, conserved chromosomal domains throughout the genome, and their well-balanced expression enables regular development from fetus to early postnatal life^[8,9].

GENOMIC IMPRINTING AND THE BRAIN

Dysregulation of imprinted gene expression can elicit complex neurodevelopmental syndromes in humans, frequently associated with mental retardation [*i.e.*, Angelman syndrome (OMIM 105830) and Prader-Willi syndrome (OMIM 176270)]^[10]. Moreover, psychotic and autistic spectrum disorders possibly result from more subtle deregulation of imprinted genes. Indeed, mice harboring altered dosage of single or multiple imprinted genes showed various defects in higher brain functions ranging from learning^[11] and memory formation^[12] to social and nurturing behaviors^[13-15].

A ROLE OF IMPRINTED GENES IN NSCS?

On a historical ground, both NSCs and imprinted genes have been studied in the context of brain development. While our insight into the role of NSCs in brain development has largely expanded over the last decade^[1], the molecular targets and cellular pathways by which imprinted genes participate in brain development remain poorly defined. Importantly, the roles of NSCs and imprinted genes have been commonly investigated independently of each other although they apparently participate in the same developmental processes. Thus, new studies should explore possible reciprocal interactions between imprinted genes and NSCs during neurodevelopment. Here, we highlight recent evidences in the scientific literature on the critical role of imprinted genes in NSCs and major cellular processes they control.

INSULIN-LIKE GROWTH FACTOR 2

Insulin-like growth factor (*Igf2*) was the first mammalian gene shown to be maternally imprinted as a result of a differentially methylated imprinting control region (ICR) located nearby and upstream of *H19*, which is imprinted in the opposite direction (see below)^[16].

The product of *Igf2* is a potent growth factor promoting cell survival, proliferation, and differentiation by binding with high affinity to the insulin-like receptors *Igf1r* or *Igf2r*, but less efficiently to the insulin receptor (*Insr*). Hereby, *Igf2r* has no signaling function and is encoded by the paternally imprinted *Igf2r* gene. This antagonistic functional relationship together with the opposite imprinting of *Igf2* and *Igf2r* originally stimulated the genetic conflict hypothesis for genomic imprinting^[17].

During CNS development, *Igf2* is for the most part synthesized by the choroid plexus, and released into the cerebrospinal fluid (CSF), which contacts the primary cilia and apical surfaces of cortical progenitors.

There, CSF-borne Igf2 binding to the Igf1r stimulates neural precursors proliferation^[18].

Neurogenesis is maintained at a low level in the adult brain in so-called neurogenic niches, which comprise the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. Adult neurogenesis resembles in many aspects embryonic neurogenesis and raises the possibility of an additional role of Igf2 in stemness maintenance in the mature brain. In support of this hypothesis, transcriptome analysis of the SGZ evidenced substantially higher expression of Igf2 in stem cells than in immature neurons^[19]. Igf2 expression localized to radial-glia like NSCs (Nestin⁺, Sox2⁺, and Gfap⁺) and to a significant fraction of dividing cells (Ki67⁺). Interestingly, Igf2 enhances *in vivo* and *in vitro* the proliferation of NSCs isolated from the DG, but not from the SGZ, indicative of a site-specific effect. Finally, the secretion of Igf2 by Nestin⁺ progenitors in the external granule cell layer (EGL) potently stimulates neuronal cell proliferation whereas overexpression of Igf2 in granule neurons facilitates tumor formation in rodents^[20].

In sum, these reports show that *Igf2* enhances in a tissue- and age-dependent manner NSC proliferation and maintenance.

***H19* ncRNA**

The maternally imprinted *H19* locus localizes in tandem with the oppositely imprinted *Igf2* gene^[16] and encodes high levels of a 2.5 kb long RNA polymerase II-derived transcript. This large intergenic non-coding RNA (lincRNA) is not involved in the imprinting process^[21,22] but inhibits *in vitro* and *in vivo* tumor growth possibly due to its participation in and regulation of an imprinted gene network (IGN, see below).

Additionally, the first exon of *H19* encodes a micro RNA-containing hairpin that serves as a template for the miRNA 675, which reduces Igf1r expression and Igf2-signalling in the placenta^[23]. These self-restraining activities of the tandem *Igf2-H19* locus are necessary for normal embryogenesis and protect against parthenogenetic development in mammals^[24].

Erasure of imprinting at the *Igf2-H19* DMR is found in primordial germ cells (PGS) and associates with overexpression of *H19* RNAs at the expense of Igf2. This epigenetic switch-off is thought to safeguard PGS quiescence and prevent from teratoma formation^[25].

A similar strategy seems to be used by very small embryonic-like stem cells (VSELs), a population of very rare early-development cells with broad differentiation potential^[26]. VSELs can give rise to neurons, oligodendrocytes, and microglia among other cell types and possibly fulfill a role in physiological tissue rejuvenation and regeneration following cell damage.

In VSELs, the paternally silenced allele of select imprinted genes (*i.e.*, *Igf2-H19* and *Rasgrf1*) is reactivated by demethylation and results in biallelic expression. Conversely, select maternally expressed

alleles (*i.e.*, *Peg1*, *Igfr2*, and *p57^{Kip2}*) undergo deactivation and silencing by DNA methylation. Overall, this cell type-specific resetting of a limited number of genomic imprints supports growth-inhibition, cellular quiescence, and preservation of the stem cell population. On the other hand, methylation at the *Igf2-H19* DMR slowly increases with aging and has been suggested to facilitate increased insulin signaling and age-related depletion of the VSELs reservoir^[27].

Taken together, integrate imprinting at the *Igf2-H19* tandem locus critically controls growth and cell proliferation in the early embryo as well as in VSELs.

DELTA-LIKE HOMOLOG 1

The imprinted *Dlk1-Dio3* domain harbors the delta-like homolog 1 (*Dlk1*) and type III iodothyronine deiodinase (*Dio3*) genes which are expressed from the paternal derived chromosome. Similarly to the *Igf2-H19* locus, *Dlk1* and the close by *Gtl2* (gene trap locus 2, *alias* maternally expressed gene 3, *Meg3*) are imprinted in an opposite manner and locate 80 kb apart from each other. Three DMRs containing specific epigenetic signatures are hypermethylated on the paternal allele in somatic tissues^[28]. Hereby, those DNA methylation marks which are deposited in the paternal germline are confined to the central DMR. This region contains tandem repeats and localizes in the intergenic region of the tail-to-head orientated *Dlk1* and *Gtl2* genes (Figure 1).

Dlk1 encodes a transmembrane protein and closely resembles the Notch/Delta/Serrate type family of signaling molecules. Due to minor albeit important structural differences *Dlk1* is thought to compete with canonical Delta-like (DLL) ligands at the Notch receptor and to inhibit downstream signaling^[29].

Dlk1 is broadly expressed in developing mouse tissues and continues to be expressed in some adult neuronal tissues (*i.e.*, ventral striatum, septum, and ventral tegmental area) including the SVZ^[29,30]. Here, *Dlk1* is detected in NSCs (Nestin⁺, Sox⁺, Gfap⁺) and astrocytes (Sox2⁺, Gfap⁺, S100b⁺) localized to the germinal niche but not in differentiated parenchymal astrocytes (Gfap⁺, S100b⁺) and neuroblasts (β III-tubulin⁺). NSCs mainly express the membrane-bound form of *Dlk1* that is poorly active on its own but necessary for the response to secreted *Dlk1* produced by nearby astrocytes.

Postnatal deletion of *Dlk1* enhances NSC proliferation in the SVZ, causing the depletion of the quiescent NSC pool and reduced neurogenesis at later ages. Interestingly, this process takes place irrespectively of the parental origin of the deleted allele^[30], suggesting that *Dlk1* can be expressed from either parental allele. Indeed, both alleles of *Dlk1* are expressed from postnatal day 7 onward in NSCs and niche astrocytes as a result of an increase in methylation at the germ line targeted ICR of the maternal allele (Figure 1). This postnatal epigenetic switch at the *Dlk1* locus is

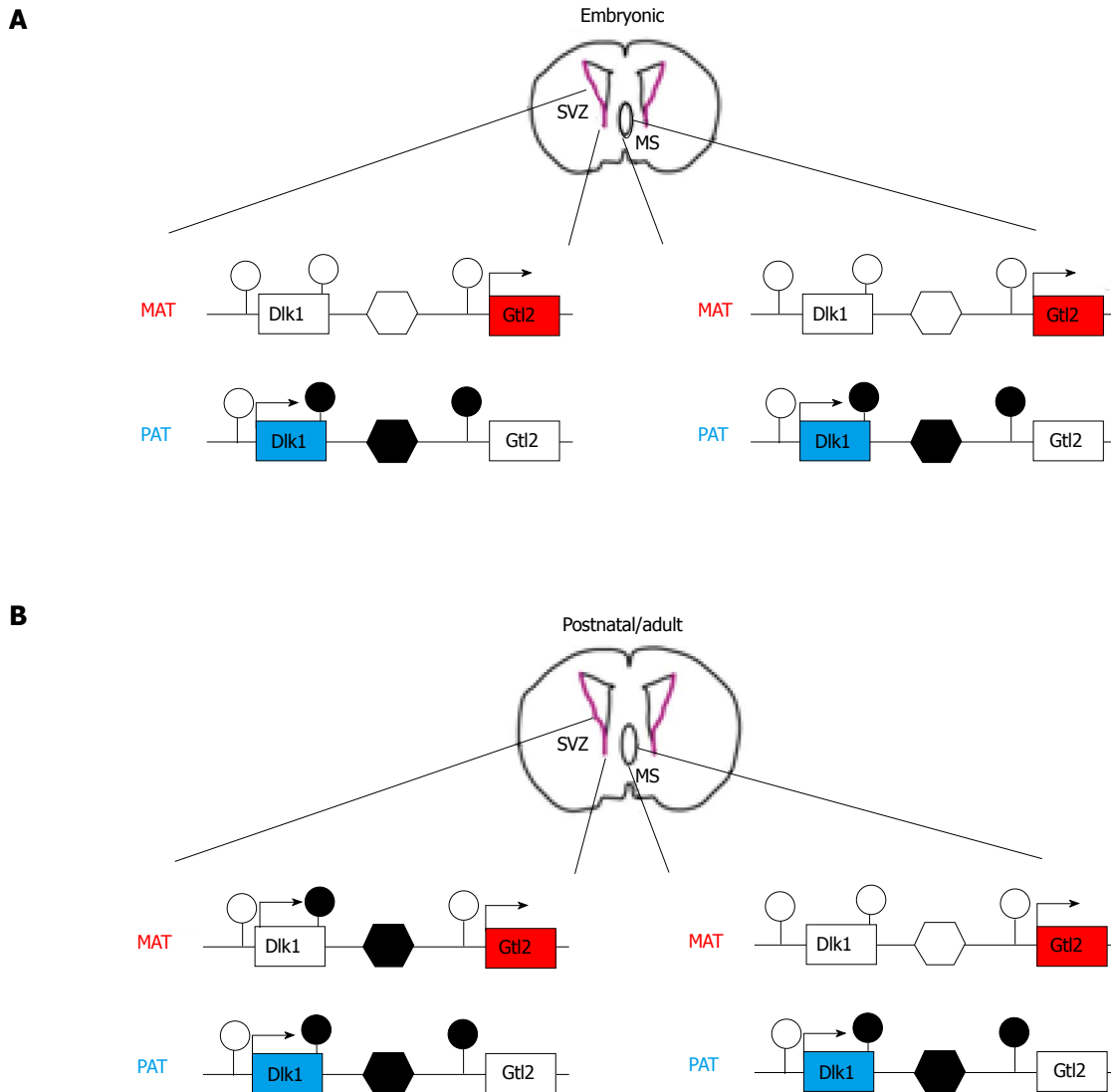


Figure 1 Genomic imprinting at the *delta-like homolog 1* locus is reset in the subventricular zone. A: *Delta-like homolog 1* (*Dlk1*) is monoallelically expressed from the paternal allele during development. Silencing of the maternal allele takes place in non-neuronal and various neuronal tissues such as the subventricular zone (SVZ) of the lateral ventricle (SVZ) and the medial septum (MS). At the molecular level, maternal silencing results from the absence of DNA methylation at the intergenic, germ line-controlled differentially methylated regions (DMR) (unfilled hexagon), which resides between the *Dlk1* and *Gtl2* genes and their associated DMRs (unfilled lollipops). Conversely, methylation at the intergenic DMR (filled hexagon), the 3' end *Dlk1* DMR and the *Gtl2* DMR (filled lollipops) associates with expression from the paternal allele. The same methylation patterns are present in the SVZ (left scheme) and MS (right scheme) at embryonic ages; B: *Dlk1* shows biallelic expression from postnatal day 7 onward towards adulthood in the SVZ, but not in the MS. Hereby, the maternal methylation pattern closely resembles the one on the paternal allele (*i.e.*, methylation at the 3' end *Dlk1* DMR, the intergenic DMR, but not the *Gtl2* DMR). At opposite, the methylation pattern in the MS is preserved and determines monoallelic *Dlk1* expression.

confined to the neurogenic areas, whereas all other tissues continue to express *Dlk1* exclusively from the paternal allele from embryogenesis to adulthood.

These findings suggest that genomic imprints at select loci (*i.e.*, *Igf2-H19* and *Dlk1*) are dynamically regulated in NSCs during specific developmental time windows, possibly to match the need for cell stemness vs cell differentiation.

NECDIN

Human and mouse *necdin* genes are maternally imprinted and localize to chromosome 15 q11.2 and a syntenic segment on chromosome 7, respectively^[10], within a cluster of paternally expressed genes.

Paternal deletion of this chromosomal segment in human underlies the neurodevelopmental Prader-Willi syndrome, which manifests with feeding anomalies, gross obesity, and hypogonadism^[10].

Necdin (*Ndn*) is broadly expressed in postmitotic neurons from early embryonic to adult ages with particular high expression in the developing hypothalamus, medulla oblongata, pons, and midbrain. Knock out mice show variable neonatal lethality, reductions in oxytocin and luteinizing hormone-releasing hormone producing hypothalamic neurons, impairments in serotonergic and catecholaminergic projections, and decreased tangential migration of neocortical interneurons from the basal forebrain^[31].

Ndn was originally discovered in a screen for

genes induced in neurally differentiated embryonic carcinoma cells^[32]. Functionally, Ndn potently inhibits proliferation in favor of differentiation by virtue of its interaction with various proteins critically involved in cell cycle progression and survival^[33,34]. Similar to the retinoblastoma tumor suppressor gene, Ndn binds to the carboxyl-terminal transactivation domain of E2F1 to repress its activity and consequently cell cycle progression^[33]. On the other hand, Ndn interacts with the amino-terminal transactivation domain of p53 to abolish its proapoptotic function without interfering with p53-dependent cell cycle arrest^[34]. Ndn also recruits the deacetylase sirtuin 1 to promote deacetylation of p53 leading to its inactivation and protection against DNA damage induced neuronal apoptosis^[35].

Furthermore, Ndn interacts and promotes the degradation of the hypoxia inducible factor-1 alpha (HIF) under normoxia^[36], whereas hypoxia enhances degradation of Ndn in primary NSCs through the HIF-associated ubiquitin-proteasome system^[37].

Interestingly, a growing number of reports (e.g.,^[38-40]) suggest that NSC proliferation is increased under hypoxia. Accordingly, Ndn-deficient NSCs show increased proliferation and apoptosis under normoxia, but not under hypoxia, which triggers degradation of endogenous Ndn in wild-type NSCs. Moreover, Ndn null mice show higher rates of NSC proliferation and apoptosis [e.g., in the embryonic (E14.5) ganglion eminence], strengthening Ndn's dual role in the suppression of proliferation and apoptosis^[33-35].

Ndn controls additionally the proliferation of NSCs in the ventricular zone of the embryonic cortex as evidenced by an upregulation of the stem cell marker Sox2, a downregulation of the cyclin-dependent kinase inhibitor p16^{Ink4}, and significantly increased proliferation rates in Ndn null mice^[41]. Interestingly, Ndn binds *in vitro* and *in vivo* to the polycomb protein Bmi1 and counteracts Bmi1-dependent inhibition of the p16^{Ink4} promoter and consequently promotes NPC proliferation. Conversely, overexpression of Bmi1 prevents Ndn-mediated inhibition of E2F1-driven Cdk1 promoter activity^[41].

Together, these findings suggest that Ndn controls NSC proliferation and apoptosis in an oxygen-dependent manner through interaction with various proteins driving cell proliferation (E2F1, Bmi1) and apoptosis (p53, Sirt1).

differentiation, quiescence, senescence, or apoptosis.

The formation of early progenitors from neuro-epithelial cells and the transition from proliferative symmetric to neurogenic asymmetric division is accompanied by a lengthening of the cell cycle, preferentially in G1-phase, implicating an involvement of CKIs^[42].

Paternally imprinted *p57^{Kip2}* gene encodes a cyclin-dependent kinase inhibitor (CKI)^[43] expressed in the VZ and SVZ, midbrain, thalamus, hypothalamus, cortical plate, septum, basal ganglia, cortex, and mantle zone of the hippocampus during development^[44,45]. By means of controlling cortical progenitor cell cycle exit, *p57^{Kip}* influences the migration and differentiation of neuronal precursors. Absence of *p57^{Kip2(+/-)}* during late embryogenesis and postnatal life gives rise to cortical hyperplasia. *p57^{Kip2(+/-)}* deficiency leads to an increased proliferation of radial glia cells (RGC) and intermediate precursors (IPC) and promotes re-entry into the cell cycle during corticogenesis^[46]. Cell cycle analysis of RGCs and IPCs evidenced an abnormal short cell cycle length favoring precursor proliferation and aberrant migration into cortical layers.

In mice adult hippocampus, *p57^{Kip2}* deficiency causes the severe depletion of the NSC reservoir by enhancing neuronal differentiation of NSCs at early stages of life^[47]. Moreover, consistent with a role of *p57^{Kip2}* in restraining NSC proliferation, neurogenic stimuli such as extensive running elicit a stronger activation of NSCs in mid-aged *p57^{Kip2(+/-)}* animals indicating that *p57^{Kip2}* might also play a critical role in the life long plasticity of brain functions^[47].

In addition to their canonical role in cell cycle control, CKIs have further functions including transcriptional regulation^[48]. As an example, nuclear *p57^{Kip2}* expression rises transiently during early telencephalic progenitor proliferation [embryonic day (E) 12.5] without inducing cell cycle exit. Instead, *p57^{Kip2}* interacts with the pro-neuronal basic helix-loop-helix (bHLH) factor Mash1 and blocks its transcriptional activity. As a result, *p57^{Kip2}* delays neuronal differentiation of telencephalic progenitors by antagonizing Mash1.

Taken together, *p57^{Kip2}* through inhibition of cell cycle progression and unrelated transcriptional mechanisms regulates many key processes in NSCs, including proliferation, cell cycle exit, differentiation, cell fate decisions, and stem cell quiescence in a cell type- and age-specific manner.

CYCLIN-DEPENDENT KINASE INHIBITOR *P57^{KIP2}*

The catalytic activity of cyclin-dependent kinases (CDK) is regulated by the binding of cyclins which oscillate periodically during the cell cycle and drive the orderly progression through consecutive phases. Conversely, inhibition of these complexes by CKIs induces transient or permanent cell cycle arrest,

ZINC FINGER PROTEIN REGULATING APOPTOSIS AND CELL CYCLE ARREST

The *Zac1* gene is maternally imprinted and maps on chromosome 10 in mice and chromosome 6q24 in human^[49,50]. The presence of a canonical C2H2 zinc finger domain and the potent induction of apoptosis and cell-cycle arrest in transformed tumor cells inspired originally the naming "*Zac1*"^[51].

An increased dosage of ZAC1 due to chromosomal anomalies or imprinting defects at the DMR is the most frequent genetic defect underlying transient neonatal diabetes mellitus (TNDM)^[52]. This disease manifests with intrauterine growth retardation (IUGR), dehydration, hypoinsulinemia, and early-onset hyperglycemia in term, newborn infants. Transgenic mice, which harbor an extra copy of the human ZAC1 locus, display key symptoms of the human condition and show at the cellular level an impaired proliferation and maturation of β -cell progenitors^[53]. In naïve pancreata, Zac1 is preferentially expressed in insulin-positive progenitor cells and increases strongly perinatally with the onset of terminal differentiation. At the same time, Zac1 confers repression following binding to specific DNA elements (see below) at the proximal promoter of the paternally imprinted *Rasgrf1* gene, an important modulator of various growth factor pathways. As a result, stimulus-induced activation of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways and, ultimately, insulin secretion is impaired under conditions of increased Zac1 dosage^[54].

Zac1 confers transcriptional regulation either by DNA-binding^[50,55-57] or as coregulator of the nuclear receptor family, in particular of those members belonging to the subgroup of steroid receptors^[58], and furthermore, by coactivation of members of the p53 family^[59,60]. Transcription factors typically comprise separable, modular DNA-binding and transcriptional domains; the latter confer gene activation and repression in a context-dependent manner^[61]. Zac1 matches well to these criteria, whereby its diverse transcriptional functions are tightly controlled by the interaction of single zinc fingers at the level of protein-protein and protein-DNA interactions^[55,62].

The N-terminal zinc finger domain, containing seven canonical C2H2 zinc fingers, is highly conserved in mouse and human Zac1 proteins and across the *Zac1* gene family^[63]. Mouse and human proteins diverge, however, by a central region rich in proline residues and a C-terminal cluster of glutamic acid residues both of which exist exclusively in mice. The proline-rich region together with the adjacent upstream region, termed linker region, confers potent transactivation^[64]. This function is further enhanced by the simultaneous recruitment of the general coactivators p300/CBP through the C-terminus. In contrast to the separable function of multiple domains in mice, transactivation and p300 recruitment map indistinguishably to the C-terminus of human Zac1^[62].

Zac1 can bind as a monomer to GC-rich palindromic DNA-elements or as a dimer to direct and reverse repeat elements to confer transactivation. Conversely, Zac1 binding to a half-site of a repeat element causes repression^[55]. Mechanistically, the zinc fingers assist in the recruitment of p300 and regulate p300's catalytic activities in a manner dependent on the nature of the bound DNA element^[62]. Hereby, single zinc fingers can

participate selectively in DNA binding and/or regulation of coactivator activities.

Moreover, Zac1 can also act as a coregulator for unrelated transcription factors comprising nuclear steroid or thyroid hormone receptors or various members of the p53 family (including p53 itself, p63, and p73). All of these transcription factors bind to specific DNA elements at their target genes to critically control cell proliferation and differentiation in a cell type-specific manner^[58,59]. For example, Zac1 is recruited jointly with the coactivators p300 and PCAF by the tumor suppressor p73 at the *p21^{Cip1}* promoter during early neuronal differentiation (see below). In addition to serving as a scaffold for coactivator assembly, Zac1 furthermore regulates PCAF's catalytic functions similar to the ones of p300^[65]. Overall, this close relationship between Zac1 DNA-binding and enzymatic regulation of transcription might contribute to the precise and efficient regulation of target genes.

In brief, Zac1's transcriptional activities are coordinated by sequence-specific DNA binding or the coregulation of unrelated transcription factors.

Zac1 expression during neurodevelopment

Zac1 is highly expressed in progenitor/stem cells of neuroectodermal and mesodermal tissues during early embryogenesis^[66]. Zones of active cellular proliferation, comprising the telencephalic vesicles and the infundibular recess of the third ventricle (the origin of the neurohypophysis) show robust Zac1 expression at E9.5 and E12.5. Moreover, Zac1 is also detected in mitotically active regions of the developing nervous system including the neural tube at E9.5 and the neural retina at E10.5. At later stage of neurodevelopment, high levels of Zac1 expression appeared in the mitotically active cell layers lining the VZ of the 3rd and 4th ventricle, the EGL of the cerebellum, and different neuroepithelia (*e.g.*, infundibulum, ventral hypothalamic sulcus)^[67,68].

As noted before, Zac1 expression induced apoptosis and cell cycle arrest in transformed tumor cells^[50,51,56] raising the question of its function in neural progenitor cells. In this respect, the analysis of Zac1 deficient mice (*Zac1^{+/-}*) provided interesting insight into the role of Zac1 in the brain^[69]. Specifically, these animals revealed a high incidence of hydrocephalus, a significant decrease in brain size, and a substantial increase in the proliferation rate of progenitor cells in the germinative zones of the dentate gyrus, the RMS of the olfactory system, and the dentate gyrus^[70]. At the same time, the number of Nestin⁺ cells was largely unaffected, compatible with the view that Zac1 rather controls the proliferation of progenitor cells than of stem cells. Yet, a limitation to this work is the fact that Zac1 deficient mice develop IUGR, various skeletal anomalies, and postnatal lung failure pointing to additional defects at the level of single organs and whole systems that are likely to impact on brain development. As an alternative to the multilayered

phenotype of *Zac1* knock out mice, we conducted genome wide expression profiling (see below) in order to identify *Zac1* target genes and analyzed their roles in well-defined *in vitro* and *in vivo* systems to elucidate *Zac1*'s function at the cellular level.

***Zac1* target genes in NSCs**

Pituitary adenylate cyclase activating polypeptide receptor 1: The first *Zac1* target gene to be identified was the G-protein coupled receptor for the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP)^[51,57,71], which controls various neuroendocrine functions in addition to its role as potent neurotrophic factor^[72-74]. Mechanistically, *Zac1* binds to an imperfect palindromic DNA element localized in the proximal polypeptide receptor 1 (*Pac1*) promoter to confer transactivation in a cell-type specific manner and to compete at the same time with the estrogen receptor dependent activation of *PAC1*^[75].

PACAP and *PAC1* are broadly expressed in the fetal brain and reduce the proliferation rate of certain neural precursor populations. At E13 in rat, PACAP can be detected hippocampus, hypothalamus, cortex, amygdala, dorsal root ganglia, and spinal cord, whereas *PAC1* is expressed in the neural plate, the neuroepithelia of the mesencephalon and rhombencephalon at E9, and in the neuroepithelia of the cortex, hippocampal formation, and cerebellum at E11, and in the basal telencephalon and olfactory bulb from E13 and E16 forward, respectively^[76]. PACAP induces a sharp increase in *p57^{Kip2}* expression in embryonic cortical precursors resulting in decreased CDK2 kinase activity, S-phase entry, and DNA synthesis. Furthermore, PACAP promotes the association of *p57^{Kip2}* with the kinase complex supporting its anti-mitogenic activity in neural progenitors. In accord with the specific role of *p57^{Kip2}* in cortical neurogenesis (see above), the expression levels of CDK2, cyclin E, or of the CKIs *p21^{Cip2}* and *p27^{Kip1}* remain unaffected by PACAP.

Coregulation of *p53* and *p73* target genes: As noted before, *Zac1* can serve as a coregulator for *p53* and *p73* due to its scaffolding function and regulation of coactivator activities^[59,65]. The *p53* family consisting of *p53* itself, *p63*, and *p73* encodes transcription factors with a key role in proliferation, differentiation, apoptosis, stem cell renewal and cell fate commitment^[77].

Zac1 coregulation of *p53* has been originally discovered in a cervical carcinoma cell line^[59] and led us to investigate its potential role in NSCs. Consistent with previous studies^[78,79], we observed high levels of *p53* in the undifferentiated state in ESCs which reside primarily in the cytoplasm^[80] and decline upon differentiation^[65]. At the same time, *p53* binds to the promoter of the pluripotency genes *Nanog* and *Oct-4*^[81,82], represses their transcription, and triggers the transition from self-renewal to differentiation.

Although *Zac1* has been reported to co-repress nuclear receptors in a cell-type specific manner, no evidence exists for a comparable role towards members of the *p53* family^[58,59].

In the developing brain, *p53* is expressed in progenitor cells of the SVZ^[83] where it induces cell-cycle arrest, DNA repair and cell death following genotoxic stress^[84]. *p53*-deficient mice show higher cell proliferation in the SVZ and enhanced neurosphere formation *in vitro*^[83,85] consistent with a role of *p53* as negative regulator of NSC self-renewal. Moreover, *p53* seems to be involved in different aspects of NSC differentiation including repression of self-renewal and promotion of gliogenesis^[86,87].

Mounting experimental evidence indicates that endogenous reactive oxygen species (ROS) play a critical role in cell signaling and NSC physiology including appropriate timing of neurogenesis. In this respect, the pattern of ROS generation matches the one of *p53* immunoreactivity in the developing telencephalon. Similarly to *Zac1*, nuclear *p53* is detected in Nestin⁺ NSCs in E11 and E13 proliferative germinal zones, and its expression decreases towards the cortical plate^[88]. Loss of *p53* function causes enhanced ROS production and premature neurogenesis, which is partly reversed by reinstatement of *p53* or antioxidant treatment^[88].

Despite these interesting findings, a role of *Zac1* in any of these *p53*-dependent processes is presently unknown and future studies are needed to address this topic.

p73 has been recognized for its critical role in brain development as evidenced by the highly penetrant phenotype in *p73* null mice which display cortical hypoplasia, hydrocephalus, and hippocampal dysgenesis^[89,90]. The hippocampal anomaly corresponds with either a complete absence or truncation of the lower blade of the neurogenic DG and an abnormal gyration of the Ammon's horn. Isoform-specific *p73* knock-out mice showed that this phenotype results in major part from the absence of the activation-proficient *p73* protein (TAp73) as opposed to the activation-deficient *p73* protein (ΔNp73). The latter isoform lacks *Zac1* coactivation^[65] and is thought to act as a potent prosurvival protein in neurons by counteracting the proapoptotic function of *p53*^[91,92]. Therefore, it would be of interest to investigate the possibility of *Zac1*-dependent coregulation of TAp73 in NSC in greater depth.

Withdrawal of leukemia inhibitory factor (Lif1) potentially induces neuronal differentiation of ESCs and strongly upregulates *Zac1* and *p73* expression concomitantly to a rapid decline in *p53* mRNA and protein^[65]. At the same time, *p73* isoforms switch from activation-deficient ΔNp73, prevailing under the undifferentiated state, to activation-proficient TAp73 and caused a strong up-regulation of the *p21^{Cip1}* and *p57^{Kip2}* genes, two well-known direct *p73* target genes^[93]. As referred to above, DNA bound *p73* recruits *Zac1* to select target genes, where it

serves as a scaffold for coactivator assembly and enhancement of catalytic functions. As a result, *Zac1* enhances p73 transcriptional activities in a site-specific manner^[65]. These findings suggest a joint role for *Zac1* and p73 in inducing cell cycle exit of ESCs and in differentiation towards neural cells.

In vivo studies evidenced a reduced proliferation of neurogenic cells isolated from the E16 and E18 VZ/SVZ and a smaller size of the perinatal SVZ in p73 deficient mice when compared to controls. As a result, p73^(-/-) mice suffer from a depletion of the stem cell compartment at birth pointing to a role of p73 in NSC self-renewal and maintenance^[94]. This function of p73 extends also to the adult neurogenesis in the DG. At the molecular level, p73 deficiency elicits perturbations in the canonical Sox2 and Notch signaling pathways driving NSC proliferation^[95]. Additionally, a reduced transcription of *Hey2*, a negative regulator of activator bHLH proteins, impairs the long-term maintenance of neural precursors in the absence of p73^[95].

Because *Zac1* is expressed in embryonic and adult neural stem cells it could potentially interact with and coregulate TAp73 functions. Presently, the actual evidence remains limited to *Zac1* coactivation of TAp73 during neuronal differentiation of ESCs. However, further studies should address the role of *Zac1* coregulation for NSC self-renewal, maintenance and differentiation at different developmental stages though.

Suppressor of cytokine signaling 3: Genome-wide expression profiling in a cerebellar neural stem cell line (C17.2) using a Tet-off system led to the identification of suppressor of cytokine signaling 3 (*Socs3*), a negative regulator of Jak/Stat3 signaling, as potential *Zac1* target gene^[96].

The transition from neuronal cell types to glial subtype-specific precursors is critically controlled by preset developmental programs and extracellular signals like the cytokine driven Jak/Stat3 pathway, which is largely inactive at early, neurogenic stages and takes on at later gliogenic stages, when the expression of neurogenic factors progressively declines^[97].

Zac1 recognizes a cluster of GC-rich DNA elements in the proximal promoter and intronic region of the mice and human *Socs3* genes to confer transcriptional activation^[96].

Two radial glial-like NSC lines, derived from E15 or the adult SVZ of mice, showed a transient upregulation of *Zac1* mRNA and protein upon neuronal or astroglial differentiation, whereby transactivation of *Socs3* occurred solely under the latter condition indicative of a role of *Socs3* as a lineage-specific target gene. Consistent with this finding, DNA methylation at the *Socs3* gene decreased during astroglial differentiation but remained unaltered during neuronal differentiation. *Zac1* and *Socs3* are expressed simultaneously exclusively during the early

stage of astroglial differentiation and associated with a strong decline in receptor activation-dependent tyrosine phosphorylation of Stat3. In agreement with these results, *Zac1* and *Socs3* are highly expressed in the neocortical ventricular zone at E18, which corresponds to the onset of astroglial differentiation^[96].

Astroglial differentiation is triggered by various cytokines namely cardiotrophin-1, ciliary neurotrophic factor, or leukemia inhibitory factor, which activate Jak/Stat3 signaling^[97-100]. Conversely, genetic deletions in this pathway (*i.e.*, gp130, LIF and Stat3) reduce astroglial differentiation^[97,101,102]. Importantly, overexpression of *Socs3* inhibits Stat3 signaling and impairs astroglial differentiation^[103], whereas conditional deletion of *Socs3* leads to enhanced astroglial differentiation in neonatal mouse brain and primary neuroepithelial cells^[100]. These findings led us to investigate whether *Zac1*-dependent induction of *Socs3* could provide a negative feedback loop to inhibit Jak/Stat3 signaling during early astroglial differentiation of NSCs.

In accord with an inhibitory role in Jak/Stat3 signaling, *Zac1* overexpression delayed astroglial differentiation, independent of a simultaneous increase in the number of cells in transition into G₁ arrest^[96]. Conversely, knock-down of *Zac1* in NSCs facilitated astroglial differentiation and postponed cell cycle arrest. Although lengthening of the G₁ phase has been suggested to initiate differentiation, this mechanism seems not to apply to *Zac1*'s cell cycle arrest function in NSCs and supports the self-sufficient role of *Socs3* in the control of astroglial differentiation. Compatible with this view, knock-down of *Socs3* in *Zac1* overexpressing cells reinstated timely astroglial differentiation. Similar results were obtained in primary E18 NSCs strengthening the role of *Zac1*-dependent regulation of *Socs3* in early astroglial differentiation of NSCs^[96].

Overall, this study provides detailed insight into the molecular mechanisms by which an imprinted gene can fine tune cell-fate decisions and differentiation in NSCs and assigns to *Zac1* a critical role in the prevention of precocious astroglial differentiation (Figure 2).

Transcription factor 4: *Zac1* expression robustly increased under astroglial as well as neuronal differentiation, which led us to question *Zac1*'s function during the latter condition^[96]. The family of bHLH proteins plays a prominent role in cell fate decisions and differentiation. These proteins share the eponymic bHLH domain which is necessary for homo- or heterodimerization and for binding to a specific DNA sequence named E-box motif^[104]. bHLH proteins expressed in the brain can be classified into two groups; the so-called specification factors (*i.e.*, Math, Mash, neurogenin, and NeuroD), which are expressed in a spatiotemporally controlled manner, and their ubiquitously expressed dimerization partners, the E proteins^[105,106]. Under the undifferentiated, proliferative state proneural factors are weakly

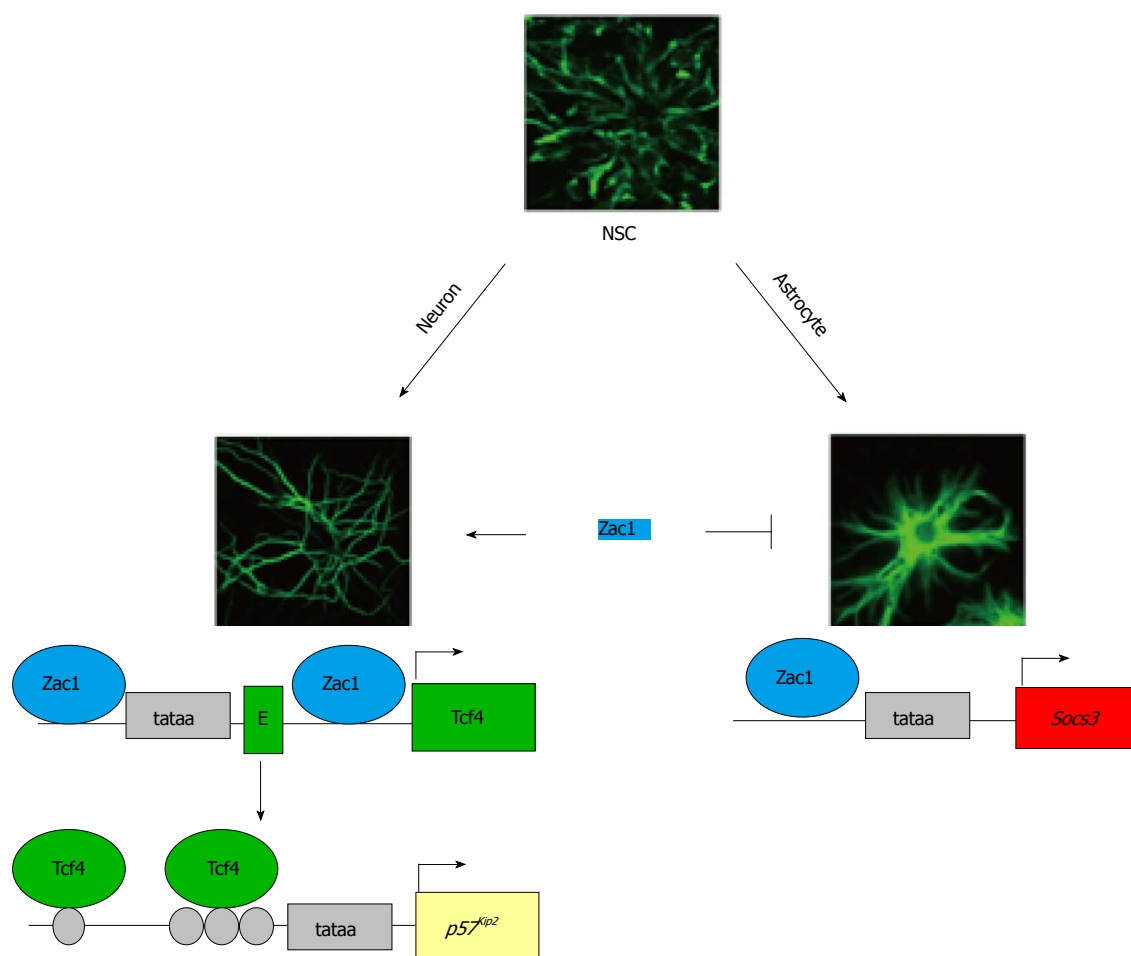


Figure 2 Imprinted *Zac1* favors neuronal differentiation of neural stem cells. The *Zac1* gene is maternally imprinted and encodes a versatile transcriptional regulator. *Zac1* expression is strongly induced during neuronal and astroglial differentiation of embryonic and adult neural stem cells (NSCs). During neuronal differentiation (left scheme), *Zac1* binds to GC-rich DNA binding sites at the *Tcf4* promoter and first intron to confer synergistic transactivation. As a result, enhanced *Tcf4* expression promotes binding to and transactivation of the cyclin kinase inhibitor *p57^{Kip2}*, which induces G1 arrest. Moreover, *Zac1* binds to GC-rich DNA elements at the *Socs3* promoter during astroglial differentiation (right scheme). *Socs3* encodes a potent inhibitor of prodifferentiative Jak/Stat3 signaling and prevents precocious astroglial differentiation. The tataa-elements are boxed in light grey and the transcriptional start sites are symbolized by arrows. The first exon in the *Tcf4* locus is depicted as a green box (labeled E) and coding exons of *Tcf4* and *Socs3* as green and red boxes, respectively. *Tcf4* binds to various E-box motifs (light grey circles) localizing to the *p57^{Kip2}* regulatory region.

expressed, prior to a rapid increase in concert with the E-proteins at the initiation of neurogenesis. E-protein family members comprise two splice variants of *E2A* (*E12* and *E47*), *HEB*, and transcription factor 4 (*Tcf4*) (*alias E2-2*, *SEF2*, or *ITF2*), which upon heterodimerization with a specification factor, bind to the promoter of their target genes, in order to promote neurogenesis, and inhibit astroglial differentiation and NSC proliferation^[107,108].

Expression profiling in a NSC line (C17.2) based on inducible *Zac1* expression showed a robust upregulation of *Tcf4* mRNA and protein^[109]. Thereby, *Zac1* bound simultaneously to the proximal promoter and first intron of *Tcf4* and binding at either site was necessary to confer synergistic transactivation.

Neuronal differentiation of ESCs caused a strong *Zac1* and *Tcf4* upregulation and associated with *Zac1* binding at the *Tcf4* gene. As noted before, *Zac1* upregulation following differentiation of embryonic and adult NSC lines occurred under either astroglial

or neuronal differentiation, whereby induction of *Socs3* was confined to the astroglial lineage. Oppositely, *Zac1*-dependent upregulation of *Tcf4* was specific to neuronal differentiation, associated with an overall increase in active chromatin marks but no change in DNA methylation, at the *Tcf4* locus^[109]. *Zac1*-dependent *Tcf4* regulation was also confined to neuronal differentiation of primary NSCs; moreover, *Zac1* binding to and transactivation of the *Tcf4* locus occurred exclusively during periods of neuronal differentiation in the neocortical ventricular zone^[109].

Among known *Tcf4* target genes, *p57^{Kip2}* is of particular interest as it is co-regulated with *Zac1* in the framework of an imprinted gene network (see below), it critically controls differentiation and migration of radial glia cells, and shares with *Zac1* and *Tcf4* a cell cycle arrest function (see above).

Zac1 and *Tcf4* are broadly expressed in neuronal progenitor cell populations during early (E11) and midneurogenesis (E15) such as in the caudal brain

regions, the pallium, and the prethalamus eminence^[109].

$p57^{Kip2}$ expression is more localized and mapped strongly to the subpallium and peduncular hypothalamus where all three factors were detected.

Functionally, *Zac1* enhances G1-cell cycle arrest in NSC lines and primary NSCs (E15) during neuronal differentiation, by inducing $p57^{Kip2}$ expression through *Tcf4*^[109].

Taken together, these results suggest a role to *Zac1* in the prevention of precocious astroglial differentiation through *Socs3* induction and in advancing at the same time neuronal differentiation through *Tcf4* induction and *Tcf4*-mediated upregulation of $p57^{Kip2}$ (Figure 2).

ZAC1 IMPRINTED GENE NETWORK

Recent evidence indicates that many imprinted genes might work in an integrated network of imprinted genes. In this respect, Arima *et al.*^[110] noted that *Zac1* and $p57^{Kip2}$ show a conspicuously similar expression pattern in mesenchymal and neuroepithelial tissues, suggesting a functional interaction between these genes. Interestingly, the Beckwith-Wiedemann syndrome (loss of $p57^{Kip2}$ imprinting) and TNDM (loss of *ZAC1* imprinting) represent with partly opposite phenotypes including gigantism vs IUGR or hypoglycemia vs hyperglycemia.

At the molecular level, *ZAC1* binds in a methylation-sensitive manner within the promoter CpG island of *LIT1* (KCNQ1OT1), which encodes a paternally expressed, anti-sense RNA thought to repress $p57^{Kip2}$ in *cis*^[16]. *ZAC1* confers transactivation to *LIT1* promoter constructs suggesting that *ZAC1* might down-regulate $p57^{Kip2}$ via *LIT1* anti-sense^[110]. Oppositely, we demonstrated that *Zac1* upregulates $p57^{Kip2}$ via *Tcf4* in NSCs^[109].

Some aspects of the *Zac1* knock-out phenotype, such as growth retardation, perinatal death, and incomplete bone formation appear difficult to reconcile with *Zac1*'s antiproliferative activities^[69]. A possible explanation to this puzzle is provided by a meta-analysis of microarray data, which indicates that *Zac1* coordinates a network of genes that consists of a remarkable huge number of imprinted genes. These include *Igf2*, *H19*, *Dlk1*, *Ndn*, and $p57^{Kip2}$, which share an important role in NSC maintenance and differentiation, among others (*Grb10*, *Gnas*, *Meg3*, *Mest*, and *Sgce*). Moreover, *Zac1*-deficient liver tissue and *Zac1* overexpression experiments in neuroblastoma cells showed an opposite regulation of *Igf2*, *H19*, *Dlk1*, and $p57^{Kip2}$. Interestingly, *Zac1* bound to the downstream *H19* enhancer and conferred transactivation to both the *H19* and *Igf2* promoters (Figure 3).

More recently, Lui *et al.*^[111] showed that IGNs are possibly involved in mammalian somatic growth control. Early postnatal life is a period of fast somatic growth, which slows down with maturation and finally arrests in adulthood. A group of 11 imprinted genes involved in cell proliferation (including *Zac1*, *Igf2*, *H19*, *Dlk1*, *Ndn*, and $p57^{Kip2}$) and part of the *Zac1*-IGN is expressed in multiple tissues at levels that

correlate with trajectories of overall somatic growth and decrease coordinately with age. Although this study does not explicitly address the role of IGNs in NSCs, it importantly suggests that the function of IGNs is not reserved to embryonic life but potentially extends across lifespan.

Another recent study focused on gene expression profiling in murine long-term hematopoietic stem cells (LT-HSC) vs their differentiated progeny. These experiments showed that imprinted genes (including *Zac1*, *Igf2*, *H19*, *Dlk1*, *Ndn*, and $p57^{Kip2}$) were more uniformly expressed in progenitors when compared to the differentiated counterparts^[112]. Moreover, stem/progenitor cells from adult skeletal muscle and epidermis show a higher expression of these genes when compared to their differentiated derivatives.

Taken together, the *Zac1*-associated IGN network comprises, among others, imprinted genes that play a critical role in NSC maintenance and differentiation. Some of them, like *Igf2* and *H19* seem to be under the direct transcriptional control of *Zac1* while others like *Dlk1* might be regulated more indirectly as exemplified by *Zac1*-dependent upregulation of $p57^{Kip2}$ via *Tcf4* (Figure 3).

CONCLUSION

A wide range of genes acts in a concerted manner during brain development to regulate NSC proliferation, migration, and differentiation. Among these, imprinted genes have gained increasing apprehension in NSC biology due to their critical roles in quiescence, stemness, and cellular differentiation.

The molecular processes controlling both NSC fate and imprinted gene expression are manifold and include transcriptional regulation, epigenetic, and genomic interactions.

For instance, imprinted genes can regulate the expression or the transcriptional activities of proneural bHLH proteins in NSCs. *Zac1* activates *Tcf4* during neuronal differentiation of NSCs, while $p57^{Kip2}$ inhibits the transcriptional activity of *Mash1*. Conversely, the proneuronal bHLH-protein neurogenin regulates the imprinted *Dlk1-Gtl2* locus in the dorsal telencephalon^[113] indicating bidirectional interactions between imprinted genes and proneuronal proteins.

Genomic imprinting defects have been originally recognized for their role in early development of the embryo and placenta^[9] and more recently for postnatal life^[114]. New evidences suggest an additional role of variation in genomic imprinting in the mediation of environmental exposures, which is thought to associate with less severe consequences than those resulting from loss of genomic imprinting and might represent an important component of complex traits^[115]. For example, newborn of obese parents show altered DNA methylation profiles of multiple imprinted genes, which may be carried onto the next generation and confer an increased risk for metabolic diseases

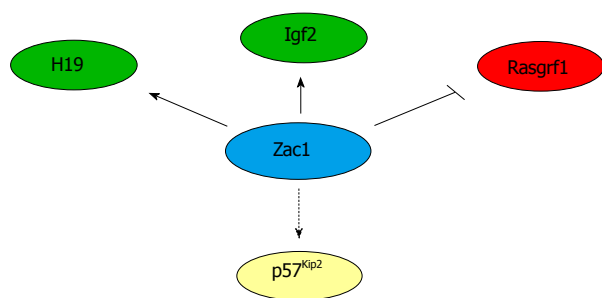


Figure 3 Zac1-dependent transcriptional regulation of imprinted genes. Zac1 binds to the downstream enhancer of *H19* and transactivates *H19* and *Igf2* expression in neuronal cells. In pancreatic progenitor cell, Zac1 binds to the promoter of the paternally imprinted *Rasgrf1* gene and confers repression. Following neuronal differentiation of NSCs, Zac1 induces *Tcf4*, which binds at the promoter of the imprinted *p57^{Kip2}* gene and enhances G1 cell cycle arrest.

in adulthood^[116]. Moreover, the degree of methylation of *ZAC1* associates with pre- and postnatal growth in healthy infants as well as with maternal nutrition and lasts at least until the first year of life^[117]. Similarly, individuals prenatally exposed to famine showed 6 decades later less DNA methylation of *IGF2* compared with their unexposed, same-sex siblings^[118], although variation in DNA methylation at this locus is thought to increase as a result of the aging process itself^[119]. Collectively, these findings indicate the need for further studies on genetic and epigenetic variation at imprinted loci in response to environmental exposures and across lifetime.

Remarkably, in NSCs and other discrete stem cell populations, recent findings indicate that genomic imprinting can be epigenetically switched off at defined developmental time windows as shown for *Igf2-H19* and *Dlk1*. Such temporary changes in allele-specific transcription of imprinted genes alter gene dosage in a cell-type and tissue-specific manner and are required to prevent precocious depletion of the stem cell pool. The influence of various environmental exposures on epigenetic switches in NSCs is presently unknown and might contribute to brain function and aging in individuals at risk.

Interestingly, recent literature suggests that imprinted genes do not operate in isolation, but as complex network of genes, whose expression is dynamically controlled by epigenetic mechanisms that extend from prenatal to postnatal development and possibly during aging. For instance, *Zac1* is a central hub in an IGN comprising *Igf2*, *H19*, *Dlk1*, *p57^{Kip2}*, and *Ndn*, which share a role in NSC maintenance and differentiation.

Though, additionally studies are necessary to explore in more detail the role of IGNs in NSCs across lifespan as well as in response to environmental exposures and to elicit their molecular basis. Collectively, a better understanding of the complex interactions governing imprinted genes expression promises new insight into the biology of NSC and associated conditions ranging from imprinting disorders to age-related diseases.

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Induced pluripotent stem cells: Mechanisms, achievements and perspectives in farm animals

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unlimited self-renewal, and they can be triggered to differentiate into desired specialized cell types. These features provide the basis for an unlimited cell source for innovative cell therapies. Pluripotent cells also allow to study developmental pathways, and to employ them or their differentiated cell derivatives in pharmaceutical testing and biotechnological applications. *Via* blastocyst complementation, pluripotent cells are a favoured tool for the generation of genetically modified mice. The recently established technology to generate an induced pluripotency status by ectopic co-expression of the transcription factors Oct4, Sox2, Klf4 and c-Myc allows to extending these applications to farm animal species, for which the derivation of genuine embryonic stem cells was not successful so far. Most induced pluripotent stem (iPS) cells are generated by retroviral or lentiviral transduction of reprogramming factors. Multiple viral integrations into the genome may cause insertional mutagenesis and may increase the risk of tumour formation. Non-integration methods have been reported to overcome the safety concerns associated with retro and lentiviral-derived iPS cells, such as transient expression of the reprogramming factors using episomal plasmids, and direct delivery of reprogramming mRNAs or proteins. In this review, we focus on the mechanisms of cellular reprogramming and current methods used to induce pluripotency. We also highlight problems associated with the generation of iPS cells. An increased understanding of the fundamental mechanisms underlying pluripotency and refining the methodology of iPS cell generation will have a profound impact on future development and application in regenerative medicine and reproductive biotechnology of farm animals.

Key words: Reprogramming; Large animal models; Stemness; Chimera; Germline transmission; Induced pluripotent stem cells; Gene delivery

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Abstract

Pluripotent stem cells are unspecialized cells with

Core tip: The generation of an induced status of pluripotency

in somatic cells by ectopic expression of core transcription factors allows to extending advanced genetic modifications and reproductive techniques to species, for which the derivation of genuine embryonic stem cells was not successful till now. The commonly employed viral gene transfer may be genotoxic and therefore non-viral methods for iPS cell derivation are intensively studied. In this review, we focus on the mechanisms of cellular reprogramming and current methods used to induce pluripotency.

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INTRODUCTION

Induced pluripotent stem (iPS) cells are defined as differentiated cells that have been experimentally reprogrammed to an embryonic stem (ES) cell-like state. The first generation of murine iPS cells was achieved^[1] by retroviral transduction of four core reprogramming factors: Oct4, Sox2, Klf4, and c-Myc. Subsequently, human iPS cells were produced by viral transduction of adult fibroblasts^[2,3]. Also a combination of Oct4, Sox2, Nanog and Lin28, was effective for the generation of human iPS cells^[4]. An overview of reprogramming cells into iPS cells is shown in Figure 1.

Subsequently, the core reprogramming factors have been successfully used to derive pluripotent cells in various other species, including rhesus monkey^[5], rat^[6], pig^[7], dog^[8], cattle^[9], horse^[10], sheep^[11], goat^[12] and buffalo^[13]. A summary of the generation of iPS cells from different species of livestock is enumerated in Table 1. Importantly, iPS cells could be isolated from several species, in which the isolation of authentic ES cells was not successful despite several attempts since many years^[14,15]. In particular, for economically important species, such as farm animals, the availability of authentic iPS cells would have important consequences for reproductive biology and approaches for genetic modification. For agricultural purposes, iPS cells from farm animal species can serve as a valuable genetic engineering tool to boost the generation of livestock with advantageous genes that are important for economic, reproductive and disease resistant traits, or for the study of functional genomics in mammals.

So far, iPS cells have been successfully produced from fibroblasts^[16], pancreas cells^[17], leukocytes^[18], hepatocytes^[19], keratinocytes^[20], neural stem cells^[21], cord blood cells^[22], and other cell types. Together these data suggest that most cell types can be reprogrammed to a pluripotent state, and that the unidirectional lineage commitment can be experimentally overwritten.

Certain cell types, such as neuronal progenitors, which exhibit basal expression of one or more of the core reprogramming factors, seem to be ideal for reprogramming^[21].

Rodent iPS cells are almost identical to their ES cell counterparts, sharing typical hallmarks of pluripotency such as colony morphology, unlimited self-renewal, *in vitro* and *in vivo* differentiation potentials, and contribution to the germline^[23,24]. Most iPS lines from farm animal species have not been tested in chimera complementation assays; however some preliminary reports suggest that chimeras and germline transmission can be achieved in sheep and pig^[25,26]. iPS cells derived from rodents, humans, monkeys and farm animals share the features of high telomerase activity, expression of alkaline phosphatase, and expression of stemness genes, such as *OCT4*, *SOX2*, *UTF1* and *REX1*. The epigenetic status of murine iPS cells has been analysed by bisulfite sequencing and chromatin immuno-precipitation DNA-Sequencing (ChIP-Seq)^[27]. Thus the hallmarks for iPS cell characterisation can be enumerated as (1) unlimited self-renewal; (2) *in vitro* differentiation capacity; (3) *in vivo* differentiation capacity; (4) chimera contribution; and (5) subsequently germline transmission.

Apart from scientific and ethical hindrances, religious concerns restricted the derivation of human ES cells. To circumvent these concerns, alternative approaches to generate pluripotent cells have been assessed. The alternative approaches include culture of somatic cells with cell extracts isolated from ES cells^[28] or oocytes^[29], and fusion of somatic cell with pluripotent cell^[30]. However, extremely low efficiencies, high technical difficulties and aberrant ploidies of the resulting cells^[31,32] did reduce the enthusiasm for these attempts. At the moment, the derivation of iPS cells from human tissues seems to be the most promising alternative. Prior to clinical application of iPS-derivatives, cell survival, functional integration of the cellular transplant and safety of the cell products have to be assessed in informative animal models.

The progress in iPS cell development in farm animals lags behind those in rodents, but large mammalian models may be instrumental for pre-clinical tests of novel cell therapies (Table 2), enhanced pharmaceutical studies and regenerative studies, including the restoration of fertility.

HISTORICAL PERSPECTIVE

Ontogenesis of an organism and cellular differentiation were thought to be a unidirectional process, where stem and progenitor cells progressively develop to terminally differentiated cells, for example neurons, muscle, and epithelial cells. During ontogenesis the nuclear DNA of most cell types is unchanged, but different epigenetic marks, such as DNA methylation and histon modifications, are set, and lock the

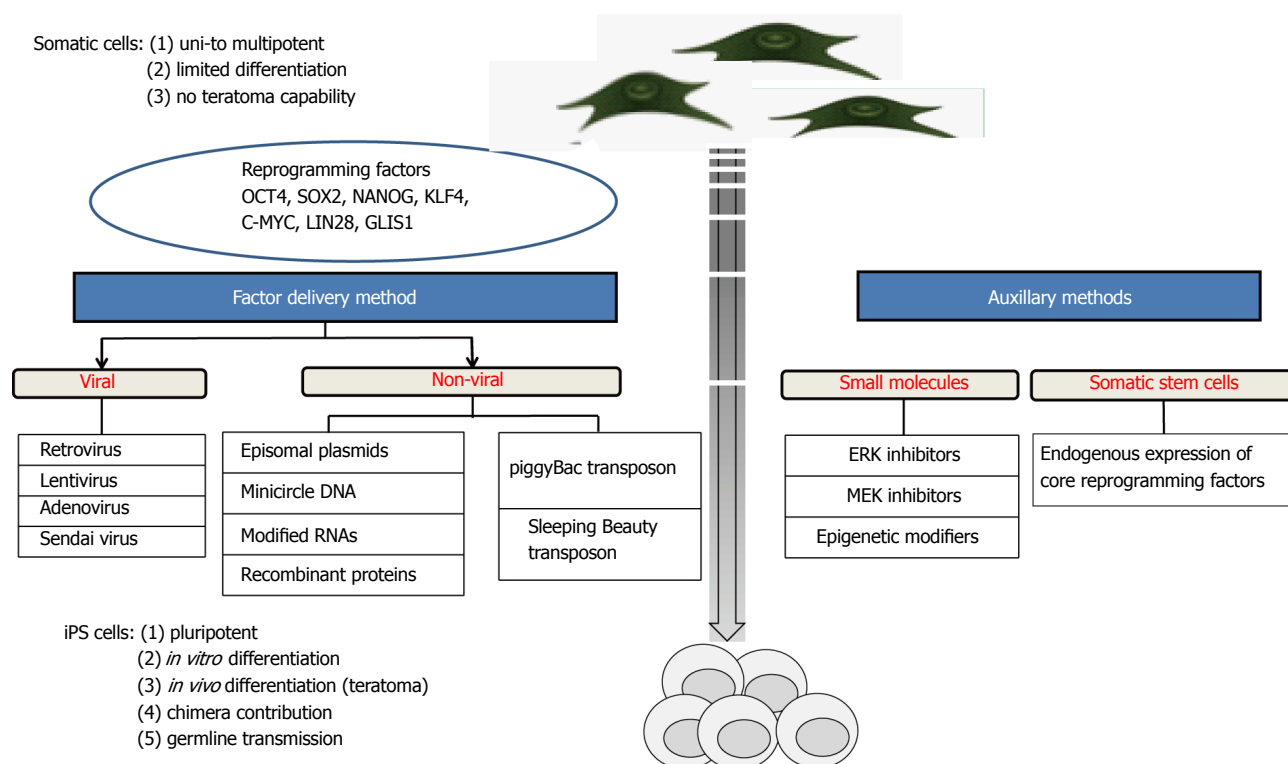


Figure 1 Methodological toolbox for generating induced pluripotent stem cells. iPS: Induced pluripotent stem.

cellular potency and cell lineage commitment. This is depicted by the “epigenetic landscape” proposed by Waddington^[33].

Already in 1962, Gurdon^[34] questioned this view by amphibian cloning; he transplanted nuclei from intestinal cells into irradiated oocytes and obtained vital tadpoles. More than three decades later, the successful cloning of a sheep (Dolly) by SCNT of a mammary epithelial cell to an enucleated oocyte, showed that even mammalian cells can be reprogrammed^[35]. This success demonstrated that differentiated cells contain the genetic information to direct ontogenesis of an entire mammalian organisms, and that enucleated oocytes contain pivotal factors for reprogramming of differentiated cell nuclei. However, the identity of the oocyte reprogramming factors remained elusive.

The discoveries that ectopic expression of Antennapedia-a transcription factor was able and sufficient to induce leg structures in *Drosophila*^[36], and that ectopic expression of the mammalian transcription factor MyoD1 converted fibroblasts into myocytes^[37] led to the concept of “master genes”. A master gene was defined as a key transcription factor that in a hierarchical manner regulates a cascade of critical genes, which in a concerted action induce the cell commitment.

DISCOVERY OF INDUCED PLURIPOTENCY

In 2006, Takahashi *et al.*^[1] proved that not a single

master factor, but a combination of four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, was sufficient to induce the pluripotent status in somatic mammalian cells. The resulting cells were called iPS cells^[1]. This discovery offers new opportunities to study developmental biology, regenerative medicine, as well as reproductive biology and biotechnology of farm animals.

iPS cells from farm animals will likely serve as a bridging link between well developed rodent iPS and poorly characterised human iPS (Table 2), supporting the translation of innovative cell therapies from experimental studies to curative treatments. At the moment, human iPS cell application seems to be too risky because of basic lack of knowledge and ethical consideration which forbid certain tests such as chimera assays.

In contrast, research on iPS cells derived from farm animal species is not tainted with ethical concerns. Furthermore, the methodology for generation of iPS cells is relative simple and is thought to be easily transferable to other mammalian species. Thus farm animal models may turn out to be ideally suited to determine required cell doses, to assess long-term performance, tumorigenicity, applications methods and fate of transplanted cells^[38-41].

Recent advances in genetic engineering of farm animals allow the generation of precise genetic modifications^[42-47], such as the production of immunodeficient pigs^[48] which will be instrumental for further advances in preclinical testings of new cell therapies. A boost of recent

Table 1 Most advanced achievements in induced pluripotent stem cells from domestic animals

Domestic species	Cell type	Transduction	Reprogramming factors	Culture medium	Differentiation		Chimera	Germline contribution	Ref.
					<i>In vitro</i>	<i>In vivo</i>			
Buffalo	Fetal fibroblasts	Retrovirus	OSKM	A	EBs	Teratoma	NA	NA	[13]
Cattle	Fetal fibroblasts	Retrovirus	OSKM, OSKMLN, OSKM	B	EBs	Teratoma	NA	NA	[9]
Dog	Fetal fibroblasts	Plasmid	OSKM	C	EBs	Teratoma	NA	NA	[53]
	Skin fibroblasts	Lentivirus	Human OKSM	J	EBs	Teratoma	NA	NA	[56]
Goat	Skin fibroblasts	Retrovirus	Mouse OKSM	K	EBs	Teratoma	NA	NA	[54]
	Fibroblasts	Inducible lentivirus	OSKM, SV40 large T and hTERT	A	EBs	Teratoma	NA	NA	[12]
Horse	Fetal fibroblasts	PiggyBac transposon	OSKM	E	EBs	Teratoma	NA	NA	[10]
	Adult fibroblasts	Retrovirus	OSK	F	EBs	Teratoma	NA	NA	[61]
Pig	Mesenchymal stem cells from bone marrow	Lentivirus	OSNKLM	G	EBs	NA	Low grade	Two offspring	[26]
	Fetal fibroblasts	Sleeping Beauty transposon	Mouse OSKM	I	Neuronal lineage	Teratoma	NA	NA	[91]
Rabbit	Skin fibroblasts	Retrovirus	Human OKSM	I	EBs	Teratoma	NA	NA	[72]
Sheep	Fetal fibroblasts	Retrovirus	MKOS	D	EBs	Teratoma	Low grade	NA	[25]

A: DMEM, ESC FBS, L-glutamine, NEAA, β -Me, bFGF, LIF and MEFs; B: DMEM, KSR, L-glutamine, NEAA, β -Me, bFGF and MEFs; C: DMEM/F12 + N2 and Neurobasal with B27, L-glutamine, hLIF, PD0325901, CHIR99021 and MEFs; D: KO-DMEM, SR, L-glutamine, NEAA, 2-Me, human bFGF and MEFs; E: DMEM, FBS, L-Glutamine, NEAA, β -Me, Sodium Pyruvate, LIF, bFGF, Doxycycline, CHIR99021, PD0325901, A83-01, Thiazovivin, B431542 and 1:1 MEFs and EEFs; F: α -MEM, FBS, deoxyribonucleosides, ribonucleoside, glutamax, NEAA, β -Me, ITS, human LIF, β FGF, EGF and MEFs; G: DMEM/F12, KSR, L-glutamine, NEAA, β -Me, FGF and MEFs; H: KO DMEM, KSR, glutamax-L, NEAA, 2-Me, pLIF, forskolin and collagen I; I: DMEM/F12, KSR, L-glutamine, NEAA, β -Me, bFGF and MEFs or gelatinized plates; J: KO DMEM, ESC FBS, bFGF, hLIF and MEFs; K: DMEM/F12, KSR, bFGF, hLIF, PD0325901, CHIR99021 and MEFs. DMEM: Dulbecco's modified Eagle's medium; LIF: Leukemia inhibitory factor; IGF1: Insulin-like growth factor 1; NEAA: Nonessential amino acids; FBS: Fetal bovine serum; KO: Knockout; MEM: Minimum essential medium; ITS: Insulin-transferring selenium; bFGF: Basic fibroblastic growth factor; DOX: Doxycycline; EB: Embryonic body; FCS: Fetal calf serum; hSCF: Human stem cell factor; KSR: Knockout serum replacement; MEFs: Mouse embryonic fibroblasts; OKSM: Oct-4, Klf4, Sox2, and c-Myc; OKSMLN: Oct-4, Klf4, Sox2, c-Myc, Lin28 and Nanog; VPA: Valproic acid; Me: Mercaptoethanol.

publications describe iPS cells from buffalo^[13], cattle^[9,49-53], dog^[8,54-56], goat^[11,57], horse^[10,58-62], pig^[7,63-71], rabbit^[72-74] and sheep^[11,75,76]. The majority of these iPS cells from farm animals showed typical hallmarks of pluripotency, such as differentiation *in vivo* and teratoma formation. However, most farm animal iPS cultures were not assessed for chimera contribution so far. Preliminary results that porcine iPS cells can contribute to chimera formation in blastocyst complementation were provided recently^[71]. Similarly, ovine iPS cells contributed moderately to chimeric lambs after injection into eight-cell stage embryos or blastocysts^[25]. These experiments represent an important step in the understanding of mechanistic nature of pluripotency in farm animals. The iPS technology may become instrumental for advanced transgenesis in large mammals (Figure 2).

METHODS TO DERIVE IPS CELLS

In recent years, several methods have been established for iPS cell generation (Figure 1), employing the core reprogramming factors as genes, mRNAs or proteins, and auxiliary chemical agents, which infer with the involved signalling pathways. Here, the main approaches for the generation of iPS cells are summarized.

Virally-induced iPS cells

There has been extensive amount of work carried out to obtain virally-derived iPS cells employing either retroviruses, lentiviruses, and non-integrating viruses. The first iPS cells have been generated through retroviral transduction of Oct4, Sox2, Klf4 and c-Myc^[1]. Disarmed, optimized retro- or lentiviruses can infect mammalian cells with high efficiencies. The use of the pantropic vesicular stomatitis virus G protein (VSVG) was instrumental for viral transduction of a broad spectrum of receptive cells. Interestingly, unstimulated T cells, B cells and hematopoietic stem cells could not be efficiently transduced with the VSVG lentiviruses^[77].

Retro- and lentiviruses integrate into the host genome allowing for high expression of the encoded cargo genes. The expression can be temporally confined by employing viral promoters, such as the 5' long terminal repeat, which are usually silenced by epigenetic mechanisms. Disadvantages of the viral approach include the limited cargo capacity of maximally 7 kb, the induction of immune responses and potential genotoxic effects. Retro- and lentiviral integrations do not happen randomly in the genome, but show a strong bias for promoter and exonic regions, which may result in dysregulation of endogenous genes. In a retrovirus-based clinical gene therapy of the X-linked

Table 2 Achievements with induced pluripotent stem cells from rodents, farm animals and humans

	Rodents	Farm animals	Human
iPS cells	√√	√√	√√
<i>In vivo</i> differentiation	√√	√√	√√
<i>In vitro</i> differentiation	√√	√√	√√
Chimera	√√	√/-	Ethically not allowed
Germline transmission	√√	√/-	Ethically not allowed
Follow up generations	√√	--	Ethically not allowed
Transplantation of iPS cell-derived cells	√√	√/-	No clinical studies to date ¹

√√: Fully proven; √/-: Partially proven; --: Not achieved yet; ¹The first clinical study was recently initiated (http://www.riken.jp/en/pr/press/2013/20130730_1). iPS: Induced pluripotent stem.

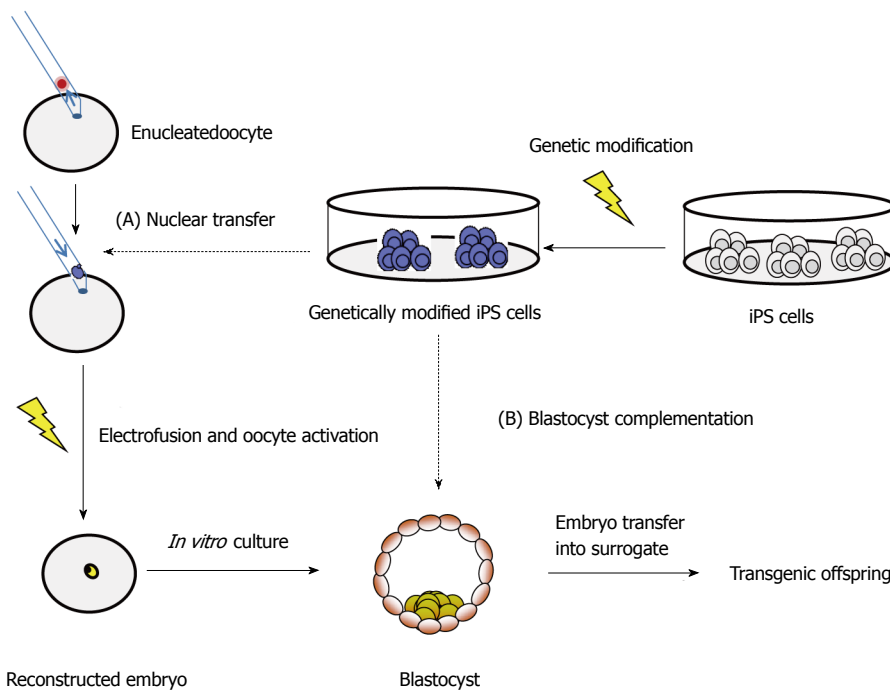


Figure 2 Application of induced pluripotent stem cells for advanced generation of transgenic animals. iPS: Induced pluripotent stem.

severe combined immunodeficiency (X-SCID), two of the treated children independently developed T-cell lymphomas due to viral integration in the neighborhood of the LIM domain only 2 gene^[78]. These data highlight the risks of viral-based therapies^[78]. Somatic cells derived from retrovirally reprogrammed iPS cells are apparently inconspicuous, provided that the c-Myc transgene is silenced^[19,79]. Retroviral reprogramming may evoke an immunogenicity of iPS cells^[80]. Human iPS cell-like cells can be formed through transduction with lentiviruses, which do not carry reprogramming factors. The “pseudo” iPS cells were induced by viral encoded microRNA expression^[81].

Alternative to integrating retroviruses, non-integrating adenoviruses can be used for reprogramming^[17,82]. Another non-integrating virus is represented by the Sendai virus system. Sendai viruses enable efficient production of iPS cells and later on elimination of the viral vector^[83]. Though viral mediated gene transfer offers high efficiency in generation of iPS cells, they require specific safety conditions for their handling.

Non-virally-derived iPS cells

The generation of iPS cells without viral transduction is preferable for regenerative medicine. Non-viral methods of reprogramming include episomal vectors^[84], minicircle DNAs^[85], plasmid vectors^[86], small molecules^[87], mRNAs^[88], recombinant proteins^[89] and transposons like piggyBac^[90] and Sleeping Beauty^[91]. In comparison to viral systems, non-viral approaches such as transposons are able to carry large DNA cargo into the host cell, they are non-infectious and do not evoke immune responses.

Episomal vectors

Episomal vectors for reprogramming of somatic cells were recently described^[84]. In this method, reprogramming of fibroblasts was carried out by transfecting with the episomal vector oriP/Epstein-Barr nuclear antigen-1. This vector was chosen because it can be removed after reprogramming by a drug selection method. The iPS cells generated through this method show similar morphology and expression patterns to ES cells. Further, they were able to form

teratomas in immunocompromised mice. As there was no integration into the host genome, transgene free iPS cells may be selected through further sub-cloning. Despite these advantages, this method yields low reprogramming efficiency in human fibroblasts at about three to six iPS colonies per 10^6 input cells^[84].

Minicircle vectors

Minicircle vectors are produced by the recombinatorial elimination of the bacterial backbone of the original plasmids. Minicircles containing the four reprogramming factors Oct4, Nanog, Lin28, and Sox2 in addition to an enhanced green fluorescent protein were used to obtain human iPS cells^[85]. The group excised the bacterial backbone from the plasmid by taking advantage of the PhiC31-based intramolecular recombination system, which cleaves away the undesired bacterial plasmid backbones, leaving minicircle DNA to be purified containing the desired reprogramming factors^[85]. It was claimed that minicircle DNA benefited from higher transfection efficiency compared to the parental plasmids. They also have longer ectopic expression, which is due to the lower activation of exogenous silencing mechanisms. Later, other groups reproduced the minicircle approach for reprogramming^[92,93].

Small molecules

Nowadays, small molecules and chemicals are assessed to enhance reprogramming efficiency and iPS cell generation. The idea behind their use is to substitute core reprogramming factors with small molecules, which will serve to enhance the reprogramming. Shi *et al.*^[94] showed that neural progenitor cells, which endogenously express Sox2, were reprogrammed only by ectopic expression of Oct4 and Klf4. They also showed that this process was supported by the G9a histone methyltransferase inhibitor, BIX-01294 (BIX). Ichida *et al.*^[95] used small molecules (RepSox2) for replacing transcription factors (Sox2) by inhibiting transforming growth factor- β signalling. In this direction, Lee *et al.*^[96] used magnetic nanoparticle-based transfection method that employs biodegradable cationic polymer PEI-coated super paramagnetic nanoparticles for iPS cells generation. Recently, the L-channel calcium agonist, BayK8644, was assessed to improve generation of iPS cells^[87] and it was claimed that BayK8644 does not directly cause epigenetic modifications as it works upstream in cell signalling pathways and can therefore avoid unwanted modifications. A more comprehensive list of small molecules involved in the iPS cells generation and their mechanism has been reviewed recently^[97].

Transposon systems

The recent development of hyperactive transposase enzymes makes transposon systems an interesting alternative to viral based methods. The commonly employed Sleeping Beauty, piggyBac and Tol2

transposon systems are relatively simple organized, and the essential components can be separated on two plasmids. One plasmid carries the inverted terminal repeats (ITR) flanking the transgene, the other plasmid carries an expression cassette for the respective transposase enzyme. Upon co-transfer of both plasmids into a cell, the transposase becomes expressed and subsequently transposes the ITR-flanked transgene into the genome. Importantly, only the desired transgenes becomes integrated by a cut-and-paste mechanism, whereas the plasmid backbones are degraded. On a genomic scale transposon integrations appear to happen at random, without a bias for promoter and gene-containing regions. The integrated transposon can be removed seamlessly by supplying the transposase in trans^[98], which makes the system more attractive and relevant in producing the safe and clean iPS cells. Up to six reprogramming factors have been connected by self-cleaving peptide sequences allowing for coexpression from a single cassette^[91,99-103]. Individual proteins are then produced by the self-cleaving peptide^[104-106].

Reprogramming with protein factors

The discussed transposon and episomal systems still require the introduction of cargo DNA into the cells^[106]. Delivery of reprogramming factors as proteins is an obvious alternative. In 2009, transgene-free iPS cells were produced with proteins of reprogramming factors^[107]. Therefor recombinant reprogramming proteins were produced as fusion proteins containing cell penetrating peptides. Repeated supplementation of the culture media of fibroblasts converted them to iPS cells. However, the protein-based reprogramming approach has not found widespread use, mainly due to relative low reprogramming efficiencies, and high costs for repeated treatments with protein factors.

mRNAs and microRNAs

The most recent trend in the field of non-viral iPS generation is reprogramming by using RNA molecules. Recently, modified mRNAs encoding reprogramming factors were employed to generate iPS cells with high efficiency^[108]. Messenger RNAs are an ideal vehicle for reprogramming, because they do not bear the risk of integrational mutagenesis, they can be transduced to cells with high efficiency, and they can be combined in desired ratios of the individual factor encoding transcripts^[108]. Disadvantages of mRNAs are the short half-life of -10 h, and that innate immune responses must be inhibited to allow for the full effects^[109].

Recently, it was shown that micro RNAs (miR) expression is sufficient to induce pluripotency^[110-112]. Two independent groups reported iPS cell generation by delivery of miR302, or miR200c, miR302, and miR369^[113,114]. These miR-derived iPS cells were indistinguishable from conventionally generated iPS cells. MicroR reprogramming seems to have advantages

for cellular reprogramming^[114-116], for example it avoids the need of transducing proto-oncogenic transcription factors^[117,118]. However, it needs to be assessed whether this approach will be successful in other species, since the underlying mechanisms are not well understood^[119].

MOLECULAR FACTORS REGULATING REPROGRAMMING

The core factors for reprogramming are Oct4, Nanog, Sox2, Klf4, c-Myc and Lin28. These genetic factors reprogram cells by regulating critical signalling pathways, epigenetic modifications and micro RNAs^[114].

Reprogramming by core transcription factors

Oct4 is the best studied regulator of pluripotency. Oct4 expression is confined to early embryonic cells, germ line cells and cultured pluripotent stem cells, where it activates the gene transcription of stemness gene^[120]. Oct4 protein cooperates with stemness factors such as Nanog and Sox2, but it also interacts with Polycomb group proteins^[120], which are important repressors of transcription. Sox2 is a transcription factor that acts as coactivator of Oct4^[121]. Binding of Oct4/Sox2 dimers to the promoter sequences of *Oct4* and *Nanog* genes upregulate their transcription^[122]. Nanog is a homeobox-containing transcription factor stabilizing the stemness network^[122]. Klf4 is a zinc finger-containing transcription factor which regulates the expression of Oct4, Sox2 and Nanog^[123-125]. Over-expression of Klf4 in ES cells increased the expression of Oct4 which further improve the self-renewal ability^[126]. c-Myc enhances the efficiency and speed of reprogramming^[127]. LIN28 promotes the expression of Oct4 at the posttranscriptional level by direct binding to its mRNA^[128]. Recently, Glis1 has been identified as a substitute for c-Myc^[129]. Glis1 transactivate the genes of Wnt ligands, Lin28a, Nanog, Myn, Mycl1, and Foxa2^[129].

The aspect of whether the species-specificity of reprogramming factors is relevant for proper reprogramming, is not well understood. In principle, the essential domains of the reprogramming factors are highly conserved between mammalian species, and several publications showed successful reprogramming with human and murine sequences in other species^[5-13,130].

APPLICATIONS OF IPS CELLS

Modeling of human diseases and preclinical trials

The potential applications of iPS cells will impact regenerative medicine, pharmaceutical industry, and animal biotechnology^[131]. Human iPS cells could be utilized for curative treatments, to studying onset and disease progression *in vitro*, and to test potential therapeutic in high throughput screens^[114,131,132]. The production of disease-specific iPS cells has found

widespread use in recent years^[133-136]. Disease-specific iPS cells provide a unique resource to obtain a molecular understanding of disease onset and progression^[131,132]. Induced PS-derived differentiated cells will allow to carry out *in vitro* drug screening (Figure 3), and to test therapeutic interventions^[131]. In mice, Fanconi anemia and sickle cell anemia have been successfully corrected by using iPS cells^[131,133-136].

However with regard to potential curative treatments, the functionality, safety, and lack of tumorigenicity of iPS-derived cells have to be assessed in appropriate animal models bearing significant physiological and anatomical similarities to humans (Table 2). Hence, animal models could be contributed tremendously to a better understanding of disease mechanisms and therapeutic interventions. In addition, iPS cells from monkey^[5], porcine^[41,26], canine^[8] and cattle^[9] would be useful in animal biotechnology such as making precise genetic engineering for improved production traits and products^[137,138].

Advanced transgenesis in large mammals

Transgenic farm animals can serve as excellent models of human diseases and during the past few years transgenic farm animals have gained renewed popularity. This is due to the availability of annotated genome depositories of the major domestic species and other organisms (for example: www.ensembl.org; or www.ncbi.nlm.nih.gov/genome), and due the introduction of active methods of transgenesis, which dramatically increased the success rates^[42,43]. The repertoire of molecular tools now allows the precise modification of large mammalian genomes at rapid pace and has led to a recent boost in this area. The development of genuine iPS cells from domestic species will contribute to these advances and allow to perform desired genetic modifications *via* high throughput screens *in vitro*, and then use either SCNT^[47] or blastocyst complementation for the generation of transgenic offspring (Figure 3). However at the moment most of the iPS cells cultures from different domestic species have not been tested for their capability to contribute to chimera formation, and only preliminary data are available^[25,26]. Thus reinforced efforts to assess the potential of current livestock iPS cell lines for chimera contribution and germ cell differentiation are required. The majority of current livestock iPS cell lines are generated with retro- or lentiviral reprogramming approaches (Table 1), and the opportunities to assess alternative non-viral approaches are not widely assessed^[10,56,106]. Also the potential of auxiliary small molecular inhibitors of stemness signaling pathway is not exploited for livestock iPS cells. Potentially, high throughput screens to identify small molecules with species-specific activity are required. It is anticipated that these approaches will lead to livestock iPS cells, which will make a significant impact for future genetic modifications of these species.

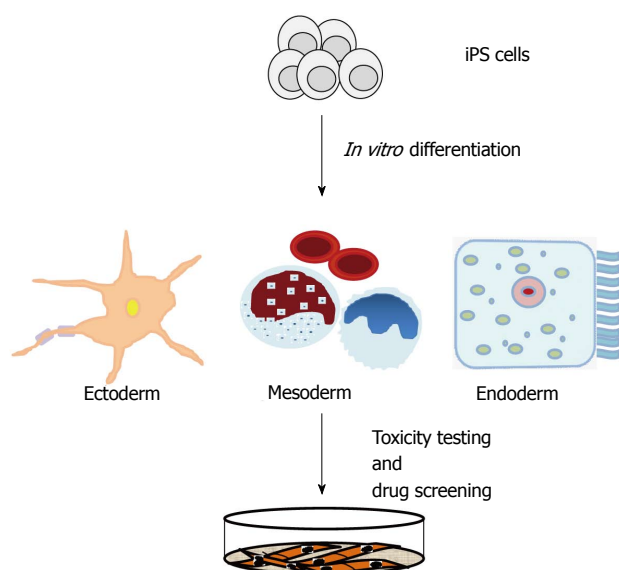


Figure 3 iPS cell technology contributes to disease modelling and drug discovery. iPS: Induced pluripotent stem.

Preservation of genetic resources and endangered breeds

The iPS technology has the potential to preserve endangered animals and highly valuable genotypes in the near future^[139]. Cryopreservation of cells and tissues is an important and useful approach for genetic preservation of valuable breeds and for conservation of endangered wild and domestic species. For highly endangered species, the derivation of iPS cells may become a method to prevent extinction. For example, iPS cells have been produced from endangered snow leopard^[140], drill and white rhinoceros^[139]. The iPS cells generated can be easily expanded for banking of genetic material, or used as donor cells for SCNT. Potentially, iPS cells from endangered species may be differentiated into mature oocytes and spermatozoa (Figure 4), which might be employed for *in vitro* embryo production^[139,140]. The differentiation of livestock iPS cells to functional gametes *in vitro* have not been achieved yet, however the current pace in developing fine-tuned protocols for *in vitro* differentiation of desired cell types, and the progress in inducing meiosis support the notion that the generation of fully functional spermatozoa and oocytes may be feasible. The possibility to obtain fully functional spermatozoa and oocytes from iPS cells of domestic and wild species would have far reaching consequences for maintenance of endangered species, as well as for breeding and genomic selection programs of domestic species. Even potential applications for infertility treatments in humans may become feasible^[141,142].

PROSPECTS OF FARM ANIMAL IPS CELLS IN PRECLINICAL STUDIES

The generation of iPS cells has opened new vista to

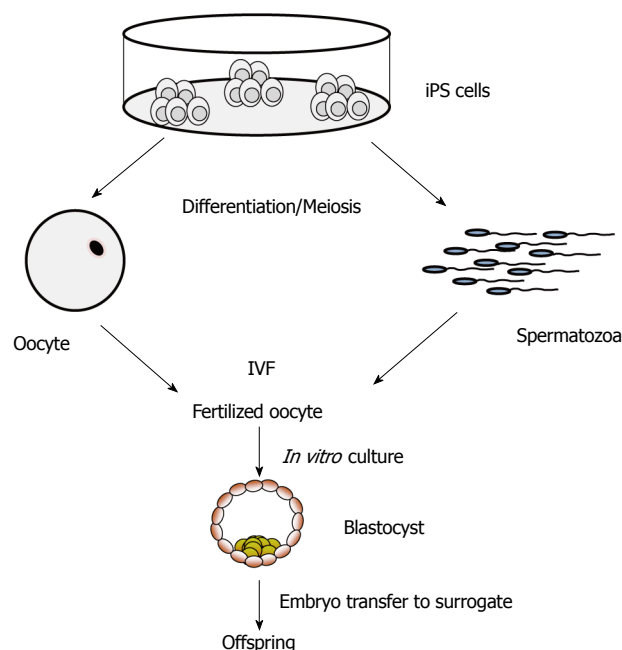


Figure 4 Application of induced pluripotent stem cells for *in vitro* generation of gametes. iPS: Induced pluripotent stem; IVF: *In vitro* fertilization.

understand pluripotency, disease onset and progression, and to develop regenerative medicine^[132]. However, before the clinical application of iPS cell-derived therapies can be envisioned, the low efficiency and kinetics of iPS cell formation, the risks of insertional mutagenesis, reactivation of silenced ectopic transgenes and potential tumor formation have to be assessed and solved^[131]. An important aspect is the biosafety of transplanted derivatives of iPS cells^[132]. A number of reports showed that iPS cell lines could contain genetic mutations, copy number variations, and epigenetic mutations^[132,143-145]. These aberrant changes may increase the tumorigenicity of iPS and iPS-derived cells. Retro- and lentiviruses are commonly used to introduce the reprogramming factors into differentiated cells, which can increase the immunogenicity^[146].

Farm animals represent informative model organisms, which seem to be suitable to assess obstacles and risks in longitudinal pre-clinical studies^[147]. In contrast to rodent models, they are more similar to humans with respect to life-span, physiology, metabolism and pathophysiology^[148,149]. Large mammalian models will allow to determine required cell doses to obtain therapeutic effects, to follow the fate of transplanted cells and their functional integration in the host tissue^[150]. Thus the research on pluripotent stem cells from farm animals will contribute to the development of innovative cell therapies for human patients.

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Kumar D *et al.* IPS cells from farm animals species

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Cardiac disease modeling using induced pluripotent stem cell-derived human cardiomyocytes

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has been carried out by expressing the mutated proteins in *in-vitro* heterologous systems. While these studies have provided a wealth of functional details that have greatly enhanced the understanding of the pathological mechanisms, it has always been clear that heterologous expression of the mutant protein bears the intrinsic limitation of the lack of a proper intracellular environment and the lack of pathological remodeling. The results obtained from the application of the next generation sequencing technique to patients suffering from cardiac diseases have identified several loci, mostly in non-coding DNA regions, which still await functional analysis. The isolation and culture of human embryonic stem cells has initially provided a constant source of cells from which cardiomyocytes (CMs) can be obtained by differentiation. Furthermore, the possibility to reprogram cellular fate to a pluripotent state, has opened this process to the study of genetic diseases. Thus induced pluripotent stem cells (iPSCs) represent a completely new cellular model that overcomes the limitations of heterologous studies. Importantly, due to the possibility to keep spontaneously beating CMs in culture for several months, during which they show a certain degree of maturation/aging, this approach will also provide a system in which to address the effect of long-term expression of the mutated proteins or any other DNA mutation, in terms of electrophysiological remodeling. Moreover, since iPSC preserve the entire patients' genetic context, the system will help the physicians in identifying the most appropriate pharmacological intervention to correct the functional alteration. This article summarizes the current knowledge of cardiac genetic diseases modelled with iPSC.

Abstract

Causative mutations and variants associated with cardiac diseases have been found in genes encoding cardiac ion channels, accessory proteins, cytoskeletal components, junctional proteins, and signaling molecules. In most cases the functional evaluation of the genetic alteration

Key words: Cardiomyopathies; Cardiac arrhythmias; Induced pluripotent stem cells; Human cardiomyocytes

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Core tip: This paper revises the cardiac genetic diseases that have been modeled so far using the technology that starts from patient somatic cells, reprogram their fate to a pluripotent state, and then proceed to cardiomyocyte differentiation. We will describe the main steps of this procedure, from pluripotent stem cells to mature cardiomyocytes, and we will discuss the main features linked to the different cardiac pathologies that this model recapitulate in a cell culture dish.

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INTRODUCTION

In 19th century, the Italian physician Giulio Bizzozero, founder of the Italian school of experimental pathology, proposed the classification of tissues depending on their regeneration rate. He divided human tissues in three categories: labile, stable, and perennial, where the labile are physiologically subjected to a continuous renewal, *e.g.*, keratinocytes, or bone marrow cells; stables, that are normally in a quiescent state but still retain the capacity to proliferate, *e.g.*, hepatocytes; and perennial, the so-called post-mitotic cells, that have completely lost their regenerative potential. The most representative cells in the latter case are neurons and cardiomyocytes, highly specialized cells that, apart from their duplication inability, share also the capacity, during development, to connect to sister cells and to build an organized network capable of an electric signal transmission.

During these years we learned the methods to cultivate and to maintain labile and stable cells in culture, but neurons and cardiomyocytes are still "problematic" cells, difficult to preserve in absence of their natural support, glial cells or cardiac fibroblasts, and, moreover, almost impossible to be obtained as sample from a healthy person.

Progress in this field came from studies of developmental biologist that were able to isolate, cultivate, and differentiate adult or embryonic stem cells (ESCs). These cells basically recapitulate *in vitro* the developmental process and ESCs in particular are indeed a continuous source of terminally differentiated post-mitotic cells that can be currently easily studied. The isolation and the use of human ESCs, although obtained from surplus of *in vitro* fertilization procedures, opened a serious and profound ethical issue. The great revolution came in 2006 when Shinya Yamanaka proposed a complex protocol designed to change cellular fate by forcing transcription factor expression^[1]. With this procedure, a murine

fibroblast was reprogrammed to a cell that assumed the main characteristics of an ESC: high proliferation rate as well as wide differentiation potential toward the three germ layers. Nevertheless, the search for an identical physiological counterpart has lead to the conclusion that these induced pluripotent stem cells (iPSCs), although very similar to ESCs, must be considered as artificial cells created in the lab. In 2007, the same process was applied successfully to human fibroblasts by Yamanaka's as well as Thomson's group^[2,3], thus creating cell lines that can be propagated almost indefinitely and can be used, in a close future, as a continuous source of differentiated cells for therapeutic purposes.

The application of this powerful technique to humans opened the possibility to reprogram fibroblasts isolated not only from healthy people but also from patients suffering of a genetic disease. The resulting human iPSCs (hiPSC) will keep the entire genetic information of the patient, including the mutation that has been linked to the pathology. The first examples of disease-related hiPSC involved patients with a range of human genetic diseases, whose DNA mutations, including the trisomy 21, were effectively maintained through all the reprogramming procedure^[4].

Since then, many laboratories started to model *in vitro* human genetic disease, succeeding to get a considerable number of post-mitotic cells for detailed functional studies. Indeed, this result is particularly important and useful when studying affected human cardiomyocytes or neurons, otherwise difficult to be obtained and maintained in culture. In most cases, despite the fact that hiPSC-derived cells are not exactly equal to the physiological counterpart, the system has attested successful *in vitro* replication of the main cellular characteristics already known to be associated with the modeled disease.

In this review we will discuss the important discoveries of the biological mechanisms underlying some genetic cardiomyopathies, made possible by the use of the cellular reprogramming technology.

FROM PLURIPOTENT STEM CELLS TO SPONTANEOUSLY CONTRACTILE CELLS

In vitro development of ESCs has been widely studied using murine ESCs (mESCs), whose differentiation procedure in culture implies the initial leukemia inhibitory factor (LIF) removal and the formation of cellular aggregates using the "hanging drop" method. These three dimensional (3D) structures, called embryoid bodies (EBs), replicate *in vitro* the different stages of murine embryonic development^[5]. Around differentiation day (dd) 8, clusters of spontaneously beating cells appear in culture; these cells express several transcriptional and structural cardiac markers and were therefore classified generically as cardiomyocytes^[6]. It should be noted that mESC differentiation starts by just removing LIF also in a 2D culture, but the resulting process does not strictly

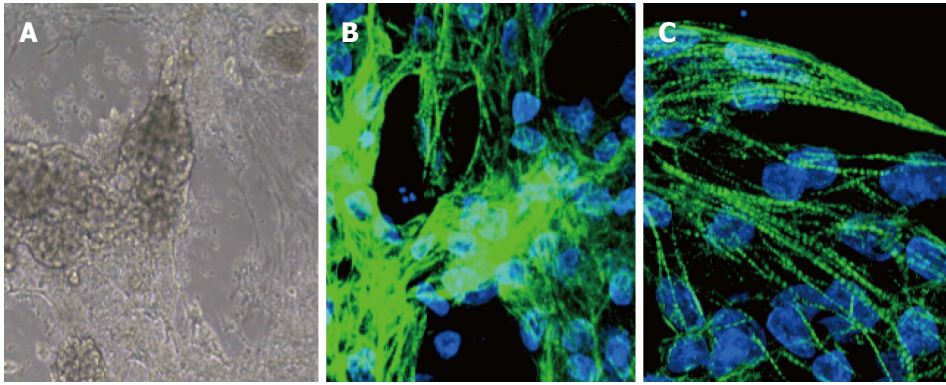


Figure 1 Cardiomyocyte at day 16 post differentiation. A: Microscopical view of beating areas; B: Expression of cardiac troponin T (green), nuclei (DAPI) in blue; C: Cardiomyocyte sarcomeric structures evidenced by cardiac troponin T staining (green), nuclei (DAPI) in blue.

follow embryonic development (P. Dell'Era, unpublished results).

Since their isolation, human ESCs have shown different culturing needs from the murine counterpart, and their behavior revealed also minor differentiation plasticity. Cardiac differentiation is the most glaring example of this statement. The spontaneous 3D differentiation of mESC leads to the easy appearance of spontaneous beating foci in all the considered EBs, while in the case of both hESC and hiPSC, only a modest proportion of EBs contains contracting cells. This occurrence leads to the setup of different methods aimed to increase cardiac differentiation during *in vitro* development of pluripotent stem cells. While some of these procedures retain an initial 3D EB formation, others start from a confluent 2D monolayer.

Differentiation protocols

During gastrulation, cardiomyocytes emerge from mesodermal tissue, in particular from the anterior region of the primitive streak. Bone morphogenetic protein produced by the adjacent endoderm induces the cardiomyocyte fate, whereas WNT-mediated signals from the underlying neural tube and notochord suppress cardiomyocyte specification. Therefore the timed addition of inducers and/or inhibitors of the cited pathways would guide PSC differentiation toward: mesoderm, cardiomyogenic mesoderm, cardiac precursors, and cardiomyocytes. This is conceptually the summary of several protocols. At variance the required instructive signals can be provided by a visceral endoderm-like cell line that can be used as a feeder layer during the differentiation process^[7].

Within these years, several efforts have been made to identify a protocol that would give rise to high percentage of cardiomyocytes following differentiation of either hESC or hiPSC. All the attempts lead to the creation of several tools such as defined cultivation media that, avoiding animal components, will facilitate therapeutic use of pluripotent stem cells; recombinant single protein matrices such as vitronectin or laminin;

aggregation plates to standardize the production of EBs; and the more recent commercial cardiomyocyte differentiation media (StemRD and Life Technologies). The main differentiation protocols that have been used over the years are outlined in Table 1.

We tested several of these procedures using iPSC derived from patients' fibroblasts and adapted to grow on Matrigel-coated dishes; in our experience, we observed beating areas only when cells were treated with modulators of the Wnt pathway, while the general *TNNT2* expression was achieved in most protocols (Figure 1B). Of particular interest for therapeutic purposes the recent setup by BurrIDGE *et al.*^[8] that employs a chemically defined medium consisting of just three components on a dish covered by synthetic biological matrices. Indeed, using this protocol we strongly increased the number of beating cells in our culture up to 50%, but the best result, around 70%, was recently obtained using the PSC Cardiomyocyte Differentiation Kit (Life Technologies).

FROM SPONTANEOUSLY CONTRACTILE CELLS TO MATURE CARDIOMYOCYTES

In early development, the primitive heart tube is composed by small myocytes without distinct region of cells conducting and coordinating the stimulus. The contractile impulse begins with the primary pacemaker area in the primitive atrium that will later evolve in the sinoatrial node, where the fast beating pacemaker myocytes will reside. As development proceeds, also the conductive system will differentiate, generating CMs in anatomical different regions that will become the atrio-ventricular (AV) node, the AV bundles, and Purkinje fibers. Each of these CM subtype has its own electrical properties such as the presence and the form of the action potential (AP), the contraction rate, and the presence of specific currents that can be measured electrophysiologically.

However, CMs differentiated *in vitro* vary considerably from cells isolated from a mature human heart, because of the absence of humoral factors and organized

Table 1 From pluripotent stem cells to cardiomyocyte: Details of main cardiac differentiation protocols

PSC type		Differentiation			Pre-treatment		Cardiac differentiation treatment					% Beating cells	% cTNT + cells	Ref.
h-iPSC	h-ESC	2D	3D	Others	ROCK-I	GSK-I	BMP4	Activin A	Wnt-I	FGF2	Others			
	X			END2							AA	< 40%	n.a.	[83]
X	X		X								Specific serum	5%-10% hiPSC 10%-25% hESC	n.a.	[20]
X	X		X				X			X		n.a.	> 80% hiPSC 60%-80% hESC	[84]
	X		X				X	X			AA	n.a.	50%-70%	[85]
X	X		X								p38-MAPK-I	> 23% hiPSC 50% hESC	n.a.	[86]
X		X		Sandwich	X		X	X		X		n.a.	98%	[87]
X	X	X			X	X	X	X	X		AA	n.a.	80%	[88]
X			X		X						END-2-CM	n.a.	< 10%	[89]
X		X			X		X	X		X	MEF-CM	n.a.	< 60%	
X			X				X	X			Tricho-statin A	< 50%	< 10%	[90]
X	X		X		X		X	X	X	X	VEGF	< 50% hiPSC < 40% hESC	n.a. 95% hESC	[91]
X		X				X			X		Albumin, AA	n.a.	90%	[8]
	X	X					X	X	X	X	VEGF	< 40%	< 20%	[92]
X	X		X				X	X	X		Blebbistatin	100%	90%	[93]

iPSC: Induced pluripotent stem cell; ESC: Embryonic stem cell; n.a.: Not available.

mechanical and electrical stress. In general, many of the features of hPSC-CMs are reminiscent of normal fetal cells. hPSC-CMs are spontaneously beating cells co-expressing atrial-, ventricular-, and nodal- markers, with unorganized sarcomeres, immature mitochondria, and an expression profile different from adult CMs^[9]. Our data indicate that, after 16 d of *in vitro* differentiation, iPSC-derived CMs start to segregate in the various subtypes, showing pronounced sarcomeric structures that reveal a certain degree of maturation (Figure 1C).

The CMs that arise during early hESC or hiPSC *in vitro* differentiation exhibit spontaneous AP, with a relatively depolarized resting membrane potential, probably due to the temporary absence of the inward rectifier potassium current (I_{K1})^[10].

The expression of the ion channels and, consequently, the ionic currents will undergo developmental maturation over time, as assessed by modifications in current density and property^[10]. hPSC-CMs immaturity is also reflected in their excitation-contraction machinery, lacking clear T-tubuli, disorganized sarcomeric striations, and immature Ca^{2+} handling^[11-13]. Unlike primary CMs that tend to undergo apoptosis or dedifferentiate, CMs derived from hPSC develop and maintain a functional phenotype in long-term culture^[14]. After surviving for 80 d, late-stage hESC-CMs show pronounced multinucleation that is accompanied by an increase in cellular perimeter, and area^[15]. Ultrastructural studies demonstrated that the sarcomere of hiPSC-CMs continue to mature through a 1-year culture^[16]. Young hiPSC-CMs contained a low number of unaligned myofibrils and immature high-density Z-bands. Within 6 mo the myofibrils became more tightly packed and formed parallel arrays accompanied by the appearance of mature Z-, A-, H-, and I-bands. M-bands were finally detected in 360-d-old

CMs, but expression levels of M-band-specific genes remained lower in comparison with those in the adult heart^[14].

Also a different gene expression accompanies these changes: late-stage CMs show increased levels of structural filaments MYH6 and MYH7, and of other specific molecules such as connexin 43, hyperpolarization activated cyclic nucleotide-gated potassium channel 4, and sarco(endo)plasmic reticulum Ca^{2+} ATPase^[14-16]. Finally, the electrophysiological profile of late-stage hESC-CMs show a significantly enhanced AP upstroke and a hyperpolarized maximum diastolic potential, and during maturation no differences were observed for AP duration (APD) to 50% or 90% (APD50, APD90) of repolarization^[15].

It must be noted that, while at early differentiation stages there are unspecified CMs that co-express different CM-subtype markers at later stages, around dd30, CMs acquire a more specific phenotype, expressing ventricular-, atrial-, and nodal-markers^[8]. The relative proportion of the three CM subtypes varies among the differentiation protocols that have been used, and can be additionally modulated by supplementing chemicals, microRNAs, or biological molecules to the culture^[17].

CARDIAC DISEASE MODELING

Despite all the discussed limitations, hPSC differentiation remains a powerful method to model *in vitro* genetic cardiac diseases, because of the capacity of these cells to give rise to terminally differentiated stable cardiac cells. The cardiac pathologies that have been modeled so far using hiPSC include some cardiomyopathies and arrhythmias, whose implicated

Table 2 Inherited cardiac diseases modeled using Induced pluripotent stem cell

Cardiac disease	Gene	Protein/current	Chr	Mutation	Differentiation method	Cardiomyocyte subtype	Maturation days	Ref.
HCM	MYH7	Myosin heavy chain β	14q12	R663H	3D spontaneous			[19]
DCM	LMNA	Lamin A	1q22	R225X GCCA insertion	END-2 co-culture	V- and A-like	20	[25]
	TNNT2	Troponin T type 2	1q32	R173W	3D, activin, BMP4, DKK1, FGF2, VEGF	V-, A-, and N-like	> 30	[27]
	DES	Desmin	2q35	A285V	END co-culture	n.d.	> 14	[26]
BTHS	TAZ	Tafazzin	Xq28	517delG c.328T>C	2D, activin, BMP4	n.d.	> 12	[30]
LQT1	KCNQ1	Kv7.1/I(Ks)	11p15	R190Q	3D spontaneous	V-, A-, and N-like	20-30	[36]
LQT2	KCNH2	hERG/I(Kr)	7q36	R176W	3D spontaneous	V- and A-like	n.d.	[42]
				A561T		V-, A-, and N-like	25-30	[38]
				A561V		V- and A-like	21	[45]
				A614V		V-, A-, and N-like	> 30	[37]
				N996I		n.d.	20-60	[43]
LQT3	SCN5A	Nav1.5 /I(Na)	3p21	F1473C	3D, activin, BMP4, Wnt-I, FGF2	V- and A-like	25-45	[50]
				V1763M	3D spontaneous	V-, A-, and N-like	> 28	[51]
				V240M, R535Q	END-2 co-culture	V-, A-, and N-like	20-30	[52]
LQT8	CACNA1C	CaV1.2/I(Ca)	12p13	G1216A	3D + Wnt3a	V-, A-, and N-like	> 37	[55]
CPVT	RYR2	Ryanodine receptor 2/I(Ca)	1q43	F2483I	END-2 co-culture	V-, A-, and N-like	20-30	[61]
				P2328S	END-2 co-culture	Mostly V-like	n.d.	[62]
				S406L	3D spontaneous	V-, A-, and N-like	> 70	[64]
				M4109R	3D spontaneous	V-, A-, and N-like	> 30	[65]
				E2311D	3D spontaneous	V/A- and N-like	> 30	[66]
	CASQ2	Calsequestrin	1p13	D307H	3D spontaneous	n.d.	25-43	[67]
ARVC	PKP2	Plakophilin-2	12p11	L614P	3D spontaneous	V-, A-, and N-like	> 28	[72]
				A324fs335X	3D spontaneous	n.d.	> 30	[73]
				T505fsX110				
				Cryptic splicing c.2013delC	3D spontaneous	n.d.	> 60	[74]

HCM: Hypertrophic cardiomyopathy; DCM: Dilated cardiomyopathy; BTHS: Barth syndrome; LQT: Long-QT; CPVT: Catecholaminergic polymorphic ventricular tachycardia; ARVC: Arrhythmogenic right ventricular cardiomyopathy; n.d.: Not defined.

genes are listed in Table 2.

hiPSC modeled cardiomyopathies

Familial hypertrophic cardiomyopathy: Hypertrophic cardiomyopathy (HCM) is a heterogeneous monogenic heart disease in which a portion of the myocardium is heavily hypertrophic. It is caused by more than 1400 mutations in at least 11 genes that encode thick and thin contractile myofilaments of the sarcomere or the adjacent Z-disc^[18]. HCM patients display abnormal thickening of the left ventricular myocardium in the absence of increased hemodynamic burden. Most people with familial HCM are symptom-free or have only mild symptoms, but their risk for clinical complications such as progressive heart failure, arrhythmia, and sudden cardiac death is strongly increased.

Efforts to elucidate the mechanisms underlying development of HCM have lead to the generation of patient-specific hiPSC-CMs that recapitulate *in vitro* a number of disease characteristics including cellular hypertrophy, and contractile arrhythmia^[19]. hiPSC were generated from a family cohort carrying a hereditary HCM missense mutation (Arg663His) in the MYH7 gene, and CMs were generated using the 3D spontaneous differentiation protocol^[20]. Mutant hiPSC-

CMs demonstrated not only cellular enlargement and multinucleation, but also other hallmarks of HCM including expression of atrial natriuretic factor, elevation of β -myosin/ α -myosin ratio, calcineurin activation, and nuclear translocation of nuclear factor of activated T cells^[19]. Using this model the authors were able to show that irregular Ca^{2+} transients and elevation of diastolic intracellular calcium $[\text{Ca}^{2+}]_i$ precedes the presentation of other phenotypic abnormalities, strongly implicating dysregulation of Ca^{2+} cycling in the pathogenesis of the disease. Pharmaceutical drug screening of mutant hiPSC-CMs further supported elevated $[\text{Ca}^{2+}]_i$ as a central mechanism for arrhythmia development. Indeed, only pharmaceutical blockade of Ca^{2+} and Na^+ entry mitigated contractile arrhythmia in HCM-CMs^[19].

Dilated cardiomyopathy: Dilated cardiomyopathy (DCM) represents the final common morphological and functional consequence of various pathological conditions in which a combination of myocyte injury and necrosis associated with tissue fibrosis results in impaired heart mechanical function. Nevertheless, primary familial forms of DCM represent a genetic condition in which the pathological involvement is predominantly limited to the myocardium. The main

hallmark of primary DCM is the presence of a left or biventricular dilatation with severely impaired systolic function in the absence of abnormal loading conditions or ischemic heart disease sufficient to cause global systolic impairment^[21]. Point mutations in 31 autosomal and 2 X-linked genes have been implicated in causing familial DCM (FDC) but account for only 30% to 35% of genetic causes^[22]. One of the more common genes identified in FDC is LMNA, which codes for lamin A/C proteins, intermediate filament proteins of the nuclear lamina^[23].

Several animal models of LMNA mutations have been generated to provide initial insights into the pathophysiology of lamin A/C-related DCM^[24]. Nevertheless the mechanism that links LMNA mutations with DCM remains uncertain.

Two different LMNA mutations have been modeled using hiPSC-CMs: an autosomal dominant non-sense mutation (R225X) in exon 4 of the lamin A/C and a GCCA insertion at base 50 in LMNA that creates a frameshift and premature stop codon, hence causing lamin A/C haploinsufficiency. LMNA^{R225X/WT} and LMNA^{Frameshift/WT} hiPSC-CMs showed normal phenotypes and basal electrophysiological properties as control hiPSC-CMs. However, when these CMs were subjected to electrical stimulation as in the cardiac environment, they exhibited typical nuclear abnormalities together with increased apoptosis^[25]. These two properties were replicated *in vitro* by shRNA knockdown of LMNA in control hiPSC-CMs, that was ineffective when the MEK1/ERK1/2 pathway was blocked pharmacologically^[25], thus identifying this pathway as a potential therapeutic target in LMNA-related DCM.

The same group also modeled a pathogenic phenotype of DCM due to a novel A285V Desmin (DES) mutation identified by whole exome sequencing (WES) using the hiPSC-CM system^[26]. Characterization of hiPSC-CMs carrying A285V-DES mutation revealed a poor co-localization of DES with several cytoskeletal proteins, including cardiac troponin-T, α -actinin and F-actin, and diffuse isolated aggregations of DES-positive protein^[26]. The electrophysiological analysis revealed that A285V-DES CMs exhibit significant functional abnormalities compared with the control-CMs as demonstrated by the diminished maximum rate of calcium ion re-uptake, slower spontaneous beating rate and failure to respond to the inotropic stress induced by isoproterenol^[26]. When control-iPSC-CMs were transduced with a lentivirus carrying the A285V-DES mutation, the resulting CMs simulated the phenotypes of DES-DCM-CMs, thus confirming the idea that abnormal DES-positive protein aggregates due to DES mutation can cause structural and functional abnormalities in cardiomyocytes. The relevance of this study is the demonstration that patient-specific hiPSC-CMs can be used to provide confirmation of a suspected genetic basis for DCM identified by WES.

Lastly, cardiomyocytes derived from patients in a

DCM family carrying a point mutation (R173W) in the gene encoding sarcomeric protein cardiac troponin T were analyzed^[27]. Compared to healthy individuals in the same family cohort, CMs from DCM patients exhibited altered regulation of Ca²⁺, decreased contractility, and abnormal distribution of sarcomeric β -actinin. When stimulated with a β -adrenergic agonist, CMs showed characteristics of cellular stress such as reduced beating rates, compromised contraction, and abnormal sarcomeric β -actinin distribution. Treatment with β -adrenergic blockers or overexpression of sarcoplasmic reticulum Ca²⁺ adenosine triphosphatase rescued the pathological phenotype^[27].

Barth syndrome: Barth syndrome (BTHS) is a rare, metabolic, and neuromuscular genetic disorder that occurs exclusively in males. Clinical features include variable combinations of pathologies including DCM, HCM, endocardial fibroelastosis, left ventricular non-compaction, ventricular arrhythmia, sudden cardiac death^[28]. The gene responsible for the disorder, tafazzin (TAZ), has recently identified: it is located on the long arm of chromosome X at Xq28, and encodes an acyltransferase that catalyzes the acylation of cardiolipin, the major phospholipid of the mitochondrial inner membrane^[28,29]. So far, more than 120 mutations have been described, but no correlation with specific phenotype has been observed^[28].

hiPSCs from two unrelated individuals with BTHS were generated, BTH-H and BTH-C, carrying TAZ frameshift (c.517delG) and missense (c.328T > C) mutations, respectively. CMs were generated by treating a cell monolayer with inducers and sorted for the surface marker vascular cell adhesion molecule-1 to obtain preparations highly enriched in CMs. Purified CMs were analyzed morphologically to evaluate sarcomere organization, and to assess mitochondrial functionality. CMs were then seeded onto thin elastomers with patterned lines of fibronectin, obtaining self-organized anisotropic myocardial tissues to study contractility properties^[30]. TAZ deficiency in BTHS-CMs impairs mitochondrial functionality as well as sarcomere assembly, generate contractile stress, and markedly increase reactive oxygen species (ROS) production. When linoleic acid (LA), an essential unsaturated fatty acid precursor of mature cardiolipin, was added to CM culture, the metabolic phenotype was corrected, the sarcomere organization and contractile defects were mitigated, and ROS production was strongly reduced^[30].

Using the hiPSC-derived model the authors showed that suppression of ROS, not only by LA but also by mitoTEMPO, normalized the metabolic, sarcomerogenesis and contractile phenotypes of BTHS- CMs, thus setting the basis for an effective pharmacological therapy of BTHS patients^[30].

hiPSC modeled arrhythmias

Long-QT syndromes: Long-QT syndromes (LQTS) are a group of heritable, usually autosomal dominant disorders with a estimated prevalence of 1:2500, characterized by an abnormally delayed or prolonged ventricular repolarization phase (prolongation of the QT interval on an electrocardiogram) and a propensity toward polymorphic ventricular tachycardia (often termed Torsades de pointes, TdP), syncope and sudden cardiac death in young patients^[31]. Clinically LQTS present a broad range of phenotypes even among family members with identical mutations, possibly as a result of genetic modifiers^[32]. To date, LQTS have been associated with over 500 different mutations in at least 13 genes encoding cardiac ion channel proteins, but the most prevalent forms are LQT1 and LQT2 caused by potassium channel mutations with a percentage of genotyped cases of > 50% and 30%-40%, respectively, and LQT3 caused by a sodium channel mutation that accounts for 10%-15%^[31].

LQT1 patients carry mutations in the *KCNQ1* gene (also known as KVLQT1 or Kv7.1), which encodes the pore-forming α -subunits of the channels generating I_{Ks} , an adrenergic-sensitive, slow outward potassium current, while LQT2 implicates hERG protein (encoded by *KCNH2* gene), which constitutes pore-forming α subunit of the rapidly-activating delayed rectifier potassium current (I_{Kr})^[33]. LQT3 is instead associated with gain-of-function mutations of the *SCN5A* gene, which encodes the α -subunit of the Na^+ ion channel $NaV1.5$ ^[34], while loss-of-function mutations in the same gene are associated with several other genetically heterogeneous disorders including Brugada syndrome, cardiac conduction disease, sick sinus syndrome sudden infant death syndrome and others^[35].

LQT1 was the first cardiac disease modeled using hiPSC and since then several hiPSC-CMs from patients carrying mutations in LQTS-associated channels have been considered^[36-42]. Indeed, patient-specific hiPSC-CMs represent a platform to investigate the functionality of ion channel mutations expressed in their complex genetic backgrounds and may provide unique insight into therapeutic approaches for disease management^[43].

LQT1: A family affected by LQT1 was screened and an autosomal dominant missense mutation R190Q in the *KCNQ1* gene was identified^[36]. hiPSC from two family members and two healthy controls were generated by retroviral vectors encoding the human transcription factors OCT3/4, SOX2, KLF4, and c-MYC (hOSKM). Using a 3D differentiation protocol, these cells were then differentiated into CMs. Spontaneously beating cells dissociated from LQT1 and control explants responded to pacing and generated three distinct types of APs, designated as "ventricular (V)", "atrial (A)", and "nodal (N)" on the basis of their similarity to the APs of human fetal heart CMs^[36].

Several disease-specific abnormalities were observed in LQT1-CMs: the duration and the rate adaptation of the AP, a 70%-80% reduction in I_{Ks} , as well as vulnerability to catecholaminergic stress^[36]. R190Q-KCNQ1 is a dominant mutation and indeed in hiPSC-CMs the mutated protein was absent on cell surface, but still retained in the endoplasmic reticulum. Similar results were obtained by expressing the wild type and the mutated protein in cardiomyoblast H9c2 cell line^[36]. Furthermore, electrophysiological studies confirmed the protective effect of β -blockade in the abnormal response to catecholamine stimulation, thus confirming the efficacy of the therapeutic approach for LQT1 patients^[36].

LQT2: The voltage-gated inwardly rectifying potassium channel that comprise KCNH2-encoded protein is composed of homo- or heterotetrameric complexes of pore-forming α subunits, like hERG, that associate with modulating β subunits. hERG consists of six transmembrane α helices, a pore helix, and N- and C-termini cytoplasmically located. The channel mediates the rapidly activating component of the I_{Kr} in heart^[44]. A large number of natural variants have been described, most of them in association with LQT2 syndrome (see <http://www.uniprot.org/uniprot/Q12809>).

A panel of control and LQT2-related hPSC were generated and characterized by several laboratories. hiPSC-CMs showing five different hERG mutations in genetically unrelated backgrounds were intensely characterized: R176W^[42], A561T^[38] and A561^[45], A614V^[37], and N996I^[46]. The aminoacids 176 and 996 reside in the N- and C-terminus cytoplasmic domains respectively, while the position 561 is in a transmembrane region of the protein, and the aminoacid 614 is located in the pore-forming segment.

Heterozygous KCNH2 mutations exert a dominant-negative effect on wild-type (WT) hERG channels associated I_{Kr} , by impairing trafficking pathways or altering channel kinetics of the resulting co-assembled hERG heterotetramers^[31,47]. Due to this behavior, it is possible to transfer them in a WT environment to verify their biological consequences in an unrelated genetic background.

The reprogramming and the following differentiation process were similar for all the laboratories: most of them used a retroviral transduction system, except for Mehta *et al.*^[45] that choose a viral-free episomal non-integrating approach, but the following differentiation involving EB formation and spontaneous differentiation was identical for all of them.

It must be pointed out that LQT2 is a disorder with incomplete penetrance where genetic background variations can confound disease traits. For this reason, the KCNH2 N996I mutation was deeply analyzed in (1) hiPSC-CMs from LQT2 patient; (2) hiPSC-CMs from LQT2 patient, previously corrected to wild-type using an homologous recombination system; and (3) NKX2.5eGFP/w hESC-CMs where the N996I-KCNH2

mutation was introduced using the same approach as before^[46].

Several observations were commonly reported to all LQT2-CMs: intracellular patch clamp recording of APD or extracellular measurement of field potential duration (FPD) using multi electrode array (MEA) showed prolonged intervals for both A-like and V-like cells^[37,38,42,45,46]. A summary of AP characteristic parameters measured by several authors in iPSC- or ESC-derived CMs is reported in Hoekstra *et al.*^[48]. Nevertheless, the AP increase was more restricted when isogenic cell lines were compared^[46], thus suggesting that a different genetic background can indeed over-estimate the differences between a control versus diseased CMs.

AP was modulated in LQT2-CMs by several drugs: pinacidil, a K_{ATP}-channel opener, significantly shortens APD₉₀, while Na-channel blocker, ranolazine, was ineffective^[37]; K channel enhancers, nicorandil and PD118057, caused AP shortening and in some cases could abolish early afterdepolarization (EAD)^[38]. In most of the papers some arrhythmogenicity of LQT2-CMs was reported, as evidenced by EAD events during spontaneous recordings^[37,42,45] or when challenged with the clinically used stressor, isoprenaline^[38].

As expected, the measurement of I_{Kr}, determined by adding the specific E4031 inhibitor, showed a reduction in LQT2-derived versus control CMs^[37,42,45,46].

Definitively, the genetic correction of the N996I-*KCNH2* mutation associated with LQT2 restores I_{Kr} density and normalizes APD in patient-specific LQT2-CMs, while the introduction of the same mutation in hESC-CMs reduced I_{Kr} and prolonged the AP duration^[46]. In addition, the molecular defects of hERG A561V and N996I mutants have been analyzed. In the NKX2.5-eGFP⁺ hESC-N996I cells, as well as in LQT2-N996I CMs, a trafficking defect was identified. Indeed, the 155-kDa protein band, representing the form transported to the cell membrane through the Golgi, was reduced by two-fold compared to the WT protein, while the 135-kDa band, which corresponds to the protein located in the ER, was unaffected^[46].

Similarly, hERG mutation A561V causes a reduced membrane localization of glycosylated/mature protein^[45]. Treatment of LQT2-A561V CMs with the calpain and proteasome inhibitor ALLN, not only increased membrane localization of mature hERG but also reduced repolarization, increased I_{Kr} and reduced arrhythmogenic events, thus suggesting a new therapeutic approach to treat LQT2 patients^[45].

LQT3: The α -subunit of the Na(v)1.5 cardiac sodium channel, encoded by *SCN5A* gene is composed by intracellular N- and C- terminus, and four homologous domains, attached one another by cytoplasmic linkers, forming a pore that conducts Na⁺ ions across membrane^[49]. In LQT3, the gain-of-function *SCN5A*-

mutations cause an increased persistent Na⁺ influx during depolarization that results in an enhanced late or persistent sodium current due to defective open-state inactivation of the channel^[35]. Four different *SCN5A* alterations have been modeled using hiPSC cellular system: a *de novo* heterozygous missense mutation F1473C associated with a polymorphism (K897T) in *KCNH2*, identified in a newborn patient with extreme prolonged QT interval^[50]. The second *SCN5A* modification was again a *de novo* heterozygous missense V1763M mutation, found in a Chinese girl^[51], and the last two V240M and R535Q alterations were from two LQT3-diagnosed patients^[52]. The F1473C mutation occurs in the channel inactivation gate of *SCN5A* while V240M and V1763M reside in transmembrane segments, and R535Q mutation occurs in the first cytoplasmic linker^[53].

In the first paper a lentiviral transduction of OSKM was used to reprogram fibroblasts, and a complex protocol rich in developmental modulators was used to achieve CMs differentiation^[50]. Ma *et al.*^[51] used instead an mRNA-based non viral non integrating reprogramming approach, followed by the spontaneous EB-based CM differentiation. Finally Fatima *et al.*^[52] used the classical retroviral infection, followed by a differentiation protocol guided by END-2 co-culture.

The data of hiPSC-CMs from the newborn and from the Chinese girl were compared with those obtained from hiPSC-CMs of healthy parents and of a healthy sister respectively that, because of the common genetic background with the patients, strengthened the obtained results^[50,51].

Terrenoire *et al.*^[50] reported a mutation-dependent increase in I_{NaL}, a right-shifted steady-state channel availability, and faster recovery from inactivation in all clones from the proband and none of the parents' clones. By using the hiPSC-CM system, the authors easily showed that the *KCNH2* heterozygous polymorphism T897 and K897, derived from homozygous parents, had no impact on electrophysiological pathology of the proband^[50]. Furthermore, the system allowed the pharmacological evaluation of the current patient's therapy, not so effective in controlling episodes of arrhythmia. Indeed, a more effective therapy has been identified as a result of the proposed study^[50].

Ma *et al.*^[51] observed significantly prolonged APD in patient-derived V-like CMs compared with control cells, while Fatima *et al.*^[52], showed a tendency to prolonged APD not statistically significant. Relevant to this point Terrenoire *et al.*^[50], comment that the relatively depolarized diastolic membrane potentials at this embryonic developmental stage inactivate sodium channels and consequently minimize contributions of sodium channel activity to AP^[53]. Relatively to sodium current Ma *et al.*^[51] found that TTX-sensitive I_{NaL} was significantly larger in patient-derived hiPSC-CMs compared with control hiPSC-CMs^[51], while Fatima *et al.*^[52]

showed that time-to-peak for sodium current and time to 90% of inactivation of the Nav1.5 were significantly longer in the LQT3-CM^[52]. In agreement with previous findings^[50], mexiletine reduced the late Na⁺ current and shortened the APD in patient hiPSC-CMs^[51].

LQT8/Timothy syndrome: Timothy syndrome (TS) is a multi-system disorder characterized by cardiac, hand, facial and neurodevelopmental features that include QT prolongation, webbed fingers and toes, flattened nasal bridge, low-set ears, small upper jaw, thin upper lip, and characteristic features of autism or autistic spectrum disorders (see www.orpha.net).

TS is caused by mutations in the *CACNA1C* gene and is inherited as autosomal dominant trait. The gene codifies for Cav1.2 channel, the main L-type channel in the mammalian heart that is essential for generating the cardiac action potential and for excitation contraction coupling^[54].

To date, just one paper reported the modeling of this syndrome using hiPSC: 16 iPSC lines from two TS patients, and 10 control lines from two unrelated individuals were generated using the classic retroviruses, and CMs were then obtained spontaneously from EBs^[55].

A prolonged AP was observed in TS-V-like CMs, while no differences were observed in A-like and N-like cells^[55]. The L-type Ca²⁺ channel current had significantly reduced voltage-dependent inactivation in TS-CMs compared to control cells^[55]. In addition, the TS-CMs exhibited a large number of depolarizing events that failed to trigger a full AP, similar to delayed afterdepolarizations (DADs) that arise after ectopic release of Ca²⁺ from the sarcoplasmic reticulum and which are associated with cardiac arrhythmias^[55]. Moreover, the Ca²⁺ elevations in spontaneously contracting TS-CMs were more prolonged and more irregular than those of control CMs^[55]. Finally, roscovitine rescued the electrophysiological properties of TS- CMs by increasing Cav1.2 voltage-dependent inactivation, reducing the APD, and decreasing the frequency of abnormal depolarizing events^[55].

Catecholaminergic polymorphic ventricular tachycardia: Another inherited cardiac disorder that was studied using hiPSC-CMs is catecholaminergic polymorphic ventricular tachycardia (CPVT) that is characterized by emotional and physical stress-induced ventricular tachyarrhythmia, syncope and sudden cardiac death in children and young adults. Two type of CPVT have been described: the autosomal dominant form (CPVT1) linked to mutations in the cardiac ryanodine receptor type 2 gene (*RYR2*) and a rare autosomal recessive form (CPVT2) caused by mutations in the calsequestrin-2 gene (*CASQ2*)^[56].

RYR2 gene encode for the principal Ca²⁺-releasing channel expressed in the membrane of the sarcoplasmic reticulum (SR). Studies based on *in vitro* expression of mutant *RYR2* in heterologous cell systems and

transgenic mice carrying specific *RYR2* mutations suggested that arrhythmias in CPVT1 are due to the diastolic Ca²⁺ leak from the SR following catecholaminergic stimulation^[57]. This leakage may lead to develop DADs through activation of the membrane Na⁺/Ca²⁺ exchanger, which can eventually result in triggered activity^[58].

The *CASQ2* gene encodes for a high-capacity, low affinity Ca²⁺ binding glycoprotein located inside the SR and involve in excitation-contraction coupling process^[59]. The functional alterations in intracellular Ca²⁺ handling resulting from the mutated *CASQ2* gene may cause DADs^[60]. In 2011, Fatima and his group described the hiPSC generation from a patient with CPVT1 carrying the mutation F2483I in *RYR2* gene^[61]. The authors differentiated hiPSC-CMs from fibroblasts of the CPVT patient and after 20-30 d of culture the cells were electrophysiologically analyzed. Frequent DADs and arrhythmias in CPVT-CMs exposed to adrenergic agonists were consistently observed. Furthermore, abnormal sensitivity to phosphorylation and cAMP-mediated regulation, together with the tendency for I_{Ca}-triggered Ca²⁺ release to continue following repolarization have been found^[61]. These first results using hiPSCs-based cardiac model validated the earlier hypothesis obtained only with animal models. Different groups have obtained similar results: Kujala has demonstrated that in addition to DADs, CPVT-CMs with P2328S *RYR2* mutation displayed also EADs which may be involved in cardiac arrhythmogenesis of their patients^[62]. Zhang reported specific analyses on calcium current (I_{Ca}) and Na⁺-Ca²⁺ exchanger current (I_{NCX}): in this case RyR2 F2483I mutant CMs have aberrant unitary Ca²⁺ signaling, smaller I_{NCX} reflecting smaller Ca²⁺-stores, higher I_{Ca}-gated Ca²⁺-release gains, and sensitized adrenergic regulation, consistent with functionally altered Ca²⁺-release profile of CPVT syndrome^[63]. Jung also reported the hiPSC generation of a 24-year-old woman with a diagnosis of familial CPVT1 with S406L missense mutation in the *RYR2* gene^[64]. In addition to presenting the electrophysiological properties of CPVT-CMs, the rescue capacity of dantrolene, which is believed to stabilize skeletal and cardiac RYRs by binding to a N-terminal sequence, is analyzed. Treatment with dantrolene restored normal Ca²⁺ spark properties in CPVT-CMs under basal conditions and corrected S406L-*RYR2* hyperactivity induced by adrenergic stimulation, with minimal effects in control cells; moreover, the same drug completely abolished DADs and triggered arrhythmias^[64].

Two more studies evaluated the effects of different drugs onto CPVT-CMs: Itzhaki reported the positive effects of flecainide, an antiarrhythmic agent, and thapsigargin, a β -blocker on CMs carrying the *RYR2* M4109R mutation^[65], while Di Pasquale *et al*^[66] reported the rescue of the arrhythmic phenotype induced by catecholaminergic stress by KN-93, an antiarrhythmic

drug that inhibits Ca^{2+} /calmodulin-dependent serine-threonine protein kinase II on CMs carrying the *RYR2 E2311D* mutation.

An additional study focused on the autosomal recessive form of the disease caused by the missense mutation D307H in *CASQ2*^[67]. In agreement with previous observations, isoproterenol stimulation caused DADs, oscillatory arrhythmic prepotentials and after-contractions, and diastolic intracellular calcium rising in *CASQ2*-derived CPVT model^[67]. Most importantly, electron microscopy showed that CMs derived from CPVT patients present an immature morphology with less-organized myofibrils, enlarged SR cisternae, and reduced number of caveolae^[67]. These data confirm previous findings derived from knock-out and knock-in *Casq2* mice that showed ultrastructural abnormalities in the SR^[68,69].

Arrhythmogenic right ventricular cardiomyopathy:

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heritable primary cardiac disease characterized by the replacement of CMs with fatty or fibrofatty tissue^[70]. ARVC has an autosomal dominant trait with reduced penetrance; approximately 40%-50% of ARVC patients have a mutation identified in one of several genes encoding components of the desmosome, which can help confirm a diagnosis of ARVC^[71].

Also ARVC has been modeled using hiPSC by three independent groups starting from skin biopsies of five patients: retroviruses were used to transduce patient's-derived fibroblasts and CMs were differentiated with the spontaneous 3D differentiation protocol^[72-74]. The five patients carried different alterations in Plakophilin-2 gene (*PKP2*), a structural component of desmosome^[75]: a heterozygous L614P mutation^[72]; a heterozygous insertion resulting in a frame shift from amino acid 324 to a stop codon in position 335 (A324fs335X) and a heterozygous deletion resulting in replacement of threonine by serine in position 50 and in a frame shift leading to a stop codon in position 110 (p.T50SfsX110)^[73]; and, finally, a homozygous mutation that causes cryptic splicing with a 7-nucleotide deletion in exon 12, leading to frame-shift of the carboxy-terminal amino acids, whose results were further confirmed with cells carrying a heterozygous c.2013delC in exon 10 of *PKP2*^[74].

Common observations of ARVC-CMs carrying the heterozygous conditions reported specific down-regulation of *PKP2* and its interactor plakoglobin both at mRNA and at protein level^[72,73]. Transmission electron microscopy (TEM) analysis displayed larger ARVC-CMs with less organized, thicker and more pleomorphic Z-bands compared with the Z-bands in control cells^[72], as well as distorted desmosomes associated with accumulation of lipid droplets, that were strongly enhanced in lipogenic media^[72,73]. Relevant to this point, the addition of the GSK-3 β inhibitor BIO strongly suppress the effects of the

lipogenic stress^[73].

The phenotypic study of CMs derived from the patient carrying the homozygous mutation led to similar conclusions: an abnormal translocation of plakoglobin proteins associated with very low β -catenin activity^[74]. In addition, the culture of these ARVC-CMs, devoid of a correct *PKP2* carboxy terminus, in a lipogenic medium additionally containing PPAR- γ activating drugs, demonstrated exaggerated lipogenesis and pronounced apoptosis, that can be prevented by the addition of PPAR- γ antagonists^[74]. Similar results were obtained with CMs derived from the patient carrying a heterozygous c.2013delC mutation^[74].

The rescue of the pathogenic phenotypes was achieved by introducing the wild-type *PKP2* gene back into mutant hiPSC-CMs, thus suggesting that mutation of *PKP2* is sufficient to induce the pathological features observed in ARVC-CMs^[74].

Interestingly, the same cells were used by Cerrone *et al.*^[76] to verify a *PKP2*-mediated modulation of I_{Na} : indeed, as already shown using other cellular systems, ARVC-CMs showed drastically reduced I_{Na} and the deficit was restored by re-introducing the wild type *PKP2* gene.

CONCLUSION

Since the discovery of the cellular reprogramming method followed by *in vitro* CM differentiation, several findings regarding human cardiac cells, especially those exhibiting a pathological phenotype, have been made possible. We can discuss how close or how far is the relation between hiPSC-CMs and the normal counterpart, but surely the possibility to obtain human CMs in a culture dish is adding a missing item to the scientific community. Every model has its own limitations and this is true also for hiPSC-CMs. Indeed, data from the literature reports the most evident and predictable phenotypes, leaving aside those perhaps more interesting but still unexplainable. However, as shown, many patients' features are found in these hiPSC-CMs, starting from the prolongation of the APD corresponding to the elongation of the cardiac QT interval, or EAD or DAD events equivalent to arrhythmic episodes, or the presence of cellular lipid droplets primarily found in cardiac biopsies of patients with ARVC^[77].

Human CMs have been derived also from cells of patients carrying other genetic disorders involving cardiac pathologies such as Leopard syndrome^[78], Pompe disease^[79], Duchenne muscular dystrophy^[80], Friedreich's ataxia^[81], and Fabry disease^[82].

In all of the cases, patients-derived CMs showed the main characteristic traits of the related pathology: hypertrophic cells in Leopard^[78]; lower β -glucosidase activity, lower markers of metabolism, and higher glycogen content in Pompe^[79]; a dystrophic gene expression profile in Duchenne^[80]; impaired mito-

chondrial homeostasis in Friedreich's ataxia^[81]; and globotriaosylceramide accumulation in Fabry disease^[82]. Moreover, patients-derived CMs represent a powerful model to test type and dosage of clinically used drugs, laying the basis for a personalized therapy.

Increasing the number and diversifying the type of modeled patients will surely improve the understanding of the biological mechanism that leads to the considered disease. Nevertheless the big ongoing efforts reside in eliminating any kind of interaction of the reprogramming technology with patient's DNA and in identifying an easily reproducible protocol that leads to CM differentiation. Then, the non integrative Sendai-based reprogramming approach, followed by a chemical defined differentiation medium will surely solve the issue of reproducible cardiomyocyte differentiation.

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Stem cell therapy in inflammatory bowel disease: A promising therapeutic strategy?

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Core tip: Inflammatory bowel diseases are inflammatory, chronic and progressive diseases of the intestinal tract. A limited experience is available with hematopoietic and mesenchymal stem cell transplantation for the treatment of these conditions. Research is ongoing with other cell lines which have been used in conditions alike to inflammatory bowel disease and which will possibly have a therapeutic role in this condition.

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Abstract

Inflammatory bowel diseases are inflammatory, chronic and progressive diseases of the intestinal tract for which no curative treatment is available. Research in other fields with stem cells of different sources and with immunoregulatory cells (regulatory T-lymphocytes and dendritic T-cells) opens up new expectations for their use in these diseases. The goal for stem cell-based therapy is to provide a permanent cure. To achieve this, it will be necessary to obtain a cellular product, original or genetically modified, that has a high migration capacity and homes into the intestine, has high survival after transplantation, regulates the immune reaction while not being visible to the patient's immune system, and repairs the injured tissue.

INTRODUCTION

Inflammatory bowel disease (IBD) mainly consists of two clinical conditions, Crohn's disease (CD) and ulcerative colitis (UC). It is mainly characterized by chronic, destructive inflammation of the gastrointestinal tract for which no curative treatment is currently available.

Its etiology is unknown, but it is accepted that it could be the result of loss of tolerance to intraluminal bowel antigens^[1]. Genetic, environmental, and microbiological factors are involved in its development,

together with morphological and functional changes in the intestinal barrier associated to an impaired immune response^[2]. Early data supporting genetic involvement in the pathogenesis of IBD come from familial clinical studies showing a greater incidence in twins^[3,4], first-degree relatives^[5,6] and given ethnic groups^[7,8]. Genome-wide association scan studies have allowed for identification of more than 163 loci associated to IBD^[9], 73 genes associated to CD and 47 to UC^[10], and overlapping genes for both conditions have also been found^[11]. Genetic factors would however account for less than 25% of cases^[12]. The exception is represented by a monogenic disorder referred to as IBD-like diseases, which are associated with severe colitis in childhood and have at most three loci alternatives^[13]. On the other hand the increase of the incidence of IBD suggests that environmental factors are more important than genetic factors in the development of IBD^[14].

Since IBD etiology is currently unknown, current treatment is intended to control the inflammatory intestinal process, thus avoiding irreversible structural damage. However, current therapeutic results are discouraging. Thirty-three percent of patients with CD do not respond to anti-TNF alfa therapy^[15-18], and one third of responders loss the response^[19]. Based on all the foregoing and on advances in understanding of the pathophysiological mechanisms involved in IBD development, new biological drugs and cell therapies are being investigated.

Future of the cellular-based therapy in IBD: Lessons from preclinical and clinical studies

Cell therapies are promising candidates for the treatment of IBD. However, inconsistent results have emerged from current clinical trials using both, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). To establish the best stem cell type, the administration route and optimal dose of cells to achieve an effective therapy and to guarantee the safety of the patient, in-depth basic research is necessary. Therefore, preclinical studies using different animal models are necessary to understand the pathogenesis of IBD. These studies will facilitate a better design of preclinical stem cell therapies that will eventually become a suitable clinical therapy for IBD patients.

ADULT STEM CELLS

At present, HSCs and MSCs have been used in several clinical trials. However, up to now, the results are unpredictable. For additional information, the reader is referred to the recent review published by our group in 2014^[20].

Hematopoietic stem cells transplantation

Use of hematopoietic stem cells transplantation (HSCT) in IBD is restricted to severe CD with few therapeutic

options. These are patients who do not respond to standard treatment in whom surgery is not an option due to extent of disease. HSCT may also be used in monogenic diseases, such as interleukin-10 deficiency, where allogenic bone marrow transplant would correct the disease by reconstituting a new immune system.

Allogenic HSCT is not currently accepted for the treatment of CD because of its high mortality rate. Current studies focus on autologous HSCT, which is intended to "reset" the immune system of the patient. Once reactive T-lymphocytes and memory cells of the patient are eliminated by chemotherapy (lymphoablation), administration of autologous HSCs generates an immunotolerant system^[21-23]. Unfortunately, this effect will probably be transient until the patient cells start the inflammatory mechanisms again.

Clinical experience is limited (Table 1), with the Burt study^[25] reporting complete remission in all their 24 patients. We are currently waiting for the results of the ASTIC study^[28]. This is a phase III clinical trial comparing two treatment arms intended to clarify whether improvement is due to reset of the immune system or to transplantation. The first arm uses chemotherapy followed by early transplantation (30 d), and the second arm chemotherapy with late transplantation (13 mo). Results reported to date include a high adverse effect rate and appear to suggest efficacy of transplantation.

Mesenchymal stem cell transplantation

MSCs seem to be a promising therapeutic strategy for IBD because of their ability to selectively home in to injury/inflammation areas after systemic administration, and their immunosuppressive and tissue healing properties^[30,31]. However, the clinical data published about MSCs transplantation in IBD patients showed conflicting results. An explanation for these inconsistent results could be the different sources used to obtain the MSCs that could have distinct differentiation and regeneration capabilities and the variety of protocols used for their isolation and culture. A better understanding of the MSCs biology and mechanisms of action and the exploration of other sources of stem cells in preclinical models of IBD are necessary.

Bone marrow and adipose tissue are the main sources of MSCs for both experimental and clinical studies. However, these sources have several disadvantages such as the invasive procedure used for their isolation, the small number of MSCs isolated, and the low proliferation and differentiation capacities related to donor age. For this reason, in the last few years the search for alternative tissue sources for MSCs has become of vital importance. Placental tissues, both fetal (amniotic fluid, Wharton's jelly, amniotic membrane, chorionic villi) and maternal (decidua) represent an important source of MSCs with some advantages including the isolation of large number of cells in a non invasive way^[32,33]. In addition, like

Table 1 Autologous hemapoietic stem cells transplantation studies in Crohn's disease

Ref.	Patients (n)	Follow up (mo)	Deaths	Remission (number of patients, time of evaluation in months)	Recurrence (number of patients or %, time of evaluation in months)
Oyama <i>et al</i> ^[24]	12	18.5 mo (7-37)	No	Clinical 11 (12 mo)	1% (18.5 mo)
Burt <i>et al</i> ^[25]	24	60 mo	1 not related	Clinical 24 (6-12 mo)	9% (12 mo) 37% (24 mo) 43% (36 mo) 61% (48 mo) 81% (60 mo)
Cassinotti <i>et al</i> ^[26]	10	56 mo (23-68)	No	Clinical 10 (3 mo) Endoscopic 5 (3 mo)	20% (12 mo) 50% (24 mo) 60% (36 mo) 70% (48 mo) 70% (60 mo)
Hasselblatt <i>et al</i> ^[27]	12	37 mo (IQR 6-123)	No	Clinical 4/8 (6 mo) Endoscopic 5/9 (9.1 mo) 3/9 mild disease	7% (10.9 mo)
Hawkey ^[28]	Data from 30 out of 45 patients 16 mobilisation + HCST (A) 16 mobilisation (B)		1 death after HCST	NA A: CDAI fell 162 (IQR 0-190) B: CDAI fell 82 (IQR 41-137)	NA
Jauregui-Amezaga <i>et al</i> ^[29]	21 evaluable	12 mo	1 after CMV infection and multiorganic failure	NA	NA

NA: Not available; CDAI: Crohn's disease activity index; IQR: Interquartile range; CMV: Cytomegalovirus.

bone marrow MSCs, placenta-derived MSCs are non-immunogenic and immunomodulatory stem cells with high expansion and differentiation capacity^[32-35].

An important issue in using MSCs is their safety. Although some studies supported that there is a risk of MSCs malignant transformation^[36,37], several recent studies using different types of MSCs supported that there is neither *in vitro* risk of development chromosomal aberrations after long term culture nor *in vivo* induction of tumors^[30,32,38,39].

For IBD treatment it is essential to increase the number of cells that migrate and home in to the intestine. A preclinical model of radiation enteritis treated with MSCs genetically modified to express the CXCR-4 receptor showed an increase of MSCs migration to intestinal site of injury and an improvement of symptoms^[40]. In the same way, MSCs coated with antibodies against vascular cell adhesion molecule VCAM-1 showed an increased cell migration of MSCs to inflamed colon and thereby an increased tissue repair capacity^[41]. A different strategy is to select a subpopulation of MSCs within the bone marrow that expresses high levels of EphrinB2. This subpopulation has an increased migration capacity to intestinal injury areas, and as a consequence, these MSCs would help to improve healing of intestinal injury^[42]. Once MSCs engraft in the intestinal damaged tissue they can proliferate and transdifferentiate into intestinal stem cells, or secrete cytokines and growth factors that will promote the proliferation and differentiation of intestinal stem cells in order to repair the injured areas of the intestinal tissue^[43].

Besides the migration, homing and tissue repair capabilities of MSCs, they also have an important function in modulating the inflammation and high

immune response within the injured tissues. These immunomodulatory properties of MSCs are of special importance in the treatment of IBD. Systemic administration of bone marrow MSCs in a mouse model of chemical-induced colitis^[43] and in a pig model of radiation-induced proctitis^[44], down-regulated autoimmune and inflammatory responses, and as a consequence, facilitated tissue regeneration.

The experience in luminal CD is limited (Table 2). Experience in UC is even smaller, and was mainly obtained in Russian studies about response of clinical activity^[50], changes in the pattern of systemic cytokines^[51] and elimination of cytomegalovirus after Mesenchymal stem cell transplantation (MSCT)^[52]. The most important work in this field is a phase III study^[48] that plans to include 330 patients who will be treated with MSCs at different doses, but final results are not expected until 2018. According to data reported to date, the safety profile appears to be favorable, and formation of aberrant tissue has not been detected.

As regards local treatment for perianal CD (Table 3), a single study using bone marrow cells is available^[55], and there is an 11-year experience of the Spanish group with MSCs taken from fat tissue (ASCs)^[53,54,56], initially autologous, except for a phase I / II trial using donor cells^[56]. We are currently waiting for completion of a phase III trial using donor cells which is planned to recruit a large patient sample. Two Korean studies using autologous ASCs have more recently been published. The first was carried out to evaluate the safety of the treatment^[57]. The second is a phase II study^[58]. A total of 43 patients were injected with ASCs. Among these, 33 were included in the modified per protocol analysis. The results showed complete sealing of 27 patients 8 wk after the final injection of

Table 2 Mesenchymal stem cell transplantation studies in luminal inflammatory bowel diseases

Ref.	Patient (n)	Procedence	Follow up (d, mo or wk)	SAEs	Response/remission (number of patients, time of evaluation)	Recurrence (number of patients, time of evaluation)
Onken <i>et al</i> ^[45]	10 CD (9 evaluable)	BM Allogenic	28 d	No SAEs	Clinical 3/1 (28 d)	NA
Duijvestein <i>et al</i> ^[46]	10 CD (9 evaluable)	BM Autologous	14 wk	No SAEs	Clinical 3/0 (6 wk) Endoscopic 0/2 (6 wk)	NA
Liang <i>et al</i> ^[47]	7 (4 CD/3UC)	BM/umbilical cord Allogenic	19 mo (range 6-32)	No SAEs	Clinical 7/3 (12 wk) Endoscopic 3/0 (3-5 mo)	1-3
Osiris Therapeutics ^[48]	Estimated 330 CD	BM Allogenic	NA	NA	NA	NA
Forbes <i>et al</i> ^[49]	16 CD (15 evaluable)	BM Allogenic	42 d	1 SAE probably not related	Clinical 12/8 (42 d) Endoscopic 7/0 (42 d)	NA
Lazebnik <i>et al</i> ^[50]	39 UC 11 CD	BM Allogenic	4-8 mo	NA	Clinical response UC 39/39 CD 11/11	NA

BM: Bone marrow; SAEs: Serious adverse events; NA: Not available; CD: Crohn's disease; UC: Ulcerative colitis.

Table 3 Mesenchymal stem cell transplantation studies in perianal Crohn's disease

Ref.	Patients (n)	Procedence	Follow up (mo)	SAEs related	Response/closure (number of patients or fistulas, time of evaluation)	Recurrence (number of patients, time of evaluation in months)
García-Olmo <i>et al</i> ^[53]	4 patients (8 fistulas)	Adipose Autologous	22 mo (range 12-30)	No	2/6 (2 mo)	NA
García-Olmo <i>et al</i> ^[54]	49 (14 CD) 25 (7 CD) fibrin glue (group A) 24 (7CD) ASCs (group B)	Adipose Autologous	12 mo	No	Group A: NA/1 (7 CD) Group B: 2/5 (7 CD)	3/17 global recurrence in group B (12 mo) Data for CD NA
Ciccocioppo <i>et al</i> ^[55]	10	BM Autologous	12 mo	No	3/7 (12 mo)	0/7 (12 mo)
de la Portilla <i>et al</i> ^[56]	22 per protocol	Adipose Allogenic	6 mo	2 SAEs possibly related Pyrexia Abscess	Closure: 5/18 fistulas (6 mo)	NA
Cho <i>et al</i> ^[57]	10	Adipose Autologous	6 mo	No SAEs	1/3 (2 mo)	0/3 (8 mo)
Lee <i>et al</i> ^[58]	33 per protocol	Adipose Autologous	12 mo	No SAEs	5/27 (2 mo) Per protocol	3/26 (12 mo)

BM: Bone marrow; CD: Crohn's disease; NA: Not available.

ASCs. No serious adverse effects were reported.

It is obvious that MSCs are a promising tool in the treatment of IBD. However, a large amount of work remains to be done to understand the mechanisms through which MSCs regulate the immune system, homeostasis and tissue repair. This knowledge will provide us with new tools to implement an effective MSCs-based treatment for IBD.

AMNIOTIC FLUID STEM CELLS

Amniotic fluid stem cells (AFSCs) are isolated from the excess of second-trimester amniotic fluid obtained during routine amniocentesis for prenatal diagnosis.

Recently, AFSCs were used in a neonatal rat model of necrotizing enterocolitis, one of the primary causes of morbidity and mortality in neonates, showed a decrease in intestinal damage, an increase in gut tissue repair and a higher survival^[59,60]. A better understanding of the AFSCs biology and mechanisms of action may help to develop strategies for their use in other IBD.

INDUCED PLURIPOTENT STEM CELLS

These are pluripotent cells derived from somatic cells by the introduction of reprogramming factors (Oct-4, Sox2, Klf4, c-Myc, Nanog and Lin28). These pluripotent cells can be differentiated to any

tissue specific cells to generate autologous cells for cell-replacement therapy^[61]. Human intestinal organoids have recently been generated from these cell lines^[62]. This will allow in the future for studying the pathophysiology of the disease and for testing new therapies, including generation of potentially viable tissues. Induced pluripotent stem cells (iPSCs) have been derived from somatic cells obtained from patients suffering a variety of diseases and important progress has been made in establishing preclinical iPSC-based disease models including IBD^[63]. Although iPSCs do not have the ethical problems of embryonic stem cells, there are many similarities between them and, as a consequence, iPSCs could develop teratomas following transplantation, hindering their use in clinical trials.

INTESTINAL STEM CELLS

Intestinal stem cells (ISCs) are a rare population of fast-cycling Lgr5⁺ cells and slow-cycling Tert⁺/Bmi1⁺ cells situated above them at the crypt base. ISCs are in charge of the renewal of the intestinal epithelium which is changed every 4-5 d and in the regeneration of the intestinal epithelium after injury or inflammation^[64]. It has been suggested that Lgr5⁺ cells and Tert⁺/Bmi1⁺ cells are two functionally different populations of ISCs^[65]. Lgr5⁺ cells are responsible for the maintenance of the normal homeostatis conditions, whereas Tert⁺/Bmi1⁺ cells are more quiescent cells responsible for the intestinal epithelium regeneration under injury or inflammation conditions^[66,67]. ISCs have proliferation and multipotency capabilities, *i.e.*, they are able to divide and later differentiate into all intestinal subtypes (enterocytes, goblet cells, Paneth cells and neuroendocrine cells). Recently, research in the ISCs field has advanced greatly and many ISCs markers have been identified^[68]. However, an exhaustive characterization of ISCs as well as the identification of specific markers still remains elusive^[64].

Transplantation of fetal and adult ISCs expanded *in vitro* presented a strong engraftment and healing potential in a colonic injury model in mice^[69,70]. However, ISCs in culture maintained as single cells have a very limited use in the study of the development of IBD and as a method for drug screening. Recently, intestinal organoids have been obtained from adult mouse and human ISCs^[66,71,72]. These organoids were able to engraft and repair murine and human epithelium and represent an important step forward in the treatment of IBD^[73]. Human organoids will be a very useful tool to study the pathological mechanisms of the disease from a specific patient and to test which is the best treatment to repair the intestinal epithelium for that patient. These organoids will be an important way to reach a more personalized medicine for IBD. These results highlight that those intestinal stem cells are a very promising source of stem cells for future patient-specific regeneration of the digestive tract^[68].

ENDOTHELIAL PROGENITOR CELLS

Besides local inflammation, IBD is characterized by anomalous angiogenesis/vasculogenesis and severe damage in epithelial cells^[74]. Important results have been obtained using endothelial progenitor cells (EPCs) for the treatment of hindlimb ischemia and myocardial ischemia. Recently, EPCs transplantation into fetal sheep showed an efficient migration and homing within the mucosal layer and a contribution to the vasculogenesis of the intestine^[75]. These results suggest that EPCs could represent an additional source of cells for IBD cellular therapy, on their own or in combination with other stem cells such as MSCs.

TOLEROGENIC IMMUNE CELL THERAPIES

In inflammatory disorders, special interest has been given to therapeutic strategies that could enhance the patient's tolerance response to intraluminal antigens. T-regulatory cells (Tregs) suppress immune responses of other cells and maintain tolerance to self-antigens. Tregs can be generated *ex vivo* by activation of both, murine and human CD4 T cells, suggesting that they could be an extra source of cells for cellular therapies in IBD. Intraperitoneal injection of induced Tregs in a mouse model of chronic colitis showed an attenuation of the preexisting gut inflammation response^[76,77].

Dendritic cells (DCs) are antigen-presenting cells involved in immunity and tolerance. DCs seem to be the most important regulators of immune tolerance in the gastrointestinal system, however, extensive studies are necessary to understand their role in this tissue and their mechanisms of action^[78]. Like MSCs, tolerogenic-DCs (tol-DCs) do not express neither MHCII nor the T-cell co-stimulatory molecules, and will not activate an immune response in the host. *Ex vivo* generated tolerogenic-DCs are available as a clinical grade product and used as therapeutic vaccines to restore antigen-specific tolerance in autoimmune diseases^[78]. Tol-DCs have been used in very few recent clinical studies such as rheumatoid arthritis and other not inflammatory diseases, and as a result conclusions about their clinical efficiency are still elusive. Several mouse models of colitis showed an important effect of tol-DCs in the prevention and reduction of symptoms of IBD^[79-83]. However, several questions must be resolved before tol-DCs can be used in IBD cellular therapy in humans, mostly due to the differences in IBD and tol-DCs between mice and humans^[84].

CONCLUSION

The goal for stem cell-based therapy is to provide a permanent cure for IBD. To achieve this, it will be necessary to obtain a cellular product (original or genetically modified) that has a high migration and homes into the intestine, has high survival after

transplantation, regulates the immune reaction which is not detectable to the patient's immune system, and will repair the injured tissue. Intestinal tissue is composed of several cell types and IBD are characterized by widespread damage. Cell-based therapies will probably be designed as a combination of several cell types that will produce a synergic therapeutic response.

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"Second-generation" stem cells for cardiac repair

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an inspiring alternative to restore cardiac function after myocardial infarction. A large body of evidence has been obtained in this field but there is no conclusive data on the efficacy of these treatments. Preclinical studies and early reports in humans have been encouraging and have fostered a rapid clinical translation, but positive results have not been uniformly observed and when present, they have been modest. Several types of stem cells, manufacturing methods and delivery routes have been tested in different clinical settings but direct comparison between them is challenging and hinders further research. Despite enormous achievements, major barriers have been found and many fundamental issues remain to be resolved. A better knowledge of the molecular mechanisms implicated in cardiac development and myocardial regeneration is critically needed to overcome some of these hurdles. Genetic and pharmacological priming together with the discovery of new sources of cells have led to a "second generation" of cell products that holds an encouraging promise in cardiovascular regenerative medicine. In this report, we review recent advances in this field focusing on the new types of stem cells that are currently being tested in human beings and on the novel strategies employed to boost cell performance in order to improve cardiac function and outcomes after myocardial infarction.

Key words: Stem cells; Cardiac repair; Myocardial infarction; Heart failure; Second generation

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Core tip: Myocardial infarction and heart failure represent two of the most prevalent and fatal diseases. Stem cell therapies represent a novel approach capable of restoring the cellular loss observed in these conditions. Data from initial human studies have been encouraging but inconclusive. However, refinements in cell populations as well as new stem cell sources are currently being tested in large phase III clinical trials after showing positive results in preclinical models and early clinical reports, thus holding a promise for the achievement of a true

Abstract

Over the last years, stem cell therapy has emerged as

myocardial regeneration after myocardial infarction. We review here recent developments in this field.

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INTRODUCTION

Cardiac heart disease is a leading cause of mortality and morbidity worldwide. Coronary heart disease (CHD) is estimated to affect near 16 million people in the United States and accounts for about 1 of every 6 deaths^[1]. CHD and myocardial infarction (MI) are also a major cause of heart failure (HF), which is an important public health issue with an estimated prevalence of 5 million in the United States^[1] and 23 million worldwide^[2].

Risk factor modification, advances in medical, device-based therapies and interventional management have improved cardiovascular outcomes^[3], but even when mortality has shown a decline in most developed countries since mid 70s^[1,4-6], CHD is the leading cause of death in the United States^[7]. Its main consequence - HF - carries a poor prognosis, even worse than many cancers^[8]. Moreover, the decline in MI acute mortality achieved with primary percutaneous coronary interventions (PCI), has augmented the prevalence of HF among survivors^[9,10], because of the development of substantial scarring despite reperfusion strategies^[11].

Current treatment of systolic HF is mainly focused on counterweighting the neurohormonal activation cascade, improving contractile function and reducing the incidence of sudden cardiac death^[3]. None of these approaches is capable of restoring the cellular loss produced after MI, which is the cornerstone of the adverse ventricular remodeling process that leads to HF. Heart transplantation is a not viable alternative in most cases, and is hampered by limited donor hearts.

Since the beginning of the past decade, regenerative cardiac therapies have emerged as an option to satisfy these unmet needs^[12-14]. Until the end of the last century, the human heart was believed to be a terminally differentiated post mitotic organ, unable to be repaired after an injury such acute MI. This dogma was challenged in 2001 by the evidence of mitosis in cardiomyocytes after MI^[15]. Beyond that, and in contrast to some lower vertebrates^[16], cardiac self-repair ability is limited in human adults^[17], and unable to replace the massive cellular loss that occurs after MI, but its discovery opened up the possibility to be externally enhanced. In order to achieve this objective, two approaches can be adopted: (1) the addition of

progenitor cells for the repopulation of the damaged heart with contractile cells and vascular structures; and (2) the enhancement of cardiac self-repair ability. Most times both mechanisms are concomitant. The former, represented by stem cell therapy, has been the most extensively investigated, being tested in several studies enrolling thousands of patients worldwide and evaluating different cell types and delivery routes. Promising results were reported in preclinical studies, leading to a quick translation into clinical trials. Initial reports in human beings were encouraging and established the safety of these therapies. But their efficacy has been subject of continuous debate, since robust evidence is lacking due to inconsistency in benefits observed in clinical trials. Differences in methodologies have been invoked to explain these discrepancies. Notwithstanding, this initial preclinical and clinical data have provided important insights into the selection and manufacturing of cell products, their delivery and the mechanisms of action that drive myocardial regeneration. The initial concept of achieving cardiac repair by differentiation of stem cells into functional cardiomyocytes and vascular structures has shifted towards a paracrine paradigm, where stem cells exert their beneficial effects by promoting myocardial salvage and self-repair mechanisms.

So far, the optimal source for stem cell therapy as well as its processing, delivery route and dosage, remains unknown and many fundamental issues need to be addressed. Ideal stem cell therapy must accomplish with several requirements including: (1) the ability to regenerate damaged myocardium; (2) the ease of obtaining, storing and delivering; (3) no tumorigenesis, immunogenicity or ongoing ethical issues; and (4) cost-effectiveness. The heterogeneous cell products employed in the early years of regenerative cardiac medicine have been replaced by more purified stem cell populations with a greater reparative potential, and *in vitro* priming of stem cells to enhance their engraftment, survival, plasticity and paracrine activity, has also been extensively investigated. All of these advances have lead to a new generation of stem cells ("second-generation" stem cells) that should overcome the hurdles found with first-generation ones. In this review we summarize recent research and novel strategies in this field, focusing on priming of "first-generation" cells and on the new cell products that are being tested for cardiac regeneration after MI.

GENETICALLY ENGINEERED SKELETAL MYOBLASTS

The first type of stem cell thought to be useful for cardiac regenerative purposes were autologous skeletal myoblasts. Their muscular phenotype and many other advantageous features including ease of isolation through muscle biopsy, rapid expansion *in vitro* and lack of ethical or immunological issues made them

an attractive option^[18]. In fact, their use in animal models^[19-21] and phase I non-randomized human trials^[22-26] described their ability to form some cardiac structures and yielded promising results regarding improvement in cardiac performance after MI.

Nevertheless, subsequent studies documented that myoblasts differentiate into skeletal myocytes instead of cardiomyocytes^[27], and the first and larger randomized controlled trial in humans, the MAGIC trial, showed no benefits on cardiac function^[28].

More worrisome is the lack of electro-mechanical coupling of these cells, that made them prone to generate ventricular arrhythmias due to their inability to express certain cardiac-specific genes codifying important proteins of the gap junctions, as N-cadherin and connexin-4^[25,28,29]. Down-regulation of these genes is induced by the transdifferentiation process^[29]. However, improved electrical coupling as well as a reduction in the arrhythmogenic potential of the transplanted cells was demonstrated by the enhancement of connexin-43 expression *via* genetic manipulation^[30-32].

Another drawback of skeletal myoblasts in their application for cardiac repair is massive apoptosis and their low survival rate when applied to the ischemic myocardium^[33]. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), have showed their ability to induce angiogenesis^[34,35]. Indeed, transfected skeletal myoblasts with augmented VEGF and FGF expression exhibit increased survival, promoted by an anti-inflammatory and angiogenic effect^[36-38]. Cell survival after transplantation can also be improved using myoblasts lacking the *MyoD* gene. These myoblasts induce angiogenesis *via* secretion of stromal cell-derived factor-1 (SDF-1) and placental growth factor^[39], and are less sensitive to apoptosis by up-regulation of a number of anti-apoptotic genes (*Pax3*, *Bcl-2*, *Bcl-xl*)^[40].

Despite of these advances, skeletal myoblasts seem far from being the optimal cell source, with safer and more efficacious novel cell types displacing its role in cardiac regenerative medicine.

BONE MARROW DERIVED STEM CELLS

Bone marrow derived mononuclear stem cells

Bone marrow derived mononuclear stem cells (BMMNCs) are the most extensively used type of cells for cardiac regeneration. They represent a heterogeneous mixture of mononuclear stem cells including hematopoietic and endothelial progenitors and mesenchymal stem cells. The landmark study of Orlic *et al.*^[41] was the proof-of-concept that a population of Lin⁻/c-kit⁺ BMMNCs was capable of transdifferentiating into myocytes and vascular structures and restore a large amount of the damaged myocardium after MI in a mice model. This finding generated great interest in this type of cells, spurring

an intense research and rapid clinical translation.

The exact mechanisms by which BMMNCs are thought to work remain poorly understood. Different explanations have been advocated including paracrine effects, transdifferentiation into cardiomyocytes and activation of resident cardiac stem cells^[42-44].

Many studies have shown a positive effect of BMMNCs in cardiac function after MI, both in the acute and chronic phases^[45-51], although some other trials reported a less evident or even absent benefit^[52-62]. The different clinical settings where cells have been tested, as well as methodology differences in these trials may explain, at least in part, the contradictory observed results. Five systematic reviews and meta-analyses concluded that BMMNCs transplantation after MI resulted in a better left ventricular function and remodeling with a modest but significant improvement in left ventricular ejection fraction (LVEF), ranging from 2.55% to 3.96%. No safety concerns have been observed and what is more provocative, better clinical outcomes have been documented in treated patients, although these trials did not include hard clinical endpoints as primary objectives^[63-67].

BMMNCs-based therapies face many limitations: (1) homing, engraftment and maintenance of cells in the transplanted tissue^[68]; (2) limited plasticity when using adult stem cells^[69,70]; and (3) depletion and impairment of their functionality associated with advancing age and comorbidities^[71,72]. Many different strategies for cell enhancement have been studied in the last years in order to overcome these problems.

MicroRNA (miRs) are small non-coding RNAs controlling gene expression, either by inducing mRNA degradation or by blocking mRNA translation^[73]. They are involved in the maintenance of the pluripotent state of stem cells and its over-expression is able to induce such a state in somatic cells^[73,74]. They also participate in the differentiation and lineage commitment processes^[73,74]. Thus, miRNAs have been investigated as a target for stem cell enhancement. For instance, cardiovascular diseases and ageing promote up-regulation of the pro-apoptotic and anti-proliferative miR-34a in human BMMNCs. Its inhibition *in vitro* has shown to improve cell survival and their therapeutic benefit in a mice model of MI^[75]. Furthermore, BMMNCs seem to regulate the expression of miRs in cardiomyocytes *in vivo*. In a mice model, intramyocardial delivery of BMMNCs mediated a paracrine effect by releasing insulin-like growth factor 1 (IGF-1) which inhibited the expression of the pro-apoptotic miR-34, thereby exerting a cardioprotective effect^[76].

But as in the case of skeletal myoblasts, new and more refined cell products are supplanting BMMNCs. In the meanwhile, definitive conclusions about the efficacy of BMMNCs after MI are expected to be elicited by the ongoing BAMi trial (NCT01569178). This large scaled, multinational, multicenter randomized and controlled trial, will test for the first time, the effect of

intracoronary infusion of BMMNCs three to six days after acute MI with LVEF < 45% on all-cause mortality as its primary endpoint. Over 3000 patients in 11 European countries are planned to be enrolled. This trial will help to trace the future directions that should be followed with BMMNCs therapies, supporting its administration, warranting further investigations or definitively leaving apart their use to focus on new generations of cells.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as multipotent stromal cells, represent a subset of BMMNCs discovered more than 40 years ago^[77]. They can be found within connective tissue in other organs, from which they can be easily isolated and cultured. They have awoken a big interest in recent years because they display a number of traits that made them an attractive cell product for cardiac repair^[78]. MSCs are multipotent and have the capability to transdifferentiate into lineages of mesodermal tissues^[79,80], including cardiomyocytes^[81]. *In vivo* studies have also proved the ability of human MSCs to differentiate into cardiomyocytes in adult mice hearts^[82]. They also display a great paracrine potential, secreting growth factors that promote endogenous healing^[83].

A number of preclinical studies have shown the benefit of these cells in cardiac function after MI^[84-86]. Clinical trials have also elicited promising results^[87,88] and a small and recent randomized phase I and II placebo-controlled trial suggested that transendocardial injection of MSCs is superior to BMMNCs and placebo in reducing scar size in chronic ischemic cardiomyopathy^[89].

But similarly to BMMNCs, autologous use of MSCs is hampered by their loss of functionality associated with ageing and comorbidities^[71,72], and their heterogeneous phenotype compromises their therapeutic effect^[90]. A variety of different strategies have been developed in order to improve MSCs regenerative potential. One of the most promising is the so-called "guided cardiopoiesis" of MSCs. This term defines the process by which a stem cell is engaged towards a cardiac differentiation program while its proliferative and self-renewal capacities remain intact^[91]. This can be achieved by mimicking the cardiogenic instructive signals that drive the embryonic development of the heart^[92]. The up-regulation of certain cardiac transcription factors such as Nkx-2.5, MEF2C, FOG-2, TBX5, MESP1 and GATA-4 is responsible of the adoption of a cardiogenic phenotype in MSCs, preserving their proliferative ability before the final differentiation step towards sarcomerogenesis begins^[92,93]. The up-regulation of these cardiac transcription factors is feasible when MSCs are cultured within a cardiogenic cocktail containing transforming growth factor beta (TGF- β), bone morphogenic protein (BMP)-4, activin A, FGF-2, IGF-1, interleukin (IL)-6, factor IIa (h-alpha-thrombin) and retinoic acid^[93]. Differentiation beyond this intermediate state is possible by exposing the cells to a 1% human

platelet lysate medium^[93]. In a murine model of ischemic cardiomyopathy, the priming of MSCs with these growth factors led to a cardiopoietic phenotype that improved their therapeutic benefit (by yielding sarcomere-containing myocytes with electromechanical response, and with mitochondrial structures closer to that seen in adult cardiomyocytes)^[93]. In another rat model of MI, transplantation of pretreated MSCs with IGF-1, BMP-2 and FGF-2 led to increased tolerance to hypoxic conditions of the MSCs and augmented expression of connexin-43 and gap junctions linking MSCs to cardiomyocytes. Indeed, MSCs exerted a protective effect over cardiomyocytes against hypoxia mediated by these gap junctions, showing a superior myocardial salvage of the infarcted heart and leading to an improvement in left ventricular function^[94]. Recently, the C-CURE clinical trial (Table 1) addressed the feasibility and the safety of cardiopoietic autologous MSCs in a randomized trial involving 48 patients with chronic ischemic HF^[95]. In this phase II study, the lineage-guided therapy with primed MSCs resulted in a total improvement in LVEF of 7% over baseline, compared to 0.2% in the standard of care group. Reductions in left ventricular volumes and increases in the 6-min walk distance were also observed. No adverse events related with the cells were documented^[95]. This study has supported the initiation of two ongoing large phase III trials, CHART-1 (NCT01768702) and CHART-2, where autologous cardiopoietic MSCs will be tested in patients with chronic HF secondary to IM.

A drawback of MSCs therapy is the poor survival and the low retention rate of transplanted cells that limit their regenerative potential. *Ex vivo* genetic modification may enhance viability of the transplanted cells by over-expression of anti-apoptotic genes. MSCs transduced with vectors encoding for genes such as heat shock protein (Hsp27)^[96], microRNA-1^[97], and protein kinase type 1 α ^[98] have shown increased survival and a more effective and efficient performance restoring cardiac function compared to conventional MSCs in rodent models of MI. Other pharmacological approaches to improve cell retention have also been evaluated. Thymosin β 4 is a protein implicated in cytoskeletal homeostasis that has been shown to protect MSCs against hypoxic injury and to increase cell retention in a rat model of MI^[99].

In summary, MSCs are among the most promising cells for cardiac reparative medicine. Novel approaches that prime cell functionality have shown encouraging results that have led to ongoing large-scaled randomized phase III clinical trials.

CARDIAC STEM CELLS

The classic concept stating that the adult mammalian heart is a post-mitotic organ without self-renewal capacity was challenged in the beginning of the past decade. The first evidence came out with the finding of mitosis among cardiomyocytes in human hearts after MI by Beltrami *et al.*^[15]. Shortly after, the same group

Table 1 Clinical trials evaluating new stem cells for cardiac repair following myocardial infarction

Study	n	Design	Type of cells	Delivery route	Clinical setting	Follow-up	Outcomes
Bartunek <i>et al</i> ^[95] (C-CURE)	47	Multicenter, randomized 2:1 (cells <i>vs</i> standard of care)	Autolo-gous bone marrow derived cardiopietic MSCs	Endo- myocardial injection	Chronic ischemic heart failure (LVEF 15%-40%)	Safety 2 yr Efficacy 6 mo	Feasible and safe ↑ LVEF ↓ LVESV ↑ 6-min walk distance and improvements in QoL and NYHA
Bolli <i>et al</i> ^[113] (SCIPIO)	23	Unicenter, randomized 2:1 (cells <i>vs</i> standard of care)	Autolo-gous c-kit+/lin- CSCs	Intra-coronary infusion	Chronic ischemic heart failure (LVEF ≤ 40% four months post CABG)	12 mo	Feasible and safe ↑ LVEF ↓ Infarct size
Malliaras <i>et al</i> ^[122] (CADUCEUS)	25	Two centers, randomized 2:1 (cells <i>vs</i> standard of care)	Autolo-gous CDCs	Intra-coronary infusion	Chronic ischemic heart failure (1.5-3 mo after MI)	12 mo	Feasible and safe ↓ Infarct size ↑ Viable myocardium and regional contractility ≈ LVEF and ventricular volumes
Hare <i>et al</i> ^[144] (POSEIDON)	30	Multicenter, randomized 1:1 (autologous <i>vs</i> allogeneic cells)	Three different doses of autologous or allogeneic bone marrow derived MSCs	Endo-myocardial injection	Chronic ischemic heart failure (LVEF ≤ 50%)	12 mo	Feasible and safe ≈ LVEF Autologous ↑ 6-min walk distance and QoL Allogeneic ↓ LVEDV

↑: Indicates increased; ↓: Indicates decreased; ≈: Indicates no change; MI: Myocardial infarction; MSCs: Mesenchymal stem cells; LVEF: Left ventricular ejection fraction; LVESV: Left ventricular end-systolic volume; QoL: Quality of life; CSCs: Cardiac stem cells; CABG: Coronary artery by-pass graft; CDCs: Cardiosphere-derived cells; LVEDV: Left ventricular end-systolic volume.

described the existence of a population of multipotent and clonogenic cells that expressed the tyrosine kinase receptor in their surface (c-kit⁺) and that were able to differentiate into the three cardiac lineages (cardiomyocytes, smooth muscle cells and endothelial cells)^[100]. Since then, strong evidence supported the concept that turnover in the adult cardiomyocyte population is provided by cardiac stem cells (CSCs)^[17,101], but the magnitude of this turnover and the exact underlying mechanisms remain unknown^[102]. CSCs are rapidly activated after myocardial injuries or physiological stimuli^[103-105]. Recently, Ellison *et al*^[106] have demonstrated in a murine model that some of the new cardiomyocytes generated after a myocardial injury are c-kit⁺ CSCs descendants. Nevertheless, this finding does not rule out other concomitant mechanisms that could be implicated in myocardial regeneration, and a recent report by van Berlo *et al*^[107] suggests that the new cardiomyocytes generated from CSCs are functionally insignificant. In fact, it is evident that self-renewal capacity of the adult human heart is unable to restore the large amount of cellular loss after MI.

Stem cell therapy with CSCs may offer some advantages over other extra-cardiac cells, because they are believed to be more prone to differentiate towards cardiac lineages. Most research has focused on transplanting CSCs in the infarcted area and various preclinical studies using this approach in different animal models have demonstrated the ability of CSCs to alleviate left ventricular dysfunction in both acute and chronic MI^[100,108-112].

These promising results warranted translation into

clinical research and led to the conduction of the first phase I clinical trial using c-kit⁺ CSCs, the SCIPIO trial^[113] (Table 1). This study reported that isolation and expansion of CSCs from cardiac tissue, harvested during coronary artery by-pass graft surgery, was feasible. Intracoronary injection of these cells in patients with left ventricular dysfunction (LVEF < 40%) after surgical revascularization improved LVEF by 12.3% compared with baseline, reduced scar size and was associated with improvements in quality of life and New York Heart Association functional class. These are preliminary data of the first eight patients enrolled in the study, which is still ongoing.

However the number of CSCs in adult hearts is small, and albeit their *ex vivo* isolation and expansion are feasible, their availability represents a major challenge. In 2004, Messina *et al*^[114] first described the *in vitro* formation of self-adherent multicellular spherical clusters from human surgical cardiac biopsies in culture, termed cardiospheres (CSs). These CSs were clonogenic and able to yield the three cardiac lineages *in vitro* and *in vivo* when transplanted in an animal model of MI, resulting in improvements in fractional shortening^[114]. CSs have a core of c-kit⁺ cells surrounded by progenitors in distinct stages of the differentiation process towards the three major cardiac lineages. They can also be obtained through percutaneous endomyocardial biopsy and used as source for cardiospheres-derived cells (CDCs), a mixed cell population that is clonogenic, express surface markers typical of stem cells (c-kit⁺, CD105⁺), negligible hematopoietic markers, and that can be expanded on fibronectin^[115]. This approach, described by Marbán *et al*^[115], has some advantages, *i.e.*,

the larger number of cells that can be obtained and the shorter time of manufacturing, in comparison to other methods to produce CSCs^[111,116,117]. In animal models of MI, human CDCs have been shown to promote cardiac regeneration and to improve LVEF^[115,118,119]. The same group conducted a direct comparison between CDCs and other extracardiac stem cells including BMMNCs, MSCs and adipose derived stem cells (ADSCs) in order to address their potency for myocardial repair. *In vitro*, CDCs showed a greater myogenic differentiation potency and higher angiogenic and paracrine potential. *In vivo*, injection of CDCs in infarcted mice provided the greatest functional benefit^[120].

This preclinical evidence led to the first phase I clinical trial using CDCs, the CADUCEUS trial (Table 1). This study randomized 25 patients with LVEF 25%-45%, two to four weeks after MI, to receive intracoronary autologous CDCs obtained from endomyocardial biopsies or standard of care. The primary safety endpoint was the proportion of patients who died due to ventricular tachycardia, ventricular fibrillation, or sudden unexpected death, or had MI after cell infusion, new cardiac tumor formation on MRI, or a major adverse cardiac event (MACE: Composite of death and hospital admission for HF or non-fatal recurrent MI). Preliminary efficacy endpoints on MRI parameters were also analyzed. Autologous CDCs treatment was safe and associated with reductions in scar size, increased viable myocardium and regional contractility. Nonetheless, ventricular volumes and LVEF did not differ between groups^[121,122], casting doubts about the true regenerative effect of CDCs.

Notwithstanding, both SCPIO and CADUCEUS trials included a small number of patients and there are some issues concerning their results, such as the low expression of cardiac markers in CSCs in the former, and the lack of benefit in terms of LVEF in the latter. Moreover, and although in both studies a true regeneration of damaged myocardium was invoked, the real underlying mechanism of action of these cells remains unproved.

Similarly to other extra-cardiac cell types, engraftment and retention rates of the transplanted CSCs and CDCs within the harsh environment of the infarcted myocardium are low, regardless of the administration route^[112,123]. Although direct differentiation of cardiac progenitor cells in cardiac lineages has been observed, this observation of low CSCs retention rates into the myocardium further supports the hypothesis of a paracrine effect, as another pathway responsible of the positive effect of the cells^[124].

In order to overcome the limited survival and retention of the transplanted cells, different strategies have been tested in animal models. Mohsin *et al.*^[125] used genetic manipulation to deliver the pro-survival gene Pim-1 kinase into human CSCs, resulting in a superior cellular engraftment and differentiation, that led to a superior reparative potential compared with conventional CSCs in an immuno-compromised

mice model of MI. Interestingly, no oncogenic issues were observed because telomere lengthening induced by Pim-1 overexpression was transitory^[126]. Another option for priming CSCs and make them less prone to apoptosis under hypoxic conditions is the preconditioning with cytoprotective pharmacological agents. For example, treatment of CSCs with cobalt protoporphyrin reduces oxidative stress-induced apoptosis, by up-regulation of heme oxygenase 1, COX-2, and anti-apoptotic proteins (BCL2, BCL2-A1, and MCL-1) and increased phosphorylation of NRF2^[127]. Hypoxic protection can also be achieved by means of β -O-linkage of N-acetylglucosamine to certain pathway proteins. Increases in this pro-survival signaling system by thiamet-G significantly improved CDCs survival after hypoxic injury^[128]. In addition, exposure of CSCs to H₂O₂ for 2 d before transplantation stimulates neoangiogenesis in the peri-infarct area following ischemic-reperfusion injury and could be a viable therapeutic option to prevent HF^[129]. CDCs engraftment and differentiation can also be enhanced by controlled release of FGF in ischemic myocardium in a pig model of MI, resulting in a significant functional improvement^[130].

Finally, previous reports suggest that one of the mechanisms by which MSCs promote cardiac repair is the stimulation of endogenous CSCs *via* paracrine effects^[131]. This finding raised the question whether combining both MSCs and CSCs could amplify the response to cell therapy. This hypothesis has been recently addressed by Williams *et al.*^[132], they found that intramyocardial delivery of human CSCs and bone marrow MSCs in a swine model of MI resulted in a 2-fold greater scar reduction and a 7-fold enhancement of engraftment of stem cells compared with each cell therapy alone, leading to improvements in hemodynamics and LVEF.

The discovery of CSC has revolutionized regenerative medicine. As they are derived from the target organ, they are supposed to be more committed with a cardiac fate, and the understanding of the molecular mechanisms that steer their mode of action is providing new insights that extend application of cell-based therapies. Early clinical experience with CSCs/CDCs warrants further investigation in larger phase II studies that will address the unresolved issues of SCPIO and CADUCEUS. At present, three clinical trials are evaluating the effect of CSCs and CSs in myocardial regeneration after MI: the RECONSTRUCT (NCT01496209), the ALLSTAR (NCT01458405) and the ALCADIA trials.

ALLOGENEIC STEM CELL THERAPY FOR CARDIAC REPAIR

Allogeneic stem cell therapy may offer many advantages in cardiac regenerative medicine. Firstly, allogeneic cells could be used as a scalable and reproducible cell product readily available "off-the-shelf"

that could be administered in the setting of primary PCI avoiding delays inherent to harvest and culture of autologous stem cells. Secondly, this type of cells can be obtained from young and healthy donors, thereby avoiding the aforementioned impairment in autologous stem cells functionality observed with advancing age and comorbidities.

Most research in allogeneic cardiac regeneration has been done with MSCs due to their immunoprivileged profile and immunosuppressive properties. MSCs lack expression of major histocompatibility class II antigens^[133], down-regulate T cells response through direct contact and secretion of anti-inflammatory cytokines^[134] and significantly affect the ability of dendritic cells to prime T-cell responses^[135]. These findings raised interest in MSCs for allogeneic stem cell transplantation. Nonetheless, recent data have challenged this assumption suggesting that under certain inflammatory environment and during their differentiation process, MSCs can switch their immune phenotype towards an immune-enhancing pattern, limiting their long term survival and benefits^[136,137]. However, and as pointed out before, autologous stem cell therapy faces the same problem, and if the advocated paracrine effect is responsible of stem cell regenerative capacity, survival and maintenance of cells in the transplanted heart could not be indispensable^[138].

There is a large body of preclinical work supporting the safety and the efficacy of allogeneic MSCs therapy^[84,139-142]. In a large animal model of MI in pigs, Amado *et al.*^[139] demonstrated that allogeneic MSCs injections in the necrotic area were feasible with no evidence of rejection and provided near-normalization of cardiac function and Quevedo *et al.*^[84] refrained these results in a swine model of chronic ischemic cardiomyopathy, where allogeneic MSCs showed to be able to differentiate in the three cardiac lineages and to improve LVEF. These findings supported the first-in-man clinical trial using allogeneic bone marrow-derived MSCs by Hare *et al.*^[143]. They conducted a randomized, double-blinded, placebo-controlled phase I trial in which 53 patients with reperfused MI were allocated to intravenous administration of allogeneic human MSCs or placebo. The study met its primary safety objective with no evidence of tumor formation or immunogenicity. It also suggested a greater benefit in cardiac function in larger MIs and improved outcomes regarding arrhythmias and pulmonary performance^[143]. The same group has recently conducted a head-to-head comparison between autologous and allogeneic MSCs in a phase I / II randomized pilot study involving 30 patients with chronic ischemic left ventricular dysfunction secondary to MI, the POSEIDON trial^[144] (Table 1). In this case, cell therapy was delivered by transendocardial injection. Both therapies were safe, with no or negligible allogeneic sensitization in the allogeneic group and no evidence of ectopic tissue formation. Both types of cells reduced infarct

size as measured by early enhancement defects in multidetector computed tomography, but no significant improvements in ejection fraction were documented. Intriguingly, allogeneic MSCs reduced LV end-diastolic volumes while autologous did not. Furthermore, improvements in quality of life, NYHA and 6-min walk test were more evident with autologous therapy. However, the small number of patients and the absence of a placebo group make these observations difficult to interpret. An interesting finding was the greater benefit regarding LV volumes and LVEF obtained with low-dose concentrations of MSCs vs high doses (20 million vs 200 million), which is consistent with preclinical data^[145] and warrants further investigation of the optimum dose before undertaking large clinical trials.

Mesenchymal precursor cells (MPCs) represent an immature subpopulation of bone marrow-derived cells that express the Stro3⁺ marker^[146]. MPCs are multipotent cells with an extensive proliferative and differentiation capacity as well as a great paracrine potential that outperforms the one of MSCs^[147,148]. They also display an immunoprivileged phenotype, appropriate for allogeneic transplantation. Previous animal studies have provided evidence on the positive effect of these cells in the setting of acute MI when delivered intramyocardially^[145,148,149] or by intracoronary infusion^[150]. Houtgraaf *et al.*^[150] have recently reported the benefit of the intracoronary infusion of different doses of allogeneic MPCs, when delivered few minutes after reperfusion in a sheep model of MI. The therapy was safe and provided reductions in infarct size *via* cardiomyocyte salvation and proliferation, and induced angiogenesis. It was also suggested that the smaller size of MPCs, compared to MSCs, prevents microvascular obstruction due to cellular aggregation observed in previous studies. Similarly to the findings of the POSEIDON trial, the benefit was more evident in the low-dose group (< 75 million vs 200 million), an observation which may be related to the better coronary flow observed after infusion with lower number of cells.

Recently, Penn *et al.*^[151] have tested a different bone marrow-derived cell product, named MultiStem, in 19 patients with acute MI in an allogeneic setting and using a novel delivery approach. Three different doses of cells were injected through a microneedle in the culprit vessel adventitia two to five days after primary PCI. The procedure was safe and LVEF was increased when compared to registry controls.

Another milestone in the field of allogeneic cell therapy is being carried out in the CAREMI clinical trial. This study represents the first-in-man experience with allogeneic CSCs in the acute setting of CHD. It is a phase I / II clinical trial that has been designed with a first dose-escalation phase and a second part with a randomized and controlled design. In this trial, 55 patients with acute reperfused MI and LVEF < 45% will be treated with intracoronary infusion of human

allogeneic CSCs, being the primary objective the rate of death from any cause at 30 d (dose-escalation phase) and MACE and magnetic resonance parameters at 12 mo (randomized phase).

In summary, allogeneic cell therapy represent a novel approach in cardiac regeneration that could overcome some of the strong barriers found by autologous cell products. Before large scaled trials are performed, some concerning issues as immunogenicity and rejection must be thoroughly examined.

OTHER SOURCES OF STEM CELLS

In contrast to adult stem cells, embryonic stem cells (ESCs) are pluripotent, *i.e.*, they are capable of differentiate into cells of the three germ layers. ESCs isolated from blastocysts demonstrate a great potential to generate functional cardiomyocytes and their transplantation into infarcted hearts in animal models have been evaluated showing improvements in LV function^[152,153]. Despite these findings, ESCs-based therapies face strong barriers that go beyond biological aspects, such as immune rejection or tumor formation. Ethical issues also remain a major limitation to the widespread use of these cells. So far, no clinical trial has tested ESCs in humans and is unlikely to be done.

Given these constraints, Takahashi and Yamanaka reported in 2006 the feasibility of generating induced pluripotent stem cells (iPSCs) from mouse adult fibroblasts by the transduction of four transcription factors that led to a phenotype similar to that of ESCs^[154]. This achievement could prevent the immunogenicity issues inherent to allogeneic ESCs application, but do not avoid oncogenic risks^[155]. On top of this, current reprogramming of somatic cells is a low efficient process that should be refined before translation to clinical trials. Therefore, nowadays, iPSCs represent a new source of cells that holds a great promise for reparative medicine but is not ready for their application in human beings yet.

ADSCs represent a population of stem cells located in the adipose tissue that are able to differentiate into multiple cell lineages including cardiomyocytes and vascular cells^[156]. They offer two major advantages over some previously mentioned types of cells: firstly, the easy and repeatable access that makes it possible to harvest large amounts of adipose tissue by a minimally invasive method and, secondly, their increased proliferative potential in culture^[157]. Preclinical reports have documented that administration of ADSCs after MI improves cardiac function^[158,159], in a range similar to BMMNCs^[159]. They have also been tested in 2 clinical trials, the APOLLO^[160] and the PRECISE trials^[161]. The APOLLO trial was a small randomized, double-blind, placebo controlled, phase I / II study designed to assess the safety and the feasibility of intracoronary infusion of ADSCs in patients with large ST-segment elevation acute MIs. The study proved the feasibility and the safety of both liposuction and ADSCs

intracoronary infusion after MI, and showed a trend towards improved cardiac function and reductions in scar formation^[160]. The PRECISE trial reported that intramyocardial injection of ADSCs in patients with refractory angina not amenable for revascularization ("no-option" patients) improved exercise capacity and myocardial perfusion, reduced scar size and preserved maximal oxygen consumption^[161]. However, the discovery of CSCs and new developments with MSCs and allogeneic therapies have somehow waned the initial interest in ADSCs.

STEMLESS APPROACHES

To conclude, stemless therapies to restore cardiac function after MI haven also been proposed. As outlined above, if stem cell benefits are mediated by a paracrine effect and by activation of self-repair mechanisms, exogenous administration of the cytokines and growth factors implicated in the regeneration process could theoretically replace cell transplantation with a readily and "off-the-self" available product, similarly to other current biological treatments. Nadal-Ginard *et al.*^[102] advocate this cell-free therapies. They have reported that intracoronary administration of IGF-1 and hepatocyte growth factor after coronary reperfusion reduced cardiac remodeling, induced myocardial regeneration and improved ventricular function in a pig model of MI. This was achieved by activation of c-kit⁺ endogenous CSCs, which expanded and generated new cardiomyocytes and microvessels^[162,163]. Other acellular approaches have tried to promote stem cell recruitment by the infarcted myocardium. Modulation of the CXC-chemokine receptor 4 and SDF-1 axis *via* gene therapy has shown to exert beneficial effects in preclinical studies and in human phase I trials^[164,165].

In spite of that, molecular, cellular and myocardial tissue regeneration mechanisms are highly complex and driven by the interplay of several factors, not yet completely understood. Therefore, it seems unlikely that the simple administration of one or two growth factors could be capable of inducing a full cardiac regeneration. Nevertheless, further research in this field is warranted in order to shed light on these mechanisms and to elucidate if stemless approaches are a feasible option in the future.

CONCLUSION

Regenerative therapies represent a novel paradigm in cardiovascular medicine, and have grown up over infancy and into adolescence. Translation of basic research and animal studies into the clinical scenario has never been so quick, driven by the enormous enthusiasm raised by the possibility of achieving heart regeneration. This initial euphoria has been dampened by some contradictory results and by the modest benefits observed in cardiac function, leading

to skepticism in a part of the scientific community. But these pioneering results should not be interpreted as the definite evidence in favor or against cell-based reparative therapies. Instead, they have established their safety and have shown the strong barriers that should be overcome, providing important insights about the source of cells to be used as well as about manufacturing processes and delivery routes. With all this background, the state-of-the-art in the field of stem cell therapy can be summarized as follows: (1) The molecular mechanisms that rule myocardial repair are highly intricate and still remain poorly understood. It is unlikely that the simple administration of "first-generation" stem cells or growth factors to the failing heart will be able to achieve a complete restoration of cardiac function; (2) As a result of a better knowledge of the molecular and genetic mechanisms that induce progenitor cell proliferation and differentiation into cardiac structures, a new generation of cell products ("second-generation" stem cells) are now being evaluated in large clinical trials after promising and encouraging results in phase I and II studies; and (3) Combining cellular, molecular and genetic basic research with preclinical studies and well-designed clinical trials, together with more collaborative research networks and with the definitive help of bioengineering, will be the keys for the definitive development of cardiac regenerative medicine.

Keeping all this in mind, the future of stem cell-based therapies is more promising than ever, and the goal of a true regeneration or repair of the damaged myocardium stands awaiting in the years to come.

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Mesenchymal stem cells as a therapeutic tool to treat sepsis

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targeting components of the derailed host response have failed. Therefore, there is a dramatic need for new and mechanistically alternative therapies to treat this syndrome. Based on their immunomodulatory properties, adult mesenchymal stem or stromal cells (MSCs) can be a novel therapeutic tool to treat sepsis. Indeed, MSCs reduce mortality in experimental models of sepsis by modulating the deregulated inflammatory response against bacteria through the regulation of multiple inflammatory networks, the reprogramming of macrophages and neutrophils towards a more anti-inflammatory phenotype and the release of anti-microbial peptides. This report will review the current knowledge on the effects of MSC treatment in preclinical experimental small animal models of sepsis.

Key words: Adult mesenchymal stem cells; Therapy; Sepsis

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Core tip: Sepsis remains as the most frequent cause of death in hospitalized patients and, therefore, new therapeutic alternatives are needed. Adult mesenchymal stem cells reduce mortality in experimental models of sepsis by modulating the deregulated inflammatory response against bacteria through the regulation of multiple inflammatory networks, the reprogramming of macrophages and neutrophils towards a more anti-inflammatory phenotype and the release of anti-microbial peptides. In this report we aim to provide a comprehensive snapshot of the potential clinical use of cell therapy with mesenchymal stem cells for sepsis.

Abstract

Sepsis is a clinical syndrome caused by a deregulated host response to an infection. Sepsis is the most frequent cause of death in hospitalized patients. Although knowledge of the pathogenesis of sepsis has increased substantially during the last decades, attempts to design effective and specific therapies

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INTRODUCTION

Sepsis is a clinical syndrome caused by a deregulated host response to an infection. Sepsis is the most frequent cause of death in hospitalized patients. Although knowledge of the pathogenesis of sepsis has increased substantially during the last decades, attempts to design effective and specific therapies targeting components of the derailed host response have failed. Sepsis will remain an important clinical problem in the future, especially in light of the ageing population and emerging antibiotic resistance. Therefore, there is a dramatic need for new and mechanistically alternative therapies to treat this syndrome. Based on their immunomodulatory properties, adult mesenchymal stem or stromal cells (MSCs) can be a novel therapeutic tool to treat sepsis. This report will review the current knowledge on the effects of MSC treatment in preclinical experimental small animal models of sepsis.

SEPSIS

Epidemiology

The incidence of sepsis varies between different reports, largely due to the use of different case definitions and diagnosis codes^[1,2]. Nevertheless, sepsis clearly is a leading cause of death, and the most frequent cause of death in non-coronary intensive care units (ICUs) in the developed world^[2]. In the United States the yearly incidence of severe sepsis is estimated at 300 cases per 100000 person-years population, which accounts for 10% of all ICU admissions^[3]. The incidence of severe sepsis was recently reported to increase^[4], although it is uncertain whether this signifies a true increase or altered coding and registration practices^[2,5]. Mayr *et al.*^[2] have recently reported that the mortality of severe sepsis and septic shock lies between 25%-50%, with the extent and number of organ failures as the strongest predictors of an adverse outcome. Notably, the case fatality rate for sepsis has declined in the past decade, most likely due to improved general care in the ICU^[6].

The most common sources of sepsis are in descending order pneumonia, intra-abdominal-, urinary tract- and soft tissue infections^[5]. Blood cultures are positive in only one third of cases, and up to a third of cases are culture negative from all body sites. The most commonly isolated Gram-positive bacterial pathogens are *Staphylococcus aureus* and *Streptococcus pneumoniae*, and the most common Gram-negative pathogens are *Escherichia coli*, *Klebsiella spp.*, and *Pseudomonas aeruginosa*^[7]. While Gram-positive infections had been reported as surpassing Gram-negative infections in recent years^[8], a recent study encompassing 14000 ICU patients in 75 countries found that 62% of positive isolates were Gram-negative bacteria, vs 47% Gram-positive and 19% fungal^[9].

Pathophysiology and host response

Sepsis occurs when the body's response to infection

injures the host's tissues and organs. The deregulated host response during sepsis entails both excessive proinflammatory and immune suppressive anti-inflammatory components^[7,10].

Immune cells can sense pathogens *via* so-called pattern-recognition receptors (PRRs), which recognize conserved motifs expressed by microorganisms called pathogen-associated molecular patterns or PAMPs^[7,11]. Four classes of PRRs have been identified: Toll-like receptors (TLRs), C-type lectin receptors, RIG-I-like receptors and NOD-like receptors^[11]. Activation of PRRs by PAMPs causes upregulation of inflammatory gene transcription and initiation of innate immunity, a response aimed at eliminating the invading pathogen. However, when bacteria overcome the ability of the innate immune system to clear the infection, resulting in progression to sepsis, the interactions between pathogens and PRRs advances into a deregulated response that no longer benefits the host. During such injurious host response inflammation can be perpetuated by stimulation of PRRs by so-called danger-associated molecular patterns (DAMPs or alarmins), which are endogenous molecules released by injured or dying cells^[12]. Alarmins are also released during sterile injury such as after trauma or severe pancreatitis, which contributes to the concept that the pathogenesis of multiple organ failure in sepsis and non-infectious critical illness is not fundamentally different^[5,13].

Cytokines are an important component of the "hyperinflammatory" response to severe infection. Experimental sepsis induced by systemic challenge with high bacterial doses is associated with enhanced release of multiple cytokines, and elimination or inhibition of several of these proinflammatory mediators [including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-12, IL-17, IL-18, interferon- γ , and macrophage migration inhibitory factor] improves survival in these models^[14]. However and importantly, these systemic challenge models do not adequately mimic the clinical syndrome of sepsis. Many trials evaluating the efficacy of proinflammatory cytokine inhibition, especially targeting TNF- α and IL-1, or other anti-inflammatory strategies have failed^[15]. Other proinflammatory mediators implicated in sepsis pathogenesis include high mobility group box 1 (HMGB1) and S100 proteins.

Activation of the complement systems forms a fundamental part of the innate immune response to infection^[16]. Sepsis is associated with systemic activation of the complement system, which can be harmful in the setting of fulminant sepsis. Indeed, neutralization or genetic absence of complement factor C5a and its receptors results in increased survival during abdominal sepsis or endotoxemia in mice. Other hallmark features of the sepsis host response include activation of the coagulation system and vascular dysfunction. The most severe manifestation of coagulopathy is the syndrome of disseminated intravascular coagulation, with an

estimated incidence between 30%-50% in severe sepsis, caused by tissue factor-driven activation of coagulation with concurrent impairment of anticoagulant and fibrinolytic mechanisms^[17]. Organ dysfunction in sepsis is at least in part caused by tissue hypoperfusion, secondary to hypotension, microvascular thrombosis and/or dysfunction of the vascular endothelium with loss of barrier function^[5]. Mitochondrial dysfunction and altered cellular bioenergetics have been implicated in sepsis-induced organ dysfunction, although further research is warranted to establish a causal relationship^[18].

While proinflammatory responses definitely contribute to sepsis pathogenesis, immune suppression is also a common feature in patients with sepsis^[7,10]. Autopsy studies have revealed strong deficiencies of splenocytes harvested from patients who had died of sepsis to produce cytokines upon stimulation^[19]. The mechanisms that underlie this phenomenon have not been fully elucidated, although likely anti-inflammatory cytokines, particularly IL-10 and transforming growth factor (TGF)- β , and inhibition of signalling by PRRs, partially due to epigenetic modifications of essential promoter regions, are involved. Moreover, apoptosis of immune cells has been implicated in immune dysfunction and mortality in sepsis^[10]. Most cells that undergo enhanced apoptosis in sepsis are of lymphoid origin (B cells, CD4 T cells), but also dendritic cells are affected. Preclinical studies have suggested that enhanced apoptosis of lymphocytes contributes to sepsis lethality^[10].

MSC

MSCs have emerged in recent years as therapeutic tools based on four important features: (1) differentiation potential; (2) capacity to modulate immune responses; (3) pro-angiogenic and repair promoting capacities; and (4) low immunogenicity; the latter feature may allow allogeneic treatments. MSCs have been found in a variety of adult tissues of mesodermal origin, such as bone marrow, adipose tissue, placenta, umbilical cord, dental pulp or synovium^[20-25]. Although sharing the main characteristics, differences between MSCs from different sources can be found, for instance at the RNA and protein expression profiles levels^[26-28]. MSCs are considered a promising tool for cell therapy, in particular for inflammatory diseases, based on their immunomodulatory properties and paracrine effects through trophic factors with anti-fibrotic, anti-apoptotic or pro-angiogenic properties^[29,30]. MSCs regulate the function of a broad range of immune cells^[30-37], and are activated by inflammatory mediators released from activated immune cells (*i.e.*, IFN γ , IL1 β and TNF α)^[38,39]. The mechanisms involved in the immunoregulatory activity of MSCs are still under investigation but rely on both cell contact-dependent mechanisms (*i.e.*, Jagged1-Notch1 interaction, Fas-Fas-L interaction)^[40,41] and

paracrine effects through the release of soluble factors including hepatocyte growth factor, prostaglandin-E2 (PGE2), TGF- β 1, nitric oxide (NO), IL-10, IL-6, heme oxygenase-1 (HO-1), HLA-G5 or the enzymatic activity of indoleamine 2,3-dioxygenase^[42]. In addition to the direct effect of these soluble factors, MSCs may also modulate immune responses through the generation of immune cells with regulatory phenotype, including regulatory T cells or anti-inflammatory macrophages^[43-45].

MSCs have also been reported to show antimicrobial activities against different pathogens upon activation with inflammatory cytokines^[46]. Noteworthy in the context of sepsis, the functionality of MSCs can also be modulated by activators of TLRs^[47]. It has been described that MSCs can be polarized *in vitro* towards either anti-inflammatory or pro-inflammatory phenotypes, depending on the TLR ligand time/concentration used for activation^[48]. Furthermore, it has been recently described that interaction of gastrointestinal bacteria (*Salmonella typhimurium* or *Lactobacillus acidophilus*) with MSCs increased their capacity to inhibit T lymphocyte proliferation *in vitro* through a PGE2-dependent mechanism, indicating that bacteria may also enhance the immunomodulating properties of MSCs^[49].

MSCs can sense inflammatory signals through the expression of cytokine/chemokine receptors and integrins, and subsequently migrate to sites of inflammation^[50]. Moreover, homing of systemically administered MSCs to lymphoid organs (draining lymph nodes and spleen) and the subsequent generation of functional Tregs have also been reported^[51-53]. MSCs do not long-term engraft at the inflammation site and cells seem to be cleared shortly after administration. This suggests that transient effects through soluble factors and cell-to-cell contacts play a main role in MSC-mediated initial controlling and balancing of local inflammation.

Allogeneic MSCs are regarded as a preferred source for treatment as they would allow treatment with a ready to use, off-the-shelf product, available for a large number of patients, specially, in acute life threatening indications like sepsis in which isolation and expansion of autologous MSCs is not an option. In that context, MSCs are considered immune privileged as they express constitutively only low levels of cell-surface HLA class I molecules and lack expression of HLA class II, CD40, CD80 and CD86 which would lead to reduced activation of the innate and adaptive immune responses^[54]. This immune privilege of MSCs therefore supports the feasibility of allogeneic treatments without the requirement of suppression of host immunity^[55,56]. However the immunogenic features of MSCs are currently under review as there is some evidence of immunogenicity in experimental animal models that coincides with immunomodulatory effects by MSCs^[57].

MSC IN EXPERIMENTAL MODELS OF SEPSIS

Sepsis being a disease that results as a consequence of deregulated inflammatory and immune responses against an infection can lead to tissue damage, multiorgan failure and death. Interest in investigating the therapeutic effect of MSCs on experimental models of sepsis emerged recently, and is based on their immunomodulatory properties^[58]. A description of the therapeutic effects of MSCs in experimental small animal models of sepsis and the mechanisms involved are described in the following sections. A summary of the data is provided in Table 1.

Experimental models of sepsis

In order to study sepsis pathophysiology, animal models of sepsis have been established. These models are normally used to preliminarily test potential therapeutic treatments prior to human clinical trials. On the basis of the initiating agent, sepsis models can be divided into three categories: toxemia models (exogenous administration of a bacterial toxin, such as lipopolysaccharide), bacterial infection models (exogenous administration of a bacteria) and host barrier disruption models (alteration of the animal's endogenous colonic protective barrier allowing bacterial leakage)^[59]. These experimental models have in common high inflammatory responses against endotoxins or bacteria, subsequent organ injury and failure and, as a consequence, high mortality rates within few hours or days. All models have contributed significantly to our understanding of sepsis pathophysiology, although no single one fully mimics the course of human disease. Two limitations of sepsis models compared to human disease are the timing of disease progression (the progression to multiorgan failure and death occurs in hours to days in most animal models, whereas in human sepsis this occurs in days to weeks) and lack of supportive therapeutic intervention (*i.e.*, intubation and mechanical ventilation, fluid therapy), in particular in small animal models. Therefore, extrapolation of efficacy results obtained in small sepsis animal models to the human disease has to be made with caution^[58,59].

The toxemia models involve the administration by intraperitoneal or intravenous injection of a bacterial toxin. Thus, a single injection of high dose LPS (normally 10-20 mg/kg) is the most commonly used toxemia model (LPS model). LPS administration induces a very rapid and transient increase in systemic cytokine levels, hypodynamic cardiovascular activity and a shock-like state. The injection of LPS may result within hours in high mortality rates that may vary with the dose, type of LPS, age and strain of animal. The bacterial infection models consist on an exogenous bacterial infection and the severity of the model may vary depending on the bacterial strain (*i.e.*, *Escherichia coli*, *Pseudomonas aeruginosa*) and route of infection

(intravenous, intraperitoneal, intratracheal) used. The clinical progression of the disease is rapid with hypodynamic cardiovascular state, high cytokine levels and progression towards death within hours. The host-barrier disruption models require the surgical disruption of the shielding barrier that protects sterile compartments from pathogens, allowing bacteria to spread. These models have become the most relevant sepsis models because they create a focus of infection that can disseminate throughout the body, mimicking the human situation. The caecal ligation and puncture model (CLP) is considered to be one of the most clinically relevant models for sepsis research. The model involves surgical ligation of the distal cecum with suture followed by one or two small punctures distal to the ligation. This allows the leakage of intestinal content into the peritoneal cavity, which results in polymicrobial sepsis (several bacterial species can be found in the blood and other organs of CLP animals). Technical variations (needle size and number of punctures) can influence the severity of the CLP model (mortality within hours or several days)^[59,60].

Effect of MSC treatment on mortality and organ injury induced by sepsis

The therapeutic effect of MSC treatment has been tested using different sepsis animal models, MSC types, dosing, timing and routes of administration. These studies have consistently reported improvement on survival rates of animals treated with MSCs (Table 1). In mice, one single dose of between 3×10^5 and 10^6 MSCs administered by intraperitoneal, intravenous or intratracheal route was able to significantly reduce sepsis-related mortality in LPS, CLP, *P. aeruginosa* peritonitis and *E.coli* pneumonia mouse models^[52,61-68]. Similar therapeutic effects have been observed using autologous, allogeneic or xenogeneic MSCs^[52,61]. Noteworthy, treatment with fibroblasts has not been reported to increase survival of septic mice, despite the shared immunomodulating properties of fibroblasts with MSCs^[61,63,64].

The effects on survival might depend on the dose (low/high, one/multiple) and the timing of administration (early/late after insult). Gonzalez-Rey *et al.*^[52] reported that one dose of 10^6 human ASCs administered intraperitoneally 30 min after LPS injection in mice had a higher protective effect on mortality than one dose of 3×10^5 cells. Mei *et al.*^[62] found that intravenous administration of one dose of 2.5×10^5 mouse BM-MSCs after 6 h of CLP did not significantly protect mice, unless MSCs were administered concomitantly with antibiotics. These results might be related to different experimental settings because, compared to other studies carried out in CLP mouse models, Mei *et al.*^[62] administered a lower dose of MSCs (2.5×10^5 vs 10^6 cells) and at a later time point (6 h after CLP vs a range between 24 h before and 4 h after CLP)^[52,61,66,68]. On the other hand, Hall *et al.* reported that three intravenous

Table 1 Efficacy preclinical studies on experimental models of sepsis using mesenchymal stem cells, mesenchymal stem cell-conditioned medium or mesenchymal stem cell-derived macrophages

Animal model	MSC type	Route/time	Dose	Number of doses	Therapeutic effects					MoA	Ref.
					Survival	Cytokines	Inflammatory infiltration	Organ injury	Bacterial load		
LPS, mouse	hASCs (xeno)	I.P./after 0.5 h or I.P./after 0.5 h	3×10^5 10^6	1 1	Improved Improved	Reduced pro-inflammatory cytokines in serum, liver, lung and intestine Increased anti-inflammatory cytokine (IL10) in liver, lung and intestine	Reduced lymphocyte, neutrophil and macrophage infiltration in peritoneum, liver, lung and intestine	ND	ND	ND	Gonzalez-Rey <i>et al</i> ^[23]
LPS, mouse	hBM-MSCs (xeno) normal/ senescent	I.P./after 0.5 h	10^6	1	Improved only by normal cells	Reduced pro-inflammatory cytokines in serum and lungs (normal cells) Reduced anti-inflammatory cytokine (IL10) in serum (normal cells)	ND	ND	ND	ND	Sepúlveda <i>et al</i> ^[61]
LPS, mouse	hASC/BM-MSC-CM	I.P./at 0 h	1 mL CM (from 2×10^6 cells per milliliter)	1	Improved only by hBM-MSC CM	ND	Reduced neutrophil infiltration in kidney (hBM-MSC CM)	Improved kidney, liver and lung damage (hBM-MSC CM)	ND	ND	Elman <i>et al</i> ^[63]
LPS, rat	hASCs (xeno)	I.V./after 0.5 h	2×10^6	1	ND	Reduced pro-inflammatory cytokines in lung No effect on anti-inflammatory cytokine (IL10)	ND	Improved kidney, liver and lung damage	ND	ND	Shin <i>et al</i> ^[73]
LPS, rat	hBM-MSCs (xeno)	I.M./at 0 h	2×10^6	1	ND	ND	ND	Improved kidney, liver and lung damage	ND	ND	Yagi <i>et al</i> ^[73]
LPS, rat	hBM-MSCs (xeno)	I.M./at 0 h	2×10^6	1	ND	Reduced pro-inflammatory cytokines in serum	Reduced neutrophil and macrophage infiltration in kidney, liver and lung	ND	ND	MSC release of sTNFR1	Yagi <i>et al</i> ^[76]
LPS, rat	mBM-MSCs (xeno)	I.P./after 1 h	2×10^6	1	ND	Reduced pro-inflammatory cytokines in serum and myocardium Increased anti-inflammatory cytokine (IL10) in serum but not in myocardium	ND	Improved myocardial damage Cells from female donors showed higher effect	ND	Higher expression of anti-apoptotic proteins in myocardium	Manukyan <i>et al</i> ^[71]
LPS, rat	mBM-MSCs (xeno)	I.P./after 1 h	2×10^6	1	ND	Reduced pro-inflammatory cytokines in serum and myocardium Increased anti-inflammatory cytokine (IL10) in serum but not in myocardium	ND	Improved myocardial damage	ND	ND	Weil <i>et al</i> ^[72]
LPS, rat	rBM-MSCs (auto)	I.V./after 1 h	2.5×10^6	1	ND	Reduced pro-inflammatory cytokines in serum and myocardium Increased anti-inflammatory cytokine (IL10) in serum but not in myocardium	ND	Improved myocardial damage	ND	ND	Weil <i>et al</i> ^[74]

CLP, mouse	hASCs (xeno) mASCs (auto/allo)	I.P./after 4 h	10 ⁶	1	Improved	Reduced pro-inflammatory cytokines in serum, liver, lung and intestine Increased anti-inflammatory cytokine (IL10) in liver, lung and intestine	Reduced lymphocyte, neutrophil and macrophage infiltration in peritoneum, liver, lung and intestine	ND	ND	ND	Gonzalez-Rey <i>et al.</i> ^[52]
CLP, mouse	mBM-MSCs (auto/allo)	I.V./24 h prior or I.V./after 1 h	10 ⁶ 10 ⁶	1 1	Improved Improved	Reduced pro-inflammatory cytokines in serum Increased anti-inflammatory cytokine (IL10) in serum	Reduced neutrophil infiltration in peritoneum, liver and kidney	Improved kidney, liver, pancreatic and spleen damage and vascular permeability	Reduced bacterial counts in blood	Anti-inflammatory Mph (IL10) induced by MSCs through PGE2	Németh <i>et al.</i> ^[61]
CLP, mouse	mBM-MSCs (auto)	I.V./after 6 h	2.5 × 10 ⁵	1	Improved	Reduced pro-inflammatory cytokines in serum and BAL No effect on anti-inflammatory cytokine (IL10) in serum and BAL	Reduced neutrophil infiltration in peritoneum, liver and kidney	Improved kidney and lung damage No effect on liver and pancreatic damage	Reduced bacterial counts in spleen	Increased phagocytic activity of macrophages and neutrophils	Mei <i>et al.</i> ^[62]
CLP, mouse	mBM-MSCs (auto)	I.V./after 2 h and I.V./after 24 h and I.V./after 48 h	5 × 10 ⁵ 2.5 × 10 ⁵ 2.5 × 10 ⁵ total: 10 ⁶	3	Improved	ND	Reduced neutrophil infiltration in bowel	Improved bowel, kidney, liver and spleen damage	Reduced bacterial counts in peritoneum and blood	Increased phagocytic activity of neutrophils	Hall <i>et al.</i> ^[64]
CLP, mouse	mBM-MSCs (auto)	I.V./after 3 h	10 ⁶	1	Improved	Reduced pro-inflammatory cytokines in serum Increased anti-inflammatory cytokine (IL10) in serum	Reduced neutrophil infiltration in kidney	Improved kidney damage	Reduced bacterial counts in blood	ND	Luo <i>et al.</i> ^[66]
CLP, mouse	ASC-derived mouse Mph	I.P./after 4 h or I.P./after 6 h or I.P./after 12 h or I.P./after 24 h	10 ⁶ 10 ⁶ 10 ⁶ 10 ⁶	1 1 1 1	Improved Improved Improved No effect	Reduced pro-inflammatory cytokines in serum (only treatment at 4 h tested)	Reduced lymphocyte, neutrophil and macrophage infiltration in peritoneum, lung, liver and intestine (only treatment at 4 h tested)	ND	ND	IL10 secreted by Mph	Anderson <i>et al.</i> ^[77]
CLP, mouse	hUC-MSCs (xeno) wt/Poly I:C preactivated	I.V./after 1 h	10 ⁶	1	Improved Better preactivated	Reduced pro-inflammatory cytokines in plasma Better preactivated	Reduced inflammatory infiltration in kidney, liver and lung	Improved kidney, liver damage and pancreatic damage Better preactivated	Reduced bacterial counts in peritoneum and blood Better preactivated	Poly I:C inhibition of MMR-143 expression by MSCs	Zhao <i>et al.</i> ^[68]
CLP, rat	rASCs (auto) living/apoptotic	I.P./after 0.5 h and I.P./after 6 h and I.P./after 18 h	1.2 × 10 ⁶ 1.2 × 10 ⁶ 1.2 × 10 ⁶ total: 3.6 × 10 ⁶	3	Higher mortality by living cells Improved by apoptotic cells	Reduced TNFα (apoptotic rASC treated rats)	ND	Improved kidney, liver, lung and myocardial damage (apoptotic rASCs)	ND	ND	Chang <i>et al.</i> ^[69]

<i>E.coli</i> pneumonia, mouse	hBM-MSCs (xeno)	I.T/after 4 h	10 ⁶	1	ND	Reduced pro-inflammatory cytokines in bronchoalveolar liquid (BAL)	Reduced neutrophil infiltration in BAL	Improved lung epithelial and endothelial permeability	Reduced bacterial counts in BAL	MSC release of the antimicrobial peptide LL-37	Krasnodems-kaya <i>et al</i> ^[70]
<i>P.aeruginosa</i> peritonitis, mouse	hBM-MSCs (xeno)	I.V/after 1 h	10 ⁶	1	Improved	No changes in serum or peritoneal fluid levels of pro or anti-inflammatory mediators	ND	ND	Reduced bacterial counts in peripheral blood, peritoneal fluid, lung, and spleen	Increased phagocytic activity of macrophages	Krasnodems-kaya <i>et al</i> ^[65]

LPS: Lipopolysaccharide; CLP: Caecal ligation and puncture; h: Human; r: Rat; m: Mouse; ASCs: Adipose mesenchymal stem cells; BM-MSCs: Bone marrow mesenchymal stem cells; UC-MSCs: Umbilical cord mesenchymal stem cells; Mphi: Macrophages; I.P: Intraperitoneal; I.V: Intravenous; I.T: Intratracheal; I.M: Intramuscular; ND: Not determined; CM: Conditioned medium.

administrations of 5×10^5 , 2.5×10^5 and 2.5×10^5 mouse BM-MSCs at 2, 24 and 48 h after CLP, respectively, also reduced mortality rates on mice, although they did not compare with the effects of one single dose^[64]. Unfortunately, so far, different timing of administration have only been compared in the same study by Németh *et al*^[61] who reported that a prophylactic intravenous treatment with mouse BM-MSCs 24 h prior to CLP had similar therapeutic effects than a therapeutic treatment after 1 h of CLP. However, due to the urgent and acute condition of sepsis, a prophylactic treatment is clinically not feasible and comparing in the same study single vs multiple dosing and different time regimens (early or late after the induction of sepsis) might be very important in order to understand if there is a time window in which MSC

treatment is most efficacious and, therefore, maximize the therapeutic benefit of MSC therapy.

In addition to the dose and time, the “fitness” of the cells at the time of administration might also affect the therapeutic effect of MSCs. Thus, Sepúlveda *et al*^[67] found that while intraperitoneal administration of normal human BM-MSCs 30 min after LPS injection reduced mortality in an LPS sepsis model in mice, senescent human BM-MSCs failed to protect them, despite the fact they conserved the capacity to modulate the function of lymphocytes and macrophages *in vitro*. However, in contrast to these positive results, Chang *et al*^[69] reported that three intraperitoneal administrations of 1.2×10^6 living rat ASCs (at 0.5, 6 and 18 h) did not reduce, but moderately increased, mortality in a rat model of CLP, whereas the administration of apoptotic rat ASCs protected rats from death. Further studies will be needed to better understand these results.

The effects of MSC treatment on survival are a consequence of the reduction of inflammation-associated organ injury and the improvement in organ function. MSC treatment has been reported to reduce damage in kidney (*i.e.*, reduced levels of apoptotic cells, serum creatinine and tubular injury score), liver (*i.e.*, reduced levels of apoptotic cells, serum liver enzymes and blood urea nitrogen), pancreatic (*i.e.*, reduced levels of serum amylase), spleen (*i.e.*, reduced levels of apoptotic cells), lung (*i.e.*, reduced levels of apoptotic cells and vascular leakage) and heart function (*i.e.*, improved cardiac depression) in a variety of sepsis models and experimental settings^[61,62,64-66,68-75]. Improvement in organ damage correlates with reduction on neutrophil infiltration and myeloperoxidase (MPO) activity in target organs^[52,61]. Notably, these effects can also be obtained by intraperitoneal administration of conditioned medium from BM-MSCs or ASCs in a LPS mouse model, suggesting that the therapeutic effects of MSCs might be mediated, at least in part, by soluble factors^[65]. Of note, Manukyan *et al*^[71] observed that female mouse BM-MSCs injected intraperitoneally had a better improvement of cardiac function of LPS septic rats than male mouse BM-MSCs. This effect correlated with a higher expression of the anti-apoptotic protein Bcl-XL in the myocardium of female MSC treated rats.

No specific side effects of MSC treatment have been reported in sepsis models (only Chang *et al*^[69] reported increased mortality when using living rat ASCs compared to the untreated group in a rat model of CLP). The fate of MSCs in sepsis models have been also investigated in some studies. When MSCs were administered intravenously, cells were always detected in the lungs and eventually, to a lesser extent, in spleen, liver, kidney or lymph nodes^[61,66,74]. When MSCs were administered

intramuscularly, cells were only detected in the muscle up to 24 h after administration^[76].

Effects of MSCs on inflammation induced by sepsis

The pathogenesis of sepsis is characterized by massive infiltration of immune cells in target organs and high pro-inflammatory cytokine levels systemically and locally, that can lead to tissue damage, multiple organ failure and death. Treatment with MSCs reduces the infiltration of neutrophils and monocyte/macrophages to target organs, including liver, lung, intestine and kidney^[52,61,62,64-66,68,70,76]. Furthermore, MSC treatment has also been reported to reduce the levels of proinflammatory cytokines (*i.e.*, IFN γ , TNF α , IL1 β or IL6) in several organs including serum, liver, lung, intestine and myocardium^[52,61,66-68,70-72,74,76]. These anti-inflammatory effects can be enhanced by preactivation of UC-MSCs with Poly I:C which results in the inhibition of miR-143 expression by MSCs^[68]. The reduction on the levels of anti-inflammatory cytokines was accompanied by the increase on the levels of the anti-inflammatory cytokine IL10^[52,61,66-68,70-72,74,76], although other authors have reported either no effect on IL10 levels or even a reduction^[62,63,67,75]. These differences might be related to differences in the experimental settings, such as the use of different animal models, MSCs, dosing and time of sample collection. Nevertheless, there is evidence that IL10 plays an important role in the therapeutic effects of MSCs in sepsis. Thus, injection of a neutralizing antibody against IL10 or IL10 receptor prior to CLP abrogated the therapeutic effects of mouse BM-MSCs^[61]. *In vitro* studies showed that IL10 was not directly produced by MSC, but by macrophages through a mechanism that required MSC-secretion of PGE2^[61,77]. Moreover, a role of IL10 in inhibiting the migration of neutrophils into the infected tissues has also been suggested^[61]. In addition to IL10, other mediators of the therapeutic effect of MSCs have been identified. Thus, Yagi *et al.*^[73] observed that blockade of sTNFR1, which is released by MSCs in response to inflammation, partially impaired the anti-inflammatory effects of MSC treatment.

The MSC-mediated reprogramming of macrophages towards a regulatory and anti-inflammatory M2 phenotype has also been reported in sepsis models by other authors. Krasnodemskaya *et al.*^[63] observed a larger population of monocytes expressing CD206 (a marker of alternative activated M2 macrophages) in the spleen of MSC-treated mice and a higher phagocytic capacity of blood monocytes. Furthermore, Anderson *et al.*^[77] provided strong evidence of the important role that MSC-induced regulatory macrophages play in the therapeutic effects of ASCs in sepsis. The authors generated "ASC-mediated regulatory macrophages" (ASC-Mph) by *in vitro* culture of mouse bone marrow macrophages and ASCs (either mouse or human) and injected 10⁶ ASC-Mph intraperitoneally in septic mice

at different time points after CLP. These treatments resulted in reduced mortality rates when ASC-Mph were administered between 4 h and 12 h (but not at 24 h) after CLP by a mechanism that required the production of IL10 by ASC-Mph^[77]. Moreover, these regulatory ASC-Mph also reduced levels of pro-inflammatory cytokines in serum and infiltration of inflammatory cells in the peritoneum, lung, liver and intestine. Finally, the relevance of monocytes/macrophages, but also neutrophils, in mediating the therapeutic effects of MSCs is highlighted by the fact that depletion of monocyte/macrophages (by using clodronate-filled liposomes) or neutrophils (by using anti-Ly6G antibody) completely abrogated the protective effects of MSCs *in vivo*^[61,64].

The effects of MSC treatment on transcriptional inflammatory pathways in target organs of CLP septic mice treated with MSCs have been investigated by microarray analysis of total RNA expression. The results show that MSC treatment affects an ample range of transcriptional networks (it was estimated that up to a 13% of total murine genome was transcriptionally reprogrammed after MSC treatment compared to control septic mice including: (1) downregulation of TLR, NF- κ B or IL6 signaling pathways; (2) upregulation of NF-AT-related genes; (3) upregulation of genes involved in phagocytosis, antigen presentation, bacterial killing, coagulation, complement regulation and platelet activation; and (4) upregulation of genes involved in cell-to-cell interaction and endothelial/vascular integrity^[62,78].

Effect of MSCs on bacterial burden in sepsis

The mechanism by which MSCs protect from sepsis is not only limited to reducing the production of inflammatory cytokines and migration of inflammatory cells to infected organs, but also includes direct anti-microbial properties, as well as the improvement of the phagocytic properties of monocyte/macrophages and neutrophils. Gonzalez-Rey *et al.*^[52] and Németh *et al.*^[61] first reported a reduction on bacterial load in target organs (*i.e.*, peritoneal cavity, blood, spleen or liver) in MSC-treated septic mice, despite the MSC-mediated reduction of the inflammatory response. Krasnodemskaya *et al.*^[70] determined that MSC have intrinsic anti-microbial activity because they secrete the anti-microbial peptide LL-37 in response to the stimulation with *Escherichia coli* or *Pseudomonas aeruginosa*. Intratracheal administration of human BM-MSCs in a mouse pneumonia model highly reduced bacterial counts in bronchoalveolar lavage (BAL). However, when a LL-37 neutralizing antibody was also administered to mice, the anti-microbial effects of MSCs were only partially lost, suggesting that additional anti-microbial mechanisms might be involved. This potential direct killing of bacteria by MSCs needs to be further confirmed as Gonzalez-Rey *et al.*^[52] did not observe direct killing of *Escherichia coli* by MSCs *in vitro* in the absence of other cells.

In addition, the enhancement of the phagocytic properties of monocyte/macrophages and neutrophils have also been reported to improve bacterial clearance by MSCs. Noteworthy, MSCs seem not to have the capacity to phagocyte bacteria *in vitro*^[62,64]. Mei *et al*^[62] found that MSC treatment in a mouse CLP model increased the phagocytic capacity of peritoneal and spleen CD11b positive cells (mainly monocyte/macrophages and neutrophils) in MSC treated mice. Krasnodembskaya *et al*^[63] observed a reduction on bacterial counts in several organs, but more significantly in peripheral blood of MSC treated mice infected with *Pseudomonas aeruginosa*, which was also associated to an increased capacity of peripheral blood monocytes to phagocyte bacteria. Hall *et al*^[64] determined that MSCs, but not fibroblasts, also enhanced the phagocytic properties of neutrophils *in vitro* and in a CLP mouse model. In fact, depletion of neutrophils *in vivo* abrogated the ability of MSCs to promote bacterial clearance^[64]. Notably, Németh *et al*^[61] noticed that while infiltration of neutrophils to target organs was inhibited in MSC treated mice, their presence in circulation was concomitantly increased and suggested that this mechanism might help to clear bacteria from circulation and minimize organ injury due to leukocyte infiltration. Interestingly, preactivation with Poly I:C increased the *in vivo* anti-microbial effects of UC-MSCs in a CLP mouse model through a mechanism that requires the inhibition of the expression of miR-143^[68].

CONCLUSION

Sepsis is a leading cause of death and the most frequent cause of death in non-coronary ICUs in the developed world and, despite improvement in treatments, the mortality of severe sepsis and septic shock remains very high, showing that current treatments are not sufficient to combat this syndrome. The use of MSCs in experimental animal models of sepsis has reported strong evidence of the therapeutic potential of MSC therapy in this indication. These studies have been mainly focused on the effects of MSCs on the pro-inflammatory phase of sepsis, while the effects of MSCs on the subsequent anti-inflammatory/immune exhaustion phase of the disease has not been elucidated so far and will need further investigation. The mechanisms by which MSCs improve survival in sepsis models rely on the collective effects of their immunomodulatory and anti-microbial properties: MSC treatment modulates inflammation in septic mice by a mechanism that requires the reprogramming of macrophages towards a more anti-inflammatory phenotype (release of anti-inflammatory IL10), resulting in reduced levels of pro-inflammatory cytokines in blood and organs and attenuated infiltration of immune cells in infected tissues (monocytes and neutrophils). Moreover, MSCs show direct (release of LL-37 peptide) and indirect (increase of phagocytic properties of

monocyte/macrophages and neutrophils) anti-microbial effects. The combined effect of reducing both the inflammatory response and the bacterial burden results in an improvement of organ function and higher survival rates. The promising results obtained in these, small animal, preclinical efficacy studies are encouraging and suggest that MSCs might be a therapeutic option to treat sepsis in patients. Importantly, efficacy of MSCs in large animal models that better replicate the inflammatory response, organ failure and disease in humans (e.g., sheep models) will be additionally relevant to support further testing of the therapeutic potential of allogeneic MSC treatment in humans. Such clinical trials should be prospective, controlled, and randomized so to guarantee a clear outcome of the MSC treatment effect. Moreover, taking into consideration the complexity and heterogeneity of sepsis and the poor results up to now in sepsis clinical trials, we believe that such trials should first be done in well defined and homogeneous sepsis patient populations.

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Transplantation of stem cell-derived astrocytes for the treatment of amyotrophic lateral sclerosis and spinal cord injury

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Abstract

Neglected for years, astrocytes are now recognized to fulfill and support many, if not all, homeostatic functions

of the healthy central nervous system (CNS). During neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI), astrocytes in the vicinity of degenerating areas undergo both morphological and functional changes that might compromise their intrinsic properties. Evidence from human and animal studies show that deficient astrocyte functions or loss-of-astrocytes largely contribute to increased susceptibility to cell death for neurons, oligodendrocytes and axons during ALS and SCI disease progression. Despite exciting advances in experimental CNS repair, most of current approaches that are translated into clinical trials focus on the replacement or support of spinal neurons through stem cell transplantation, while none focus on the specific replacement of astroglial populations. Knowing the important functions carried out by astrocytes in the CNS, astrocyte replacement-based therapies might be a promising approach to alleviate overall astrocyte dysfunction, deliver neurotrophic support to degenerating spinal tissue and stimulate endogenous CNS repair abilities. Enclosed in this review, we gathered experimental evidence that argue in favor of astrocyte transplantation during ALS and SCI. Based on their intrinsic properties and according to the cell type transplanted, astrocyte precursors or stem cell-derived astrocytes promote axonal growth, support mechanisms and cells involved in myelination, are able to modulate the host immune response, deliver neurotrophic factors and provide protective molecules against oxidative or excitotoxic insults, amongst many possible benefits. Embryonic or adult stem cells can even be genetically engineered in order to deliver missing gene products and therefore maximize the chance of neuroprotection and functional recovery. However, before broad clinical translation, further preclinical data on safety, reliability and therapeutic efficiency should be collected. Although several technical challenges need to be overcome, we discuss the major hurdles that have already been met or solved by targeting the astrocyte population

in experimental ALS and SCI models and we discuss avenues for future directions based on latest molecular findings regarding astrocyte biology.

Key words: Neuroprotection; Stem cell; Cell therapy; Astrocyte; Transplantation; Amyotrophic lateral sclerosis; Spinal cord injury

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Core tip: Amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI) result in incurable neurological dysfunction due to loss of spinal motor neurons and axonal degeneration, amongst other mechanisms. Astrocytes are increasingly recognized as being necessary for neuroprotection and regeneration in the central nervous system as they promote axonal growth and deliver essential neurotrophic factors under both physiological and pathophysiological conditions. Given the central role played by astrocytes, we gathered convincing results from ALS and SCI literature that argue in favor of stem cell-based astrocyte replacement therapies and stress the scientific community to investigate more deeply the molecular understanding of astrocyte biology.

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MULTIPLE FACETS OF ASTROCYTES IN THE CENTRAL NERVOUS SYSTEM

Astrocyte functions

Astrocytes are the most abundant cells in the central nervous system (CNS), outnumbering neuronal cells by several fold in some CNS regions. They have long been relegated to a secondary position, behind neurons or oligodendrocytes, as many thought that astrocytes were barely by-standers of the CNS. Accounting for a large fraction of the brain volume, their particular shape and tissue distribution are known to elaborate an extensive network of fine interconnected processes. Their star-shaped morphology projecting long branched processes provides a large coverage of CNS structures and the ensheathment of the brain or spinal cord in the pia matter. They draw the whole brain microanatomy by secreting astrocyte-derived extracellular matrix proteins (*e.g.*, chondroitin sulfate proteoglycans, hyaluronan, tenascin proteins family, thrombospondin), thereby providing structural support for nervous system cells. Beside their structural role, astrocytes are recognized to fulfill and support many, if

not all, functions of the healthy CNS^[1]. Astrocyte end-feet cover more than 90% of the CNS vasculature and come in contact with endothelial cells. Expressing key glucose transporter-1, astrocytes convey glucose from blood vessels to nervous system cells, most of them being devoid of direct access to this high-end source of energy. Hence, astrocytes synthesize *via* specific metabolic pathways glycogen and lactate, main energy fuels for neurons or distant synapses. Through humoral factors released at the perivascular space, astrocytes control local cerebral blood flow and blood-brain barrier (BBB) integrity. Transforming growth factor-beta, glial-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2) and angiopoietin 1 (binding the endothelium-specific receptor TIE2), all secreted at the vascular end-feet, act on endothelial cells in order to induce or maintain an operational BBB^[2,3]. Astrocytes-released growth factors [*e.g.*, brain-derived neurotrophic factor, BDNF; ciliary neurotrophic factor (CNTF)] exert beneficial effects far beyond the perivascular space and act as neurotrophic factors on all CNS cells^[4,5]. The perivascular end-feet of astrocytes show other specialized features characteristic of this location, including a high density of orthogonal arrays containing the water channel aquaporin 4 (AQP4) and the Kir4.1 K⁺ channel^[6,7]. Those proteins closely interact with endothelial basal lamina and regulate ions/water fluxes through the BBB^[7-10]. Astrocytes finely tune the dynamics of cerebral blood flow in order to increase the availability of oxygen and glucose locally to highly active brain areas; this process is mediated via secreted vasoactive compounds (*i.e.*, endothelin-1, arachidonic acid, prostaglandins, nitric oxide) and intercellular Ca²⁺ signaling^[11-13]. As a member of the tripartite synapse, astrocytes are responsible for proper neurotransmission. Astrocyte end-feet express Kir4.1 channel, which is responsible for the rapid uptake of K⁺ released in the interstitial CNS fluid and thus for spatial extracellular potassium siphoning *needed* during high-frequency firing of neurons^[14-16]. Astrocytes also express membrane-bound transporters and receptors involved in the re-uptake and the response to neurotransmitters (*i.e.*, glutamate, GABA, glycine), whose synaptic concentrations must be tightly controlled in the synaptic cleft. In the case of glutamate homeostasis, two major CNS glutamate transporters, GLT1/EAAT2 and GLAST/EAAT1, are expressed almost exclusively by astrocytes in the adult mammals. GLT1 accounts for upward of 90% of glutamate uptake in most CNS regions^[17]. By regulating extracellular glutamate homeostasis, GLT1 assures proper synaptic function and prevents excitotoxic insult to susceptible neurons, axons and oligodendrocytes. In addition, astrocytes form a gap junction-coupled network of very narrow overlapping domains throughout the CNS^[18,19]. Each domain covers a CNS area encompassing from 20000 neuronal synapses in rodents up to 2000000 in humans and connects neighboring astrocytes by

junctional complexes made of connexin (Cx) family proteins. On a functional point-of-view, the astrocyte syncytium supports ATP-mediated long-distance propagation of Ca^{2+} waves^[20,21], which is involved in the control of local cerebral blood flow and the fine tuning of electrical neuronal activity^[22]. Cx channels allow the intercellular passage of monovalent ions (Na^+ , K^+) or small molecules up to 1.5 kDa (e.g., ATP, glutamate, d-serine), contributing to signaling, metabolic cooperation and ionic spatial buffering^[23,24]. Astrocytes express Cx26, Cx30 and Cx43 that interact with homologous connexins in astrocyte-to-astrocyte (A/A) gap junctions and that can also bind in a heterologous fashion Cx29, Cx32 and Cx47 on oligodendrocytes partners^[24-26]. In mammals at least, the presence of these 6 connexins suggests a high level of complexity in coupling partners within the glial cell populations and reveals close cellular interactions between astrocytes and oligodendrocytes. Astrocyte-oligodendrocyte (A/O) coupling via connexins is necessary to sustain myelin formation and maintenance throughout life. In humans, mutations in Cx29, Cx32 and Cx47 genes lead to myelin formation abnormalities and are linked to Charcot-Marie-Tooth disease and several forms of leukodystrophies^[27]. Beyond mutations targeting oligodendroglial connexins, the effect of loss-of-function of astrocyte connexins has been experimentally investigated using knockout mice. Double Cx30^{-/-} and Cx43^{-/-} knockout mice showed white matter pathology comprising vacuolated oligodendrocytes and intramyelinic edema^[28]. Histopathological changes were accompanied by significant sensorimotor and cognitive deficits. Similar findings were shown when double-deleting Cx43 and Cx32 in mice^[29]. All of these findings suggest an essential role of A/A and A/O coupling in maintaining overall CNS functions^[30] and pave the way for developing integrated therapies targeting the astrocyte syncytium and its dysfunction(s) during neurodegenerative conditions.

Wealth through diversity

A rapid look at the morphology of white matter astrocytes compared to gray matter astrocytes reveals the complexity and heterogeneity of this class of cells. Their different morphologies are most likely to be related with their wide range of functions, their neuroanatomical sites and the stem cells from which they derive (reviewed by^[31]). During development, astrocytes mainly arise from radial glial cells located in the brain and spinal cord. During adulthood, astrocytes are still generated from differentiating progenitors in stem cell niches^[32] or from dividing mature astrocytes in specific brain regions^[33]. Historically, two classes of astrocytes were described: "type I" fibrous astrocytes mostly found in white matter tracts and "type II" protoplasmic astrocytes found in the grey matter^[34,35]. Nowadays, the scientific community agrees that astrocyte complexity, in particular within

the protoplasmic subfamily, has increased along with phylogenetic evolution. As far as we know, this complexity culminates in the human CNS. Compared to rodents^[19], human astrocytes have a greater size, a more complex morphology, a large pleiomorphism^[36] and are able to propagate calcium waves five times more rapidly^[31,37,38]. Although mice represent a useful tool to study astrocytes and their *in vivo* functions through genetic manipulation, one limitation of rodent-to-human extrapolations is the wider diversity of human counterparts. For instance, primate brain contains two types of astrocytes not found in rodent brain: interlaminar and varicose astrocytes, whose functions are as of yet undetermined but seem to be related to the higher complexity of neuronal layers inside the human cortex^[37]. Despite this heterogeneity, astrocytes share similarities such as the expression of several common proteins. Intermediate filament proteins are very abundant in the cytoplasmic compartment among all astrocytes types: glial fibrillary acidic protein (GFAP), vimentin, desmin and synemin. Recently, the cell surface marker CD44, the receptor of extracellular matrix hyaluronan, has been described to distinguish, more accurately than GFAP, protoplasmic astrocytes from fibrous-like astrocytes^[36]. Resting astrocytes also display specific immunoreactivity for aldehyde dehydrogenase 1 family member L1, AQP4, S100, GLT1, GLAST and glutamine synthetase. Under pathological conditions affecting CNS [e.g., Alzheimer's disease, multiple sclerosis, stroke, amyotrophic lateral sclerosis (ALS), spinal cord injury (SCI)], astrocytes can switch to an activated phenotype. Activated astrocytes, which can in some case include a population of proliferating astrocytes, are commonly observed around focal CNS lesions, in areas of neuronal loss/damage, in demyelinating areas, during neuroinflammation or during CNS repair. Astrocyte hypertrophy and upregulation of GFAP, vimentin, nestin or S100 are hallmarks of activated astrocytes. For instance, new protein markers may also be expressed. Expression of S100A6, a calcium binding protein, is drastically increased in astrocytes from ALS spinal cord^[39,40], after traumatic CNS injury^[41] or in Alzheimer brain tissue^[42]. Given that ALS and SCI affect either diffuse or discrete areas, respectively within the CNS (*i.e.*, in ALS from motor cortex to bulbar respiratory nuclei to the lumbar spinal motor neurons; in SCI, both gray and white matter astrocytes are lost over a distance of several millimeters), therapeutically-targeting astrocytes would undoubtedly take into account their diversity of shape and function, with respect to their location. Our knowledge about astrocyte heterogeneity now allows for generating *in vitro* subpopulations of stem cell-derived astrocytes endowed with specific fates or defined functional abilities. These cells, isolated and expanded *in vitro*, can be further used in transplantation paradigms aiming at providing neuroprotection or replacing astrocyte-deficient

functions observed during neurodegenerative and traumatic diseases of the spinal cord such as ALS or SCI.

SOURCES OF STEM CELL-DERIVED ASTROCYTES

Glial-restricted precursor cells

Use of lineage-restricted progenitors as a source for deriving astrocytes has gained wide popularity in recent years due to their ability to generate specific glial types following transplantation. Glial-restricted progenitors are present in the embryonic spinal cord and through their ability to self-renew and differentiate give rise to oligodendrocytes and astrocyte populations. The first type of glial restricted progenitor that was originally characterized was the A2B5⁺ O-2A progenitor cell. This was isolated from optic nerve of embryonic and post-natal rats and through *in vitro* differentiation was shown to be able to differentiate into oligodendrocytes and a particular type of astrocyte called the type-2 astrocyte^[43-45]. More recently, a different type of progenitor cell was characterized that is able to differentiate into oligodendrocytes and two different populations of astrocytes. This cell type was isolated from E13.5 rat spinal cord and is also positive for the A2B5 antigen. *In vitro* conditions that supported their above mentioned differentiation included culture medium supplemented with fetal calf serum or platelet-derived growth factor (PDGF) and T3 thyroid hormone. Clonal analysis confirmed that these individual GRPs are tripotent. Furthermore, these cells are capable of extensive self-renewal in the presence of PDGF and FGF2. These GRPs differed from the original A2B5⁺ O-2A progenitor cell in several aspects such as absence of mitogen requirements, responsiveness to PDGF and morphologies^[46].

Neural stem cells or radial glia

Using cell genesis and fate specifying processes, neural tube-derived neural stem cells give rise to 10¹¹ neurons and at least 10¹² glial cells of many different phenotypes in the adult brain^[47-49]. Neural stem cells (NSCs) are an attractive source for generating astroglial cells through their multipotency and high potential of self-renewal. NSCs represent an endogenous population existing during brain development and to a lesser extent in adult brain niches. In adult CNS, they are found around the sub-ventricular zone, the sub-granular layer of the hippocampus and spinal ependymal canal. Their phenotype is characterized by an astrocyte-like morphology and GFAP expression^[50,51] together with other markers of an undifferentiated state (e.g., nestin, SOX2). The most common sources for NSC isolation include embryonic brain cortex and adult sub-ventricular zones. One of the basic features of NSCs is that they can easily divide *in vitro* either as

spherical aggregates called “neurospheres” or adherent layers of cells when supplemented with epidermal growth factor and fibroblast growth factor 2. Culture conditions involving growth factor withdrawal and serum exposure elicit a differentiation program into either neurons, oligodendrocytes or GFAP⁺ astrocytes, with various yields^[52-54]. Transplantation of NSCs into the CNS results in the generation of multiple cell types, including astrocytes^[53] and oligodendrocytes^[55]. Another source of astrocytes in the adult CNS is radial glial cells^[56,57]. Several lines of evidence support the existence of a small pool of such cells expressing classic radial glial markers (*i.e.*, NG2, RC1, 3CB2, brain lipid-binding protein)^[58] and located in limited regions of ongoing neurogenesis in the adult CNS^[59-61].

Embryonic or Induced-pluripotent stem cells

Embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSCs) represent an ideal pluripotent source for deriving human astrocytes. Even though early reports demonstrated differentiation of astrocytes from human iPSCs or ESCs, there was a great diversity in terms of reported methods and the total yields of neural cells were not high. Furthermore, astrocytes generated by these protocols were not defined in terms of their subtype identity. On the other hand, specific protocols to generate astrocytes with regional or subtype identity were not reported^[62-68]. This gap was fulfilled by a recently reported protocol, which allows directed differentiation of astrocytes *via* a prior differentiation of human stem cell derived neuroepithelial cells into glial progenitor cells by repression of neurogenesis. Astrocytes generated by this protocol were characterized by specific gene expression profiles, neurotransmitter and ionic uptake, support of neuronal maturation, and importantly expression of specific sets of homeodomain transcription factors that specify functional identity^[69]. On the basis of this, another protocol was recently reported for an efficient generation of immature astrocytes from human pluripotent stem cells (hPSCs)^[70]. The protocol consists of three steps. In the first stage, hPSCs were differentiated into neuroepithelial cells either by co-culturing with mouse embryonic fibroblasts or by differentiation in feeder free conditions. These appeared in the form of columnar epithelia that organized into neural tube-like rosettes at about day 10-15 of differentiation. Addition of morphogens at days 8 to 15 allows differentiation of specific astrocyte subtypes. In the second stage, floating neural progenitor aggregates were expanded with epidermal growth factor, FGF2 and mitogenic factors. Neuronal differentiation was repressed and potential for gliogenic progenitor was enhanced by weekly mechanical trituration. By around 90 d, most of the progenitors expressed markers for astrocyte progenitors or astrocytes such as CD44 and S100. Thus at this stage, they are referred to as “astrospheres”. In the third and

final stage, astroglial progenitors were differentiated into functional astrocytes by removal of mitogens. Addition of CNTF for 6 d stimulated gliogenic gene expression. If neuroepithelial cells were patterned in stage 1, terminally differentiated astrocytes expressed specific markers such as Hoxb4 for posterior identity or Nkx2.1 for ventral identity.

In vivo or in vitro transdifferentiation

Some cases of *in vivo* transdifferentiation into astrocytes have been described in the literature following transplantation of bone marrow-derived stem cells or mesenchymal stem cells into rodent CNS. It is not yet clear whether transdifferentiation normally occurs in healthy CNS and, if so, whether it requires a de-differentiation step. Several criteria must be filled to truly establish transdifferentiation into astrocytes: original cells must lose their committed-lineage differentiation markers; they have to adopt a astrocyte-like morphology and express astrocyte markers (*e.g.*, GFAP, S100). Several reports showed that GFP reporter-expressing bone marrow stem cells transplanted into adult rodent CNS gave rise to GFP⁺ GFAP⁺ astrocytes^[71-73]. Similarly, mesenchymal stem cells (MSCs) isolated from bone marrow, when grafted into neonatal mouse brain, have been shown to migrate extensively and differentiate into olfactory bulb granule cells and astrocytes^[74]. In an original experimental approach, Boucherie and colleagues intrathecally grafted MSCs in ALS-affected rodent spinal cord and observed that MSCs were able to extensively migrate while differentiating mostly into astrocytes at the sites of neurodegeneration^[75]. In recent years, *in vitro* transdifferentiation, starting from single skin fibroblasts, has also made possible the modeling of CNS diseases in a dish, bypassing invasive neurosurgical procedures to get human diseased tissue. Recent cell reprogramming protocols describe the direct conversion of human fibroblasts to "induced" neural stem cells, able to further differentiate into the three CNS lineages (neurons, astrocytes and oligodendrocytes) following appropriate culture conditions. Artificial and temporal expression of specific transcription factors such as Oct-3/4, Sox2, Klf4, Brn4 and c-myc seems to govern the cell conversion towards a neural fate^[76-78]. Molecular pathways and key transcription factors making transdifferentiation possible are currently under investigation for different cell types^[79].

RATIONALE FOR ASTROCYTE REPLACEMENT IN ALS AND SCI

What is amyotrophic lateral sclerosis?

Amyotrophic lateral sclerosis (ALS) is a common adult-onset neurodegenerative disease of the motor system, with a prevalence of 2-5/100000 people. It is characterized by a rapidly progressing neurode-

generation selectively affecting cortical, brainstem and spinal motor neurons. ALS remains an incurable disease, leading to fatal respiratory failure usually within 5 years following diagnosis^[80]. Pathophysiology of ALS is poorly understood and likely multifactorial. Proposed starting points for this complex disease targeting the motor neuron population include mitochondrial dysfunction, intracellular protein aggregation, disturbances of RNA metabolism, extracellular toxic environment, impairment at the level of axonal transport and at the neuromuscular synapse (reviewed by^[81]), together with extrinsic events: blood-brain barrier breakdown, glial cell reaction/dysfunction and neuroinflammation^[82-88]. It is known that dying motor neurons influence surrounding cells, of which astrocytes are most commonly investigated. Astrocytes shift from an anti-inflammatory and neuroprotective role to one that is pro-inflammatory and neurotoxic, thus adding to complexity of this fatal cascade^[80]. In 5%-10% of patients, ALS is inherited (familial ALS). In those cases, every fifth patient carries a detected mutation in superoxide dismutase 1 (SOD1), which is at present the most reliable and most widely used genetic animal model for ALS^[89]. Among others, the most commonly described mutations include genes encoding the DNA/RNA-binding proteins FUS, TDP43^[90], the ubiquitin-like protein Ubiquilin 2^[91] and optineurin^[92]. On the other hand, an intronic hexanucleotide repeat in the C9ORF72 gene was discovered in both familial and sporadic cases of ALS^[93].

What are spinal cord injuries?

SCI are devastating and diverse set of conditions that result from damage to spinal cord grey matter and white matter, as well as corresponding spinal nerves. The National Statistics for SCI estimates approximately 12000 new cases each year in the United States alone, most of which result from preventable causes (*e.g.*, motor vehicle accidents, falls, sports or violence). Some common outcomes of SCI include dysfunctions in the musculoskeletal, respiratory, uro-genital and gastrointestinal systems. The diversity of this condition results from differences in the location, type and severity of trauma, as well as on the consequent types and degree of functional impairment. Despite this diversity, all forms of SCI are linked to specific phenotypic changes in populations of spinal cord astrocytes. Some examples of these changes are: (1) acquisition of specific protective functions such as glial scar formation to constrain the secondary expansion of the lesion; (2) loss of certain crucial homeostatic functions such as the astrocyte glutamate transporter system that are key to normal CNS physiology; and (3) gain of toxic functions such as the generation of pro-inflammatory signaling molecules that contribute to degeneration, neuronal hyperexcitability and other detrimental effects. The response of astrocytes is a graded response such that there is diversity in these changes that vary with the type and severity of trauma and with proximity

to the lesion. Furthermore, even within a population of astrocytes within the spinal cord, the response of individual astrocytes to the same injury can vary, likely reflecting the normal heterogeneity amongst astrocytes that is now becoming increasingly appreciated.

Defective astrocyte glutamate handling: one rationale among others

ALS and SCI are both spinal cord disorders involving all CNS cells: neurons, oligodendrocytes, microglial cells, endothelial cells and astrocytes. In both cases, there is significant motor, as well as sensory, dysfunction, although sensory involvement is still a matter of debate in ALS. To some extent, they share histopathological features in the spinal cord tissue: death of spinal motor neurons, axonal damage in white matter tracts, anterograde axonal degeneration, BBB dysfunction, impaired astrocyte function and neuroinflammation. In the case where neurodegeneration reaches the cervical level of the spinal cord, the loss of phrenic motor neurons that control contraction of the diaphragm leads to severe respiratory deficits and represents a life-threatening condition. ALS, historically characterized by the loss of upper and lower motor neurons, is more and more recognized as endowed with a non-cell-autonomous component, in which microglia and astrocytes act as significant contributors to overall spinal cord dysfunction. It has been shown that microglia carrying ALS-linked mutant SOD1 drive ALS disease progression by fostering neuroinflammation and a toxic environment for spinal motor neurons^[81,94]. With respect to astrocytes in ALS, independent groups demonstrated that astrocytes bearing mutant SOD1 are key determinants of disease progression^[95-97]. In the absence of any pathology, normal astrocytes uptake glutamate, released at the synaptic cleft, through their glutamate transporters (GLT1/EAAT2 and GLAST/EAAT1). Furthermore, normal astrocytes protect motor neurons from glutamate excitotoxicity by stimulating the neuronal expression of AMPA-GluR2, which is less permeable to calcium making motor neurons less sensitive to excitotoxicity^[98]. During ALS disease course, it is known from human and animal model samples that astrocyte-based glutamate uptake is compromised due to an early loss of astrocyte GLT1. Drastic GLT1 downregulation is most likely to be linked with deleterious extrasynaptic glutamate accumulation, which gave rise to the theory of glutamate-induced excitotoxic motor neuron death and to the unique FDA-approved drug that slows ALS: Riluzole, based on its anti-glutamatergic action.

Following SCI, the entire architecture of the spinal cord is disrupted at the lesion site; the trauma produces both immediate and delayed cell death affecting all nervous system populations: neurons, astrocytes and oligodendrocytes. Shortly after injury, it has been demonstrated that glutamate starts to accumulate in

the extracellular space^[99], and can persist for over a week, depending on injury severity^[100]. Overload of CNS extracellular glutamate may cause excitotoxic damage to neurons, axons and oligodendrocytes *via* overactivation of both AMPA and NMDA receptors^[101,102]. Failure of long-term extrasynaptic glutamate clearance is suspected to be one major cause of secondary cell loss following SCI. Noteworthy, we and others demonstrated that astrocyte GLT1 was chronically lost at the injury epicenter following SCI but also downregulated in spinal cord regions distant from the lesion core^[103-106]. Furthermore, experimental data showed that the newly-generated astrocytes arising during the SCI repair phase lacked GLT1 expression, possibly compromising long-term astrocyte glutamate homeostasis^[107]. Other consequences of astrocyte dysfunction or loss should be considered in ALS and SCI: shortage of neurotrophic factors important for neuronal survival, overwhelming of anti-oxidative defenses, lack of support to maintain endothelial BBB integrity, impaired water, ionic and metabolic transport, release of harmful proinflammatory cytokines and synthesis of glial scar-related ECM proteins that block axonal regrowth^[1]. Regardless of whether astrocyte dysfunction is a cause of disease or a consequence of neuronal loss, altered physiology of pathologic astrocytes likely results in further susceptibility to CNS tissue loss, justifying the rationale for transplantation-based astrocyte replacement^[108]. Numerous studies indicate that transplantation of stem cell-derived astrocytes has exhibited beneficial effects on histological/functional outcomes in ALS and SCI animal models. In this review, we summarize the data from recent literature regarding transplantation of stem cell-derived astrocytes targeting the replacement of deficient or lost astrocytes during ALS and SCI.

METHODOLOGICAL CONSIDERATIONS

Feasibility of astrocyte transplantation in healthy spinal cord

Pilot studies were done on spinal GRPs isolated and characterized from the human aborted embryos^[46,109,110]. Upon transplantation into neonatal and adult rodent brain, human GRPs survived, migrated and differentiated into (im)mature oligodendrocytes and into GFAP-expressing astrocytes^[111]. These results were later confirmed by another group who analyzed the fate of GRPs harvested from alkaline phosphatase-expressing transgenic rats injected into intact and injured spinal cord. In the first set of experiments, GRPs survived at least 6 wk^[112] and in the second experiment up to 15 mo post-transplantation^[113], demonstrating their long-term integration. Cells showed morphological maturation and differentiated along astrocyte and oligodendrocyte lineages and not into neuronal lineages. Interestingly, cells grafted into the intact spinal cord showed a particular tropism for white matter tracts and robust migration capacities

since they were found more than 15 mm away from the injection sites^[112,113]. Transplantation of GRP-derived astrocytes seems to be a safe procedure, at least experimentally, since no tumor formation or pronounced immune response were observed at the graft sites. Isolated human GRPs express *in vitro* functional glutamate transporters EAAT1, EAAT3 and EAAT4 but not GLT1/EAAT2^[114]. According to the nature of *in vitro* pre-differentiation signals, GRPs do not give rise to homogenous astrocyte populations upon transplantation^[115,116]. Following bone morphogenic protein 4 (BMP4) driven differentiation, GRPs start to express EAAT2/GLT1 together with AQP4, AKAP12, Cx43 as markers of mature astrocytes^[116]. On the contrary, *in vitro* GRP exposure to CNTF give rise to mature astrocytes expressing FGF receptor-3 and several axon growth inhibitory proteoglycans, such as neurocan, brevican and phosphacan, suggestive of a phenotype of reactive astrocytes.

Routes of administration

Considering the route of administration for a stem cell therapy product is a significant factor when targeting the CNS and the spinal cord in particular. Stem cell transplantation into the spinal cord was successfully reported, in terms of homing and long-term engraftment, using different cell populations and different route of administration. Intraparenchymal injections of MSCs and NSCs into the thoracolumbar region^[117-123] and of GRPs into the cervical region^[104,124] have been successfully reported in animal models of ALS. Although direct targeting of the spinal cord is technically challenging and an invasive application, this route promises to be the most versatile and accurate method of targeted CNS therapeutic delivery, and therefore has been chosen for clinical translation of stem cell-based therapies in ALS patients. As both ALS and SCI affect long segments of spinal cord (several millimeters long and sometimes independent regions within the cord), a need for multiple injections adds to the complexity of the procedure. Aware of this issue, neurosurgeons are developing new surgical techniques or tools (e.g., radially-branched cannulae) able to deliver therapeutic agents to large CNS areas in a single shot^[125]. Other attempts to find the optimal delivery route used intranasal^[126], intrathecal^[127-129], intraperitoneal^[130], intramuscular^[131], intravenous^[132-135] and intra-bone marrow cell^[136] transplantation. Major shortcomings are poor CNS homing of the therapeutic product compared to the initial dose given (dilution of cells in unwanted organs/sites) and adverse effects such as blockade of intravenously-injected stem cells in lung capillaries or spleen (reviewed by^[137]). In many cases, researchers reported beneficial effects of peripherally-delivered stem cells associated with improved histological and functional outcomes in ALS animal models. While various routes of stem cell

delivery have been investigated in ALS therapy (Table 1), experimental SCI pathology was mainly targeted by focal stem cell injections in and around the lesion core, regardless of the spinal level affected (Table 2). Although the most efficient delivery method is still a matter of debate, the dose of transplanted cells, the timing, the location, the type of cells for particular needs, and their migratory abilities are additional parameters to take into account in designing CNS stem cell-based therapies. Numerous preclinical studies using animal transplantation paradigms will still be required to assess biodistribution, viability, integration into host tissue, differentiation into functional cells, lack of tumorigenicity and safety of delivery before broad clinical application^[138-140].

LESSONS FROM TRANSPLANTATION PARADIGMS IN ALS MODELS

Taking into account that neurons represent only a portion of the various nervous system cell types, it is not surprising that during onset and especially during progression of disease non-neuronal cells contribute significantly to neuronal dysfunction and death. For example, it has been shown that wild-type motor neurons in close proximity to mutant SOD1-containing non-neuronal cells became affected by pathological chain reactions specific for ALS and eventually die with many features of this disease^[141]. In the same way, wild type non-neuronal cells extend survival of SOD1 mutant motor neurons^[142]. It has been clearly shown that reduction of mutant SOD1 selectively in astrocytes results in a prolongation of disease duration, but has no effects on disease onset. At least a part of this process is modulated by dysfunction and decrease in levels of the primary astrocyte glutamate transporter, GLT1, in areas of motor neuron loss^[143]. Taken together, these results suggest a particular role for astrocytes in later progression of disease^[81,97]. Main studies that have assessed replacement of astrocytes based on transplantation in ALS animal models have been gathered in Table 1.

Transplantation of wild-type astrocytes or their precursors into CNS tissue affected by ALS represents a promising experimental approach. With the aim to rescue motor neurons responsible for breathing, which is the primary cause of death in human ALS, GRPs were transplanted into the cervical spinal cord of the SOD1^{G93A} rat model. GRPs survived in diseased tissue (32.2% ± 4.6% of transplanted cells survived at least 80 d post-transplantation), differentiated efficiently into astrocytes, and reduced microgliosis in the cervical spinal cord. Most notably, GRPs extended survival and disease duration, attenuated motor neuron loss, and slowed declines in forelimb motor and respiratory function. Since GRPs that did not express the glutamate transporter GLT1 did not have similar effects on behavioral measures or

Table 1 Transplantation-based astrocyte replacement in amyotrophic lateral sclerosis animal models

Animal	Type of cells	Delivery	Effect on disease	Main outcomes	Ref.
SOD1 ^{G93A} ALS rats (80 d-old)	GRPs from rat E13.5 spinal cord, wild-type and overexpressing GLT1	Injections into C4-C6 cervical spinal cord, 6 sites, bilateral 1.5×10^5 cells/site	Delayed decline in motor function and survival extension	Differentiation into functional astrocytes. Prevented motor neurons loss independently from growth factors secretion, sustained GLT1 levels, alleviated microgliosis	[104]
SOD1 ^{G93A} ALS mice (75 d-old)	Human neural precursors (hNPs) overexpressing BDNF, IGF-1, VEGF, NT-3, or GDNF	Injection in cisterna magna and cerebral ventricles	No effect on motor function or animal survival	Differentiation in GFAP ⁺ GLT1 ⁺ expressing and growth factors-secreting astrocytes. Prevented motor neurons loss	[147]
SOD1 ^{G93A} ALS rats (90 d-old)	Rat adult MSCs	Intrathecal delivery in lumbar cisterna magna, 1.95×10^6 cells	Preserved motor function and survival extension	Differentiation into astroglial cells. Decreased neuroinflammation	[75]
SOD1 ^{G93A} ALS mice (24-26 wk-old)	Human umbilical cord blood cells overexpressing VEGF and FGF2	Intravenous delivery, 1×10^6 cells	Not investigated	Differentiation in S100 ⁺ astrocytes	[146]
SOD1 ^{G93A} ALS rats (14-26 wk-old)	NSCs from rat E16 brain cortex	Intravenous delivery, 1×10^7 cells	Not investigated	Preferential homing to late symptomatic ALS brain and spinal cord. Differentiation into neurons and astrocytes	[135]
SOD1 ^{G93A} ALS mice (50-60 d-old)	hGRPs from fetal cadaver brain tissue (week 17-24 of gestational)	Injections into C4-C5 cervical spinal cord, 4 sites, bilateral 1.2×10^5 cells/site	No effect on histological or functional outcomes	Poor cell survival	[124]

ALS: Amyotrophic lateral sclerosis; BDNF: Brain-derived neurotrophic factor; C: Cervical; E: Embryonic; FGF2: Fibroblast growth factor 2; GDNF: Glial cell-derived neurotrophic factor; GFAP: Glial fibrillary acidic protein; GLT1: Glutamate transporter 1; GRP: Glial-restricted precursor; IGF-1: Insulin-like growth factor 1; MSC: Mesenchymal stem cell; NP: Neural precursor; NSC: Neural stem cell; NT-3: Neutrophin-3; SOD1: Superoxide dismutase 1; VEGF: Vascular endothelial growth factor.

animal survival, this highly suggested that glutamate-relevant pathways contribute to the cascade of events leading to cell death in this model and that the focal beneficial effects of GRP transplantation could be explained, at least in part, by increases in glutamate transporter expression^[104].

With the aim to translate astrocyte-based transplantation towards treatment of patients, human GRPs have been transplanted into SOD1^{G93A} mice. At disease end-stage, 10% of initial hGRP transplants survived and distributed throughout of the cervical spinal cord and up to 80% of all human derived cells co-expressed the astrocyte marker GFAP. Unlike the results obtained with rodent-derived GRPs, this xenograft transplantation paradigm using human GRPs did not provide a phenotypic preservation of motor function^[124].

In another study, Boucherie and colleagues tested a different approach: they injected MSCs into the cerebrospinal fluid of symptomatic SOD1^{G93A} rats. They successfully penetrated the CNS parenchyma and accumulated at sites of motor neuron degeneration. They differentiated into astrocytes and, most notably, decreased motor neuron loss in the lumbar spinal cord, preserving hindlimb motor function and extending the survival of SOD1^{G93A} rats. This neuroprotection correlated with decreased inflammation, as shown by a decrease in the proliferation of microglial cells and reduced expression of inflammatory-related genes, COX-2 and NOX-2^[75]. Recent publications confirmed that transplantation of stem cells indeed reduces neuroinflammation and suggests that alleviating

astrocytosis or microgliosis is an important parameter leading to both histological and functional improvement in ALS animal models^[144,145].

Another approach was tested by Mitrećić *et al.*^[135]. They transplanted NSCs into the rat model of ALS using the intravascular route. It was clearly shown that in animals affected by disease cells did cross the blood-brain barrier and accumulated in the regions affected by disease (motor cortex, ventral horns of the spinal cord) more than in healthy non-diseased animals. In the diseased CNS tissue, although only 6% of the initial stem cell dose was found at 7 d post-infusion, the transplanted cells differentiated into neurons and astrocytes^[135]. Transplantation of genetically-engineered cells is another strategy to provide additional support or growth factors to ALS-affected cells and could also unexpectedly influence (stem) cell differentiation. This is what happened in a study from Rizvanov *et al.*^[146] who showed that wild-type human umbilical cord blood cells differentiated into endothelial and microglial lineages after transplantation into SOD1^{G93A} mice, while the same cells genetically modified to overexpress vascular endothelial growth factor (VEGF) and FGF2 exhibited preferentially an astrocytic differentiation^[146]. In the same vein, human neural precursors were also genetically engineered to express insulin-like growth factor-1, neurotrophin-3, BDNF, VEGF or GDNF. These cells were then transplanted into the spinal cord or brain of SOD1^{G93A} mice where they migrated and differentiated into neurons, oligodendrocytes or GLT1-expressing astrocytes. Unfortunately, although cells of interest

Table 2 Transplantation-based astrocyte replacement in spinal cord injury animal models

Animal and type of SCI	Type of cells	Delivery	Effects on disease	Main outcomes	Ref.
Rat, aspiration of C3 fasciculus gracilis	E14 rat spinal cord astrocytes	Intraparenchymal graft at lesion site	Worsened hindlimb function compared to controls	Migration of grafted GFAP ⁺ astrocytes toward the nucleus gracilis of the host medulla	[184]
Rat, L3 hemisection	Neonatal rat cortical astrocytes	Intraparenchymal injection at lesion site, 2.5 × 10 ⁵ cells, in suspension or in gelfoam	Not investigated	Migration more than 4 mm away from the injection site, reduced glial scarring	[185]
Rat, photochemically-induced infarction of dorsal funiculus	Neonatal rat mixed glial cells (close to type-1 astrocytes) Neonatal kitten mixed glial cells (giving rise to type-2 astrocytes) CG4-mixed glial cells (differentiated into type-2 astrocytes)	Intraparenchymal injection at lesion site	Not investigated	Produced dense clusters of astrocytes surrounded by meningeal cells within the cyst Produced cells that filled the cyst with a loose network devoid of meningeal cell infiltration at the lesion Filled the cyst with a loose network and increased the density of blood vessels in the lesion core	[186]
Rat, T9/T10 contusion	GRPs from rat E13.5 spinal cord	Intraparenchymal injection at lesion site, 5 × 10 ⁵ cells	Not investigated	Differentiated into oligodendrocytes and astrocytes. Reducing glial scar and proteoglycans synthesis. Supported axonal regrowth in the lesion but not on long-distance	[155]
Rat, T8 dorsal hemisection	P3 rat neonatal cortical astrocytes (mainly type 1 astrocytes)	Intraparenchymal injection at lesion site, 2.5 × 10 ⁵ astrocytes in a collagen I scaffold	Modest temporary improvements of locomotor function	No migration of astroglial cells out of the implant. Significant increase in the number of ingrowing axonal fibres	[152]
Rat, T8/T9 contusion	Mixed NRPs and GRPs (ratio 1:3) from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 3 sites, 1 × 10 ⁶ cells	Improvement of bladder, sensory and motor functions	Differentiation into neurons and glia. Volume of spinal cord spared was increased and local lumbosacral circuitry was modified	[187]
Rat, T8 complete transection	Adult rat cortical astrocytes	Intraparenchymal injection below lesion site (T11), 1.5 × 10 ⁵ cells	Not investigated	Massive rostral migration (8 mm)	[188]
Rat, C1/2 or C3/4 dorsal hemisection	GDAs ^{BM/IN} from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 6 sites, 2-3 × 10 ⁴ cells/site	Functional locomotor recovery	Significant axonal regrowth, decreased synthesis of inhibitory proteoglycans, suppression of axotomized neurons atrophy	[156]
Rat, C1/2 or C3/4 dorsal hemisection	GDAs ^{BM/IN} from rat E13.5 spinal cord GDAs ^{CNTF} from rat E13.5 spinal cord GRPs	Intraparenchymal injections at and around lesion site, 6 sites, 3 × 10 ⁴ cells/site	Same for GDAs ^{BM/IN} as in [156], GRPs and GDAs ^{CNTF} caused mechanical allodynia and thermal hyperalgesia	Same results for GDAs ^{BM/IN} as in [156], GRPs and GDAs ^{CNTF} failed to support axonal regrowth	[115]
Rat, C3/4 dorsal hemisection	GDAs ^{BM/IN} and GDAs ^{CNTF} from human embryonic spinal cord tissue (week 9 of gestation)	Intraparenchymal injections at and around lesion site, 6 sites, 3 × 10 ⁴ cells/site	GDAs ^{BM/IN} promoted locomotor recovery	GDAs ^{BM/IN} supported axonal regrowth, neuronal survival more efficiently than GDAs ^{CNTF}	[116]
Mouse, T9/10 contusion	Mouse iPSC-derived astrocytes	Intraparenchymal injection at lesion site, 1 × 10 ⁵ cells	No improvement of locomotor or sensory functions	No tumor formation. Long GFAP ⁺ processes from transplanted cells. No interaction with host cells.	[189]
Athymic rats, T10 contusion	hGRPs from fetal cadaver brain tissue (week 18-24 of gestational)	Intraparenchymal injections at and around lesion site, 3 sites, 1 × 10 ⁶ cells	No significant improvements in motor function recovery. hGRP grafts attenuated hyperactive bladder reflexes	Differentiation for 80% of grafted cells into GFAP ⁺ astrocytes	[159]
Athymic rat, C4/5 dorsal hemisection	GRPs, GDAs ^{BM/IN} and GDAs ^{CNTF} from human and rat embryonic tissue	Intraparenchymal injection at lesion site, 1 site, 6 × 10 ⁵ cells	Not investigated	In all 3 groups differentiation into astrocytes generating a permissive environment for axonal regrowth, but not out of the lesion	[157]
Rat, T9 contusion	GDAs ^{BM/IN} from rat E14 spinal cord and overexpressing D15A	Intraparenchymal injections around lesion site, 4 sites, 4 × 10 ⁵ cells	Improved locomotor function. No changes in neuropathic pain	Differentiation into GFAP ⁺ astrocytes not secreting CSFG and allowing robust axonal regeneration. Increased spared white matter and decreased injury size compared to controls	[181]
Athymic rat, C4/5 dorsal hemisection	GRPs, GDAs ^{BM/IN} and GDAs ^{CNTF} from fetal cadaver brain tissue (week 20-21 of gestation)	Intraparenchymal injection at lesion site, 1 site, 6 × 10 ⁵ cells	Not investigated	Differentiated astrocytes from all 3 groups generated a permissive environment for axonal regrowth	[160]
Rat, T8 contusion	GDAs ^{BM/IN} from rat E13.5 spinal cord	Intraparenchymal injection at and around lesion site, 12 sites, 1.5 × 10 ⁴ cells/site	Improved hindlimb motor function	Promoted axonal regrowth, reduced glial scarring, inhibited neuroinflammation	[190]

BM/IN: Bone morphogenic protein 4; CNTF: Ciliary neurotrophic factor; E: Embryonic; CSFG: Chondroitin sulfate proteoglycans; GDA: GRP-derived astrocyte; GFAP: Glial fibrillary acidic protein; GRP: Glial-restricted precursor; h: Human; iPSC: Induced-pluripotent stem cells; L: Lumbar; NRP: Neural-restricted precursor; P: Post-natal; SCI: Spinal cord injury; T: Thoracic.

were generated and produced substantial amount of growth factors, they were unable to modify ALS disease onset or progression^[147].

In the last few years, we have been witnessing translation of the basic research into clinical trials for ALS. Maybe too prematurely, clinical trials have been launched around the world, without consensus on the cell types used, the delivery methods, or targeted outcome measurements. The majority of the these studies have focused on safety measurements, and all of them have reported transplantation of stem cells as a safe procedure (reviewed by^[148]). As we are approaching the stage when some of these trials are moving from phase 1/2 to phase 2/3, the field is eagerly awaiting detailed reports of the clinical benefits of these cellular interventions. Currently, there are approximately 15 ongoing clinical trials in the EU and United States. Most of them are focused on intraspinal delivery of fetal/mesenchymal/neural stem cells (United States, Italy, Spain) and some used intrathecal delivery of autologous bone marrow-derived cells; so far, none of them is specifically addressing the replacement of ALS-affected astrocytes.

LESSONS FROM TRANSPLANTATION PARADIGMS IN SCI MODELS

Some of the major experimental studies addressing the replacement of astrocytes based on transplantation paradigm in SCI animal models are summarized in Table 2. Historical studies conducted in the laboratories of George Smith and Jerry Silver tested the therapeutic potential of rodent neonatal astrocytes by transplantation following CNS insults. In this paradigm, cerebral midline was lesioned causing severing of callosal axons. A nitrocellulose bridge was introduced at the site of injury. When conducted at postnatal day 8 or younger ages, no necrosis was observed within 24 h. Furthermore, glial cells migrated and integrated into the graft, which provided a substrate for axon growth. However, when conducted later, there was extensive tissue degeneration and the implant was covered with a scar like mixture of fibroblasts and astrocytes. This glial scar failed to promote axon regeneration. Interestingly, when this paradigm was modified such that glial cells from younger pups were grafted into lesions of the older pups, they retained their ability to promote axon outgrowth. These pioneering studies demonstrated an age dependent decrease in the ability of endogenous and transplanted astrocytes to promote axon regeneration^[149].

Successive *in vitro* studies conducted by these researchers confirmed age dependent changes in intrinsic properties and molecular basis for supporting axon growth in astrocytes. The rate and extent of chick, as well as rodent, axon outgrowth was consistently greater over the surface of immature compared to mature astrocytes. In terms of molecular properties,

NCAM and G4/L1 antibodies reduced the rate of outgrowth over immature but not mature astrocytes, whereas integrin B1 receptor antibody reduced outgrowth on immature, and to a lesser extent, over mature astrocytes^[150].

Furthermore, there are differences in the motile properties of the two cell populations such that following transplantation into adult brain immature astrocytes demonstrate extensive migration and interaction with host blood vessels, unlike mature astrocytes. This ability may be linked to their potential to limit glial scar formation and support axon outgrowth^[151]. Transplantation of neonatal astrocytes at the site of thoracic SCI resulted in outgrowth of axons along the transplants, as well as some motor recovery^[152].

Even though these early studies produced promising results, because of the difficulty in harvesting these beneficial immature astrocytes from human tissues, further studies explored more practically relevant cell types such as multipotent NSCs as a viable source to generate transplantable astrocyte populations. In studies conducted by the Whittemore lab, NSCs isolated from E14 rat cortex were transplanted into intact and injured spinal cord, without prior differentiation. In the intact spinal cord, over a 2-mo period, a 50% of multipotent stem cells differentiated into GFAP positive astrocytes and a small percentage differentiated into oligodendrocytes and neurons. Following injury to the spinal cord, astrocyte differentiation dropped to 35% and neuronal differentiation was not observed at all^[153], demonstrating an effect of the micro-environment on the differentiation pattern of the transplanted stem cells. To overcome this, in a separate study, NSCs were transfected with Ngn-2, followed by transplantation into injured spinal cord. This resulted in a significant reduction in astrocyte differentiation and an increase in differentiation into oligodendrocytes and neurons. Furthermore, Ngn-2-NSC treated rats showed improved motor function. These studies also showed that transplantation of control NSCs enhanced neuropathic pain associated with SCI, whereas this unexpected side effect was not observed in Ngn-2-NSC transplanted rats^[154].

While NSCs provide the benefit of being able to give rise to neurons as well as glia, the use of GRPs harvested from fetal CNS has gained wide popularity in recent years for targeting astrocytes. Early studies documented extensive migration of transplanted GRPs in injured and intact spinal cord and differentiation into cells of the glial lineage, predominantly into astrocytes^[112]. Transplantation of these cells into thoracic SCI reduced scar formation and expression of growth-inhibiting proteoglycans; however, no axon outgrowth was observed^[155]. This obstacle was overcome by pre-differentiating GRPs into astrocytes (GDAs) by treatment with BMP-4. These GDAs were able to promote axon outgrowth when transplanted into lesioned spinal cord without causing neuropathic

pain^[156]. These investigators also differentiated GRPs into astrocytes by treatment with a different estrogenic signaling molecule, CNTF. This type of GDA however failed to promote axon regeneration and promoted thermal hyperalgesia and mechanical allodynia following transplantation into lesioned spinal cord^[115]. These studies highlighted that heterogeneous astrocyte populations may have distinct therapeutic potentials. Contrasting results from the Fischer lab indicated that, although heterogeneous, both BMP-4 and CNTF GDAs promoted axon outgrowth to a similar extent^[157]. Although it is not possible to compare in detail the precursor populations used by the two labs, the properties of specific glial precursor populations, differences in the culture conditions, and different transplantation and tracing protocols may have led to these contrasting results. Advantages derived from the graft are manifold. For example, a recent report from the Proschel lab illustrated an underlying mechanism through which BMP4-GDAs promote axon regeneration involving secretion of periostin^[158].

While the above-mentioned studies involving rodent GRPs provided an understanding of their properties and therapeutic potential, further studies involving more clinically relevant human GRPs were designed based on these previous studies. hGRPs harvested from fetal human brain were pre-differentiated into astrocytes (hGDAs) by treatment with BMP-4 and transplanted into injured spinal cords of athymic rats. Graft expansion was seen over an 8 wk time period, and in both hGRP and hGDA transplant groups, cyst and glial scar formation was reduced. Although there was no significant motor recovery, lack of neuropathic pain and permissive properties of these cells make them promising therapeutic candidates after SCI^[159]. Based on their rodent GRP work, the Fischer group generated GDAs by treatment with either CNTF or BMP-4 and showed that these cells have similar therapeutic potential in terms of promotion of axon generation^[160], whereas the Davies and Proschel groups again reported contrasting results^[116]. These differences may be related to the age, region and technique of isolation of hGRPs, as well as differences in the culture conditions used by the two labs.

In recent years, the discovery of induced pluripotent stem cells (iPSC) cells has generated great excitement as large quantities of clinically relevant mature cell types can be generated from these pluripotent cells, without facing the ethical concerns presented by pluripotent embryonic stem cells (ESCs). However, this field is in its infancy, especially in the context of astrocytes. Recently, the Zhang laboratory developed a protocol for producing large quantities of regionally specific astrocytes from ES and iPSC cells^[69]. A shortcoming of this study however is that it required 6 mo of culturing. Shorter duration protocols were subsequently reported, but these produced mainly populations of immature astrocytes^[161,162]. Astrocyte maturation takes place in

two distinct phases and marker expression for these phases has been characterized, which led to the development of protocols that generate astrocytes with mature phenotypes. While the treatment of immature astrocytes with FGF1 or FGF2 promoted up-regulation of maturation markers such as glutamate transporters and downregulation of GFAP, treatment with tumor necrosis factor- pushed cells toward a reactive astrocyte phenotype. These cells can be used to model human pathological processes *in vitro* and *in vivo* as well as for therapeutic transplantation^[163]. More recently the Zhang laboratory reported another shorter duration protocol for generating astrocytes from iPSCs by differentiation of these cells into neuroepithelial cells, followed by removal of mitogen and treatment with CNTF to induce differentiation into astrocytes^[70]. Recently, the Maragakis lab conducted transplantation of iPSC- and ESC-derived immature astrocytes into rodent spinal cord. Although limited, the grafts showed long-term survival, with less than 5% of the transplanted cells surviving at 12 wk post-transplantation. Furthermore, graft derived astrocytes expressed markers of mature, quiescent astrocytes such as AQP4, EAAT1, EAAT2, as assessed by immunostaining analysis and gene expression profiling, suggesting that these cells are a promising resource for transplantation therapies^[164]. Additional reports suggest that it is important to evaluate the *in vivo* safety of these cells, especially in terms of their tumor formation tendency, prior to their use for clinical transplantation^[165,166]. Recent work from the Lepore lab, conducted as an extension of their work related to rodent GRPs, generated glial progenitors from human iPSCs, followed by further differentiation into astrocytes. Following transplantation into injured spinal cord, these cells showed long-term survival, no tumor formation, and efficient differentiation into GFAP expressing cells. These cells could even be engineered to express GLT1, suggesting that hiPSCs could be used as a safe source for transplantation therapies for targeted replacement of astrocytes in SCI and other CNS diseases (unpublished data).

Until recently, the SCI field primarily focused on the role of astrocytes with regard to scar formation and their inhibitory effects on axon re-growth. Recent studies however have elucidated their heterogeneity and the changes exhibited by these cells following SCI, thereby underscoring the need to preserve their normal physiological functions. Studies mentioned above, as well as other studies that are not detailed here, are indicative of the growing appreciation of cell transplantation therapies for therapeutically targeting astrocytes in SCI.

With a special focus on chronic SCI conditions, many clinical trials based on the transplantation of unmodified mesenchymal, neural or bone-marrow stem cells have been launched around the world^[167-171]. Among phase 1 studies, most of them have focused on safety measurements and graft survival according to different routes of administration (mostly intrathecal

or focal intraparenchymal delivery). All of these studies have reported transplantation of stem cells as a risk-free procedure. To our best knowledge, none of the current human trials are specifically addressing the replacement of lost astrocytes following SCI.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We must admit that, despite numerous preclinical animal studies demonstrating improvement in a variety of functional outcomes following astrocyte transplantation, the variability and the poor reproducibility of the results still hinder full clinical translation. The variability might be partly explained by the limited survival of transplanted cells in diseased spinal cord tissue. In published preclinical ALS and SCI studies, cell graft survival was found to be usually between 5% and 32% of the initial dose and was often assessed at early time points post-transplantation. Inter-laboratory discrepancies might also be due to the source or type of astrocytes (or precursors) used in the transplantation paradigms. Indeed, astrocytes, in rodents and even more in humans, are a broad class of CNS cells endowed with specific functions according to their location, their morphology, their degree of maturity and their activation state. Finally, lack of standardization, different type of cells, different animal species and single targeting strategies of multifactorial diseases may also be part of the failures. Therefore, future protocols should aim at standardizing the culture conditions, verifying the astrocyte population homogeneity, the cell purity and, above all, at enhancing cell survival after *in vivo* transplantation. Researchers are also encouraged to provide preclinical data about long-term follow-up of engrafted cells, including survival time, differentiation pattern and CNS integration several months post-transplantation.

During this last decade, detailed molecular dissection of astroglial populations has shown that astrocytes are active players in CNS homeostasis and far more than basic fibroblast-like cells of the CNS. Understanding the molecular mechanisms underlying the normal biology of astrocytes as well as their behavior during pathological conditions will provide us with undoubtedly novel astrocyte-specific therapeutic targets. Especially regarding ALS and SCI that share several common pathophysiology mechanisms, targeting astrocytes, whose dysfunction is now well established from animal and human studies, might be a promising therapeutic strategy. As far as we know, oxidative stress, neuroinflammation and glutamate excitotoxicity are all pathological events in which astrocytes are actively involved. Therapeutics that address each separately or all-in-one these specific pathological events are of great relevance to ALS and SCI.

Stem cell based therapy for ALS and SCI includes three main aims: (1) replacement of lost nervous cells for restoring homeostatic functions of the CNS;

(2) neuroprotection of unaffected surrounding tissue *i.e.*, by secretion of neurotrophic factors or anti-inflammatory molecules; and (3) enhancement of the endogenous repair process. Based on the biology and the known functions of astrocytes, here are a few examples of neuroprotection-based mechanisms that are under investigation and might deserve particular attention in future transplantation paradigms targeting astrocytes.

Increased oxidative stress has been implicated in the pathogenesis of ALS and SCI, leading to the testing of different antioxidants in affected patients. Several studies have demonstrated that astrocyte-secreted glutathione greatly contributes to neuroprotection against oxidative stress. One of the main regulators of glutathione production and release is the transcription factor Nrf2. ALS mice with astrocyte driven Nrf2 overexpression showed increased lifespan, better functional outcomes and lower glial reactivity in spinal cord tissue^[172]. Accordingly, it would be interesting to investigate the effect of transplantation of Nrf2-overexpressing astrocytes. Stat3 is another transcription factor regulating GSH synthesis^[173], as well as a "master-switch" that mediates transition from quiescent to reactive astrocyte. Following SCI, transgenic mice ablated for Stat3 in the astroglial population showed less reactive astrocytes in the spinal cord, resulting in more widespread neuroinflammation, motor neuron loss and axonal damage and thus worsened motor deficits compared to wild-type counterparts. This study and others demonstrates that Stat3 is a key transcription factor controlling astrocyte reactivity during neurodegenerative processes, providing a potential target for intervention^[174,175].

Far from being science fiction, researchers can genetically modify (stem) cells to make them produce more growth factors or proteins of interest, as Trojan horses to deliver molecules to specific CNS locations. For instance, NSCs engineered to overexpress CNTF exert neuroprotection by *in situ* differentiation into supportive astrocytes in a mouse model of photoreceptor degeneration^[176]. Similarly, NSCs overexpressing nerve growth factor showed beneficial effects on cognitive functions after CNS transplantation and astrocyte differentiation in a learning deficit mouse model^[177]. Another example is illustrated in a recent report from Gowing^[178]. This group engineered GDNF-secreting human neural progenitors that were able to survive up to 7.5 mo after intraspinal transplantation, differentiate mainly into astrocytes and still maintain locally high levels of GDNF. More specifically, intranigral transplantation of astrocytes transduced with a lentiviral vector expressing GDNF resulted in sustained local production of growth factor and provided neuroprotection in a rat model of Parkinson^[179]. Lastly, genetically-modified astrocytes designed to secrete BDNF promoted retinal ganglion cell survival^[180], and transplantation of neurotrophin-releasing GRPs improved functional and histological outcomes after

SCI^[181]. Growth factors released from transplanted cells act in a paracrine manner on surrounding host tissue but are likely to also act in an autocrine manner on grafted cells to promote survival and/or a specific differentiation pattern. All aforementioned examples should encourage us to investigate more deeply the therapeutic potential of grafted exogenous astrocytes or stem cell-derived astrocytes in neurodegenerative and traumatic CNS diseases, as well as to think about novel genetically-modified cells that specifically target pathogenic events occurring during ALS or SCI.

Inspired from encouraging results obtained following transplantation of GLT1-overexpressing GRPs in the rat model of ALS^[104], the Lepore lab is studying their applicability and evaluating their efficacy in clinically-relevant SCI models^[182,183]. The rationale behind such an approach is based on the findings that, following SCI, astrocyte GLT1 expression and function are compromised, resulting in excitotoxicity-induced cell death during the delayed secondary injury phase^[105]. Unpublished data from our group show that transplantation of astrocytes engineered to overexpress GLT1 can prevent excitotoxicity and protect respiratory phrenic motor neurons following cervical SCI. While unmodified transplants showed robust survival, they expressed relatively low levels of GLT1 in injured spinal cord. Excitingly, GLT1 overexpressing transplant-derived astrocytes continued to express high levels of GLT1 protein following transplantation into the injured spinal cord and promoted survival of these important phrenic motor neurons and preservation of diaphragm function (unpublished work).

Finally, although ALS and SCI share many similarities including the death of spinal motor neurons, it must be emphasized that important differences between two diseases exist. ALS is a progressive neurodegenerative condition starting with discrete and asymptomatic loss of motor neurons in remote specific regions of the CNS (upper and lower motor neurons), whose symptomatology becomes apparent at approximately 50% of motor neuron loss and fatally progresses when disease affects bulbar and/or spinal respiratory centers. Therefore, therapeutic strategies in ALS, once diagnosed, should focus on astrocyte-mediated neuroprotection that prevent further motor neuron loss, worsening of functional outcomes and delayed fatal respiratory failure. Unlike ALS, SCI is characterized by different temporal sequence of events. First, acute and sub-acute phases following the initial trauma in the first hours-by-days post-injury result in a hostile environment in the spinal cord around the lesion epicenter, including inflammation, excitotoxicity and oxidative stress that lead to more cell loss and thus extension of initial lesion. This narrow time window would be appropriate for interventional strategies targeting astrocytes that favor neuroprotection or compensate for lost astrocyte-specific functions. At later time points, during the chronic stage of SCI, characterized by the resolution of subacute events, glial scarring and reorganization of spinal tissue architecture, one can imagine designing astrocyte transplants to make

scar tissue permeable to axonal regrowth, to promote invasion by remyelinating oligodendroglial precursors, or to facilitate overall rewiring of ascending/descending tracts in the spinal cord.

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Tooth-derived stem cells: Update and perspectives

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Abstract

Tissue engineering is an emerging field of science that focuses on creating suitable conditions for the regeneration of tissues. The basic components for tissue engineering involve an interactive triad of scaffolds, signaling molecules, and cells. In this context,

stem cells (SCs) present the characteristics of self-renewal and differentiation capacity, which make them promising candidates for tissue engineering. Although they present some common markers, such as cluster of differentiation (CD)105, CD146 and STRO-1, SCs derived from various tissues have different patterns in relation to proliferation, clonogenicity, and differentiation abilities *in vitro* and *in vivo*. Tooth-derived tissues have been proposed as an accessible source to obtain SCs with limited morbidity, and various tooth-derived SCs (TDSCs) have been isolated and characterized, such as dental pulp SCs, SCs from human exfoliated deciduous teeth, periodontal ligament SCs, dental follicle progenitor cells, SCs from apical papilla, and periodontal ligament of deciduous teeth SCs. However, heterogeneity among these populations has been observed, and the best method to select the most appropriate TDSCs for regeneration approaches has not yet been established. The objective of this review is to outline the current knowledge concerning the various types of TDSCs, and discuss the perspectives for their use in regenerative approaches.

Key words: Cell-based therapy; Mesenchymal stem cells; Dental stem cells; Differentiation; Tissue engineering

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Core tip: Stem cells (SCs) present the characteristics of self-renewal and differentiation capacity, which make them promising candidates for regenerative approaches. Although they present some common markers, SCs derived from various tissues have different patterns of proliferation, clonogenicity, and differentiation. Tooth-derived tissues are an accessible source of SCs with limited morbidity. However, heterogeneity within populations of tooth-derived SCs has been observed, and the best method to select the most appropriate SCs for regenerative approaches has not yet been established.

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INTRODUCTION

Stem cells (SCs) are cells that present two distinctive characteristics: they are able to continuously self-renew, and they can be induced to differentiate into multiple specialized cell types^[1]. SCs have therefore been a subject of interest to researchers and the general public, as a way to regenerate damaged tissues and improve the resolution of some illnesses, such as Parkinson's disease^[2] and diabetes^[3], that current approaches in the medical field have not yet achieved. In this context, many studies have been conducted to identify and isolate SCs and to understand their biologic aspects.

SCs can be isolated in the earliest stages of embryogenesis (embryonic SCs)^[4-9] or in various postnatal tissues (adult SCs)^[2,10,11] (Table 1). Although embryonic SCs present interesting properties, such as the ability to differentiate into hundreds of other cell types, the bioethical aspects involved in the study of these cells, especially for human embryos, have hindered advances in this field and research has thus been focused on adult SCs^[1,10-12]. Adult SCs can be obtained from adult specialized tissues, such as bone marrow^[13-16], skin^[17,18] and fat^[19-23], where they likely act to renew cell populations and maintain tissue homeostasis, or help to repair the tissue in case of injury^[18,24,25]. Even though adult SCs can be obtained from less ethically concerning sources, they have some limitations compared to embryonic SCs, such as more limited lifespan and differentiation potential^[1,11,24,26]. In order to overcome these drawbacks, adult SCs can be reprogrammed by the insertion of SC-associated genes, forming induced pluripotent SCs (iPSCs)^[3,27-31].

Within the medical field, mesenchymal SCs (MSCs) have been widely studied to understand their role in skeletal tissue development, physiology and repair^[14], and because of their promising therapeutic potential^[2]. MSCs are characterized by the capacity to differentiate into multiple types of skeletal tissues^[14,32-36]. They were first described as adherent, clonogenic, self-renewing, fibroblast-like cells (colony-forming unit fibroblasts) obtained from bone marrow^[35,37,38]. Subsequently, several studies were performed to identify other sources and to understand how these cells can give rise to distinct cell types, for the purpose of using these cells in regenerative procedures^[39-43].

In this context, dental tissues have also been investigated as niches of MSCs, and many tooth-derived SCs (TDSCs) have been identified and characterized, including dental pulp SCs (DPSCs)^[44-48], SCs from human exfoliated deciduous teeth (SHED)^[49-53],

periodontal ligament SCs (PDLSCs), dental follicle progenitor cells (DFPCs)^[54-56], SCs from apical papilla (SCAP)^[19,56-59], and periodontal ligament of deciduous teeth SCs (DePDL)^[50,51,60-62] (Figure 1). Dental tissues are an accessible source of MSCs that can be obtained with limited morbidity and without additional risks to the donor, as extracted/exfoliated teeth represent a waste product of dental procedures^[13,63-65]. However, the properties of these TDSCs and their feasibility for regenerating tissues still need to be investigated in greater detail. Thus, the aim of the present review is to describe the current knowledge concerning TDSCs, and to consider the perspectives for their use in regenerative approaches.

ISOLATION, CHARACTERIZATION, AND DIFFERENTIATION POTENTIAL OF TDSCs

Because of the variety of methodologies used to isolate and characterize MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human bone marrow SCs (BMSCs) and other types of MSCs *in vitro*^[32]. Briefly, MSCs must adhere to plastic under standard culture conditions, express cluster of differentiation (CD)105, CD73 and CD90, but not CD45, CD34, CD14, CD11b, CD79a, CD19, or human leukocyte antigen-DR surface molecules, and have the potential to differentiate along osteogenic, chondrogenic and adipogenic lineages^[32]. Therefore, studies characterizing TDSCs usually evaluate these criteria, as well as clonogenicity (capacity to form adherent colonies derived from one single cell) and differentiation potency, in order to compare them to each other and to BMSCs^[66,67] (Table 2).

DPSCs

DPSCs were the first TDSCs isolated and characterized in 2000^[44]. Obtained from permanent third molars, these cells were found to be more proliferative than BMSCs, and had the capacity to form mineral deposits *in vitro*, though in reduced amounts compared to BMSCs^[44]. DPSCs also failed to form lipid-laden adipocytes *in vitro*, whereas BMSCs are capable of differentiating into adipocytes^[44]. However, more recent studies demonstrate that DPSCs can differentiate into adipocyte cells when other supplements are added to the adipogenic induction medium^[46,65].

When transplanted *in vivo*, some DPSC clones differentiate into aligned odontoblast-like cells, with prolonged processes oriented into newly formed dentin-like structures^[44,46], whereas BMSCs form distinct lamellae of bone^[44]. DPSCs can also form reparative dentin-like tissue on the surface of human dentin *in vivo*^[68].

SHED

In 2003, progenitor cells were isolated from the remnant pulp of exfoliated deciduous teeth^[49]. SHED

Table 1 Classification of stem cells according to their plasticity^[1,2,41,66]

Stem cell type	Description
Totipotent	Stem cells able to differentiate into cells of all three germ layers (ectoderm, mesoderm and endoderm) and extra-embryonic tissues (<i>e.g.</i> , zygote)
Pluripotent	Stem cells able to differentiate into all cells of the body, but that cannot form extra-embryonic tissues (<i>e.g.</i> , embryonic stem cells and induced pluripotent stem cells)
Multipotent	Stem cells that have differentiation abilities restricted to some cell types, usually from the germ layer they are derived from (<i>e.g.</i> , mesenchymal stem cells)

Table 2 Characterization of tooth-derived stem cells

TDSCs	Ref.	Location	Expression markers		Differentiation capacity	
			Positive	Negative	<i>In vitro</i>	<i>In vivo</i>
DPSCs	[13,44,46,51,65]	Permanent tooth pulp	CD29, CD44, CD73, CD90, CD105, CD146, STRO-1, Oct-3/4, Sox-2, nanog	CD14, CD34, CD45	Osteoblast, adipocyte, chondrocyte, hepatocyte, neuron	Dentin-like structures
SHED	[49,51,53,69,70]	Deciduous tooth pulp	CD29, CD105, CD146, STRO-1	CD31, CD34	Osteoblast, odontoblast, adipocyte, neural cell	Dentin formation, induce bone formation by murine host cells
SCAP	[13,56,57,65]	Apical papilla of developing tooth	CD24, CD29, CD31, CD44, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Oct-3/4, Sox-2, nanog, survivin	CD14, CD18, CD34, CD45, CD150	Osteoblast, adipocyte, chondrocyte, hepatocyte, neuron	Dentin-like tissue
DFPCs	[13,54,56,65,72]	Dental follicle of developing tooth	CD29, CD44, CD73, CD90, CD105, nestin	CD14, CD31, CD34, CD45, CD117	Osteoblast, adipocyte, chondrocyte, hepatocyte, neuron	Bone/cementum-like tissue
PDLSCs	[13,57,60,73]	Permanent tooth periodontal ligament	CD44, CD90, CD105, CD166, CD146, STRO-1, Oct-3/4, Sox2, nanog, nestin	CD14, CD34, CD34, CD45	Osteoblast/ cementoblast, adipocyte, neuron	Periodontal ligament/ cementum-like tissue
DePDL	[60]	Deciduous tooth periodontal ligament	CD105, CD166, STRO-Oct-4	CD34, CD45	Osteoblast, adipocyte	

CD: Cluster of differentiation; DePDL: Periodontal ligament of deciduous teeth stem cells; DFPCs: Dental follicle progenitor cells; DPSCs: Dental pulp stem cells; Oct: Octamer; PDLSCs: Periodontal ligament stem cells; SCAP: Stem cells from apical papilla; SHED: Stem cells from human exfoliated deciduous teeth; Sox2: SRY-box containing gene 2.

were found to be more proliferative than BMSCs and DPSCs^[49,51], and showed higher capability for osteogenic and adipogenic differentiation than DPSCs *in vitro*^[51]. SHED can also differentiate into neural cells^[69]. When 12 single-colony-derived SHED clones were transplanted into immunocompromised mice, only three clones demonstrated the potential to generate ectopic dentin-like tissue on the hydroxyapatite/tricalcium phosphate (HA/TCP) carrier equivalent to that generated by multiclonal-derived SHED^[49]. When SHED were seeded into human tooth slices and transplanted into immunodeficient mice, they were also able to form a dentin-like structure^[70]. Although some researchers claim that SHED have the ability to differentiate into osteoblasts *in vivo*^[51], Miura *et al.*^[49] reported that, in fact, SHED act as an osteoinductive factor, inducing the host cells to form bone.

SCAP

The apical papilla is the tissue located at the apex of the root of developing teeth^[66], and is distinct from the pulp^[71]. As this tissue is associated with root formation, it potentially provides a source of MSCs for

this purpose. SCAP are the cells isolated from this tissue that present characteristics of MSCs, and can give rise to odontoblastic, osteoblastic and adipocyte-like cells when cultured under appropriate conditions^[57]. SCAP also have the capability to form a dentin-like structure when transplanted into immunocompromised mice, using HA/TCP as a scaffold^[57]. Sonoyama *et al.*^[57] evaluated whether SCAP and DPSCs are the same or distinct MSC populations based on their cDNA microarray profile, and observed that many genes were differentially expressed by these MSC populations. In particular, CD24 and survivin were highly expressed in the SCAP population. Additionally, SCAP also showed other favorable characteristics, such as higher proliferative rate and telomerase activity, and improved migration capacity^[57].

DFPCs

The dental follicle is a condensation of ectomesenchymal cells that surrounds the tooth germ in early stages of tooth formation and contains cells that form the three tissues that constitute the periodontium: periodontal ligament, cementum and alveolar bone^[54,72]. When the

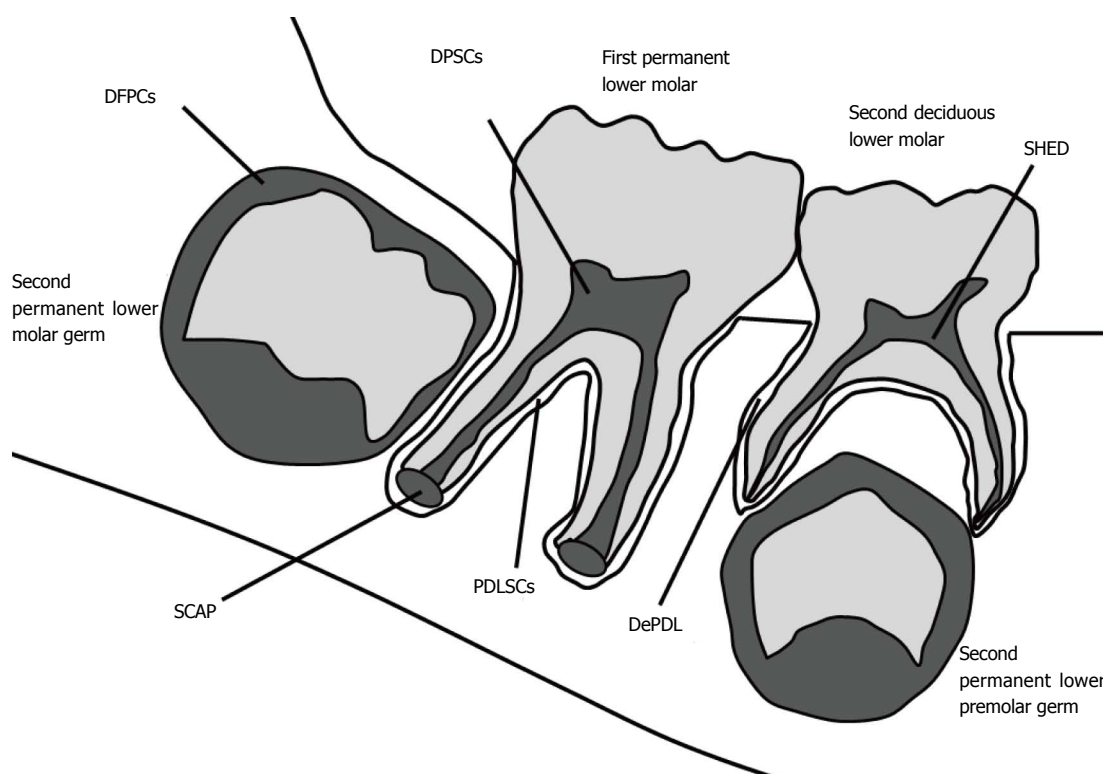


Figure 1 Sources of tooth-derived stem cells. DePDL: Periodontal ligament of deciduous teeth stem cells; DFPCs: Dental follicle progenitor cells; DPSCs: Dental pulp stem cells; PDLSCs: Periodontal ligament stem cells; SCAP: Stem cells from apical papilla; SHED: Stem cells from human exfoliated deciduous teeth.

heterogeneity of DPFCs was analyzed, it was observed that, although all cloned cell lines were positive for MSC-related surface markers (CD105, CD44, CD29) and negative for hematopoietic markers (CD34, CD117), they were different in terms of proliferation and mineralization patterns, indicating that they could be committed to distinct lineages^[72].

In order to avoid donor variability, TDSCs from follicle, pulp and papilla were isolated from a single donor tooth and the morphology, proliferation rate, expression of MSC-specific and pluripotency markers, and *in vitro* differentiation into osteoblasts, adipocytes, chondrocytes and hepatocyte-like cells were compared^[65]. Adherent, fibroblast-like morphology was observed in all TDSCs cultured under the same standard conditions, and DFPCs were more proliferative than DPSCs and SCAP^[65]. Although all three cell types were able to differentiate into the osteoblast lineage, DFPCs and DPSCs showed higher potentials than SCAP to form mineralized nodules *in vitro*^[65]. Additionally, when cultivated under chondrogenic-inducing conditions, DFPCs expressed all three chondrogenic-specific markers (aggrecan, and type I and type III collagen), whereas DPSCs and SCAP only expressed aggrecan^[65].

PDLSCs

The periodontal ligament harbors a heterogeneous cell population, with subsets of cells in various stages of commitment to fibroblastic and osteoblastic/cementoblastic lineages^[34,73,74]. Within these subsets,

it was supposed that putative MSCs would be present in the periodontal ligament, which was confirmed in 2004^[73]. PDLSCs show expression of STRO-1 and CD146, SC markers previously reported to be expressed in BMSCs and DPSCs, and also express scleraxis, a tendon-specific transcription factor^[73].

PDLSCs are more proliferative and clonogenic than BMSCs^[34]. PDLSCs can also differentiate into adipocytes^[34,73] and chondrocytes^[34], as well as osteoblasts/cementoblasts^[34,60,73]. Although PDLSCs are able to form mineralized tissue *in vitro* when osteoblastic/cementoblastic differentiation is induced, they form fewer mineralized nodules than BMSCs^[34,73]. Additionally, when transplanted into immunocompromised mice, some clones of PDLSCs have been shown to form periodontal ligament-like structures *in vivo*^[73].

DePDL

As MSCs can be isolated from the pulp of deciduous teeth, it was thought that the periodontal ligament of deciduous teeth may also harbor MSCs^[60]. In 2010, DePDL were isolated and compared with their permanent counterparts and found to be more proliferative than PDLSCs^[60]. Moreover, it was observed that, although DePDL and PDLSCs have the ability to differentiate into both adipocyte-like and osteoblast-like cells *in vitro*, DePDL show a higher potential for adipogenic commitment, and PDLSCs have a higher potential for osteogenic commitment^[60].

iPSCs from TDSCs

Following reports of reprogramming of dermal fibroblasts to behave like embryonic SCs^[27,28], studies were conducted to evaluate if other cell types could also be reprogrammed, including TDSCs^[29,30]. It was reported that human gingival and periodontal ligament fibroblasts^[29], SHED, SCAP and DPSCs^[30] can be reprogrammed as iPSCs, with formation of teratomas after implantation in immunocompromised mice^[29,30].

PERSPECTIVES ON TDSCs IN REGENERATIVE APPROACHES

Tissue engineering is an emerging field based on basic science and engineering technology, designed to create suitable conditions to regenerate damaged tissues^[75-78]. The basic components for tissue engineering involve an interactive triad of scaffolds^[79-81], signaling molecules^[82-84] and cells^[24,33,41,85], which play a fundamental role in the regeneration process^[24,76,86,87]. Scaffolds serve as a three-dimensional template mimicking the extracellular matrix; signaling molecules enhance this cellular activity by stimulating cells to migrate, proliferate and differentiate; and cells provide the machinery synthesis of the extracellular matrix and tissue regeneration^[24,75,76,88,89]. Due to their interesting properties, including self-renewal and differentiation abilities, MSCs are considered important for tissue maintenance and renewal, and, therefore, a promising candidate for tissue engineering^[24,90-94].

Some studies demonstrated that cell-based therapies are able to regenerate dental tissues^[48,57,70,95-98]. In a study in dogs, complete pulp regeneration was achieved when CD105⁺ DPSCs with stromal cell-derived factor-1 were transplanted into pulp, and this was not observed when total pulp cells or CD105⁺ adipose-derived cells were used^[48]. Supplementation of guided tissue regeneration with periodontal ligament cells for the treatment of class II and III furcation defects in dogs enhances periodontal regeneration^[95,97]. Twelve weeks after PDLSCs with an HA/TCP scaffold were transplanted into periodontal defects in a minipig model, new bone, cementum and periodontal ligament formation was observed^[98]. Sonoyama *et al.*^[57] also explored the potential of human PDLSCs and SCAP to generate a root-periodontal ligament complex in minipigs. They were able to obtain engineered roots capable of supporting porcelain, though with lower compressive strength. Nakahara^[96] reported the formation of root and periodontal ligament in a new culture system using one tooth crown collected from a neonatal mouse, which was referred to as a "test-tube dental implant". The author stated that cell therapy will be the next generation of dental medicine, but further information regarding human SCs is necessary for safe and reliable clinical applications^[96].

In regard to human clinical trials, autologous progenitor cells obtained from periodontal ligament have been used to treat intrabony defects^[99]. These progenitor cells were of a later cell lineage with

decreased capacity for osteogenic and adipogenic differentiation compared to PDLSCs *in vitro*. Despite this, these progenitors were able to promote improvement in clinical and radiographic parameters. Another study reported the cultivation of periodontal ligament cells on titanium pins that were subsequently implanted in patients and in dogs^[91]. Clinical evaluation of the implants placed in the patients showed satisfactory mechanical function, and radiographs revealed bone filling and formation of a lamina-dura around the implants. Additionally, histologic evaluation of the implants placed in dogs revealed a ligament-like formation. Although these studies are not directly related to MSCs, they indicate that cell therapy can be a feasible clinical approach in the near future.

Despite these promising studies in cell-based tissue engineering, it is important to highlight that the best method to select the most appropriate MSC type for regenerating dental tissues is not yet clear. Although BMSCs, DPSCs, SHED, SCAP, DFPCs, PDLSCs, and DePDL present a common marker profile, they differ in their clonogenicity, proliferative ability, and differentiation potential *in vitro* and *in vivo*, suggesting that these properties are related to the microenvironments of origin of each cell lineage^[13,24,34,44,60,65,67,73]. Additionally, it has been noted that, even in the same population of MSCs, there are heterogeneous cell subpopulations with distinct differentiation potentials^[100]. This heterogeneity in relation to the ability to differentiate *in vitro* and to form dental tissues *in vivo* has also been reported in some TDSCs lineages, including DPSCs^[44,46,67], SHED^[49], DFPCs^[72], and PDLSCs^[34,73,74,101-104]. Therefore, it can be concluded that, although there are MSC-related surface markers, such as STRO-1, CD146 and CD105, specific surface markers associated with the hierarchical commitment to differentiation pathways of TDSCs are not yet well established. In this context, further advances in understanding the regulation of MSCs during differentiation and dental development are required in order to develop new approaches for dental tissue regeneration with predictable outcomes^[19,26,67,89].

CONCLUSION

The interest in organ regeneration using SCs has increased in the last decade. In this context, TDSCs are promising candidates, as they are readily available, highly proliferative, and present multi-differentiation abilities. Research on cell therapy for regenerating dental tissues has already been done, and shows promising results. Nevertheless, further research is needed to better characterize TDSCs and to understand their differentiation pathways in order to develop the most appropriate approaches for SC-based tissue engineering-therapies in dental practice.

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Could cancer and infection be adverse effects of mesenchymal stromal cell therapy?

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article reviews the effect of MSCs on tumor establishment, growth and metastasis and also susceptibility to infection and its progression. Data published to date shows a paradoxical effect regarding MSCs, which seems to depend on isolation and expansion, cells source and dose and the route and timing of administration. Cancer and infection may thus be adverse or therapeutic effects arising from MSC administration.

Key words: Cancer; Infection; Mesenchymal stem cells; Therapy; Biosafety

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Core tip: Mesenchymal stem cells (MSCs) derived from different origins have recently received much attention as potential therapeutic. However, such cells also appear to have essential functions in building and supporting tumor microenvironments. Here, we review the effect of MSCs on tumor establishment, as also susceptibility to infection and its progression. The literature reveals incongruity regarding the impact of MSCs on the development of cancer and infection; such paradoxical effect might be attributed to differences in isolation and expansion conditions, the source and dose of the cells, the administration route and its timing and host characteristics. MSCs immunomodulatory potential seems to be the leading mechanism responsible for such effects.

Abstract

Multipotent mesenchymal stromal cells [also referred to as mesenchymal stem cells (MSCs)] are a heterogeneous subset of stromal cells. They can be isolated from bone marrow and many other types of tissue. MSCs are currently being tested for therapeutic purposes (*i.e.*, improving hematopoietic stem cell engraftment, managing inflammatory diseases and regenerating damaged organs). Their tropism for tumors and inflamed sites and their context-dependent potential for producing trophic and immunomodulatory factors raises the question as to whether MSCs promote cancer and/or infection. This

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INTRODUCTION

Multipotent mesenchymal stromal cells, also referred

to as mesenchymal stem cells (MSCs), were described for the first time half a century ago^[1]. Such cells are distributed throughout the stroma of several organs *in vivo* whilst MSCs adhere to plastic *in vitro* and proliferate when stimulated by fetal bovine serum^[1]. MSCs differentiate into mesodermal cells *in vitro* and *in vivo* (*i.e.*, adipocytes, chondrocytes, osteocytes and myocytes)^[2]. They can also cross the germ line barrier and produce cells from endo- and ectodermal lineages, such property being known as cell plasticity^[3].

MSCs are an ideal tool for cell therapy because they are easily procured from live donors^[4] and can be efficiently expanded *ex vivo*^[5]. The receptors do not need to have been conditioned before cell administration transplant^[6], as in total bone marrow or hematopoietic stem cell transplant. Once administered intravenously, they are able to home onto and engraft into damaged tissue where they could become differentiated into tissue-specific cells, release trophic factors, promote neovascularization, manage oxidative stress and fibrosis, or trigger an anti-inflammatory response^[7-11].

MSCs from the same individual (autologous) were administered into a human for the first time in 1995^[12]; MSCs were safely allogeneically transplanted seven years later^[13]. More than 350 clinical trials involving the use of MSCs are currently under way (www.clinicaltrials.gov) and no serious adverse events have been reported to date. Nevertheless, MSCs biosafety is still a major concern, particularly regarding the development of adverse event-related cancer and infection.

MSCs AND CANCER

MSCs might form tumors

Like any other cell, when MSCs are manipulated in the long-term they might have chromosomal aberrations and produce tumors in healthy animals^[14]; this has mainly been reported regarding mouse cells, which require extensive cultures for producing a significant number of hematopoietic-free MSCs^[14]. For instance, it has been shown that intravenously administered NOC/SCID bone marrow-derived MSCs embolize within the lung capillaries, expand and invade the lung parenchyma and form tumor nodules^[15]. These lesions rarely contain lung epithelial cells but they have the characteristics of cartilage and immature bone resembling well-differentiated osteosarcoma.

No transformation has been proven so far for human MSCs when expanded properly *ex vivo* (*i.e.*, non-exhausted and not forced to cell crisis)^[14]. The Canadian Critical Care Trials Group has recently published a meta-analysis of randomized, non-randomized, controlled and uncontrolled, phase I and phase II clinical trials^[16]; no association between autologous or allogeneic MSCs administration and tumor formation was reported in the 36 studies reviewed by them. Nonetheless, longer follow-up is required to draw a final conclusion regarding human

MSCs' tumorigenic potential.

MSCs may promote tumor growth

Human bone marrow-derived MSCs have increased the growth of ERA α positive breast cancer cell lines (T47D, BT474 and ZR-75-1) in an *in vitro* three-dimensional tumor environment, but have had no effect on an ERA α negative cell line (MDA-MB-231)^[17]; however, the growth rate of another ERA α negative cell line (MDA-MB-468) was high in the presence of human MSCs. Another study has shown that both human fetal MSCs and human adipose-derived MSCs transplanted subcutaneously into BALB/c-nu/nu mice alone or together with tumor cell lines F6 or SW480 (ratio 1:1 or 1:10), favored the growth of these tumor cell lines^[18].

Tumor cells obtained from primary breast cancer grown in the presence of human bone marrow-derived MSCs (ratio 1:1) and tested in secondary mice have been seen to have greater tumor-producing ability than cells obtained from primary tumors and grown in the absence of MSCs^[19]. Besides, tumor incidence and/or size^[18,20,21] as well as tumor vascularity^[22] have all increased when breast, lung, colon or prostate tumor cells have been co-injected with human adipose-derived or bone marrow-derived MSCs. The same has been proven for osteosarcoma, melanoma and glioma tumor cells^[23]. Another interesting observation concerned adipose tissue implant adjacent to lung cancer or Kaposi sarcoma xenografts resulting in a substantial increase in tumor size along with the appearance of stromal cells from the implant; adipose-derived MSCs can thus promote tumor growth^[24].

MSCs' innate tropism for established tumors has been widely reported^[24], yet the mechanism behind it still remains to be fully elucidated^[25]. The explanation advanced to date is that tumors behave as unresolved wounds as their stroma closely resemble healing granulation tissue and they produce cytokines, chemokines and other chemoattractants^[26] and MSCs chemotactic properties are similar to those of leukocytes^[27,28]. MSCs tropism for tumors has been successfully exploited for the delivery of antitumor agents in animal models of lung and breast cancer and melanoma and glioma^[25].

MSCs might promote metastasis

Breast cancer cells co-cultured with human bone marrow-derived MSCs (ratio 1:1) up-regulate the expression of oncogenes and proto-oncogenes associated with tissue invasion, angiogenesis and apoptosis (*i.e.*, N-cadherin, vimentin, Twist, Snail and E-cadherin)^[29]. Such molecular changes have been accompanied by morphological and growth alterations, these being features of a more metastatic phenotype. It has been seen that 0.5×10^5 breast cancer cells co-injected subcutaneously with 1.3×10^6 human bone marrow-derived MSCs have significantly increased lung metastasis rate in NOD/SCID mice. This effect was lost when bone marrow-derived MSCs were injected

separately from tumor cells^[20]. On the other hand, it has been shown that bone marrow-derived MSCs facilitate cancer cells [MCF-7, T47D low invasive cell lines and stromal cell-derived factor 1 (SDF-1)^{null} MDA-MB-231 highly aggressive ones] homing into bone marrow and have modified the metastatic niche through trophic factor secretion (SDF-1 and CXCR4) and improved neovascuogenesis in a xenogeneic mouse model^[30].

MSCs might inhibit tumor growth

It has been shown that human bone marrow-derived MSCs interfere *in vitro* with small cell lung cancer (A549), esophageal cancer (Eca-109), Kaposi's sarcoma and leukemic cell line proliferation kinetics^[31]. The foregoing was observed when 0.5×10^5 tumor cells were co-cultivated with 0.5×10^5 human bone marrow-derived MSCs but also when they were exposed to MSCs-conditioned medium; cells were arrested during the cell cycle G₁ phase in both cases by the downregulation of cyclin D2 and induction of apoptosis^[32,33]. MSCs from other sources, including human fetal skin-derived MSCs and adipose-derived MSCs, have also inhibited the growth of human liver cancer cell lines^[34], breast cancer (MCF-7)^[35] and primary leukemia cells by reducing their proliferation, colony formation and oncogene expression^[22]. The intravenous injection of 4×10^6 human bone marrow-derived MSCs into Kaposi's sarcoma-bearing nude mice has inhibited tumor cell growth^[36]. A similar effect has been observed in an animal model of hepatocellular carcinoma and pancreatic tumors as altering cell cycle progression has led to decreased cell proliferation^[22,37]; the same has happened with melanoma due to increased apoptosis of capillaries^[38] and rat colon carcinoma growth has been inhibited when rat MSCs (the MPC1cE cell line) were co-implanted with tumor cells in a 1:1 or 1:10 ratio^[39].

Human fetal skin-derived MSCs (Z3 cell line) have also delayed liver tumor growth and decreased tumor size when injected with the same number of cells from the H7402 cell line in SCID mice^[34]. Injecting human adipose-derived MSCs (1×10^3 cells/mm³) into established pancreatic cancer xenografts has led to apoptosis and the abrogation of tumor growth in female Swiss nude (athymic) mice^[37].

The role of MSCs in cancer thus remains paradoxical. Evidence to date has suggested that they are pro- as well as anti-tumorigenic^[40-42] such discrepancy seems to depend on isolation and expansion conditions, cell source and dose, the administration route and the tumor model used.

MSCs AND INFECTION

MSCs might increase infection

MSCs can be recruited into inflamed sites secondary to microbial infection where they promote potent immune-suppressive activity^[43,44]. For instance, it has

been shown that administering MSCs (1.25×10^5 cells/kg) to animals infected by *Trypanosoma cruzi* (*T. cruzi*, protozoa) or *Mycobacterium tuberculosis* (*Mtb*, bacteria) has worsened the natural course of infection. Activated macrophages play an essential role in host defense against *T. cruzi* as they can destroy intracellular parasites *via* interferon (INF)- γ - and tumor necrosis factor (TNF)- α -stimulated nitric oxide (NO) production^[45]. It has been shown that mice bone marrow-derived MSCs switch macrophages to an anti-inflammatory profile, thereby suppressing inflammatory cytokine production and enhancing interleukin (IL)-10 production^[46]. An immune response to *Mtb* depends on IFN- γ -producing T-lymphocytes activating macrophages to produce NO^[47,48]. Bone marrow-derived MSCs (2.5×10^5 /kg) infusion into animals, which are normally resistant to this infection [transforming growth factor β (TGF- β) RIIDN transgenic mice], has resulted in making them susceptible to disease. Furthermore, it has been observed that donor MSCs have been recruited to the periphery of live bacteria-containing granuloma and have induced regulatory T-cell differentiation, thus resulting in immunosuppression.

A recent study aimed to prove the safety and feasibility of autologous bone marrow-derived MSCs infusion (1×10^6 cells/kg, two doses) into kidney allograft recipients, showing that three out of six enrolled patients developed an opportunistic viral infection^[49].

MSCs might decrease infection

Regarding fungal infection, the intravenous administration of an IL-17-producing sub-population of bone marrow derived-MSCs (1×10^6 cells) significantly reduced the fungal burden of kidneys in immunocompetent mice, which had suffered invasive candidiasis^[50].

Both un-stimulated and IFN- γ stimulated human MSCs can inhibit the growth of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, as well as the growth of Gram-positive pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, group B *Streptococci* and *Enterococcus faecium*^[46,51]. MSCs' antimicrobial effect depends on whether they have been stimulated^[51]; while cathelicidin LL-37 antimicrobial peptide is critical to un-stimulated human MSCs, tryptophan-catabolizing enzyme heme oxygenase-1 and indoleamine-2,3-dioxygenase (IDO) are needed in IFN- γ -stimulated human MSCs. Tryptophan depletion and toxic kynurenine accumulation leads to the inhibition of bacterial growth in the latter case. Differences between human and murine MSCs antibacterial activity have also been reported; murine MSCs do not produce cathelicidin LL-37 and cannot express IDO, even after stimulation with a combination of cytokines, but they do produce lipocalin 2 (an antimicrobial molecule)^[52].

Little data has been published concerning the

impact of MSCs on viral pathogens. One study has reported that IFN- γ -stimulated human MSCs have reduced intracellular replication of cytomegalovirus and herpes simplex virus type 1 *in vitro*^[46], such effect being attributed to IDO activity^[46].

Together with MSCs' direct antimicrobial effect, it has been shown that they play an important role in the complex network of host immune response against pathogens, particularly regarding the dynamic coordination of the immune system's pro- and anti-inflammatory components^[53].

MSCs' antimicrobial activity observed *in vitro* has been clearly supported by animal models of experimental infection, such as polymicrobial sepsis^[42], lipopolysaccharide (LPS) administration^[54] and pulmonary respiratory distress syndrome^[55]. Regardless of MSCs source, administration route (intravenously *cf* intraperitoneally) or strategy (prophylaxis *cf* therapy), their administration leads to reduced pathogen burden and significantly improved survival rate^[41,42,53,56].

It has been shown that administering 2.5×10^5 mouse bone marrow-derived MSCs led to decreased mortality, controlled multi-organ dysfunction/injury and reduced pulmonary and systemic inflammation in a clinically relevant model of polymicrobial sepsis where infection was settled after the inoculation of Gram-negative and Gram-positive organisms^[42]. An endotoxemic rat model (involving intravenous LPS injection) has been used to demonstrate that administering 2.5×10^5 human adipose-derived MSCs decreased inflammatory cytokine level in serum and the lungs, reduced inflammatory changes in the lungs, prevented apoptosis in the kidneys and reduced multi-organ injury^[54]. A pulmonary respiratory distress syndrome model (induced by intratracheal endotoxin administration) has been used to show that the intrapulmonary delivery of mouse bone marrow-derived MSCs has down-regulated an LPS-induced inflammatory response and reduced lung injury, while direct lung injury by toxins or pneumonitis led to severe pulmonary edema and inflammation^[57,58].

MSCs' antimicrobial effect has also been demonstrated in the blood, peritoneum, liver and spleen, using a Gram-negative pneumonia model involving immunocompetent mice^[42,44,56].

As MSCs might lessen the development of infection, they have been used recently in a clinical study aimed at treating patients suffering acute respiratory distress syndrome (NCT01902082); one intravenous dose of 1×10^6 cells/kg allogeneic adipose-derived MSCs proved to represent a safe and feasible therapeutic tool for this infection^[59].

MSCs have been seen as an innovative therapeutic tool for preventing or treating graft-versus-host disease (GvHD) following allogeneic hematopoietic stem cell transplant (HSCT)^[60,61] owing to their immunosuppressive properties, such as not eliciting immunological responses from alloreactive T-lymphocytes and/or other immunological effector cells. However, it is not

known whether using immunosuppressive MSCs may inadvertently inhibit antimicrobial immune responses and ultimately result in an increased risk of infection in allogeneic HSCT recipients^[62], considering that infection is one of the major complications following an HSCT contributing to high morbidity and mortality indexes^[63,64]. One open randomized clinical trial has demonstrated that acute grade II-IV and chronic GVHD incidence in 10 patients receiving a median 3.4×10^5 /kg MSCs dose from a human leukocyte antigen-identical sibling donor was lower than in 15 patients who did not receive MSCs (11% *cf* 53% and 14% *cf* 29%, respectively)^[65]. Unfortunately, this did not mean a lower risk for infectious complications. Early and mid-phase severe infection incidence was even higher in patients who had received a co-transplant of hematopoietic stem cells and MSCs compared to a control group, which did not receive MSCs, although differences were not statistically significant [4/10 (40%) *cf* 5/15 (33%)]. Patients receiving MSCs suffered from cytomegalovirus (CMV) interstitial pneumonia and bacterial and/or fungal infection whereas this was only seen in two of the patients who did not receive MSCs^[65]; no patient treated with MSCs died because of infectious complications, whereas this happened in two control group patients who did not receive MSCs. This raises the question of whether infection severity is lower when MSCs are co-transplanted with a graft.

By contrast, another two non-randomized clinical trials, involving 20 patients^[66] and 14 pediatric patients^[67], showed that co-transplanting MSCs did not result in higher infection incidence and severity when compared to historical controls.

On the other hand, multivariate analysis regarding a retrospective cohort study of 691 HSCT patients showed that GVHD grade II-IV, CMV infection and having received human bone marrow-derived MSCs were factors which were associated with overall pneumonia-related deaths^[68].

Thus, the role of MSCs in infection is paradoxical. Evidence reported to date suggests that there may be pro- as well as anti-microbial effects^[40-42] and this seems to depend on isolation and expansion conditions, cell origin and dose and administration route and timing.

MECHANISMS BEHIND MSCs CANCER-INDUCING EFFECT

MSCs modify cancer cells

Although the cancer stem cell (CSC) concept was first introduced in hematological malignancies (chronic and acute leukemia)^[69], it has been identified during recent years in a variety of solid tumors such as glioblastomas, medulloblastomas and carcinomas^[70]. It has been demonstrated that MSCs interact with CSC in human cancer and regulate their own self-renewal through cytokine networks involving IL-6 and CXCL7^[19]. CSC-produced IL-6 interacts with IL6R/gp130 expressed on MSCs to produce CXCL7; this molecule

interacts with CSCs through the CXCR2 receptor where it induces the synthesis of others cytokines (*i.e.*, IL-8, IL-6, CXCL6, and CXCL5)^[19]. These cytokines trigger CSC self-renewal and enhance their invasive properties while IL-6 mediates chemotaxis, which may facilitate MSCs homing to primary tumor growth sites. It has been shown that MSCs administered subcutaneously in mice having had a breast tumor xenograft became recruited to the tumors and produced IL-6 and IL-8, which accelerated their growth by regulating the CSC population^[20].

MSCs might induce epithelial-to-mesenchymal transition

Most malignancies have an epithelial origin, and cancer progression is often associated with epithelial-to-mesenchymal transition (EMT)^[71]; this is a physiological process, which is recognized as being crucial for embryogenesis and wound healing. It involves epithelial cell conversion to mesenchymal cells through the disruption of cell-cell junctions and the reorganization of the actin cytoskeleton; EMT has gained much attention recently due to its role in converting benign lesions into invasive and metastatic tumors^[72]. It is governed by complex networks, which are influenced by signals from the neoplastic microenvironment, such as collagen, cytokines and TGF β , epidermal growth factor, fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and platelet-derived growth factor^[71-73]. Interestingly, all the aforementioned factors are secreted by MSCs^[9].

MSCs modify vasculogenesis

Vasculogenesis plays a critical role in tumor growth^[74]; MSCs could contribute towards tumor vasculogenesis because they act as pericytes but may also differentiate into endothelial cells and secrete provasculogenic factors^[75-77], thereby allowing blood vessel formation^[75]. Vascular endothelial growth factor (VEGF) and FGF-2 are the two main MSCs-secreted vasculogenic factors involved in tumor neovascularization^[76]. VEGF is known to regulate MSCs mobilization and recruitment to neovascularization sites and directs MSCs differentiation to vascular cell^[78,79]. VEGF expression in MSCs can be enhanced by hypoxia, a common phenomenon in tumor tissue^[80] whilst FGF-2 is a potent mitogen which is produced and secreted by endothelial cells and MSCs^[81]. This factor has been implicated in cell proliferation and endothelial cell migration during tumor growth^[81]; conversely, MSCs appear to reduce vascular density due to endothelial cell cytotoxicity in certain conditions^[38].

MSCs modify anti-cancer immune response

MSCs suppress both innate and adaptive immune responses^[82,83]; they inhibit CD4⁺ and CD8⁺ T-cell proliferation^[84] by producing a wide range of mediators, including TGF β 1, HGF, insulin-like growth factor, prostaglandin E₂, NO, heme oxygenase-1 and IDO^[85-89]. MSCs also inhibit monocyte and hematopoietic progenitor proliferation and differentiation into mature dendritic

cells^[32,90]. Other MSCs-induced effects regarding dendritic cells would be a loss of their ability to stimulate allo-responses^[91], acquiring a regulatory phenotype due to the production of large amounts of IL-10^[91] and changing dendritic cells' cytokine secretion profile by MSCs-derived PGE₂^[91]. MSCs alter the natural killer (NK) cell phenotype besides suppressing their proliferation and cytokine secretion^[92]; this requires cell-to-cell contact and soluble factors (TGF β 1 and PGE₂). Hence, MSCs could promote an anti-inflammatory response within a tumor, thereby allowing its enlargement^[93]. Systemically administered MSCs have promoted immune-tolerance in damaged organs, irrespective of whether donor cells home into them^[7,8,94]. It is expected that MSCs would worsen the immune-destruction of tumor cells and thus facilitate tumor growth and metastasis. Conversely, increased macrophage and granulocyte infiltration in MSCs-injected tumors has been shown, suggesting that allogeneic MSCs immunogenicity might contribute towards their antitumor effect^[32,39].

Changes in MSCs microenvironment, together with changes in transformed cells, would also seem to contribute towards carcinogenesis^[95].

MECHANISMS BEHIND MSCs INFECTION ADVERSE EFFECTS

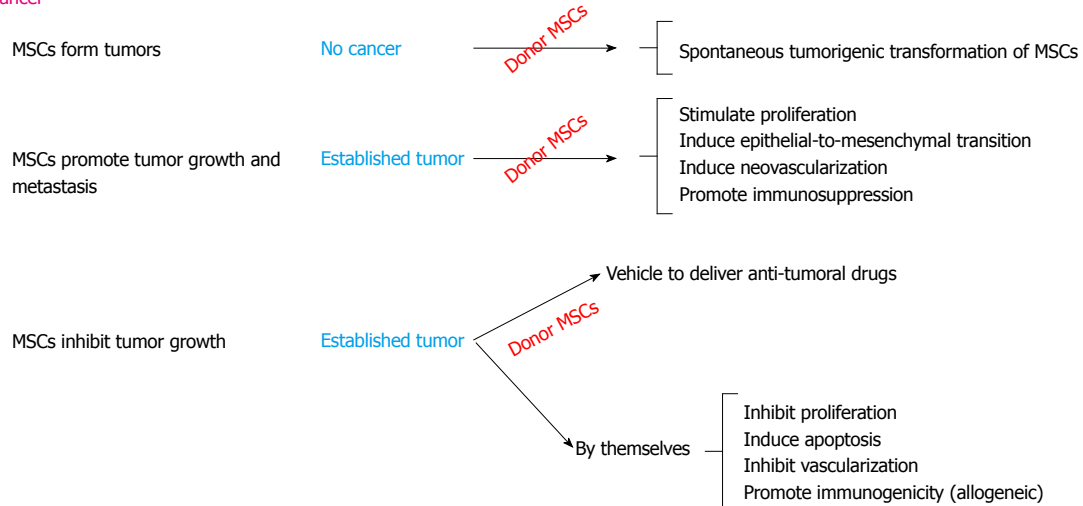
MSCs modify bacterial growth inhibition and clearance

MSCs can participate in host defense through the secretion of antimicrobial peptides (cathelicidin LL-37^[51] and lipocalin 2^[52]), which can directly inhibit bacterial growth or kill the pathogens. The secretion of these soluble peptides improves resident phagocyte ability to clear bacteria through the up-regulation of pathways associated with monocyte/macrophage, phagocytosis, NK cell activity and antigen presentation^[42] while MSCs antifungal activity means an increased amount of TH17 cells in the blood, thereby promoting TH1-type immune responses and restraining TH2-type ones^[50].

MSCs modify anti-microorganism immune response

MSCs induce a marked decrease in Toll-like receptor 2 expression, which plays a fundamental role in pathogen recognition and activation of innate immunity^[96]. MSCs induce a marked increase in macrophage susceptibility to infection by parasites and bacteria. The mechanisms so involved appear to be linked to the production of inflammatory cytokines TNF- α , IL-12p70 and IFN- γ which drive NO production^[43]. MSCs switch activated macrophages into regulatory ones producing low levels of pro-inflammatory cytokines. MSCs could modify an immune response against microorganisms by inducing apoptosis and cell-cycle arrest of T-cells by producing NO, TGF β or IDO^[91]. MSCs can inhibit cellular immune responses and promote regulatory T-lymphocyte production, thereby establishing T-cell tolerance for microorganisms^[97-100].

MSCs and cancer



MSCs and infections

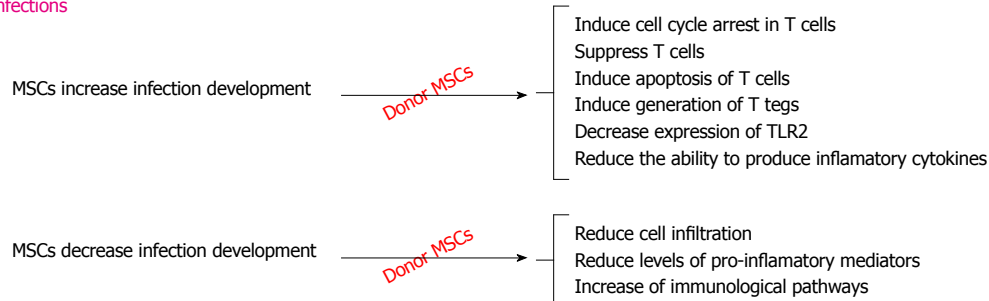


Figure 1 Paradoxical effect of donor mesenchymal stem cells in cancer and infection development. MSCs: Mesenchymal stem cells; TLR2: Toll-like receptor.

ARE CANCER AND INFECTION ADVERSE EFFECTS ARISING FROM USING MSCs IN THERAPY?

In vitro and *in vivo* studies have demonstrated MSCs' pro- and anti-cancer and pro- and anti-infection effects nevertheless, most clinical trials have reported that MSCs-based therapy appears safe and has not been associated with severe adverse events. Together, due to MSCs' context-dependent potential to produce immune-modulatory factors they seem to be an ideal therapeutic tool for both cancer and infections.

CONCLUSION

The pertinent literature reveals incongruity regarding the impact of MSCs on the development of cancer and infection (Figure 1); such paradoxical effect might be attributed to differences in isolation and expansion conditions, the source and dose of the cells being used, the administration route and its timing and host characteristics. MSCs immunomodulatory potential seems to be the leading mechanism responsible for such effects. Until conclusive data becomes available, cancer and infection will still be seen as adverse effects and therapeutic targets for using MSCs-based therapy.

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Mitochondria as therapeutic targets for cancer stem cells

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somatic stem cells and are responsible for tumor initiation, chemoresistance, and metastasis. Evidence for the CSCs existence has been reported for a number of human cancers. The CSC mitochondria have been shown recently to be an important target for cancer treatment, but clinical significance of CSCs and their mitochondria properties remain unclear. Mitochondria-targeted agents are considerably more effective compared to other agents in triggering apoptosis of CSCs, as well as general cancer cells, *via* mitochondrial dysfunction. Mitochondrial metabolism is altered in cancer cells because of their reliance on glycolytic intermediates, which are normally destined for oxidative phosphorylation. Therefore, inhibiting cancer-specific modifications in mitochondrial metabolism, increasing reactive oxygen species production, or stimulating mitochondrial permeabilization transition could be promising new therapeutic strategies to activate cell death in CSCs as well, as in general cancer cells. This review analyzed mitochondrial function and its potential as a therapeutic target to induce cell death in CSCs. Furthermore, combined treatment with mitochondria-targeted drugs will be a promising strategy for the treatment of relapsed and refractory cancer.

Key words: Cancer stem cells; Mitochondria; Relapsed and refractory cancer; Therapeutic target; Mitochondrial energy metabolism

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Core tip: This review is devoted to the analysis of mitochondrial function as a therapeutic target to induce cell death in cancer stem cells (CSCs). In particular, we focused on the differences in energy metabolism and features between CSC and non-CSC mitochondria, and between CSCs and normal stem cells. We described the roles of mitochondria that may make CSCs more susceptible to anti-cancer treatment and apoptosis, and how these may be useful to develop novel strategies for cancer treatment, such as through combined therapy

Abstract

Cancer stem cells (CSCs) are maintained by their

with specific mitochondrial-targeting drugs.

Song IS, Jeong JY, Jeong SH, Kim HK, Ko KS, Rhee BD, Kim N, Han J. Mitochondria as therapeutic targets for cancer stem cells. *World J Stem Cells* 2015; 7(2): 418-427 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i2/418.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i2.418>

INTRODUCTION

Over the last decade, cancer therapies have improved the quality of life of cancer patients. However, although almost all developed anti-cancer drugs are apparently successful following initial therapy, secondary tumors development and disease relapse is common. The limitation of classical anti-cancer therapies has been attributed recently to the existence of cancer stem cells (CSCs), which are quiescent, have relatively small population, and highly drug-resistant cells. CSCs act like stem cells (SCs) and are responsible for cancer growth and metastasis^[1]. Through the continued effort of many researchers, CSCs features have been revealed, such as anti-cancer drug resistance, metastasis, proliferation, hypoxic tolerance, and the capacity for neovessel induction^[2,3].

Mitochondria-targeted drugs may overcome potentially the drug-resistance mechanisms that have progressed toward conventional chemo-therapeutics in cancer^[4-7]. Mitochondria produce ATP, but they also mediate cell death and produce reactive oxygen species (ROS). Although ROS are affected in the regulation of various cellular responses, excessive production may be harmful to the cell^[8]. Cancer cells also exhibit extensive metabolic rearrangement that makes them more susceptible to alteration of mitochondria than normal cells^[9,10]. However, mitochondrial properties of CSCs in tumors remain unknown.

This review analyzed the potential role of mitochondria as a therapeutic target for inducing cell death in CSCs. In particular, we focused on the differences in energy metabolism and mitochondrial features between CSCs and non-CSCs, as well as between CSCs and normal SCs, and how these unique features of CSCs may increase the susceptibility of CSCs to anti-cancer treatment and apoptosis induction. We described how CSC mitochondria may be useful targets for the development of novel cancer treatment strategies, such as targeting CSCs *via* combination therapy with specific mitochondrial-targeting drugs.

CURRENT STATUS OF CSCs

History

The concept of CSCs is many decades old^[11]. In the middle of 1800s, the embryonal rest theory of cancer introduced the idea that cancer arises from SCs, but

the existence of CSCs in tumors could not be verified due to a lack of techniques. Furth *et al.*^[12] first alluded to CSCs in 1937 when they showed that a single cell within a tumor initiates the generation of new tumor in a recipient mouse^[12]. This finding was defined in the 1960s and 1970s by the development of quantitative methods to measure the tumorigenic ability able to sustain tumor growth *in vivo*. In the middle of 1900s, Radiolabeling permitted the measurements of cellular phenotype such as cell proliferation, lifespan, and hierarchical organizations within normal tissues^[13]. Al-Hajj *et al.*^[14] and Singh *et al.*^[15] represented that a small subset of cells within breast and brain tumors can be isolated prospectively and can generate phenotypically heterogeneous tumor *in vivo*. Thus, these various evidences represent that diverse solid tumors are organized hierarchically and sustained by a distinct subpopulation of CSCs.

Identification of CSCs

CSCs are classified according to several properties such as the presence of cell surface markers and their occupancy in the Fluorescence Activated Cell Sorting (FACS) analysis. Flow cytometry with antibodies against cell surface antigens has been the preferred method for characterizing and sorting normal stem cells. However, differences between CSC and normal SC markers are not well defined, and CSCs and normal SCs share some surface markers.

Most of CSCs studies isolate CSCs marker or a combination of markers, which is expressed heterogeneously in a certain tumor type. Based on this marker heterogeneity, subpopulations including CSCs are isolated from original tumors and injected into immunodeficient mice, after which tumor growth is assessed several weeks or months later. Table 1 shows current CSC markers according to cancer types, as FACS markers allow for consistent sorting according to marker expression. For example, Al-Hajj *et al.*^[14] used a marker combination of the CD24 and CD44 as an indicator of breast CSC, and the CD133 marker has been shown to be both normal SC and CSC marker^[16-20].

Stem cells and CSCs

The first embryonic SC lines were developed from the inner cell mass of early embryos in 1998^[21]. In 1999 and 2000, it was discovered that it could produce different cell types through manipulating adult mouse tissues, indicating that stem cell differentiation and proliferation could be controlled externally. Both somatic SCs and CSCs generate numerous daughter cells, differentiate into a variety of cell types, actively express telomerase, activate anti-apoptotic pathways, increase active membrane transports, and metastasize^[22]. Moreover, SCs are induced to differentiate by niche signaling and outer environmental stimuli. Niche signaling keeps the undifferentiation of SCs until they are stimulated to

Table 1 Markers used to identify stem cells and cancer stem cells

Marker	Cancer origin	Marker properties	Ref.
ALDH1	Breast	Catalyzes the oxidation of aliphatic and aromatic aldehydes Converts retinol to retinoic acid	[81]
ABC135	Melanomas	AdSC ATP binding cassette family Involved in transport of sterol and other lipids	[82]
Bmi-1	Breast, prostate, leukemias, neuroblastomas	HSC, NSC, and AdSC marker	[83,84]
CD20	Metastatic melanomas	Hematopoietic marker	[85]
CD29	Breast, colon	AdSC marker	[86,87]
CD34	Leukemias, sarcomas	HSC, MSC marker	[88-91]
CD44	Breast, pancreas, colon, head and neck, prostate	Adhesion molecule related to metastasis HSC and pluripotent stem cell marker Normal prostate epithelial stem cell marker	[91-96]
CD49f	Prostate	Adhesion to extracellular matrix	[97]
CD90	Liver, breast, glioblastomas	Glycoprotein, role in stem cell differentiation MSC marker	[98-100]
CD113	Lung, pancreas, colon, glioblastoma, melanomas, etc.	HSC, NSC AdSC (colon) marker	[16-18,101-104]
CD117	Breast, ovarian, lung, glioblastoma	Progenitor cell marker	[105,106]
Oct4	Many carcinomas	Embryonic stem cell and induced pluripotent stem cell marker	[107,108]
Sca-1	Lung	Skin epithelial stem cell and HSC marker	[109]

AdSC: Adult stem cell marker; HSC: Hematopoietic stem cell; NSC: Neuronal stem cell; MSC: Mesenchymal stem cell.

generate new cells, suggesting a similarity with signaling pathways that govern normal SC proliferation. Local environment signaling can initiate CSC proliferation, and thus, trigger tumor initiation and growth^[23]. Therefore, SC markers and features may not be effective therapeutic targets for inhibiting CSC growth.

MITOCHONDRIA AND CANCER

Roles of mitochondria

As the main energy producers, mitochondria produce ATP using the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). However, they also generate ROS during this process, which are harmful to the cell if produced excessively. In addition, mitochondria play a crucial role for the regulation of cell death pathways and intracellular Ca²⁺ homeostasis. Mitochondria activate apoptosis by regulating the releasement of proapoptotic proteins space to the cytosol from the mitochondrial intermembrane^[7], and they also play a crucial role in non-apoptotic cell death^[24].

Key regulators related to cell death and other cellular processes in the mitochondria are frequently altered in cancer cells^[8], as cancer cell mitochondria differ functionally and structurally compare with that of normal cells^[25]. Fast growing tumors result in hypoxia because of an inadequate amount of oxygen from the local vasculature. In addition, cancer cells include the DNA mutation of mitochondria and nucleus, which affect the OXPHOS components and result in ROS overproduction, wasteful ATP production, and mitochondrial oxidative damage^[25]. Warburg^[26] pioneered research on the cancer-related alterations in mitochondrial respiration and suggested a mechanism to explain how they progress during the tumorigenesis.

The proposed mechanism differs from that in non-malignant cells utilizing OXPHOS. Although aerobic glycolysis has been corroborated in cancer cells, the function of mitochondria has been controversial^[27]. In cancer cells, the aerobic glycolysis generate glycolytic intermediates to the pentose phosphate pathway. Moreover, the glycolytic ATP generation is important for survival in hypoxic conditions^[28]. In OXPHOS, the ATP synthesis requires much oxygen, which leads to continuous the ROS production such as superoxide anion, organic peroxide, and hydrogen peroxide^[29]. If the generated ROS are not eliminated by redox regulating system, they may cause cellular damage.

Mitochondrial antioxidant system

Mitochondria have a multi-level network of redox-defense systems for the elimination of hydrogen peroxide (Figure 1). Glutathione and glutathione peroxidases require nicotinamide adenine dinucleotide phosphate (NADPH) for the reduction of H₂O₂ and other peroxides generated in the mitochondria. Mitochondrial redox balances are also regulated by the mitochondrial inner membrane electrochemical gradient, which mitochondrial Complex V (ATP synthase) uses to produce ATP from ADP and inorganic phosphate (Pi).

Moreover, the physiological significance of mitochondrial redox balance has been highlighted by the antioxidant genes-deletion and over-expression. As antioxidant defense system, Peroxiredoxin (Prx) 3, Prx5, superoxide dismutase 2, and thioredoxin 2 eliminates ROS produced in mitochondria^[30,31]. Knockout (KO) of Prx3 mice result in induction of oxidative damage^[32], KO of thioredoxin 2 mice showed an embryonic lethal phenotype^[33] and KO of superoxide dismutase 2 mice die within 3 wk of birth because of mitochondrial

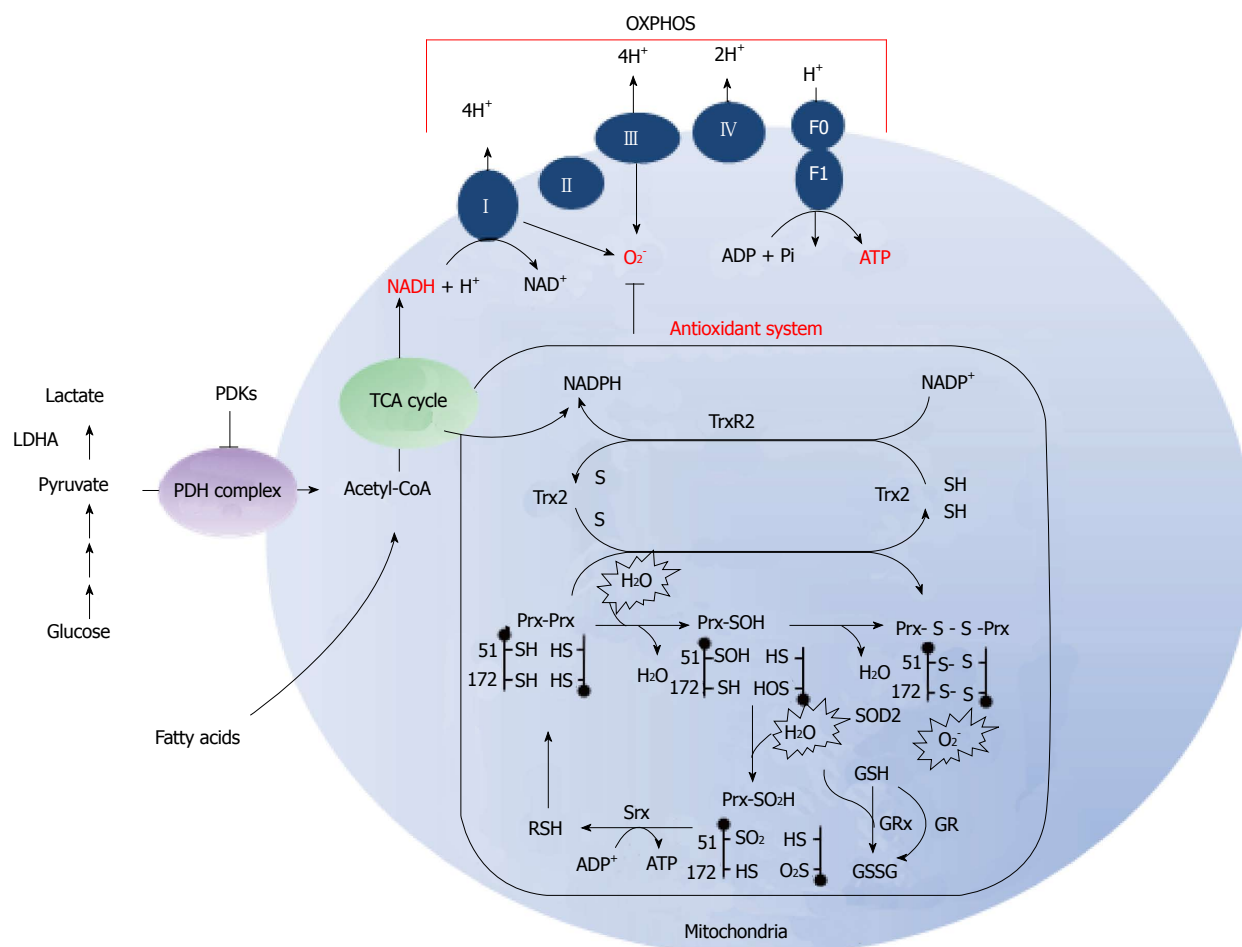


Figure 1 Antioxidant and oxidative phosphorylation systems in mitochondria. Under normal conditions, normal cells rely primarily on oxidative phosphorylation for ATP synthesis, whereas cancer cells rely more on glycolysis. Pyruvate from glycolysis is converted to acetyl-CoA, CO₂, and NADH by pyruvate dehydrogenase (PDH). Acetyl-CoA enters the TCA cycle by the citrate synthase-mediated reaction with oxaloacetate to generate citrate. NADH is oxidized first by Complex I in the electron transport chain (OXPHOS). Electrons from Complex I and II are transferred to coenzyme Q10, then passed on to Complex III, cytochrome c, Complex IV, and finally to O₂ to generate H₂O. O₂ is converted to H₂O₂ through the action of SOD2 and/or spontaneous dismutation. H₂O₂ is eliminated by three mechanisms: (1) glutathione (GSH) peroxidase (GPx) coupled to GSH and GSH reductase (GR); (2) Prx3 coupled to Trx2 and Trx reductase (TrxR 2); and (3) non-enzymatic eliminating by redox compounds. The H₂O₂ selectively oxidized cysteine Cys-SH to Cys-SOH, which then reacts with the resolving cysteine Cys-SH of the other subunit in the homodimer to form an intermolecular disulfide bond. The disulfide bond is reduced by Trx2. Moreover, the generated Cys-SOH is oxidized to Cys-SO₂H. Reactivation of the enzyme is achieved by reduction of the Cys-SO₂H moiety and is catalyzed by sulfiredoxin (Srx). Nicotinamide adenine dinucleotide phosphate (NADPH) is utilized by the reductases in the peroxidase system (GR and TrxR) to reduce disulfide bonds formed in proteins during the elimination of H₂O₂. TCA: Tricarboxylic acid; ATP: Adenosine triphosphatase; ADP: Adenosine diphosphate.

oxidative damage and severe neurodegeneration^[34,35]. Therefore, the inhibition of antioxidant systems may provide a targeted therapy that leads to mitochondrial dysfunction and cell death.

Mitochondrial membrane potential

Mitochondria harbor a robust mitochondrial trans-membrane potential ($\Delta\Psi_m$), and the exchange of small metabolites between the mitochondrial matrix and the cytosol is induced by the low conductance of permeability transition pore complex (PTPC)^[36]. The rupture of mitochondrial membranes leading to functional impairment result in the release of toxic mitochondrial intermembrane space proteins, such as apoptosis-inducing factor and cytochrome *c*, into the cytosol^[37]. Under apoptotic conditions, including ROS and Ca²⁺ overload, the PTPC presumes a high conductance state allowing uncontrolled influx of small solutes

into the matrix of mitochondria. This mitochondrial permeability transition (MPT) leads to osmotic swelling of the mitochondrial matrix and dissipation of the $\Delta\Psi_m$ ^[38,39], and eventually cell death occurs due to mitochondrial outer membrane permeabilization^[40]. The MPT is triggered by reagents increasing ROS generation, cytosolic Ca²⁺ concentrations, or acting on the PTPC. Therefore, the induction of mitochondrial membrane permeabilization are attractive targets to develop drug for cancer therapy.

Mitochondria-targeted cancer therapy

As mentioned above, mitochondria play important role in apoptosis, but also trigger cell death through various mechanisms^[41-43]. Various mitochondria-targeted strategies for cancer treatment have been developed over the last decade^[6,44] that focused on the development of agents that regulate the MPT,

Bcl-2 family proteins, and ROS production in cancer^[6]. Numerous molecules, acting on mitochondria, are currently used or being tested in clinical trials^[45]. Several experimental anti-cancer drugs, such as ceramide^[46], CD437^[47], and MKT077^[48], and clinically approved anti-cancer drugs, such as etoposide^[49], paclitaxel^[50], and vinorelbine^[51], induce apoptosis *via* mitochondria dysfunction. Furthermore, determining of pathophysiological differences of mitochondria between cancer cells and normal cells, will improve the selectivity of mitochondria-targeted anti-cancer agents.

MITOCHONDRIA OF CSCs

Because mitochondria play a key role in the alteration of oxidative stress, energy status, and apoptotic stimuli, scientists have assumed that they are also involved in the regulation of stemness and differentiation in SCs. Researchers have attempted to employ mitochondrial properties in the selection of SCs^[52]. Lonergan *et al.*^[53] and Bavister^[54] suggested that functional mitochondrial characteristics, such as subcellular localization and metabolic activity could verify stemness, SC stability, and pluripotency. Mitochondria are localized in perinuclear sites in embryonic stem cells (ESCs) and have a more scattered distribution throughout the cytoplasm after differentiation and senescence^[55].

Mitochondrial metabolic activity is also related to cell differentiation, as early passages of an adult primate stromal cell line have a higher oxygen consumption rate (OCR) and a low ATP/ mitochondrial DNA content compared with long-term cultured cells^[53]. In CD34⁺ hematopoietic SCs, a low mitochondrial OCR and mitochondrial mass result in a predominantly perinuclear mitochondrial arrangement^[56].

Antioxidant enzyme expression also shows a dramatic change during differentiation^[57]. Moreover, ROS play an agonistic role in the differentiation of ESCs. Enhanced intracellular ROS as the differentiation stimulus may act on transplanted SCs into the cardiovascular lineage^[58], indicating that mitochondrial redox metabolism act as a crucial regulator in cardiac differentiation of SCs. Furthermore, Plotnikov *et al.*^[59] suggest a correlation of the mitochondrial function and the status of neural SCs.

SC mitochondria play important roles in maintaining stemness and differentiation. However, whether the roles of CSC mitochondria are similar to SC mitochondria or cancer cells in general is uncertain. Two hypotheses on the origin of CSCs, both of which contribute to acute myeloid leukemia^[1,60], have been proposed. One hypothesis of the origin of CSCs is that they are derivatives of SCs residing in various organs. Genetic mutations and epigenetic changes, which are crucial for initiation and progression of tumor growth, accumulate in long-lived stem cells, and the transformation of SCs into CSCs initiates carcinogenesis.

CSCs may also have a greater differentiation potential than other SCs. (SCs can be divided into the following groups based on differentiation potential: the totipotent, pluripotent, multipotent, and unipotent group). Another hypothesis assumes the existence of ESC-like cells that convert into CSCs when they are exposed to damaging environmental factors. Additional differentiation and mutation of these cells may also contribute to development of CSCs^[61]. Based on these reports, the CSCs may be more differentiated than normal SCs and likewise, the mitochondrial properties of CSCs are different from those of SCs or general cancer cells.

Recently, Ye *et al.*^[62] determined the mitochondrial features between lung CSCs and non-CSCs. As a results, it is showed a lower mtDNA contents, lower OCR, glucose consumption, intracellular ATP and ROS level in the lung CSCs compared to non-CSCs. Leukemia CSCs showed a low ROS level and reduced OXPHOS compared with that of non-CSCs^[63]. However, Pastò *et al.*^[64] reported that CSCs exhibited over-expressed genes related to glucose uptake, oxidative phosphorylation, and fatty acid β -oxidation, indicating higher ability to direct pyruvate towards the TCA cycle. As reported, ovarian CSCs showed higher mitochondrial ROS production and $\Delta\Psi_m$ than non-CSCs. In addition, targeting mitochondrial biogenetics induced caspase-independent cell death in ovarian CSCs^[65]. In glioma CSCs, a higher mitochondrial reserve capacity was measured as compared to the differentiated cells^[66]. Glioblastoma CSCs also depend on OXPHOS for their energy production and survival^[67]. Besides, breast CSCs have higher ATP content compared to their differentiated progeny^[68]. Based on these studies, CSCs mitochondria showed the different roles and features according to the cancer type. A summary of the mitochondrial features between CSCs and non-CSCs according to cancer origin is highlighted in Table 2. Although the mitochondrial features of CSCs in several cancers are not identical, CSCs mitochondria obviously differ from those of non-CSCs. Moreover, mitochondrial features of CSCs have not been clearly defined in other cancer types. Most importantly, little has been known about the mitochondrial features related to energy metabolism and the ROS/antioxidant enzyme system of CSCs in colon, stomach, liver, bone, and prostate cancer. Therefore, defining these features will be essential for developing a mitochondria-targeted therapeutic drug that induces death of CSCs, and therefore, reduces the risk of relapsed or refractory cancer.

CLINICAL IMPLICATION AND THERAPEUTIC TARGETS OF CSCs

Despite the recent surge of published studies on CSCs, the clinical significance of this population remains unclear and has been slow in progression of the development of clinical agents to eliminate CSCs. However, most experts agree that effective

Table 2 Mitochondrial features of cancer stem cells according to cancer origin

Cancer origin	Mitochondria features			Energy metabolism of CSC	Target/drug for CSCs	Ref.
	Feature	CSC	Non-CSC			
Breast	Glucose uptake	High	Low	OXPHOS		[68]
	ATP contents	High	Low			
	OCR	High	Low			
	Lactate production	Low	High			
Glioma	Membrane potential	High	Low	OXPHOS		[66]
	Glucose consumption	Low	High			
	ATP contents	High	Low			
	Lactate production	Low	High			
Leukemia	OCR	High	Low	OXPHOS	IMP-2	[67]
	ATP contents	High	Low			
	ROS	Low	High			
	Proliferation rate	Slow	Fast			
Lung	OCR	Low	High	Low glycolysis Low OXPHOS	Bcl-2/ ABT263	[63]
	Lactate production	Low	High			
	ATP contents	Low	High			
	Glucose consumption	Low	High			
Ovarian	OCR	Low	High		NV-128	[65] [64]
	ROS level	Low	High			
	ATP contents	Low	High			
	Membrane potential	High	Low			
	Mitochondrial DNA	Low	High	OXPHOS		
	ROS	High	Low			
	Membrane potential	High	Low			
	ATP contents	High	Low			
	Glucose deprivation	Resist	Sensitive			

CSC: Cancer stem cell; OCR: Oxygen consumption rate; ROS: Reactive oxygen species; OXPHOS: Oxidative phosphorylation; ABT263: Bcl-2 inhibitor; NV-128: Isoflavone derivative (play a role as inhibitor of mitochondrial function); IMP-2: Insulin-like growth factor 2 mRNA-binding protein 2.

anti-cancer drugs should be targeted toward CSCs in addition to non-CSCs. Current cancer treatments such as conventional chemotherapy and radiotherapy target rapidly proliferating cells that make up the bulk of the tumor, but do not specifically target CSCs. Thus, the hypotheses on the origin of CSCs may explain the development of relapsed and metastatic cancer. In cancer therapy, the new paradigm requires development of novel anti-cancer drug molecules and drug targets to assess drug responses of CSCs.

Altered expression of genes involved in apoptosis, survival, and DNA repair machinery are among the multiple mechanisms responsible for the chemoresistance of leukemia^[69], brain^[70], pancreatic^[71], breast^[72], melanoma^[73,74], and colon cancer^[75] CSCs. Liu *et al.*^[23] reports that CD133⁺ glioblastoma cells isolated from patients have a high expression of genes in the Bcl-2 and inhibitor of apoptosis (IAP) families. Moreover, several types of CSCs have upregulated ATP binding cassette (ABC) pumps that make them resistant to various chemotherapeutics^[73,74]. Therefore, finding targets that efficiently promote CSC cell death is important and a focus of intensive research. Dong and colleagues demonstrate that loss of fructose-1,6-bisphosphatase in breast CSCs induces glycolysis, as well as inhibiting oxygen consumption and ROS generation, through the suppression of mitochondrial Complex I activity^[76]. The report implies

that overproduction of ROS and reduction in glucose metabolism may be effective against breast CSCs. Hirsch *et al.*^[77] showed that metformin, an AMPK activator and Complex I inhibitor often used as the first-line drug for treating diabetes, and selectively kills CSCs in breast cancer cell lines. The novel isoflavone derivative NV-128 significantly decreased mitochondrial function, as shown by a decreases in ATP, Complex I, and Complex IV levels, and induced cell death in ovarian CSCs^[65]. These results demonstrate that specific mitochondrial targeted compounds can induce cell death in chemoresistant CSCs and may be a new venue for treating ovarian cancer patients with relapsed or metastatic cancer. The new-generation taxoid SB-T-1214 significantly inhibited stemness gene expression profiles and induced cell death in both CSCs and general cancer cells, indicating its promise in overcoming relapsed and refractory cancer due to CSCs^[78]. Finally, mitochondria-targeted vitamin E succinate (MitoVES), which includes the positively charged triphenylphosphonium group, may be the most well-characterized toxic agent in its ability to induce apoptosis in breast CSCs^[79]. Meanwhile, it was reported that a drug which inhibits the self-renewal of CSCs by targeting of Notch and Hedgehog pathway has been developed^[80]. It was also reported that has been developed a drugs, which can eliminate CSCs by targeting cell surface markers such as CD133 and EpCAM. However, the use of these drugs increases

the exposure to side effects due to the sharing of signaling pathway and cell surface marker with normal SCs. Thus, it is important to understand how CSCs differ from normal SCs and differentiated cells. Moreover, a full understanding of the mitochondrial function and energy metabolism in CSCs contributes to the development of the agents targeting mitochondrial functions (such as ROS overproduction, energy metabolism inhibition, and antioxidant protein inhibition), and presents a need to develop new strategies to target CSCs in the clinical field^[80].

CONCLUSION

In summary, the mitochondria are an important tool to investigate CSCs properties and to develop anti-cancer drugs. However, the properties and clinical significance of mitochondria in CSCs have not been verified. Because mitochondria-targeted therapy may open new strategies for the treatment of relapsed and refractory cancer, mitochondrial properties unique to CSCs need to be defined. Furthermore, combined treatment with mitochondrial-targeted and anti-cancer drugs may specifically induce the death of both CSCs and general cancer cells and promises to be a novel cancer therapy.

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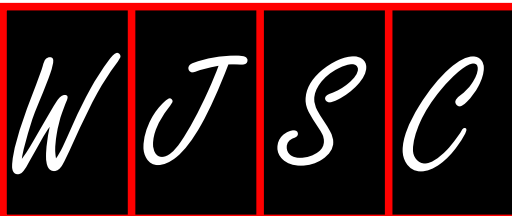
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Vital roles of stem cells and biomaterials in skin tissue engineering

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Tissue engineering was first defined in 1987 by the United States National Science Foundation which critically discussed the future targets of bioengineering research and its consequences. The principles of tissue engineering are to initiate cell cultures *in vitro*, grow them on scaffolds *in situ* and transplant the composite into a recipient *in vivo*. From the beginning, scaffolds have been necessary in tissue engineering applications. Regardless, the latest technology has redirected established approaches by omitting scaffolds. Currently, scientists from diverse research institutes are engineering skin without scaffolds. Due to their advantageous properties, stem cells have robustly transformed the tissue engineering field as part of an engineered bilayered skin substitute that will later be discussed in detail. Additionally, utilizing biomaterials or skin replacement products in skin tissue engineering as strategy to successfully direct cell proliferation and differentiation as well as to optimize the safety of handling during grafting is beneficial. This approach has also led to the cells' application in developing the novel skin substitute that will be briefly explained in this review.

Key words: Hair follicle stem cells; Skin repair; Tissue engineering; Chitosan; Collagen

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Core tip: Biomaterials and epithelial stem cells, and especially hair follicle stem cells are vital components for successful skin tissue engineering. Ignoring one of these components will decrease the opportunity for skin tissue engineering to foster complete healing through skin repair and will increase the failure of skin grafting in the clinical setting. The latest technology, new raw biomaterials and information on the significant contribution of stem cells are likely to be of great benefit to skin tissue engineering.

Abstract

Tissue engineering essentially refers to technology for growing new human tissue and is distinct from regenerative medicine. Currently, pieces of skin are already being fabricated for clinical use and many other tissue types may be fabricated in the future.

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INTRODUCTION

In classical bioengineered skin, the engineered epidermis is reconstructed from a skin autograft or allograft serving as a cellular dressing. The limitations addressed by this technique are pain and scar formation at the donor site, impaired wound healing and non-healing wounds, insufficient material to cover a large defect area and autoimmune rejection in the case of an allograft. The emergence of skin tissue engineering has led to robust innovation in skin substitutes and skin replacement products for burn and wound management. Diseased or injured skin may rule out a particular treatment because of wound depth. Superficial, partial and full-thickness wounds require diverse skin substitutes that simultaneously function as a primary dressing. Although various commercial skin substitutes are available, novel findings on fabrication techniques for biomaterials and on regulators of wound healing have highly encouraged scientists to develop new engineered skin substitutes that offer an effective remedy for wound care and wound management. The combination of stem cells or other cells with a specifically designed novel biomaterial has resulted in different impacts on engineered skin after wounding. An ideal biomaterial with multiple combinations of cultured cells and a collectively established broad knowledge of the healing process are the main criteria for the future development of skin substitutes. Eventually, skin substitutes that can be kept frozen, that are ready for use, inexpensive in cost, less labor intensive, and permanently adherent to the wound bed, that yield an impressive cosmetic outcome and that do not contain animal or human serum will definitely be in increased demand for skin tissue engineering purposes.

DEFINITION OF STEM CELLS

Stem cells have at least two advantageous properties. First, these cells are capable of dividing or renewing themselves for long periods of time with the identical morphology and phenotypic characteristics as their parent cells. This self-renewal mechanism is crucial for recovering degenerated and damaged cells, tissues or organs. Second, the differentiation of stem cells is a mechanism that can maintain the cellular and molecular integrity of adjacent or other cells. Stem cells differentiate into specialized cells to perform a specific function^[1] after receiving internal or external signals, which are signs of signalling pathway changes in the

cells' microenvironment. Differentiation starts when the cells discontinue renewal. Differentiation abilities are used to rank stem cells' potency (Figure 1). Stem cells that are able to differentiate into one cell type are known as unipotent stem cells, such as epidermal stem cells which regenerate differentiated epidermis^[2]. Hair follicle stem cells (HFSC) are able to differentiate into multiple types of structures with specialized functions in the body including hair follicles^[3], epidermis^[4], sebaceous glands^[5] and neurons^[6]. These stem cells are known as multipotent stem cells. Pluripotent stem cells are stem cells that have the ability to differentiate into ectoderm, mesoderm and endoderm germ layers as demonstrated by embryonic stem cells and induced pluripotent stem cells^[7]. Totipotent stem cells have the excellent ability to differentiate into whole tissues. This total potency can be observed in the fertilized eggs of humans or animals.

SKIN STEM CELLS

The skin is known as cutaneous or integumentary and its appendages constitute the integumentary system. The skin is the largest organ, contributing 15%-20% of body mass to form the external covering of the body. The skin has two main components: the epidermis and the dermis. The epidermis is derived from ectoderm, composed of a keratinized stratified squamous epithelium that grows continuously and simultaneously maintains its normal thickness by the process of desquamation^[8]. The dermis is derived from mesoderm, composed of a connective tissue that provides mechanical support, strength and thickness to the skin. The hypodermis is not a part of the skin but lies beneath the dermis and is equivalent to the subcutaneous fascia. This layer contains adipose tissue arranged into lobules that are separated by connective tissue septa. The epithelial skin appendages are composed of the hair follicles and hairs, sweat glands, sebaceous glands, nails and mammary glands. The skin forms an effective barrier against pathogen invasion, chemicals and ultraviolet light^[9]; participates in homeostasis^[10] by regulating the body temperature and water loss; conveys sensory information; and function in pigmentation^[11], physical appearance, wound repair and regeneration^[12]. The skin is considered as a stem cell zoo, as it accommodates a variety of stem cell niches. A niche is the microenvironment in which stem cells remain quiescent. The dermal papilla is a stem cell niche for mesenchymal stem cells (MSC) which initiate hair follicle growth^[13]. The superior bulge is a stem cell niche for HFSC^[14] and melanocyte stem cells (MeSC)^[15]. Multipotent HFSC not only function to maintain the hair growth cycle^[16], but also give rise to sebaceous glands^[17] and contribute to skin re-epithelialization^[18], which form neo-epidermis by differentiating into keratinocytes within the wound. MeSC supply melanin for hair and skin pigmentation^[19] and are concurrently involved in skin re-epithelialization^[20]. The abundant fat

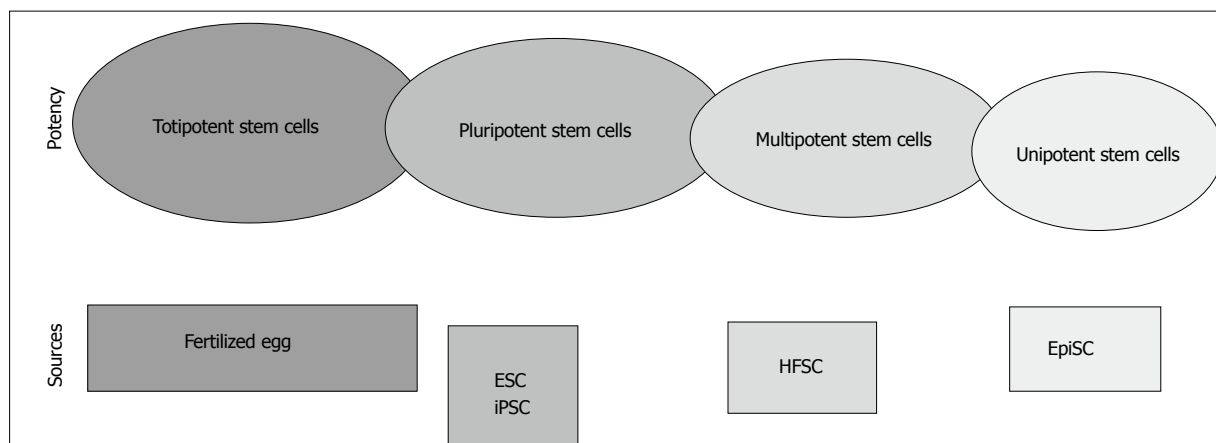


Figure 1 The potency of stem cells. ESC: Embryonic stem cells; iPSC: Induced pluripotent stem cells; HFSC: Hair follicle stem cells; EpiSC: Epidermal stem cells.

in the subcutaneous layer contains adipose stem cells (ASC)^[21] which are efficient in promoting skin repair^[22]. Collectively, the evidence suggests that skin's distinct resident stem cell pools have a crucial function in skin repair and regeneration. Therefore, it is very important for scientists to develop a good understanding of this idea before skin substitutes can be engineered in the future.

SKIN TISSUE ENGINEERING

Skin tissue engineering is the first field within tissue engineering that successfully engineered tissue in the laboratory. As the largest organ, the skin has high potential risk of injuries and diseases. Skin damaged requires a skin replacement product from either a natural or an artificial source. Skin replacement products are in high demand for the treatment of burns and wounds, leading the industrial sector to highly invest in skin tissue engineering. Skin tissue engineering utilizes biomaterials, stem cells, connective tissues and an established broad knowledge of the mechanism of the acute and chronic healing processes. The main target of skin tissue engineering is to produce an excellent skin replacement product for application in wound repair, especially in the case of full-thickness skin loss, with the goal of less or no scar formation after wounding. However, skin replacement products are not able to replace damaged skin as completely as native skin can, leaving a scar. Consequently, the field of skin tissue engineering is challenging.

BIOMATERIALS

Collagen gel is the first biomaterial or skin replacement product that has been used in skin tissue engineering to replace the use of allografts and autografts. Essentially, to produce an excellent skin substitute, a biomaterial must be able to support cell growth and differentiation in a similar manner as in the

cells' original microenvironment. Most importantly, biomaterials with multiple graded pore sizes allow the acceleration of tissue reconstruction^[23]. For example, microscopic pores improve cell attachment, proliferation and responses^[24] and macroscopic pores that are at least 100 μm in diameter play a role in enhancing the ingrowth of cells and blood capillaries^[25]. To allow three-dimensional (3-D) tissue reconstruction *in vitro*, a biomaterial should support uniform cell spreading into interconnected pores for the engineering specific tissue^[26]. Biomaterials with a single pore size have certain limitations, only allowing one cell type to grow according to the pore size whereas, biomaterials with multiple graded pore sizes can reconstruct various types of tissues simultaneously^[27]. A previous study has suggested that a pore size 20-120 μm in diameter is suitable for skin tissue engineering^[28]. Biomaterials must have the ability to absorb the nutrients involved in wound healing and the exudate of the wound bed which are important factors in skin tissue engineering. The absorption ability is known as the water uptake ratio (WUR). Biomaterials with a high WUR are suitable for use in full-thickness wounds that contain excess exudates and inflammation that lead to impaired wound healing. Meanwhile, a lower WUR is suitable for partial thickness wounds^[29]. An appropriate WUR enhances the biological activity of skin equivalents and contributes to hydrophilicity and the maintenance of 3-D structure. A moist wound bed is required to enhance dermal regeneration and wound closure which can be achieved if a scaffold has suitable water vapor permeability (WVP). The WVP depends on the scaffold thickness and the ratio of the scaffold area to the water surface area^[30]. A suitable WVP is an important factor for wound dressing. If the WVP is too high, the wound bed will be dry and it may increase metabolic activity. In contrast, if the WVP is too low, the accumulation of exudates will trigger the onset of bacterial growth. Following the criteria mentioned above, a fabricated skin replacement product will retain the cell-cell and cell-biomaterial signaling, allowing the complete layer

Table 1 Current skin replacement products

Brand	Cell and biomaterial	Wound type
Apligraf®, United States	Human keratinocytes and fibroblasts cultured on collagen	Full thickness
Biobrane®, United Kingdom	Silicone membrane attached to a nylon mesh	Partial thickness
CellerateRX®, United States	Gel containing 65% collagen	Superficial
Cryoskin®, United Kingdom	Keratinocytes cultured on silicone	Superficial
Dermagraft®, United States	Cryopreserved human fibroblasts from foreskin cultured on a polyglactin mesh scaffold	Full thickness
EpiDex®, Switzerland	HFSC cultured on silicone	Full thickness
EpiFix®, United States	Human amniotic membrane	Full thickness
Integra®, United States	Semi-permeable silicone membrane composed of bovine tendon collagen	Full thickness
MyDerm™, Malaysia	Human fibroblasts and keratinocytes cultured on fibrin-silk	Full thickness
OASIS™, United States	Porcine small intestinal submucosa	Full thickness
OrCel™, United States	Human keratinocytes and fibroblasts cultured on bovine collagen	Full thickness
PriMatrix, United States	Collagen from fetal bovine dermis	Full thickness
Transcyte™, United States	Human newborn fibroblasts cultured on nylon mesh	Full thickness

HFSC: Hair follicle stem cells.

of skin to be engineered. Subsequently, the engineered skin substitute can be used for grafting purposes, this is how skin tissue engineering works. Most importantly, the engineered skin must be tolerated by the host, be retained permanently and later be able to degrade slowly over time. A permanent skin replacement product is a primary dressing that is ideal for application in great skin loss because it is used only once during treatment, until healing is completed. In contrast, a temporary skin replacement product must be changed many times during treatment which may increase the cost of wound management and wound care. Commercial skin replacement products are collagen, chitosan or fibrin-based and these products have been broadly used in clinical applications as shown in Table 1.

Apligraf is a permanent cellular based skin replacement product for the treatment of non-healing wounds including diabetic foot ulcer and venous leg ulcer. It was approved by the United States Food and Drug Administration's guidelines (FDA) for use in non-infected, partial and full-thickness skin ulcers.

Biobrane is a temporary acellular based skin replacement product. It originated from porcine dermal collagen which is bonded to a nylon mesh and nylon membrane to form a biosynthetic skin dressing for use in superficial wound, partial-thickness wound and donor site wound dressing.

CellerateRX is a patented form of Type I bovine collagen for the treatment of diabetic or other impaired healing wounds. It is provided in a powder and a gel form which can be used as alone or a powder-gel combination.

Cryoskin is a cell spray-based skin replacement product and is prepared upon request by clinicians. It is made from allogeneic donor cells which are originally isolated from a newborn foreskin biopsy. Cryoskin spray is suitable for the treatment of chronic wounds and burns.

Dermagraft is a permanent cryopreserved cellular based skin replacement product. It composed of collagen which is isolated from neonatal foreskin biopsy and cultured on biodegradable mesh. Dermagraft was

approved by the FDA for use in diabetic foot ulcers.

EpiDex is a permanent autologous epidermal skin replacement product that is isolated directly from epithelial stem cells of patients. The patient's own hair was plucking using non-surgical procedure and then grafted onto a chronic wound.

EpiFix is a dehydrated amniotic membrane allograft. It is provided in sheets form, does not require FDA approval as human amniotic membrane is considered not significantly changed from its original structure. EpiFix is used in the treatment of acute and chronic wounds.

Integra is a bilayer engineered skin replacement product. It composed of porous matrix derived from bovine tendon collagen, glycosaminoglycan and a semi-permeable polysiloxane. Integra is used in the treatment of chronic and traumatic wounds.

MyDerm is an autologous bilayer engineered skin replacement product. It contained the mixture of fibrin-keratinocytes that seeded onto a piece of medical grade of silk. The mixture of fibrin-fibroblasts is then seeded onto fibrin-keratinocyte skin equivalent. MyDerm is used for the treatment of wounds and burns.

OASIS is a xenogeneic porous collagen matrix which derived from porcine small intestinal submucosa. It was approved by the FDA for the management of pressure ulcers, venous ulcers, diabetic ulcers and chronic vascular ulcers.

OrCel is an allogeneic bilayered cellular based matrix. It is biodegradable, made of composite bovine collagen which contained skin cells. OrCel was approved by the FDA for use in patients with dystrophic epidermolysis bullosa.

PriMatrix is a xenogeneic acellular based dermal matrix. It is derived from fetal bovine dermis. PriMatrix is provided in meshed, fenestrated and solid form. It was approved by the FDA for pressure and venous stasis ulcers.

Transcyte is an allogeneic bilayer skin replacement product for the treatment of burn. It made of human fibroblasts grown on nylon mesh and then combined with a synthetic epidermal layer. Transcyte has been

approved by the FDA for temporary covering over burn wounds.

Collagen

Collagen is the main protein component in connective tissue and its most abundant sources are found in mammals and marine animals. Collagen is easily obtained from porcine and bovine sources which provide bone, skin, tendon and many other parts of the body as raw materials. Unfortunately, prion disease that may transfer to humans after these materials' use^[31] has led scientists to have seek an alternative source. Currently, fish skin, seaweed, jellyfish and other marine sources are in high demand for isolating collagen and are the ideal sources for skin tissue engineering because prion disease transmission to humans is eliminated if marine-based sources are used compared with mammalian-based sources. Collagen-based biomaterials encode antimicrobial activity^[32] and do not support colonization by bacteria in a full-thickness wound. The prevention of infection in wounds leads to minimized scar formation and promote wound healing^[33]. Due to their biocompatibility and biodegradability, collagen biomaterials have been established for use in skin repair in the clinical setting since last decade with various forms such as a gel^[34], sponge^[35], film^[36] and paste^[37].

Chitosan

Chitosan is a biopolymer obtained from deacetylated chitin and the second most abundant biopolymer sources after collagen. Crude chitin can be found in shellfish. Chitosan is naturally insoluble unless in acidic solutions, and possesses a positive charge and low cytotoxicity which broadly imply excellence as a DNA carrier^[38], protein nanocarrier^[39], drug delivery system^[40], siRNA nanovector^[41] and growth factor carrier^[42]. Based on its composition, the structure of chitosan is similar to that of glycosaminoglycans. Glycosaminoglycans are a component of the extracellular matrix and are an important substrate for tissue constructs^[43]. Chitosan arrests bleeding in major hepatic injuries and is effective for use in patients who suffer major injury with clotting dysfunction^[44]. Therefore, chitosan is preferable for tissue engineering and especially for engineered skin applications. The used of a novel technique to develop a chitosan sponge by electrospinning has produced a chitosan nanofiber that provides excellent substrate-cell adhesion, proliferation and differentiation^[45]. During skin repair and regeneration, nanofibers contribute to increased vascularization, re-epithelialization and enhanced granulation tissue formation. The major skin cells, particularly epithelial and epidermal cells and also fibroblasts grow well on this type of biomaterial.

Fibrin

Fibrin-based biomaterials are well-established biological

sealants for skin tissue engineering. However, the use of raw blood to isolate the fibrin has limited fibrin's use as a novel skin substitute. Commercial fibrin in kit form can successfully heal a wound but is relatively expensive for the majority of patients. Fibrin gel is continuously in demand in plastics and reconstructive surgery as it minimizes subcutaneous seroma formation and decreases wound morbidity^[46]. The combination of fibrin with bone marrow mesenchymal stem cells can improve the condition of scalded skin, providing strong self-repair capability and promising an acceptable cosmetic appearance with hair follicle formation. This application is more suitable for the treatment of patients with burns in emergency cases^[47]. Long-lasting fibrin biomaterials ensure stable and functional angiogenesis by highly tunable and sustained delivery of growth factors^[48].

MESENCHYMAL STEM CELLS

Mesenchymal stem cells also known as mesenchymal stromal cells, have broadly contributed to cellular therapy and skin tissue engineering. MSC are spindle-shaped, or similar to morphology to but phenotypically different from fibroblasts. Bone marrow derived-MSC were first isolated in 1981^[49] before MSC from other tissues were explored. Skin derived-MSC were first isolated from mice in 2001, and were initially named skin-derived precursors^[50]. In the skin, MSC are predominantly found in the dermal papilla, functioning in secreting diverse growth factors after wounding to promote fibroblast proliferation and collagen formation and to elicit intrinsic stem cell differentiation^[51], serving as a modulator to activate macrophages^[52], and directly affecting hair follicle morphogenesis^[53] and neo-dermis reconstruction^[54]. MSC elicit leukocyte migration for skin homeostasis and produce hepatocyte growth factor and basic fibroblast growth factor to inhibit scar formation at the wound site^[55]. The MSC-based skin substitute constructs have increased paracrine factor levels in promoting skin repair^[56].

FIBROBLASTS

Apart from stem cells, other components of the dermis such as fibroblasts are important in skin tissue engineering^[57]. Fibroblasts synthesize and deposit extracellular protein after wounding, producing growth and angiogenic factors that accelerate cell proliferation and angiogenesis^[58]. Fibroblasts are also transformed into myofibroblasts that express contractile fibers. As a myofibroblast contracts, forces are applied to the collagen fibers, thus reducing the size of the wound^[59]. Cultured fibroblasts in biomaterials have been reported to improve regeneration of the dermis^[60] and epidermis^[61] especially by re-epithelialization^[57]. This specific role of fibroblasts, especially in improving scarring and the contraction of skin wounds has encouraged researchers



Figure 2 The rapid proliferating of hair follicle stem cells. At hour 0, cells were separated (A) and then closely adhered to a plate (B) until one of the cells doubled (arrow head) at 12.59 h post-culture (C). Magnification 400 $\times$.

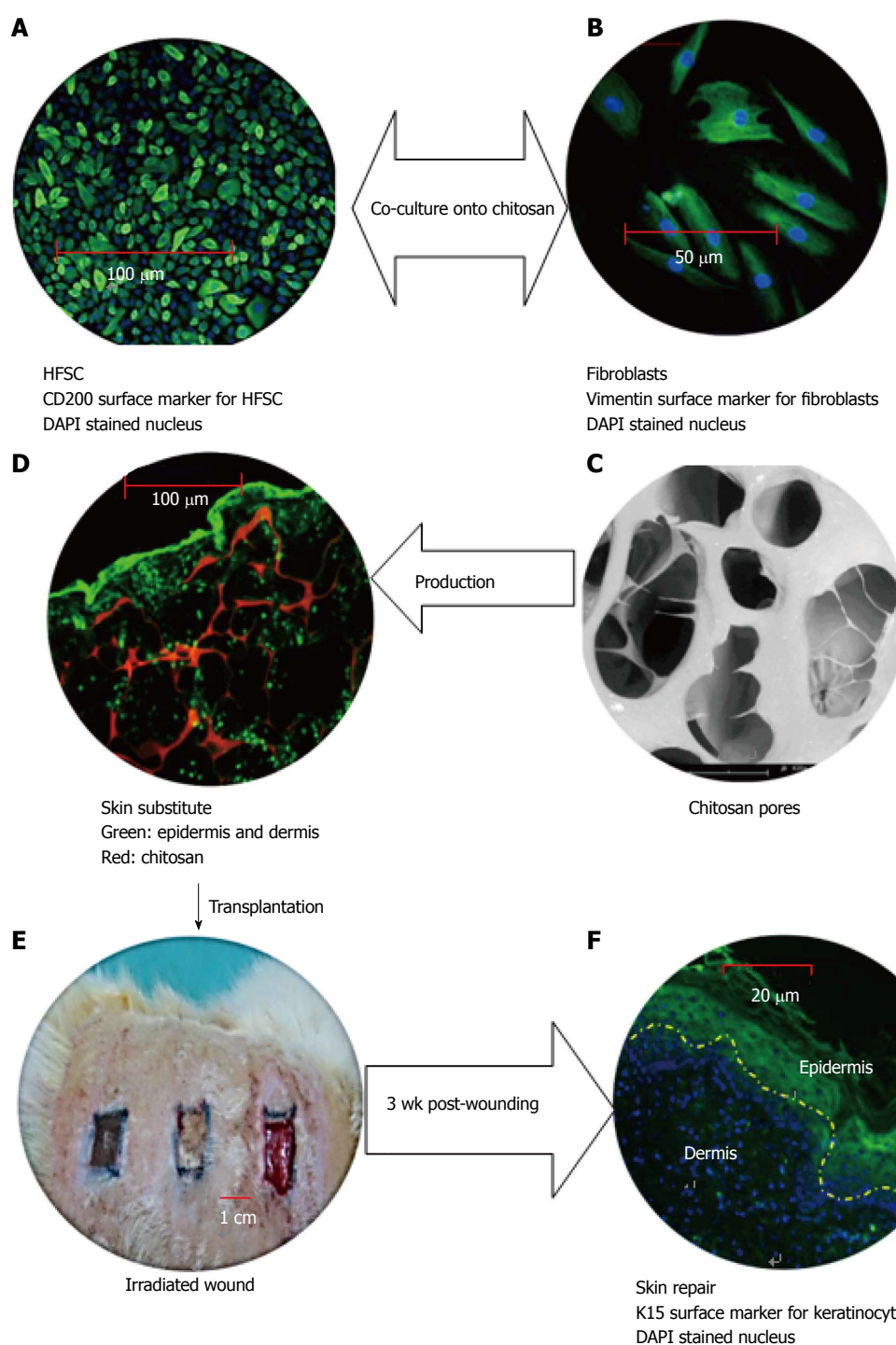


Figure 3 The vital role of hair follicle stem cells in skin tissue engineering. HFSC: Hair follicle stem cells; DAPI: 4', 6-diamidino-2-phenylindole.

to highly prioritize facilitating these cells' use in skin replacement therapies^[62]. Additionally, a previous study has demonstrated that fibroblasts from the dermis are the best source for skin tissue engineering applications compared with fibroblasts from other sources based on yields and reduced contraction of wounds^[63].

ADIPOSE STEM CELLS

Fat accommodates adipose tissue-derived stem cells usually known as adipose stem cells^[21]. ASC are actually MSC therefore, ASC express the same surface markers and highly contribute to skin repair as well. ASC improve neo epidermis formation and skin aging^[64] by accelerating angiogenesis^[65] and fibroblast proliferation^[66]. The use of ASC in skin repair increases the deposition of extra cellular matrix components such as collagen type-VI which has the potential to improve the protein deficiency^[67]. A mixture of fibroblasts and ASC can improve the epidermal morphogenesis of tissue-engineered skin^[68]. The ability of ASC to grow on porous biomaterial and to differentiate into fibrovascular, endothelial and epithelial tissues for skin repair^[69] has proven these cells' excellent capability as a cellular source for skin tissue engineering.

HFSCs

HFSC and their progenitors are directly involved in hair and skin regeneration during skin homeostasis^[70]. Based on their anatomical classification and function, HFSC have mainly been called epithelial stem cells^[71,72] and have been broadly known as epidermal stem cells^[73,74] as well as bulge stem cells^[75,76] in diverse studies. The critical role of HFSC in epidermal homeostasis^[77] has led to their application in fabricating neo epidermis in skin tissue engineering. Due to their vital characteristic, HFSC are capable of proliferating rapidly and this phenomenon is shown in Figure 2.

The use of HFSC in skin tissue engineering was successfully performed in chronic wounding in 2002, when Limat and Hunziker demonstrated that their HFSC-silicone constructs contributed to accelerated the healing of patients' leg ulcers^[78]. To date, the contribution of HFSC to ameliorating impaired healing due to radiation has stimulated the interest of tissue engineers in using epithelial stem cells for other tissue reconstruction. In Figure 3 shows that the chitosan based skin substitute which composed of HFSC-fibroblasts is an ideal source for skin tissue engineering. The chitosan composite was then used for transplanting into irradiated wound. Full-thickness wounds 1 cm by 1 cm in size were excised and treated using the novel skin substitute. As a result, a complete re-epithelialization occurred during skin repair after 3 wk post-wounding.

CONCLUSION

This review has summarized the fundamentals of

stem cells and especially the terminology for stem cell potency which has occasionally created confusion in certain experiments. Stem cell potency provides the necessary guideline for skin engineering experiments. The importance of fibroblast, HFSC, MSC, ASC, chitosan, collagen and fibrin in skin tissue engineering highlights their critical role in the repair process. The use of stem cells and biomaterials is especially important in the case of impaired wound healing and in cases involving major excisional skin defects.

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Neural differentiation from embryonic stem cells *in vitro*: An overview of the signaling pathways

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particularly those associated with *in vitro* differentiation. The inducers and pathways explored include retinoic acid, Wnt/ β -catenin, transforming growth factor/bone morphogenetic protein, Notch, fibroblast growth factor, cytokine, Hedgehog, c-Jun N-terminal kinase/mitogen-activated protein kinase and others. Some other miscellaneous molecular factors that have been reported in the literature are also summarized and discussed. These include calcium, calcium receptor, calcineurin, estrogen receptor, Hox protein, ceramide, glycosaminoglycan, ginsenoside Rg1, opioids, two pore channel 2, nitric oxide, chemically defined medium, cell-cell interactions, and physical stimuli. The interaction or crosstalk between these signaling pathways and factors will be explored. Elucidating these signals in detail should make a significant contribution to future progress in stem cell biology and allow, for example, better comparisons to be made between differentiation *in vivo* and *in vitro*. Of equal importance, a comprehensive understanding of the pathways that are involved in the development of neurons from ESCs *in vitro* will also accelerate their application as part of translational medicine.

Key words: Neurons; Differentiation; Embryonic stem cells; Signaling; Pathways

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Core tip: Neurons are derived from embryonic stem cells (ESCs) and have been a focus of research recently, particularly because of their application in regenerative medicine. Here we review and summarize the signaling pathways that have been reported to be involved in neuronal differentiation of ESCs, mainly *in vitro*. The inducers and pathways explored include retinoic acid, Wnt/ β -catenin, transforming growth factor/bone morphogenetic protein, Notch, fibroblast growth factor, cytokine, Hedgehog, c-Jun N-terminal kinase/mitogen-activated protein kinase and others. Some miscellaneous factors are also explored. Elucidating these signals in

Abstract

Neurons derived from embryonic stem cells (ESCs) have gained great merit in both basic research and regenerative medicine. Here we review and summarize the signaling pathways that have been reported to be involved in the neuronal differentiation of ESCs,

detail should make a significant contribution to future progress in stem cell biology and should also accelerate the application of stem cells in translational medicine.

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INTRODUCTION

Having been established from the inner cell mass of the blastocyst, embryonic stem cells (ESCs) possess pluripotency and can theoretically differentiate into all kinds of embryonic tissue. During the last decade, a number of studies have reported the differentiation of ESCs into a range of embryonic tissues. These compelling results were done either by stimulating the cells with particular molecules or by simulating the environmental cues of the early embryo. Importantly, these differentiated cells can then be used in regenerative medicine and for drug discovery. Hence, it is important to elucidate the detailed involvement of signals and signaling pathways in these processes before these cells are used for therapeutic purposes. Compared to development *in vivo*, neurons that differentiate from ESCs *in vitro* seem to develop *via* a similar pattern and thus have become a promising field in terms of the medical applications in stem cell research.

Basically, a default model for development *in vivo* is hypothesized as that induction of neural differentiation reflects the earliest fate in determining neurons in the ectoderm. The neural inducers involved in the process have been found to be bone morphogenetic protein (BMP)-binding molecule called noggin in *Xenopus*^[1] and FGF in chicken^[2]. Utilizing a serum-free and patterning factor-free condition to cultivate mouse ESCs (mESCs), Tropepe *et al.*^[3] found that neural progenitors differentiated specifically from these stem cells. Neural differentiation occurs spontaneously and does not require the presence of any extrinsic neural inducer in a special culture system named serum-free culture of embryoid body-like aggregates with quick reaggregation (SFEBq)^[4]. ESCs were found to selectively differentiate into neural progenitors efficiently (> 95% of total cells). The endogenous signals can also be minimized by adding the inhibitors of Wnt and/or Nodal such as dickkopf-1 and/or lefty-1, respectively. Furthermore, utilizing transforming growth factor β (TGF β) antagonists to abolish the SMAD signaling is shown to enhance neural differentiation, especially in induced pluripotent stem cells and human ESCs (hESCs)^[5]. All the results are in agreement with the "neural default

model" of embryonic development.

Neural cells were one of the first lineages to be developed from ESCs *in vitro*^[6,7]. Many studies have effectuated subsequently to differentiate ESCs into the creation of different cellular subtypes such as neurons and glial cells. These ESCs can be surgically transferred back into vertebrate embryos and are then participated in brain development^[8]. Work on ESCs over the last three decades has been concentrated on elucidating the characteristics of stem cells, particularly the differentiation approaches to obtain specific cell types such as neurons. Neural differentiation by ESCs is able to provide insights into the mechanisms involved in neural induction. In this review, we focus on obtaining an improving understanding of the overall signaling that underlies neural differentiation from ESCs *in vitro*. The inducers and pathways explored include retinoic acid (RA), Wnt/ β -catenin, TGF/BMP, Notch, FGF, cytokine, Hedgehog, JNK/mitogen-activated protein kinase (MAPK) and others (Figure 1). This should help researchers with exploring the various areas of stem cell biology and also help to enhance the application of ESCs differentiation in regenerative medicine.

THE RETINOIC ACID INDUCTION PATHWAY

RA typically is used at a range of concentrations from 5 μ mol/L to 5 mmol/L in order to facilitate the differentiation of ESCs into neural progenitor cells (NPCs). Shan *et al.*^[9] found that the active cyclic AMP response element-binding protein (CREB) protein was obviously increased after treatment with 5 μ mol/L RA treatment during the differentiation/formation of embryoid body (EB). Inhibition of CREB activity was found to result in EBs switching the differentiation to other germ layers fate, while enhanced expression of CREB was found to augment NPCs differentiation. RA promoted the expression of active CREB by increasing the activity of c-Jun N-terminal kinase (JNK). These results show that JNK/CREB is likely to be a crucial factor in the RA-induction of NPCs differentiation^[9]. In addition, Theus *et al.*^[10] found that neurons derived from ESCs after RA induction led to significant neurite growth. This event is associated with an spread expression pattern of Src kinase from the cell body to the neurite processes and an up-regulated expression of Src, Fyn and Lck^[10]. These neurons were characterized by the expression of neurofilament, synaptophysin and NMDA as well as the presence of kainate currents; they were found to have become vulnerable to excitotoxicity and went on to form functional excitatory synapses. Since these events were abolished when the cells were grown in the addition of the Src family kinase inhibitor PP2, it seems likely that the pathway induced by RA is

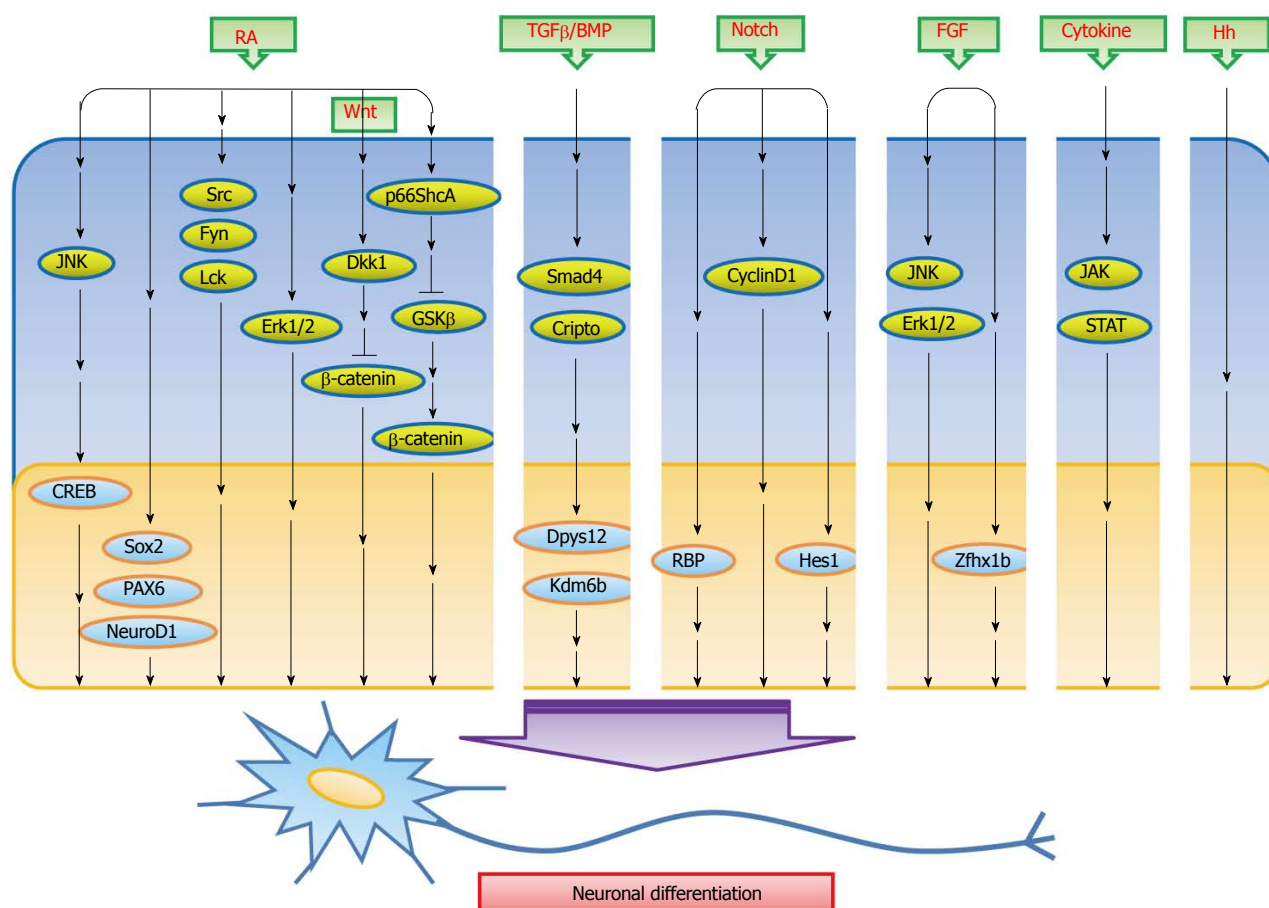


Figure 1 A simplified scheme outlining the signaling pathways described in the text. The inducers that induce embryonic stem cells to differentiate into neurons reported thus far including RA, Wnt/ β -catenin, TGF/ β /BMP, Notch, FGF, cytokine, Hedgehog, JNK/MAPK and others. The inducers in the middle part of figure mediate signaling molecules that bring about the differentiation into neurons. $\rightarrow$ means stimulation. Some miscellaneous molecules/factors mentioned at the end of this text are not included in this figure. RA: Retinoic acid; TGF: Transforming growth factor; BMP: Bone morphogenetic protein; CREB: cAMP response element-binding protein; Dkk-1: Dickkopf-related protein 1; Erk: Extracellular signal-regulated kinase; FGF: Fibroblast growth factor; Hh: Hedgehog; JAK: Janus kinase; JNK: C-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase.

mediated *via* Src^[10]. Furthermore, Tonge *et al.*^[11] demonstrated that neural differentiation of hESCs and embryonal carcinoma cells induced by RA needs both prolonged exposure of RA and cellular interaction that is done by the presence of a high cell density. These factors are required for the increase of the expression of various neural genes (*NeuroD1*, *PAX6* and *Sox2*) and the development of a neuronal appearance. They also found that inhibition of GSK3 β activity was able to block the RA-induced differentiation of neural lineage derived from ESCs. This finding suggests a role for properly modulated Wnt signaling in this process^[11]. After RA induction for 1-5 d, Li *et al.*^[12] found that there is a dramatic increase in extracellular signal related kinase 1/2 (Erk 1/2) phosphorylation (p-Erk 1/2) and that this can be attenuated by treatment of U0126, a p-Erk 1/2 inhibitor. Furthermore, both the expression of associated cytoskeletal proteins and the number of NeuN-positive cells are dramatically reduced after the inhibition of p-Erk 1/2. As a result there was an increase in the differentiating ESCs of the nuclear translocation of STAT3, together with a

decrease in the expression of NGF, BDNF and GDNF molecules. These results imply that phosphorylation/activation of Erk 1/2 is a key signaling that is essential for survival of ESCs and early neural differentiation^[12]. Recently, Glaser *et al.*^[13] found that when mESCs were initiated to progress into neural differentiation with RA, the expression of Oct-4 and the P2X7 receptor, an ATP-gated cation channel, were reduced. Utilizing KN-62, a specific P2X7 receptor inhibitor, they found an increased number of SSEA-1 and type III β -tubulin expressing double-positive cells. This confirms the appearance of neuroectodermal differentiation and it would seem that the neural fate determination of mESCs is dependent on suppression of P2X7 receptor activity^[13].

RA could also mediate crosstalk among other signaling pathways such as the Wnt/ β -catenin, FGF, and Erk pathways in order to induce neural differentiation. This is based on the finding that 4-d of RA treatment substantially increases the synthesis of the Dickkopf-related protein 1 (Dkk-1), a Wnt antagonist, and induces the expression of the Wnt/

Dkk-1 co-receptor LRP6^[14]. When recombinant Dkk-1 was utilized, the EBs presented in a similar manner to treatment with RA, namely there was an induction of two neural markers, the distal-less homeobox gene (*Dlx-2*) and nestin gene. Dkk-1 overexpression was found to be able to block the Wnt pathway, as evidenced by a decrease of β -catenin protein in the nucleus. These findings show that the prevention of the canonical Wnt pathway is a prerequisite for neural differentiation of ESCs when this is induced by RA treatment^[14]. Conversely, judging from the expression of neural marker *Hoxc4*, Otero *et al.*^[15] found that neural differentiation can be initiated by overexpressing β -catenin alone or combination with RA. Nevertheless, RA treatment was found to inhibit the β -catenin-induced production of tyrosine hydroxylase positive neurons, which suggests that the effects of RA are only partially dependent on β -catenin signaling. These results also suggest that β -catenin signaling enhances determination of neural lineage in ESCs. Moreover, β -catenin signaling could play a role of required co-factor in RA-induced pathway so as to permit the neural differentiation^[15]. Papadimou *et al.*^[16] reported that p66ShcA is increased during neural induction of ESCs *in vitro*. Overexpression of p66ShcA in ESCs ablates GSK-3 β kinase activation which in turn to stabilize β -catenin protein. In parallel, p66ShcA over-expression was found to result in both mESCs and hESCs undergoing neural induction as predicted and accelerated neural differentiation. Thus there seems to be a role for p66ShcA in the regulation of Wnt/ β -catenin pathway as well as in ESCs neutralization. Based on the above, p66ShcA would seem to also participate in a part of the RA-induction pathway^[16]. Furthermore, Engberg *et al.*^[17] monitor ESCs containing reporter genes that allowed the detection of markers associated with the early neural plate and the primitive streak and its progeny. When RA signaling is inhibited, they found that the change from neural to mesodermal fate develops. In addition, neural induction in ESCs needs RA to block Nodal signaling. Thus, the mechanism by which Wnt signaling pathway inhibits neural development could be interpreted as *via* facilitation of Nodal signaling pathway^[17]. Stavridis *et al.*^[18] shows that retinoid repression of fibroblast growth factor (FGF) signaling is able to promote the onset of neural differentiation. Induction of FGF8 by RA and subsequent Erk activity under early differentiation conditions could function to ascertain the loss of self-renewal. Nevertheless, a progressing inhibition of FGF4 by RA would seem to be associated with an overall decrease in Erk activity at the later stage. The admission of a neural or a non-neural fate is therefore decided by an inhibition of FGF signaling. Hence, inhibition of FGF/Erk activity would enhance ESCs self-renewal, but a subsequent abolishment of FGF signaling seems to have the opposite effect and act as a driver for differentiation^[18].

THE TGF β /BMP PATHWAY

It has been speculated that a default mechanism for neural differentiation might be involved in regulating the property of neural stem cell identity directly from ESCs. As above-mentioned, Tropepe *et al.*^[3] characterized that the neural lineage of differentiation from a nascent stem cell is modulated negatively by TGF β -related signaling. Moreover, differentiated mESCs *in vitro* with *Smad4* or *Cripto* genes knockout have been found to produce increased numbers of neurons^[19]. The profiles analysis of gene expression *in vitro* further demonstrates that cells bearing *Smad4* gene deletion were inclined to possess expressing patterns of mid-hindbrain and anterior hindbrain. However, the *Cripto* knockout cells tended to express gene markers of rostral central nervous system (CNS) in addition to other previous genes. Thus it would seem that *Smad4*^{-/-} ESCs exhibit differentiation of mesoderm while *Cripto*^{-/-} ESCs develop into epidermal/neuroectodermal cell types^[19]. To investigate the role of BMP-4 in the determination of either epidermal or neural fate, Gambaro *et al.*^[20] demonstrated that treatment of BMP-4 on murine ESCs results in the significant apoptosis of neural precursor cells which contain Sox-1 expression. Furthermore, counteraction of the SMAD pathway by overexpression of SMAD6, an inhibitor SMAD (I-SMAD), hinders the BMP4-induced apoptosis. Utilizing Noggin and SB431542, Chambers *et al.*^[5] shown that these two inhibitors of SMAD signaling are sufficient to allow the induction of neural differentiation derived from hESCs.

Genome-wide mapping was used to obtain plausible downstream candidates within the TGF β /BMP pathway that are involved in ESCs differentiation. Fei *et al.*^[21] mapped the gene promoters on a genome-wide scale to search for the target sequences bound with SMAD1, SMAD4, and SMAD5. They found that these molecules were associated with many developmental regulators and these were abundant in terms of H3K4 and H3K27 trimethylation bivalent markers. These promoters were found to be repressed when cells were in the self-renewing state, whereas these promoters underwent rapid induction upon differentiation. In the same context, the results from SMAD loss-of-function experiments further supported the hypothesis that BMP mediating signaling *via* SMAD does not directly affect self-renewal, whereas is necessary for various processes relevant to differentiation. Within the various SMAD-associated genes, they were able to identify two regulators which have been known to participate in the early neural differentiation regulated by BMP. These genes are *Dpysl2* (also known as *Crmp2*) and the H3K27 demethylase *Kdm6b* (also known as *Jmjd3*). Bertacchi *et al.*^[22] also adopted a global gene expression approach and were able to show that mESCs produce, secrete, and respond to BMPs during neural differentiation *in vitro*. Utilizing the analysis of

several markers of dorsoventral and anterior/posterior identity, they found that the gene expression pattern of differentiated ESCs reflects the midbrain identity. They also revealed that the endogenous BMPs during neural differentiation principally function to inhibit the expression of genes with a telencephalic profile. This phenomenon was evidenced by treating ESCs with a number of BMP inhibitors or Noggin.

THE NOTCH PATHWAY

Lowell *et al.*^[23] explored the role of Notch receptors and ligands in mESCs. They found that genetic manipulation that the constitutively activated Notch does not change the phenotype of stem cells. Nonetheless, these cells differentiate exclusively and promptly into the neural lineage upon abolishment of self-renewal stimuli. Conversely, genetic or pharmacological interference with Notch signaling inhibits the determination of neural fate. The neural commitment enhancing by Notch needs parallel signaling through the FGF receptor. Since expression of Notch ligand in stromal cells also induces the neural differentiation of hESCs, it indicates this pathway is conserved within pluripotent stem cells^[23]. Das *et al.*^[24] investigate the role of the Notch pathway by engineering a mESCs line such that there were short pulses of activated Notch. The alteration of Notch protein could be induced at the various stages of neural differentiation *in vitro*. The results show that activation of Notch signaling for 6 h specifically at day 3 during neural induction from ESCs was found to lead to dramatically increase cell proliferation. This outcome is associated with the cyclin D1 expression induced by Notch. In contrast, a decrease of cyclin D1 was observed during the development of the CNS in mouse embryos without Notch signaling. The ESCs containing a dominant negative form of cyclin D1 was found to abrogate the Notch-induced cell proliferation. These seem to indicate the presence of a special function for Notch in regard of temporal context. These findings also confirm that cyclin D1 is a crucial signaling molecule in Notch-induced differentiation/proliferation in ESCs^[24].

Downstream of the Notch pathway, the protein RBP was also show to play a key role. Main *et al.*^[25] obtained RBP^{+/-} mice using RBPJK^{loxP/loxP} mice^[26] bred with CMV-cre mice. After RBP^{-/-} mESCs had been obtained and cultured at low density, they were found to behave in the same way as wild-type cells in terms of the origin of apical specification and neural progenitors. When ESCs undergo development through the rosette formation, RBP was found to be required for the modulation of neuronal differentiation and for the appropriate preservation of rosette structure. Utilizing inhibitors of Notch and/or loss-of-function analysis of Notch signaling resulted in the disintegration of neural rosettes and an acceleration of neuronal differentiation. Rosette integrity was

also found to demand Rho kinase activity and actin polymerization in addition to requiring normal Notch signaling. However, it is worth noting that rosette maintenance is not required as a prior condition for regular neuronal differentiation. Various results demonstrate that Notch signaling also plays a role in the maintenance and organization of polarity during early nervous system development^[25]. Furthermore, it was also showed that Hes1-high ESCs are inclined to a mesodermal fate, whereas Hes1-low ESCs are inclined to a neural fate^[27]. Kobayashi *et al.*^[28] further showed that Hes1-low and Hes1-high ESCs are respectively correlated with cells that have undergone activation and inactivation of Notch signaling. Although Notch and Hes1 function in the same direction in most other cell types, the abovementioned results show that both signaling leads to opposite effect during ESCs differentiation. That is, Hes1 would seem to be not the downstream signaling molecule of Notch pathway during ESCs differentiation.

It was known that both activation of the Sonic Hedgehog (Shh) pathway and inhibition of the Notch pathway induce the neural differentiation during the neural tube development *in vivo*. To distinguish the effect of Shh and/or Notch signaling on ESCs-derived EBs, Crawford and Roelink used N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), an inhibitor of Notch, to investigate how critical the Shh signaling is in the EBs formation and neural differentiation *in vitro*. It was found that DAPT led to promote neuronal differentiation. On the other hand, more interneurons were identified in the absence of Shh. They also found that the effect of DAPT on EBs with an activated Shh signaling is associated with the premature disappearance of markers involved in the ventral neuronal precursors^[29].

THE FGF PATHWAY

Chen *et al.*^[30] showed that FGF family includes FGF1, FGF2, and FGF4, but not FGF8b, are able to enhance the neurogenesis of mESCs during serum-free neural induction. They found that the enhanced neurogenesis by FGF is not mediated through a promotion of the proliferation of Sox1⁺ cells or *via* a rescue of apoptosis. It was found to involve the inactivation of JNK-1 and Erk-2, but did not involve p38 MAPK, which is known to inhibit neural formation by inhibiting ESCs differentiation. Furthermore, ESCs that lacked FGF4 or have been treated with a FGF receptor inhibitor were found to be resistant to neural and mesodermal induction^[30]. Kunath *et al.*^[31] found that activated Erk 1/2 induced by FGF4 is a stimulus by which naive ESCs are able to be released from the self-renewal program. FGF4 is capable of initiating differentiation activity. The important role of Erk downstream signaling was further explored by an examination of Erk2 deficient

ESCs, which fail to proceed to either mesodermal or neural differentiation and retain their pluripotency. On the other hand, FGF2 which functions for the maintenance of epiblast stem cells and hESCs was found to inhibit development of early neural cells by epiblast intermediates. Nevertheless, FGF2 alone is sufficient to enhance self-renewal of epiblast stem cells. Conversely, FGF8, the endogenous inducer for embryonic neural differentiation, promotes more homogenous neural induction that is accompanied by transient self-renewal of early neural cells. They also found that completely blocking of FGF signaling in epiblast cells enhances prompt neural induction as well as the succeeding neurogenesis. Therefore, FGF signaling pathway seems to play a variety of roles during the different stages of ESCs differentiation^[31].

Dang *et al.*^[32] investigated the downstream molecules involved in FGF signaling. Sox1, enhancing the colony formation of definitive neural stem cells, is regularly used as a marker of neural precursors. Under typically culture conditions of ESCs in which the medium contains leukemia inhibitory factor (LIF) and serum, overexpression of *Zfhx1b* in these cells is essential to initiate the expression of Sox1. When mESCs were initiated to the neural differentiation, a prompt increase of *Zfhx1b* gene expression is observed and can be further potentiated by FGF signaling^[32]. In the same content, utilizing siRNA to knockdown *Zfhx1b* in ESCs leads to decrease the developmental capability of these neural cells although the initial transition of ESCs to a neural cell fate is not affected. Taken together, these findings show that intercellular FGF signaling induces *Zfhx1b* and this is able to promote the development of definitive neural stem cells after an initial neural specification event has occurred.

THE CYTOKINE PATHWAY

Under culture conditions, LIF is used to prevent the differentiation of mESCs. However, He *et al.*^[33] found that utilizing inhibitors to abolish the STAT3 signaling which was activated by LIF could block the neuronal differentiation of ESCs. Furthermore, inhibition of the MEK signaling which was activated by LIF decreased the differentiation of ESCs into glial cells. During ESCs differentiation, LIF enhanced proliferation of cells and inhibited apoptosis of cells. In addition, LIF promoted the determination of neural progenitors although inhibited the differentiation of mesoderm and extraembryonic endoderm fates. Foshay *et al.*^[34] further examine the role of STAT3. The loss of STAT3, brought about by generating STAT3 dominant negative ESCs, led to the production of significantly fewer neural stem cells and this was associated with a decrease in the expression of the neural stem cell marker nestin. Further investigation revealed that the Sox2 promoter is directly regulated by STAT3. The decreased expression of Sox2 would induce the expression of

nestin and result in a commitment to a neural stem cells fate. Using mouse embryonal carcinoma P19 cells, Pacherník *et al.*^[35] demonstrated that the effects of LIF on inducing neural differentiation were inhibited by blocking the JAK2/STAT3 signaling pathway. This is also partially true for the pro-neural effects of RA. Conversely, inhibition of the MEK1/Erk signaling pathway was found not to show any such effect. These data suggest that the cooperation between LIF and other factors, such as RA, may eventually converge into the STAT3 signaling pathway.

THE HEDGEHOG PATHWAY

Maye *et al.*^[36] demonstrated that mutants in the signaling molecules of the Hedgehog pathway within ESCs could lead to defect in the generation of neuroectoderm-forming EBs. These mutant ESCs are incapable to produce nestin positive neural stem cells or differentiate into mature neurons in respond to RA treatment. Moreover, ESCs lacking *Odf1*, a gene that causes Oral-Facial-Digital 1 (OFD1) Syndrome when mutated, were found to show an increased tendency on neuronal differentiation. Furthermore, the OFD1 mutant ESCs-derived neurons are unable to differentiate into V3 interneurons, a cell type dependent on Hedgehog signaling^[37]. These findings show a role for Hedgehog signaling in producing the neuronal and glial progenitors derived from ESCs.

THE JNK/MAPK PATHWAY

Amura *et al.*^[38] showed that there is an essential role for the JNK pathway during ESCs neurogenesis. ESCs lines containing homozygous destruction of the *JNK1*, *JNK2*, or *JNK3* genes were introduced into differentiation protocol *via* the procedure at the EB formation. The outcome shows that neural differentiation was observed in wild-type EB cultures, *JNK2*^{-/-} EB cultures, and *JNK3*^{-/-} EB cultures except in *JNK1*^{-/-} EB cultures. The identified inhibitors of ESCs neurogenesis, Wnt-4 and Wnt-6, were found to be increased their expression in *JNK1*^{-/-} cultures as compared to wild-type, *JNK2*^{-/-}, and *JNK3*^{-/-} cultures. Furthermore, a genetic approach using JNK knockout ESCs has revealed a role for JNK1 in neural differentiation that involves repression of Wnt expression using a murine ESCs model^[38]. Na *et al.*^[39] reexamined the role of Erk 1/2 in hESCs by using a chemically defined culture system. The results demonstrate that when the activity of Erk 1/2 is inhibited, the differentiation of neurons and mesendoderm is inhibited. However, these cells are still able to differentiate after BMP stimulation.

OTHER MOLECULES/FACTORS

Calcium, calcium receptor, and calcineurin

A number of studies have shown that calcium

homeostasis is a crucial factor in determination of neural fate. This has been thoroughly discussed in a previous review^[40]. It is worth noting that utilizing proteomic analysis or gene screening during neural induction in mESCs, four Ca^{2+} -related proteins, namely neuronatin, translationally controlled tumor protein, pyruvate dehydrogenase E1/E2 subunits, and calreticulin, have been found to be altered in expression^[41,42]. When neuronal cells are differentiated from ESCs lacking the Ca^{2+} release channel type 2 ryanodine receptors ($\text{RyR2}^{-/-}$), Yu *et al.*^[43] found that the rate of neurogenesis was significantly blocked. Meanwhile, the expression of NeuroD, a neuronal transcription factor, and the activity of intracellular Ca^{2+} signaling were also inhibited in the $\text{RyR2}^{-/-}$ deficient mESCs. Moreover, neuronal differentiation in $\text{RyR2}^{+/+}$ cells enhanced by activation of L-type Ca^{2+} channels or of GABA receptors was inhibited by RyR inhibitors. Therefore it would seem that cooperation between RyR2 channels and L-type Ca^{2+} is important for activity-dependent neurogenesis. Recently, Cho *et al.*^[44] found that neural induction is dependent on a Ca^{2+} -activated phosphatase, calcineurin. They also have shown that calcium entry mediated by FGF stimulation activates calcineurin, which then directly and specifically dephosphorylates BMP-regulated Smad1/5 proteins.

Estrogen receptor

Utilizing an estrogen receptor (ER) agonist, Zhang *et al.*^[45] found that $\text{ER}\beta$, but not $\text{ER}\alpha$, stimulated calcium oscillations in neurons derived from ESCs. The increase of calcium oscillations and the phosphorylation of PKC, AKT and Erk1/2 induced by the $\text{ER}\beta$ agonist in ESCs derived neurons could be blocked by nifedipine, an inhibitor of L-type calcium channels. The result demonstrates that $\text{ER}\beta$ could modulate neuron activity via L-type voltage gated calcium channels.

Hox protein

In order to investigate the role of the *Hox* gene in neuronal differentiation, Bami *et al.*^[46] used a mESCs cellular model by combining efficient neural differentiation with inducible *Hoxb1* expression. The profile of gene expression indicates that *Hoxb1* could function as both activator and repressor in the short term, whereas as a repressor in the long term. Such a pattern of *Hoxb1* activity was observed in the regulation of mESCs after RA induction.

Ceramide

It has been previously showed that bioactive lipids are important regulators of stem cell survival and differentiation^[47]. It was found that the sphingolipid ceramide and its derivative, such as sphingosine-1-phosphate, are able to function synergistically during ESCs differentiation and the guided differentiation of mESCs toward neural and glial lineages^[48].

Glycosaminoglycan

mESCs that lack heparan sulfate (HS) cannot process into neural specification whereas this phenomenon can be recovered by adding a highly sulfated glycosaminoglycan, one kind of soluble heparin^[49]. Pickford *et al.*^[50] demonstrated that specific heparin polysaccharides or HS support the formation of Sox11 neural progenitor cells from wild-type ESCs. They also found that a number of receptor tyrosine kinases were affected by HS during the differentiation.

Ginsenoside Rg1

Ginsenoside Rg1, a saponin and major component in ginseng, has been shown to possess neuroprotective effects. Wu *et al.*^[51] explored the effect of Rg1 on the promotion of mESCs differentiation towards the neuronal lineage. They found Rg1 increased the phosphorylation of Akt and Erk 1/2 in a time dependent pattern through glucocorticoid receptor. Treatment with either LY294002, an inhibitor of PI3K, or U0126, an inhibitor of MEK, blocked the Rg1-induced neuronal differentiation.

Opioids

Kim *et al.*^[52] measured μ -opioid receptor and κ -opioid receptor expression in various cell types including ESCs and neural progenitors induced by RA. In the RA-induced ES cells, a biphasic profile of Erk activation after opioid stimulation was observed. Nevertheless, the proliferation of the neural progenitors was inhibited after opioid stimulation in which this phenomenon was Erk independent. The findings indicate that opioids could have opposite effects on ESCs self-renewal and ESCs differentiation.

Two pore channel 2

The nicotinic adenine acid dinucleotide phosphate (NAADP), located on membranes of lysosome, has a potent effect on mobilizing endogenous Ca^{2+} . Two pore channel 2 (TPC2), voltage-gated ion channels, is shown to be the receptor of NAADP. Zhang *et al.*^[53] found that expression of TPC2 was decreased dramatically when the ESCs entry differentiation towards neural progenitor cells. During the late stages of neurogenesis, the expression of TPC2 reoccurred. Analysis of loss-of-function mutants of TPC2 found that TPC2 knockdown in mice accelerated mESCs differentiation into neural progenitors. This contrasted with the situation where there was TPC2 gain-of-function in a mouse model; this revealed that gain-of-function inhibited mESCs from entering the early neural differentiation. These findings suggest that TPC2 signaling plays a vital role in regulating the differentiation of mESCs into the neural lineage.

Nitric oxide

Employing various approaches, including ESC-derived neural precursor cells, Arnhold *et al.*^[54] studied the

role of nitric oxide in initiating the differentiation of neurons. They found that specific blocking of the NOS- II isoform was able to bring about the inhibition of neurite outgrowth.

Chemically defined medium

When chemically defined medium (CDM) is used for growth, ESCs differentiation is highly neurogenic. Neural differentiation in CDM is shown to be dependent on endogenous FGF signaling. This process is able to be inhibited by BMP4 or LiCl in which they simulate Wnt pathway. The neural differentiation in CDM could be terminated by blocking Hedgehog activity endogenously. Therefore, a common developmental mechanism could be processing since the profile change of gene expression in stem cells cultivation in CDM and the ones in the early embryos are extremely similar^[55].

Cell-Cell interactions

Parekkadan *et al.*^[56] observed that the presence of a previously specified Sox1-GFP⁺ cell in contact with undifferentiated ESCs was able to initiate a similar specification. This induction relied on the age of previously specified cells before co-culture. Further search for the cell adhesion molecules, it was found that connexin (Cx)-43 expression was associated with the age-dependent effect of cell contact in the experiments of cell pair. Both aberrant neuroectodermal specification and lineage commitment were seen in ESCs in which Cx-43 was knockout. Such an observation highlights the important role of gap junction signaling in the neuronal development.

Physical stimuli

Interestingly, physical stimuli are also able to affect the differentiation of ESCs and these phenomena have gained some attention recently. Piacentini *et al.*^[57] reported that the percentages of cells expressing type III β -tubulin, microtubule-associated protein 2, and calcium channel proteins (Cav1) were dramatically increased when differentiating neural stem cells are exposed to extremely low-frequency electromagnetic fields (ELFEFs, 1 mT, 50 Hz). An obviously increase in spontaneous firing were also found in these ELFEF-exposed neurons. Furthermore, they found that stimulation of ELFEF during the early differentiation could induce an increase of cells expressing CREB phosphorylation by which it is calcium channel dependent^[57]. In another study, Maioli *et al.*^[58] created a Radio Electric Asymmetric Conveyer (REAC) that is able to deliver wireless fidelity radiofrequency (Wi-Fi RF) at 2.4 GHz. This radio wave is delivered by immersing the conveyer electrodes into the culture medium. Using such a device allows mESCs to be exposed to REAC and in such circumstances it was found that transcription of genes involved

in differentiation, such as neuronal commitment (neurogenin1), were upregulated, while other genes, such as *Sox2*, *Oct4*, and *Nanog*, were downregulated. These findings mean that the physical environment is also able to regulate the fate of stem cells.

CONCLUSION

Some canonical pathways involved in cell size such as Hippo/Yap pathways and/or growth such as PI3K/Akt pathways seem to have little relationship with the initiation of neuronal differentiation from ESCs *in vitro*. The PI3K/Akt pathway is viewed as important to the maintenance of neuronal survival, but not to the differentiation process. In this context, Watanabe *et al.*^[59] show that the membrane bound Akt through myristoylation (myr-Akt) could maintain mESCs at the undifferentiated status without supplement of LIF in the medium. Once the myr-Akt was deleted, the dependence of LIF and ability of differentiation were recovered. They found that the PI3K/Akt signaling could regulate "stemness" of many stem cell systems. Zhao *et al.*^[60] also found that insulin can rescue ESCs-derived neural progenitor cells from hypoxia-induced cell death. Such an effect is able to be inhibited by LY294002, an inhibitor of the phosphatidylinositol 3-kinase (PI3K). Nevertheless, Chuang *et al.*^[61] have recently reported that the mTOR pathway, a downstream pathway of PI3K, would seem to play a role in ESCs-derived neuronal differentiation. In order to reveal the role of raptor/mTOR in neurons differentiated from ESCs, we established raptor gene-trap mESCs and raptor knockdown mESCs using raptor RNAi infection followed by puromycin selection. Embryonic body growth in both cases was greatly reduced and the result was an unsuccessful differentiation of neurons. Furthermore, treatment with 1 μ mol/L rapamycin over 48 to 72 h of treatment starting at the point when neuronal precursors began to differentiate from mESCs was found to bring about a gradual loss of neuritis together with a shrinkage of the soma and a decreased ratio of neurite length to cell number. Knockdown of raptor during neuronal differentiation from mESCs also resulted in a gradual loss of neurites and cell body shrinkage. The loss of neurite density that results from rapamycin treatment is able to be reversed by overexpression of S6K T389E. Therefore, raptor/mTORC1/S6K would seem to play a critical role in the differentiation and survival of neurons derived from mESCs^[61]. Therefore, it seems likely that the mTOR pathway plays a pivotal role in neuronal differentiation of ES cells *in vitro*.

To comprehensive understand these pathways will definitely contribute greatly to stem cell biology and translational medicine. In conclusion, the pathways outlined here are simple and linear. However, it is still unclear how these pathways crosstalk with each other and/or what is the level of

interplay between the pathways both temporally and spatially. The integration of these pathways into a comprehensive network will probably require more incisive investigative approaches. Generating specific types of neurons from ESCs *in vitro* has created high expectations in terms of possible medical treatments and/or potential cures for neuronal pathological diseases. Knowledge obtained by the research related to ESCs derived neurons *in vitro* should also provide a technical basis for regenerative medicine, applied medical research, and drug discovery.

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Importance of the stem cell microenvironment for ophthalmological cell-based therapy

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Abstract

Cell therapy is a promising treatment for diseases that are caused by cell degeneration or death. The cells for clinical transplantation are usually obtained by culturing healthy allogeneic or exogenous tissue *in*

vitro. However, for diseases of the eye, obtaining the adequate number of cells for clinical transplantation is difficult due to the small size of tissue donors and the frequent needs of long-term amplification of cells *in vitro*, which results in low cell viability after transplantation. In addition, the transplanted cells often develop fibrosis or degrade and have very low survival. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS) are also promising candidates for cell therapy. Unfortunately, the differentiation of ESCs can bring immune rejection, tumorigenicity and undesired differentiated cells, limiting its clinical application. Although iPS cells can avoid the risk of immune rejection caused by ES cell differentiation post-transplantation, the low conversion rate, the risk of tumor formation and the potentially unpredictable biological changes that could occur through genetic manipulation hinder its clinical application. Thus, the desired clinical effect of cell therapy is impaired by these factors. Recent research findings recognize that the reason for low survival of the implanted cells not only depends on the seeded cells, but also on the cell microenvironment, which determines the cell survival, proliferation and even reverse differentiation. When used for cell therapy, the transplanted cells need a specific three-dimensional structure to anchor and specific extra cellular matrix components in addition to relevant cytokine signaling to transfer the required information to support their growth. These structures present in the matrix in which the stem cells reside are known as the stem cell microenvironment. The microenvironment interaction with the stem cells provides the necessary homeostasis for cell maintenance and growth. A large number of studies suggest that to explore how to reconstruct the stem cell microenvironment and strengthen its combination with the transplanted cells are key steps to successful cell therapy. In this review, we will describe the interactions of the stem cell microenvironment with the stem cells, discuss the importance of the stem cell microenvironment for cell-based therapy in ocular diseases, and introduce the progress of stem cell-based

therapy for ocular diseases.

Key words: Microenvironment; Niche; Stem cell; Cell-based therapy; Ocular diseases; Ophthalmology

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Core tip: Cell therapy is a promising treatment for the diseases caused by cell degeneration or death. However, the transplanted cells often develop fibrosis or are absorbed and cannot survive long. It is not simply because of seed cells, but also due to the cell microenvironment. How to reconstruct the stem cell microenvironment and strengthen its combination with the transplanted cells is the key to successful cell therapy. We will discuss the importance of the stem cell microenvironment for cell-based therapy in ocular diseases and introduce the progress of cell therapy for ocular diseases.

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INTRODUCTION

Limbal stem cell deficiency, corneal endothelial decompensation, corneal grafts endothelial decompensation, retinitis pigmentosa, age-related macular degeneration, Stargardt disease and other hereditary retinal diseases are all caused by cell degeneration or death. There is no effective clinical treatment currently available. Unlike traditional medicine or surgical therapy, cell therapy is a promising treatment and can treat the abnormal cells most directly and efficiently.

The road to cell therapy has been a long and tortuous process. In the early days, most of the efforts to obtain sufficient therapeutic seed cells were based on establishing cell lines or improving tissue culture methods with the expectation that they will provide therapeutic effects after the cell transplantation. The former long-term passaged cell lines were mainly obtained by transgenic or nuclear atypia. Because of biosecurity risks and allograft immune rejection, cells obtained by this method are mainly used for basic research^[1]. The cells for clinical transplantation are usually obtained by culturing healthy allogeneic or exogenous tissue *in vitro*. It is hard to get large tissue from the eye to obtain a sufficient amount of cells and the culture often needs to go through long-term amplification *in vitro*, resulting in low cell viability after transplantation. Cells often develop fibrosis or are absorbed and cannot survive long. Thus, the desired clinical effect of the cell therapy cannot be accessed

and so it was shelved for a while.

Considerable progress has been made in the induction and differentiation of embryonic stem cells (ESCs) since the last century and it brought new vitality to cell therapy. However, the differentiation of ESCs can bring immune rejection, tumorigenicity and differentiation of uncertainty, which limits its clinical application. Although induced pluripotent stem cells (iPS) can avoid the risk of immune rejection caused by ESC differentiation post-transplantation, the low conversion rate and the risk of tumor formation still exists and the potentially unpredictable biological danger achieved through genetic manipulation hinders its clinical application. Thus, cell therapy returns to the transplantation of the cultured autologous cells, especially the enriched stem cells and the methods and the results have been greatly improved.

With the development of research, it is increasingly recognized that the reason the implanted cells cannot survive long-term in cell therapy is not simply because of seed cells, but also because of the cell microenvironment which determines cell survival, proliferation and even reverse differentiation. The birth of Dolly the sheep is the best example. In a good embryo environment, mature breast cells can be re-developed into a new individual sheep. From a biological point of view, whether it is a cell or an organism, it must exist in its surroundings with exchange material, energy and information. In terms of cell therapy, the transplanted cells need a specific three-dimensional structure to anchor and specific extra cellular matrix (ECM) components and cytokine biological information transfer to support their growth. These structures are present in the matrix in which the stem cells located and are called the stem cell microenvironment. The microenvironment interacts with the stem cells and they are interdependent, mutually promote and complement each other, working together to maintain the stem cell homeostasis^[2-5]. A large number of studies suggest that exploring how to strengthen the organic combination of the transplanted cells and the stem cell niche and reconstructing the stem cell microenvironment is the key to successful cell therapy.

In this review, we will recount the interactions of the stem cell microenvironment with the stem cells, discuss the importance of the stem cell microenvironment for cell-based therapy in ocular diseases, and introduce the progress of stem cell treatment for ocular diseases.

THE ROLE OF THE STEM CELL MICROENVIRONMENT

Obtaining autologous cells for cell therapy aided by the stem cell microenvironment

In the process of inducing embryonic stem cells and iPS cell differentiation, it was discovered that the adult cells can induce embryonic stem cells to differentiate

and the embryonic stem cells can promote somatic cell proliferation, repair the defects of co-cultured cells, or even reverse the differentiation state of somatic cells^[6-9] by improving the microenvironment *via* secreting a variety of factors and cell interactions, *etc.*

We began to work on the induction and differentiation of embryonic stem cells and epidermal stem cells^[10-12] in 1997 and found that when the adult cells were treated with supernatant from cultured embryonic stem cells or co-cultured with the embryonic stem cells, the aging process of adult cells can be slowed down or even reversed into progenitor cells and their self-renewal and proliferation capacity can be increased significantly^[13-18], whereas the adult cells can also induce embryonic stem cells to differentiate. We found a phenomenon that the corneal epithelial cells maintain long-term proliferative capacity and tissue-specific cell phenotype by factors secreted from murine ESCs. Rabbit corneal epithelial cells, cat corneal endothelial cells, rabbit skin epithelial cells and rabbit conjunctiva epithelial cells grew very well in culture medium with addition of ESC conditioned medium. These corneal epithelial cells were serially subcultured for more than 20 passages and maintained high cell purity, cobblestone-like morphology, enhanced colony forming efficiency, normal diploid and capacity to regenerate a functional stratified corneal epithelial equivalent. The rabbit corneal epithelial cells cultured in the embryonic stem cell microenvironment can be continuously passaged over 55 generations in 22 wk, gradually restoring its precursor characteristics, such as: decreased corneal epithelial cell specific differentiation markers K3/K12 expression, increased corneal epithelial precursor cell markers *P63* and *ABCG2* expression, but the expression of *Oct-4* was not detected, indicating that the embryonic stem cell microenvironment treated cells obtained a strong proliferative capacity without the potential tumorigenicity and uncertain differentiation^[13-18]. Zhang *et al.*^[13] also found that the proliferation and maturation of the dendrite cells co-cultured with the bone marrow mesenchymal cells were able to be significantly promoted, with the enhanced precursor cell marker expression and reduced expression of differentiation markers, so that the mature dendritic cells were reversed to the original progenitor cell stage. Pearton *et al.*^[9] reported that mouse embryonic skin can induce the terminal rabbit central corneal epithelial cells to reverse to the limbal stem cells by gradually losing specific marker K12 and K3. These results strongly suggest that the stem cell microenvironment can significantly regulate adult cell proliferation. It has the potential to become a more effective and safe method to access autologous seed cells with high proliferative activity which are close to pluripotent stem cells or transient amplifying cells without uncertain differentiation direction or tumorigenicity and render them more

suitable for clinical use.

Repair of the stem cell microenvironment is the basis for long-term efficient cell-based therapy

Stem cell microenvironment is the general term of the three-dimensional structure and a variety of signaling molecules (growth factors and their receptors, hormones and signaling molecules) present in the stroma where the stem cells reside and it can regulate the fate (proliferation/differentiation) of the stem cells. Because of its specific three-dimensional structure, it is vividly called niches (niche), which consists of three components: the extracellular matrix (ECM), niche cells (supporting cells, stem cells) and soluble factors derived from the niche cells (Figure 1). The proliferation and differentiation of stem cells are pre-programmed by themselves and are also affected by the microenvironment where they are residing. The stem cell microenvironment can anchor stem cells *in vivo* and regulate the self-renewal and production of their progeny cells through cell-cell, cell-ECM and cytokine-cell interactions. The different macromolecules or properties of the cells and ECM interact with each other in a complex and dynamic network^[19,20]. Nowadays, there is increasing evidence showing that the ECM is not only the supportive scaffold but also plays a fundamental role in cell biology. It plays important roles in the development of the cells and can regulate their behavior^[21] by the production, degradation of its components and the remodeling of the structure^[22,23] and the direct and indirect signaling properties^[21]. The polarity, division and migration of the cells can be influenced by the physical properties of the ECM, such as rigidity, porosity, topography and insolubility^[24]. Cytokines play an important role in exchanging information from cell-cell and cell-ECM. The changes of the extracellular matrix components also affect the differentiation of the stem cells and the induced differentiation *in vitro* is accomplished by mimicking the cell microenvironment. So, it is difficult to obtain a long lasting therapeutic effect in cell-based therapy without the support of a good stem cell microenvironment, even when excellent cells are transplanted.

The importance of stem cell microenvironment in tissue engineering has also been verified. How to rebuild the stem cell microenvironment becomes the biggest challenge currently for constructing tissue engineered tissues and organs. In the past, because the scaffolds for tissue engineering of organs and tissues had no such sophisticated stem cell microenvironment, the desired therapeutic effect could not be achieved and the structure and function could not be completely recovered after transplantation. In 2009, we introduced phospholipase A2, which can specifically hydrolyze the phospholipids of the corneal stroma cell membrane, can destroy the cell structure and be used to prepare acellular porcine corneal stroma acellular porcine corneal stroma

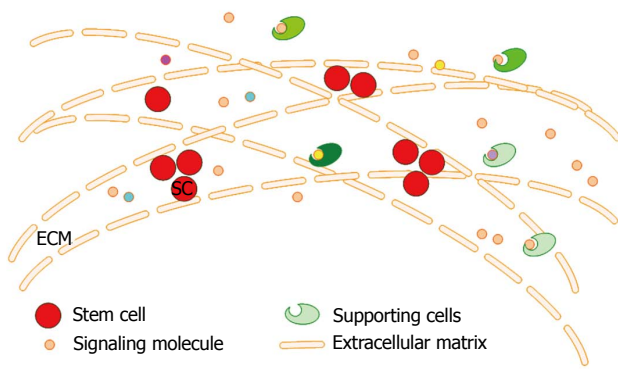


Figure 1 Schematic overview of the stem cell microenvironment components. ECM: Extra cellular matrix; SC: Stem cell .

(APCS) for biological tissue engineering cornea^[25]. The natural corneal collagen structure and 80% of the extracellular matrix components can be retained by this means. This APCS not only has good biocompatibility and biomechanical strength, but also keeps the limbal stem cells microenvironment necessary for their long-term proliferation. The grafts of APCS maintained good biomechanical strength and high transparency post-transplantation in animal experiments and it can help to rebuild the limbal stem cell microenvironment^[26-30]. Nakayama *et al.*^[31], Ott *et al.*^[32], Petersen *et al.*^[33], Uygun *et al.*^[34] and Conrad *et al.*^[35] have also reported the use of acellular matrix as a scaffold in tissue engineering in kidneys, lungs, heart, trachea and bladder. As Song *et al.*^[36] stated, using an acellular matrix scaffold with natural extracellular matrix to build tissue engineering products can mediate organ development, reparation and regeneration which cannot be accessed by synthetic materials. There are further experiments that proved that the acellular materials with extracellular matrix play an important role in the regulation of stem cells^[37]. These studies showed that a decellularized tissue with the natural ECM scaffold can induce the stem cells to differentiate into cell types present in certain tissue^[31] and thus the decellularized organ has already been used in tissue engineering and cell therapy^[31,36].

The microenvironment has a great effect on the fate of stem cells. In recombination experiments, hair follicle stem cells were induced to differentiate into corneal epithelial cells when they were cultured in a limbus-specific-like niche^[38]. These cells grow into stratified epithelium and express the cornea-specific markers K12 and Pax6 while the epidermal specific K10 obviously down-regulated^[38]. While in other studies the rabbit epithelial cells from the central cornea differentiated into epidermal keratinocytes when recombined with mouse embryonic dermis, it lost corneal-specific marker K3/K12 together with the down-regulation of Pax6 and expressed keratinocyte marker K5/K14^[9]. All of these changes showed that

the microenvironment has an important regulation role in the fate of the stem cells.

Stem cells can sustain and repair the structure and the function of the niche

On the other hand, evidence *in vivo* showed the relevance of the ECM and the stem cell behavior in that the altered properties or the aged niches' ability of maintaining stem cells' stemness can be reduced^[39] and it further affected stem cell proliferation and updated, which created a vicious cycle. Scientists from Harvard University attempted to study the relationship between human aging and microenvironmental changes between stem cells via the stem cell microenvironment (niche) and they found that altering the aged microenvironment with the young microenvironment can improve the ability of the proliferation and renewal of the stem cells. The study also found that adding "insulin-like growth factor-1" (IGF-1) can also improve the state of the aging stem cell microenvironment.

Cell receptors can directly mediate the interactions between the stem cells and the ECM where they are located. It was also found that integrins, a large family of heterodimeric transmembrane receptors, are important receptors for the ECM-stem cell interactions and can regulate cell survival, migration, proliferation and differentiation by connecting the ECM to the intracellular cytoskeleton^[40] as well as the adhesion, anchorage and homing of the stem cells. Different kinds of integrins bind to different ECM components or different cell surface adhesion molecules and receptors^[41-43]. Integrins can regulate the self-renewal and proliferation of the stem cells by directly activating focal adhesion kinase (FAK) and the phosphoinositide 3-kinase (PI3K) signaling pathway^[40,44,45]. The $\alpha\beta1$ integrin can bind to the ECM protein laminin and help the spermatogonial stem cells home in to the testicular niche^[46] and NSCs (neural stem cells) adhere to the vascular niche^[47]. The $\alpha9$ integrin is essential for the proliferation of the HSC^[48] and NSC microenvironment^[49] *via* binding protein tenascin-C in the ECM. The $\alpha4$, $\alpha6$, $\alpha9$ and $\beta1$ integrin chains are also important to the HSCs in their homing in to the bone marrow niche^[50-53]. HSC homing and proliferation are also regulated by $\alpha\nu\beta3$ integrin^[54-56]. Furthermore, $\beta1$ integrins can control the balance between symmetric and asymmetric divisions in skin and brain, as well as the differentiation and self-renewal of the stem cells^[57-60] by regulating the activity of the Notch pathway and EGF receptor^[61,62]. They are also important for the proliferation of intestinal stem cells (ISCs) *via* the Hedgehog signaling pathway^[63]. Signaling pathways of the growth factors and cytokines, such as IL-3 and TGF- β ^[43,64-67], can also be regulated by integrins and these signaling pathways can regulate the expression of integrins conversely^[46]. Therefore, the receptors

have specific activities in a certain kind of stem cell microenvironment.

Some ECM components can regulate their availability by setting a biochemical gradient and binding growth factors^[21]. On the one hand, the ECM can also make the growth factors insoluble, unavailable or not bioactive and serve as the reservoir for them, like proteoglycans, collagens, fibronectin and vitronectin, which bind VEGFs, FGFs, HGFs, TGF- β and BMPs. On the other hand, ECM proteins and proteoglycans can be induced to be soluble and remodeled to release and distribute growth factors under the action of enzymes, such as metalloproteinase^[21]. The function NSC can be favored by the ECM components in its niche by promoting growth factor activity *via* capturing FGF-2 from it^[68,69]. Similarly, in the procedure of the regulation of muscle satellite cells, all kinds of growth factors bind to the cell surface or the basal lamina proteoglycan and then they can be activated in a certain signaling pathway^[70].

The biophysical properties of the ECM can determine the behavior of stem cells. The balance of the internal forces generated by cell cytoskeleton tension and the external forces from the compression of the neighboring cells and the stiffness of the surrounding ECM maintains the shape of the cells and makes them stay in their anatomical localization^[71], which can regulate the cell behavior finally^[72-74]. Recently, the YAP/TAZ transcriptional factors were found to have key biological effects on the ECM elasticity, cell geometry and cytoskeletal modulation^[71,74,75] among the mechanotransduction pathways (Wnt/ β -catenin, PI3K/Akt, TGF- β , Ras/MAPK, and RhoA/ROCK pathways). In fact, ECM organization and composition can regulate tissue stiffness and then have an influence on the stem cell behavior^[72,76]. The human mesenchymal stem cells can express organ-specific transcription factors and differentiate into myoblasts, neurons and osteoblasts^[77] when they are cultured on ECMs with similar stiffness of the muscle, brain or bone respectively. Culture on hydrogel with the same elastic modulus as the bone marrow can increase the self-renewal ability and maintain multipotency of the hMSCs (human mesenchymal stem cells) compared to those cultured on stiffer substrates^[78]. NSCs cultured on hydrogel with similar stiffness to the brain tissue gain the neuronal differentiation potential, while the stiffer gels make them differentiate into glial cells^[79]. That the stiffness gradients of the hippocampus regulate NSC behavior *in vivo* confirmed that ECM and its biomechanical properties play important roles in the fate of the stem cell^[80]. This opens new insights in to the role of ECM mechanical properties in the stem cell microenvironment.

Stem cells in ocular tissues: Several studies showed the conjunctiva epithelial stem cell niche located in the fornix by growth potential assays, label-retention analyses and keratin expression detection^[81-84]. They have bipotential to differentiate

into both epithelial cells and goblet cells^[85].

Corneal epithelial stem cells resident at the basal limbal epithelium and called limbal stem cells were first applied to clinical use in ophthalmology. Tung-Tien Sun's group did immunostaining with monoclonal antibodies against the corneal-specific K3^[86] and showed the negative expression of K3/K12 in the limbal basal layer, which gave rise to a number of experiments that verified that the corneal stem cells were located in the limbus^[86-88]. These label-retaining limbal cells^[89] have a higher proliferative potential^[90] and colony-forming ability^[91] compared with central ones.

People made efforts to find specific molecules markers of the limbal stem cells. p63^[92], vimentin^[93-95], α -enolase^[96,97], α 9 β 1 integrin^[98], tenascin-C and EMILIN1^[99], ABCG2^[100-103] and ABCB5^[104] are all highly expressed in the basal layer of limbal epithelium but none of them are the specific markers of the limbal stem cells as expected. This is because the early differentiating cells^[105,106] still have the stem cell markers and show intermediate profiles between stem and differentiated cells until the stem cell markers are down-regulated with the expression of the differentiated phenotype^[107]. Thus, we can only enrich the stem cells while separating them with these molecules markers^[108].

Since it is complex and difficult to characterize the corneal stem cells, people try to identify them by analyzing their niches and the regulatory functions of the niche.

The limbus is a specific region which is characterized by the palisades of Vogt with the papillae-like projections and the vascular net in the peripheral cornea^[109]. It enables the epithelial cells to interact with ECM and chemical signals diffused from the vascular network^[110]. Some studies showed that the limbus has a specific anatomic structure such as the niche, the LEC (limbal epithelial crypt) or LC (limbal crypt), which consists of a cord or finger of cells that are located between the palisades of the limbal stroma and extends radically to the conjunctiva stroma^[92,93]. The high expression of K14^[111], ABCG2^[112] and p63^[92,93] in cells at the LEC^[112]/LC^[113] suggest that they are the microenvironment of the limbal stem cells but the LEC/LC structure has not been found in other species besides humans and pigs^[94].

This tissue with unique cellular properties can synthesize different kinds of ECM substrates. Several studies on the ECM components of the cornea were performed regarding the aspect of the biochemical and immunological characteristics. Corneal stroma comprises collagen type I-VI^[114-117], glycosaminoglycans (chondroitin, heparin, dermatan) and keratan sulfates^[118-122], fibronectin and laminin^[105,123] and hyaluronic acid^[124] and the limbal epithelial cells are more likely to adhere to a rougher surface than those in the central cornea^[125]. To further study the interaction between corneal stem cells and their

microenvironment and the different functions between the central cornea and the limbus, the corneal basement membrane components were analyzed by several studies. These studies found that the conjunctiva, limbal and central corneal epithelia have a heterogeneous composition of the basal membrane (BM)^[126]. Some studies reported that there was no collagen IV in the central cornea BM^[127], while others had the controversial results that collagen IV presented both in the limbal and the central corneal BM^[126]. Later, people found that collagen IV $\alpha 1$ (IV) and $\alpha 2$ (IV) chains show more intense staining at the corneal limbus and the $\alpha 3$ (IV) chain shows an abrupt decrease at the limbus^[128,129], while collagen types IV ($\alpha 3$ - $\alpha 4$ chains) and XII are only expressed in the central cornea^[128]. Then, other components of the limbal BM were studied further. Laminin $\alpha 2$ - $\alpha 5$, $\beta 1$ - $\beta 3$, $\gamma 1$ - $\gamma 3$, nidogen-1, -2, SPARC/BM-40, as well as agrin are preferentially expressed in the limbal BM^[128], which colocalized with the ABCG2/p63/K19-positive and K3/Cx43/desmoglein/integrin- $\alpha 2$ -negative stem cells and early progenitor cell clusters^[128,129]. The BM components, such as type XVI collagen, fibulin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate and versican, are colocalized with the putative vimentin-positive late progenitor cells^[128-130] at the limbus. On the contrary, type V collagen, fibrillin-1 and 2, and thrombospondin-1 were almost only found in the corneal BM^[128]; others, such as type IV collagen $\alpha 5$ and $\alpha 6$ chains, collagen types VII, XV, XVII and XVIII, laminin-111, laminin-332, laminin chains $\alpha 3$, $\beta 3$ and $\gamma 2$, fibronectin, matrilin-2 and 4, and perlecan, were expressed throughout the epithelial layer on the ocular surface^[129,130]. All these studies showed that the BM at the limbus has a specific ECM composition which is different from that in the peripheral or central cornea. This suggested that the EMC at the LEC/LC created a microenvironment that regulates stem cells and their progeny by supporting stemness while inhibiting the differentiation and preserving the proliferative abilities in limbal cells.

The stem cells of the corneal endothelium and the trabecular meshwork are believed to be located at the transition zone between the peripheral corneal endothelium and the anterior non-filtering portion of the trabecular meshwork^[131]. Corneal stroma stem cells are located in the limbal stroma, play roles in visualization and have corresponded to the limbal niche cells^[132].

At the early stage, the stem cells of the lens were assumed to be the label-retaining cells which are located at the anterior central region of the lens^[133]. Yamamoto *et al.*^[134] concluded that the germinative zone of the lens epithelium contains transient amplifying cells with the positive expression of proliferation markers, such as A1, B1, C and D1 cyclins and PCNA (proliferating cell nuclear antigen), and can be labeled by BrdU (5-bromo-2'-deoxyuridine). On the

other hand, other studies showed that they were probably located in the region anterior to the germinative zone. However, Remington *et al.*^[135] assumed that lens stem cells resided in the ciliary body because the lens is non-vascular, its epithelium does not have the morphology of other stem cells and no type of tumors are derived from the lens. Thus, the existence of the lens stem cells remains controversial and needs to be elucidated.

Previously, it was believed that there were no stem cells in the mammalian retina since it cannot regenerate^[136] but von Leithner *et al.*^[137] found retinal precursors in the peripheral retinal pigment epithelium later. However, the cells from the pigmented ciliary margin were later found to have the ability to form spherical colonies and produce various types of differentiated retinal cell^[138]. These results gave the evidence that retinal stem cells are located in the pigmented ciliary margin epithelium.

CELL-BASED THERAPY IN OCULAR DISEASES

Progress in the research of stem cell therapy for corneal diseases

Limbal stem cell deficiency can be caused by a myriad of insults that present with the following pathological states: damaged corneal barrier function, persistent corneal epithelium defects or recurrent corneal erosions, chronic inflammation associated with corneal stromal scarring, visualization, conjunctivalization and eventually blindness. Some researchers speculate that this occurs due to gradual deterioration of the limbal stromal niches^[139]. Thus, limbal epithelial stem cell transplantation and the reestablishment of the limbal stem cell microenvironment are necessary for ocular surface reconstruction in these diseases^[139].

Autologous or allogenic limbal transplantation has achieved good clinical efficiency in the treatment of limbal stem cell deficiency. However, in successful cases post limbal transplantation, there is a doubt as to whether there is a causal relationship between the survival of the donor limbal stem cells and the clinical effect. Shimazaki *et al.*^[140] detected the presence of donor-derived epithelial cells in 60% of cases (10 eyes/9 cases) post human limbal transplantation with fluorescence in situ hybridization assay, with 77.8% by RFLP analysis. At the same time, the authors stated that there was no difference in the postoperative clinical outcomes whether the presence of survived donor-derived cells was detected. Thus, more studies should be carried out to confirm the clinical significance of the survival of donor cells. Furthermore, it was reported that donor-derived cells were undetected in cases with objective clinical improvement after three to five years post limbal transplantation, even with DNA fingerprinting analysis^[141]. This suggested that the survival of

donor-derived cells is not essential for improvements of the clinical symptoms. These results indicated that limbal transplantation might simply serve as the corneal stroma transplantation. Presumably, limbal transplantation improves the residual stroma stem cell microenvironment, making residual stem cells in the patient regenerate on the ocular surface and allowing for improvement of the ocular surface^[142]. Therefore, some scholars believe that the essence of limbal transplantation may be the restoration of the limbal stroma resulting in increased stability of the limbal stem cell "niche"^[143]. So, limbal epithelial stem cell deficiency treatment lies in the application of various methods to restore the normal limbus matrix.

Although traditional corneal transplantation, limbal transplantation, has already achieved good results in ocular surface reconstruction for corneal diseases, there still several problems that have hindered clinical application, such as the shortage of donors, the immune rejection post-transplantation and other issues. Therefore, the construction of tissue engineering cornea *in vitro* with appropriate biomaterials and synthetic materials is the hope for solving these questions. How to get a sufficient amount of high activity seed cells for tissue engineering has been the challenge for tissue engineering product construction and cell therapy. The microenvironment plays an important role in the survival and development of cells and tissues. The microenvironment or simulated microenvironment can effectively induce ES and iPS differentiation in a certain direction and the embryonic microenvironment also has the effect of reverse differentiation of the adult cells. We have considered two aspects in our studies: (1) cellular microenvironment: embryonic stem cell microenvironment culture systems can make the differentiated corneal epithelial cells, conjunctiva epithelial cells^[15] and even human corneal endothelial cells^[17] obtain a strong proliferation ability and can be passaged long-term with de-differentiation cell marker expression, normal cell morphology and karyotype, but no tumorigenicity. Our preliminary findings showed that the ES microenvironment may inhibit the apoptosis of the cells by activating telomerase *via* integrin the b1-FAK-PI3K/Akt, telomerase-p21-mitochondrial axis and FAK/Wnt signaling pathway^[18,144]; and (2) stromal microenvironment: the APCS limbal produced by lipase (not existing protease digestion)^[25,30] can retain the normal extracellular matrix, collagen lamellar micro ultrastructure. It can repair and maintain the stemness and proliferation ability of the limbal stem cells after it is transplanted to the limbal stem cell deficiency rabbit model. In short, the microenvironment can be used to obtain sufficiently pure seed cells with strong proliferation capability but no immunogenicity. The microenvironment plays an important role in cell therapy and is the basis for the long-term efficacy of the treatment. Establishment of seed cells using the microenvironment will make cell therapy return to

autologous cell transplantation. Maintaining the specific three-dimensional structure and the extracellular matrix to promote the proliferation and long-term survival of the transplanted cells^[145] has great meaning. Professor Ott *et al.*^[32] stated that acellular matrix has a natural extracellular matrix which can mediate and guide organ development and mediate repair and regeneration. The key steps for the best clinical efficacy of cell transplantation are the long-term survival of the seed cells and the reconstruction of the stem cell niche.

Progress in the research of stem cell therapy for the retinal and optic nerve diseases

Retinal stem cells and optic nerve repair: Retinal diseases such as age-related macular degeneration, Leber congenital amaurosis and cone rod dystrophy are caused by lesions of retinal neuronal cells, which have an irreversible pathological process of degeneration and damage of the retinal neuronal cells, causing serious visual impairment or even blindness which currently cannot be effectively treated. Since the stem cells have self-renewal and multi-differentiation potential abilities, using stem cells as donor cells for retinal diseases treatment has become a hot topic.

In 2000, Wirtschafter *et al.*^[83] found that groups of self-renewing cells in the ciliary epithelium of the adult mice can form neurospheres when they were cultured *in vitro*. They can be induced to differentiate into specific types of neuronal cells in the retina, such as the rod cells, bipolar cells and glial cells, indicating that retinal stem cells exist in adult mammalian eyes. Ballios *et al.*^[146] found that retinal stem cells (retinal stem cell, RSC) also exist in the ciliary margin zone in people of different ages. These cells have proliferative capacity *in vitro* and can be induced to differentiate into different retinal neurons. Some of these stem cells can migrate and integrate into the host retina and can even differentiate into photoreceptor cells. These studies suggested that RSCs from different sources of animals or humans can survive and migrate to the host retina layers after transplantation. Although the implanted RSCs can migrate into the retina and differentiate into a variety of retinal cells, most transplanted RSCs remain in the subretinal space or the vitreous body, while less can be integrated and induced to differentiation, so the efficiency is not ideal.

Meyer *et al.*^[147] transplanted GFP-labeled ESCs into mouse vitreous after induced differentiation and found that the transplanted cells can migrate into the entire retina layers and differentiate into retinal neurons cells with the expression of markers such as NeuN, calretinin and cPKC- α . In 2010, Parameswaran *et al.*^[148], using a similar method as embryonic stem cell differentiation, completed the differentiation of mouse iPS cells into retinal ganglion cells, which not only highly express the retinal ganglion cells

regulation gene *Ath5*, *Wtl*, *Brn3b*, *Rpfl* and *Irx2*, but also can specifically project upwards to the superior colliculus with the synapse structure formation which has a sensitive tetrodotoxin voltage-dependent sodium current. This fully proved that iPS cells can differentiate into retinal ganglion cells. In glaucoma and traumatic optic neuropathy, the regeneration of retinal ganglion cells may be the only way to restore vision. The finding that iPS cells can differentiate into retinal ganglion cells provides a new method for the treatment of such diseases.

Arnhold *et al.*^[149] found that the cones cannot survive without healthy rod cells. Therefore, it is difficult to achieve the aim of the treatment by simply transplanting the induced iPS cells into the retina of patients with retinitis pigmentosa because the rod cells will eventually die.

Stem cell treatment for retinitis pigmentosa:

Retinal pigment epithelium (RPE) is a single layer of epithelium with polarity and a rich pigment that is located between the neural retina and choriocapillaris layer. It can support the metabolism and activities of the retinal photoreceptor cells and phagocytose the outer segment photoreceptors. Recent studies show that the retinal pigment epithelium also has self-renewing stem cells that can be induced to form other cell types under suitable conditions.

RPE degeneration diseases caused by age-related macular degeneration (AMD), hereditary retinal degeneration, macular dystrophy and Stargardt disease result in the death of photoreceptors and neural retina and eventually cause blindness. There is currently no effective treatment for retinitis pigmentosa. The transplantation of the induced differentiated stem cells to establish the retinal pigment epithelium membrane *in vivo* has been studied extensively but it is still far from functional reconstruction. Thus, looking for new ways to stimulate the repair of RPE is an important research direction for the future. With the rise of regenerative medicine research, stem cell transplantation as a regenerative therapy becomes a hotspot for research. In the past decade, scholars induced bone marrow mesenchymal stem cells, iPS cells and embryonic stem cells to differentiate into retinal pigment epithelial cells but the process of differentiation into RPE cells is not clear and only a small fraction of cells can be differentiated into RPE cells. So, the research of finding the desired factors to directly induce cell differentiation is still very popular.

In 2010, Geron biopharmaceutical company (Geron, United States) sponsored the world's first clinical trial on using hESC to repair damaged nerves. Currently, the United States Food and Drug Administration has approved the ACT company (Advanced Stem Cell Technologies) to carry out two Phase II clinical trials on using embryonic stem cells to treat macular degeneration in the United States, dry AMD and juvenile macular dystrophy (Stargardt disease). This

time, they induced hESCs to differentiate into retinal epithelial cells with purity over 99%. Approximately 50000 retinal pigment epithelial cells were isolated and injected into the retina of two patients. Four months later, the researchers found that RPE had been completely replaced by the injected retinal epithelial cells. They measured the visual acuity of the two female patients and the data confirmed that the injected cells survived and largely improved their vision. Early data suggest that hESC therapy is not only safe but also efficient. The research paper was published in the world's oldest and most respected peer-reviewed medical journal, "The Lancet"^[150]. These results from ACT have brought new optimistic hope for stem cell research. This same study found that it is crucial that the transplanted cells can attach to the Bruch's membrane and integrate into the host RPE layer and survive to have a successful therapeutic effect^[149].

CONCLUSION

In summary, recent studies show that real cell therapy is not a simple supplement of cells. The interaction, interdependence, mutual promotion and supplementation between the transplanted cells and the microenvironment are more important. The microenvironment of the recipient can regulate the transplanted cell behavior and decide their fate. The transplanted cells cannot survive long-term without the support from the microenvironment of the recipient. The survived transplanted cells can not only completely replace the recipient's cells, but also supplement the sufficient quantity and function of the recipient's cells. More importantly, they are involved in the cellular microenvironment reconstruction so the stem cells obtain a stronger self-renewal capacity and proliferation ability. Therefore, in order to improve clinical cell therapy, we should pay more attention to the characteristics and components of each stem cell microenvironment and put efforts into understanding the regulation mechanisms of the stem cell microenvironment^[151].

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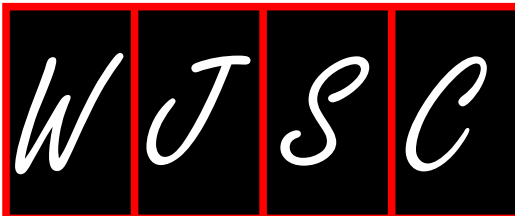
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Using induced pluripotent stem cells as a tool for modelling carcinogenesis

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Abstract

Cancer is a highly heterogeneous group of diseases that despite improved treatments remain prevalent accounting for over 14 million new cases and 8.2 million deaths per year. Studies into the process of carcinogenesis are limited by lack of appropriate models for the development and pathogenesis of the disease based on human tissues. Primary culture of patient samples can help but is difficult to grow for a number of tissues. A potential opportunity to overcome these barriers is based on the landmark study by Yamanaka which demonstrated the ability of four factors;

Oct4, Sox2, Klf4, and c-Myc to reprogram human somatic cells in to pluripotency. These cells were termed induced pluripotent stem cells (iPSCs) and display characteristic properties of embryonic stem cells. This technique has a wide range of potential uses including disease modelling, drug testing and transplantation studies. Interestingly iPSCs also share a number of characteristics with cancer cells including self-renewal and proliferation, expression of stem cell markers and altered metabolism. Recently, iPSCs have been generated from a number of human cancer cell lines and primary tumour samples from a range of cancers in an attempt to recapitulate the development of cancer and interrogate the underlying mechanisms involved. This review will outline the similarities between the reprogramming process and carcinogenesis, and how these similarities have been exploited to generate iPSC models for a number of cancers.

Key words: Induced pluripotent stem cells; Cancer; Model; Reprogramming

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Core tip: Human induced pluripotent stem cells (iPSCs) represent a novel method for studying the mechanisms of cancer development and progression. Recently, a number of studies have generated iPSCs from human cancer cells and cell lines, which can then be used as a model for carcinogenesis. This review outlines the similarities that exist between pluripotent and malignant cells and summarizes available studies that have generated iPSC models of cancer.

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INTRODUCTION

Cancer is a heterogeneous group of diseases that accounts for an estimated 14.1 million new cases and 8.2 million deaths annually, with the number of cases expected to increase to 24 million annually by 2035^[1]. Surgical removal, chemotherapy and/or radiotherapy have become the mainstay treatment for cancer. In recent decades research has focused on improving current drugs and developing targeted therapies specific to the defining biological properties of tumours. Despite the development of these therapeutic interventions, the death rate from cancer remains high. One major reason for this is the limitations of current representative pre-clinical models for human carcinogenesis, including the difficulty in growing and expanding primary cultures. Induced pluripotent stem cells (iPSCs) offer a relevant and unlimited system to study the development and progression of cancer. This review will discuss the current models for carcinogenesis, similarities between cancer and stem cells, and recent iPSC models of human cancers.

CURRENT MODELS

Cell lines developed from human tumours are often used to study carcinogenesis. These are inexpensive and can be maintained over lengthy periods; however prolonged culture can alter the characteristics of cells resulting in them becoming less representative of primary tumours^[2]. Alterations in gene expression with prolonged *in vitro* culture are also associated with the use of cell lines, with studies using microarray data from snap-frozen normal human tissue, primary tumour biopsy tissue and tumour-derived cell lines identifying that only 2% of tissue specific and 5% of tumour specific genes were expressed when compared to their equivalent tissue or tumour^[3]. Primary cell cultures better represent inter-patient heterogeneity which exists due to differences in tumorigenic cell properties and numbers, variation in cell of origin and frequency of mutations^[4,5], however cultures have a limited life span and are difficult to obtain, maintain and expand. A number of animal models for carcinogenesis exist and have greatly increased our knowledge of cancer. However, animal models are not fully representative of carcinogenesis in the human setting due to inherent species differences including organism size and longevity as well as cancer susceptibility^[6].

STEM CELLS IN DISEASE MODELLING

Stem cells are defined by their ability for self-renewal and differentiation into a range of cell types. Their ability to replicate indefinitely overcomes the limitations of current human tissue models as they are able to generate a limitless supply of human cells. Somatic stem cells are present within many organs

and are defined by their ability for both self-renewal and differentiation to maintain homeostasis^[7]. These cells could be used to model development and disease; however adult stem cells comprise rare populations that are not easily identifiable.

The first human embryonic stem cell line was derived from human blastocysts in 1998 by Thomson *et al*^[8]. Stem cells have two defining features; self-renewal and indefinite proliferation, meaning a limitless supply of tissue can be derived from these cells. Due to these properties, it is hoped that stem cells can be used as a system for disease modelling and drug discovery. However, research using human embryonic stem cells (hESCs) is hampered due to the ethical issues surrounding ESCs and the stringent restrictions enforced as a result.

An alternative to hESCs and adult stem cells are induced pluripotent stem cells (iPSCs), generated in a landmark study by Takahashi *et al*^[9]. From a screen of 24 candidates, 4 factors were identified which were able to reprogram mouse somatic cells to pluripotency; Oct3/4, Sox2, c-Myc and Klf4. These cells showed characteristics of ESCs including morphology, marker expression and the ability to form all three embryonic germ layers^[9]. Subsequently, the same four factors were shown to also have the ability to reprogram human adult dermal fibroblasts to iPSCs^[10]. An alternative cocktail of factors consisting of Oct4, Sox2, Nanog and Lin28 was also shown to generate iPSCs from human fibroblasts^[11]. Human iPSCs possess a number of features that are typical of ESCs including self-renewal and expression of ESC marker genes. Importantly, as for hESCs, iPSCs have the ability to differentiate both *in vitro*, via the formation of embryoid bodies (EBs) comprising all three germ layers and *in vivo*, as demonstrated by the formation of teratomas^[10]. The use of iPSCs also resolves the ethical issues associated with hESCs, a controversial topic due to the use of blastocyst stage human embryos to derive the cells^[12]. As iPSCs can be generated from somatic cells, they also show great potential for developing patient specific models of diseases, which can be used to study the underlying mechanisms of disease development and the efficacy of treatments^[13].

REPROGRAMMING VS CARCINOGENESIS

A number of similarities exist between the processes of reprogramming and carcinogenesis. Cancer cells have a number of defined characteristics including sustained proliferative signaling and replicative immortality^[14]. Stem cells also possess this intrinsic ability for both self-renewal and proliferation, highlighting their similarity to cancer cells.

Cancer stem cells

Tumours, like normal tissue, are heterogeneous populations of cells, varying in phenotype, function

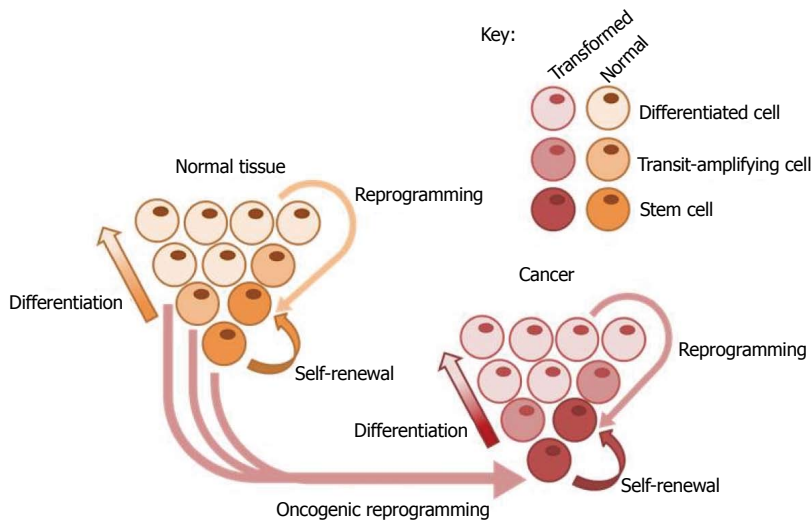


Figure 1 Schematic illustrating normal stem cell expansion and the cancer stem cell model. Normal stem, transit-amplifying or differentiated cells can undergo oncogenic reprogramming resulting in generation of cancer stem cells (CSCs). Non-CSCs also demonstrate plasticity and can dedifferentiate to CSCs.

and gene expression^[15]. Furthermore, studies from a number of cancers have shown that not all cells can regenerate tumours upon injection into immunodeficient mice, a functional assay which is now used to identify cells termed cancer stem cells (CSCs)^[16]. CSCs can be defined as tumour cells which have the ability for both self-renewal to maintain the stem cell pool, and differentiation to the heterogeneous cell types which maintain the tumour^[17], and therefore share the quintessential properties of normal stem cells (Figure 1). Importantly, due to these properties, CSCs which survive chemotherapy are able to re-establish tumours^[18]. Whilst the origin of CSCs is not fully known, it has been suggested that CSCs could arise as a result of mutations in stem, transit-amplifying, differentiated or cancer cells leading to acquisition of malignant and/or stem cell properties^[19]. Understanding the process by which CSCs are generated and how they maintain tumours is critical to develop therapies which target this cell population and therefore prevent tumour recurrence.

CSCs were originally identified in acute myeloid leukemia, however putative CSCs have now been identified in solid cancers including breast, colon, brain, prostate, and lung cancer^[20]. These cells could be used to study tumours however they are difficult to isolate, particularly in solid tumours which are very heterogeneous and therefore are not fully represented by tissue samples taken from a single site within the tumour^[21]. Reprogramming of primary cells of interest derived from patients into iPSCs could allow generation of disease- and patient-specific models to study carcinogenesis and to further interrogate the CSC model. An interesting addition to the CSC model is the concept of tumour cell plasticity. A number of studies have shown that non-CSCs can dedifferentiate and acquire properties of stem cells, converting them to CSCs which can then maintain the tumour. In colorectal cancer, Wnt signaling induced by the tumour microenvironment activates CSC properties in differentiated cancer cells^[22], whilst transformed

human mammary epithelial cells can spontaneously convert to cells with a CSC phenotype both *in vitro* and *in vivo*^[23].

Cell reprogramming, clonal expansion and evolution

Carcinogenesis is a multi-step process consisting of initiation, promotion, conversion to malignancy and progression^[24]. Cancer is known to undergo clonal expansion throughout this process, resulting from an initial change or mutation in a single cell which confers a growth advantage allowing for enhanced proliferation and expansion of this population. Further mutations then occur within cells forming subpopulations which expand and continue to accumulate changes and mutations which further alter proliferation, cause loss of differentiation and enhance invasive potential, resulting in a malignancy which is genetically and phenotypically divergent from the normal tissue counterpart^[25]. Clonality is also a defining feature of stem cells, and is often used to measure the ability of such cells to self-renew^[26].

Role of epithelial-mesenchymal transition and its significance

Epithelial-mesenchymal transition (EMT) is defined as a conversion of cells from an epithelial to mesenchymal phenotype with loss of epithelial properties such as apical-basal polarization and cell-cell adhesion and gain of mesenchymal properties such as motility, degradation of the extracellular matrix and resulting invasiveness^[27,28]. This process is critical for normal development- during gastrulation for formation of the mesoderm, and also at subsequent stages such as neural crest formation and heart development^[29]. Similarly, EMT occurs during differentiation of ESCs^[30], whilst its opposing process mesenchymal-epithelial transition is necessary to reprogram cells to pluripotency^[31]. EMT has also been implicated in cancer with roles in a number of cancer hallmarks. The EMT inducers SNAIL1 and SNAIL2 correlate with relapse and survival in breast, ovarian and colorectal

cancers, suggesting that EMT is associated with poor outcome^[28]. Expression of EMT genes is also associated with cancer progression as these expression profiles are identified at the invasive front of a number of cancers including colon carcinoma, papillary thyroid carcinoma and some breast carcinomas^[28]. Furthermore, an EMT expression profile was shown to associate with development of metastasis in cutaneous malignant melanoma^[32].

TGF β , an inducer of EMT, has a dual role in cancer, acting as a tumour suppressor and activator of apoptosis whilst also promoting immune tolerance, invasion and metastasis^[33]. TWIST1/2 proteins, which also act as EMT inducers, are increased in a number of human cancers and prevent senescence in cancer cells by inhibition of the p53 and Rb pathways^[34]. Finally, EMT has also been implicated in resistance to chemotherapies, with evidence that a number of chemoresistant cell lines are induced to undergo EMT^[35,36].

Role of reprogramming factors in cancer

Transcription factors which play a critical role in reprogramming cells to pluripotency have also been identified in human cancers. Oct4 is a transcription factor with roles in embryogenesis^[37] which is expressed in all testicular germ cell tumours and also pre-malignant carcinoma *in situ* lesions^[38], as well as in breast, pancreas and colon cancer cDNA panels^[39]. Furthermore, induced expression of Oct4 in the somatic cells of mice results in epithelial dysplasia, providing further evidence of a role for stem cell genes in carcinogenesis^[40].

Immunohistochemical staining of a panel of oral squamous cell carcinoma (OSCC) patients identified an increased incidence of Oct4 expression with advanced stages of the disease, with enhanced nuclear staining in higher grade oral cancers^[41]. Transduction of primary human breast epithelial cultures with Oct4 generated colonies with self-renewal capacity, which similarly to oral squamous cells with overexpressed Oct4 showed enhanced expression of mesenchymal markers and a loss of epithelial markers. These cells were also highly tumorigenic, forming poorly differentiated, high grade tumours after injection into nude mice^[42]. Oct4 has also been implicated in cervical cancer with increasing numbers of Oct4 expressing cells in both carcinoma *in situ* and invasive cervical cancer compared to normal cervical tissue, suggesting a role for Oct4 in the development of cervical cancer. Overexpression of Oct4 in cervical cancer cell lines resulted in increased tumour volume and weight upon injection into mice, and decreased apoptosis both *in vitro* and *in vivo* compared to controls^[43].

MYC encodes the Myc transcription factor which regulates genes involved in cell growth and proliferation^[44]. Myc is a known oncogene and one of the most commonly altered genes in human cancers with copy number

alterations in 14% of cancer samples^[45]. Translocation of c-Myc from chromosome 8 to chromosome 2, 14 or 22 in Burkitt lymphoma cells was the first evidence for the role of Myc in cancer development^[46]. Myc translocation is also common in multiple myeloma where the oncogene is fused to an IgH or IgL locus in early carcinogenesis^[47]. Abnormalities at the c-Myc or I-Myc locus have been identified in 19/20 multiple myeloma cell lines and 50% of advanced primary cancers^[48]. Finally, Myc has also been implicated as a downstream target of deregulated Notch signalling which is apparent in T cell leukaemia, with the two molecules concurrently regulating leukaemic cell growth and proliferation^[49].

Myc has also been implicated in solid tumours. c-Myc amplification occurs in 25% of primary breast carcinomas with protein overexpression in 45% of breast tumours, and was significantly correlated with poorly differentiated and highly proliferative tumours^[50]. Overexpression of c-Myc mRNA is also thought to occur in 60%-80% of human colorectal adenocarcinomas^[51] and has been shown to enhance colon cancer cell angiogenesis *via* inhibition of HIF-1 α degradation and promotion of VEGF expression^[52].

Myc is overexpressed or activated in over 50% of human cancers, but in most human cells cannot independently induce tumorigenesis, instead cooperating with other events such as loss of p53 or overexpression of Bcl2 to bypass normal cell checkpoints and initiate carcinogenesis^[53]. Interestingly, the level of Myc expression has been shown to produce varying effects, with low level deregulated Myc resulting in proliferation alone whilst high levels of Myc overexpression are required to activate tumour suppressor mechanisms such as ARF induction leading to cellular apoptosis^[54].

As is the case with Oct4, Nanog expression correlates with poor survival in OSCC and also in nasopharyngeal carcinoma if co-expressed with Oct4^[41,55]. High levels of Nanog expression are also present in breast, lung, ovarian and colon cancer cell lines, and furthermore in cervical cancer Nanog expression increases with progression from cervical intraepithelial neoplasia to invasive cervical carcinoma and is associated with poorer prognosis. Inhibition of Nanog by siRNA decreased proliferation in a mouse model of colon cancer, implicating Nanog as a potential therapeutic target in the disease^[56].

Sox2 is contained within the most significantly amplified peak in lung and esophageal squamous cell carcinoma (SSC), identifying it as an oncogene for these cancers^[57]. Sox2 is also expressed in both mouse and human pre-neoplastic skin lesions and squamous cell carcinomas, but not in normal epidermis. Deletion of Sox2 in melanoma or SSC caused regression of tumours, and identified a number of genes involved in proliferation, stemness and cell survival which are regulated by Sox2, providing further evidence of a role for Sox2 in carcinogenesis^[58].

A number of studies have suggested a role for

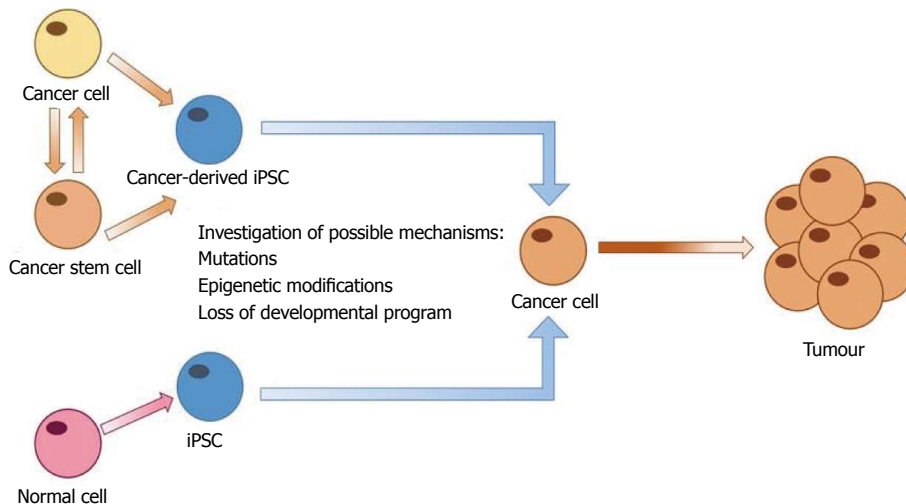


Figure 2 Generation of induced pluripotent stem cells from both normal and malignant patient tissue could be used to model carcinogenesis and elucidate the mechanisms which underlie the stages of cancer development. iPSC: Induced pluripotent stem cell.

typical stem cell genes in carcinogenesis. An 11-gene stem cell-like signature found in primary prostate tumours can be used as a predictive tool for prostate, breast and lung cancer patients, amongst other cancer types^[59]. Construction of a gene module map by Chang *et al.*^[60] identified modules specific to adult and murine ESCs and found that the ESC module was activated in human epithelial cancers. Activation of these genes was associated with poorly differentiated breast tumours with an increased risk of metastasis and death, and was also associated with an increased risk of death in lung adenocarcinomas. The c-Myc oncogene was subsequently shown to induce the ESC-like module both *in vitro* and *in vivo*^[60], consistent with the well-described role of c-Myc in stem cell self-renewal^[61]. An ESC signature developed by Weinberg's group showed enrichment of stem cell genes in poorly differentiated and larger breast tumours and again this was associated with a poorer survival. The ESC signature was also associated with high grade gliomas and bladder carcinomas^[62].

Metabolism

Altered metabolism has been implicated in cancer due to the "Warburg effect" whereby cancer cells use glycolysis for energy production rather than mitochondrial oxidative phosphorylation^[63]. Aerobic glycolysis occurs in cultured cancer cells and pluripotent stem cells^[64]. This is mediated by uncoupling proteins (UCPs) which uncouple oxidative phosphorylation from glycolysis, including UCP2 which is thought to be important for pluripotency^[65] and is also increased in most human color cancers^[66] as well as showing high expression in 90% of ovarian carcinomas and 94% of breast carcinomas^[67]. Recent studies have found that during induction of pluripotency in somatic cells, genes involved in glycolysis are upregulated whilst those involved in oxidative pathways are downregulated, mediating a switch from oxidative phosphorylation to glycolysis^[68] in a similar manner to that seen in carcinogenesis.

CURRENT MODELS OF CARCINOGENESIS

The generation of iPSCs from human somatic cells heralds a new era in disease modelling, allowing the development of patient specific models. As previously mentioned, despite improvements in cancer treatment the disease is still a major cause of morbidity and mortality. The lack of a relevant model to study the development of cancers and their progression has limited research which is suitable for translation to the clinical setting. Generation of iPSCs from human cancer cells represents an opportunity to develop *in vitro* models of carcinogenesis for specific cancer types (Figure 2).

Recently, a number of studies have generated iPSCs from cancer cells with the hope of developing such a model (summarized in Table 1). One of the earliest attempts to generate iPSCs from malignant cells used retroviral transduction of Oct3/4, Sox2, Klf4 and c-Myc into cancer cell lines from pancreatic, liver, stomach and colorectal cancers. These cancer-derived iPSCs had slower proliferation and increased sensitivity to chemotherapeutic agents in comparison to their parental cells^[69].

iPSCs and chronic myeloid leukemia

Carette *et al.*^[70] generated iPSCs from the KBM7 chronic myeloid leukemia (CML) cell line. Interestingly, despite the sensitivity of the KBM7 cell line to imatinib, the iPSCs generated lost their BCR-ABL dependence and became resistant to imatinib^[70], the tyrosine kinase inhibitor which targets the BCR-ABL protein which defines CML^[71]. In a further study, Kumano *et al.*^[72] derived iPSCs from primary cultures from two CML patients and showed stem cell morphology and markers along with the ability to differentiate into haematopoietic progenitors which expressed the BCR-ABL fusion protein. Again, these iPSCs were generated from imatinib-sensitive patients but became resistant. Once differentiated, immature cells (CD34⁺38⁺90⁺45⁺) were identified which were resistant

Table 1 Current induced pluripotent stem cells models of carcinogenesis

Malignancy	Study	Ref.
PDAC	Model of early PDAC and progression	Kim <i>et al</i> ^[73] , 2013
CML	iPSCs from CML cell lines and primary patient samples	Carette <i>et al</i> ^[70] , 2010 Kumano <i>et al</i> ^[72] , 2012
JML	Derived iPSCs from 2 JML patients	Gandre-Babbe <i>et al</i> ^[73] , 2013
Gastrointestinal cancer	Generated iPSCs from multiple GI cancer cell lines	Miyoshi <i>et al</i> ^[69] , 2010
Glioblastoma	iPSCs generated from glioblastoma-derived neural stem cells	Stricker <i>et al</i> ^[76] , 2014; Stricker <i>et al</i> ^[77] , 2013

iPSC: Induced pluripotent stem cells; PDAC: Pancreatic ductal adenocarcinoma; CML: Chronic myeloid leukaemia; JML: Juvenile myelomonocytic leukemia.

to imatinib and demonstrated phenotypic similarities to CML stem cells. This cell population may therefore be useful to interrogate the role of stem cells in CML and the mechanisms underlying the development of resistance^[72].

iPSCs and pancreatic ductal adenocarcinoma

iPSCs have also been used to study pancreatic ductal adenocarcinoma (PDAC), which currently has no suitable model. iPSC lines were generated from human tumours and injected into immunodeficient mice. After 3 mo, pancreatic intraepithelial neoplasia-like structures could be identified in 9 out of 10 teratomas, and by 9 mo solid tumours were present, suggesting that PDAC derived iPSCs can capture the process of carcinogenesis from pre-malignant lesions to the malignant phenotype. *In vitro* 3D culture of cells harvested from mice at 3 mo identified 25 proteins which were secreted by all teratomas, of which 8 have been previously reported in PDAC, pancreatic epithelial neoplasia or intraductal papillary mucinous neoplasms^[73]. However, although 9 patient samples were used in the study, only 1 iPSC line with a cancer genotype was generated from a single patient, which is consistent with other studies suggesting the difficulty of reprogramming malignant cells^[74].

iPSCs and juvenile myelomonocytic leukemia

A model of juvenile myelomonocytic leukemia using iPSCs derived from two patients has also recently been generated. Cells were able to differentiate to myeloid cells which showed phenotypic similarities to the primary tumours including enhanced proliferation, suggesting they could be a useful resource to model the disease^[75].

Gastrointestinal cancer and glioblastoma

iPSCs have also been generated from a number of gastrointestinal cell lines using retroviral transduction. These cancer-derived iPSCs showed reduced tumorigenic potential *in vivo* as well as increased sensitivity to anti-cancer drugs and decreased proliferative rate^[69]. This decrease in tumour forming ability is a concern for the use of cancer-derived iPSCs as it is thought that reprogramming cells removes epigenetic marks which are important for lineage identity and malignancy. However, lineage specificity may remain due to incomplete

reprogramming and can also be induced by differentiation. For example, in iPSCs derived from glioblastomas both lineage and cancer associated methylation marks were reset however during differentiation along the neural lineage cells maintained their malignant phenotype^[76,77].

Modelling carcinogenesis using non-malignant cells

An alternative method to study carcinogenesis using iPSCs is to reprogram normal cells to pluripotency and follow their development to interrogate the processes which contribute to cancer development (Figure 2). iPSCs are able to differentiate into all adult cell types and can therefore be used to model development of human tissues^[78]. iPSC models of organ and tissue development can then be monitored in real-time to identify any changes which may induce the onset of carcinogenesis. Recently, an *in vitro* model of skin was developed using iPSCs differentiated to keratinocytes and fibroblasts, providing an iPSC-generated *in vitro* model of a human organ^[79].

Alternatively, iPSCs from normal somatic cells could also be manipulated to study carcinogenesis through overexpression or silencing of oncogenes, tumour suppressor genes and other factors thought to play a role in carcinogenesis, or alteration of the microenvironment. The response of cells to these changes can then be studied to determine the roles of such factors in cancer initiation and progression.

CONCLUSION

Pluripotent cells and cancer cells share a number of characteristics including the ability for continual proliferation and self-renewal. iPSCs offer an opportunity to develop disease-specific models for carcinogenesis by reprogramming malignant cells. A number of studies have successfully generated iPSCs from human tumours and cancer cell lines and used them to study the underlying mechanisms of cancer. Generation of iPSCs from other cancer types is necessary to develop relevant *in vitro* models for carcinogenesis.

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CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture

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purification of human bone marrow mesenchymal stem cells. This marker has been shown to be specifically expressed by these cells. Thus, CD271 has been proposed as a versatile marker to selectively isolated and expand multipotent mesenchymal stem cells with both immunosuppressive and lymphohematopoietic engraftment-promoting properties. This review focuses on this marker, specifically on identification of mesenchymal stem cells from different tissues. Literature revision suggests that CD271 should not be defined as a universal marker to identify mesenchymal stem cells before culture from different sources. In the case of bone marrow or adipose tissue, CD271 could be considered a quite suitable marker; however this marker seems to be inadequate for the isolation of mesenchymal stem cells from other tissues such as umbilical cord blood or wharton's jelly among others.

Key words: Mesenchymal stem cells; CD271; Low-affinity nerve growth factor receptor; p75; Bone marrow

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Core tip: CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture.

Abstract

Mesenchymal stem cells, due to their characteristics are ideal candidates for cellular therapy. Currently, in culture these cells are defined by their adherence to plastic, specific surface antigen expression and multipotent differentiation potential. However, the *in vivo* identification of mesenchymal stem cells, before culture, is not so well established. Pre-culture identification markers would ensure higher purity than that obtained with selection based on adherence to plastic. Up until now, CD271 has been described as the most specific marker for the characterization and

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INTRODUCTION

In recent decades researchers have focused on regenerative medicine as a interesting option

for the treatment of different pathologies^[1,2]. Mesenchymal stem cells (MSC) are ideal candidates for cellular therapy due to their characteristics^[3]. MSC are viewed as clinically promising because of their differentiation capacity and pro-regenerative features^[4,5]. Moreover, MSC could be used for both autologous and heterologous transplant due to their low immunogenicity^[6]. Friedenstein *et al.*^[7] discovered bone marrow (BM)-MSC. They demonstrated that BM contains a population of cells with a high proliferative capacity that adhere to plastic. These authors were also the first to propose the capacity of these cells to form colonies from a single cell (the fibroblast-colony forming unit F-CFU)^[7-9]. Since Friedenstein *et al.*^[7] described MSC in the BM in the 70's several researchers have focused their attention on this type of adult stem cell. After their initial isolation from humans, MSC have since been successfully harvested from many other species including: mouse, rat, dog and horse^[10]. They have also been isolated from almost every type of tissue, including: BM, adipose tissue, liver, skeletal muscle, amniotic fluid, umbilical cord blood (UCB) or dental pulp^[11-14]. Due to the heterogeneity of the results obtained by many groups, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC. They proposed three criteria to define MSC in culture: adherence to plastic, specific surface antigen expression and multipotent differentiation potential^[15]. All these criteria perfectly define MSC in culture, however the *in vivo* identification of MSC, before culture, is not as well established. In 1999, Pittenger *et al.*^[16] described a small percentage of MSC present in BM (0.001%-0.01%) so this could explain the difficulty in establishing the exact phenotype of MSC before culture^[16].

A suitable method of selection would allow the employment of MSC in different pathologies directly after their isolation or after their *in vitro* expansion. Pre-culture identification markers would ensure higher purity than that obtained with selection based on adherence to plastic. Many investigators direct their efforts towards find a marker or a combination of markers to ensure their selection.

Up until now, CD271 (LNGFR) has been described as one of the most specific markers for the purification of human BM-MSCs^[17,18]. CD271, also known as low-affinity nerve growth factor receptor (LNGFR), nerve growth factor receptor (NGFR), or p75NTR (neurotrophin receptor), belongs to the tumor necrosis factor superfamily^[19]. Taking into account that this marker has been the most commonly used in the isolation of BM-MSC this review will focus on to the identification of MSC from different tissues (Table 1).

BM

Several independent studies have confirmed the

specificity of CD271 on MSC isolated from BM. At the beginning of the last decade, Quirici *et al.*^[18], (2002) published that the anti-CD271 antibody is specific for a population of multipotent BM cells and suggested the use of this marker as an option for the selection of MSC from BM^[18]. The same year, Jones *et al.*^[20], conducted a study in which they carried out a cellular purification from BM with antibody-conjugated magnetic beads. They found that the D7-FIB-positive fraction contained not only all the CFU-F activity but also a unique population of CD45^{low}/LNGFR⁺ cells. These cells were adherent, proliferative, and multipotent following cell sorting and standard expansion^[20]. In 2006 the same group, suggested the need to find a way to count MSC based on the use of surface markers considering that up to this point researchers were using CFU-F assays for the enumeration of MSC. Published results demonstrate the use of flow cytometry as a rapid MSC detection method and suggested *in vivo* selection of the phenotype CD45^{low}/LNGFR⁺/D7FIB⁺^[21]. Following the studies started in 2002, Jones's group continued providing more data about this marker. In a comparative study, Jones *et al.*^[22] demonstrated that CD271 antigen (followed by CD146, CD106, D7-FIB, CD13 and CD166) remained one of the most selective markers for enriching progenitor cells from MSC of human BM. These results are supported by Kuçi *et al.*^[23,24], who published a study which demonstrated that the CD271 is an adequate marker for the selection of multipotent BM cells with immunosuppressive properties. Afterwards, the same group published another study in which they demonstrated that CFU-F activity was found only in the CD271⁺ cell fraction, whereas no CFU-F was observed in the CD271⁻ population^[24]. Flores-Torales *et al.*^[25], (2010) proposed the use of a single marker, CD271, for the selection of MSC from BM before culture. These authors maintain that the use of this marker would reduce costs and provide a rapid and simple way of obtaining MSC. However, a high percentage of CD271⁺ cells in BM and synovium co-express CD34, which disqualifies CD271 as a unique marker for the isolation of MSC. Nevertheless, these studies, among others, have confirmed the usefulness of CD271 in combination with other markers such as CD45 to isolate fresh BM-MSC. Poloni *et al.*^[26], (2009) carried out a selection of CD271 positive cells and cultivated them in a media supplemented with 10% allogeneic human sera, cells maintained the capacity to differentiate and no karyotypic variations were observed. Our group utilized this marker (CD271⁺/CD45⁻) to quantify the MSC population in BM samples obtained for cell therapy using flow cytometry^[27]. Recently Mabuchi *et al.*^[28] performed a comprehensive screening of putative surface markers to select the most useful ones for prospectively identifying a pure MSC population in human BM. They concluded that the combination marker CD271⁺CD90⁺CD106⁺ can

Table 1 Summary of references documenting CD271 expression in different sources

Source	CD271	Ref.
Bone marrow	Positive	Alvarez-Viejo <i>et al</i> ^[27] , 2013 Flores-Torales <i>et al</i> ^[25] , 2010 Jones <i>et al</i> ^[20] , 2002; Jones <i>et al</i> ^[21] , 2006; Jones <i>et al</i> ^[22] , 2008 Kuçi <i>et al</i> ^[23] , 2011; Kuçi <i>et al</i> ^[24] , 2010 Poloni <i>et al</i> ^[26] , 2009 Quirici <i>et al</i> ^[18] , 2002
Adipose tissue	Positive	Cuevas-Diaz Duran <i>et al</i> ^[31] , 2013 Quirici <i>et al</i> ^[33] , 2010
Umbilical cord blood	Negative	Attar <i>et al</i> ^[46] , 2013 Alvarez-Viejo <i>et al</i> ^[44] , 2013 Watson <i>et al</i> ^[47] , 2013
Wharton's Jelly	Low/negative	Alvarez-Viejo <i>et al</i> ^[44] , 2013 Margossian <i>et al</i> ^[35] , 2012
Placenta	Positive/negative	Battula <i>et al</i> ^[50] , 2008 Soncini <i>et al</i> ^[52] , 2007
Trabecular bone cavity	Positive	Jones <i>et al</i> ^[56] , 2010
Dermis	Positive	Vaculik <i>et al</i> ^[57] , 2012
Peripheral blood	Negative	Attar <i>et al</i> ^[55] , 2011

be used selectively to isolate the most potent and genetically stable MSC^[28]. Therefore, CD271 may be considered a suitable marker for determining MSC in BM.

It has been demonstrated that as the age of donors increases the number and potential for differentiation of BM MSC diminish^[29]. Taking into consideration that it is not easy to obtain such blood donors other sources for the obtention of MSC are required.

Adipose tissue is an interesting option for this end. MSCs can be isolated from fat tissue easily obtained by liposuction. It has been demonstrated that these cells are easily cultivated and have the capacity to differentiate into various cell lines^[30].

ADIPOSE TISSUE

In 2002 a type of stem cell from adipose tissue was isolated for the first time: adipose-derived stem cell (ADSC)^[31]. Since then, it has been demonstrated that adipose tissue provides an abundant source of MSC with similar yields to those obtained from BM. Furthermore, ADSC have a similar differentiation capability, morphology, and phenotype to MSC collected from BM^[31]. It has been shown in mice that p75NRT seems to be a useful marker for ADSC isolation^[32]. After this, Quirici *et al*^[33] selected CD271⁺ cells immune-magnetically from ADSCs in humans in a similar way as has been done from BM by others authors. CD271⁺ cells showed higher clonogenic and differentiation potential compared to plastic adherent ADSC. Thus these authors demonstrated the utility of this marker in the selection of ADSC cells^[33]. Cuevas-Diaz Duran *et al*^[31] used this marker to analyze if there was a relationship between donor's age and CD271⁺ cell yield in freshly isolated ADSC. They suggested that the proportion of CD271⁺ ADSC decreased with

age; however, positive cells were present in all age groups and their frequency was higher than what has been found in BM. Therefore, CD271⁺ cells from adipose tissue were proposed as the primary choice for tissue regeneration and autologous stem cell therapies in older subjects^[31].

The umbilical cord, placenta and placental membrane have been presented as cheap and attractive alternatives for the obtention of MSC^[34].

UMBILICAL CORD

The UC is the structure that connects the foetus to the placenta. The UC has two arteries and a vein wrapped in a gelatinous connective tissue called Wharton's jelly (WJ). The UC has characteristics ideal for procuring of MSC, because its use would not generate ethical conflicts, it is easily obtained and it is an abundant resource which is discarded^[35]. As described below, MSCs have been described from UCB and from WJ.

UCB

UCB was found to contain different populations of stem cells, a unique feature not shared with peripheral blood. Some authors propose that UCB derived MSC show high morphological and molecular similarities to BM derived MSC including the lack of hematopoietic surface antigens^[36]. The presence or absence of MSC in UCB has been quite controversial. A few researchers have denied the presence of MSC in UCB such as Yu *et al*^[37], (2004) whose results suggest that early fetal blood is rich in MSC however, the term UCB is not used. On the other hand, some authors propose the difficulty in obtaining MSC from UCB to be due to their low frequency, inferior to that in BM. Up until now, the literature does not

show interesting results regarding the obtention, isolation and purification of UCB MSCs. Some publications show that the MSC can be obtained in a low percentage of UCB units (20%-63%)^[38,39]. These data bring into question the possibility of considering the UCB as a resource for acquiring MSC^[40]. However, several authors have differentiated UCB-MSC *in vitro* to osteogenic, chondrogenic, neural and hepatic lineages successfully^[41-43]. Our group has not been able to identify MSC from UCB using conventional methods of adherence to plastic. We observed a heterogeneous population of adherent cells, with a rounded and spindle-shaped appearance^[44]. Our results are in concordance with Perdikogianni *et al.*^[45], (2008). We also used CD271 for the presence of cells from UCB expressing this marker. Our results showed no validity for this marker in this tissue and these data are in agreement with two studies published in parallel. Attar and coworkers showed that CD271 did not contribute to isolation of MSC from UCB^[46]. Watson *et al.*^[47], (2013) published that, CD271 is an efficient marker for MSC isolation from BM but failed to isolate MSC from UCB. Therefore, our experience and results from the literature suggest that CD271 is not a suitable marker for the identification of MSC from UCB without culture.

WJ

Thomas Wharton was the first to describe WJ in 1656. WJ is a gelatinous substance composed of various isoforms of collagen and proteoglycans. The principal function of WJ is to protect the arteries and veins from the compression and torsion that they can be subjected to. These provide a bidirectional flow, providing oxygen and nutrients that contribute to the adequate development of the foetus and eliminating waste and carbon dioxide^[48]. It has been demonstrated that human WJ-MSC present an elevated capacity for autoregeneration and have been compared with those obtained from BM^[39]. MSC derived from the discarded UC, more precisely WJ, offer a low-cost and pain-free collection method of MSC that may be cryogenically stored, and are considered extremely favorable for tissue engineering purposes^[35]. Data published by our group highlight the ineffectiveness of CD271 as a marker for the isolation of MSC from WJ before culture^[44]. These results are supported by data published by Margossian *et al.*^[35]. They studied the expression of CD271 *in situ* on fresh fragments of WJ and they observed it to be weakly expressed^[35]. Although few studies have focused on determining the effectiveness of this marker in WJ, those that have been carried out suggest that CD271 is not a suitable marker to identify MSC from WJ before culture.

PLACENTA

Human placenta, plays a fundamental and essential

role in fetal development, nutrition, and tolerance^[49]. Various reports have demonstrated that human term placenta is a plentiful source of MSC^[50,51]. Considering the complexity of the structure of the placenta, Parolini *et al.*^[49] published a paper in order to define, as clearly as possible, the region of origin and methods of isolation of cells derived from this tissue. This work arose out the first international Workshop on Placenta Derived Stem Cells, (March 2007). One of the main characteristics is the existence of four regions in the placenta: Amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal and chorionic trophoblastic. From these regions, the following cell populations are isolated: amniotic epithelial cells, amniotic mesenchymal stromal cells, chorionic mesenchymal stromal cells, chorionic trophoblastic stromal cells^[49].

In 2007, Soncini *et al.*^[52], achieved the isolation of amnion mesenchymal cells (AMSC) and chorion mesenchymal cells (CMSC). These MSCs were isolated by a mechanical separation followed by enzymatic digestion. AMSC and CMSC show MSC characteristics, such as adherence to plastic, fibroblastic morphology and the capacity to form colonies. Both types of cells when analysed by flow cytometry show phenotypes similar to BM-MSC. Also, AMSC and CMSC demonstrate high plasticity when cultivated in adequate differentiating media, showing that these can differentiate into fat, bone and cartilage.

In order to isolate cells with MSC characteristics from human fetal membranes, AMSC and CMSC expressing CD271 were enriched by immunomagnetic isolation. CD271⁺ cells were demonstrated to possess higher clonogenic and osteogenic differentiation potentials than CD271-depleted fractions. Based on these findings, these authors suggest that amnion and chorion can be considered as a novel and convenient sources of adult MSC^[52]. Another study published in 2007 by Battula *et al.*^[50] confirmed the possibility of obtaining MSC from chorion; however, their results showed that CD271 is not an adequate marker for the identification of MSC. They demonstrated that CD271 is expressed only at negligible levels on naive placenta MSC. Nevertheless, they identified FZD9 (frizzled-9) as a novel marker for isolation of MSC from chorionic placenta and showed that cells with CFU-F capacity reside exclusively in the FZD9⁺ population^[50]. FZD proteins comprise a family (FZD1-10) of seven transmembrane-spanning receptors^[53]. Based on the literature, it is still not possible to confirm CD271 as a suitable marker for the isolation of MSC from placenta.

Because of their attractiveness, researchers have attempted to isolate MSC from many tissues and their existence has been documented in several of them. Here, we discuss some studies which have referred to the use of CD271 for the identification of MSC from tissues other than those discussed above.

OTHER SOURCES

Chong *et al.*^[54] in 2012 demonstrated that MSC from peripheral blood maintain similar characteristics and have similar chondrogenic differentiation potential to those derived from BM. Based on the literature, we have found just one reference which documents that CD271 positive selection can not help isolation of MSC from granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood^[55]. Another source is published by Jones *et al.*^[56], (2010). They showed that CD45^{low}CD271⁺ MSC are abundant in the trabecular bone cavity and indistinguishable from aspirated CD45^{low}CD271⁺ MSC. Moreover, MSC are found in human dermis. Vaculik *et al.*^[57] distinguished dermal MSC from differentiated fibroblasts. For this they selected CD271⁺ and SSEA-4⁺ cells from adherent dermal cells and checked their differentiation capacity. They observed that a CD271⁺ dermal population presented a greater potential for adipogenic, osteogenic and chondrogenic differentiation.

Besides CD271, several markers have been used in order to identify MSC before culture. Possibly one of the most commonly used markers for this purpose is Stro-1. After a complete review, Lv *et al.*^[58], concluded that Stro-1 expression appears not be a universal marker for MSC from different tissues. Another marker used to identify MSC is SSEA-4. It is an embryonic stem cell marker and it was documented in the isolation of genuine MSC from BM^[59]. Conversely, other authors reported no detection of SSEA-4 expressing cells in unsorted BM^[60,61].

Taken together, the data discussed in this review suggest that CD271 would not be considered as a universal marker to identify MSC before culture. In the case of BM or adipose tissue, CD271 could be considered a quite suitable marker for the isolation of MSC. As described in this review, several independent studies confirm the specificity of this marker in different tissues. However, CD271 is not adequate in the isolation of MSC from other tissues such as UC or UCB. Moreover, in the case of placenta contradictory results have been obtained by different groups. These contradictory results could be due to variations in the methodologies used by different laboratories. Hines *et al.*^[62] published an interesting study in which they demonstrated that membrane markers are notoriously dynamic and their expression can often be dependent on minor technical issues. Technical issues, indicating that the reproduction of results is the corner-stone of science^[62].

Due to the interest in MSC for their potential applications in the clinic, it is necessary to continue research in this field, in order to find a marker or markers for optimal selection and identification of MSCs without culture. This would allow the generation of purer cultures than those obtained by adherence to plastic alone and possibly direct application avoiding cultivation costs, time and risk of contamination.

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Adult stem cells in neural repair: Current options, limitations and perspectives

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that defy doctors and researchers around the world. Stem cells can be divided into three main groups: (1) embryonic stem cells; (2) fetal stem cells; and (3) adult stem cells. In terms of their capacity for proliferation, stem cells are also classified as totipotent, pluripotent or multipotent. Adult stem cells, also known as somatic cells, are found in various regions of the adult organism, such as bone marrow, skin, eyes, viscera and brain. They can differentiate into unipotent cells of the residing tissue, generally for the purpose of repair. These cells represent an excellent choice in regenerative medicine, every patient can be a donor of adult stem cells to provide a more customized and efficient therapy against various diseases, in other words, they allow the opportunity of autologous transplantation. But in order to start clinical trials and achieve great results, we need to understand how these cells interact with the host tissue, how they can manipulate or be manipulated by the microenvironment where they will be transplanted and for how long they can maintain their multipotent state to provide a full regeneration.

Key words: Stem cells; Stem cell therapy; Adult stem cells; Neural stem cells; Bone marrow stem cells; Mesenchymal stem cells; Olfactory ensheathing cells

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Core tip: Adult stem cells are useful tools to treat various diseases, but we must first comprehend how they work to use their capacity at maximum. Ageing, inflammation, other stem cells of the host tissue and the co-transplantation with another stem cells type can change their profile and compromise the regeneration process. Having these barriers in mind, several researchers started to look more closely to adult stem cells. In this review we will show some interesting results from experimental and clinical trials at this group of stem cells.

Abstract

Stem cells represent a promising step for the future of regenerative medicine. As they are able to differentiate into any cell type, tissue or organ, these cells are great candidates for treatments against the worst diseases

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INTRODUCTION

Stem cells represent a promising step for the future of regenerative medicine. As they are able to differentiate into any cell type, tissue or organ, these cells are great candidates for treatments against the worst diseases that defy doctors and researchers around the world.

Stem cells can be divided into three main groups: (1) embryonic stem cells, which are cells of the inner layer of the blastocyst and form the three primary germ layers (ectoderm, mesoderm and endoderm); (2) fetal stem cells, which are found in the embryo and form the various organs of the human body; fetal stem cells include neural crest cells, hematopoietic cells, fetal mesenchymal stem cells, fetal neural stem cells and pancreatic islet progenitors; and (3) adult stem cells, which are found in various regions of the adult organism, such as bone marrow, skin, eyes, viscera and brain (adult stem cells are also known as somatic stem cells).

In terms of their capacity for proliferation, stem cells are also classified as totipotent, pluripotent or multipotent. Totipotent stem cells have a high capacity for differentiation and can form a complete individual, but they lack the capacity for self-renewal. Pluripotent cells, on the other hand, are capable of differentiating into any of the 200 or more kinds of cells that make up the human body. Finally, multipotent cells give rise to cells of a specific tissue, *e.g.*, hematopoietic stem cells give rise to blood cells, while neural stem cells give rise to neurons and glia^[1,2]. Another group of stem cells that has been extensively studied in recent years includes induced pluripotent stem cells (IPS). IPS cells are adult differentiated cells that return to their pluripotent state by genetic engineering mechanisms. Some researchers believe these cells have great potential, but there have been numerous reports of extensive epigenetic and transcriptome aberrations with these cells, as well as tumor formation^[3,4].

ADULT STEM CELLS

Adult stem cells are multipotent cells that can differentiate into unipotent cells of the residing tissue, generally for the purpose of repair. These cells can be obtained from several different regions of the adult organism, they can be used for autologous transplantation and their great advantage resides in the fact that embryos do not have to be destroyed in order for these cells to be obtained^[5,6].

Regardless of the cell type chosen, there is a

relative degree of difficulty in extracting viable stem cells from their *in vivo* niche. There is approximately one hematopoietic stem cell for every 10000 bone marrow cells, and one mesenchymal stem cell for every 10000-100000 cells^[7]. For this reason, researchers use substances that can stimulate the growth of these cell populations as well as facilitate the process by which they are obtained. For example, G-CSF (granulocyte stimulating factor) stimulates the production of CD34⁺ hematopoietic stem cells that can be extracted by simple puncture of peripheral blood, which is less invasive than bone marrow aspiration. Other substances that enhance the isolation of adult stem cells are currently under investigation^[8].

Another issue of concern is the cell senescence related to the expansion time *in vitro*. Cell proliferation is decreased mainly due to telomerase activity, and several researchers have focused on telomerase activation in an attempt to increase and maintain population growth *in vitro*. However, it focusing only on correcting the shortened telomeres is not sufficient, and studies should focus on the role of other factors that participate in telomere maintenance, which may also play an important role in cell differentiation^[9].

Senescence of adult organisms has to be considered, given that cell function, potency and replication decrease with time, while mutations may increase. In the case of the nervous system, mutations may result in neuronal loss, abnormal cell function, increased neuronal longevity (resulting in an even greater accumulation of mutations) and decreased regenerative capacity after an injury. These findings have been reported in studies investigating cell proliferation during learning: while young animals showed better performance than older animals, pharmacological stimulation in the older animals led to improved performance, with the newborn neurons integrating into the existent neural circuits^[10-12]. Moreover, the aging of stem cell niches may contribute to the aging of stem cells, changing the organism's regulatory microenvironment, which in turn influences the aging process of other cells. To summarize can be quoted the influence of protein p53 expression levels: when expressed at low levels, it contributes to stem cell metabolic maintenance, but when expressed at high levels, it decreases the number of stem cells by inducing cell death, and the role of energetic metabolism and the production of reactive oxygen species that acts directly in stem cell niches^[13].

Another important feature of stem cells is their immunomodulatory power, which allows them to "feel" the environment and change their patterns of migration, interaction and survival. When transplanted into the lesion site, some stem cells interact with the proinflammatory environment, migrate to the site of injury and differentiate into the required cell type in order to effect repair. Moreover, some stem cells play an important role in the proliferation and migration of new cells to the site of injury. This helper profile has

been observed in neural stem cells and mesenchymal stem cells. In the case of mesenchymal stem cells, this function can play an important role in changing the macrophage proinflammatory profile into a proneurogenic role, once there is no longer an urgent need for a robust immune response^[14-16].

Understanding the biology of stem cells and their niches, as well as the mechanisms involved in their multipotent state, are basic criteria to achieve great experimental results and to take the next step towards clinical trials.

MESENCHYMAL STEM CELLS/BONE MARROW STEM CELLS

The bone marrow is the only organ in which two different types of stem cells coexist: the bone marrow stem cell (BMC, also called hematopoietic stem cell) and the mesenchymal stem cell (MSC, also called mesenchymal stromal cell). Bone marrow cells are responsible for generating blood cells, while mesenchymal stromal cells are responsible for supporting hematopoietic progenitors by regulating the niche microenvironment and by facilitating the maturation of blood cells. Mesenchymal cells can also be extracted from adipose tissue, umbilical cord, skin and placenta^[17,18].

Experimental studies have shown that MSCs can survive and migrate to the lesion site, prevent astrogliosis and microglial activation, and delay the loss of motoneurons. A comparative study between BMCs and MSCs showed that BMCs are relatively more effective in motor function recovery and have a higher survival rate^[18,19]. In clinical trials with BMCs, authors reported motor and sensory improvement, as well as improvement in patients' quality of life, with some being able to sit again and even get dressed with partial assistance^[19-23]. MSCs also showed great results when transplanted with T cells, controlling inflammatory activity in order to create the proper microenvironment for cell transplantation^[24]. The immunomodulating effect of MSCs has also been reported in other clinical trials and MSCs have been shown to play an important role as immunomodulators and angiogenic agents in drug resistant patients as well as in patients with cerebral palsy, critical limb ischemia and kidney transplants^[25-32]. In another study conducted with 40 patients, bone regeneration was achieved when a great number of CD34⁺ cells were transplanted to the lesion site, 20 patients achieved mature bone regeneration, even in the group that received a low number of cells 4 patients achieved bone regeneration. However, when transplanted together with MSCs, these cells were able to use paracrine and autocrine cross-talk up-regulation to achieve bone regeneration^[33].

Despite these positive results, several side effects were reported when MSCs from adipose tissue were

administered *via* the cephalic vein, including chest pain and tightness, mild fever, furuncle on the upper thigh, musculoskeletal pain, painful neck and shoulder, increased sputum, upper respiratory infection, urinary incontinence, urinary tract infection, aggravation of spasticity, neuropathic pain, pain exacerbation, headache, low thyroid stimulating hormone and somnolence^[34].

OLFACTORY ENSHEATHING CELLS

Neurogenesis in the olfactory system continues to take place even in the adult. Stem cells proliferate in the subventricular zone of the forebrain, generating neural progenitors that migrate to the olfactory bulb to create new interneurons. If an injury occurs, these neurons are immediately replaced through a surge in neurogenesis. Olfactory ensheathing cells (OECs) surround the axons of the sensory neurons in the olfactory epithelium and form synapses in the olfactory bulb in the brain. Due to their ability to guide the connections between the peripheral nervous system and the central nervous system, as well as their ability to differentiate into non-olfactory cell types, these cells are excellent candidates for cell transplantation^[35]. These multipotent cells have been extensively studied in cases of spinal cord injury, and authors have reported that transplants were safe and patients experienced motor and sensory improvement, as well as recovered bladder function and activity of several muscles below the injury level^[36,37]. In an amyotrophic lateral sclerosis (ALS) clinical trial conducted with OECs in China, researchers reported that patients experienced no benefits, two patients had severe side effects and one even died following transplantation^[38]. One theory contends that OECs should be transplanted together with neural stem cells in order to potentiate the growth of neural processes. OECs have been shown to stimulate axon regeneration by secreting growth factors, axon guidance molecules and basement membrane components. They also aid in tissue repair by effecting structural remodeling and support, modulating the immune system, enhancing neurotrophic and antigenic stimuli and metabolizing toxic macromolecules. Finally, OECs may be transplanted together with growth factor (*e.g.*, bFGF, or basic fibroblast growth factor) to sustain cell survival and proliferation^[39-41].

NEURAL STEM CELLS

Neural stem cells were first described by Altman in 1960 and have the potential to differentiate into any cell type in the central nervous system^[42-45]. It is known that adult neurogenesis occurs in two brain regions, the subventricular and subgranular zones of the dentate gyrus, and the spinal cord^[45].

Neural stem cells have been used to treat several neurologic conditions such as spinal cord injury, ALS, Parkinson's disease, traumatic brain injury, Huntington's

Table 1 Adult stem cells transplantations: Published clinical trials from the past 4 years

Ref.	Year	No. of patients	Age range	Type of cell grafted	Adjuvant treatments	Follow-up
Dai <i>et al.</i> ^[53]	2013	20	22-54 yr	MSCs	-	6 mo
García-Santos <i>et al.</i> ^[54]	2013	11	33-61 yr	MSCs	-	12 mo
Tian <i>et al.</i> ^[55]	2013	97	21.1-38.2 ¹ yr	MSCs	-	14 d
Frolov <i>et al.</i> ^[56]	2012	20	18-55 yr	HSCs	-	48 mo
Karamouzian <i>et al.</i> ^[20]	2012	31	10-50 yr	BMCs	-	33 mo
Martínez <i>et al.</i> ^[57]	2012	67	49.2 ± 10.3 ¹ yr	CD133+	-	12 mo
Mazzini <i>et al.</i> ^[28]	2012	19	20-75 yr	MSCs	-	108 mo
Moviglia <i>et al.</i> ^[47]	2012	7	33-78 yr	NSCs	T-cell vaccine	12 mo
Prasad <i>et al.</i> ^[58]	2012	11	30-70 yr	MNCs	-	52 wk
Brazzini <i>et al.</i> ^[59]	2010	53	38-81 yr	BMCs	-	1-18 mo
Karussis <i>et al.</i> ^[26]	2010	19	53.0 ¹ yr	MSCs	-	25 mo
Lee <i>et al.</i> ^[60]	2010	16	64.0 ± 11.6 ¹ yr	MSCs	-	5 yr
Venkataramana <i>et al.</i> ^[61]	2010	7	22-62 yr	MSCs	-	12-36 mo

¹The authors only provide mean values. MSCs: Mesenchymal stem cells; HSCs: Hematopoietic stem cells; BMCs: Bone marrow stem cells; NSCs: Neural stem cells; MNCs: Mononuclear cells from bone marrow.

s disease and several demyelinating diseases. The main issues to consider when using neural stem cells are how the transplanted cells will interact with the host microenvironment, how the local immunological response will interfere with and prevent neurorestoration, and how the transplanted neural stem cells will modulate the microenvironment *via* paracrine and autocrine effects. These mechanisms need to be clarified before moving on to clinical trials. Several investigators have questioned the behavior of transplanted cells and several transplantation strategies have been tested, including co-transplantation with other stem cell types, T cells or neurospheres. Nevertheless, much work needs to be done in order to better comprehend how neural stem cells interact with the host tissue^[45-52]. Clinical trials have not shown statistically significant results, Moviglia *et al.*^[47] transplanted neural stem cells in seven patients with ALS and only in five patients were observed motor improvements. In their combined protocol the local immunological response were controlled by a T-cell vaccination before the transplants of NSCs, but authors agree that the transplantation of neural stem cells is safe and feasible^[46-49] (Table 1).

CONCLUSION

Adult stem cell transplantation represents a promising choice of treatment for the field of regenerative medicine, but several aspects must still be clarified before proceeding with clinical trials. More studies are needed to establish how to obtain a large population of adult stem cells and to ensure the safety and viability of the transplants. We must also understand these cells' mechanisms of interaction and how we can use these mechanisms to achieve full regeneration.

Based on the studies cited here, it is possible to affirm that in the near future we will have effective therapies against various diseases that affect and challenge the medical community and the population

at large.

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Development of cancer-initiating cells and immortalized cells with genomic instability

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epigenetic aberrations. The development of genomic instability is associated with mutations that contribute to cellular immortalization and transformation. Cancer occurs when cancer-initiating cells (CICs), also called cancer stem cells, develop as a result of these mutations. In this paper, we explore how CICs develop as a result of genomic instability, including looking at which cancer suppression mechanisms are abrogated. A recent *in vitro* study revealed the existence of a CIC induction pathway in differentiating stem cells. Under aberrant differentiation conditions, cells become senescent and develop genomic instabilities that lead to the development of CICs. The resulting CICs contain a mutation in the alternative reading frame of *CDKN2A* (ARF)/p53 module, *i.e.*, in either ARF or p53. We summarize recently established knowledge of CIC development and cellular immortality, explore the role of the ARF/p53 module in protecting cells from transformation, and describe a risk factor for genomic destabilization that increases during the process of normal cell growth and differentiation and is associated with the downregulation of histone H2AX to levels representative of growth arrest in normal cells.

Key words: ARF/p53 module; Cancer stem cells; Cancer-initiating cells; Differentiation; Genomic instability; H2AX

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Core tip: Cancer usually develops in conjunction with genomic instability and multiple genetic mutations. Only a small number of cells, called cancer-initiating cells (CICs), are the progenitors of cancerous tissue; but how genomic instability and genetic mutations prompt CICs to develop is still unclear. Recent investigations have uncovered the existence of a pathway that could be responsible. This review explores how that pathway might induce the development of CICs, the tumor suppression mechanisms that must be abrogated in order for malignancies to occur, and the role of the alternative reading frame of *CDKN2A* (ARF)/p53 module/p53 module in protecting

Abstract

Cancers that develop after middle age usually exhibit genomic instability and multiple mutations. This is in direct contrast to pediatric tumors that usually develop as a result of specific chromosomal translocations and

normal cells from oncologic transformation.

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INTRODUCTION

Somatic stem cells are responsible for the development and homeostasis of organs and other bodily tissues. Cancer-initiating cells (CICs), or cancer stem cells, are responsible for the development^[1,2], metastasis, and drug resistance^[3-6] of tumors. Although CIC characteristics are increasingly being well defined, how CICs develop remains unclear.

Unlike pediatric tumors, which usually develop as a result of very specific chromosomal translocations^[7,8] and epigenetic aberrations^[9,10], cancers that become more common around the age of 40 years exhibit extreme chromosomal instability (CIN) or microsatellite instability (MSI)^[11-14]. MSI occurs in cells that do not have adequate mismatch repair systems^[15-17], and CIN occurs in the presence of other types of repair deficiencies. The hereditary versions of myelodysplastic syndrome^[18], breast and ovarian cancers^[19-22], and skin cancers^[23-25] are examples of CIN. CIN can even occur in normal senescent cells^[26,27].

MSI and CIN usually do not occur together and are considered mutually exclusive. For example, 15% of colorectal cancers exhibit MSI, but most of the remainder exhibit CIN. There are also tumors that do not exhibit either MSI or CIN and result from DNA polymerase ϵ mutations, which interfere with proofreading functions and produce hypermutations^[28,29]. Massive genomic rearrangements occur in a wide variety of cancers^[30-32], and the unstable structures that result vary widely^[33,34]. Since only a very small number of CICs are necessary to produce a malignancy, what causes CICs to develop in the first place is an important question^[35,36].

In malignant cells, mutations in either alternative reading frame of *CDKN2A* (ARF) or p53 are common. The ARF/p53 module plays a major role in keeping normal cells from transforming into malignancies, so these mutations that interfere with ARF/p53 functioning show us how the barrier reactions performed by the ARF/p53 module normally work and the way genomic instability promotes the development of CICs.

EFFECTS OF GENOMIC INSTABILITY

Like cancer cells that develop because of genomic instability *in vivo*^[37-40], cells cultured *in vitro* can also be transformed and/or immortalized in association with either CIN- or MSI-type genomic instabilities^[26] and

mutations in the ARF/p53 module^[41]. Following serial proliferation, normal mouse embryonic fibroblast cells (MEFs) stop reproducing after a growth arrest command issued by the ARF/p53 module^[41]. This command prevents cells from immortalizing^[27,42], but immortality can develop if the genome is destabilized^[43], clearly demonstrating that genomic instability is a triggering event in cellular immortalization.

Genomic instability probably contributes to the induction of mutations in the ARF/p53 module^[27,42]. In MEFs, immortalization can result if genomic instability is induced and/or mutations occur in either ARF or p53 (Figure 1), but is blocked in cells with stable genomes that are under the continuous regulation of the ARF/p53 module^[27]. Healthy ARF/p53 regulation is essential for the prevention of cellular transformation and immortalization, but which functions of ARF and p53 are responsible for tumor suppression is controversial.

It was generally believed that p53 suppresses tumors by inducing apoptosis and senescence^[44-47], but recent studies have cast doubt on this hypothesis. For example, in multiple transgenic mouse models in which p53 cannot induce cell-cycle arrest and apoptosis after DNA damage, the number of malignancies that form as a result is not significantly higher than the rate of malignancies in normal mice^[48-50]. These observations raise questions regarding the exact role of p53 in tumor suppression. One hypothesis is that p53 regulates metabolism by inhibiting glycolysis^[51-53] and/or activating the mammalian target of rapamycin pathway^[52,54,55].

EFFECT OF MUTATIONS IN THE ARF/P53 MODULE

Because ARF and p53 are mutated in cancer cells in a mutually exclusive manner, it is likely that the p53 functions that are essential for cancer suppression are expressed under the control of ARF^[56]. Intriguingly, transgenic mice with an extra copy of *Arf* and *p53* (super-*Arf/p53* mice) exhibit both reduced rates of cancer and slower overall aging^[41]. Furthermore, MEFs derived from these mice rarely immortalize spontaneously. Therefore, it is likely that the roles of ARF and p53 in cancer suppression are involved in the maintenance of homeostasis *in vivo* and are primarily regulated at the level of individual cells. This cellular-level protection against transformation is generally associated with growth arrest and low levels of the histone H2A variant H2AX^[27,57], which is required for active cell growth^[58].

The downregulation of H2AX is governed by the ARF/p53 module^[27,42]. Growth-arrested cells in the liver, spleen, and other organs of healthy adult mice generally exhibit downregulated levels of H2AX, whereas immortalized/transformed cells, which have mutations in the ARF/p53 module, have normal H2AX levels and exhibit active growth^[27,57].

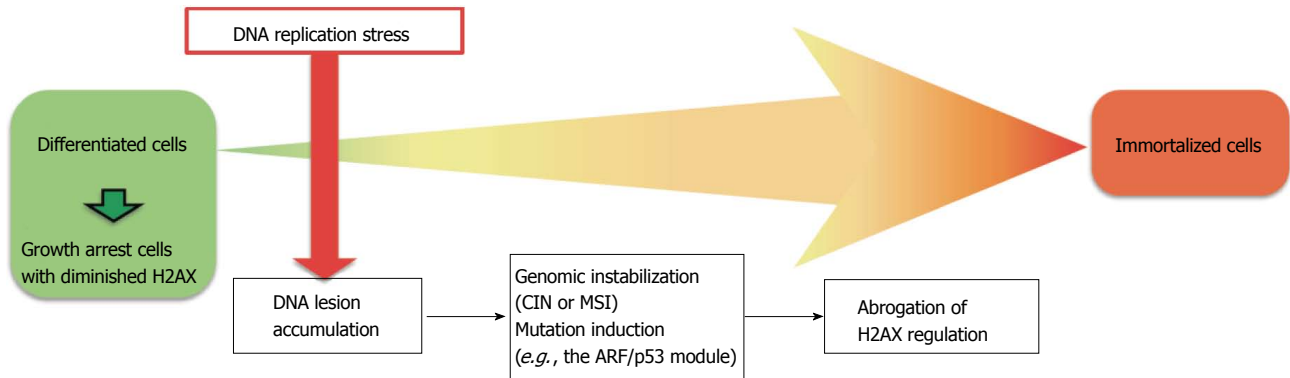


Figure 1 Model of cellular immortalization in association with genomic instability. As illustrated in mouse embryonic fibroblasts, immortality is induced by genomic instability and mutation of either Arf or p53. Growth arrest and the downregulation of H2AX protect mouse embryonic fibroblasts against immortalization. Because H2AX downregulation is dependent on the ARF/p53 module, cells with mutations in this module recover H2AX expression and growth activity. CIN: Chromosomal instability; MSI: Microsatellite instability.

As described above, cellular transformation is suppressed primarily by the induction of growth arrest and the downregulation of H2AX under the control of the ARF/p53 module. It is promoted by genomic instability and the mutations it produces. Unfortunately, when H2AX is downregulated, the mechanisms that repair lesions fail and this is a risk factor for the genomic instability and tumorigenesis seen in many hereditary cancers^[15-25]. Despite the aforementioned protective effect of H2AX downregulation, the risk of sporadic cancer development is probably due, in large part, to a reduction in H2AX levels and the associated repair deficiencies^[27]. In fact, cells without H2AX exhibit faulty homologous recombination and non-homologous end-joining during DNA repair^[59,60], which results in elevated genomic instability^[58].

CIC DEVELOPMENT IS DISTINCT FROM IMMORTALIZATION

Although immortalized MEFs develop in association with genomic instability and mutations in the ARF/p53 module^[26,41], the resultant cells do not exhibit robust tumor-forming ability unless they are pre-transformed by oncogenes^[61,62]. This observation illustrates the difference between immortalized MEFs and CICs. MEFs are differentiated cells and do not exhibit stem cell characteristics, but CICs act like stem cells in cancer-tissue development. In fact, CICs share a number of common characteristics with normal stem cells, including embryonic stem (ES) cells and induced pluripotent stem cells, but do not resemble immortalized MEFs with their high dependence on normal glycolysis^[63,64], sphere-forming ability, and expressed stem cell marker genes^[65-67]. Cancer tissues generally exhibit the "Warburg Effect" seen in normal stem cells that produce elevated glycolysis.

Transformation of normal stem cells into CICs after genomic destabilization

To truly understand cancer, we must discover how

genomic instability promotes the development of CICs. CICs may develop *via* multiple pathways. A recent study using normal murine ES cells as a model showed that one of these possible pathways might be created when normal stem cells are developing under unstable genomic conditions. Under aberrant differentiation conditions, ES cells with the ability to form normal mice develop high levels of DNA lesions. These lesions induce cellular senescence and genomic instability, ultimately leading to renewed growth in cells that harbor mutations in the ARF/p53 module^[68]. These cells exhibit a number of stem cell characteristics, including sphere formation and the expression of undifferentiated marker genes such as *Nanog*, *Klf4*, *Oct3/4*, and *Sox2*, even in a growth factor-poor medium containing 10% newborn bovine serum with no leukemia inhibitory factor. Furthermore, these cells possess tumor-forming ability and express c-Myc and CIC markers such as CD133, CD33, and CD34.

The fact that oncogenesis can be triggered by niche disruption supports this theory. For example, both leukemia and myelodysplasia develop after dysregulation of the stem cell niche^[69], and cancers often develop from stem cells that are injected at heterotopic sites^[70]. Stem cells growing in such an aberrant environment do not get what they need to maintain themselves properly and often transform into CICs^[70]. Another example is embryonal carcinomas that can develop from cells transplanted from the inside of blastocysts and from primordial germ cells derailed from the migration track^[71,72]. These *in vivo* and *in vitro* findings suggest that the genomic instability that leads to the development of CICs can be triggered by aberrations in the environment when stem cells are differentiating.

Because cancer development is initiated by CICs, these cells are considered promising targets for chemotherapy aimed at either killing them directly or getting them to differentiate^[73-75]. However, this strategy is not always successful, partly because CICs are so adaptable and plastic^[76,77]. In one study in

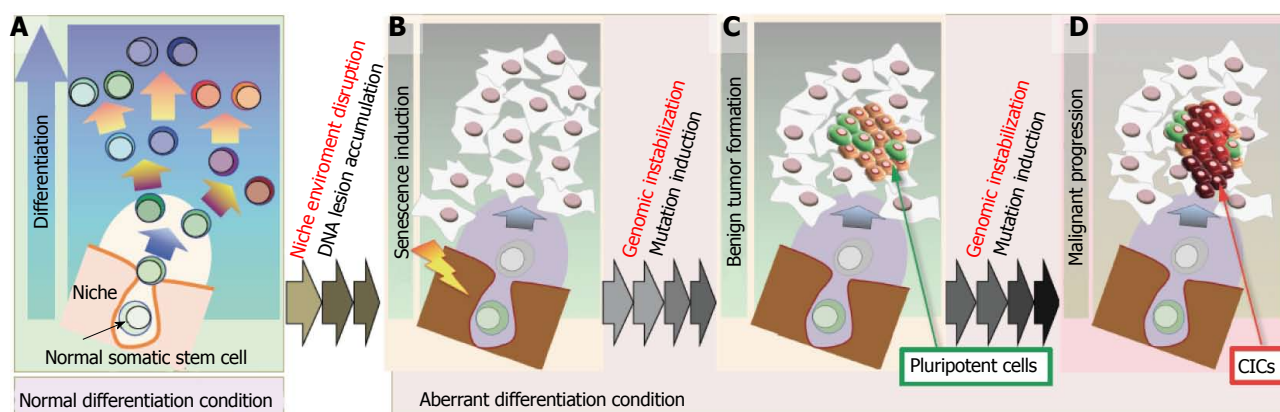


Figure 2 Model of cancer-initiating cell development. A: In normal tissues, stem cells are maintained in a specific niche and the niche environment is important both for stem cell maintenance and organ homeostasis; B: Niche disruption deranges the differentiation environment of stem cells, leading to the accumulation of DNA lesions and the induction of cellular senescence. Such DNA lesions trigger genomic destabilization, leading to the development of benign tumors that contain a small number of C: Pluripotent cells that eventually develop into; D: Cancer-initiating cells (CICs) that cause malignancies.

which CICs developed from ES cells, transformation was coupled with the acquisition of plasticity. ES cells start to differentiate in response to leukemia inhibitory factor withdrawal and become senescent when differentiation conditions are aberrant; but the CICs that result re-acquire stem cell characteristics as they develop genomic instability, even under conditions in which stem cells ordinarily do not thrive and cannot be cultivated^[68]. CICs that re-acquire stemness under these conditions maintain stem cell characteristics with great persistence, and it is difficult to get them to differentiate completely.

RELEVANCE OF *IN VITRO* MODELS TO CANCER DEVELOPMENT

Unlike *in vitro* malignancy transformation, *in vivo* cancer development has multiple steps that include abrogating organic regulation, which results in the development of benign tumors such as adenomatous polyposis coli mutations in the colon^[78,79], and losing the ARF/p53 module that leads to uncontrolled growth and the development of malignancies^[80,81]. Each organ in a complex animal is regulated by its own specific mechanisms that keep the organ in homeostasis, which can malfunction and allow tissues to become precancerous or malignant when the genome becomes unstable or the mutations affect the ARF/p53 module^[40,56].

CONCLUSION

Genomic instability contributes to the development of CICs by directly transforming somatic stem cells, reprogramming differentiated cancer cells, and a number of other mechanisms. Several lines of evidence suggest that one of these pathways is triggered by the disruption of the niche environment (Figure 2). Maintenance of the niche environment is essential for

somatic stem cell maintenance and differentiation and homeostasis (Figure 2A). The stem cell niche can be damaged by exogenous stresses that cause irregular stem cell differentiation, the accumulation of DNA damage, and the induction of senescence (Figure 2B). DNA damage often triggers genomic destabilization, which can promote the development of precancerous (Figure 2C) and cancerous (Figure 2D) lesions through the maintenance of small pockets of pluripotent stem cells that eventually become CICs.

If stem cells start to differentiate under unfavorable differentiation conditions, they become senescent, but can start developing again and reacquire stem cell characteristics when conditions change. Stemness recovered under these circumstances is associated with robust plasticity and the ability of these cells to self-renew, even under conditions in which normal stem cells do not thrive. Unfortunately, such cells do not differentiate completely and remain permanently in less developed states that often lead to malignancies.

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Adult stem-like cells in kidney

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cell or system is reportedly presents suggesting that adult stem-like cells in kidney can be practical clinical targets for kidney diseases. However, it is still unclear if kidney stem cells or stem-like cells exist or not. In general, stemness is defined by several factors such as self-renewal capacity, multi-lineage potency and characteristic gene expression profiles. The definite use of stemness may be obstacle to understand kidney regeneration, and here we describe the recent broad findings of kidney regeneration and the cells that contribute regeneration.

Key words: Stem cell; Label-retaining cells; rKS56; SP cells; CD24; CD133; Sca-1; Induced pluripotent stem; ES cell

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Core tip: Controversies still persist whether kidney stem cells exist or not, but renal progenitor cell or system is reportedly presents suggesting that adult stem-like cells in kidney can be practical clinical targets for kidney diseases. In this mini-review, we describe the recent broad findings of kidney regeneration and the cells that contribute regeneration.

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Abstract

Human pluripotent cells are promising for treatment for kidney diseases, but the protocols for derivation of kidney cell types are still controversial. Kidney tissue regeneration is well confirmed in several lower vertebrates such as fish, and the repair of nephrons after tubular damages is commonly observed after renal injury. Even in adult mammal kidney, renal progenitor

INTRODUCTION

Recent developments in human pluripotent cells including both embryonic stem cells and induced pluripotent stem (iPS) cells^[1] are promising for clinical development of cell therapies and tissue

engineering^[2-4]. There is an urgent need for stem cell and regenerative medicine approaches to kidney diseases, because patients with end-stage kidney disease require lifelong dialysis treatment or transplantation that incurs a significant cost. Several established protocols for derivation of cardiomyocytes and neurons were already reported, but that for kidney cell types are still controversial. Recently human iPS cells were successfully differentiated into kidney lineage *via* OSR1 (+) cells^[5,6]. However metanephric progenitors were not induced from OSR1(+) cells^[7], and efficient differentiation of human pluripotent cells into intermediate mesoderm was reported by treatment the cells with glycogen synthase kinase-3 β inhibitor^[8]. Different from mammals, the capacity to regenerate kidney tissue is well confirmed in several lower vertebrates such as fish^[9]. Kidney regeneration, such as the repair of nephrons after tubular damages, is commonly observed after renal injury even in humans^[10,11]. Concerning adult mammal kidney, there are lots of evidences that suggest existence of renal progenitor system^[12-19]. Collectively, these results suggest the potential role of adult stem-like cells in kidney for practical clinical treatment of kidney diseases. In this review, we describe the localization and recent functional findings of kidney regeneration and the cells that contribute regeneration.

TUBULAR CELLS (LRC, RKS56, NFATC-1⁺, ALDH^{HIGH}, CD24⁺/CD133⁺, MRPC)

To identify the stem cells, BrdU-DNA labeling is commonly used to find slow-cycling cells because stem cells have a slow cell cycle. Slow cycling cells were also called label-retaining cells (LRC), and LRC in tubules were confirmed by several groups^[20-22] (Table 1). In rat kidneys, LRC were distributed among renal epithelial tubular cells^[20]. In 3 dimensional culture system, LRC formed tubule-like structure. Moreover, injected LRC into cultured metanephros formed nephrons and collecting ducts^[23]. rKS56 cells were established from proximal tubules (S3) by using microdissection of a single nephron^[24]. rKS56 cells expressed markers of an immature progenitor state such as c-Kit and Sca-1. When rKS56 cells were transplanted into acute kidney disease models, the cells differentiated into epithelium. A resident progenitor cells in proximal tubular (PTC) cell was identified by Nfatc1-P2-Cre reporter system^[25]. Nfatc-1-labeled PTC cells were apoptosis-resistant and proliferated to repair the damaged proximal tubule segment. ALDH activity was used to isolate cells with progenitor-like characteristics from the tubular fraction of the renal cortex^[26-29]. ALDH^{high} cells displayed typical stem cell properties such as sphere

formation and anchorage-independent growth. CD24/CD133 double-positive cells were localized in the tubular epithelium, and demonstrated clonogenic multipotency and self-renewal ability^[28]. Bombelli *et al.*^[30] recently proposed existence of CD133⁺/CD24⁻ renal stem cells but Romagnani reported that CD133⁺ renal stem definitely co-express CD24 in human kidney^[31]. Gupta *et al.*^[32] reported localization of stem-like cells around the tubules. They named the cells multi-potent renal progenitor cells (MRPC). By using similar culture condition used for culture of bone marrow-derived multi-potent adult progenitor cells, MRPCs were isolated from rat kidney. The plasticity of MRPC was confirmed by expression of endothelial, hepatocyte, and neural markers by RT-PCR and protein expression. When MRPC were injected under the capsule of an uninjured kidney or arterially into acute kidney injury model, the cells differentiated into renal tubules. However, differentiation/induction of matured kidney cells from MRPC *in vitro* was not confirmed yet.

RENAL PAPILLA (LRC, CD133⁺)

To identify the stem-like cells in kidney, Oliver *et al.*^[33,34] performed pulse label of rat and mouse pups by BrdU, and confirmed the existence of LRC cells in the kidney. LRC cells were very sparse in the kidney, but they found numerous LRC cells in renal papilla^[33]. In 3 dimensional culture, LRC cells in renal papilla spontaneously formed spheroids, and clones from single cell of LRC cells expressed both mesenchymal and epithelial markers. The papillary cells also differentiated to myofibroblasts and neuronal cells. In acute kidney injury model such as ischemic injury, LRC cells in papilla migrated to the upper papilla and formed a compartment of rapidly proliferating cells suggesting that the cells contributed to repair of kidney tissue^[34]. Papillary cells that expressed CD133 were also expressed nestin and embryonic cell markers (Oct3/4, Nanog, SOX2 and SSEA-4)^[35].

INTERSTITIAL SPACE (SCA-1⁺CD45⁻, SP, CD133⁺)

Several groups have found adult stem-like cells in murine kidney interstitial space using different approaches. Stem cell antigen-1 (Sca-1)-positive and CD45-negative cells were isolated from whole kidney tissue by magnetic assisted cell sorting and fluorescence activated cell sorting (FACS) sorting^[36]. The cells were negative for hematopoietic stem cell and lineage markers and located in the renal interstitial spaces. The differentiation of the cells into multi-lineage (myogenic, osteogenic, adipogenic and neural) was also confirmed. In acute kidney injury model, injected Sca-1⁺CD45⁻ cells contributed

Table 1 Localization and characteristics of adult stem-like cells in kidney

Localization	Characteristics	Species	Ref.
Tubular cells	LRC	Rat	[20-23]
	rKS56	Rat	[24]
	Nfactc-1 ⁺	Mice	[25]
	ALDH ^{high} /CD24 ⁺ /CD133 ⁺	Human	[26-29]
	MRPC	Rat	[32]
Renal papilla	LRC	Rat, mice	[31,32]
	CD133 ⁺	Human	[33]
Interstitial space	Sca-1 ⁺ /CD45 ⁺	Mice	[34]
	SP cell	Rat, mice, human	[38-41]
Bowman's capsule	CD133 ⁺	Human	[27]
	CD24 ⁺ /CD133 ⁺ /PDX ⁺	Human	[44-48]
	CD24 ⁺ /CD133 ⁺ /PDX ⁻	Human	[44-48]

LRC: Label-retaining cells; MRPC: Multi-potent renal progenitor cell; PDX: Podocyte marker.

kidney repair. To isolate hematopoietic stem cell-rich population in a single step, Goodell *et al.*^[37] stained cells with Hoechst 33342 dye and isolated the cells by FACS. The cells isolated by this method were named side population (SP) cells. This method was also used to purify a stem cell-rich population in various kinds of tissue. SP cells isolated from adult kidney demonstrated ability of self-renewal and differentiation into multiple lineages^[38]. SP cells isolated from adult kidney located in interstitial spaces, and secreted reno-regenerative/protective factors (HGF, VEGF, and BMP-7)^[39-42]. The injection of SP cells isolated from adult kidney cells into a model of acute kidney injury demonstrated the recovery of renal function^[39,40]. Interestingly, Inowa *et al.*^[43] confirmed the existence of kidney SP cells in human. Concerning CD133 positive cells, Bussolati *et al.*^[27] reported that the cells were localized to the interstitium, but not in glomeruli. Kidney CD133 positive cells lacked expression of hematopoietic markers and expressed Pax-2, an embryonic renal marker. Intravenous injection of kidney CD133 positive cells in SCID mice with glycerol-induced tubulonecrosis, the cells homed into the injured kidney and integrated in tubules.

BOWMAN'S CAPSULE

(CD24⁺CD133⁺PDX⁺, CD24⁺CD133⁺PDX⁻)

Some parietal epithelial cells (PEC) are reported to be adult stem-like cells^[44-47]. Sagrinati *et al.*^[45] confirmed the existence of PEC that expressed CD24, CD133 Oct-4 and Bmi-1 in the Bowman's capsule. CD24⁺CD133⁺ PEC were isolated by culture of capsulated glomeruli plated on fibronectin-coated dishes. CD24⁺CD133⁺ PEC showed potential of self-renewal and a high cloning efficiency. Transplantation of CD24⁺CD133⁺ PEC into acute kidney injury model significantly improved not only morphologic but functional kidney damage. Further characterization

using podocyte marker (PDX) of CD24⁺CD133⁺ PEC revealed a hierarchical population of the cells in a precise sequence with Bowman's capsule and exhibited heterogeneous potential such as differentiation and regeneration^[48]. CD24⁺CD133⁺PDX⁻ cells localized to the urinary pole could differentiate into both tubular cells and podocytes, but CD24⁺CD133⁺PDX⁺ cells localized between the urinary pole and vascular pole could differentiate into only podocytes. Transplantation of CD24⁺CD133⁺PDX⁻ cells reduced proteinuria and improved chronic glomerular damage in adriamycin-induced nephropathy models.

FUNCTIONAL REGULATION OF ADULT STEM-LIKE CELLS IN KIDNEY

As mentioned above, different kinds of adult stem-like cells in kidney have been reported, but their functional regulations were poorly understood. If it is possible to regulate multi-potent adult stem like-cell *in situ*, this can be a good regenerative treatment. Recently MyoR was reported to regulate regenerative function of kidney SP cells^[49], and such a molecule can be a good target for pharmacological treatment for kidney disease.

CONCLUSION

Several adult stem-like cells in kidney reportedly demonstrated multi-potency. However, it is impossible to get enough cells for cell therapy from the patient. The adult stem-like cell in kidney is expected to play key role to preserve kidney function, and the cells may be the good targets for pharmacological treatment. For cell therapy, iPS or ES cells might be applicable as in the case with neural and cardiac regeneration.

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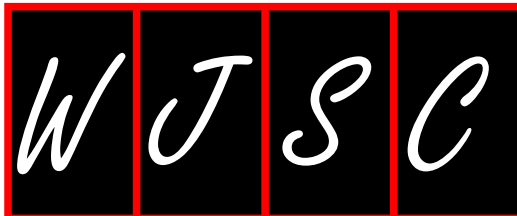
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Niche interactions in epidermal stem cells

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skin diseases. Thus, strategies for manipulating cell-ECM interactions are critical for treating wounds and a variety of skin diseases. Many of these strategies focus on epidermal stem cells, which reside in a unique niche in which the ECM is the most important component; interactions between the ECM and epidermal stem cells play a major role in regulating stem cell fate. As they constitute a major portion of the ECM, it is likely that integrins and type IV collagens are important in stem cell regulation and maintenance. In this review, we highlight recent research-including our previous work-exploring the role that the ECM and its associated components play in shaping the epidermal stem cell niche.

Key words: Stem cell niche; Epidermal stem cells; Integrins; Type IV collagen; MiR135b

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Core tip: Epidermal stem cells reside in a unique niche within the skin, which is shaped by interactions between stem cell-associated integrins and components of the extracellular matrix. Here, we review literature evaluating the role that integrins play in epidermal stem cell maintenance and proliferation, and highlight methods that have been used to enrich for epidermal stem cells. We stress that by understanding the epidermal stem cell niche, new regenerative medicine applications can be developed.

Abstract

Within the epidermis and dermis of the skin, cells secrete and are surrounded by the extracellular matrix (ECM), which provides structural and biochemical support. The ECM of the epidermis is the basement membrane, and collagen and other dermal components constitute the ECM of the dermis. There is significant variation in the composition of the ECM of the epidermis and dermis, which can affect "cell to cell" and "cell to ECM" interactions. These interactions, in turn, can influence biological responses, aging, and wound healing; abnormal ECM signaling likely contributes to

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INTRODUCTION

The skin has epithelial and mesenchymal components^[1].

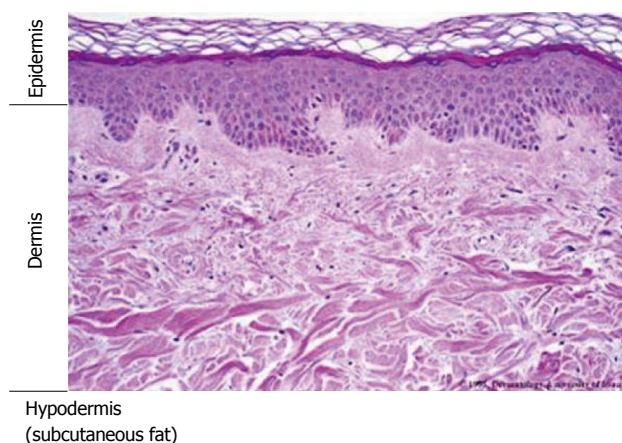


Figure 1 Skin structure involves the epidermis, dermis and hypodermis.

Normally, tissues consist of differentiated cells and a small number of stem cells localized to specific niches^[2]. Stem cell niches are specific microenvironments that determine stem cell number and affect stem cell mobility^[3]. Furthermore, the complex nature of stem cell niches allows for the formation of distinct structures specialized for different stem cell types^[4]. Epidermis is multilayered and is continually renewed and replaced by new cells, a process that results from the action of stem cells. It is known that the extracellular matrix (ECM) is a key component of the stem cell niche in the skin, and biophysical properties of the ECM (such as stiffness) also can affect cell fate determination^[4]. As epidermal stem cells express high levels of integrins, these cells are able to interact directly with the ECM^[1,4]. Thus, the interaction between stem cell-associated integrins and the ECM may be important in shaping the epidermal stem cell niche. In addition to the ECM and integrins, growth factors or mechanobiological factors can also affect stem cell fate^[4,5]. Furthermore, low oxygen tensions (hypoxia) are necessary to maintain undifferentiated stem cell phenotypes, and also influence proliferation and stem cell fate^[6]. However, among all of these factors, the ECM seems to play the largest role in shaping stem cell niches and stem cell behavior, and may thus have a major underlying role in physiological and pathological conditions.

SKIN STRUCTURE: A BRIEF OVERVIEW

Skin is composed of the epidermis, dermis, and hypodermis (Figure 1). Multilayered epidermis serves as the outer layer of the skin. Additional structures such as hair follicles and sebaceous glands are also found within the skin. Stem cell proliferation and differentiation are vital for the maintenance of the epidermis, and different pools of skin stem cells are located in the hair follicle (the bulge) and the interfollicular epidermis^[7,8]. Histologically, the lower layer of the epidermis is attached to the basement

membrane (BM), which separates the epidermis from the underlying connective tissue layer known as the dermis.

THE BASEMENT MEMBRANE AND THE EPIDERMAL STEM CELL NICHE

The BM of the epidermis contains distinct subtypes of laminin, type IV collagen, nidogen, and perlecan (a heparan sulfate proteoglycan); immunohistochemical staining and gene expression studies have demonstrated that there is considerable regional variation in the BM^[1]. The mechanical support provided by the BM is determined primarily by its type IV collagen scaffold; however, laminin is essential for the initial assembly of the BM *in vivo*^[9]. Integrins also demonstrate variable expression in different regions of the epidermis^[10], and these transmembrane proteins themselves have different kinds of combination (*i.e.*, $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 4$). Integrins are the major receptors for ECM proteins, and interact with growth factors and cytokine receptors^[4]. Growth factors and morphogens can also directly bind to the ECM; this suggests that the ECM might integrate different types of signals in biological microenvironments. As a result, clinical applications of stem cells have emphasized the relationship between epidermal stem cells and the ECM^[4]. Recent research has sought to understand how the ECM and integrins cooperate with one another to form the architecture of the epidermal stem cell niche, and how they maintain the balance between stem cell renewal and differentiation^[4]. It is possible that variations within the ECM may create distinct environments that can potentially affect the properties of different stem cell populations^[1].

EPIDERMAL STEM CELLS: IDENTIFICATION, REGULATION, AND APPLICATIONS

Markers of epidermal stem cells and the epidermal stem cell niche

Stem cells are localized to “niches”—specific sites that regulate how stem cells participate in tissue maintenance and repair. In a niche, stem cell proliferation is regulated and these cells are protected from depletion^[11]. Interestingly, evidence suggests that cancer—a common cause of death—is also a stem cell-based disease. Cancer stem cells (CSCs) are a subset of cancer cells that demonstrate stem cell-like abilities—abilities that may induce tumor growth and tumor recurrence. CSCs also require a special “CSC niche” to regulate their stemness and proliferation, and as with endogenous stem cells, this niche prevents CSCs from being depleted^[12]. Thus, the stem cell niche is a key factor in the maintenance of stem cells.

In the skin, an increased level of integrins expression is considered to be a marker of epidermal stem cells^[13]. Although the expression levels of integrins in stem cells are only 2-3-fold higher than in stem cell progeny, this is sufficient to make stem cells more adhesive to ECM proteins^[14]. Based on these findings, we developed a simple method to isolate epidermal stem cells based on integrin expression. As epidermal stem cells express high levels of integrins, type IV collagen is a good candidate to bind epidermal stem cells^[14]. To purify epidermal stem cells, three keratinocyte populations were first characterized by their ability to bind type IV collagen: rapidly adhering (RA), slowly adhering (SA), and non-adhering (NA) cells. The expression levels of *p63* and *c-Myc* were also compared between cells in these three populations. It was concluded that RA cells could be easily isolated from the epidermis, and that these cells were likely epidermal stem cells^[15]. This research suggests that integrins may play an important role in the regulation of epidermal stem cells. Type IV collagen has not only been used to isolate epidermal stem cells, but has also been suggested to be directly involved in maintaining epidermal stem cells. Very recently, we reported that inhibition of miR135b could increase the proliferative potential of normal human keratinocytes^[16]. Our data demonstrated that type IV collagen is a target of miR135b, and that the inhibition of miR135b may improve the epidermal stem cell microenvironment and increase the proliferative potential of basal cells (Figure 2). These findings suggest that restoration of type IV collagen to the basement membrane is important for stem cell maintenance in skin. Microarray analysis also revealed differences in the expression of ECM proteins between hair follicle stem cells and other epidermal stem cells^[17]. These results suggest that integrins and type IV collagen are not the only proteins involved in determining the epidermal stem cell niche; ECM proteins are also important in shaping the niche within the interfollicular epidermis. Although no specific markers are available for the identification of epidermal stem cells, *p63* is commonly regarded as a potential epidermal stem cell marker, as *p63*^{-/-} mice fail to develop stratified squamous epithelia^[18].

The stem cell niche in regenerative medicine

An effective response of the body to injury depends on regenerative processes that maintain proper cell numbers and replace damaged cells. This "reparative" potential is determined by the presence of stem and progenitor cells, which respond to exogenous cues such as tissue damage. Therapeutic applications of stem cells address the biological constraints of these cells, the stem cell niche, and the location to which these cells are summoned to respond. Understanding the role of the stem cell niche is essential for the application of stem cells in regenerative medicine^[19].

A major concern in regenerative medicine is obtaining an adequate number of stem cells to treat

an injury or disease. For practical applications, one of the most important problems is the small number of stem cells. In order to overcome this challenge, researchers often expand the number of stem cells by *in vitro* culture; however, most stem cells begin to lose their stem cell character during this process. In many cases, replicating the niche environment in culture is critical to maintain stem cell characteristics^[20]. Similarly, "mimicking the native niche" has been shown to be important in tissue engineering of human corneal limbal crypts^[21]. As wound healing is a critical process that re-establishes the epithelial barrier following disease or injury, poor wound healing increases infection risk, causes patient morbidity, and may lead to the scar formation. If there is a defect in the stem cell niche, wound repair could be delayed by the abnormal response of stem cells to injury^[5]. Thus, understanding how to recreate the stem cell niche is key to regenerative medicine applications.

Integrins and niche interactions

Integrins are a complicated group of proteins that function as transmembrane receptors; these proteins either connect cells to one another, or to the ECM. Such connections trigger signal transduction pathways, which result in biological responses such as cell proliferation or cell motility. Fibronectin, vitronectin, collagen, and laminin are all ligands for integrins. However, other proteins can mediate cell-cell or cell-ECM interactions; these include cadherins, the immunoglobulin superfamily of cell adhesion molecules, namely, selectins, and syndecans.

Stem cells, whether multipotent or tissue-specific, embryonic or adult, are controlled by intrinsic transcriptional regulation and extrinsic signals^[22]. The extrinsic signals originate from the local microenvironment in which stem cells reside. It is well known that the ECM is an important component of the epidermal stem cell niche (see above). In the skin, high integrin levels can distinguish epidermal stem cells^[14], and now integrin expression can be used to enrich for stem cells in mixed cell populations^[23,24]. In our reports, on skin equivalents (SEs), addition of IGFBP-2 to culture media increased the expression levels of both $\alpha 6$ integrins and $\beta 1$ integrins in cultured keratinocytes, which are associated with an increased number of epidermal stem cells (*p63* positive cells)^[25] (Figure 3). Furthermore, it has been reported that activation of $\beta 1$ integrin decreases terminal differentiation^[26]. Signaling studies have demonstrated that the Erk/MAP kinase pathway, which acts downstream of $\beta 1$ integrins, provides a signal which inhibits differentiation^[26,27]. Collectively, these findings are consistent with previous reports that integrins are related to stem cell maintenance.

Integrin regulation and stem cells

Few studies have dissected how epidermal integrin expression and *ECM* gene expression are regulated. In our previous study, we reported that when hair

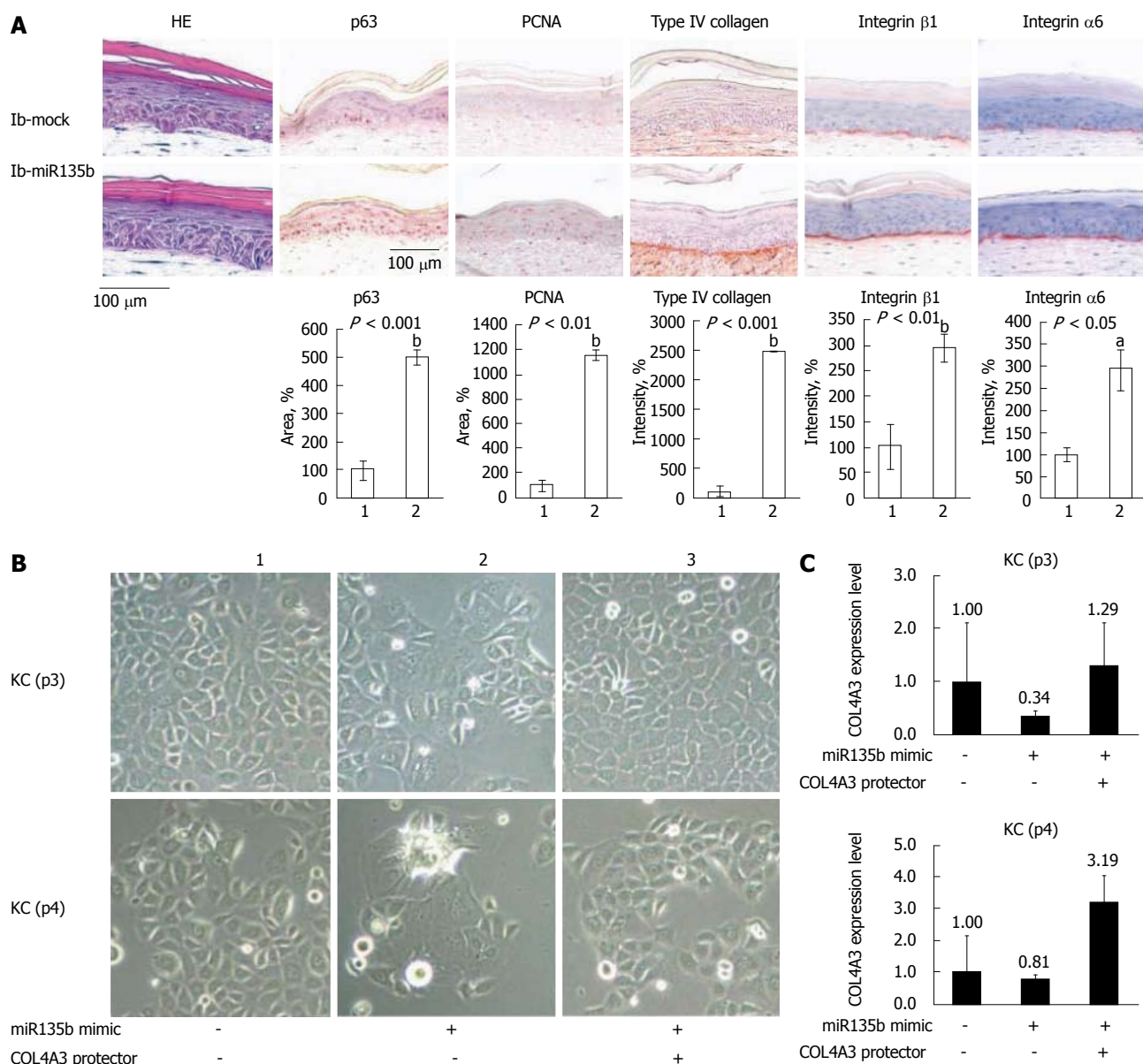


Figure 2 The inhibition of miR135b prolongs the stemness and proliferative potential of epidermal keratinocytes in skin equivalents models. **A:** Histology of skin equivalent (SE) models constructed with ib-mock-transfected or ib-miR135b-transfected keratinocytes, and immunohistochemical staining of these models for p63, proliferating cell nuclear antigen, type IV collagen, and integrin $\beta 1$, and integrin $\alpha 6$. Expression levels of p63, PCNA, type IV collagen, integrin $\beta 1$, and integrin $\alpha 6$ were increased in SEs generated from ib-miR135b-transfected cells; 1: ib-mock; 2: ib-miR135b; **B:** Fast-forward co-transfection of keratinocytes with “miRNA mimic” and “target protector”. 1: Negative control of miRNA mimic (10 nmol/L) and negative control of target protector (500 nmol/L); 2: miRNA mimic (10 nmol/L) and negative control of target protector (500 nmol/L); 3: MiRNA mimic and target protector (500 nmol/L). The inhibition of miR135b increases proliferation of keratinocyte via targeting type IV collagen; **C:** Real-time PCR analysis of gene expression in co-transfected keratinocytes. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. The result was same as (B). HE: Hematoxylin and eosin.

follicle dermal sheath cells were used to construct SEs, this resulted in increased epidermal thickness and increased expression of $\alpha 6$ integrins, compared to SEs generated from fibroblasts^[28] (Figure 4). Building upon this study, it was found that IGFBP-2 is a major factor produced by dermal sheath cells, which regulates the regenerative capacity of the skin; IGFBP-2 plays an important role in maintaining stem cell characteristics in human epidermal keratinocytes^[25]. These findings suggest that levels of integrins, which can be regulated by IGFBP-2, are vital for the maintenance of epidermal stem cell characteristics in the skin.

As it is well known that fetal wounds heal rapidly, we also evaluated the regenerative potential of and expression of integrins in neonatal keratinocytes and fibroblasts. When neonatal keratinocytes were used for epidermis reconstruction, expression of p63 was increased compared to adult keratinocytes; levels of integrins were also increased in this model^[28]. Recently, we also demonstrated that when neonatal fibroblasts were used to make a dermal substitute, both integrin and p63 expression increased (personal communication). Collectively, these results demonstrate that neonatal cells maintain a higher

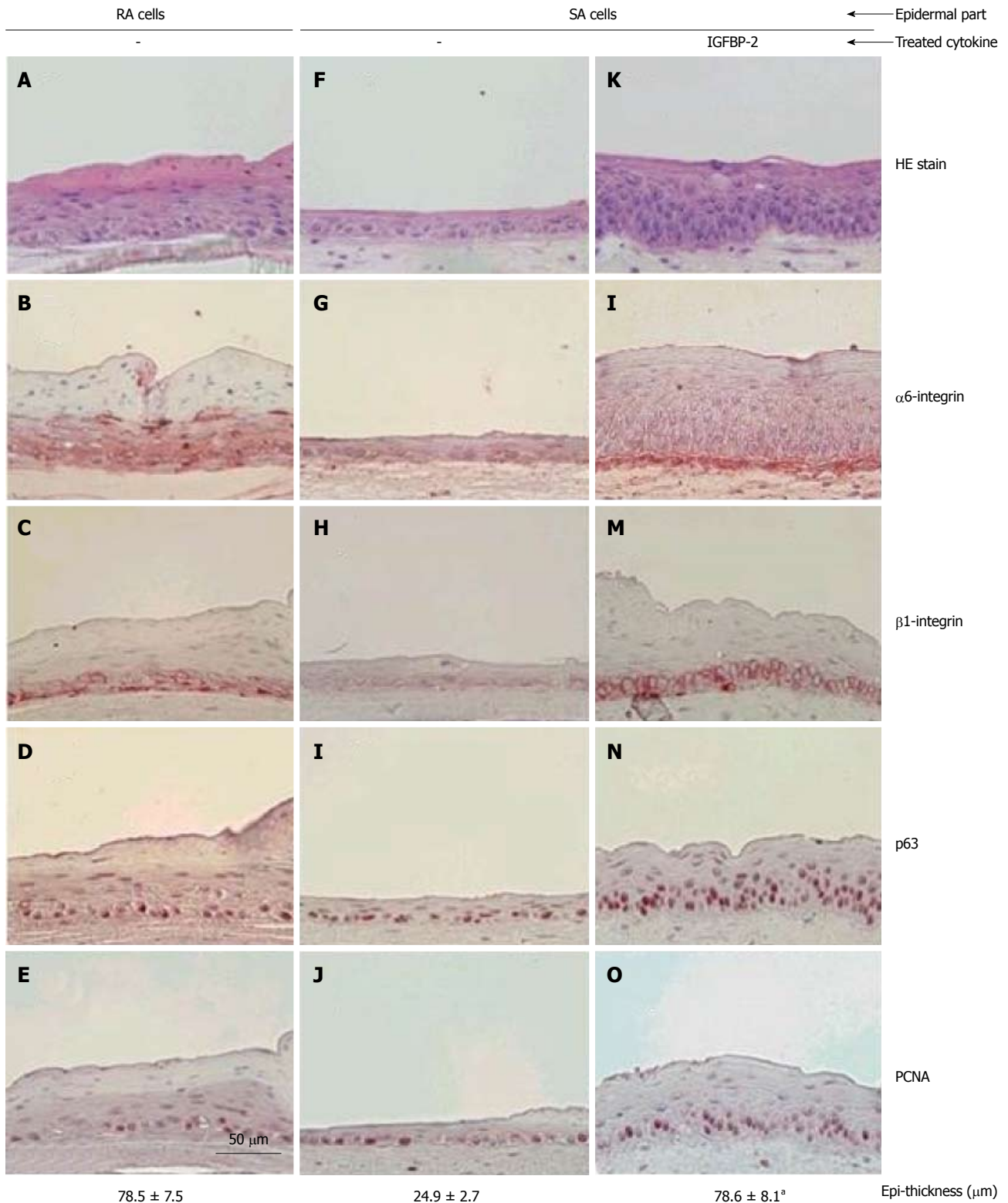


Figure 3 Effects of IGFBP-2 on skin equivalents reconstruction. Skin equivalents (SEs) were cultured with rapidly adhering (RA) cells (A-E) or slowly adhering (SA) cells (F-O). Sections of SEs cultured under different treatments [media only (A-J) and 200 ng/mL IGFBP-2 (K-O)] and stained for hematoxylin and eosin (HE) (A, F, K), $\alpha 6$ integrin (B, G, I), $\beta 1$ integrin (C, H, M), p63 (D, I, N), and proliferating cell nuclear antigen or proliferating cell nuclear antigen (PCNA) (E, J, O). Addition of IGFBP-2 to culture media increased the expression levels of $\alpha 6$ integrins, $\beta 1$ integrins, p63, and PCNA in cultured keratinocytes. Original magnification 400 $\times$. Epidermal thickness was measured at 10 randomly selected locations using an Olysia Soft Imaging System (Olympus). Statistical analysis was performed using the Student's *t*-test. ^a*P* < 0.01 vs SA + no cytokine model.

regenerative potential compared to adult cells. This may result from the fact that neonatal cells have the

potential to express more integrins, which favors the environment of the epidermal stem cell niche.

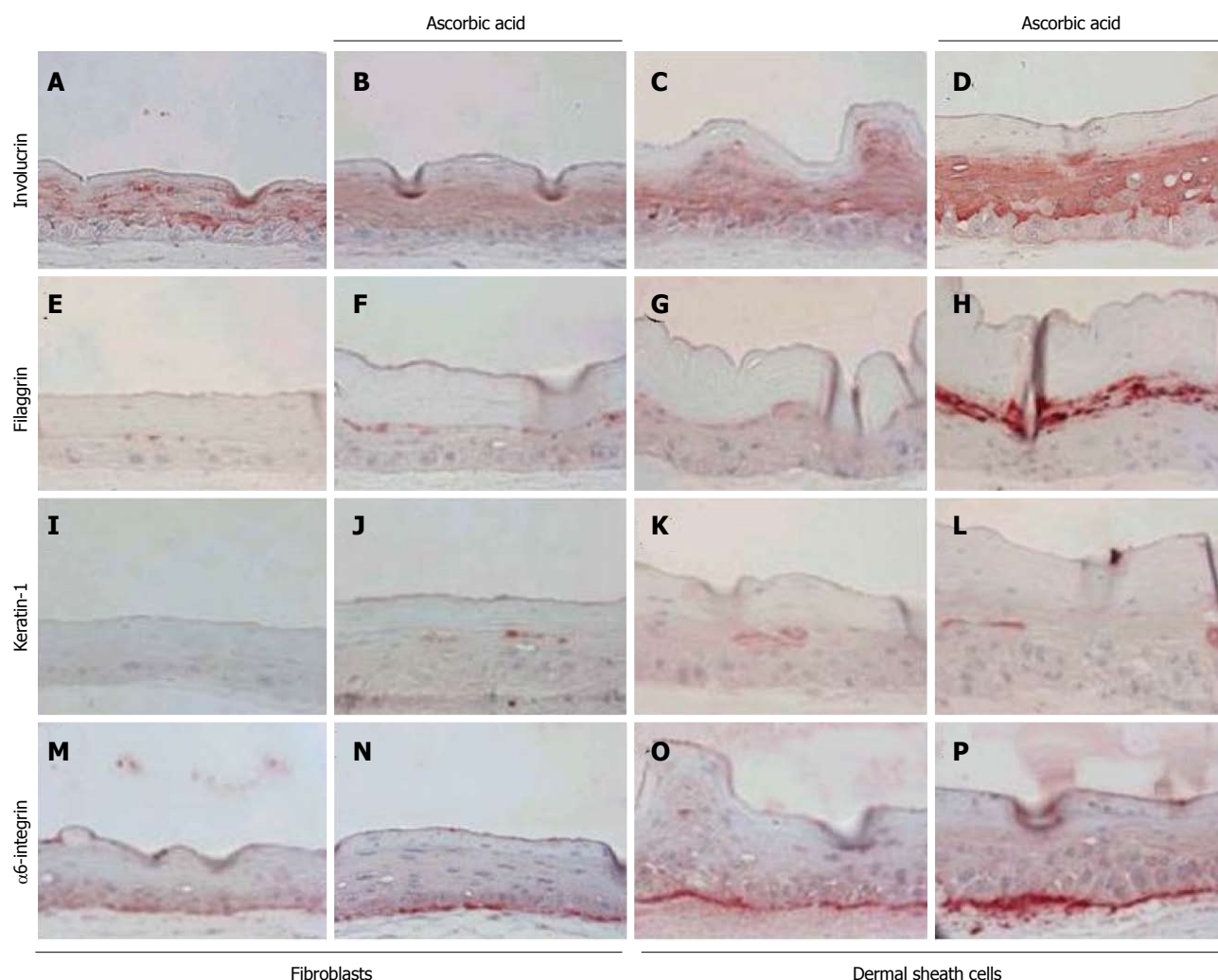


Figure 4 Immunohistochemical staining for involucrin, filaggrin, keratin 1, and $\alpha 6$ integrin in skin equivalents generated from fibroblasts or dermal sheath cells. Sections of skin equivalents (SEs) produced using fibroblasts (A, B, E, F, I, J, M, and N) or dermal sheath cells (C, D, G, H, K, L, O, and P) were stained for involucrin (A-D), filaggrin (E-H), keratin 1 (I-L), and $\alpha 6$ integrin (M-P). SEs were cultured in complete medium in the presence (B, D, F, H, J, L, N, and P) or absence (A, C, E, G, I, K, M, and O) of ascorbic acid. Expression of involucrin, filaggrin, keratin 1, and $\alpha 6$ integrin was up-regulated in SEs of dermal sheath cells compared with SEs of fibroblasts. Original magnification: 400 $\times$ (A-P).

CONCLUSION

Many ECM genes are up regulated in epidermal stem cells. These proteins act as extrinsic signals that may affect stem cell fate. In this review, we have presented a general overview of ECM interactions, and discussed our previous reports demonstrating the important role that integrins and type IV collagen play in shaping the epidermal stem cell niche.

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Neural stem cells could serve as a therapeutic material for age-related neurodegenerative diseases

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Abstract

Progressively loss of neural and glial cells is the key event that leads to nervous system dysfunctions and diseases. Several neurodegenerative diseases, for instance Alzheimer's disease, Parkinson's disease, and Huntington's disease, are associated to aging and suggested to be a consequence of deficiency of neural stem cell pool in the affected brain regions. Endogenous neural stem cells exist throughout life and are found in

specific niches of human brain. These neural stem cells are responsible for the regeneration of new neurons to restore, in the normal circumstance, the functions of the brain. Endogenous neural stem cells can be isolated, propagated, and, notably, differentiated to most cell types of the brain. On the other hand, other types of stem cells, such as mesenchymal stem cells, embryonic stem cells, and induced pluripotent stem cells can also serve as a source for neural stem cell production, that hold a great promise for regeneration of the brain. The replacement of neural stem cells, either endogenous or stem cell-derived neural stem cells, into impaired brain is highly expected as a possible therapeutic mean for neurodegenerative diseases. In this review, clinical features and current routinely treatments of age-related neurodegenerative diseases are documented. Noteworthy, we presented the promising evidence of neural stem cells and their derivatives in curing such diseases, together with the remaining challenges to achieve the best outcome for patients.

Key words: Alzheimer's disease; Huntington's disease; Neural stem cells; Parkinson's disease; Cell therapy; Neurodegenerative diseases

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Core tip: Neural stem cells present throughout life of human. The cells can be distinguished from differentiated progeny by acknowledging a few key features, including proliferation, self-renewal, multipotency and molecular markers. These features make neural stem cells as an essential component in the development of nervous system and in maintaining cell number of adult nervous tissues following injury and diseases. Besides conventional treatments, neural stem cells have been proposed as a promising approach to cure patients with neurodegenerative diseases. Several animal studies showed the efficiency of neural stem cells in treating age-related neurodegenerative diseases, in particular

Alzheimer's, Parkinson's and Huntington's diseases.

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INTRODUCTION

Neurodegenerative diseases are a global burden and often affect elderly population worldwide. The central and shared characteristics of neurodegenerative diseases are the loss of neuronal cells in either the central or peripheral nervous systems. Aging can cause chronic neurodegeneration, which results in the loss of particular neuronal subtypes over time. Parkinson's, Alzheimer's and Huntington's diseases are the three most dominant neurodegenerative diseases, found worldwide. In the brain, Alzheimer's and Huntington's diseases result in widespread loss of neurons, while Parkinson's disease involves the specific loss of midbrain dopaminergic neurons. In spite of an extensive effort to develop treatment against these neurodegenerative diseases, effective treatments still do not exist. Recently, cell therapy has been proposed as an attractive option, and the application of stem cell technology to regenerative medicine is rapidly progressed. Stem cell-based approaches are applicable for therapeutic purposes, for instance it might be possible to replace lost neurons or glia by transplantation of stem cell-derived nervous cells. Cell replacement might also be succeeded by activating endogenous neural stem cells in patients own brain to form new neurons and glia. In this review, we summarized clinical manifestations of the major age-related neurodegenerative diseases, in particular Parkinson's, Alzheimer's, and Huntington's diseases, together with their currently available treatments. The properties and characteristics of neural stem cells were presented, highlighting key features that enable neural stem cells to be a promising therapeutic material. Lastly, we documented evidence of neural stem cells and other stem cells to cure neurodegenerative diseases, especially Parkinson's, Alzheimer's, and Huntington's diseases.

CLINICAL MANIFESTATIONS OF AGE-RELATED NEURODEGENERATIVE DISEASES: ALZHEIMER'S, PARKINSON'S, AND HUNTINGTON'S DISEASES

Parkinson's disease (PD) is one of the most common movement malfunctions and affects nearly 1% in elderly population age over 60. The average age

of onset at 55 years, and the frequency increases markedly with age^[1,2]. There are several lines of studies proposing that genetic mutations or polymorphisms are correlated and can contribute to an increased risk of PD in senior population^[3]. Although genetics are only influenced a minor group of PD, the studies of genetic factors improve an understand of the pathophysiology of PD. An increasing number of genetic risks appear to be connected with PD, and many of them followed Mendelian inheritance rules, including alpha-synuclein gene (*SNCA*, PARK1/4), parkin gene (*Parkin*, PARK2), P-TEN-induced putative kinase 1 gene (*PINK1*, PARK6), Daisuke-Junko 1 gene (*DJ-1*, PARK7), and leucine-rich repeat kinase 2 gene (*LRRK2*, PARK8). These genes have been conclusively proven as a monogenic etiology for familial parkinsonism^[4]. To characterize PD, patients can be diagnosed by several key manifestations, including bradykinesia (slowness of establishing the voluntary movement and declination in speed of repetitive manners), resting tremor (4-6 Hz), and rigidity^[5]. A group of clinical outcome usually presents in an asymmetrical manner. The presences of a change in handwriting with micrographia, reduced sense of smell and facial expression, and loss of arm swing on one side of the body are often noted as early clinical features^[6,7]. When the disease progressively developed, gait instability, drooling of saliva, and impairment of postural reflexes will appear. Non-motor symptoms, such as neurobehavioral dysfunctions, insomnia, cognitive and sexual impairment may present in early or late stages of the disease progression. Depression and dementia are also common symptoms in non-motor presentation of PD's patients. A good response to levodopa treatment can differentiate PD from Parkinsonism due to other causes. The standard diagnosis for PD still relies on the neuropathological examination. All the cardinal signs of PD involve to motor disability in asymmetrical onset, without other causes of Parkinsonian syndrome and non-motor manifestations, including psychiatric symptoms^[8]. The UK Parkinson's Disease Society Brain Bank criteria is commonly used as a reference.

Alzheimer's disease (AD) is a progressively degeneration of the brain cortex, manifested by memory and intellectual decline, and progressive deficiency of daily-living activities^[9]. AD affects about 6% of the population aged over 65 years old worldwide. Age is considered the single utmost vital risk factor for AD ontogeny. Amyloid- β , oxidative stress, inflammation, and vascular injury appear to play an essential function in the neurodegeneration of AD^[10]. Multiple genetic defects have been associated to the development of AD. Initially, the molecular genetics of AD can be obtained from early-onset AD families with autosomal dominant patterns of inheritance. Highly penetrant mutations were identified in three genes: *APP*, *PSEN1*, and *PSEN2*^[11-13]. Interestingly, these genes are functionally participated in the processing of amyloid precursor protein. This suggests a central role of amyloid

precursor protein in AD pathology. Clinically, AD patients gradually evolve from the mildest to the most severe clinical manifestations of illness. Primarily, AD's patients present as an inability to restore newly acquired information, which can be examined by repetitively questioning. This short term memory impairment results from the atrophy of the hippocampus, especially the temporal horn^[14]. The clinical features are an amnesic type of memory dysfunction, deterioration of visuospatial, and language deficits^[15]. When the disease advances, devastation of other brain domains of cognition, including abstract thinking, language, judgment, calculation, and executive function, will develop. This impairment usually coincide with the aberration of art and social interpretations. AD patients evolve their symptoms from the loss of basic activities (e.g., dressing, eating, and bathing) to higher functions of activities in daily living (e.g., shopping, business management). Psychiatric and behavioural instabilities also progress over the course of AD. Emotional disorders (e.g., depression 25%-30%, anxiety 15%-25%, delusions in 20% of cases) and loss of initiation or apathy (up to 40%) commonly develop at early stage of AD and continue during disease progression. Motor and sensory dysfunctions, gait disorders, and other abnormal movements will appear at the late stage of the disease. The diagnosis of AD is frequently referred to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) and the National Institutes of Neurologic and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria^[16]. The gold standard of AD diagnosis is based on neuropathological findings of neuritic plaques (extra-neuronal β amyloid-containing plaques) and intra-neuronal neurofibrillary tangles in patient's brain tissues.

Huntington's disease (HD) is a rare inherited neurological disease, manifested by unwanted choreiform movements, psychiatric instabilities and cognitive deficiency^[17]. Disease onset is commonly between 30 and 50 years old and the patients with HD can present disease conditions at any time points during the disease progression. HD is a disease triggered by a repeated trinucleotide, CAG (more than 36 repeats), of Huntingtin gene located on the short arm of chromosome 4^[18]. Large duplication of CAG repeats are susceptible to replication error of the chromosome during meiosis, leading to extension or reduction of the CAG repeats^[19]. Choreiform movement is an early symptom, which causes limb incoordination and impaired function. This movement disorder is as a consequence of a selective neural cell death and atrophy in the putamen and caudate of the brain. Another significant feature in HD patients is an incapability to sustain a voluntary muscle contraction. The pattern of this symptom tends to change over time with other abnormal movements, such as rigidity, dystonia, and bradykinesia. HD patients might confront non-motor symptoms that could be more disturbing than motor symptoms, such as personality

changes, impulsivity, abnormal perceptions, lack of insight, and disability to learn new information or knowledge^[20]. Depression is also found and appears as a part of disease. Metabolic signs, for example weight loss and sleep problems, may contribute to depression of HD patients. A diagnosis of HD is relied principally on questionnaires, physical examination, family medical history, and neurological and psychiatric investigations. Brain imaging technology, in particular magnetic resonance imaging, can show structural alterations at specific locations in the brain affected by mutant Huntingtin protein. In spite of identification of the Huntingtin gene and other causative proteins, the mechanisms contributed to the pathogenesis and symptoms of HD remain largely elusive and this greatly hampers efficient development of clinical treatment.

CURRENT THERAPEUTIC APPROACH OF AGE-RELATED NEURODEGENERATIVE DISEASE

The early success in developing treatment strategies relied on the early understanding that PD is a DA deficiency disorder. Dopamine therapy is effective for improving both motor and non-motor symptoms in initial PD stage. Levodopa (LD) is considered as the most efficient and best accepted antiparkinsonian compound^[21]. Initiating levodopa as first-line therapy may achieve optimal outcomes in terms of patient function in the early years of the disease. Catechol-O-methyltransferase (COMT) inhibition increases the peripheral bioavailability of LD and reduces 3-O-methyldopa formation. The administration of COMT inhibitors with LD ensures a more stable plasma LD level and, consequently, it improves motor fluctuations. The use of COMT inhibitors improve the half-life of LD, and the triple combination carbidopa (CD)-LD-entacapone provide the most sustained LD plasma level^[22]. However, in long-term, this combination treatment is accompanied with the fluctuations of motor functions, dyskinesias and neuropsychiatric manifestations^[23]. Switching to alternative LD formulations, or supplementation of therapy with additional agents are general strategies for managing motor complications associated with LD. In addition, severe staged patients continually developed features that do not respond well to LD treatment, such as motor fluctuations, freezing episodes, autonomic dysfunctions, gait instability, dementia, and symptoms related to side effects of other medications^[24]. Surgical treatment is considered as an option for these patients who have motor fluctuations and dyskinesias, because these symptoms cannot be adequately managed by medications. The principal surgical option is deep brain stimulation, which has largely replaced neuroablative lesion surgeries for improving motor control^[25]. Next, the principal risk factor for AD is age. Several routinely-used drugs

can alleviate diseased conditions, but do not prove significant disease-improving effects^[26]. A number of therapeutic approaches intended at inhibiting disease progression are now developed into clinical trials. With disease advancement, brain degeneration in areas of memory, cognition, psychiatry, and movement, occurs to varying degrees. In clinical practice, non-medical interventions should be firstly tried, especially when symptoms are not causing distress to impact patients in daily living activity. Medical treatment for improving cognition, for instance acetylcholinesterase inhibitor, offers cognitive improvement (e.g., memory deterioration, language dysfunctions, and executive dysfunction,) and psychiatric repairment (e.g., depression, hallucination, agitation and delusion) in all stages of disease progression. The treatment with N-methyl-D-aspartate antagonist presents similar result, and this molecule can be applied in combination with acetylcholinesterase inhibitors in severe stage of AD patients^[27]. Many clinical trials aim to target at the generation and eradication of amyloid- β ($A\beta$) peptide, a dominant element of AD pathogenesis. Currently, the anti- $A\beta$ strategies are advancing with the increasing number of potential drugs. Direct interaction with $A\beta$ of small molecules could diminish $A\beta$ aggregation and deposition within the brain, thus reducing $A\beta$ -mediated synaptic dysfunction and neuronal cell death. The primarily anti- $A\beta$ drug to accomplish a clinical study was tramiprosate. Tramiprosate is a glycosaminoglycan derivative that binds to a single molecule of $A\beta$. The interaction of tramiprosate and $A\beta$ reduces the aggregation and toxicity of $A\beta$, while promote the clearance of $A\beta$ from the patient's brain. Although the treatment with tramiprosate was well tolerated, it fell short of an advantageous effect on the primary outcomes, which are the improvement on cognitive functions and clinical stages of AD patients. Another strategy to reduce $A\beta$ level within the brain is to minimize its production. Gamma secretase, a transmembrane enzyme, is reportedly required for $A\beta$ generation in several tissues, including brain. It cleaves at one end of the amyloid precursor protein, in which $A\beta$ is generated. Tarenflurbil, a non-steroidal anti-inflammatory drug, was found to lower the activity of gamma secretase enzyme, and, consequently reduce $A\beta$ production^[28]. In a Phase II of clinical trial in AD patients with mild-to-moderate symptoms, tarenflurbil was considerably safe and well tolerated; however, the beneficial effect of tarenflurbil on cognitive repairment was not observed^[29]. One possible explanation for the disappointing outcome of tarenflurbil is that oral administration cannot elevate the concentration of tarenflurbil inside the brain to be at sufficient level to reduce $A\beta$ production. An alternative approach focuses at modulating the abnormal aggregation of neurofibrillary tau protein, which is also another key feature of AD^[30]. Tau protein is a microtubule-associated protein, and found predominantly in neurons. Tau protein functions in

promoting and stabilizing the assembly of tubulin protein into microtubule filaments. In AD patients, hyperphosphorylation of tau protein is found in the cortex and hippocampus of the brain, and this could disrupt the original function of Tau protein. The hyperphosphorylated Tau results in self-assembly into paired helical filament structures that accumulate and form intraneuronal tangles, a neurotoxic agent. There are several compounds that can inhibit Tau-self aggregation. For instance, methylene blue, an extensively used histology dye, has been tested for its interference effect on Tau-self aggregation^[31]. This compound is currently experimented as a potential effective molecule for AD treatment, since a Phase II clinical study of methylene blue has been completed, and some outcomes suggested a drug benefit in a group of AD participants^[32]. Nevertheless, it is noted that there are no effective treatments available for AD at the present. Thus, the current strategy of AD management is to decrease symptoms and improve patient's quality of life. It is sensible to expect that, in the coming years, an optimal synergistic combination of these therapeutic compounds will be formulated, which will be able to modify neurodegenerative cascade and, therefore, reduce the global impact on the brain of this dreadful disease.

Lastly, the treatment of HD has been focusing on relieving motor dysfunctions (chorea), cognitive decline and psychiatric manifestations^[33]. Pharmacological treatment of Huntington's disease might not be necessary if symptoms are mild or not troublesome. Clinically, there are various potential drugs that show the capacity to correct the conditions of HD patients. Treating chorea is an important part of HD management, because it interferes to the quality of life of HD patients. Tetrabenazine was approved in 2008 by the United States Food and Drug Agency to control the involuntary periodic movements of the limbs and face, associated with chorea^[34]. Tetrabenazine is a central monoamine extingisher that reversibly binds to the type-2 vesicular monoamine transporter^[35]. Dopamine and glutamate transmission and interaction are one of the affected pathway in the HD brain. The aberrations of this can result in striatal and cortical connection dysfunctions^[36]. Therefore, most treatments, investigated at chorea, have targeted to these neurotransmitters and their receptors. Antipsychotic drugs, for instance clozapine, haloperidol, olanzapine, and, chlorpromazine, which act by reducing dopamine levels, are occasionally prescribed to lower chorea symptom^[37]. A side benefit of these antipsychotic drugs is that they also help controlling the HD-associated psychotic behaviors. Besides pharmacological treatment, physical and occupational therapy at the initial stages of the disease can correct motor dysfunction and locomotion, which is due to the fact that exercises can reinforce muscle strength and improve balance and posture of HD patients^[38]. Psychiatric therapy can help reducing stress, anxiety

Table 1 Characteristics and conventional treatment for Alzheimer's, Parkinson's and Huntington's diseases

Disease	Characteristics	Treatment	Ref.
Alzheimer's	Memory impairment Impaired reasoning and handling of complex tasks, poor judgment Changes in personality, behavior, or comportment	Medications: Cholinesterase inhibitors NMDA antagonist Non-medicals Psychological supports	[9,15,16,26,27,30]
Parkinson's	Motor symptoms (1) Rest tremor (2) Bradykinesia (3) Rigidity (4) Loss of postural reflexes Non-motor symptoms (1) Autonomic dysfunction (2) Cognitive/ neurobehavioral abnormalities (3) Sleep disorders (4) Sensory abnormalities such as anosmia, paresthesias and pain	1 Medications Dopaminergics: (1) Levodopa (2) Ergot dopamine agonists (3) Non-ergot dopamine agonists Non-dopaminergic: (1) COMT inhibitors (2) MAO-B inhibitors 2 Non-medicals Ablative lesions Deep brain stimulation	[1,6-8,23-25]
Huntington's	Choreiform movement Cognitive impairment Behavior and psychological disorders	Medications Symptomatic agents	[17,18,20,33,36]

NMDA: N-methyl-D-aspartate receptor; COMT: Catechol-O-methyltransferase; MAO: Monoamine oxidase.

and depression, as well as help managing with the emotional changes associated with the disease. Table 1 summarized characteristics and conventional treatment for Alzheimer's, Parkinson's and Huntington's diseases.

NEURAL STEM CELLS: PROPERTIES AND CHARACTERISTICS

Several lines of studies suggest that stem cells exist in the central nervous system (CNS)^[39]. Neural stem cells exist not only in the embryonic brain, but also in the adult nervous system of all mammals, including human. Neural stem cells can be distinguished from differentiated neurons by acknowledging a few key features, including proliferation, self-renewal, multipotency and molecular markers^[40]. These features make neural stem cells as an important element in CNS development and in maintenance cell number following injury and diseases or natural cell turnover.

Proliferation and self-renewal

To reach the correct number of differentiated cells in the CNS, neural stem cells have to be precisely controlled a balance between cell proliferation and differentiation during the embryonic CNS development. This balance is regulated by both stimulatory and inhibitory signals to converge the requirements of the tissues in which neural stem cells provide newly differentiated progenies. Proliferation potential is one of the most essential characteristics of neural stem cells and it was shown that neural stem cells in adult brain can be propagated *in vitro* for years^[41]. In order to generating a satisfactory number of neural stem cells, it is assumed that cell proliferation should be prevalent

in the early developmental timing, and that more cells differentiate into a specific cell type during the latter phases. This indicates that there is a high possibility for producing two undifferentiated daughter cells at early stages of development (symmetric division), and later cell division prefers the production of differentiated neurons and glial cells (asymmetric division). Neural stem cells residing in the developing neocortex undertake both symmetrical and asymmetrical divisions throughout their life span^[42]. Several pathways that interconnect to control cell proliferation have been well documented. Perhaps the best comprehensive studies are those cell signalling pathways that are triggered by growth factors. All types of neural stem cells are generally responsive to multiple family of growth factors; however, the exact set of growth factors should be exclusively required for neural stem cells at specific stages and could distinguish stage-specific neural stem cells. Early neural stem cells entirely respond to fibroblast growth factor2 (FGF2 or bFGF), and the loss of FGF ligands or FGF receptors results in a significant diminution of neural stem cell proliferation^[43]. On the other hand, the late emerging neural stem cells demand either FGF2 or epidermal growth factor for their proliferation^[44]. It is noted that cell self-renewal is tightly connected to this growth factor responsive potential. Self-renewal is considered as a pivotal identity of neural stem cells because it is indispensable for the cells to preserve themselves, therefore at least one of the progeny retains similar molecular characteristics to the mother stem cells. It is important to note that while a process of self-renewal occurs, neural stem cells may undergo changes in their abilities to produce different progeny during development^[45].

Multipotency

To be characterised as a neural stem cell in the CNS, a cell must contain a differentiation potential to give rise to neurons, astrocytes and oligodendrocytes^[41,46]. It is noted that neural stem cell plasticity is progressively restricted as development advances, for example early neural stem cells appear to be specified a wide range of phenotypes, from anterior to posterior parts of the brain, while late neural stem cells is only restricted to its origin^[47]. It was presented that adult neural stem cells exist mainly in two areas of the brain, subventricular zone (SVZ) and sub granular zone (SGZ), can be propagated *in vitro* for years^[41]. Neural stem cells in the SVZ can differentiate into olfactory neurons, while neural stem cells of SGZ differentiate into granular neurons of the dentate gyrus. However, when transplanting SVZ neural stem cells into dentate gyrus, they differentiate into calbindin-positive granular cells, while transplanting SGZ neural stem cells into the olfactory bulb, tyrosine- and calretinin hydroxylase-positive cells were observed. Furthermore, when transplanted into the developing eyes, hippocampal neural stem cells exhibited several morphological and immunological properties of retinal cells, including photoreceptors^[48]. This implies that the fate of adult neural stem cells could be influenced by environmental cues^[49]. In addition to the effects from environment, cell intrinsic programs also influence cell differentiation capacity. The robust intrinsic differences, with respect to distinct differentiation potential, has been shown to exist between neural stem cells isolated from different brain regions^[50,51].

Molecular markers

Many efforts have attempted to define neural stem cells according to their biological properties and molecular markers. In addition to those biological parameters, a series of immunoreactive antigens could also distinguish neural stem cells from others. Markers that define this population are now being developed; thus, they are commonly characterised retrospectively on the foundation of their behaviours. The evaluation of self-renewal of neural stem cells can be initially accomplished by the expression of specific molecular markers that can distinguish them from postmitotically differentiated cells. The successive expression of various intermediate filament genes has offered a useful system to classify distinct cell types during the early embryonic neural stem cells. The intermediate filament Nestin and Vimentin are exclusively expressed in the mitotically active cells during neural tube formation^[52]. Embryonic neural stem cells express several astrocytic markers, for instance brain lipid-binding protein, glutamate transporter, S100 β , RC2 and 3CB2^[53]. Together with their differentiation potential, these astrocytic features distinguish embryonic neural stem cells from other types of astrocytes and differentiated cells. On the

other hand, adult SVZ neural stem cells appear to be slowly proliferating and long-term BrdU-retaining cells, which express GFAP and the glycoprotein CD133 (Prominin-1)^[54]. Anatomical structures and comprehensive set of immunohistochemical markers help to ensure adult SVZ neural stem cell identity. A subset of these cells is identified by the expression of the intermediate filament Nestin, GFAP, transcription factor SOX2, and the RNA binding protein Musashi1, while absence of expression of the differentiated markers CD24, NeuN, and O4^[55]. In the SGZ of the dentate gyrus, a comparable subset of neural stem cells expressing GFAP, SOX2 and Nestin resembles to dormant or quiescent adult neural stem cells which can give rise to mature astrocytes and neuroblasts^[56].

An evolving regulatory networks governing neural stem cell identity is delineated by an integration of cell internal transcription factors with the cell extrinsic stimuli from an environment or culture conditions. Unrevealing how these regulatory network functions to control neural stem cells is essential to better understand of neural stem cell biology. Ultimately, it will also accelerate the progression of a novel targeted therapy by using neural stem cells for curing neurological disorders, for instance brain tumours, brain injuries, and also neurodegenerative diseases, for example HD, AD and PD.

POTENTIAL OF NEURAL STEM CELLS FOR TREATING AGE-RELATED NEURODEGENERATIVE DISEASES

Cell replacement by neural stem cell transplantation in the brain could alleviate pathological or functional deficits associated with diseases in the CNS. In addition to conventional clinical treatments, cell replacement therapy has offered the novel basis for the development of effective therapeutic strategies for human neurodegenerative diseases (Figure 1). It is hypothesised that the release of neurotransmitters and the production of neurotrophic factors from transplanted cells are possible mechanisms that promote neuronal regeneration in the damaged brain areas. PD is characterized by a vastly loss of midbrain dopaminergic neurons in the substantia nigra pars compacta and the striatum. It was reported that human fetal ventral mesencephalic tissues was successfully transplanted into the striatum of PD patients with advanced disease; however, this human tissue transplantation contains some limitation^[57]. For example, a sufficient amount of fetal tissues was challenging to obtain and the survival and integration of transplanted cells in patient's brains was found to be minimal^[58]. Besides, the development of abnormal movements was noticed in PD patients who have received embryonic nigral transplants. The potential propagation of Lewy body from host-to-graft tissues is also a serious concern^[59]. The generation

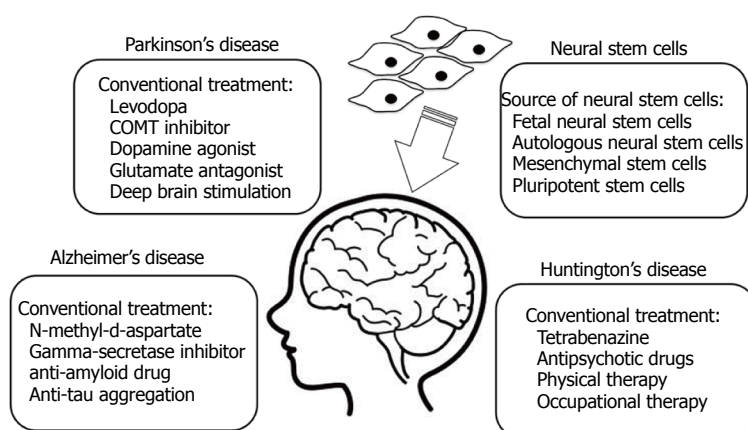


Figure 1 Neural stem cells as a therapeutic material for neurodegenerative diseases. Schematic image presents conventional treatments for Alzheimer's, Parkinson's, and Huntington's diseases. Neural stem cells serve as a potential therapeutic material, which can be obtained from several sources.

of dopaminergic neurons from stem cells could be considered as a practical and effective alternative for PD treatment. Previous studies demonstrated that dopaminergic neurons derived from human pluripotent stem cells contained comparable molecular profiling, biochemical and electrophysiological properties to human midbrain dopaminergic neurons^[60]. Importantly, the transplantation of dopaminergic neurons derived from human pluripotent stem cells can restore pathological conditions of the diseased animals, which are mice, rats and monkeys, without overgrowth of the neurons^[61]. Histological analysis of the brain indicated that the transplanted cells were well survived and integrated within the experimented animals. In addition to the derivation from human pluripotent stem cells, dopaminergic neurons can be generated by the direct conversion of human fibroblasts by overexpression of five key transcription factors, including *Mash1*, *Ngn2*, *Sox2*, *Nurr1*, and *Pitx1*^[62]. The direct reprogrammed dopaminergic neurons were positively stained for several markers for dopaminergic neurons, and showed characteristics of dopamine uptake and production. Moreover, dopaminergic neurons generated from fibroblasts provided symptomatic improvement when transplanted into a rat PD models^[62].

AD is classified by the degeneration and death of neurons throughout the brain, in particular in the basal forebrain, amygdale, hippocampus, and cortical areas. To date, AD therapy has been relied on small molecules designed to increase acetylcholine (ACh) concentration by inhibiting acetyl cholinesterase^[63]. Since these drugs are only supportive without potential protection or regeneration against progressive brain destruction, there is a need for an effective treatment and stem cell-based therapeutic approach should satisfy this requirement. Rodent AD models that received neural stem cells grafts exhibited an increased hippocampal synaptic density and improved cognitive function^[64]. Intraventricular transplantation of human neural stem cells overexpressing choline acetyltransferase (ChAT) sufficiently restored learning and memory ability of kainic-induced AD rats^[65]. In addition, a recent study demonstrated that human mesenchymal stem

cells can enhance autophagy in amyloid β -treated neurons and mice, thus promoting amyloid β clearance and increasing neuronal survival against amyloid β toxicity^[66]. Transplantation of human adipose tissue-derived mesenchymal stem cells into the brains of aged mice enhance the levels of ACh, and consequently improve the cognitive and locomotor functions of the mice^[67]. Interestingly, it was studied that ChAT-positive neurons could be directly generated from mesenchymal stem cells. These cells after transplantation could significantly improve learning and memory capacity of AD animal models^[68]. Nevertheless, there is still an argument raising whether mesenchymal stem cells can cross lineage boundaries and transdifferentiate into neuronal cells.

HD is an autosomal dominant neurodegenerative disorder, identified by uncontrolled choreic movement, cognitive disruption, and emotional instability. Initial cell-based therapy was examined by using human fetal striatal grafts and showed some clinical positive outcome in HD patients^[69]. However, the latter study found unpleasant results; grafted striatal tissues contributed to neural overgrowth and then tumour in an HD patient, who survived 5 years post-transplantation^[70]. The transplantation of neural stem cells and striatal grafts into rodent HD models demonstrated that the medium spiny neurons of transplanted cells could integrate and form neuron circuitry in the host brain^[71]. Transplantation of neural stem cells has been used not only to replace degenerated neurons, but also to protect striatal neurons against excitotoxic insults. Striatal injections of human neural stem cells into HD rodents showed incorporation into host tissues as well as migration to secondary sites associated with the disease pathology^[72]. In addition to primary cells, human neural stem cells derived from human embryonic stem cells could provide a viable source for cell therapy in HD. Previous works showed that neurons expressing striatal markers could be induced from human embryonic stem cells and brain transplantation of these human embryonic stem cell-derived neurons leads to behavioural recovery in the diseased animals^[73].

FUTURE PERSPECTIVE OF NEURAL STEM CELL-BASED THERAPY

Neurodegenerative diseases affect human well-being worldwide due to their devastating nature, cost, and lack of effective therapies. Although neural stem cells offer a great promise of treating these ailments, there are still several issues needed to be solved prior to the translation of neural stem cells into clinical setting. Several research groups around the world presented supportive data of the effectiveness of neural stem cells in correcting pathological conditions of neurodegenerative diseases; nevertheless, the exact mechanisms of how transplanted cells recover host brain function is not yet elucidated. Another important concern of neural stem cells after transplantation is the ability to migrate to the correct niche and the proper differentiation and maturation into desired neuronal cell types. And, importantly, if their growth cannot be appropriately controlled, neural stem cells may form tumor, which is more devastating condition than the original disease. Recent advance in cellular reprogramming can convert skin fibroblasts toward neural progenitor cells by chemical cocktail and hypoxia condition^[74]. These induced neural progenitor cells express multiple neuron-specific proteins, generate action potentials, and give rise to several neuronal subtypes. Generation of induced neural progenitor cells from non-neural lineages could have important implications for several purposes, including neurodevelopmental study, neurological disease modeling, and regenerative medicine application. In the near future, we might have a better understanding of pathogenesis of neurodegenerative diseases through patient-specific induced neural stem cells. The combination gene therapy with induced neural stem cell transplantation could formulate a new paradigm of therapeutic strategy to cure mutation-caused diseases^[75]. Altogether, while number of questions for neural stem cell application remain unanswered, the concerted efforts on neural stem cell research have already made a great progress toward cell replacement therapy in order to assure the best safety for patients.

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We apologize to those whose works are not cited.

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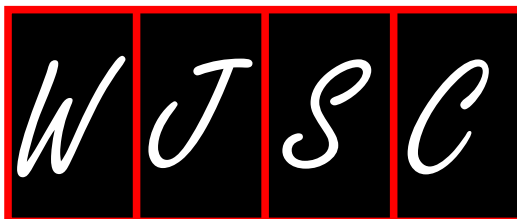
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Molecular mechanism of extrinsic factors affecting anti-aging of stem cells

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hypoxic environment, and intracellular factors including genome stability, mitochondria integrity, epigenetic regulators, calorie restrictions, nutrients, and vitamin D. Secreted tumor growth factor- β and fibroblast growth factor-2 are reported to play a role in stem cell quiescence. Extracellular matrices may interact with caveolin-1, the lipid raft on cell membrane to regulate quiescence. N-cadherin, the adhesive protein on niche cells provides support for stem cells. The hypoxic micro-environment turns on hypoxia-inducible factor-1 to prevent mesenchymal stem cells aging through p16 and p21 down-regulation. Mitochondria express glucosephosphate isomerase to undergo glycolysis and prevent cellular aging. Epigenetic regulators such as p300, protein inhibitors of activated Stats and H19 help maintain stem cell quiescence. In addition, calorie restriction may lead to secretion of paracrine cyclic ADP-ribose by intestinal niche cells, which help maintain intestinal stem cells. In conclusion, it is crucial to understand the anti-aging phenomena of stem cells at the molecular level so that the key to solving the aging mystery may be unlocked.

Key words: Stem cells; Anti-aging; Quiescence; Micro-environment

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Core tip: This review approaches anti-aging from aspect of stem cells. Stem cells may interact directly with their extracellular surroundings through caveolin-1, a lipid raft protein, to carry out and maintain stem cell functions. Mechanisms through hypoxia-inducible factor-1, glycolysis, and epigenetic regulators such as p300, protein inhibitors of activated Stats and H19 play crucial role in stem cell quiescence, and anti-aging regulations. Conversely, genomic instability such as DNA double-strand-breaks modulate cellular aging through the mammalian target of rapamycin pathway, which may lead to decreased lifespan.

Abstract

Scientific evidence suggests that stem cells possess the anti-aging ability to self-renew and maintain differentiation potentials, and quiescent state. The objective of this review is to discuss the micro-environment where stem cells reside *in vivo*, the secreted factors to which stem cells are exposed, the

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INTRODUCTION

Definition of stem cells and stem cells aging

In 1881, Weismann *et al.*^[1] discovered that organ performance was influenced by the finite cell division of tissues. Followed by Hayflick *et al.*^[2] in 1961, who discovered that normal human cells have finite cell replication and lifetime which represents human aging. When cells reach maximal replicative capacity, it is a phenomenon termed cellular senescence, or cellular aging. Recent evidence supports the model that stem cells *in vivo* are retained in a quiescent state, which can be reactivated into cell cycle progression in response to extracellular stimuli even after prolonged period of quiescence. Upon stimulation, stem cells divide to yield undifferentiated progeny and also differentiated cells through subsequent rounds of proliferation^[3]. With self-renewal capability, stem cells pool assures a constant supply of stem cells, and also differentiated cells throughout an organism lifespan. However, loss of stem cells number or functionality with age can lead to profound consequences on tissue viability^[4]. Recent data suggests that stem cells aging is partly due to heritable intrinsic event such as DNA damage, changes in their niches and micro-environment^[3]. Through systemic influences, old tissues might be rejuvenated to a young state^[5]. The significant sign of aging in stem cell culture is a diminishing replicative capacity, in which the maximal population doublings of mesenchymal stem cells (MSC) are reported as 30-40^[6]. *In vitro*, MSC cellular aging is associated with the age of donor^[7]. Conversely, *in vitro* culture of embryonic stem cells (ESC) showed no loss of proliferative potency^[8]. Human MSC cultured *in vitro* have spindle-shaped fibroblastic morphology and usually cease to proliferate no more than 40 population doublings, with the cells becoming enlarged and more flattened^[9]. The increase in cell size is associated with aging *in vitro*^[10]. Unravelling these distinctive features of aging stem cell phenotype is critical to the success of therapeutic application of stem cells in the field of regenerative medicine with respect to tissue injury, degenerative diseases or organ declines that accompany aging^[11].

Anti-aging effects of stem cells

The involvement of MSC in cell replenishment and lifespan regulations^[12] is evident since aging is defined as the sum of primary restrictions in regenerative mechanisms of multicellular organisms^[13]. Recently, the best strategy suggested to "cure" aging due to

DNA damage is a rapid and effective elimination of the damaged stem cells by apoptosis^[14]. This theory implies that stem cells may conceive ability to limit and repair intracellular damage. For example, stem cells possess robust cell surface transporters that exclude toxins^[15], the machinery for double-stranded DNA breaks repair^[16], and the production of telomerase to ameliorate telomere shortening^[17]. Also, stem cells appear to have evolved multiple mechanisms such as senescence and apoptosis to sense damaged stem cell genome with malignant potential in order to limit replicative expansion^[3,18]. These tumor-suppressor mechanisms may contribute to stem-cell irreversible growth arrest^[3]. It is worthy to understand the impacts of aging on stem cells to propose valuable strategies, gather information for potential medical anti-aging interventions.

Definition of extrinsic factors that alter stem cells properties

Aging can lead to changes of not only properties and number of stem cells, but also the niche cells, which gradually lead to decline in homeostasis and tissue regeneration. Aging has both quantitative and qualitative effects on stem cells. The qualitative changes affect self-renewal, developmental potential, and interactions with extrinsic signals^[19]. Changes in the niche or micro-environment include amount and composition of extracellular matrix (ECM), altered expression of membrane proteins and lipids in niche cells that are in direct contact with stem cells, and changes in secretion of soluble paracrine or endocrine factors (Figure 1B). In the case of tissue injury or disease, factors released from damaged cells are altered, leading to inflammatory response^[11]. Micro-environment *in vivo* is comprised of a stem cell compartment, niche cells that support stem cells, and complex ECM including fibronectin, collagen (I and IV), heparin sulphate, chondroitin sulphate, and hyaluronan^[20]. The three different micro-environments: environmental enrichment, physical exercise and calorie restriction increased the number of newly regenerated neural cells in the dentate gyrus^[21]. Hyaluronan preserved differentiation potentials of MSC derived from mouse^[22]. Hyaluronan also maintained MSC derived from human placenta in a slow-cycling mode, which was similar to quiescent state^[23]. Besides, hyaluronan is widely reviewed to regulate stem cells behavior *via* cluster determinant 44, receptor for hyaluronan-mediated motility, lymphatic vessel endothelial hyaluronan receptor, hyaluronan receptor for endocytosis, liver endothelial cell clearance receptor, or toll-like receptor 4^[24]. A report implied that the activation of cells for productive tissue regeneration required a systemic micro-environment that maintains stem cells^[5].

The micro-environment where stem cells reside *in vivo* plays important role in maintaining stem cells properties, and in regulating stem cells aging. *In vivo*, the stem cells are tightly linked to their micro-

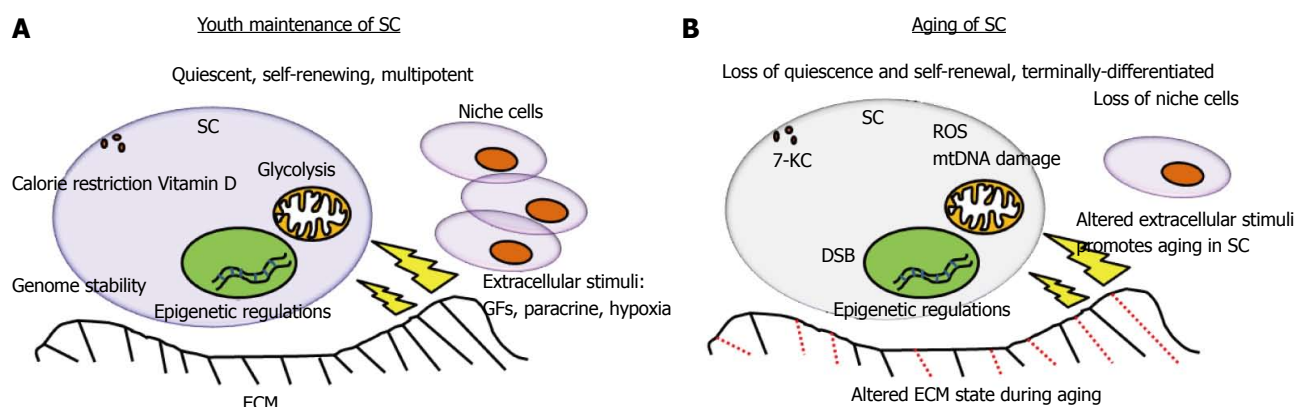


Figure 1 Stem cell maintenance and altered state during aging. A: Secreted growth factors, paracrine, calorie restriction, hypoxic micro-environment, ECM, and niche cells maintain stem cell functions. Quiescent, self-renewing, and multipotent status of stem cell are further maintained through intracellular hypoxia-responsive element, epigenetic and genomic regulators, and mitochondria; B: However, the status of stem cell is altered with decreased niche cells, fragmented ECM, increased genomic and mitochondria DNA damage, and increased ROS during aging. ECM: Extracellular matrix; ROS: Reactive oxygen species; SC: Stem cell.

environment such as ECM, and surrounding niche cells which promote self-renewal, maintain differentiation potential, maintain quiescence, regulate cell survival, homeostasis and also cellular lifespan (Figure 1). The adhesive protein N-cadherin on niche cells support stem cells; whereas integrins on stem cells and extrinsic regulators such as angiopoietin-1 (Ang-1), tumor growth factor- β (TGF- β), bone morphogenetic protein (BMP), thrombopoietin (TPO), wntless/integrated (Wnt), β -catenin (CTNNB) and osteopontin (OPN) play a role in stem cells quiescence^[25]. Hematopoiesis of hematopoietic stem cells (HSC) required the secreted factors, including stem cell factor, Ang-1, TPO, Wnt, NOTCH, OPN, chemokine stromal cell-derived factor-1 α (SDF-1 α), and direct interaction with integrin^[26]. Using genome-wide chromatin and transcriptional profiling of hair follicle stem cells *in vivo*, the hair follicle micro-environment triggered LIM homeobox-2 (LHX2) expression, which trans-activated genes that orchestrated cytoskeletal dynamics and adhesion^[27]. The removal of LHX2 led to disastrous cellular disorganization and polarization, loss of quiescence for hair follicle stem cells, loss of normal hair anchoring, as well as progressive transformation of the niche into a sebaceous gland^[27]. Since the extrinsic micro-environmental factors are required for maintaining the youth of stem cells, their interactions with stem cell membrane may also play important roles. Recently, a membrane lipid raft protein known as caveolin-1 (Cav-1), was reported to be involved in HSC quiescence and self-renewal, wherein Cav-1-deficient mice had impaired self-renewal and altered quiescent state^[28].

Furthermore, stem cells require growth factors, cytokines and mitogens for maintenance. The activation of the Wnt-Frizzled receptor with co-receptor LRP5/6 which target cytosolic CTNNB, and activation of the TGF- β receptor kinases 1/2 that phosphorylate Smads 2/3 helped maintain differentiation potential^[29]. TGF- β is a potent inhibitor of stem cell growth and cell cycle *in vitro*, and is suggested to be a regulator of stem cell

quiescence *in vivo*^[30]. Another micro-environmental factor, insulin-like growth factor-binding protein-2 (IGFBP2), used its C-terminus domain to support HSC survival and cell cycle^[31]. In addition, IGFBP2 promoted hMSC osteogenesis *via* interaction with integrin α -5^[32]. The BMP also interacted with fibroblast growth factor-2 (FGF2) through FGF receptor, which in turn activated Smad1, Smad5 and Smad8 to inhibit differentiation, and maintain quiescence in rat neural stem cells^[33]. Meanwhile, FGF2 signaling alone suppressed terminal astrocytic differentiation, and maintained rat neural stem cell potency during quiescence^[33].

MOLECULAR MECHANISMS OF STEM CELL ANTI-AGING

Hypoxia induced by micro-environment

Stem cells are maintained in a low oxygen environment or hypoxia in their native state. In general, 5% of pO₂ is the physiologically-relevant O₂ concentration in stem cells micro-environment. When 5% of pO₂ was exposed to the micro-environment, Wharton Jelly derived-MSC culture rejuvenated toward less differentiated, more primitive phenotypes^[34]. It also promoted and stabilized expression of OCT4A, NANOG, SOX2, and REX1 in induced pluripotent stem cells (iPSC)^[35]. Because 2% O₂ decreased mitochondrial DNA content^[36], this suggests that hypoxia-induced stem cell rejuvenation may be linked to energy metabolism *via* the mitochondria. Under condition of 1% O₂, hypoxia-inducible factor-1 (HIF-1) activation led to decreased extracellular signal regulated kinase, followed by decreased p16 expression^[37]. The decreased expression of the aging marker p16 helped MSC escaped cellular aging *in vitro*^[37]. A recent study further confirmed that 1% O₂ down-regulated DNA damage responsive molecules, including ATM/ATR, Chk1, and Chk2, and also cellular aging markers, including senescence-associated- β -

galactosidase, H3K9me3, heterochromatin protein 1- γ (HP1 γ), p53, p21, and p16^[38]. Since cellular aging is often accompanied with telomere length loss, it was observed that bone marrow MSC expanded under hypoxia (3% pO₂) for 15 d demonstrated telomere length maintenance; however, telomere length decreased over time under normoxia^[39]. Recently reported a major pathway that inhibits MSC senescence due to hypoxia is the HIF-1 α -TWIST pathway which down-regulated E2A-p21^[40]. In addition, hypoxia induced an immediate and concerted down-regulation of genes involved in DNA repair and damage response pathways (including *MLH1*, *RAD51*, *BRCA1*, and *Ku80* genes)^[36], which suggests that hypoxia plays a role in genome stability. Furthermore, p53 may suppressed HIF-1 translation through targeting pro-myelocytic leukemia (PML) protein, which activated the mammalian target of rapamycin (mTOR) pathway to induce aging (Figure 2)^[41,42]. Conversely, HIF-1 interacted with the aging marker p53 that led to p53 stabilization^[43], resulting to a pro-aging phenomena. Taken together, hypoxia provides stem cells with an anti-aging micro-environment and is required for healthy aging progression.

Genome stability linked to stress induced by micro-environment

The genome is like an internal turbine engine inside a cell that works perpetually throughout the life of cell. Without it, cell survival is impossible, and youth of cells cannot be maintained. In the 1950s, researchers Mortimer and Johnston stated that aging in yeast is accompanied by genome instability^[44]. In principle, there are three different modes of genome instability: mutations, mismatch repair deficiency and chromosomal instability^[45]. Mechanisms of genome instability linked to mammalian aging are stress-induced reactive oxygen species (ROS), telomere loss, and germ-line genetic variations for DNA repair^[46,47]. In addition, DNA damage caused by ROS was stated to elicit transient growth arrest, apoptosis and cellular senescence^[48]. In adult hematopoietic stem and progenitor cells (HSPCs), the histone deacetylase Sirt1 is linked to longevity^[49], DNA damage accumulation, and loss of progenitor cells. Under stress of Sirt1 ablation, HSPCs increased *Hoxa-9* expression, leading to increased DNA damage^[50]. Another sirtuin family protein, SIRT6, also regulates aging and genome stability. During DNA double-strand break repair, SIRT6 promoted DNA end resection in order to maintain genome integrity^[51]. Interestingly, a family member of p53, TAp63, prevented premature aging in skin cells as observed in TAp63^{-/-} mice by maintaining properties of epidermal and dermal precursors^[52]. The most prominent evidence that links genome stability with aging is the human disease known as progeroid syndrome. Defects in the DNA repair system known

as transcription-coupled excision repair (TCER) leads to progeroid syndrome^[53], wherein lesions in transcribed strand of active genes are repaired with defective TCER^[54]. In contrast to what is known about quiescence, the stem cells are prone to DNA damage accumulation while in quiescence. A previous study stated that following DNA damage, stem cells utilized non-homologous end-joining (NHEJ) repair system during quiescence; however, the NHEJ response is normally associated with genome rearrangement and abnormalities in HSC^[55]. In brief, genome stability is crucial for stem cells maintenance.

Mitochondrial property changes upon stem cells aging

Mitochondria play a significant role in the aging-induced signaling pathway since aging is generally characterized as a progressive decline of tissue and organ function accompanied by increased oxidative damage and mitochondrial dysfunction. Stem cells in the quiescent state appear to be less metabolically active and may be subjected to lower levels of cellular metabolism byproducts, mainly ROS. Since mitochondria are the sites for oxidative metabolism, ROS may inflict damage to its DNA and the organelle itself. Previously, functional analysis demonstrated altered mitochondrial morphology, decreased antioxidant capacities and elevated ROS levels in long-term cultivated young MSC as well as aged MSC^[56]. Another previous study stated that mice expressing a mutant form of mitochondrial DNA polymerase- γ led to HSC with compromised functions, indicating that mitochondrial integrity is crucial for stem cell maintenance^[57]. In addition, long-lived post-mitotic cells with acquired stem cell damage may be inherited by progeny cells since the mitochondrial DNA damage is passed down the generations. Recent evidence indicates that loss of a negative regulator of the *Ink4a/Arf* locus, also known as *Cdkn2a* or *BMI1*, is associated with decreased mitochondrial function, decreased electron transport flux, increased ROS concentrations, and loss of stem cell functions^[58]. Furthermore, hypoxic stimulation of glycolytic flux as well as inhibition of the p53 pathway^[59] partly stimulated glycolysis to improve stem cell pluripotency^[60] and also resulted in a younger cell phenotype. Interestingly, direct pharmacological modulation of energy metabolism or supplementation with glycolytic intermediates augmented reprogramming efficiency through glycolysis^[61]. Over-expression of the glycolytic enzyme, glucosephosphate isomerase (GPI), showed enhanced glycolysis and bypassed senescence with an increased resistance to oxidative DNA damage. In addition, depletion of GPI shortened cellular lifespan. Coincidentally, mouse embryonic stem cells, which are stem cells highly resistant to oxidative stress demonstrated a high level of glycolysis^[62]. Overall, these findings suggest that stem cells can utilize mitochondria to escape restrictions of cellular lifespan *via* an anti-aging mechanism (Figure 2).

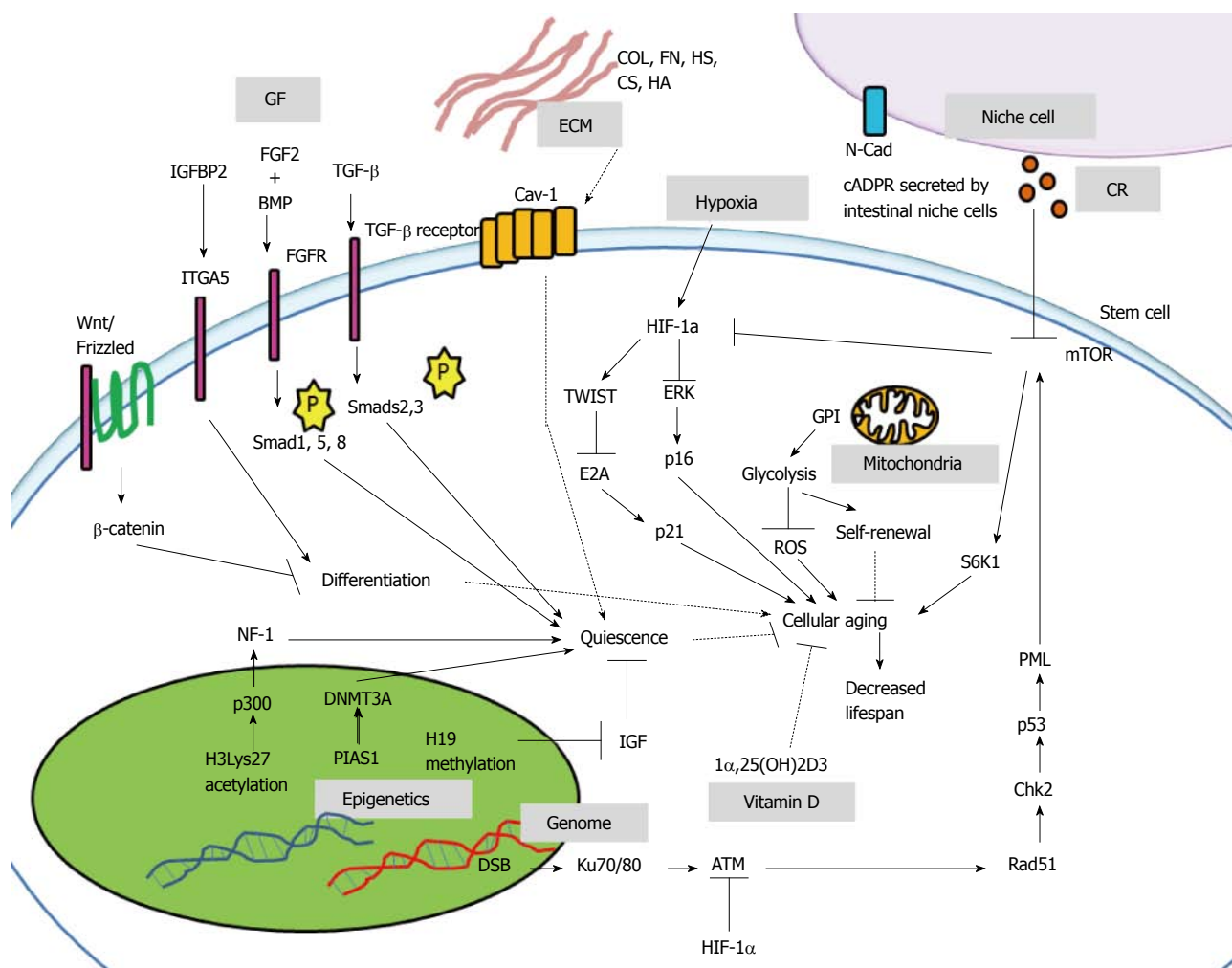


Figure 2 Signals and regulators of anti-aging in stem cell. Up-regulation of phosphorylated Smad1, Smad5, Smad8 and Smad2, Smad3 proteins maintains stem cell quiescence. Epigenetic regulators p300, DNMT3A, and H19 also maintain stem cell quiescence. Hypoxia induces HIF-1 α , a key regulator of p21 and p16 to prevent cellular aging. Under hypoxic condition, mitochondria undergo glycolysis to maintain self-renewal, and lower ROS production. With genome integrity maintained, the mTOR pathway is deactivated. In addition, stem cells interact with ECM, secreted factors from niche cells, and adhesive proteins to further maintain stem cell functions. GF: Growth factors; ECM: Extracellular matrix; CR: Calorie restriction; Wnt: Wingless/integrin; IGFBP2: Insulin-growth factor binding protein 2; ITGA5: Integrin alpha-5; FGF2: Fibroblast growth factor 2; FGFR: Fibroblast growth factor receptor; BMP: Bone morphogenetic protein; Smad: Mothers against decapentaplegic; TGF- β : Transforming growth factor- β ; Cav-1: Caveolin-1; Col: Collagen; FN: Fibronectin; HS: Heparin sulphate; CS: Chondroitin sulphate; HA: Hyaluronan; HIF-1: Hypoxia-inducible factor-1; E2A: Transcriptional factor E2; p21: Peroxidase 21; ERK: Extracellular signal-regulated kinase; p16: Peroxidase 16; GPI: Glucosephosphate isomerase; ROS: Reactive oxygen species; N-Cad: N-cadherin; cADPR: Cyclic ADP-ribose; mTOR: Mammalian target of rapamycin; S6K1: Ribosomal S6 kinase 1; DSB: Double-strand break; ATM: Ataxia telangiectasia-mutated protein kinase; Chk2: Checkpoint kinase 2; PML: Promyelocytic leukemia protein; NF-1: Neurofibromin-1; PIAS1: Protein inhibitors of activated Stats; DNMT3A: DNA(cytosine-5)-methyltransferase 3A; IGF: Insulin-like growth factor; 1 α , 25(OH)2D3: 1 α , 25-dihydroxyvitamin D3.

Epigenetics regulations

The process of aging is accompanied by nuclear architectural instability in the cell^[63,64]; however, there is a lack of evidence to show that DNA damage reduction can extend lifespan of cells *in vitro*. As a result, scientists have suggested that aging may also be caused by epigenetic regulations^[65]. Recently, protein inhibitors of activated Stats (PIAS1), a SUMO E3 ligase was shown to regulate HSCs self-renewal via GATA1 suppression by binding to GATA1 promoter region to recruit DNMT3A^[66]. Using genome-wide mapping at an enhancer associated histone mark H3Lys27 acetylation and p300 binding at promoter site, the nuclear factor-1 (NF1) protein family seemed to be required for neural stem cell (NSC) entry into quiescence^[67]. In addition, integrin alpha-6 was down-

regulated when the NSC entered quiescence^[67]. Interestingly, the imprinting alleles H19-Igf2 helped maintain HSC quiescence, wherein the maternal H19 region is differentially methylated (H19-DMR). Deletion of the H19-DMR led to Igf-Igfr1 activation and loss of quiescence^[68]. Previous findings suggested that Mym1 modulated histone modification of the Gfi1 promoter region in order to recruit Gata2 and Runx1 while maintaining HSC quiescence^[69]. Moreover, the rejuvenation of muscle cells, skin cells and bone marrow cells of mice were observed through systemic connection of one old mouse to another young mouse and were suggested to be modulated by epigenetics^[65].

Calorie intake

Apart from these, caloric restriction due to limited

calorie intake is also responsible for extrinsic signaling in stem cell maintenance and aging. In general, mechanisms involved during the stem cell quiescent state are different from those during active proliferative state. Calorie restriction led to up-regulation of cyclic ADP-ribose signaling in the intestinal niche cells known as Paneth cells, and induced proliferation of leucine rich repeat-containing G protein-coupled receptor 5-positive intestinal stem cells by inhibiting mTOR signaling^[70]. From the perspective of energy metabolism for various calorie consumptions, stem cells usually undergo glycolysis rather than oxidative phosphorylation. Under normoxia, MSCs switched to oxidative phosphorylation that led to three- to fourfold increase in senescence; however, hypoxia induced glycolysis in order to prevent oxidative stress-induced senescence and preserve MSC long-term self-renewal^[71]. Surprisingly, study stated that over-expression of phosphoinositide-dependent kinase may restore glycolytic metabolism in glycolytic-defective HSC^[72]. In general, native stem cell metabolisms are linked to increased glycolysis, limited oxidative metabolism, and resistance to oxidative damage^[73].

Nutrients

Nutrient-sensitive signaling pathways that are known to regulate organismal aging include the insulin-PI3K, Akt-FOXO, mTOR and AMPK pathways, which regulate the balance between quiescence and proliferation of stem cells during aging^[74-78]. Decreased activity of the mTOR and its target S6K1 homologs increased life span in yeast, nematodes, and fruit flies^[79]. The S6K1 is a ribosomal S6 protein kinase targeted by mTOR, and is a determinant of mammalian aging in the nutrient-sensitive signaling pathways^[80]. For the AMPK pathway, deletion of the AMPK regulator LKB1 (serine/threonine protein kinase 11) may lead to loss of mouse LT-HSC quiescence^[81]. In human ESCs and iPSCs, methionine deprivation resulted in rapid decrease of intracellular S-adenosylmethionine, which triggered stem cell differentiation, p53 and p38 activation, and reduced NANOG expression^[82]. With regard to lipid metabolism, various lipids and fatty acids regulated proliferation and differentiation of pluripotent stem cells and adult progenitors^[83], and albumin-associated lipids promoted self-renewal of hESCs^[84]. Biosynthesis of lipid such as cholesterol may influence progenitor cell differentiation, and inhibitors of cholesterol biosynthesis enhanced differentiation of mouse C2C12 myoblasts^[85]. Cholesterol is easily oxidized and may be converted to various oxidation products known as oxysterols. One of the major oxysterols is 7-ketocholesterol (7-KC), wherein increased 7-KC led to gradual changes in morphology of human adipocyte MSC, beginning with the loss of unidirectional alignment at lower concentrations, and leading towards loss of actin organization and loss of intracellular contact at higher concentrations^[86].

Vitamin D

Vitamins are important part of our diets and play some role in anti-aging of stem cells. It was reported that $1\alpha,25$ -dihydroxyvitamin D₃ ($1\alpha, 25(\text{OH})_2\text{D}_3$) delayed replicative senescence in primary hMSC; however, its pro-aging effects due to elevated systemic phosphate levels were seen in mouse models^[87]. Other findings suggested that vitamin D metabolism played a role in human osteoblastogenesis in hMSCs, in which both $25(\text{OH})\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ mediated osteoblast differentiation in hMSCs through up-regulation of insulin-like growth factor-1^[88].

CONCLUSION

The direct connection between stem cell aging and organismal aging, and lifespan is yet to be defined. The onset of aging process is often believed to influence cellular lifespan based on lowered *in vitro* replicative capability of old MSC from older compared to MSC from younger individual. Whether the influence on replicative capability is caused by systemic organismal aging, or the altered state of MSC during aging process, remains a question. Stem cells are empowered with self-renewal and differentiation potentials, and stay quiescent for long period of time while preserving their functions. The dormant state, or quiescence, seems to preserve the youth of stem cells despite the passing of time. Nevertheless, evidence suggests that stem cell population and functions may decrease in old individuals. It is likely that the youthful phenotypes of stem cells are preserved as long as it takes in order to allow healthy aging process. In times of trauma, injury, environmental stress such as ultra-violet over-exposure, and psychological stress, the process of aging may be affected, and lifespan may be shortened. Through understanding the prospect of stem cell anti-aging phenomena, the therapeutic application of stem cells can be broadened, and perhaps, a direct link between stem cell anti-aging and longevity may be established.

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Basic Study

Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair

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Data sharing: Technical appendix, statistical code, and dataset are available from the corresponding author (Benjamin. Gantenbein@istb.unibe.ch).

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Abstract

AIM: To investigate collagen patches seeded with mesenchymal stem cells (MSCs) and/or tenocytes (TCs) with regards to their suitability for anterior cruciate ligament (ACL) repair.

METHODS: Dynamic intraligamentary stabilization utilizes a dynamic screw system to keep ACL remnants in place and promote biological healing, supplemented by collagen patches. How these scaffolds interact with cells and what type of benefit they provide has not yet been investigated in detail. Primary ACL-derived TCs and human bone marrow derived MSCs were seeded onto two different types of 3D collagen scaffolds, Chondro-Gide® (CG) and Novocart® (NC). Cells were seeded onto the scaffolds and cultured for 7 d either as a pure populations or as "premix" containing a 1:1 ratio of TCs to MSCs. Additionally, as controls, cells were seeded in monolayers and in co-cultures on both sides of porous high-density membrane inserts (0.4 µm). We analyzed the patches by real time polymerase chain reaction, glycosaminoglycan (GAG), DNA and hydroxyproline (HYP) content. To determine cell spreading and adherence in the scaffolds microscopic imaging techniques, *i.e.*, confocal laser scanning microscopy (cLSM) and scanning electron microscopy (SEM), were applied.

RESULTS: CLSM and SEM imaging analysis confirmed cell adherence onto scaffolds. The metabolic cell activity revealed that patches promote adherence and proliferation of cells. The most dramatic increase in absolute metabolic cell activity was measured for CG samples seeded with tenocytes or a 1:1 cell premix. Analysis of DNA content and cLSM imaging also indicated MSCs were not proliferating as nicely as tenocytes on CG. The HYP to GAG ratio significantly changed for the premix group, resulting from a slightly lower GAG content, demonstrating that the cells are modifying the underlying matrix. Real-time quantitative

polymerase chain reaction data indicated that MSCs showed a trend of differentiation towards a more tenogenic-like phenotype after 7 d.

CONCLUSION: CG and NC are both cyto-compatible with primary MSCs and TCs; TCs seemed to perform better on these collagen patches than MSCs.

Key words: Anterior cruciate ligament rupture; Anterior cruciate ligament tenocyte; Dynamic intraligamentary stabilization system; Resazurin red assay; Mesenchymal stem cells; Real-time polymerase chain reaction; Histology; Scanning electron microscopy microscopy; Scanning electron microscopy

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Core tip: Commercially available porcine or bovine-derived collagen type I and type III patches are cyto-compatible for anterior cruciate ligament derived tenocytes (ACL-TCs) and for bone-marrow derived mesenchymal stem cells (MSCs). The combination of commercially available collagen patches with MSCs and ACL-TCs seems to be suitable for application with a dynamic intraligamentary stabilization system, which represents a novel technique to fix a ruptured ACL. However, co-culture of MSCs with ACL-TCs did show signs of differentiation of MSCs towards a more TC-like phenotype after a short-term culture of 7 d.

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INTRODUCTION

Anterior cruciate ligament (ACL) injuries represent one of the most common ligament injuries, estimated to impact 100000 to 200000 people annually in the United States^[1-4]. In Germany, the incidence of ACL ruptures is estimated at 32 per 100000 in the general population; in the active sports community, this rate is more than twice as high, at 70 per 100000^[1]. The ACL provides an essential mechanism for ensuring stability in the knee joint^[5]. The instability resulting from ACL injury not only impairs the functionality of the joint, but can also result in secondary damage to other structures, leading to arthritic changes^[1,6,7]. Current surgical treatment methods are thus focused on restoring stability of the knee, reducing pain and preventing osteoarthritis^[1,8,9]. The cruciate ligaments do not heal spontaneously; this has been attributed to a lack of a fibrin-platelet clot formation in ACL defects, which may be due to the synovial fluid surrounding the

cruciate ligaments into which blood from the ruptured stumps disperses, as well as to high levels of plasmin concentrations known to be circulating in the synovial fluid^[10]. Lack of spontaneous healing necessitates intervention to provide for alternative options to restore stability.

Given the clinical success of the dynamic intra-ligamentary stabilization approach in ACL repair^[11-13], the aim of this study was to provide insight as to whether the collagen patches-which are currently approved and marketed for cartilage and meniscus repair-are also suitable for ACL repair, and elucidate the extent to which they impact and interact with cells. For this purpose, we tested two commercially available collagen scaffolds with regards to cell viability, adherence and proliferation of seeded tenocytes (TCs), the primary cell type in ACL tissue^[14]. TCs are considered to be a specific subset of the category of fibroblasts^[14]. Ideally, the cells seeded on the scaffold would proliferate and modify the matrix, slowly reconstructing the original tissue.

Considering the difficulty of obtaining autologous ACL-derived TCs for common surgical use, we also used bone-marrow derived mesenchymal stem cells (MSCs), which can be easily harvested through bone marrow aspiration, cultured and stimulated to differentiate into a fibroblast phenotype^[2,6,15]. Several studies propose MSCs proliferate faster than TCs, and also exhibit a higher survivability *in vivo*^[15]. Additionally, current literature suggests that MSCs may have an anti-inflammatory effect, providing an additional benefit for their use in tissue engineering^[16]. We were particularly interested whether the MSCs would be able to stimulate the TCs in this *in vitro* experiment set up.

Due to the composition of the scaffold materials, we hypothesized that the patches would provide a suitable environment for TCs and lead to cell adherence as well as proliferation and collagen deposition. Furthermore, we expected MSCs to differentiate into a TCs phenotype in co-culture and even more so if cultured on collagen scaffolds rather than cultured on culture inserts. Currently, no unique marker profile for ligaments exists; thus, tendon-like cells are commonly distinguished using a combination of markers, including scleraxis, tenomodulin, tenascin-C, collagen I and collagen III^[6,14].

Here, we present analyses on cell compatibility for primary ACL-derived TCs and bone-marrow derived MSCs seeded on collagen scaffolds. We investigated the phenotype of these cells using real time real time polymerase chain reaction (RT-PCR) and biochemical analysis in order to judge the suitability of cell-seeded collagen scaffolds for the Ligamys surgery application to "boost" the healing process and possibly to improve the surgery's outcome. To the best of our knowledge, no studies have been previously published with this aim.

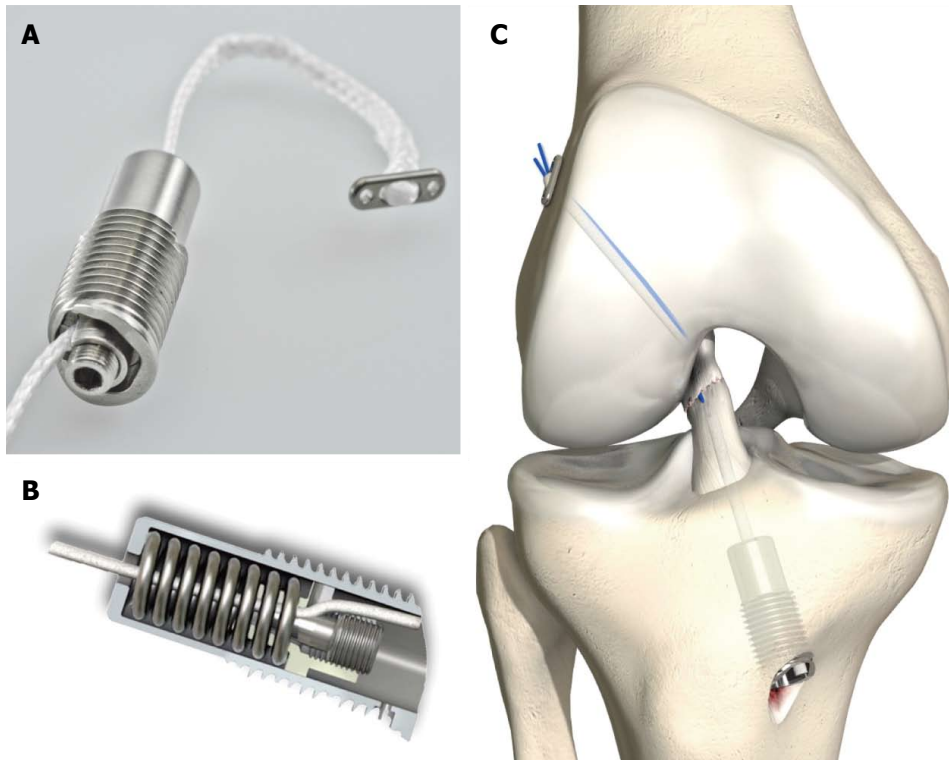


Figure 1 Dynamic intraligamentary stabilization screw called Ligamys® (Mathys, inc. Bettlach, Switzerland). A: Close-up of the outside of the screw made of titanium and illustrating with a mounted lace which mimics the polyethylene string that is mounted in the real surgery to stabilize the knee joint in case of an ACL rupture; B: Inside of the dynamic fixation screw with the spring that takes the dynamic load of the ACL and stabilizes the joint; C: Illustration of the exact position of the dynamic intraligamentary stabilization *in situ* in the knee joint. ACL: Anterior cruciate ligament.

MATERIALS AND METHODS

Scaffolds and sample preparation

Two types of commercially available and CE label approved bio-resorbable collagen scaffolds were tested in the experiments: Novocart® (NC) produced by Tetec AG (Reutlingen, Germany), a B. Braun AG Company, and Chondro-Gide® (CG), produced by Geistlich Pharma AG (Wolhusen, Switzerland). NC is a biphasic collagen-chondroitin sulfate matrix of bovine origin; CG represents a porcine collagen bilayer matrix. Both scaffolds were specifically developed for human chondrocytes and are used clinically in connection with autologous chondrocyte transplantation and autologous matrix-induced chondrogenesis for cartilage regeneration. Here, we investigated these scaffolds for application in ACL repair (Figures 1 and 2). The NC and CG scaffolds comprise a porous sponge and an adhering compact membrane to protect the cells and the lesion. Cells were seeded on the porous side as intended by its application. The sponge itself consists of fibers, building a three-dimensional structure with interconnecting pores.

Prior to cell seeding, the scaffolds were cut in equally sized samples using an 8 mm sterile biopsy punch (Kai Medical, Polymed Inc., Glattbrugg, Switzerland) and then soaked in high glucose Dulbecco's Modified Eagle's Medium (HG-DMEM), (Gibco, Life Technologies, Zug, Switzerland) with 10% fetal calf serum (FCS) and 1 ×

penicillin-streptomycin (P/S) (all Sigma-Aldrich, Buchs, Switzerland). The patches were seeded with 40000 cells; in addition, material only controls were also cultured under the same culture conditions.

Cell source and culture

Primary ACL derived TCs were isolated from ACL tissue obtained from full-knee prosthesis surgery (ethical approval was obtained from the local committee: KEK registration 22-12-13). Primary TCs were isolated by cutting the tissue in smaller pieces of approximately 4 mm³ pieces, then washed in phosphate buffered saline (PBS) twice and digested overnight in collagenase 2 (Worthington, London, United Kingdom) at 37 °C, under constant shaking at 10 RPM. The released cells were filtered with a 100 µm cell strainer (BD Falcon, Switzerland) and seeded at a density of 1000 cells/cm² for monolayer expansion in HG-DMEM + 10% FCS. With written consent human bone marrow was obtained from patients ages 55-84 undergoing hip or spine surgery. The procedure was approved by the Ethics Office of the Canton of Bern (KEK # 187/10), all patients gave their informed consent prior to their inclusion in the study. Human MSCs were amplified from the mononuclear cell fraction after density gradient centrifugation (Histopaque-1077, Sigma) by selection for plastic adherence for 2-3 passages. The MSCs were expanded using α-Minimal Essential Medium (α-MEM) with 10% FCS, 100 µg/mL penicillin, 100 UI/mL

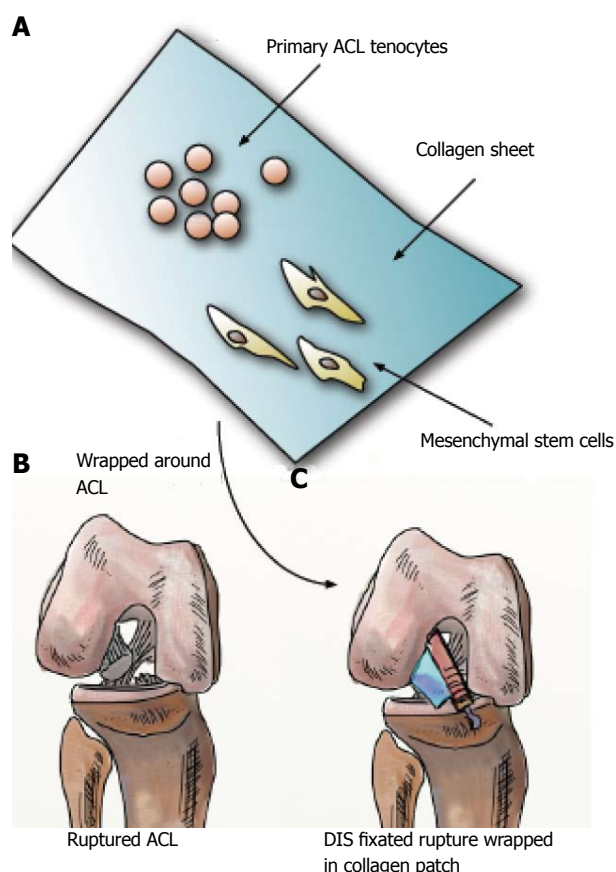


Figure 2 Overview of current regenerative approaches to improve anterior cruciate ligament rupture treatment in combination of the dynamic intraligamentary stabilization approach. A: First mesenchymal stem cells and/or primary ACL-tenocytes are pre-seeded on collagen patches (shown in blue); B: Ruptured ACL; C: DIS approach takes over major mechanical load and stabilizes the knee, whereas collagen patch wrapped around the ACL provides an ideal solution to improve clinical outcome. ACL: Anterior cruciate ligament; DIS: Dynamic intraligamentary stabilization.

streptomycin, and 5 ng/mL bFGF-2^[17].

Scaffold seeding

Scaffolds that were seeded contained either a single cell type-human MSCs or human TCs- or a combination of both cell types (Figure 3). For the samples containing co-cultures, the two cell types were mixed in a ratio of 1:1 prior to seeding; recent literature demonstrates that this provides the most effective ratio^[16]. All patches were placed in 12 well plates, and the samples were incubated for approximately one hour prior to adding 2 mL medium to each well, in order to give the cells time to adhere to the patch.

Monolayer cultures

In addition to the scaffold samples, we also grew monolayer cell cultures as well as co-cultures using translucent inserts (Falcon, 6 well cell culture inserts, 0.4 μ m PET high density pores, Falcon™, BD Biosciences, Allschwil) in 6 well plates (Falcon™). The single cell population monolayer controls served as reference samples for the real-time PCR results, while the

inserts allowed us to evaluate changes in MSCs gene expression in co-culture. Separating the MSCs and TCs cultures with inserts made it possible to attribute the relative gene expression results of cells in co-culture to the correct cell type. The pore size was chosen to facilitate cell-cell contact between the two cell types, without allowing for cell migration between the two cell populations. This setup allowed us to observe whether cell-cell contact permits differentiation of MSCs into TCs phenotype.

The co-culture for the gene expression analysis was set up as follows: culture inserts were seeded on the bottom with 100000 MSCs. Two hundred microlitre HG-DMEM medium with 10% FCS and 1 $\times$ P/S was added to the layer of MSCs. The inserts, bottom side facing up, were placed on a petri dish, covered with a beaker and placed in a 37 °C incubator for 1 h. Within this timeframe, the MSCs adhered to the insert, as has been shown in previous studies by our group^[18]. Subsequently, the other side of the insert was also seeded with the same number of TCs.

Resazurin sodium salt metabolic cell activity assay

Patches were incubated at 37 °C on a shaker for 5 h with resazurin red solution to measure mitochondrial activity as described in^[19]. Resazurin is a nontoxic, cell-permeable compound that is blue in color and virtually non-fluorescent. Upon entry into cells, resazurin is reduced to resorufin, which emits very bright red fluorescence. The amount of fluorescence or absorbance is proportional to the number of living cells (the linearity of transformation was evaluated based on primary cells) and corresponds to the cell's metabolic activity. The amount of fluorescence was monitored with a Softmax® M5 Pro Multi wavelength fluorescence reader (Molecular Devices, distributed by Bucher Biotec, Switzerland) at excitation 547 nm and emission wavelength 582 nm.

Quantification of DNA, glycosaminoglycan and HYP content

For biochemical analysis, dry-weight of each sample was measured and then each sample was digested with papain. We used the dimethylmethyle blue (DMMB) dye method to analyze the digested samples for sulphated glycosaminoglycans (GAGs), using bovine chondroitin sulfate as standard. Total collagen content was determined based on the hydroxy-proline content after acid hydrolysis and reaction with p-dimethyl-amino-benz-aldehyde and chloramine-T^[20,21].

RT-PCR

All samples were frozen down on day 0 and day 7 of each experiment, except for two samples collected during the pilot experiment, which were collected on day 8 (NC patch with TCs and NC patch with MSCs). RNA extraction was performed with TRI reagent [Molecular Research Center (MRC), Cincinnati,

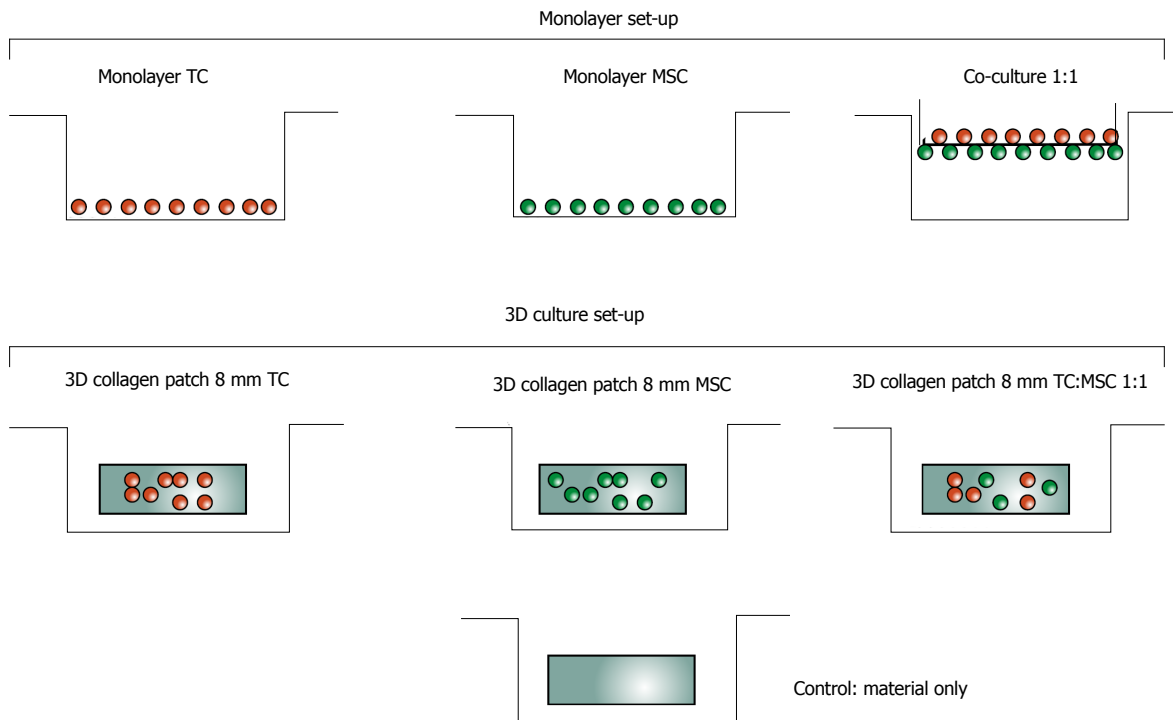


Figure 3 Experimental design of *in vitro* testing of cyto-compatibility. All monolayer and collagen patch cultures were run for 7 d under standard culture conditions (37 °C at 100% humidity and 5% CO₂) in high glucose Dulbecco's Modified Eagle Medium and 10% fetal calf serum. MSC: Mesenchymal stem cell; TC: Tenocyte.

MA, United States], polyacrylcarrier (MRC), 100% molecular grade ethanol, 1-bromo-3-chloropropane (BCP, Sigma Aldrich), GenElute miniprep kit, and the AMP-D1 DNase I Kit which contains both DNase I and 10 × DNase Digest Buffer (all Sigma Aldrich). The same process was followed for all samples, except that the scaffold samples were subject to an additional step, since those samples had been snap frozen in liquid nitrogen on day 7 of the experiments and placed in -80 °C without any RNA protective solution. In order to perform RNA extraction on those samples, the scaffold samples were first ground using a mortar pre-cooled with liquid nitrogen, then immediately placed in a solution containing 1 mL TRI and 5 µL polyacrylcarrier. The samples were then centrifuged at 16000 g to separate the scaffold debris from the solution. cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Cressier, Switzerland). For each sample, 20 µL reaction mix was set up containing 10 µL RNA template (about 0.5-1 µg total RNA), 4 µL 5 × iScript Reaction Mix, 1 µL iScript reverse transcriptase and 5 µL nuclease-free water. After the incubation, the cDNA samples were diluted with 40 µL 1 × TE Buffer.

We evaluated the relative gene expression for the genes we were interested in using human primers (all Microsynth, Balgach, Switzerland; see Table 1 for a detailed list of primers). Separate forward and reverse primers were used to set up the reaction mix, which also contained iQ SYBR Green Supermix2X (Bio-Rad) and RNase free water. We tested ligament fibroblast markers including scleraxis, tenomodulin, tenascin-C,

collagen I and III^[6,22]. In addition, we tested for gene expression of two matrix metalloproteinases (MMPs, enzymes which regulate matrix turnover) MMP3 and 13, aggrecan and collagen type II. MMP3 (stromelysin 1) and MMP13 (collagenase 3) break down collagen and proteoglycans quickly and are known to play a role in ligament remodeling^[23]. Collagen II was used to test that the cells did not differentiate into chondrocyte-like cells. There has been some evidence of increased amounts of proteoglycans in pathological tendons; therefore, gene expression of aggrecan was also evaluated^[22]. To calculate relative gene expression, the Livak method ($2^{-\Delta\Delta Ct}$)^[24] was used, which makes use of both a calibrator sample and a normalizing reference gene; we applied 18S (ribosomal RNA) as the reference gene for all samples^[25]. Relative gene expression levels for samples were calibrated to day 0 reference samples. Day 7 TCs samples were compared relative to TCs at day 0; day 7 MSCs samples were compared relative to MSCs at day 0 (*i.e.*, expression = 1.0).

Microscopy techniques

Confocal laser scanning microscopy (cLSM) was used to confirm visually that cells are able to adhere and proliferate on the scaffolds in the first two experiments. Samples for the cLSM were stained using Vybrant membrane tracker dyes DiO (DiO = green, for MSCs) and DiI (DiI = red, for TCs) (both Molecular Probes, Life Technologies, Basel, Switzerland); these dyes are lipophilic carbocyanines, which integrate into the membrane^[26]. In addition, we also imaged the cells

Table 1 Real-time polymerase chain reaction primers used for real-time polymerase chain reaction

Abbreviation	Gene name	Forward	Reverse
18S	18S Ribosomal RNA	CGA TGC GGC GGC GTT ATT C	TCT GTC AAT CCT GTC CGT GTC C
HsACAN	Core protein	CAT CAC TGC AGC TGT CAC	AGC AGC ACT ACC TCC TTC
Hs_col1	Collagen 1 A2	GTG GCA GTG ATG GAA GTG	CAC CAG TAA GGC CGT TTG
Hs_col2	Collagen 2 A1	AGCAAGAGCAAGGAGAAG	GGGAGCCAGATTGTCATC
Hs_col3	Collagen 3 A1	GTG AGC CTG GTA AGA ATG G	CCT GGA ACA CCT GGA ATA C
Hs_TNC	Tenascin C	CAC GCT GAG GTT GAT GTT C	GTT GAT GGT CGC TGG ATT G
Hs_SCX	Scleraxis A	CAC CAA CAG CGT GAA CAC	GCA GCG TCT CAA TCT TGG A
Hs_TNMD	Tenomodulin	ACA AGC AAG TGA GGA AGA A	GAC GGC AGT AAA TAC AAC AAT
Hs_MMP13	Collagenase 3	AGT GGT GGT GAT GAA GAT	CTA AGG TGT TAT CGT CAA GIT
Hs_MMP3	Matrix metalloproteinase 3	CAA GGC ATA GAG ACA ACA TAG A	GCA CAG CAA CAG TAG GAT

All primers were run at 61 °C Ta (annealing temperature) and a two-step protocol (95 °C for 10 s and 61 °C for 30 s) and 45 cycles.

on both sides of the culture inserts. This allowed us to confirm that the cells adhered to both sides of the culture insert and could therefore be evaluated with real-time PCR.

Further, additional samples were prepared during one experiment to be processed with scanning electron microscopy (SEM), as this method permits visualization of 3D structures on a very small scale. This technique also allowed us to see how the cells attach to the scaffold, as well as provided a more detailed visualization of the scaffolds themselves. On day 7, the samples were first washed with PBS to remove traces of serum, then fixed with 2.5% glutar-aldehyde. On the day of SEM sample preparation, the specimen were again washed with PBS prior to following a dehydration protocol using an alcohol series of increasing concentration, *i.e.*, 50%, 70%, 80%, 90% and 2 × 100%, with incubation periods of 10 min. Subsequently, the specimen were critical point-dried in a Bal-Tec CPD 030 (BalTec inc., Balzers, Liechtenstein) and sputtered with approximately 10 nm (30 mA for 30 s at 50 mm working distance), producing a 10 nm gold layer in a SCD 004 (BalTec inc.). Samples were then stored in an exsiccator until examination at 5 kV accelerating voltage in a Zeiss scanning electron microscope DSM 982 (Carl Zeiss, Jena, Germany).

Statistical analysis

Data were analyzed using the Kruskal-Wallis test unless otherwise noted, followed by Dunn's multiple comparisons test using the PRISM software package (GraphPad, La Jolla, United States, version 6.0e). RT-PCR data was tested using a *t*-test statistic and mean gene expression was tested if significantly different from a hypothetical value of 1.0. A significance value of $P < 0.05$ was specified to be significant. Where applicable groups were tested in 2-way ANOVA for treatment and culture time, multiple comparisons were then performed using Bonferroni's multiple testing. The statistics was checked by a biostatistics expert.

RESULTS

The experiments were meant to provide initial insight

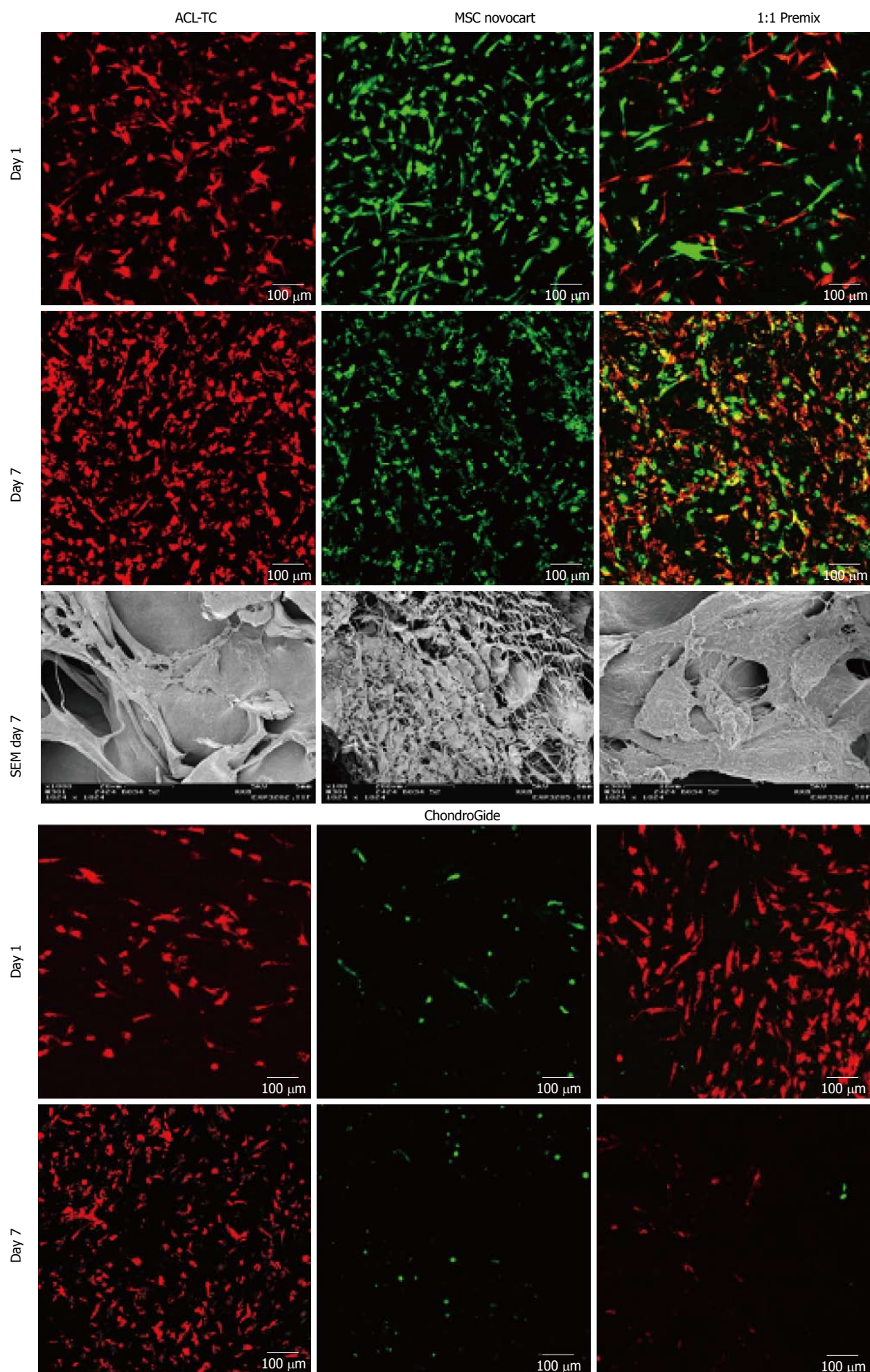
into whether NC and CG collagen patches, which are currently being used in cartilage repair, are suitable for ACL repair. Thus, adherence and proliferation of cells was tested, as well as the collagen deposition and differentiation potential of cells.

Adherence, proliferation and matrix changes

CLSM and SEM imaging and the metabolic cell activity assay confirmed that both cell types, TCs and MSCs, adhere on the substrate and seem to proliferate on both patch types (Figure 4). The images seem to show, however, that MSCs decreased in cell number after 7 d.

Noteworthy, DNA content (Hoechst) of collagen scaffolds contained a relatively high amount of porcine/bovine DNA (Figure 5), which was confirmed by DNase digestion (data not shown). There was no significant change in DNA content among the groups over the 7-d culture period. The metabolic cell activity assay (see Figure 6) revealed increased cell activity on day 7 vs day 1 for all cell treatment groups, and a slightly higher absolute level of cell activity for all CG samples versus NC patches on a given day. The relative increase in cell activity level from day 1 to day 7 in both patch types was similar (Figure 6).

The non-parametric Kruskal-Wallis tests showed that cell type did not have a significant impact on the relative change in metabolic activity for either of the two patch types (Figure 6). Furthermore, we found no significant differences between NC and CG samples in terms of the relative changes in metabolic cell activity between the two patch types. While both patch types also showed an increase in absolute cell activity, as measured in RFUs, from day 1 to day 7 for every cell type, this trend was not significant for NC patches, but significant at an overall level for CG patches ($P = 0.0298$, $n = 4$). Dunn's multiple comparisons test for CG however revealed no significant P values for individual treatment groups (TCs, MSCs, premix) with different treatment lengths (Figure 6). However, after testing for 2-way ANOVA for time and treatment we found a significant effect ($P = 0.0001$) of culture time and found that activity increased over time for TCs and premixes ($P = 0.0237$ and $P = 0.0120$, respectively)



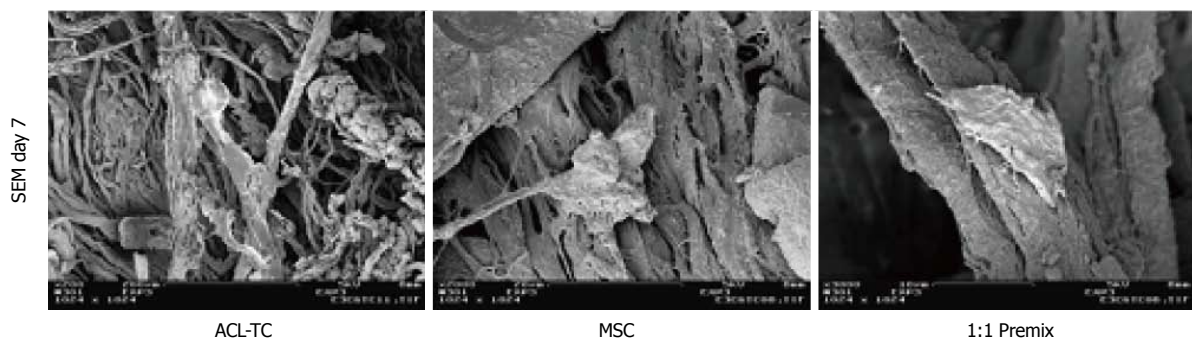


Figure 4 Z-projections of confocal laser scanning microscope images of 200 μm 3D-stacks through the collagen patches of mesenchymal stem cells and anterior cruciate ligament-derived tenocytes on day 1 and after 7 d of culture on NovocartTM and Chondro-GideTM. Cells were stained with VybrantTM DiO (DiO = green, MSCs) and DiI membrane tracker dyes (DiI = red, TCs) prior to seeding. Last row are scanning electron microscope pictures of the cell-seeded scaffolds after 7 d of culture showing adherence and shape of the MSCs and tenocytes on the scaffolds. Note that for Chondro-GideTM patches these are only images from TCs seeded scaffolds. For the NovocartTM patches the right most image is from an experiment containing a premix of cells, and the left most and center SEM image are from experiments with MSCs seeded scaffolds. MSC: Mesenchymal stem cell; ACL-TC: Anterior cruciate ligament-derived tenocyte; SEM: Scanning electron microscope.

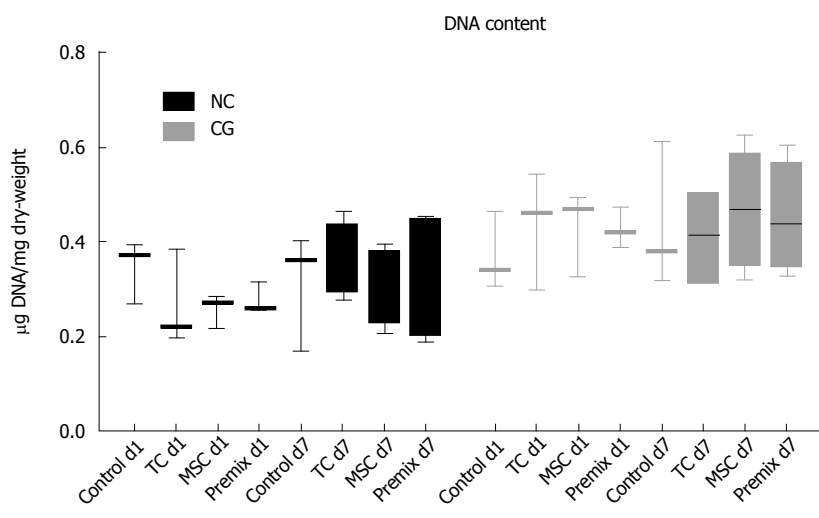


Figure 5 DNA content of collagen patches. DNA content of collagen patches seeded with anterior cruciate ligament-derived tenocytes (TCs) and/or MSCs on NovocartTM and Chondro-GideTM normalized per dry weight per carrier for the collagen patches for day 1 and day 7 of the experiment (values are means $\pm$ SEM, $n = 3$ experiments for day 1, 4 experiments for day 7). Note the large amount of DNA present in material controls. MSCs: Mesenchymal stem cells.

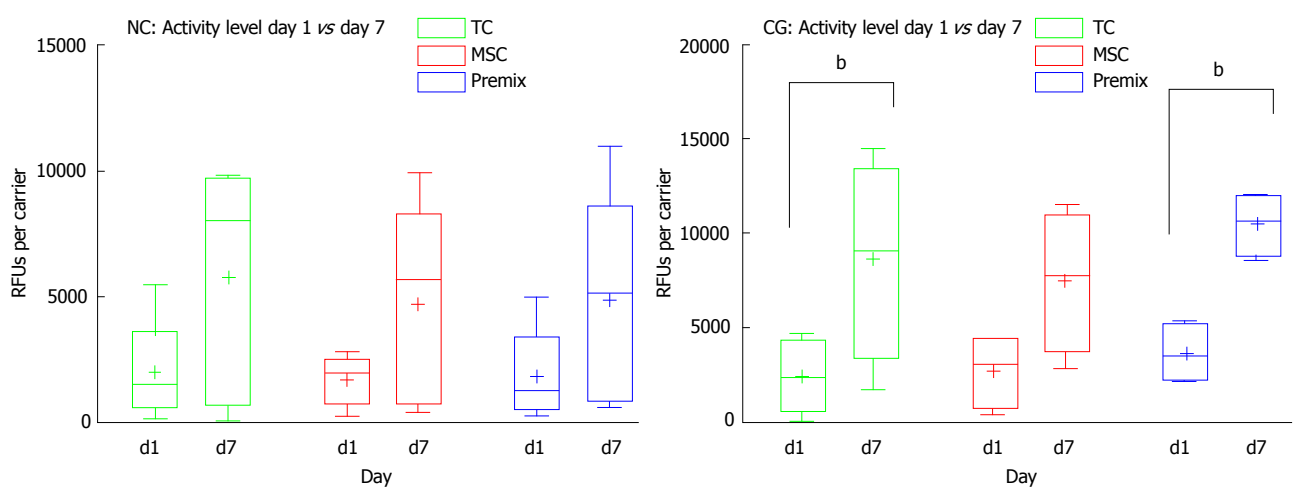


Figure 6 Mitochondrial cell activity. Mitochondrial cell activity measured by resazurin red assay of collagen patches seeded with anterior cruciate ligament-derived tenocytes (TCs) and/or MSC on NC and CG normalized per dry weight carrier for the collagen patches for day 1 and day 7 of the experiment (values are means $\pm$ SEM, $n = 4$ experiments for CG and $n = 5$ experiments for NC). b denotes $P < 0.01$. MSC: Mesenchymal stem cell; NC: NovocartTM; CG: Chondro-GideTM; SEM: Scanning electron microscope.

(Figure 6). Previous studies suggested that pathologic tendons exhibit higher GAG content^[22]. We, therefore, measured

GAG content on day 1 and day 7 ($n = 3$). In all samples, there was a decrease in the amount of GAG from day 1 to day 7 (μg per mg of dry-weight, corrected for controls

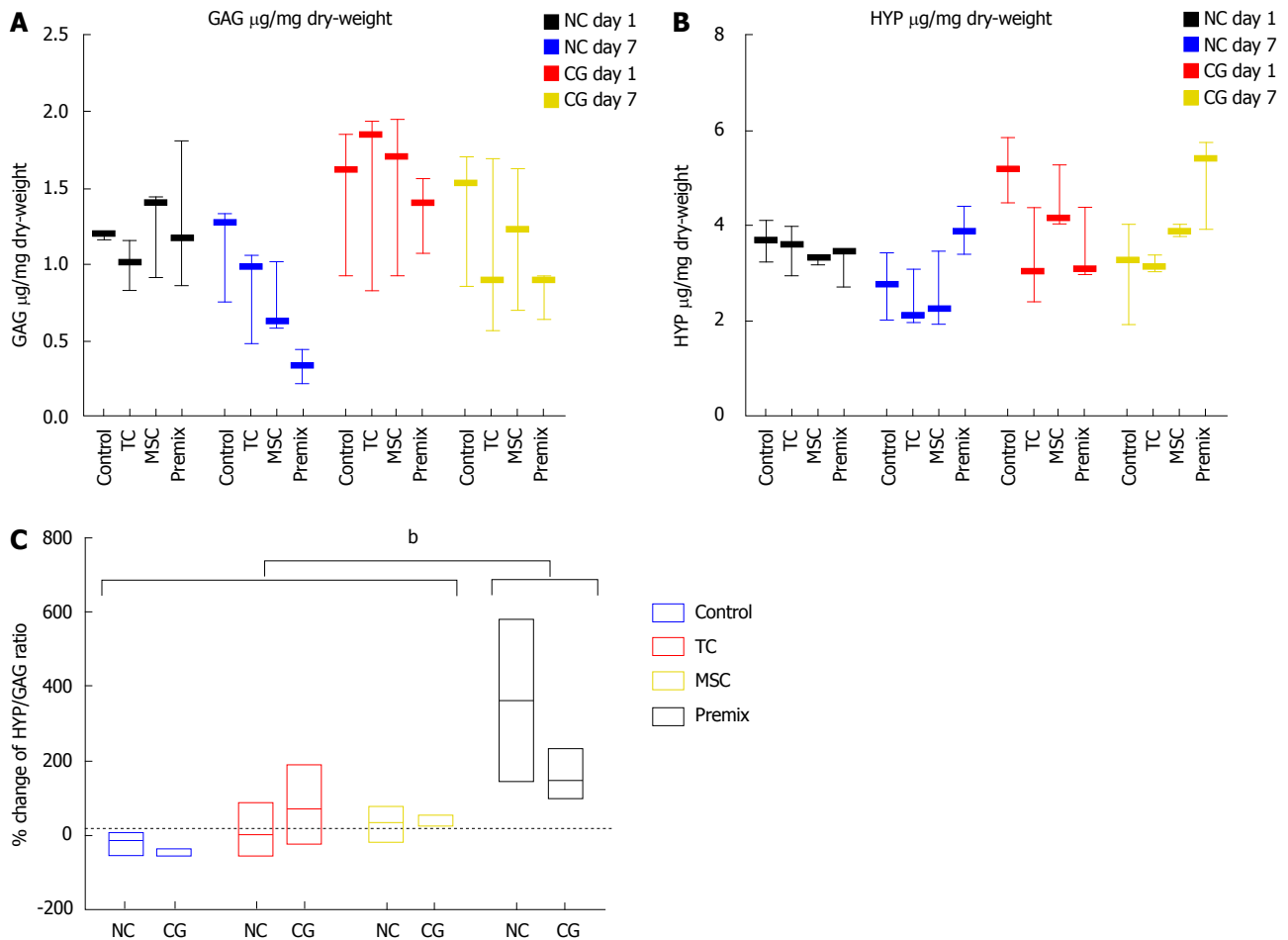


Figure 7 Hydroxy-Proline and glycosaminoglycan content. A: HYP content assay; B: GAG of collagen patches seeded with anterior cruciate ligament-TCs and/or MSCs on NC and CG normalized per dry weight carrier for the collagen patches for day 1 and day 7 of the experiment (values are means $\pm$ SEM, $n = 3$ experiments for day 1, 4 for day 7); C: Percentage change of HYP to GAG ratio relative to day 1 of NC and CG patches. b denotes a significant $P < 0.01$. GAG: Glycosaminoglycan; HYP: Hydroxy-proline; MSC: Mesenchymal stem cell; TC: Tenocytes; NC: Novocart™; CG: Chondro-Gide™; SEM: Scanning electron microscope.

using day 7 sample with no cells as a baseline for all samples). No significant difference was found between the two patch types or cell types in terms of the relative changes of GAG measured (Figure 7A).

Hydroxy-proline (HYP) levels were measured in order to assess collagen deposition of the different cell types on the scaffold (Figure 7B). Data revealed no significant change in HYP over time. In line with the other analyses, the different cell types were not found to have a significant effect on the percentage change of HYP measured, neither for NC patches nor for CG patches. Also, the Kruskal-Wallis test did not reveal a significant difference between the two patch types. However, when the ratio of HYP/GAG was considered, the 2-way ANOVA for scaffold and cell treatment showed a significant relative change for the Premix group ($P = 0.005$) (Figure 7C).

Both cell types (TCs and MSCs) were analyzed individually with regards to the effect of different substrates (NC patch, CG patch, inserts or just well plates) on the relative gene expression for the genes of interest. A summary of the relative gene expression measurements is shown in Figure 8.

Relative gene expression revealed a significant down-regulation of TNMD for TCs grown on NC scaffolds ($P < 0.0001$) and for col2 in TCs grown on culture inserts ($P = 0.0121$) relative to day 0 controls (Figure 8A). Monolayer MSCs significantly down-regulated MMP3 ($P = 0.0065$) after 7 d of culture, whereas MSCs grown on culture inserts significantly up-regulated col3 ($P = 0.006$) and down-regulated MMP3 ($P = 0.0065$).

DISCUSSION

TCs gene expression

In line with expectations, both patch types showed a slight up-regulation of collagen I gene expression for TCs in comparison to TCs grown in monolayers (Figure 8). Scleraxis expression trended up on patches vs d0 but was in line with monolayer results (Figure 8). At the same time, both patch types did not result in a significant up-regulation of collagen II or aggrecan, compared to monolayer (Figure 8). These factors suggest that the cells did not differentiate into cells of a chondrocyte phenotype. Based on the non-parametric Kruskal-Wallis test statistic, the differences in relative

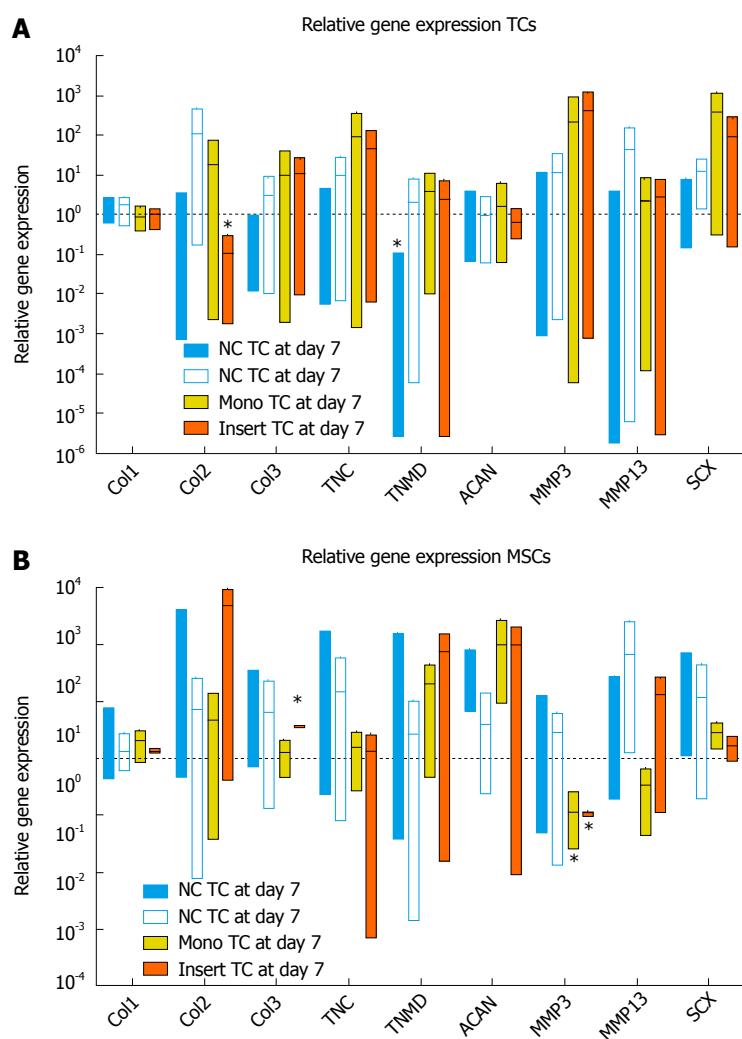


Figure 8 Relative gene expression of major extracellular matrix genes important for anterior cruciate ligament repair and mesenchymal stem cells differentiation grown on 3D collagen patches, i.e., Novocart™ and ChondroGide™. A: Gene expression of anterior cruciate ligament-derived tenocytes (TCs) relative to day 0 TCs; B: Gene expression of bone marrow-derived MSCs relative to day 0 MSCs (values are means $\pm$ SEM, $n = 4$ experiments for CG and $n = 5$ for NC). MSC: Mesenchymal stem cell; NC: Novocart™; CG: ChondroGide™; SEM: Scanning electron microscope.

gene expression between TCs cultured on NC or CG patches or in monolayers are not significant, which is in line with our initial hypothesis. We did observe significant changes of relative gene expression when tested with one-sample *t*-test. While gene expression levels for TCs on NC patches were not vastly different from those cultured on CG or in monolayers, TCs grown on NC significantly expressed less tenomodulin (TNMD) and do seem to express slightly lower levels for the ligament markers collagen type 3 (col3), tenascin- C (TNC) and scleraxis (SCX) relative to those two substrates (see Figure 8A).

At first sight, especially when considering these data in light of the metabolic cell activity results, it may seem that the CG patches provide a better environment for TCs. The metabolic cell activity measurements showed that cells grown on CG patches vs NC patches exhibited a slightly higher (yet statistically insignificant) absolute cell activity on any given measurement day, as well as a somewhat higher relative increase in cell activity from day 1 to day 7 of the experiments. However, this aspect needs to be carefully examined further.

It is possible that the culture time was not long enough to clearly discern significant differences

between patch types. MSCs and TCs were cultured for up to 28 d and checked for cell adherence with the use of CLSM and the cell tracker dyes. Studies related to osteogenesis show that osteogenic differentiation of MSCs can take up to four weeks, with an initial early differentiation phase taking place between day 5 and day 14^[27]. Conversely, it has also been shown that MSCs can show signs of differentiation into tenogenic phenotype after as little as seven days^[28]. It is also possible that the cells on the NC scaffolds first partially de-differentiated. The down-regulation of tenomodulin (TNMD) has been observed for cells that were cultured two-dimensionally^[29]. Since we observed a stronger down-regulation for cells cultured on NC than in monolayers, this may be interpreted as an indication of de-differentiation. This de-differentiation has been previously observed in monolayers as well as in organ cultures^[15].

Furthermore, scleraxis has been shown to modulate TNMD expression, such that the up-regulation of TNMD occurred several days after an up-regulation in scleraxis was observed^[30].

It is known that the structure of scaffolds can impact the cell morphology; ACL fibroblasts for instance orient parallel to collagen fibers. Cell shape

Table 2 Summary of the donor tissue that was used to harvest and expand primary cells, *i.e.*, bone marrow derived mesenchymal stem cells and anterior cruciate ligament-derived tenocytes and stage of passage for 3D culture experiment

MSCs donor characteristics						TCs donor characteristics					
Experiment	ID	Passage	Donor gender	Donor age	Source	Experiment	ID	Passage	Donor gender	Donor age	Source
1	62	1	m	75	Lumbar	1	66	1	f	62	ACL
2	62	2	m	75	Lumbar	2	66	2	f	62	ACL
3	65	1	f	84	Lower limbs	3	67	1	f	38	ACL
4	47	2	m	64	Thoracal	4	67	2	f	38	ACL
5	48	2	m	55	Thoracal	5	70	1	f	74	ACL

MSC: Mesenchymal stem cell; TC: Tenocyte; ACL: Anterior cruciate ligament.

on the other hand can impact cell differentiation^[30,31]. In fact, both patches were primarily constructed for use with chondrocytes. The manufacturer of NC patches clearly states that the patches are meant to induce a spherical morphology, which describes a chondrocyte phenotype. However, with SEM imaging we observed flat networks covering the NC patches. This observation was in line with the gene expression results, which demonstrate that the cells do not up-regulate genes, which are typical for chondrocyte phenotype. It would be interesting to re-evaluate all variables in experiments running for 14 or 21 d, to test whether this still holds true.

MSCs phenotype

MSCs cultured on patches showed a trend towards up-regulation of ligament markers versus MSCs monolayers, particularly on NC scaffolds. For instance, collagen III tended to be up-regulated on NC, with a more inconsistent expression for MSCs on CG (Figure 8). Scleraxis tended to be higher expressed on both patches compared to MSCs cultured in monolayers or on inserts (Figure 8). These results seem to indicate that MSCs are developing into a TCs phenotype on the patches. MSCs cultured with TCs, however, consistently up-regulated col 3 expression, a sign for early differentiation of the MSCs towards a more TC-like phenotype. Again, additional time might be required in order to draw more conclusive insights about the potential for MSCs to differentiate on the various substrates. However, interpreting the results of the RT-PCR data demonstrating a significant increase of col 3 in presence of TCs on culture inserts together with the significantly increased HYP/GAG ratio of "premix" of TCs and MSCs on the 3D patches (Figure 7C) could be interpreted as an early differentiation of MSCs shutting down gene expression of GAG and increasing collagen expression. A study related to tenogenic differentiation of rat MSCs showed that a significant change in expression levels for some ligament markers could already be detected after 7 d (tenascin-C as well as scleraxis); however, other markers were not up-regulated until a few days later (collagen I and III)^[28].

There were some inherent limitations in the cell types used in our study. Cells had to be obtained from

patients whom may already exhibit certain morbidities. TCs were provided from patients undergoing knee surgeries– either total knee arthroplasties or ACL reconstruction. Patient number 67, for instance, underwent ACL reconstruction due to a traumatic rupture, where the stumps were intra-operatively removed prior to graft insertion. It is possible that the cells cultured from these stumps may naturally exhibit slightly different characteristics, a topic that should be explored further. Moreover, it is also possible that the age of the donor plays a role in terms of the differentiation potential of the MSCs.

In this respect, it is also worth mentioning the existence of native ACL-derived autochthonous stem cells (ACL-SC), which already may exist in the tissue^[32]. Zhang *et al.*^[32] described the isolation and characterization of such plastic-adherent cells, which possess stem cell characteristic surface markers from healthy tissue samples. However, it is unknown whether these cells might still be present in more degenerated human tissue as analyzed in this study. An additional aspect that should be considered is the donor characteristics of cells (Table 2). In four out of five trials we had male MSC donors and in all five experiments, we had female TC donors (Table 2). The gender and age of the donors may have a hidden effect on the outcomes, including proliferation rates or differentiation ability. However, current evidence of the impact of gender and age is inconclusive^[33,34].

Imaging analysis

Confocal laser scanning microscopy (CLSM) images of CG patches showed less MSCs than TCs, both in the single cell culture of MSCs as well as when cultured together with TCs. The MSCs appear in a rounded shape in the CLSM images, as opposed to the TCs, which have a more elongated appearance. This is in line with the SEM results for the CG patches, on which we found rather roundish morphologies.

There seems a conflict in the data that the cell density on CG patches was decreasing for MSCs (Figure 4 lower panel) but DNA content (Figure 5) and cell activity (Figure 6) showed a steady-state and a trend of increased activity, respectively. We interpret this as a donor-specific phenomenon in the CLSM data.

Extracellular matrix and collagen patch environment

GAG and HYP values were also corrected for control values, as we registered significant amounts of initial GAG and HYP in our controls (Figure 7A and 7B), which was expected considering that collagen patches consist primarily of collagen 1 and 3. Correcting for the controls, we see a decrease in GAG per mg of dry-weight. This suggests that the MSCs are changing in phenotype and possibly produce less GAG over time if in direct 1:1 co-culture. Interestingly, comparing the relative change of the HYP/GAG ratio of day 7 relative to day 1 showed a significant effect of “premix” cells but there was no effect of the differences between the two scaffolds (Figure 7). The effect of cells was highly significant in the 2-way ANOVA ($P = 0.005$, Figure 7). Thus, premix of cells shifted the ratio between HYP/GAG towards lower production of GAG and slight increase of HYP.

In conclusion, the results of these 3D cell culture experiments demonstrate the potential of collagen patches for ACL repair^[4]. Using biochemical and imaging methods, we were able to demonstrate that primary TCs and MSCs can both be grown on the NC and CG patches, which were originally designed to induce a chondrogenic phenotype. Both TCs and MSCs adhere to and proliferate on the patches. Moreover, MSCs seem to show a tenogenic differentiation potential on these scaffolds as indicated by the significant increase of collagen type 3. Future work might consider the importance of alternative stimuli to accelerate healing such as the application of platelet rich plasma (PRP) or platelet rich fibrin (PRF). The problem with ACL-derived TCs and MSCs, which have to be expanded first *ex vivo* on plastics, is their clinical approval. With PRP on the other hand are unsolved issues about the reproducibility and the ideal dose of platelet concentrations^[35,36].

Further studies should be performed to evaluate additional aspects. First of all, it would be beneficial to run the experiments with a longer culture time. This would permit a more in depth analysis of the induced cell morphologies on the different patches, which could alter the survivability of the cells as well as the differentiation potential. Additionally, it would allow us to observe whether this changes the gene expression profile of the cells. It is also likely that longer culturing duration would lead to more pronounced differences between cell and patch types and be able to further support the trends we have observed so far.

Furthermore, studies should also be performed using mechanical loading. It has been shown that mechanical strain induces MSCs to differentiate into a TCs phenotype^[37,38]. It would be interesting to see whether the cells respond differently on the two patch types, subjected to equal parameters. Also, the mechanical properties of a scaffold are known to impact cell differentiation. In fact, several prior attempts at ligament or tendon repair have resulted in ectopic bone formation^[30]. Thus, further experiments

testing the impact of the mechanical properties would be helpful in evaluating the full potential of these patches. However, in these studies, it would be crucial also to look at bone-specific markers such as osteocalcin^[30].

In conclusion, while certain aspects should be examined in more depth, the use of collagen scaffolds for ACL regeneration is promising^[4]. The usage of a biological ACL repair model including collagen patches in a non-loading repair model seems a genius option to improve healing. What remains to be shown is whether mechanical loading improves the healing process or whether platelet rich fibrin PRF or PRP might be another option to be included at the site of rupture for the healing or whether it might be even hindering the healing process as has been recently proposed^[35,36,39,40].

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COMMENTS**Background**

Mesenchymal stem cells (MSCs) and anterior cruciate ligament (ACL)-derived tenocyte (TCs) are both potential source cells to be applied for the cell seeding of the dynamic intraligamentary stabilization for the fixation of ruptured ACLs. Current treatment options for non-fusions of ruptured ACLs are very limited.

Research frontiers

ACL injury is a common disease and the repair of ACL remains a challenge due to its limited self-regenerative ability. Despite the progression of reconstructive techniques for ACL repair, an ideal biological scaffold for ACL reconstruction is needed to improve clinical outcome, especially in the case of non-unions.

Innovations and breakthroughs

In this study, the authors present novel data on the regenerative technique to use commercially Food and Drug Administration (FDA)-approved collagen 1 and 3 scaffolds along with ACL-derived TCs and MSCs. They provide microscopy imaging data, biochemical data, and gene expression data of human derived primary bone-marrow derived MSCs and ACL-derived tenocytes. There was not much known how cell therapy could accelerate and improve healing for ACL ruptures in combination with a recently developed dynamic anchoring technique called Ligamys®. The combination of regenerative approaches involving TCs and MSCs transplantation along with commercially available FDA-approved collagen patches seems a straightforward approach, as the membrane is highly biocompatible and does not carry the mechanical force.

Applications

Cyto-compatibility for the collagen patches is a tool to explain good clinical outcome.

Peer-review

This paper compared two different collagen patches seeding with either bone marrow mesenchymal stem cells or primary tenocytes for possible anterior cruciate ligament repair. Chongrogide is composed of porcine collagen I and III as a bilayer membrane. Novocart is a biphasic, three-dimensional collagen-based matrix from bovine. Both collagen sponges have been used for cartilage repair. ACL injury is a common disease and the repair of ACL remains a challenge due to its limited self regenerative ability. Despite the progression of reconstructive techniques for ACL repair, an ideal biological scaffold for ACL reconstruction is needed. Thus, this study is of importance and experiments strongly suggest that the patches of collagen may be useful for anterior cruciate ligament repair.

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**EDITORIAL**

- 535 Origin of cells and network information
Tanabe S

REVIEW

- 541 Repressors of reprogramming
Popowski M, Tucker H
- 547 New insights into pancreatic cancer stem cells
Rao CV, Mohammed A
- 556 Mesenchymal stem cells in the treatment of inflammatory and autoimmune diseases in experimental animal models
Klinker MW, Wei CH
- 568 Vibration stimuli and the differentiation of musculoskeletal progenitor cells: Review of results *in vitro* and *in vivo*
Edwards JH, Reilly GC
- 583 Role of *Hox* genes in stem cell differentiation
Seifert A, Werheid DF, Knapp SM, Tobiasch E
- 596 Use of human pluripotent stem cells to study and treat retinopathies
Ben M'Barek K, Regent F, Monville C
- 605 Role of nitric oxide in the maintenance of pluripotency and regulation of the hypoxia response in stem cells
Beltran-Povea A, Caballano-Infantes E, Salguero-Aranda C, Martín F, Soria B, Bedoya FJ, Tejedo JR, Cahuana GM
- 618 Stem cell therapy in intracerebral hemorrhage rat model
Cordeiro MF, Horn AP
- 630 Recent advances in bone regeneration using adult stem cells
Zigdon-Giladi H, Rudich U, Michaeli Geller G, Evron A

MINIREVIEWS

- 641 Current focus of stem cell application in retinal repair
Alonso-Alonso ML, Srivastava GK

- 649** Search for naïve human pluripotent stem cells

Fonseca SAS, Costas RM, Pereira LV

SYSTEMATIC REVIEWS

- 657** Smart scaffolds in bone tissue engineering: A systematic review of literature

Motamedian SR, Hosseinpour S, Ahsaie MG, Khojasteh A

Contents

World Journal of Stem Cells
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Origin of cells and network information

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Core tip: The cells in the body orchestrate the unique roles of each organ through a cellular network. It is important to investigate alterations in cellular phenotypes and the regulation of genes, the genome and molecules in order to understand the origin of the cells. Insights into the changes in cellular features, including epithelial-mesenchymal transition, and recent database advances are described in this editorial.

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Abstract

All cells are derived from one cell, and the origin of different cell types is a subject of curiosity. Cells construct life through appropriately timed networks at each stage of development. Communication among cells and intracellular signaling are essential for cell differentiation and for life processes. Cellular molecular networks establish cell diversity and life. The investigation of the regulation of each gene in the genome within the cellular network is therefore of interest. Stem cells produce various cells that are suitable for specific purposes. The dynamics of the information in the cellular network changes as the status of cells is altered. The components of each cell are subject to investigation.

THE GENOME AS A BLUEPRINT

Recently, pluripotent stem cells have played an increasing role in disease and developmental models, including the challenge of generating novel organs such as intestines^[1]. Stem cell differentiation is one of the mechanisms by which regenerative tissues are produced. In each cell, the genome encodes the plan for the life of the cell and the path for organizing each tissue. The gene segments travel through the genome to settle at the gene loci^[2]. Variations within the genome produce individual differences. Dramatic transitions of cellular phenotypes, such as the Warburg effect, occur in disease states such as cancer^[3,4]. Epigenetic alterations provide cellular identity and phenotypic diversity. RNA transcription is altered in cancer; this alteration is caused by somatic DNA translocation or mutation^[5]. Variants of genes such as *BRCA2* and *CHEK2* increase the risk of lung cancer^[6]. Genome sequencing of normal

cells has revealed the accumulation of mutations and differences in each cell lineage and tissue^[7]. Genome editing has recently been developed. Additionally, gene therapy using clustered regularly interspaced short palindromic repeats/Cas9 is an emerging technique^[8]. The construction and architecture of the genome are important for understanding the cell.

STEM CELL DIFFERENTIATION AND REPROGRAMMING

Definition of stem cells

Emerging roles for stem cells as sources for cell-based therapy remind us of the importance of the definition of stem cells^[9]. Stem cells are generally defined as cells with self-renewal and differentiation potential^[10]. Accumulating knowledge and insights have shown that stem cells are able to differentiate into several cell types in the body. However, a paradigm shift occurred after the discovery of induced pluripotent stem (iPS) cells that can be created by reprogramming differentiated cells with several factors^[11]. This finding may allow for a shift in the cell type of stem cells derived from differentiated cells in the body. Thus, the range of stem cells needs to be defined. Stem cells can be classified into two categories (Figure 1): (1) pluripotent stem cells, such as embryonic stem cells or iPS cells^[12-15]; or (2) tissue multipotent stem cells such as neural stem cells, hematopoietic stem cells or mesenchymal stem cells^[16]. Recently, *SNAIL* (*SNAIL*) has been reported to localize to the nucleus and to play a role in epithelial-mesenchymal transition (EMT) during the early stage of reprogramming of differentiated cells^[17]. EMT and mesenchymal-epithelial transition processes may promote the reprogramming of differentiated cells toward stem cells^[17]. Altered phenotypes and gene networks of stem cells have been reported, suggesting that the cells themselves have various gene dynamics during culture^[18]. Cancer stem cells may be included as stem cells in cancer states. In some cases, engineered differentiated cells with gene modification or genome editing may also be included as stem cells if the cells are reprogrammed.

Cancer stem cell phenotype transition

The cell phenotype transition has been observed in cancer stem cells (CSCs)^[19]. *SOX2*, which is a reprogramming factor, is a CSC biomarker in embryonal carcinoma cells and is related to stem-like cancer cells^[20]. Genome analysis of *SOX2*-silenced human embryonal carcinoma cell lines has revealed that the cellular networks of these cells are enriched for microRNAs that are regulated by *SOX2* and that are associated with EMT markers^[20]. In contrast, an epidermal growth factor receptor exon 19-deleted lung cancer cell line was induced to exhibit CSC-like phenotypes and EMT by *DDX3X* transfection^[21]. Moreover, *DDX3X* overexpression was reported to

induce *Sox2* up-regulation^[21].

CSCs are related to chemotherapy and radiation resistance in squamous cell carcinomas (SCCs)^[22]. The CSC population is diverse in SCCs; this diversity contributes to difficulty in cancer treatment^[22]. Understanding the mechanisms of CSCs and EMT are important for the development of novel therapeutics.

CELLULAR NETWORK INFORMATION

Epithelial-mesenchymal transition

Cellular networks characterize both cells and the body, and gene combinations are critical for the presentation of phenotypes^[23]. EMT is one of the mechanisms by which the cell phenotype transitions; dihydropyrimidine has been reported to induce EMT^[24]. EMT is associated with metastasis in tumor progression and is induced by Notch activation and p53 deletion in mice^[25]. Erythropoietin-producing hepatoma (EPH) receptors, which are receptor tyrosine kinases related to cancer, may be related to EMT signaling^[26]. EPH receptor A2 induces EMT via β -catenin activation, followed by Snail expression and *cadherin 1, type 1, E-cadherin (epithelial) (CDH1)* suppression^[26]. Wnt/ β -catenin signaling is inhibited by *SOX10*, leading to the inhibition of the growth and metastasis of digestive cancers^[27]. *SRY* (sex determining region Y)-box 10 (*SOX10*) inhibits EMT, which may be one of the possible mechanisms of cancer inhibition^[27]. *Frizzled2*, the Wnt receptor, induces EMT and cell migration through the noncanonical pathway^[28]. EMT is monitored by cell rigidity, and human equilibrative nucleoside transporter-1 suppression induces EMT in pancreatic cancer cells^[29]. EMT characterization is needed for further understanding cell type transition and cancer progression.

Classification of EMT features

EMT can be characterized by the following three features: (1) changes in cellular morphology; (2) increases in cellular motility; and (3) alterations in the expression of E-cadherin and N-cadherin^[29]. Cellular morphological changes are typically observed in the transition from connective-like cells to mesenchymal-like cells^[29]. The expression of *CDH1* is usually up-regulated in connective- or epithelial-like cells, whereas the expression of N-cadherin (*CDH2*) is up-regulated in mesenchymal-like cells^[29,30]. EMT is associated with tumor metastasis^[30]. The metastasis potential or invasiveness of cancer can be measured by the mechanical rigidity of the cells^[31,32]. Several genes are involved in EMT, including *BMI1 proto-oncogene, polycomb ring finger (BMI1)*, *hypoxia inducible factor 1, alpha subunit (HIF1A, HIF-1 α)* and *twist family bHLH transcription factor 1 (TWIST1, Twist)*^[33]. *HIF-1 α* , which is a key transcription factor, is up-regulated in gastric cancer. Additionally, network pathway genes, such as *NF κ B1*, *BRCA1*, *STAT3* and *STAT1*, and network hub genes, such as *MMP1*, *TIMP1*, *TLR2*,

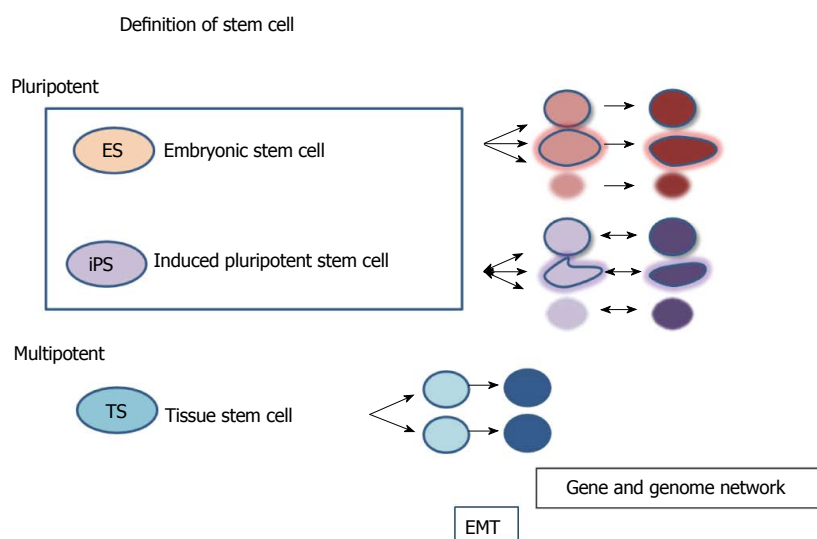


Figure 1 Current variations and definitions of stem cells. Pluripotent stem cells include embryonic stem cells and induced pluripotent stem cells, which will differentiate into all cells in the body. Multipotent stem cells, including tissue stem cells, differentiate into several types of cells to create the parts of organs or of the body. EMT: Epithelial-mesenchymal transition; iPS: Induced pluripotent stem; ES: Embryonic stem; TS: Tissue stem.

FCGR3A, *IRF1*, *FAS* and *TFF3*, have been identified^[34].

GENE REGULATION IN DISEASE

Gene and molecule alterations

An abundant number of genes are regulated in cancer. Genes are regulated not only by transcription factors but also by microRNAs (miRNAs). miRNA-9 is up-regulated in esophageal squamous cell carcinoma, which may induce EMT and metastasis in cancer^[35]. CD151, which is a regulator of laminin-binding integrin function and signaling, represses EMT and canonical Wnt signaling, leading to the inhibition of ovarian tumor growth^[36]. Wnt/ β -catenin signaling is involved in EMT induction by the parathyroid hormone in human renal proximal tubular cells^[37]. Endothelin-1 and endothelin A receptor signaling, together with Wnt signaling, regulate EMT in epithelial ovarian cancer^[38]. Endothelin/ β -arrestin signaling and Wnt/ β -catenin signaling may be involved in chemotherapy resistance in cancer^[38]. Hypoxia-inducible factors (HIFs) play roles in Wnt signaling in human colon cancer cells^[39]. HIF-1 α depletion induces the reversal of EMT, and HIF-2 α silencing affects the expression of stem cell markers and increases β -catenin transcriptional activity under hypoxic conditions^[39]. The roles of HIFs in Wnt/ β -catenin signaling and in the surrounding networks are essential for understanding cancer cell phenotypes. The silencing of β -catenin *via* promoter methylation is also involved in the enhancement of non-small cell lung cancer invasiveness^[40].

Notch1, which is one of the important molecules in cancer signaling, is involved in Ras/phosphoinositide 3 kinase (PI3K)/Akt signaling in T-cell acute lymphoblastic leukemia (T-ALL)^[41], and PI3K and Notch1 may be targets for drug resistance in T-ALL^[41]. Sox2, which

is one of the reprogramming factors used to produce iPS cells, may be a regulator of EMT during neural crest development^[42]. The Wnt pathway induces the EMT pathway, and the inhibition of the Wnt pathway may be involved in the re-differentiation of human islet β -cells^[43]. Thus, the investigation of the molecules associated with EMT and disease is of interest^[44].

MOLECULAR COMMUNICATION

The gene and genome networks

Several megaprojects have been established in response to the genome projects, one of which is called the ENCyclopedia Of DNA Elements (ENCODE) Project, which aims to translate the human genome sequence into biological and health mechanisms^[45]. The ENCODE Project has identified functional elements in the genome (<http://www.genome.gov/ENCODE/>)^[46,47].

The cross-cancer alteration of genes and their networks can be examined in cBioPortal, which is a cancer genomics database (<http://www.cbioportal.org/public-portal/>)^[48,49]. The cBioPortal includes network analysis for the visualization of networks that are altered in cancer^[49]. The precise information obtained through network analysis has been reported in several studies^[50-53]. The sources of the networks are derived from pathways and interactions from the Human Reference Protein Database^[53], Reactome^[51], the Pathway Interaction Database created by the National Cancer Institute in collaboration with Nature Publishing Group (<http://pid.nci.nih.gov/>)^[52], and the Memorial Sloan-Kettering Cancer Center Cancer Cell Map, which are all included as source information in the Pathway Commons Project (<http://www.pathwaycommons.org/>)^[50]. Pathway Commons is an open pathway that includes interaction information for multiple species,

such as humans and model organisms^[50].

The web interface called Gene Expression Commons is an interesting tool for gene expression analysis and microarray data that can be analyzed with reference data to model biological relationships (<https://gexc.stanford.edu/>)^[54]. The amount of data available in these databases is increasing and includes data from microarrays, next-generation sequencing, and clinical data.

CONCLUSION

The cell is the fundamental unit of life. The investigation of gene and genome regulation is critical for a deep understanding of phenotypic alterations and of the origin of cells. The transition of cell characteristics, including differentiation, reprogramming and EMT, and cell-to-cell communications requires further investigation to reveal the cell of origin.

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Repressors of reprogramming

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Abstract

Induced pluripotent stem cells (iPSCs) have been the focal point of ever increasing interest and scrutiny as they hold the promise of personalized regenerative medicine. However, creation of iPSCs is an inefficient process that requires forced expression of potentially oncogenic proteins. In order to unlock the full potential of iPSCs, both for basic and clinical research, we must broaden our search for more reliable ways of inducing pluripotency in somatic cells. This review surveys an area of reprogramming that does not receive as much focus, barriers to reprogramming, in the hope of stimulating new ideas and approaches towards

developing safer and more efficient methods of reprogramming. Better methods of iPSC creation will allow for more reliable disease modeling, better basic research into the pluripotent state and safer iPSCs that can be used in a clinical setting.

Key words: Reprogramming; Induced pluripotency; Stem cells

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Core tip: This review addresses an underappreciated aspect of cellular reprogramming, repressors of reprogramming. We review current literature focusing on inhibitors that modify cellular reprogramming.

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INTRODUCTION

The interest in cellular reprogramming spans basic science to clinical studies with the aim to understand the pluripotent state as well as to harness its potential to generate patient-specific therapies. These goals, while lofty, seem increasingly within reach. The first evidence of somatic cellular reprogramming was provided by the experiments performed by Gurdon *et al*^[1,2] which employed somatic cell nuclear transfer (SCNT) in *Xenopus laevis*. SCNT was later shown to be effective in mammals with the cloning of "Dolly" the sheep^[3,4] and has recently been shown to work in humans^[5,6]. SCNT requires enucleated oocytes to mediate virtually complete reprogramming of differentiated nuclei. Induced pluripotent stem cells (iPSC) were first described by Takahashi *et al*^[7,8] by overexpressing *Oct4*, *Sox2*, *c-Myc* and *Klf4*-transcription factors shown to

be essential for embryonic stem cell pluripotency. The discovery that forced expression of defined factors in differentiated cells could reprogram them to a stem-cell like state has had an enormous impact on the field of stem cell research. Advances in the creation of disease-specific stem cells are allowing us to gain previously unobtainable insights into a wide variety of diseases. Understanding the mechanics of cellular reprogramming will allow a greater understanding of developmental biology but will also translate into safe, reliable patient-specific cellular therapies.

Reprogramming is often thought of as a forward-moving process that relies on exposing the differentiated nucleus to factors that induce de-differentiation. Many groups have made strides in improving the methods of enforcing the expression of reprogramming factors^[9], increasing their efficiency^[10-12] and employing alternative reprogramming factors^[13-15]. Problematically, iPSC and to a lesser extent even SCNT-derived stem cells have been shown to be incompletely reprogrammed, leading to possible differentiation bias or unregulated growth during later differentiation^[16]. Incomplete reprogramming may also lead to unknown biases when studying disease-specific stem cells, potentially confounding results. Efficient, safe, and completely reprogrammed cells are a necessary step towards moving iPSC into the clinic. Toward this end, far less attention has been focused on eliminating endogenous factors (or their activities) which enhance cellular differentiation. Inhibition of these "negative" factors, rather than or in combination with enforcement of positive-acting factors, could lead to safer, more efficient and more completely reprogrammed iPSC. Below we summarize a number of such targets, including, as best understood, their mechanisms and their pros and cons for clinical implementation.

TRANSCRIPTION FACTORS

After the first description of iPSC, focus was turned towards finding modifiers of reprogramming. Repression of *p53*^[17], as well as its primary target gene *p21*, or addition of mutant *p53*^[18] markedly facilitates and expedites reprogramming of mouse and human cells^[19-23]. These studies proposed mechanisms by which p53 inhibits iPSCs generation, including induction of cell-cycle arrest, apoptosis and senescence. By virtue of its apoptotic induction following cellular DNA damage, p53 is a master regulator of genomic integrity and serves as a major tumor suppressor^[24-26]. p53, either directly or *via* pathway members, undergoes loss of function mutations in nearly every cancer. Often the cellular programs that are co-opted during the development of cancer are, in fact, reactivated features of stem cells^[27,28]. Reprogramming differentiated cells to pluripotent cells involves massive genomic changes that are not unlike some of the genomic changes that occur during cancer development. Indeed, despite the ability of p53 repression to enhance reprogramming

efficiency, p53-deficient cells can yield defective iPSCs with chromosomal aberrations, genome instability and malignant transformation^[18,20,29], further highlighting the link between reprogramming and tumorigenesis. Genomic integrity is a necessity for somatic cell reprogramming in order to create viable iPSC cells as well as in maintaining cellular identity. Inhibition of p53, while an interesting target for repression because of its enhancement of reprogramming, has inherent and serious risks.

The transcription factor Bright/ARID3A regulates reprogramming through direct de-repression of key pluripotency factors Oct4, Sox2 and Nanog^[30]. It has been shown to be a binding partner of Nanog^[31] as well as Oct4 and Sox2^[30] and is believed to function as a direct repressor of key developmental promoters. In culture, loss of Bright, either in through germline deletion, dominant negative inhibition, or shRNA mediated knockdown, is able to enhance standard reprogramming, induce pluripotency related genes and partially reprogram somatic cells to an ES-like state. Further, loss of Bright can induce complete reprogramming in the presence of LIF^[30]. Bright was first discovered as a B-cell specific factor^[32-34] and is critical for hematopoietic stem cell formation^[35]. Bright is known to interact directly with p53^[36] and is regulated by p53^[37]. It is possible that part of p53's influence on reprogramming may be a function of its regulation of Bright. Loss of Bright has further been shown to inhibit senescence in culture^[38], perhaps enhancing its reprogramming abilities. Bright is a tantalizing target for further analysis but there may be still as yet unknown factors which, like Bright, have multiple functions that work towards maintaining the differentiated state of the cell.

EPIGENETIC MODIFIERS

Epigenetic regulation of the genome is essential for proper gene expression and lineage commitment. As cells shift from a stem cell state into a lineage committed state, the genome goes through massive epigenetic changes from an "open" conformation to a "closed" conformation^[39]. During differentiation, global acetylation marks, typically associated with euchromatin, are reduced and global methylation markers that are associated with condensed or heterochromatin, are increased^[40], including H3K27^[41,42] and H3K9^[43,44] methylation, typically associated with inactive promoters and H3K4 methylation^[45,46], which is associated with active promoter regions. Heterochromatin is tightly packed chromatin that generally inhibits gene expression. This shift in chromatin structure and epigenetic marks is a necessary part of the differentiation process, silencing of genes that are no longer expressed in that tissue type. Underlying the critical nature of these epigenetic marks, it is often incorrect expression of silenced genes that contribute to the formation of cancer^[47], demonstrating their importance in gene regulation. Reprogramming can occur when "pioneer" factors such as Oct4, Sox2,

and Klf4 bind to enhancers near silenced chromatin and can then direct the binding of other transcription factors to these regions^[48]. Regions of the genome in fibroblasts, marked by H3K9me3, have been shown to be refractory to binding by Oct4, Sox2 and Klf4, however knockdown of H3K9 methylases increased reprogramming efficiency^[49] and the binding of “pioneer” factors to these regions^[50]. Thus, the structure and epigenetic marks of heterochromatin itself inhibits reprogramming by exogenous factors.

Stem cells often use bivalent domains to create “poised” promoters of important developmental genes^[51]. These bivalent domains are characterized by the repressive H3K27me3 and the activating H3K4me3 marks, thus repressing expression of these genes but allowing for quick upregulation in response to developmental signals^[52]. Bivalent domains are an important regulatory feature, allowing stem cells to repress the expression of key genes while keeping them “poised”. Epigenetic regulation of the genome is necessary for appropriate gene expression in both stem cells and differentiated cells^[40,41] and thus erasure of established epigenetic marks is a necessary part of reprogramming. Several histone deacetylase inhibitors have been shown to modulate reprogramming in both SCNT and iPSC^[53]. Valproic acid (VPA) is an extremely effective histone deacetylase (HDAC) inhibitor^[54] and is commonly used to enhance reprogramming^[55,56]. Other HDAC inhibitors have similarly been shown to enhance reprogramming in many different contexts^[53]. The complexities of the histone code, in particular methylation of histones, leads to an unclear picture of how inhibitors of methylation effects reprogramming^[57]. However, DNA demethylation agent 5'-Azacytidine has long been known to enhance reprogramming^[58]. Repression of AID, a cytidine deaminase, inhibits reprogramming in interspecies heterokaryons^[59] via promoter CpG demethylation^[59,60]. Inhibition of both DNA and histone methylating components of the polycomb repressive complexes 1 and 2 (PRC1 and PRC2) have been shown to inhibit reprogramming. Repression of histone methyl transferases SUV39H1^[61] and Dot1L^[62] as well as YY1^[63] enhanced reprogramming. Specifically, repression of the catalytic activity Dot1L reduced H3K79 methylation and increased reprogramming efficiency by 3-4 fold^[49]. DNA methylation and demethylation are crucial bottlenecks during reprogramming, but because of the complexities of the signals and their importance to appropriate gene expression, in looking for effective enhancers of reprogramming, targeted inhibition of specific methylation inhibitors may give a better results.

KINASES

The focus of understanding the mechanisms underlying reprogramming have typically concentrated on transcription factors and chromatin modifying factors. However, inhibition of select kinases has also been shown to increase reprogramming efficiency^[13,64].

Examples include the TGF- β r1 and SRC kinases, although only inhibition of TGF- β r1 was able to enhance reprogramming in the absence of VPA^[13]. Inhibition of TGF- β r1 allowed reprogramming to occur in the absence of Sox2 by inducing Nanog expression in intermediately reprogrammed cells that had been exposed to Oct4, Klf4 and c-Myc^[13]. A small chemical screen identified additional kinase barriers to reprogramming that include the MAP p38 kinase^[65], IP3K, which catalyzes synthesis of a crucial secondary lipid messenger required for Ca⁺ signaling^[66], and Aurora A kinase, which is essential for cell division^[67]. Inhibition of each of these enhanced iPSC cell formation, indicating that factors that may have more selected specificities beyond the transcription factors that activate them may be worthy factors for inhibition.

CONCLUSION

The potential of human embryonic stem (hESC) cells in the clinic has been understood long before science was able to deliver on that potential. The issues that may limit using hESC as a therapeutic, such as potential immune rejection and ethical concerns, has made iPSC cells a very attractive alternative approach. iPSC appear to have the same ability as hESC cells to differentiate *in vitro* and so it is hoped that they will be able to replace hESC cells therapeutically. The underlying problem is efficient creation of reliable, stable iPSC without gene integration^[68-71]. While there have been massive strides made in the field of reprogramming, there is still a lack of clinically useful methods for producing iPSC that have been shown to be safe in the long term^[16,72-74]. Future work must expand on creating more robust reprogramming protocols, rather than staying limited to established reprogramming methods. We must look at all the proteins and microRNAs in the cell, not just known transcription factors. We must look at both enhancers of pluripotency and repressors of pluripotency as possible targets. The field has advanced enormously in only a few short years; however in order to fully manipulate cell fate, we must be more inclusive when searching for cellular reprogramming factors and broaden our understanding of how cell fate is determined.

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New insights into pancreatic cancer stem cells

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of pancreatic cancer. Even after the emergence of recent new targeted agents and the use of multiple therapeutic combinations, no treatment option is viable in patients with advanced cancer. Developing novel strategies to target progression of PC is of intense interest. A small population of pancreatic cancer stem cells (CSCs) has been found to be resistant to chemotherapy and radiation therapy. CSCs are believed to be responsible for tumor initiation, progression and metastasis. The CSC research has recently achieved much progress in a variety of solid tumors, including pancreatic cancer to some extent. This leads to focus on understanding the role of pancreatic CSCs. The focus on CSCs may offer new targets for prevention and treatment of this deadly cancer. We review the most salient developments in important areas of pancreatic CSCs. Here, we provide a review of current updates and new insights on the role of CSCs in pancreatic tumor progression with special emphasis on DclK1 and Lgr5, signaling pathways altered by CSCs, and the role of CSCs in prevention and treatment of PC.

Key words: Pancreatic cancer; Cancer stem cells; DclK1; Lgr5; Prevention; Treatment

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Core tip: Despite aggressive treatment modalities, pancreatic cancer represents most lethal malignancy with uniform lethality. The pancreatic cancer stem cells (CSCs) have been found to be resistant to chemotherapy and radiation therapy. This review summarizes the important role of CSCs in pancreatic cancer tumor progression with emphasis on DclK1 and Lgr5 CSCs, molecular signaling altered by CSCs and the important role of pancreatic CSCs in prevention and treatment of pancreatic cancer.

Abstract

Pancreatic cancer (PC) has been one of the deadliest of all cancers, with almost uniform lethality despite aggressive treatment. Recently, there have been important advances in the molecular, pathological and biological understanding

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INTRODUCTION

Pancreatic cancer (PC) is a deadly disease with least survival compared to any other cancers. PC including pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer deaths in both genders (men and women) in the United States and the seventh world-wide^[1,2]. Despite tremendous scientific effort for over three decades, PC remains almost uniformly lethal devastating disease with < 6% five-year survival. The incidence of PC has been increasing over the past ten years. In 2013, of 46420 people (23530 men and 22890 women) diagnosed with PC, 39590 people (20170 men and 19420 women) were expected to die in the United States^[1]. Worldwide, 338000 people were diagnosed with PC in 2012^[2]. Lack of early diagnosis and effective interventions are the major factors in the poor prognosis and dismal survival rates^[3-5].

Recently, stem cells of pancreatic cancer (CSCs)/ or cancer/tumor initiating cells that often are resistant to treatment have been identified^[6-9]. The CSCs occupying a very small part of the entire cancer tissue are the reasons for tumor resistance against conventional chemotherapy and re-growth of untreatable tumors. Although the CSCs exists a small population in the cancer tissues, very recent evidence shows that CSCs contribute to tumor initiation, growth, metastasis, and resistance to therapy^[6-10]. Cancer stem cell (CSC) research has achieved much progress in a variety of solid tumors, including pancreatic cancer to some extent^[11]. The identification of specific cell surface markers enabled purification of CSCs from different cell lines or clinical tissues/samples, leading to evaluate the nature of CSCs^[12-14]. Understanding of pancreatic CSCs may improve existing therapies and deepen insight into the role of CSCs in pancreatic cancer progression^[11].

In this mini-review, we will comprehensively focus on pancreatic CSCs with emphasis on DclK1 and Lgr5. Also, we review the signaling pathways altered by CSCs, and the role of CSCs in prevention and treatment of PC (Figure 1).

ROLE OF CSC IN PC

Little has changed over last three decades for 5-year pancreatic cancer survival despite introduction of new and targeted chemotherapeutic agents^[15]. It is primarily due to highly aggressive nature of the PC disease as majority of the patients' present late stage disease and doesn't respond to therapy thereby showing chemoresistance^[15]. Recently, attention has been focused on pancreatic CSC levels to find better ways to combat PC. Cell membrane markers have made possible the identification of CSCs. Pancreatic CSC populations account for less than 1% of all pancreatic cancer cells^[14]. The pancreatic CSCs identified so far are CD133, CD24, CD44, EPCAM, ESA, c-Met, Aldh1, and more recently DclK1 and Lgr5^[9-18]. These

pancreatic CSC markers are well known based on several xenograft studies. All these markers may mark themselves or co-express to show the CSC properties^[19-21]. CD133 pancreatic CSC cells co-express the CXCR4 receptor at the invading margins of human pancreatic ductal tumors^[14,19]. Other CSC markers that co-express (CD24⁺/CD44⁺/EPCAM and CD133⁺) show CSC properties making them often resistant to chemo and radiotherapy. However, complete overlap may not be found between these different CSC markers^[22].

Most drugs are unable to eradicate these pancreatic CSCs, and this is postulated to be one of the primary reasons for failure of chemotherapy that will lead to tumor relapse and metastasis. The pancreatic CSCs markers CD24, CD44, CD133, CXCR4, ESA and nestin were reported in pancreatic intraepithelial neoplasia and PDAC^[23]. Pancreatic CSC populations expressing the cell surface markers CD24, CD44, and ESA^[14,19,24] were observed to maintain their cell surface marker phenotype after repeated passages in immunocompromised xenografts mice^[12,14,19]. In another study, c-Met positive cells readily formed *in vitro* spheres but not the c-Met negative cells^[14,25]. Similarly, it was noted that cells expressing combinations of CD44 and c-Met showed increased tumor formation ability and to renew^[25]. Kim *et al.*^[18] reported that irrespective of CD133 status, the pancreatic cancer cells with high ALDH1 activity were resistant to chemotherapy-induced cell death and are highly tumorigenic^[14,18]. The MiaPaCa-2 sphere derived CSC like cells obtained from the xenograft tumors of the mice showed high expression of EpCAM, CD44 and EZH2^[14,26]. All of these CSCs have been reviewed elsewhere for pancreatic cancer. However, there are recent studies showing the CSC properties for two important makers DclK1 and Lgr5 in pancreatic cancer.

DclK1 and pancreatic cancer

DclK1 (Doublecortin-like kinase 1, formerly known as DCAMKL-1 or doublecortin and CaM kinase-like 1) initially was identified as a putative intestinal stem cell marker. Earlier studies have suggested that DclK1 may mark tumor-initiating cells in a variety of tumor types^[27-39]. DclK1 regulates several key oncogenes like c-MYC, KRAS, NOTCH1 and EMT^[32,33,39]. Moreover, DclK1 is also reported to be a pancreatic cancer stem cell marker and its expression is found to be upregulated in PDAC^[9,39]. In the pancreas and intestines, DclK1 has also recently been described as an undefined tuft/brush cell marker^[36,37]. We have empirically shown DclK1 expression in both murine and human pancreatic intraepithelial neoplasia (PanIN) and PDAC^[9]. DCLK1 is also expressed by isolated cells in the pancreatic duct and islets of normal mouse pancreas, and prior studies have suggested that these non-neoplastic DclK1-expressing pancreatic cells were associated with progenitor-like function^[31]. Further, it was shown that DCLK1HI/AcTubHI mouse PanIN cells display specialized morphology, unique patterns of gene expression, and enhanced PanIN

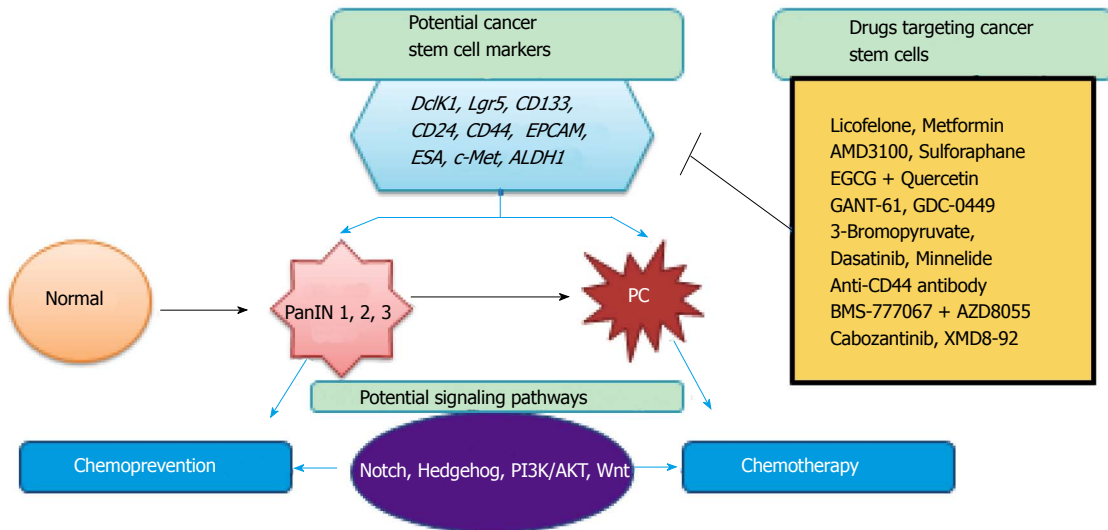


Figure 1 Schematic representation of the role of cancer stem cells markers during pancreatic cancer development, signaling pathways involved and cancer stem cells inhibition by various drugs by chemoprevention and/or chemotherapy. PC: Pancreatic cancer.

sphere-forming ability^[6]. The lineage tracing techniques using alternate DNA recombinases (e.g., Dre, Flp) or the development of non-Cre/lox-based methods for Kras activation in the murine pancreas will further the progress in this area. Multiple pathways that contribute to the CSC properties of DCLK1HI/AcTubHI pancreatic cancer cells were identified by using whole genome transcriptome analysis^[6]. Studies identified ABL1 and IGF1R to be highly expressed in DCLK1HI/AcTubHI cells, complementing ongoing preclinical and clinical evaluation of ABL1 and IGF1R pathway inhibition as new forms of targeted therapy for pancreatic cancer^[38,39]. These studies suggest that both invasive and preinvasive PC may depend on DCLK1 expressing cells with cancer stem cell capabilities. As in the case of intestinal tumorigenesis, targeting this cell population may have therapeutic potential in the treatment and/or chemoprevention of pancreatic cancer. Further, NPsiDCLK1 (nanoparticle-encapsulated siRNA) knockdown of DclK1 in the mouse xenograft lead to downregulation of important markers of pluripotency like SOX2, NANOG, OCT4 and KLF4 and EMT, and angiogenic factors^[40]. These data clearly supports a central regulatory role of DclK1 in pancreatic tumorigenesis^[39]. However, further studies are underway to evaluate the potential of DclK1 as pancreatic CSC and specifically target DclK1 along or in combination with other CSC markers.

Lgr5 and pancreatic cancer

Lgr5 also known as G-protein coupled receptor 49 (GPR49) or G-protein coupled receptor 67 (GPR67) or Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) is the Wnt target gene that marks Wnt driven actively dividing stem cells^[41-43]. In the stem cell hierarchy, LGR5 is on a higher level than CD133, however, its expression and function in the tissues of pancreatic cancer is unclear. Recent studies investigated tissue expression of LGR5 and CD133

in resected pancreatic ductal adenocarcinoma who underwent resection and demonstrated cytoplasmic expression of LGR5 in the PC cells^[17].

The maturation lineages of proximal peribiliary glands to the distal pancreatic ductal glands start near duodenum where cells express pluripotency markers (SOX2, OCT4 and NANOG), self-replication, proliferation and early hepato-pancreas commitment (Lgr5, PDX1, SOX17, SOX9)^[44]. A biological framework for life-long pancreatic organogenesis is constituted by the biliary tree-derived stem cells and their connections to committed progenitors of the pancreas^[40]. In the normal pancreas, LGR5 is co-localized with insulin and Nanog in the beta cells clusters and is exclusively expressed in the islets of the Langerhans^[45].

Since, Lgr5 is a stem cells marker in various organs and acts as Wnt-agonistic R-spondins receptor, the Lgr5 positive stem cells from intestine, liver and stomach form organoids in three dimensional cultures that resemble the tissues of origin^[42,46,47]. In a normal adult pancreas, Lgr5 is not expressed when Wnt signaling is inactive under normal conditions^[48]. However, upon the injury, Wnt pathway is activated by partial duct ligation, and Lgr5 is concomitantly expressed in the regenerating ducts^[49]. It was observed that pancreatic duct or fragments in the RSPO-1 cultures initiate Lgr5 expression and develop into organoids that can clonally expand containing Lgr5 stem/progenitor cells^[49]. The histoanatomical distribution, prevalence, and tumor biological significance of LGR5 in gastrointestinal tract tumors (including pancreatic cancer) was tested on transcriptional and translational level in tissues of 127 patients (malignant and non-malignant). It was noted that the non-neoplastic tissue usually had very few scattered LGR5 positive cells. The corresponding malignant pancreatic cancers showed significantly more LGR5 positive cells at protein and mRNA levels compared with the non-neoplastic tissue^[50]. Lgr5 co-

expressed with other CSC markers Mushashi-1, CD44 and ADAM17 and the patients with high Lgr5 positive cells had shorter median survival^[50]. Future studies are required to understand the role and clarify the prognostic value of Lgr5 in the pancreatic cancer.

SIGNALING PATHWAYS ALTERED BY CSCS

Pancreatic cancer cells and pancreatic CSCs share many characteristics of the carcinogenesis process including self-renewal, proliferation and immortality and several signaling pathways. Researchers have identified several CSC specific markers. In a recent study, investigators detected high expression of CD24, CD44, Dcl1, CXCR4, ESA, and Nestin-positive cells in the low-grade PanIN, high-grade PanIN, and PDAC (pancreatic ductal adenocarcinoma) tumors with fewer in normal ducts^[9,12,13,51,52]. These studies clearly demonstrate that pancreatic CSCs correlate with the step-wise progression of pancreatic intraepithelial neoplasias (PanINs) (PanIN1-2-3) to PC (Figure 1). During this process of early tumorigenesis after Kras mutations, several of the transcription factors like Oct4 and Nanog gets overexpressed linearly with the PanIN progression to PDAC^[53]. Oncogenic Kras mutations in the pancreas activate important signaling pathways such as sonic hedgehog, Wnt, PI3K/AKT, Notch and PTEN, Bmi1 that lead the transformation of the normal pancreas to the malignant PanINs and PDAC^[54-59].

Researchers have observed aberrant expression of Hedgehog during the PC initiation followed by progression of the pancreatic precursor PanIN lesions to pancreatic adenocarcinoma^[60]. This clearly underscores the importance of hedgehog signaling in a sequential development of PC. Recently, mounting evidence supports hedgehog signaling activation in pancreatic CSCs. Hedgehog pathway plays a significant role by regulating pluripotency maintaining factors like Oct4, Nanog, c-Myc, and Sox2 to the maintenance of stemness^[61-66]. Apart from hedgehog, increasing evidence suggests that Notch signaling activation was associated with molecular characteristics of CSCs in PC cases^[67,68]. This was shown by the studies that overexpression of Notch1 leads to increasing in the formation of pancreatospheres along with expression of the CD44 and EpCAM CSC surface markers. Other studies reported that expression of Oct4, Nanog, and PDX1 in Notch2 positive human pancreatic adenocarcinoma cells^[69]. The Oct4, Nanog and PDX1 are also considered as markers of self-renewal of pancreatic CSCs. Also, the expression of ALDH1 was associated with poor overall survival durations in PDAC patients^[70].

Newer studies illustrated involvement of PI3K/AKT signaling in pancreatic CSCs proliferation. In this direction, a recent study showed that CSCs like CD133 showed high levels of mTOR signaling in pancreatic adenocarcinoma. This underlines that the PI3K/AKT

pathway plays a prominent role in pancreatic CSCs^[71] consistent with significant inhibition of CD133 cells by the mTOR inhibitor rapamycin^[71]. However, the functions of Wnt signaling in pancreatic CSCs are less and poorly understood. Lgr5 is the Wnt target gene that is being recently explored as pancreatic CSC. Like other important signaling pathways, the Wnt/ β -catenin signaling pathway play a critical role in the pancreatic cancer development and progression.

ROLE OF PANCREATIC CSCS IN PREVENTION AND TREATMENT

Advances in the identification and characterization of pancreatic CSCs have created new opportunities for genetic targeting in treatment applications. Pancreatic CSCs are essential drivers for cancer progression and metastasis and are responsible for therapeutic resistance. Targetable cellular markers including CD24, CD44, CD133, ESA, ALDH1, c-Met, EPCAM, nestin, Lgr5 and Dcl1 have been employed to characterize pancreatic CSCs^[9-14,64]. Studies suggest that the use of the drugs that target PI3/AKT/mTOR, Hedgehog, c-Met and other developmental signaling pathways might deplete the populations of CSCs. Conceivably, multidrug combinations and multitargeting approaches will produce maximal anti-tumor efficacy by depleting resistant CSCs in PC^[72]. So far, no clinical studies have been aimed at targeting pancreatic CSCs specifically in PC patients. The pancreatic cancer precursors, PanINs, progress slowly over many years to the development of invasive PC^[3-5]. Hence, developing novel strategies to delay or inhibit progression of pancreatic cancer targeting CSCs are warranted. The K-rasG12D genetically engineered mouse (GEM) is an excellent model that shows the development of lesions closely resembling human PanIN's with progression to PDAC and it has been used successfully for chemoprevention studies^[5,8,72-78]. We have shown that licoferone, a dual COX-5-LOX inhibitor, exhibits chemopreventive efficacy against pancreatic cancer in p48^{Cre/+}-LSL-Kras^{G12D/+} mice in part by inhibiting CSCs^[72]. We have also shown that metformin, an anti-diabetic drug significantly prevents progression of pancreatic cancer by targeting in part CSCs^[8].

To date, there are no specific CSC inhibitors tested preclinically using GEMs or clinically in PC patients. Pharmacological or genetic inhibition of JNK leads to loss of stem cell properties in the pancreatic CSCs both *in vitro* and tumor. Furthermore, loss of Kras in kras mutant pancreatic CSCs led to the loss of stem cell characteristics by downregulation of the JNK pathway^[79]. Pancreatic cancers contain 1%-3% of CD133 positive cancer cells, some of which show high expression of CXCR4, a pro-invasive marker^[14]. The selective inhibition of CXCR4 signaling in CXCR4⁺ CSC cells by AMD3100 blocks tumor tissue invasion^[13,14], suggesting a potential role of CXCR4 in pancreatic tumor metastasis^[14]. Accordingly, it remains

possible that there is more than one type of CSC sub-population in pancreatic cancer tissues, which would be consistent with the known heterogeneity of most-human tumors^[14,24]. More preclinical animal and human clinical studies are warranted to evaluate the drugs for their specificity in targeting CSCs to inhibit the progression of PC. Combination of epigallocatechin-3 gallate (EGCG) and quercetin, and, Sulforaphane inhibited the self-renewal capacity of pancreatic CSCs *via* attenuation of the Hedgehog pathway^[62-65]. The GANT-61 is a Gli transcription factor inhibitor that inhibits pancreatic CSC viability and induces apoptosis^[61]. Together, blockade of hedgehog and mTOR signaling along with standard chemotherapy was found to eliminate pancreatic CSCs^[80]. Another hedgehog antagonist Vismodegib (GDC-0449), inhibits pancreatic CSC characteristics *in vitro*^[81]. Using GDC-0449, a human phase I clinical trial is ongoing^[82], which preliminarily suggest that GDC-0449 has good safety profile and antitumor activity for some of the locally advanced or metastatic solid tumors. Pancreatic cancer progression was inhibited by sulforaphane, green tea catechins and quercetin by inducing let7a miRNA and inhibiting Kras and ALDH1 activity^[82]. The 3-Bromopyruvate, a glycolysis inhibitor blocked CSC signaling in PC cell lines and the spheroids derived from patients^[83]. Also, chloroquine (antimalarial agent) decreased CSCs *in vitro* and *in vivo* in combination with gemcitabine by inhibiting the hedgehog signaling^[84].

An SRC inhibitor, dasatinib in combination with gemcitabine significantly reduced ALDH1 in MiaPaCa parental and gemcitabine resistant cells^[85]. A drug under phase 1 trial, Minnelide efficiently decreased CD133 cells in the tumors^[86]. Triptolide (plant derived Chinese medicine) is generally used for arthritis and cancer. In clinical trials it inhibited hypoxia-induced transcriptional signaling and EMT and CSC features in cell lines, decreased tumor growth *in-vivo*, and inhibited CSCs from patient tumors^[87].

Vaccine or antibody approaches are recently explored for pancreatic cancer inhibition. In the xenograft models and *in-vitro* cultures, anti-CD44 monoclonal antibodies decreased growth, metastasis and CSCs including downregulating CSC genes *Sox-2*, *Nanog* and *Rex1*^[88]. BMS-777067 (tyrosine kinase inhibitor) in combination with AZD8055 (mTOR inhibitor) successfully inhibited chemoresistant cancer cells and CSCs. However, BMS-777067 alone induces the polyploidy in the CSCs making them insensitive to the therapy^[89]. Cabozantinib downregulated c-Met, SOX2 and CD133 in the drug resistant PC cells and CSC spheroids and induced apoptosis^[90]. A kinase inhibitor XMD8-92 was shown to down-regulate Dclk1 and its downstream targets including KRAS, ZEB1, ZEB2, c-MYC, NOTCH1, OCT4, SOX2, SNAIL, SLUG, NANOG, KLF4, LIN28, VEGFR1, and VEGFR2 in the tumors of the xenografts^[90,91].

IMPLICATIONS AND FUTURE DIRECTIONS

It is now well-established that pancreatic CSCs contribute

to aggressive tumor phenotype resistant to radiation and chemotherapy^[8,14]. The molecular and pathological characteristics of the pancreatic CSCs are yet to be fully elucidated. Knowledge gained from the GEM models, and, isolation and identification of specific cancer stem cell markers and their characterization in tumor tissues will provide deep insights that will be of value in designing effective strategies for the development of chemoprevention and chemotherapy drugs that reduce tumor aggressiveness by specifically targeting CSCs^[8,14]. Although some pathways such as Notch and c-Met signaling are important for pancreatic CSC maintenance, other pathways like Wnt (Lgr5) and pathways related to Dclk1 that are required for pancreatic CSC activity must also be elucidated. Drugs targeting CSCs and their markers might modulate function of these CSCs^[8,14]. The validity of this approach was suggested by the finding that metformin, an anti-diabetic drug, displays anti-tumor effects due to the partially targeted elimination of CSCs in pancreatic cancer^[8,14]. Additional clinical and preclinical work is required to demonstrate conclusively the therapeutic benefit of metformin^[8,14,88], and CSC-targeting drugs in general, for the management of particular cancers^[8,14]. As such, CSCs are one of the best targets for cancer treatment. However, we need to improve our understanding of pancreatic CSCs and pancreatic cancer biology to develop optimal treatment (Chemoprevention and Chemotherapy) regimens. Effective treatment of pancreatic cancer could require the continued administration of conventional chemotherapeutic agents in combinations or along with agents that specifically deplete pancreatic CSCs. Pancreatic cancer could be effectively treated if the above conditions are met.

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Mesenchymal stem cells in the treatment of inflammatory and autoimmune diseases in experimental animal models

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mesenchymal stem cells (MSCs)] are currently being studied as a cell-based treatment for inflammatory disorders. Experimental animal models of human immune-mediated diseases have been instrumental in establishing their immunosuppressive properties. In this review, we summarize recent studies examining the effectiveness of MSCs as immunotherapy in several widely-studied animal models, including type 1 diabetes, experimental autoimmune arthritis, experimental autoimmune encephalomyelitis, inflammatory bowel disease, graft-*vs*-host disease, and systemic lupus erythematosus. In addition, we discuss mechanisms identified by which MSCs mediate immune suppression in specific disease models, and potential sources of functional variability of MSCs between studies.

Key words: Mesenchymal stromal cells; Mesenchymal stem cells; Autoimmunity; Animal models; Inflammation; Immunotherapy

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Core tip: In this review, we summarize recent studies examining the effectiveness of mesenchymal stromal cells (MSCs) as immunotherapy in several widely-studied animal models, including type 1 diabetes, experimental autoimmune arthritis, experimental autoimmune encephalomyelitis, inflammatory bowel disease, graft-*vs*-host disease, and systemic lupus erythematosus. In addition, we discuss mechanisms identified by which MSCs mediate immune suppression *in vivo* and potential sources of functional variability of MSCs between studies.

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Abstract

Multipotent mesenchymal stromal cells [also known as

INTRODUCTION

Multipotent mesenchymal stromal cells [also known as mesenchymal stem cells (MSCs)] are currently being studied as a cell-based treatment for a variety of human diseases. MSCs initially received attention for their somewhat restricted multipotency, as MSCs can differentiate *in vitro* into cells of the mesoderm lineage such as adipocytes, chondrocytes, and osteocytes, but not cells of other lineages such as hematopoietic cells^[1]. In addition to multipotency, MSCs have demonstrated capacity for immunosuppression, suggesting that MSCs may be useful for the treatment of immune-mediated disorders^[2]. Importantly, several attributes of MSCs contribute to their practicality as a source for cell-based therapies. First, MSCs have a wide anatomical distribution and can be isolated from several human tissues such as bone marrow, adipose tissue, and dental pulp^[3-6]. Second, although MSCs are relatively rare within these tissues, their great capacity for self-renewal allows for the efficient expansion of these cells *in vitro* using simple cell culture methods^[7]. Thirdly, MSCs are non-immunogenic due to their low expression of antigen-presenting molecules^[8]. This allows MSCs to avoid generating an allogeneic response when transferred into an un-matched recipient, and therefore donor and recipients do not need to be well-matched for histocompatibility. Due in part to the efficiency with which MSCs can be prepared for clinical use, they have received much attention from the medical research community and their utility for use in regenerative medicine and immunotherapy has been tested in more than 100 clinical trials worldwide^[9].

Despite this intense interest, important questions regarding MSCs remain unanswered. Notably, clinical trials testing the effectiveness of MSC-based immunotherapy in treating inflammatory disorders and autoimmune diseases have produced mixed results^[10,11]. In some trials administration of MSCs helped to alleviate symptoms, while in others no effects relative to placebo were detected. These discrepant results may be due to variability in the immunosuppressive capacity among MSCs obtained from different tissue sources or expanded under different conditions *in vitro* prior to their injection into subjects^[12]. Understanding the source of this functional variability and finding ways to predict or alter the activity of a given lot of MSCs is an essential step toward achieving consistent clinical results from MSC-based therapies. Additionally, while multiple mechanisms have been identified as the means by which MSCs can mediate immune suppression, those most important for suppression *in vivo* are not well-characterized. Indeed the mechanisms most important for clinical efficacy may differ between diseases, and identifying those which are most important for a given disease will allow for more effective MSC-based therapies, and potentially give important insights into the disease itself.

Pre-clinical animal models of human diseases

have already been instrumental in the study of MSCs, and are likely to be useful tools for answering these remaining questions. Mouse studies were first used to demonstrate that MSCs possessed immunosuppressive function *in vivo*, and subsequently have helped to identify some mechanisms used by MSCs to mediate these effects. Importantly, MSC-mediated immune suppression is cross reactive between species in many animal models, allowing the effectiveness of human MSCs in suppressing inflammatory disorders to be evaluated experimentally in animal models. In this review article, we summarize what is currently known about the therapeutic effectiveness of MSCs *in vivo* in several widely-studied animal models of immune-mediated diseases. Additionally, we discuss mechanisms identified by which MSCs mediate immune suppression in specific disease models, and highlight important areas of research in the general field of MSC-related immunotherapy research. To avoid confusion, this review focuses exclusively on results obtained from animal studies, with an emphasis on MSC-mediated effects that have been demonstrated *in vivo*. A thorough and informative review of clinical studies using MSC-based cellular therapies has recently been published elsewhere^[12].

ANIMAL MODELS AND TREATMENT WITH MSC

Type 1 diabetes

Type 1 diabetes (T1D) is the result of an immune-mediated response against insulin-producing pancreatic β -cells^[13]. The autoimmune attack is largely mediated by T cells and in most patients will result in the complete loss of β -cell function. Individuals with T1D are therefore unable to produce insulin in response to increasing blood glucose levels and dependent upon exogenous insulin treatment for the remainder of their lives. Even with insulin replacement therapy, individuals with T1D are at risk of long-term complication arising from improperly-regulated blood glucose levels, such as retinopathy, kidney damage, and heart disease. Several animal models of T1D exist that are used to study both the immunological pathogenesis of the disease as well as the long-term consequences of β -cell loss^[14]. The non-obese diabetic (NOD) mouse strain is used as an animal model for spontaneous autoimmune diabetes, and disease in these animals appears to share many features with T1D in humans. Alternatively, autoimmune diabetes can be induced in several strains of mice and rats by treatment with a compound toxic to β -cells. Iterative treatment with low doses of streptozotocin (STZ) damages β -cells, and this damage attracts immune cells to the islet leading to insulinitis and eventually immune-mediated β -cell destruction. In all models of T1D disease progression can be monitored by measuring glucose levels in the blood or urine, with increases in glucose levels being associated with

progressive loss of β -cell function. Disease severity between groups can also be compared by histological examination of the pancreas of experimental animals for immune cell infiltration.

MSC-based treatment has been largely successful in treating the symptoms of autoimmune diabetes in animal models. In NOD mice, treatment with MSCs has been reported to protect mice if administered before disease onset^[15,16], and to reverse disease when administered after the onset of hyperglycemia^[17]. Interestingly, MSCs derived from NOD mice were unable to suppress disease in recipients, but those derived from BALB/c mice or the non-obese diabetes resistant strain were able to do so, suggesting that MSCs in the NOD mouse may have a defect in their ability to suppress immune responses^[16]. In these studies, MSC treatment was associated with a reduction in the frequency of inflammatory CD4⁺ T cells^[16,17] and an increase in the frequency of regulatory T cells^[15-17]. Similar results have been reported using an STZ-induced model in C57BL/6 mice, with syngeneic bone marrow-derived MSCs again reverting hyperglycemic animals to normal blood glucose levels^[18]. In a rat model of STZ-induced T1D, autologous bone marrow-derived MSCs treatment led to increased insulin secretion and sustained normoglycemia, with a shift in T cell cytokine production toward that of T_H2 cells^[19]. There is also evidence that MSC treatment can aid in tissue repair after the onset of disease, as human-derived MSCs homed to the pancreatic islets and led to an increase in islet frequency and insulin production in NOD/scid mice treated with STZ^[20]. Mouse-derived MSCs displayed similar behavior, homing to both the pancreas and kidney when injected into STZ-treated mice, and were associated with an increase in renal function relative to diabetic mice not receiving MSCs^[18]. The mechanisms used by MSCs to mediate these anti-inflammatory and tissue repair effects *in vivo* have yet to be determined.

The transplantation of allogeneic islets has been suggested as a treatment for patients with T1D, but the long term engraftment of these transplanted tissues has been difficult to achieve^[21]. Here the immunosuppressive capacity of MSCs may prove useful for preventing the rejection of transplanted islets as well as mediating islet repair immediately after transplantation. In STZ-induced diabetes models in rats^[22] and mice^[23], the long-term survival of islet allografts was significantly enhanced by co-transfer with MSCs. Notably, in a model of non-human primates (cynomolgus monkey), co-transplant of allogeneic bone marrow-derived MSCs intraportally with islets significantly enhanced islet engraftment and function, which was associated with increased number of regulatory T cells^[24]. In rats, MSC co-transfer led to a reduction in T_H1-associated cytokines and an increase in IL-10-producing regulatory T cells^[22]. In contrast, mouse MSCs reportedly mediated their immunosuppressive effects by the production of metalloproteinases that cleave the α chain of the IL-2 receptor (CD25)

from the surface of activated T cells, leaving these T cells hyporesponsive to IL-2^[23]. Collectively, MSC-based therapy has performed well in treating mouse models of T1D, both in suppressing the autoimmune attack of endogenous β -cells and in improving the maintenance of allogeneic islet allografts.

Experimental autoimmune arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic joint inflammation. It is thought that the disease is initiated when autoreactive T cells infiltrate the synovial tissue and secrete cytokines and chemokines, recruiting other immune cells to the joint^[25]. The resulting inflammatory milieu progressively damages the joint and surrounding tissues, a process that can be extremely painful and debilitating. The most commonly used animal model for the study of RA is the collagen-induced arthritis (CIA) model^[26]. In this model, a susceptible strain of mouse is injected subcutaneously at the base of the tail with an emulsion of complete Freund's adjuvant and type II collagen. This injection produces a potent T cell-mediated immune response to type II collagen that eventually leads to inflammation in the joints of the paws. In some cases a second booster immunization is given several weeks after the initial injection to increase disease incidence. The severity of disease can be quantified by scoring each paw on a scale from 0 (no inflammation) to 3 (severe inflammation), or by directly measuring any increase in paw width using a caliper. CIA shares many similarities with RA such as the presence of autoantibodies, the involvement of T_H1 and T_H17 cells in disease progression, and a strong link between disease susceptibility and the genomic region encoding for class II MHC molecules^[26].

Several groups have studied the efficacy of MSC immunotherapy in mouse models for RA and have generally found that MSCs can reduce both the incidence and severity of disease. In the CIA mouse model, intraperitoneal injection of allogeneic bone marrow-derived MSCs at the time of initial immunization significantly reduced incidence of disease, and administration of MSCs after disease onset had a therapeutic effect as well^[27]. A study using adipose-derived MSCs reported similar results, as mice receiving multiple doses of MSCs were less likely to show signs of joint inflammation and overall had a milder form of arthritis than those animals not receiving MSCs^[28]. Notably, this study also found that both human and murine MSCs could suppress autoimmunity in the CIA model. These results, along with another study showing that MSCs derived from rat bone marrow are effective as well^[29], suggests that at least some immunosuppressive mechanisms employed by MSCs are cross-reactive between species. Human MSCs derived from umbilical blood suppress CIA as well^[30]. Additionally, murine MSCs injected directly into inflamed joints reduced inflammation in an antigen-induced arthritis model^[31]. These results are in contrast with an earlier report which instead

found that administering MSCs after disease onset exacerbated disease rather than ameliorating it^[32]. It should be noted, however, that these experiments used an immortalized MSC-derived cell line rather than conventional MSCs. Additionally, this study and another using conventional murine MSCs found that despite their failure to suppress CIA *in vivo*, MSCs did suppress immune responses *in vitro*^[32,33].

The mechanisms through which MSCs inhibit experimental arthritis are still in question. Several groups have reported a decrease in levels of pro-inflammatory cytokine such as TNF α ^[27,29,30] or IL-6^[30], and increases in the anti-inflammatory cytokine IL-10^[29,30]. MSCs may also modulate the nature of the T cell response in CIA by reducing the frequency of T_H1 and T_H17 cells^[28,30], and/or increasing the frequency of T_H2 cells^[30,34]. MSCs appear to promote regulatory T cells as well, as several studies have observed an increase in regulatory T cell frequency after administration of MSCs^[27,28,30]. While T cells from MSC-treated mice are generally hyporesponsive, MSC treatment does not appear to effect T cell priming, suggesting that the immunosuppressive effects of MSCs occur after the autoimmune response is initiated^[30]. MSCs from mice deficient in IL-6 or iNOS production have partially impaired immunosuppressive function^[34]. IL-6-deficient MSCs released significantly lower levels of prostaglandin E2, suggesting that autocrine IL-6 may induce the production of anti-inflammatory lipid mediators by MSCs^[34]. Although MSC treatment clearly modulates the T cell response in CIA, it is currently not known if this effect is directly attributable to MSCs or rather an indirect effect of MSCs acting on other cells of the immune system.

Graft-versus-host disease

Allogeneic hematopoietic stem cell transplantation is used for the treatment of blood-derived malignancies and immune deficiencies^[35]. Graft-versus-host disease (GVHD), a major complication that occurs after transplantation, is the result of donor-derived immune cells mounting an alloreactive response against the recipient's tissues or organs. GVHD occurs in up to 50% of transplant recipients, and therefore is a significant source of the mortality associated with these therapies^[36]. Animal models are widely used to study the GVHD response by depleting endogenous hematopoietic cells in recipient animals by radiation or chemotherapy and reconstituting the immune system in these animals with allogeneic bone marrow cells^[37]. The progression of GVHD in animal models is frequently assessed by weight loss, survival, and histological examination of organs and tissues commonly affected by GVHD. It should be noted that experimental GVHD models can vary greatly, both in the details of the protocol used and in the degree of MHC mismatch between donor and recipient strains^[37].

The effects of MSC-based therapies in these animal

models have been widely-studied, with most reports observing that MSC infusion was effective in treating or delaying experimental GVHD. An early study reported that transfer of bone marrow cells from MRL/lpr mice into irradiated recipients resulted in a GVHD-like wasting disease, but recipient animals survived much longer when the bone marrow transfer was accompanied by a bone graft (presumably containing MSCs)^[38]. In a MHC-mismatched model (bone marrow from C3H/He into irradiated BALB/c mice), a single dose of bone marrow-derived MSCs at the time of bone marrow infusion allowed recipient mice to survive much longer than those receiving only bone marrow cells^[39]. Experiments using these same strains found that an immortalized MSC cell line could suppress GVHD in this model as well^[40]. MSCs derived from adipose tissue were also able to potentially extend the life of animals in a haploidentical GVHD model^[41]. It should be noted that these authors found that MSC treatment early in the course of disease was effective, but when MSCs were administered later little benefit was observed, suggesting that the timing of MSC treatments are critical in GVHD models^[41]. T cell-derived IFN γ may be an important factor as well, as MSCs treated with IFN γ prior to infusion are reportedly superior to untreated MSCs in increasing survival after bone marrow transplant^[42]. These authors also found that MSCs were unable to enhance survival if recipients received a transplant containing IFN γ -deficient T cells, suggesting that activation-induced production of inflammatory cytokines may be required for maximal immunosuppression by MSCs *in vivo*^[42].

A xenogeneic model has also been used to investigate the effects of human-derived MSCs on the progression of GVHD^[43-45]. In this model, human peripheral blood mononuclear cells (PBMCs) are infused into irradiated NSG mice^[46], where they proliferate and initiate a GVHD-like response against mouse tissues in the recipient. Three weekly doses of umbilical cord-derived human MSC starting at the time of PBMC infusion completely prevented GVHD and strongly reduced T cell proliferation *in vivo*^[43]. Timing was again critical, as treatment started several weeks after PBMC infusion failed to prevent GVHD^[43]. In other studies using a similar xenogeneic model, bone marrow-derived human MSCs^[44] or cord blood-derived MSCs^[45] were able to suppress GVHD as well. Treating MSCs with IFN γ prior to infusion enhanced the immunosuppressive capacity of MSCs, as IFN γ -treated MSC suppressed GVHD even when far fewer cells were administered^[44].

Other reports failed to observe any positive effect of MSC treatment in GVHD^[47-51]. Although these studies failed to observe *in vivo* effects, most reported that MSC were immunosuppressive *in vitro*^[47,48,50,51], and one found that animals receiving MSCs had reduced GVHD-mediated tissue pathology despite a lack of survival enhancement^[49]. These seemingly conflicting outcomes are potentially the result of variation in experimental

design as well as in the immunosuppressive capabilities of MSCs. As mentioned above, several studies found that the timing of MSC infusion was crucial for GVHD suppression^[41,43]. MSCs in general appear to intrinsically vary in their immunosuppressive capabilities depending upon the source animal, tissue of origin, isolation methods, passage number, and route of infusion. Variation can exist between MSC lots even when these variables are held constant, as demonstrated by a report showing that MSC lines isolated from the same mouse strain in the same laboratory can have drastically different immunosuppressive capabilities *in vivo*^[52]. Interestingly, these authors found no difference in the *in vitro* immunosuppressive capabilities of these independent MSC lines despite the difference in their effectiveness *in vivo*, suggesting that *in vitro* capabilities do not necessarily reflect *in vivo* function^[52]. The specific GVHD model chosen can also have an impact on experimental results, as murine MSCs are reportedly effective in preventing GVHD in a sibling transfer murine GVHD model, but can only delay disease when donors and recipients are MHC-mismatched^[53]. While most studies find that MSCs are capable of suppressing GVHD in animal models, it is essential to better understand the source of these discrepant results and find ways to predict if a given lot of MSCs will have immunosuppressive function *in vivo*. Elucidating the mechanisms MSCs use to mediate immune suppression in GVHD models should also be the focus of future work. Several groups have reported that T cells are inhibited in animals receiving MSCs, but the molecular mediators responsible for this phenomenon are largely unknown.

Experimental autoimmune encephalomyelitis

Multiple sclerosis (MS) is a disorder of the central nervous system (CNS) characterized by the progressive demyelination of nerves in the spinal cord and brain^[54]. The resulting loss of neurological function can lead to a wide variety of symptoms including pain, loss of sensation, muscle spasms, loss of coordination, vision problems, ataxia, difficulty of thinking, and emotional distress. The cause of demyelination in MS is thought to be an inflammation of the CNS caused by infiltrating immune cells^[54]. Autoimmunity in the CD4⁺ T cell compartment appears to play a central role in the pathogenesis of MS based upon evidence from animal studies and genetic association studies in humans identifying haplotypes of the MHC region associated with both susceptibility and protection from disease^[55]. The most commonly-studied animal model for MS is experimental autoimmune encephalomyelitis (EAE), an induced model for CNS inflammation^[56,57]. In these models, mice are immunized to induce an immune response to CNS associated antigens such as myelin oligodendrocyte glycoprotein (MOG) or myelin proteolipid protein (PLP). Alternatively, EAE can be introduced by the adoptive transfer of activated

myelin-specific CD4⁺ T cells. EAE can follow distinct clinical courses depending upon the mouse strain used and the specific antigen against which the immune response is raised, each reflecting different aspects of MS in humans. Immunizing C57BL/6 mice against MOG results in a progressive disease course, whereas SJL mice immunized against PLP display a relapsing/remitting form of the disease^[56]. The first sign of disease is usually a limp tail followed by weakness or paralysis in the hind limbs as demyelination progresses. Disease severity is often represented by a paralysis scoring system where individual animals are scored based upon the level of paralysis observed. Histological examination of the spinal cord for immune cell infiltration can also be used to give a quantification of disease severity.

The impact of MSC-based therapy in EAE has been extensively studied, with most of studies reporting a potent immunosuppressive effect. Mice receiving syngeneic bone marrow-derived MSCs showed a significantly milder disease course than that of untreated animals in a progressive EAE model^[58]. This effect was evident when MSCs were administered either before disease onset or at the peak of disease severity, but not when administered later in the course of disease. This result suggests that the improvement in neurological function mediated by MSCs is due to their immunosuppressive functions rather than their multipotency. MSCs were effective in suppressing EAE in a relapsing/remitting model as well^[59]. Allogeneic MSCs suppressed EAE with an efficiency similar to that of syngeneic MSCs^[60]. Human MSCs are effective in EAE models as well, as MSCs from both bone marrow^[61,62] and adipose tissue^[63] have been reported to suppress either progressive or relapsing/remitting forms of disease.

As in other T cell-mediated autoimmune mouse models, questions still remain as to the mechanisms of immune suppression mediated by MSCs in EAE. A reduction in the frequency of T cells secreting inflammatory cytokines accompanies the reduction in disease activity^[59,64], and T cells in mice receiving MSCs appear to be hyporesponsive to antigenic stimulation or anergic^[58,59]. Murine MSCs appear to mediate immune suppression at least in part by a novel pathway inhibiting the CCL2 chemokine axis^[64]. MSCs secrete several matrix metalloproteinases that can cleave MSC-derived CCL2, resulting in an isoform that inhibits rather than activates CCR2-expressing immune cells^[65]. This mechanism plays a key role in MSC-mediated inhibition of EAE, as MSCs from CCL2^{-/-} mice are unable to suppress disease^[64]. While this mechanism is clearly important in the EAE model, its role in other T cell-mediated autoimmune animal models remains to be tested. CCL2 signaling is especially important for infiltration of the CNS by inflammatory T cells, but MSCs may rely on other immunosuppressive mechanisms in other disease models where the CCL2/CCR2 signaling

axis is less involved in disease pathogenesis^[66,67]. Finally, a defect in MSC function may be important in the pathogenesis of EAE, as MSCs derived from mice with ongoing disease are unable to suppress disease upon transfer to autologous recipients^[68].

Inflammatory bowel disease

Inflammatory bowel disease (IBD) in humans refers to a family of disorders characterized by destructive inflammation of the colon or small intestine^[69]. Damage caused by chronic inflammation leads to a host of gastrointestinal symptoms such as severe cramps and abdominal pain, diarrhea, and weight loss. In severe cases affected parts of the intestines are removed surgically, sometimes requiring a permanent colostomy. While the causes of IBD are not well understood, CD4⁺ T cells are thought to play a key role in the disease^[70]. The most commonly studied animal models of IBD involve administering an agent that causes chemical damage to the intestine such as dextran sulfate sodium (DSS) added to drinking water or intrarectally-administered trinitrobenzene sulfonic acid (TNBS)^[71]. The intestinal injury allows immune cells of the lamina propria to come in contact with luminal contents such as enteric bacteria, triggering an inflammatory cascade that bears some resemblance to human IBD. Severity of disease can vary depending upon the strain of mouse used as well as the route and frequency of administration of the chemical used. The disease course can be monitored by progressive weight loss or the appearance of diarrhea and/or bloody stools, or assessed post mortem by measuring colon length and histological examination looking for damage and immune cell infiltration^[71].

Therapies using MSCs have been largely successful in treating disease in animal models of IBD. In a DSS-induced acute colitis model, adipose-derived MSCs suppressed most measurable disease outcomes and improved survival^[72]. This effect was observed when MSCs derived from either human or mouse tissues were used, and MSCs from allogeneic donors were as effective in treating disease as those from syngeneic donors^[72]. Similar results were obtained when colitis was induced by intrarectal administration of TNBS^[73]. Human MSCs derived from bone marrow, gingiva or cord blood were also effective in treating DSS-induced colitis in mice^[74,75]. MSCs from rats are capable of suppressing experimental colitis in both rats^[76] and mice^[77].

MSCs appear to suppress disease in IBD models through multiple mechanisms. Several studies have reported that MSC infusion increased the frequency of regulatory T cells accompanied by a reduction in T cells secreting inflammatory cytokines^[72,73,78]. In the DSS-induced colitis model, MSCs from mice deficient in Fas ligand (FasL) were unable to suppress disease^[77,78]. FasL and its receptor Fas are both essential molecules for maintaining T cell homeostasis, and signaling

downstream of Fas leads to the rapid induction of apoptosis in susceptible cells, including activated effector T cells^[79]. During the course of experimental colitis MSCs can induce FasL-mediated apoptosis in T cells, and this increase in the frequency of apoptotic cells indirectly leads to an increase in regulatory T cells, as macrophages engulfing the apoptotic T cells increase their production of TGFβ^[78]. Interestingly, MSCs from Fas-deficient animals were also unable to suppress colitis despite being able to induce T cell apoptosis *in vitro*. The loss of Fas in MSCs disrupted their ability to produce CCL2, suggesting that non-apoptotic Fas signaling is required for CCL2 secretion in MSCs^[78]. Therefore Fas-deficient MSCs are incapable of attracting T cells into close proximity for FasL-mediated killing^[78]. In this study, CCL2 secreted by MSCs acted as a chemoattractant, while in the EAE model MSCs were reported to secrete an isoform of CCL2 that antagonized CCR2 signaling and inhibited CCL2-mediated chemotaxis^[64]. How MSCs can use two forms of the same chemokine with opposing activities to mediate immunosuppression is currently unknown, as are the conditions that cause MSCs to produce one form of CCL2 over the other.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a complex autoimmune disease that causes progressive and profound damage to a variety of organs and tissues^[80]. A hallmark of SLE is the presence of antibodies against nuclear antigens such as DNA or chromatin-associated proteins^[80]. Intranuclear antigens are rarely found in the extracellular space under normal conditions as dying cells are quickly cleared by phagocytic cells before intracellular contents are released. In patients with SLE, however, it is thought that an increase in the rate of cell death and/or defects in the mechanisms responsible for the clearance of dying cells results in the release of intranuclear contents. Once exposed, free chromatin is bound by autoantibodies resulting in large immune complexes. These immune complexes accumulate in certain places in the body and induce inflammation at these sites. Immune complexes frequently accumulate in the blood vessels surrounding glomeruli in the kidney in patients with SLE, leading to extensive tissue damage and loss of renal function^[80]. Other complications arising from SLE include increased risk for atherosclerosis, neurological problems, and inflammation of the heart or lungs.

The most commonly-studied animal models for SLE are mouse strains in which a lupus-like disease arises spontaneously. The model thought to best approximate the disease course seen in humans with SLE uses the first generation progeny of a breeding pair consisting of a New Zealand Black (NZB) mouse and a New Zealand White (NZW) mouse^[81]. The resulting hybrid mice (NZB/NZW F₁) develop anti-nuclear and anti-DNA antibodies along with a form of glomerulonephritis that resembles

that seen in patients with SLE. Another model uses mice with a mutation in the gene encoding Fas (lpr) on the MRL strain background. The immune cells of MRL/lpr mice cannot undergo Fas-mediated apoptosis, resulting in a pronounced lymphoproliferative disorder that leads to the development of anti-nuclear antibodies and subsequent glomerulonephritis^[81]. In both of these spontaneous models of SLE, progression of the disease can be tracked by assessing renal function and testing for the presence of anti-nuclear antibodies in the serum.

The effectiveness of MSC-based therapy in the treatment of animal models of SLE is still in question. Initial studies in the MRL/lpr model reported positive results. The infusion of allogeneic bone marrow-derived MSCs reduced serum levels of anti-DNA antibodies and improved renal function^[82]. Clinical improvement was accompanied by an increase in the frequency of regulatory T cells and a reduction of IL-17-producing CD4⁺ T cells^[82]. MSCs from human tissues suppressed disease in the MRL/lpr model as well, as infusion of human MSCs derived from either bone marrow^[83] or umbilical cord blood^[84] led to a reduction in anti-DNA antibodies, proteinuria, and renal pathology. Results obtained in the NZB/NZW F₁ model are more ambiguous, however. Human MSCs from umbilical cord blood had only a moderate effect on disease parameters and animal survival, despite markedly reducing serum levels of pro-inflammatory cytokines (IL-2, TNF α , and IL-12) and increasing anti-inflammatory cytokine concentrations^[85]. Similar results were obtained when MSCs derived from human embryonic stem cells were used, although only animal survival was considered in this study^[86]. In a study using MSCs from murine bone marrow, however, allogeneic MSCs unexpectedly exacerbated the disease, increasing titers of anti-DNA antibodies and deposition of immune complexes in the kidney^[87]. The MSCs used in this study were able to suppress T cell proliferation *in vitro*, suggesting that grossly dysfunctional MSCs are not to blame for this result^[87]. Considering these discrepant observations, it is premature to draw any conclusions regarding the effectiveness of MSCs in treating animal models of SLE.

Experimental autoimmune uveitis

Autoimmune uveitis is a group of heterogeneous and complex human eye diseases that are collectively a major source of vision impairment and blindness^[88]. Genetic and experimental studies suggest that autoimmune uveitis is mediated by a response against retina-associated antigens, with T_H1 and T_H17 cells playing an especially crucial role^[89,90]. Experimental autoimmune uveitis (EAU), the most commonly-studied animal model for human uveitis, can be induced in susceptible animals by immunization with retina antigens or transfer of retina-specific T_H1 or T_H17 cells^[91]. Most studies of MSC-based treatment in EAU have observed a potent immunosuppressive effect. In a mouse model

of EAU induced by immunization with a self uveitogenic peptide derived from the intraphotoreceptor binding protein, treatment with a single intraperitoneal injection of syngeneic bone marrow-derived MSCs was able to significantly attenuate EAU^[92]. Human-derived MSCs were effective in preventing EAU in this mouse model as well^[93]. EAU can be induced in rats using a similar protocol, and bone marrow-derived MSCs from both syngeneic and allogeneic donors were able to suppress EAU in recipient animals^[94,95].

While MSC treatment appears to be effective in EAU, little is currently known about how MSCs mediate disease suppression. Most studies report a decrease in production of pro-inflammatory cytokines accompanied by an increase in regulatory T cell frequency^[92-95]. T cells from MSC-treated rats showed reduced IFN γ and IL-2 production relative to those from untreated animals, along with a moderate increase in production of IL-10 and TGF β ^[95]. Additionally, MSC treatment reduced T_H17 cell frequency in the eye and the spleen while increasing the frequency of regulatory T cells in both locations^[95]. Whether MSCs modulate the T cell response in EAU directly or indirectly has not yet been conclusively demonstrated. Importantly, the mechanisms used to mediate immunosuppression may differ between species, as murine and rat MSCs induced regulatory T cells^[92,94,95], but human MSCs induced IL-10-producing B cells while having little effect on regulatory T cell frequency^[93].

UNANSWERED QUESTIONS: FUTURE PERSPECTIVES

MSCs are currently being studied for use in cellular immunotherapy, but the performance of MSC-based therapies in clinical trials has been inconsistent. In mouse studies MSCs have more frequently shown an immunosuppressive effect (Table 1), although several studies found that treatment with MSCs either had no effect on disease or worsened disease outcomes^[32,33,47-51,87]. These exceptions are informative and, considering the general bias against publishing negative outcomes, suggest that MSC performance in mouse models may be just as inconsistent as it has been in the treatment of human disease. Understanding the sources of this inconsistency is an essential step in improving MSC-based therapies, and using mouse models provides a practical means of doing so.

The variable outcomes seen in these studies could come from many different sources. First, the functional characteristics of MSCs may differ based upon the species or tissue or origin. Among mouse studies there currently exists no evidence that MSCs from a particular tissue are consistently more effective in suppressing immune responses, and MSCs from human, mouse, and rat tissues have all effectively treated disease in mouse models. Instead, intrinsic variation in MSC

Table 1 Immunomodulatory effect of mesenchymal stromal cells in animal disease models

Disease model	Anti-inflammatory MSC effects	Negative/neutral studies?
Type 1 diabetes	↑ Regulatory T cells (15-17, 19) ↓ Inflammatory T cells (16, 17) T _H 1 → T _H 2 (16, 19) ↑ Tissue repair (18-20)	None reported
Pancreatic islet transplantation	↑ Islet survival (22-24) ↑ Regulatory T cells (22, 24) ↓ T _H 1 cytokines (22) ↓ T cell responsiveness (23)	None reported
Experimental autoimmune arthritis	↓ Inflammatory cytokines (27, 29-31) ↑ Regulatory T cells (27, 28, 30, 34) ↓ T cell responsiveness (27) ↑ IL-10 (28-30) ↓ T _H 1/T _H 17 cells (28, 30) ↑ T _H 2 cells (30, 34)	No suppression (31-33)
Graft-vs-host disease	↓ Auto-antibodies (38) ↓ Inflammatory cytokines (39, 42, 44, 53) ↑ Regulatory T cells (40) ↓ T cell proliferation (44) ↓ T _H 1 cells (49)	Prevented disease, but no effect after disease onset (43, 47) No suppression (48, 50, 51) Reduced pathology but no effect on survival (49)
Experimental autoimmune encephalomyelitis	↓ T cell responsiveness (58) ↓ CNS infiltration (59-61, 64, 68) ↓ Auto-antibodies (59) ↓ Inflammatory cytokines (60, 64, 68) T _H 1 → T _H 2 (61) ↓ T _H 17 cells (64)	None reported
Inflammatory bowel disease	↓ Inflammatory T cells (72, 73, 78) ↓ Inflammatory cytokines (72-76) ↑ Anti-inflammatory cytokines (72-75, 78) ↓ Intestinal CD4 ⁺ T cell infiltration (72-74) ↑ Regulatory T cells (72-75, 78) ↓ Growth factor expression (76) ↑ FasL-mediated T cell apoptosis (78) ↓ T cell responsiveness (73)	None reported
Systemic lupus erythematosus	↓ Anti-DNA antibodies (82-85) ↑ Regulatory T cells (82) ↓ T cell frequency (83) ↓ T _H 17 cells (82) ↓ Plasma cells (82) ↓ Inflammatory cytokines (85) ↑ Anti-inflammatory cytokines (85)	Worsened disease, increased auto-antibodies (87)
Experimental autoimmune uveitis	↓ Inflammatory cytokines (93-95) ↑ IL-10 (93, 94) ↑ Regulatory T cells (92-95) ↓ Inflammatory T cells (95) ↓ T _H 1 cells (93) ↓ T _H 17 cells (93, 95) ↑ IL-10-producing B cells (93)	Not effective late in disease course, but effective before and shortly after disease onset (94)

MSC: Mesenchymal stromal cell.

lots may be the source of functional differences, as differences in immunosuppressive function can be seen even between MSC lots derived independently from the same mouse tissue in the same laboratory^[52]. This type of variation is likely to arise stochastically and therefore identifying biomarkers associated with *in vivo* MSC function may be useful in identifying particular lots of MSCs that are more likely to be effective *in vivo*. Mouse models provide a practical means of performing these experiments as human MSCs have proven effective in treating disease in many of these models.

While many immunosuppressive mechanisms have

been reported from *in vitro* studies of MSC function, far fewer have been identified using *in vivo* experiments^[96]. Several elegant studies reviewed here have shown that a particular mechanism is important for *in vivo* suppression in a particular disease model, but these mechanisms have yet to be confirmed in additional models^[23,34,65,77,78]. Indeed it may be the case that different immunosuppressive mechanisms are important in different disease models, and that MSC lots effective at suppressing one immune-mediated disorder are not necessarily effective in other models. Further work is required to establish if certain immunosuppressive

mechanisms are essential in multiple disease models, or if MSC immunosuppression is mediated by unique mechanisms depending upon the inflammatory context.

Collectively, the studies reviewed above demonstrate that MSC-based therapies are generally effective in treating animal models of several inflammatory conditions. Although most studies observed an immunosuppressive effect of MSC treatment, a few did not—mirroring the overall effectiveness seen in clinical trials of human MSC treatments. Understanding the sources of these inconsistent results may lead to means of improving the reliability of MSC-based therapies. Mouse models of immune-mediated disease provide a practical means of studying the variability of MSC immunosuppressive function and identifying general and model-specific mechanisms of immune suppression essential for effective MSC-based immunotherapy.

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Vibration stimuli and the differentiation of musculoskeletal progenitor cells: Review of results *in vitro* and *in vivo*

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Abstract

Due to the increasing burden on healthcare budgets of musculoskeletal system disease and injury, there is a growing need for safe, effective and simple therapies. Conditions such as osteoporosis severely impact on

quality of life and result in hundreds of hours of hospital time and resources. There is growing interest in the use of low magnitude, high frequency vibration (LMHFV) to improve bone structure and muscle performance in a variety of different patient groups. The technique has shown promise in a number of different diseases, but is poorly understood in terms of the mechanism of action. Scientific papers concerning both the *in vivo* and *in vitro* use of LMHFV are growing fast, but they cover a wide range of study types, outcomes measured and regimens tested. This paper aims to provide an overview of some effects of LMHFV found during *in vivo* studies. Furthermore we will review research concerning the effects of vibration on the cellular responses, in particular for cells within the musculoskeletal system. This includes both osteogenesis and adipogenesis, as well as the interaction between MSCs and other cell types within bone tissue.

Key words: Mesenchymal stem cells; Mechanobiology; Osteogenesis; Whole body vibration; Adipogenesis, osteoporosis; Low magnitude, high frequency vibration loading

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Core tip: Low magnitude, high frequency vibrations are attracting growing interest as a non-invasive therapy for a variety of different disorders. The number of studies aimed at elucidating the effects of vibration both *in vivo* and *in vitro* is increasing rapidly. This review aims to provide an introductory overview of the *in vivo* data for a broad range of human applications and animal models. *In vitro* work is covered in more detail, focusing in particular on studies concerning the effects of vibration on cells derived from the musculoskeletal system.

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INTRODUCTION

Vibration or low-magnitude-high-frequency vibration (LMHFV) loading can be induced clinically by standing a subject on a vibrating platform or *in vivo* by applying LMHFV to cells within culture plates or 3D constructs. It is emerging as a potential stimulus for repair and regeneration of the musculoskeletal system with some preliminary evidence that the mechanism of action is *via* effects on mesenchymal stem cells^[1,2]. Clinical trial results are variable, however, as are the results in animal models and cell cultures. Differing loading procedures and parameters make it very difficult to compare between experiments. In this review we aim to provide a series of snapshots of human, animal and cell based trials on the effects on LMHFV loading and related stimuli, with a focus on mesenchymal cells and effects on the musculoskeletal system. This is not intended to be a meta-analysis or a comprehensive review of all studies in this area, but rather to indicate how much further interdisciplinary research needs to be done before we can elucidate the effects of vibration on stem cells.

Within the musculoskeletal (MSK) system, there are a variety of different tissues which work together to allow movement. These include bone, muscle, tendon and ligament, as well as other associated tissues such as blood vessels and nerves. All of these tissues are important in locomotion and posture and are affected by a variety of diseases and by age-related degeneration. It has been well demonstrated and reviewed elsewhere^[3-5] that mechanical stimuli are involved in maintaining the structure of these tissues. Too little or too much mechanical stimulation can perturb the cells' mechanobiological signalling pathways, ultimately leading to disruption in tissue structure. In bone, such studies have shown changes in bone formation due to unloading^[6], increased osteoblast activity with single periods of dynamic loading^[7] or the well-known example of humeral hypertrophy in the playing arm of professional tennis players^[8]. Due to an increasing population which is living longer, there is a growing burden on healthcare providers of age related diseases and disorders. In addition to this, many of the aging population are remaining active longer into their lives, putting more stress on their musculoskeletal systems and desiring more effective treatments for injury or degeneration.

One of the major problems for healthcare systems with an aging population is the incidence of osteoporosis, particularly in post-menopausal women. It is estimated to affect one third of women over 50, with changes in

oestrogen levels causing a reduction in the bone mass, leading to an increase risk of fractures. In Canada in 2010, it was estimated that \$2.3 billion dollars was spent on osteoporosis treatment (1.3% of the total healthcare expenditure). This estimate rises to \$3.9 billion when those in long term care with osteoporosis are included. Between 2007 and 2008, Canadian healthcare systems dealt with over 57000 acute care admissions and over 800000 hospitalised days^[9]. In Europe, there were 3.79 million osteoporotic fractures and 0.89 million hip fractures in the year 2000. The direct costs of treatment for such fractures is estimated at €31.7 billion and these costs are expected to rise to €76.7 billion by 2050^[10]. There is therefore great economic benefit to be derived from improving osteoporosis treatment and care.

There are many other MSK malfunctions which can lead to problems in mobility. Some, such as stroke-induced paralysis, may happen later in life and require suitable rehabilitation methods for older patients. Others, such as cystic fibrosis and osteogenesis imperfecta occur at birth and treatments are required to improve the quality of life in disabled children and adults. Degeneration of the MSK system is also a problem in patients with restricted movement, such as those under long term hospital care. There is a need in all of these situations to improve or maintain the structure and function of the different MSK tissues. It is also desirable in many cases that this is done without the need for an intensive exercise, particularly in those at high risk of fractures or falls or with limited mobility.

In the MSK system, many of the cells which produce and maintain the tissues come from a lineage of progenitor cells contained within the bone marrow. These cells are known as mesenchymal stem cells (MSCs) and similar cells are found in umbilical cord blood^[11] and vein^[12] and many adult tissues including skin^[13] and adipose tissue^[14,15]. Therefore, interventions that act on the whole body to improve MSK tissue structure likely have an effect on MSCs as well as mature MSK cells. MSCs are a multipotent stem cell, capable of differentiation along osteogenic, chondrogenic, adipogenic and myogenic lineages^[16-18]. Because they are multi- rather than pluripotent some researchers in the field prefer to use terms such as "multipotent stromal cell" or "mesenchymal stromal cell". It is thought that MSCs could be a potent tool for regenerative medicine, allowing the repair of many tissue types within the body. Regenerative medicine aims to develop methods to recruit or guide the MSC pool of cells *in vivo*, enabling them to more effectively augment healing. The related field of tissue engineering is a repair strategy in which stem cells are stimulated to differentiate towards a required lineage, usually *in vitro* and then implanted into a patient. Expansion of a patient's own cells may necessary for such therapies, due to the relatively low frequency with which these cells are encountered within the bone marrow (0.01%-0.001%)^[19,20]. These cells are also known to be immunomodulatory, reducing strong

inflammatory responses within tissues^[21-23]. There is some hope that allogeneic MSCs may be of use during therapy, allowing for a more immediate source of cells than patient expanded MSCs.

In vitro, the most commonly used methods to induce differentiation of MSCs involve glucocorticoids such as dexamethasone^[24], growth factors such as bone morphogenic proteins (BMPs)^[25,26] and other biochemicals such as ascorbic acid^[27] and β -glycerophosphate^[28] (for the deposition and mineralisation of bone matrix). For a general review of such methods, see Delaine-Smith and Reilly (2011)^[29]. For transplantation of stem cells or their use in tissue engineering, it may be possible to use chemical means to direct differentiation. For *in vivo* applications, the use of chemical agents will have other systemic effects and the cost of growth factors is prohibitive for most health services. Therefore non-biochemical means of stimulating MSCs could be very useful.

There is growing evidence that mechanical stimuli can be used to direct stem cell differentiation towards a variety of different tissue lineages, reviewed elsewhere^[30-33]. For example, in MSCs it has been demonstrated that the application of compression^[34], tension^[35] or bending of scaffolds^[36] can increase osteogenic differentiation. Other work has shown that microgravity can reduce the osteoblastic differentiation of human MSCs^[37]. *In vitro* work on such stimulation techniques may allow the development of methods to enhance recruitment and differentiation of such cells *in vivo* and allow more targeted therapies. The principle behind LMHF loading is that such vibrations are similar in to the tiny, short duration forces which are applied to bones through muscle action in resting. There is evidence that these small motions may be more important than high impact events in maintaining bone mass and aiding repair^[38].

The following sections of this review will cover the *in vivo* and *in vitro* use of LMHFV. The range of measures for *in vivo* treatments will be covered briefly, with a more in depth look at studies concerning cell behaviour and progenitor recruitment or differentiation in the MSK system. The final *in vitro* section will include studies on differentiated cells before continuing to consider the growing body of literature on progenitor differentiation.

WHOLE BODY VIBRATION - LMHFV *IN VIVO*

In the treatment of bone breakages, there is a need to ensure that appropriate mechanical cues are provided to the tissue in order that it is properly maintained and able to regenerate. Fixation devices, both internal and external, may be used to maintain the position of the broken bones in order to facilitate healing. Such devices can reduce the need to avoid weight-bearing on the limb, preventing disuse which is detrimental to the bone tissue. There are also defects that will not heal with fixation alone, leading to non-union

fractures. Where standard treatments have proved ineffective in repairing the bone tissue, there is a need to find alternative therapies for such patients. In addition, people with osteoporosis are at high risk of fracture and the best method of fracture prevention in this group is to maintain bone mass.

These clinical problems have led to the investigation of methods of mechanical stimulation to improve bone mass and healing, including LMHFV, applied by having the subject stand on a vibrating platform^[39-41]. The acceleration used during these experiments is usually very low (less than the acceleration due to gravity) and the magnitude is typically less than 1 mm. The frequency varies between studies, commonly ranging from 10 to 150 Hz. In addition to different inputs, a variety of different outcomes have been measured. These include bone mineral density (BMD), trabecular width or trabecular spacing and bone formation rate (BFR), but also include measures of balance^[42], likelihood of falls^[43] and jump height^[44]. In addition, other groups have considered the effects on MSC populations, such as number, location and differentiation capability^[1,45].

There are many *in vivo* studies which have looked at the amount and quality of bone after LMHFV stimulation. Large and small animal models have been used, as well as clinical human studies. In such clinical studies, LMHFV is often referred to as "whole body vibration"^[43,46-49]. Such studies have covered healthy patients or those with compromised skeletal systems, in particular post-menopausal women. The efficacy in treating other conditions, such as recovery after stroke^[50], improving the quality of life for disabled children (see Matute-Ilorente *et al.*^[51] for a review on this topic) or limb function in patients with chronic obstructive pulmonary disease^[52] have also attracted interest. Some studies have also included the ability of LMHFV to improve performance and training in athletes^[53] and cognitive performance in adults with or without attention deficit hyperactivity disorder^[47]. Studies have also used microvibration (20 Hz rocking platform) to improve the implantation rates of embryos undergoing IVF treatment^[54].

In the case of *in vivo* murine studies, animals are usually confined in a box or cage which can be vibrated in order to apply the LMHFV stimulation^[1,55-59]. It is worth noting that in such experiments the animals are able to move about freely during the stimulation period. As human participants are usually asked to stand still on a vibrating platform, this may explain some differences in results between the two species. Compliance in animal trials is assured as their whole environment is often vibrated, but is often low with human patients and may affect the potency of the treatment. The normal movement of the animals during the stimulation, such as running, resting or standing on two legs, will affect the transmissibility of the vibrations through the body, further altering the response to loading. The breadth of

studies reported in the literature cover different vibration conditions, with differing frequencies and accelerations (often provided as a multiple of the acceleration due to gravity, g). Ranges include 0.3-1.5 g and up to 90 Hz, applied over different timescales, in mice ranging from juvenile to old. With murine studies, researchers often consider several sites of interest (several bones for example) due to the ease of analysis after sacrifice. mRNA levels of markers of osteogenic activity are often measured, as well as the density of bone in different regions.

In young healthy patients, a study by Torvinen *et al.*^[44] noted an increase in jump height for patients after 8 mo of LMHFV treatment [25-40 Hz (2-8 g), 4 min/d, 3-5 d/wk]. This was not accompanied by any changes to bone mass, structure or turnover however. In studies of post-menopausal (PM) women, reported results include small increases in bone mineral density with high compliance (0.2 g , 30 Hz, up to 20 min per day, every day for one year)^[40], slight increase in BMD and no change in bone markers (0.2 g , 30 Hz, 2/d for one year)^[39] or improvements in femoral neck BMD and balance of the participants (12.6 Hz, 3 cm amplitude, 6 min x 1 min bouts, 3 sessions per week for 8 wk)^[42]. In another study combining exercise and LMHFV, a reduction in the number of falls in PM women was noted^[43]. The study included 15 min of LMHFV during a 60 min exercise program and also found no significant differences in the BMD of the spine or hip in the subjects. Such studies suggest that the LMHFV may be having effects on other parts of the musculoskeletal system to improve a variety of measured outcomes. LMHFV has also been shown to cause a small improvement in muscle power in PM women in a short term study, despite low compliance^[60]. Such studies vary in terms of length of treatment, outcomes measured and conditions applied however, creating many difficulties in analysing the usefulness of LMHFV. These differences are often compounded by low compliance and small study size, making it difficult to draw conclusions about the efficiency of LMHFV on the MSK system.

DISUSE AND DISABILITY

Vibration has been shown to be effective in the prevention of bone loss due to bed rest. Healthy students were subjected to 56 d of bed rest with or without treatment of vibration combined with resistive exercise^[46]. Vibration treatment was found to prevent the changes in muscles size and function, as well as bone volume, compared to the start of the study. Bone mineral content was also reduced in the control subjects but not those treated.

Muir *et al.*^[61] studied the effects of 30 Hz LMHFV (10 min per day, 0.3 and 0.5 g) during periods of bed rest and found that subjects retained muscle flexion strength but not extension strength. Subjects were also found to have better postural stability compared to

controls. Miokovic *et al.*^[62] looked at vibration combined with resistive exercise over 60 d bed rest, finding slightly improved pain scores in the vibrated compared to exercise along groups but no other significant differences. Holguin *et al.*^[63] found that LMHFV (30 Hz, 10 min per day) was able to reduce swelling in the intervertebral disc of human volunteers due to 90 d of bed rest, as well as reducing the reported incidence of back pain by 46%. Other work by the authors has shown that LMHFV (90 Hz, 0.2 g , 15 min/d for 4 wk) can preserve spinal disc mechanics during periods of unloading^[64]. This work was carried out using an animal model however, other examples of which are discussed later in this review.

From the current body of clinical data, LMHFV shows promise in a variety of disease states to improve tissue structure or quality of life. There are fewer studies showing beneficial effects of LMHFV in healthy patients, suggesting that LMHFV may be not be suitable for improving already healthy tissue.

TRANSMISSIBILITY

An important factor in the use of whole body vibration as LMHFV is the transmissibility of the vibrations through the rest of the skeleton. Work in human subjects by Rubin *et al.*^[65] studied the transmissibility of LMHFV through the spine and hip (15-35 Hz, 36 N). They found that for frequencies lower than 20 Hz, there was low transmission of the forces through the body. However, resonances of the vibrations occurred at higher frequencies, which may help explain why higher frequencies appear to be more effective. Kiiski *et al.*^[66] also investigated transmissibility of LMHFV, ranging from 0.05-3 mm amplitude and 10-90 Hz. They found amplification of the peak amplitude at low frequencies at the ankle, knee, hip and spine. At the spine, this occurred only for 10 Hz acceleration, rising to include 40 Hz by the ankle. Above 40 Hz, transmissibility was reduced 10 to 1000-fold. All subjects reported discomfort for vibration between 20 and 25 Hz for displacements larger than 0.5 mm (peak acceleration of 0.8-7.5 g), with one suffering numbness in the feet at 40 Hz (3 mm amplitude, 19 g). These observations suggest that high acceleration vibrations may not be suitable for LMHFV therapy as patients may be less likely to comply if the treatment was uncomfortable. The effects of these high acceleration conditions over the longer term are not well studied and it is not known whether this discomfort will translate to negative effects on the MSK system. The trial had a very small number of participants however and the use of external accelerometers may have affected the results. A recent review of the literature concerning osteoporosis treatments^[67] included a section on LMHFV, recommending parameters of 0.3 g , 25-45 Hz, as most positive studies fall within this range of frequencies stated, and upright posture. There were also several recommendations regarding contraindications, including cancer, severe diabetes and

recent surgery or implantation. The authors suggest that lower frequencies may be unsuitable as they can cause vibration in the internal organs and notes that accelerations higher than 1g have been observed to cause side effects such as back pain.

One study in disabled children used short bouts of LMHFV treatment (0.3g, 90 Hz, 10 min/d) 5 d per week over 6 mo and found increases in BMD in tibial and spinal regions compared to a placebo device^[68]. There were however no observed changes in diaphyseal bone or muscle parameters. The study noted problems with compliance and stance of the children during the vibration and speculated that variation in posture of the children during vibration therapy may have been one factor in the relatively minor benefits seen. This suggests that transmissibility is an important and unresolved issue for the successful application of LMHFV for therapeutic use.

ANIMAL MODELS AND LMHFV

Much as there have been a range of conditions tested in human trials of LMHFV, there are a broad spread of *in vivo* animal studies. These cover the use of large animal models in sheep to small animal models in rats and mice. Some studies use healthy animals while others have included ovariectomised animals to emulate post-menopausal conditions. Outcome measures and study lengths also vary greatly and this next section provides an overview of the animal models used so far with LMHFV studies.

In a large animal model of LMHFV, the application of vibration to the hind legs of sheep (30 Hz, 0.3g, 20 min/d) 5 d a week for one year showed improvements in the bone structure^[69]. Bone mineral content and trabecular number were increased, as well as the longitudinal stiffness and strength of the tissue. The trabecular spacing was seen to decrease, demonstrating bone adaptation and strengthening. Following on from this work, Judex *et al.*^[70] looked at the same vibration conditions and modelled the trabecular stiffness in different directions from the femoral condyle. The models predicted increases in the stiffness in longitudinal, anterior-posterior and medial-lateral directions through the tissue and more uniform distribution of off-axis loading. They also noted that the bone volume and connectedness of the tissue were increased in the vibrated group, demonstrating adaptations requiring bone remodelling.

OSTEOPOROSIS MODELS AND AGING MICE

As LMHFV offers a simple, non-invasive treatment with the potential to improve bone mass and structure, there many studies investigating its use for the treatment of osteoporosis. Therefore there are a large number of animal studies attempting to clarify the

possible benefits of vibration by using Ovariectomised (OVX) or aged rodents.

OVX rats or mice are a commonly used animal model of osteoporosis, where the animals develop osteoporosis-like symptoms (such as reduced BMD and decrease in trabecular number) in the months after ovariectomy^[71]. Work by Flieger *et al.*^[72] followed OVX rats for 5 or 12 wk post-surgery, some of which were treated with LMHFV 5 d per week (50 Hz, 2g, 30 min/d). Significant increases in BMD were found in the femur and tibia (vibrated compared to non-vibrated), which gave a non-significant increase in the fracture load of the femur. The strength of the femur, however, was lower for vibrated animals than for any of the SHAM groups, although there was a non-significant increase for OVX combined with vibration compared to OVX only. Oxlund *et al.*^[73] found that vibration was able to prevent the loss of bending and compressive strength seen in OVX rats. Vibration (0.5g 17 Hz, 1.5g 30 Hz, 3.0g 45 Hz) given for 30 min per day over 90 d prevented endocortical resorption and increased bone formation. Although an increase in BFR was also seen in the OVX only animals, the increase was greatest for the 45 Hz vibration condition.

A later study also demonstrated the prevention of the detrimental effects of ovariectomy by LMHFV^[58]. 3-mo-old rats underwent ovariectomy (OVX) or a sham surgical procedure (SHAM), after which they were left untreated for 3 mo such that the OVX group developed osteoporosis. At this time, half of the animals from the OVX and SHAM treatment groups received LMHFV therapy at 90 Hz (2 × 15 min/d, 7 d/wk for 35 d). Yield load and Young's modulus of the fourth lumbar vertebra were restored to the level of SHAM animals. These changes were accompanied by increases in density (particularly for lumbar trabecular bone). Increases were also found in trabecular bone area, trabecular number and width and both the percentage of cortical bone and BMD. The vibration stimulus was found to be anabolic regardless of the oestrogen levels in the animals.

LMHFV has also been tested to try and reduce the effects of secondary osteoporosis caused by glucocorticoid treatment^[74]. Three-month-old mice were placed into three groups-control, glucocorticoid treatment and glucocorticoid treatment with LMHFV. Five days per week for 9 wk animals were given saline (control group) or methylprednisolone (glucocorticoid groups) injections. Vibrations were applied at 60 Hz during the same period (1g, 30 min per day, 5 d per week for 9 wk). Glucocorticoids alone reduced weight gain, tibial bone mineral content (BMC) and trabecular number and increased trabecular spacing compared to controls. The animals given vibration therapy showed no difference in BMC compared to controls but higher trabecular number and lower spacing than the glucocorticoid only group. This suggests that LMHFV may be a suitable therapy for limiting drug induced

changes in bone quality, although further studies on this topic are necessary.

A longer term study by Wenger *et al.*^[59] in elderly mice (18-mo-old) found no change in the bone volume or strength but increased mineralisation in vibrated animals (0.5 and 1.5g, 32 Hz, 30 min/d for 12 wk). Mineralising surface was calculated histologically and the research examined the femur, radius and lumbar vertebrae of the animals. No changes due to vibration were seen in the lumbar spine, however in the femur there was an increase in high density bone. Vibration also reduced the number of pyridinoline crosslinks at both accelerations, suggesting a reduction in the breakdown of collagen. High density bone volume was increased at 0.5g, with an increase in osteoclast numbers at the higher acceleration. Both vibration conditions showed significant increases in mineralisation surface compared to controls. There was however no significant difference in failure load or stiffness of the radius between any vibration or control condition. This demonstrates that not only are the correct conditions necessary to have an effect on bone turnover, but slight changes to a regimen may be enough to alter the outcome.

Other studies have considered the site specificity of the responses to LMHFV^[75]. They used 0.7g LMHFV at a variety of different frequencies applied for 4 wk (10 min per day, 5 d per week) and studied several skeletal sites in mature male mice. They found that the low frequency used (8 Hz) increased resorption and reduced mineral apposition, weakening the skeleton. 90 Hz vibrations increased trabecular reorganisation and mineral apposition without affecting resorption. These effects occurred for different magnitudes in the vertebrae, tibia and femur, demonstrating the variable effects of LMHFV through the MSK system. The results also demonstrate that, under the wrong conditions, LMHFV may have negative effects on bone tissue.

Judex *et al.*^[76] studied vibration (45 Hz, 0.3g) for 10 min per day (5 d/wk, 21 d) compared to hindlimb unloading in a murine model. They found increased bone formation and mineralisation in the trabecular and periosteal regions of the vibrated tibia. After 4 d in the disuse condition, there were lower levels of mRNA expression for several markers of osteogenesis (col-I, osteonectin, osterix and MMP-2) but no changes were seen for the vibrated group. At 21 d, significant increases were seen for nitric oxide synthase, MMP-2 and RANKL in the vibrated group. The work suggests that short periods of vibration may be able to prevent the bone loss and reduction in osteogenesis which occurs during disuse. As has been seen in human studies, the best results in animal models are obtained in subjects with a lower than normal BMD. Animal models often show better results than human studies, which may be due to guaranteed compliance in the animals and lower rates in human cohorts. There are still many unanswered questions, particularly regarding the efficacy of higher acceleration vibration stimuli.

OSTEOGENESIS IMPERFECTA

Osteogenesis imperfecta (OI), in which the structural protein type 1 collagen is mutated, is another condition which may benefit from LMHFV intervention to improve bone mass and structure. The "brittle bones" which characterise the condition can cause limited to severe changes in stature, skeletal deformities and moderate to severe bone fragility^[77]. The condition is difficult to treat and while bisphosphonate drugs can be used to inhibit resorption, these affect the normal bone remodelling process and can prevent maintenance of healthy tissue. In a young mouse genetic model of OI, LMHFV (0.3g, 45 Hz) was applied 5 d a week for 5 wk, starting when the mice were 3-wk-old^[57]. They found that cortical bone thickness and area was increased for both OI and wild type mice in the tibia and femur, whereas trabecular bone volume was increased for the vibrated mice in the tibia. These changes were accompanied with an increase in femoral stiffness and yield load. They noted that there were only minor differences in bone apposition between the vibrated and sham groups, suggesting that the vibration reduced bone resorption.

FRACTURE HEALING

Several groups have studied the effects of LMHFV on the healing of fractures in murine models. Leung *et al.*^[78] used LMHFV at 35 Hz (0.3g, 20 min per day, 5 d per week) beginning 5 d after the creation of femoral fractures in 3 mo old rats. They found that a larger callus was formed by 2 wk of treatment and increased remodelling had occurred after 4 wk in the vibrated animals compared to those in a sham treatment group. The mechanical strength of the femur was also higher in the LMHFV group, suggesting that the treatment was able to enhance the fracture healing by altering accelerating callus formation and turnover.

Later work using the same vibration conditions studied the effects of LMHFV on fracture healing in rats treated with ibandronate (a bisphosphonate) to suppress bone remodelling^[79]. In this study, 6 mo old rats underwent ovariectomy and were left for 3 mo to develop osteoporosis. Rats then underwent surgery to create closed femoral fractures before receiving ibandronate treatment (with or without LMHFV), LMHFV alone or sham treatment. The vibration only group showed improved speed of callus reduction, increased mineral apposition and increased serum markers of bone turnover compared to all other treatment groups. This remodelling was disrupted by the bisphosphonate treatment, but the application of LMHFV during bisphosphonate treatment was able to reduce the delay in bone remodelling caused by the drug. This provides further evidence that LMHFV may be useful in mitigating the effects of bisphosphonate treatment by reducing the negative effects on bone remodelling.

In an OVX rat model, Shi *et al.*^[80] used LMHFV to

augment fracture healing in OVX rats compared to SHAM control animals. Animals were treated with LMHFV (35 Hz, 0.3g, 20 min/d) for 5 d per week up to 8 wk. In OVX animals, callus formation and mineralisation, as well as remodelling activity and energy to failure were reduced compared to the SHAM animals. LMHFV was found to improve fracture healing, with OVX + vib showing improved fracture healing compared to SHAM + vib. This suggests that the mechanical stimulus is more effective in animals with reduced oestrogen levels, but is anabolic regardless, similar to the findings by Sehmisch *et al.*^[58]. The effects of LMHFV on fracture healing in osteoporotic and normal bone have also been investigated in terms of angiogenesis^[81]. Vibration treatment improved blood flow and angiogenesis for both sham and OVX animals, and overall improved fracture healing.

Work at a higher vibration frequency (90 Hz, 4g, 15 min twice per day, 30 d) demonstrated some beneficial effects of LMHFV on fracture healing in OVX rats^[82]. Three months old rats underwent ovariectomy and showed severe osteopenia 10 wk post-surgery. At this point, both OVX and untreated rats underwent bilateral metaphyseal osteotomy of the tibia, before beginning LMHFV or sham treatment 5 d later. After 30 d of LMHFV or sham treatment, the group found that LMHFV had improved bridging of the fracture in the OVX but not the intact rats, as well as improving the densities of cortical bone and callus. They found a reduction in the stiffness and yield load of the tibia after vibration and suggest that this may be due to the high acceleration used for the LMHFV treatment, which is similar to the result seen by Flieger *et al.*^[72]. These studies suggest that it may be possible to use LMHFV to improve fracture healing, whilst reinforcing the idea that the conditions must be carefully tailored in order to prevent detrimental effects of LMHFV.

TENDON AND MUSCLE

Work investigating the effects of LMHFV on tendon structure has shown an increase in cross-sectional area, stiffness and strain to ultimate load. Sandhu *et al.*^[83] used a rat model and 5 wk of LMHFV (0.3g, 30 Hz, 5 d/wk) to study the flexor carpi ulnaris tendon. Their results suggest that LMHFV may also affect tendon tissue within the MSK system which may be useful for tendon healing if damage has occurred to a joint.

LMHFV has also been shown to affect muscle tissue. In skeletally immature mice, LMHFV was found to affect muscle structure in the soleus muscle^[56]. After 6 wk of stimulation for 15 min per day (0.3g, 45 Hz), the number of arterioles, venules and capillaries within the muscle fibre were reduced. This was significant in the end regions of the muscle. Although a reduction in vasculature to the muscle tissue may appear to be an undesirable adaptation to LMHFV, a study by Xie *et al.*^[84] under the same conditions showed increases in

the moment of inertia and area of the soleus muscle. The same study also found increases in trabecular and periosteal bone volume, bone marrow and cortical bone area but no change in osteoclast activity. These studies provide early evidence that LMHFV is capable of effecting different parts of the MSK system and may be able to aid regeneration in complex injuries to multiple tissues.

The variety of animal studies considered above demonstrate the range of interest in LMHFV as a therapy to affect the musculoskeletal system. These studies have been conducted in small, quadruped animals and as such may not be directly applicable to human conditions. Many of the studies also use 0.3g acceleration or 45 Hz, with results suggesting that these conditions in particular may provide beneficial effects. This matches with the recommendations of Katsuri for the use of LMHFV to treat osteoporosis in humans^[67]. The research does suggest that the technique is able to affect many different cell types to alter the properties of a variety of tissues including bone, tendon and muscle. In the case of bone, evidence suggests that LMHFV may be able to improve poor bone structure or aid in healing. If the conditions used in these studies are translated directly into human trials, such as in the work by Luu *et al.*^[85] discussed in the next section, it may help develop the understanding of LMHFV as a therapy.

LMHFV AND CELLS *IN VIVO*

The effects of LMHFV on cells *in vivo* have also been investigated in a range of studies, often in combination with measures of bone properties. Over 5 wk of vibration, Christiansen and Silva^[1] found several site specific responses to loading for a range of stimulation conditions. Adult mice (7-mo-old) were vibrated with accelerations of 0.1, 0.3 and 1g at 45 Hz (15 min/d, 7 d/wk) for 5 wk before sacrifice. Bone marrow was extracted and tibiae, femurs and L5 vertebrae scanned using micro CT. The 0.3g vibration condition showed no differences in bone volume over total volume compared to controls at any site, but was significantly lower than other vibration conditions in the L5 vertebra and proximal tibial metaphysis. Researchers noted that the distance from the vibration platform did not dictate the efficacy, nor was the response seen dose dependant. Although there was a decrease in the presence of progenitor cells, no increase in bone formation was seen. The ALP activity of adherent BMSCs, extracted from the bone marrow of these animals and cultured for 2 wk was not significantly different between any groups, however alizarin red staining showed a reduction in mineralisation for 0.1g (compared to 1.0g) and 0.3g (compared to control and 1.0g samples).

Work in mice has demonstrated that LMHFV may be used to inhibit adipogenesis, with 7 wk old mice undergoing a 15 wk treatment protocol^[86]. They applied LMHFV at 90 Hz (0.2g, 15 min per day, 5 d

per week) and found lower volumes of fat (normalised to body mass) for the vibrated compared to sham control groups. Their fat also contained lower levels of triglycerides, indicating a lower risk for the development of diabetes. The work also looked at the differentiation of adipogenic precursors in a separate experiment. Eight weeks old mice were irradiated with 15 kGy of gamma irradiation to kill the bone marrow before receiving an injection of MSCs from donor mice. The donor mice express green fluorescent protein in all their tissues, allowing the donor cells to be monitored *in vivo*. Mice were allowed one week to recover before receiving LMHFV or sham treatment for 6 wk. The group found that the vibrated mice had a lower ratio of GFP expressing adipocytes to MSCs compared to controls, as well as reduced weights of the epididymal fat pad, suggesting the MSCs were less disposed to adipogenic differentiation.

Work by Luu *et al.*^[85] demonstrated the effects of LMHFV on the osteogenic and adipogenic differentiation of MSCs in a diet-induced obesity mouse model. They also applied LMHFV at 90 Hz (0.2g, 15 min per day, 5 d/wk) to 7-wk-old mice, in this case for 12 wk and compared this to a sham control with no vibration. Twelve mice received a high fat diet only during the vibration period (prevention of dietary obesity), while another 8 started the diet at 4 wk of age (reversal of obesity). In the prevention group, mice treated with LMHFV for 6 wk showed increased MSC numbers compared to controls, upregulated transcription of Runx2 and downregulation of peroxisome proliferator-activated receptor γ (PPAR γ), suggesting a more osteogenic lineage with reduced adipogenic behaviour. After 12 wk of treatment this translated to increased bone volume fraction and reduced weight gain compared to the controls. However, in the mice starting the high fat diet at 4 wk of age, there were no differences in adiposity or bone volume between control and vibrated animals, suggesting LMHFV is not able to reduce existing obesity.

Interestingly, the group translated this work into a human trial in young women with low BMD, reported in the same study^[85]. After 12 mo treatment they found an increase in BMD in the spine and no significant change in visceral fat area compared to the start of the study in LMHFV treated patients. In the control group, there was no change in BMD and an increase in fat, suggesting that the technique is also able to increase osteogenesis and reduce adipogenesis in humans. Both this work and that by Rubin *et al.*^[86] suggest that LMHFV is able to inhibit adipogenesis in MSCs as well as increasing osteogenesis. This reduction in adipogenesis may be a beneficial side effects of LMHFV treatment, but the research also suggests it will not be suitable as a method to reduce pre-existing obesity.

In a hindlimb unloading murine model, Ozcivici *et al.*^[45] compared 15 min of weight bearing each day (HU animals) to the same period of vibration (HU + vib, 0.2g, 90 Hz). This was carried out for 3 wk, at which point

animals were sacrificed or underwent a further 3 wk period of reambulation, continuing with the same sham (RA) or vibration (RA + vib) treatments. For animals sacrificed prior to reambulation, the study found no difference between sham and HU + vib conditions for trabecular bone fraction. However, the animals which had received the vibration stimulus showed several changes at a cellular level. The osteogenic MSC and osteoblast populations were found to be greater and HU + vib animals also showed a smaller osteoclast surface. Reambulation magnified these differences, with a greater increase in bone volume, MSC population, osteoblast surfaces, BFR and a higher ratio of bone-lining osteoblasts to marrow adipocytes (RA + vib compared to RA). They conclude that, although vibration does not prevent the bone loss due to disuse, there are improvements in cellular function which may enhance long term recovery. The work suggests that the overall effects on bone fraction may be due to an effect of LMHFV on MSCs within the bone marrow.

A recent study investigated the use of LMHFV to accelerate wound healing in a diabetic mouse model^[55]. 30 min of LMHFV (0.4g, 45 Hz) was applied for 7 or 14 d after wounding of the skin and compared to sham loading controls. There was an increase in angiogenesis and granulation tissue formation seen at day 7 in vibrated animals. By day 14, this had led to accelerated wound closure and re-epithelialisation. The study monitored levels of growth factors and cytokines associated with promoting healing, with increases in the vibrated group for IGF-1, VEGF, monocytes chemotactic protein-1. Additionally, while there was no effect of vibration on CD11b⁺ cells in the wounds, CD11b⁺ cells displayed a less inflammatory phenotype.

In a model of granulosa cell tumours in mice, LMHFV has been shown to help mitigate the associated osteopenia^[2]. LMHFV (0.3g, 90 Hz, 15 min bouts) was applied 5 d per week over the course of a year and found no significant changes in survival rates of the animals. They found increases in the bone volume in vibrated mice, as well as reductions in tumour incidence and the number of organ systems involved. The research also found that the number of MSCs in the bone marrow were significantly lower in vibrated mice and those lacking pathology, suggesting that they may play a role in the disease progression.

All of these studies suggest that LMHFV is having effects on a variety of cell types *in vivo*, particularly the proliferation and differentiation of MSCs. Possible benefits include increasing osteogenesis and reduction of adipogenesis. The studies suggest that higher frequency LMHFV (90 Hz) at a lower acceleration (0.2g) may be able to stimulate progenitor cells within the bone marrow. However, more studies at 0.3g covering the effects on MSCs *in vivo* will help to clarify the effects.

LMHFV AND *IN VITRO* CELL STUDIES

In order to better understand the mechanisms of

responses to LMHFV and to identify which cells types are involved, many *in vitro* studies have been undertaken. For bone, there are several possible responsive cell types including osteoblasts, osteoclasts or their progenitors. As a variety of other effects have been seen *in vivo*, such as those on muscle and tendon, there may be further cell types which are responsive to LMHFV. *In vivo* stimulation of tissue forming cells also has applications in the field of tissue engineering where mechanical forces have been well demonstrated to modulate tissue formation by cells, but it is difficult to design bioreactors and stimulation regimens that could be scaled up for clinical and commercial use. LMHFV stimulation may be an appropriate method for the application of mechanical forces to variety of scaffold types and complex geometries, as well as allowing the stimulation of multiple samples within standard culture apparatus.

Experiments conducted *in vitro* include the use of different cell types as well as vibration parameters (frequency, amplitude, duration, number of applications, timeframe). One cell type often used to study osteogenic differentiation with LMHFV is the MC3T3-E1 cell line, a murine osteoblast precursor line. A variety of different outcomes have also been measured, including mRNA expression of different molecules, matrix expression and deposition and prostaglandin E₂ (PGE₂) release.

Dumas *et al.*^[87] looked at the effects of LMHFV on MC3T3-E1 cells and also the ability of the ECM they deposited to affect MSC attachment and differentiation. The cells were stimulated with 15-40 microstrain (measured as deformation of the cell culture dish) at 400 Hz (20 min per day for 1, 3 or 7 d) and demonstrated increased nitric oxide (NO) secretion, upregulated mRNA for fibronectin, osteopontin, bone sialoprotein and collagen I, with no changes to cell number or viability. The matrix produced by these cells was then decellularised and used to culture C3H10T1/2 cells, a murine MSC cell line. The cells on these matrices were cultured for 7 d with or without LMHFV treatment and exhibited increases in cell attachment, focal adhesions and osteogenic mRNA on the matrices from vibrated cells. The cells also reduced expression of adipogenic mRNA, suggesting the matrix and vibration treatment may promote osteogenesis of recruited MSCs as well as inhibiting adipogenesis. This is in agreement with the *in vivo* work discussed earlier within this review^[86,88], providing further evidence for the effects of LMHFV on adipogenesis in MSCs.

Rat BMSCs have also been seeded into scaffolds derived from human bone and subjected to LMHFV (0.3g, 40 Hz, $\pm$ 50 μ m) for 30 min every 12 h, up to 2 d^[89]. Cells were found to attach to the scaffolds and produced higher levels of ALP than those cultured in 2D. LMHFV decreased the cell proliferation rate (measured on days 7 and 10), but increased expression of Cbfa1, ALP, col I and OCN. They also studied extracellular signal-regulated kinase 1 and 2 (ERK1/2) involvement and found that ALP activity was

reduced when the ERK1/2 pathway was inhibited.

Short bouts of LMHFV (5 min, 5, 30, 60 or 100 Hz) of varying amplitude were shown to promote the release of NO and PGE₂ in MC3T3-E1 cells^[90], molecules involved in the *in vivo* response of bone to mechanical loading. The study found no changes in cell shape, alignment or detachment with any of the treatments. When measured 30 min post vibration, there were correlations with increasing maximum acceleration for NO (increasing) and PGE₂ (decreasing) secretion. mRNA for Cyclooxygenase 2 (COX-2), a signalling molecule downstream of PGE₂, was also increased 2 fold by 100 Hz vibration compared to 5 and 30 Hz and 1.5 fold vs 60 Hz. It may be important to note that the vibration in this case was applied along the plane of cell attachment, whereas in many studies the LMHFV are applied perpendicular to this. It is not yet known whether this may produce differential effects as there have been no studies directly comparing the two planes of vibration.

After one hour treatment of MLO-Y4 osteocyte-like cells with LMHFV (0.3g, 30, 60 or 90 Hz), Lau *et al.*^[91] found increases in the amount of mRNA for COX-2, particularly at a 90 Hz vibration condition (344% increase). mRNA levels of receptor activator of nuclear factor kappa-B ligand (RANKL), an osteoclastogenic factor, as well as the soluble receptor protein RANKL and PGE₂ secretion, were found to be reduced in the vibrated cells. Conditioned media from the vibrated cells was also found to inhibit osteoclast formation (number of multi-nucleated cells present) and resorption. This suggests that LMHFV may be able to stimulate osteocytes to inhibit resorption in response to the LMHFV. Li *et al.*^[92] investigated the effects of low intensity pulsed ultrasound (LIPUS) on the interaction between osteocytic and pre-osteoblast like cells. They used 1.5 MHz stimulation on MLO-Y4 murine osteocyte-like cells (3 mm from the LIPUS source) and placed the conditioned media onto MC3T3-E1 pre-osteoblastic cells. Twenty four hours after vibration they saw increased NO and PGE₂ secretion in the MLO-Y4 cells. Conditioned media (collected one hour post vibration) inhibited proliferation in the MC3T3-E1 cells, also causing an increase in the ALP activity of these cells, suggesting osteoblastic differentiation.

Work looking directly at osteoclast precursors found a possible inhibition of osteoclast formation^[93]. They used murine osteoclast-like cells RAW264.7 and applied LMHFV (4 Hz, 20 μ m displacement) for one hour a day over 3 d and examined the mRNA and protein levels for DC-STAMP (dendritic cell-specific transmembrane protein) and the P2X7 receptor P2X7R, both membrane bound markers of osteoclast fusion. They saw inhibition of mRNA and protein production for DC-STAMP, which suggests the inhibition of osteoclast fusion had occurred. In a model of a BRU using scales taken from goldfish and placed in culture, LMHFV of varying acceleration (0.5, 1, 2, 4, 6g) for 5 or 20 min per day showed changes in osteoblast and osteoclast activity after 6 and 24 h^[94]. The ALP activity in scales was found to

increase with increasing acceleration, accompanied by a decrease in osteoclast activity (measured by tartrate-resistant acid phosphatase activity). These results were evident at 24 h, with the largest decrease in osteoclast activity for 2g. However, although the goldfish scale contains both osteoblasts and osteoclasts, it has a very different macrostructure to that of bones making it difficult to compare with other model systems.

Other studies have compared the effects of LMHFV with high magnitude strain (HMS) on the adipogenic differentiation of MSCs. Sen *et al.*^[95] used C3H10T1/2 cells and 90 Hz vibration (< 10 microstrain) compared to low frequency HMS (0.17 Hz, 20000 microstrain). They compared pauses within the loading regimen to longer periods of vibration and followed adiponectin expression as a measure of adipogenesis. They found that HMS over 2 or 6 h prevented adipogenesis in the cells, as well as two 20 min bouts separated by at least one hour rest period. The LMHFV was only able to suppress adipogenesis with the two 20 min bouts separated by more than one hour, with a 3 h pause proving better than one or two hours. The inclusion of indomethacin to induce adipogenesis inhibited the effects of the LMHFV but not the HMS treatment. The addition of BMP-2 promoted an osteogenic response with the LMHFV treatment.

A further study was carried out investigating the effects of LMHFV (termed sub sonic vibrations) on 3T3-L1 pre-adipocytes, an embryonic derived cell line^[96]. This study used horizontal vibration looking at cell proliferation (3 d, 10, 20, 30 and 40 Hz) and adipogenic maturation (6 d, 10, 20, 30 and 45 Hz). They found that 20 and 30 Hz LMHFV decreased cell proliferation and increased adipogenic gene expression. Increased triglyceride levels within cells were seen for the 10, 20 and 30 Hz vibration conditions. Although the work discussed previously^[86-88] showed a reduction in adipogenic gene expression in MSCs, this evidence suggests that LMHFV may increase adipogenesis in cells that are already of the adipose lineage, which would not be a desirable effect *in vivo*. The *in vivo* data on obesity and LMHFV, which demonstrated a lack of reduction in already obese animals^[85,86], seems to confirm that LMHFV cause more activity in cells of the adipose lineage, but may be able to mitigate their commitment to this lineage initially.

Interestingly and contradicting the theory that vibration promotes osteogenesis; MSCs from rat bone marrow were found to have lower matrix mineralisation and mRNA for osterix after 6 d period of vibration^[97] compared to controls. The cells were subjected to vibration at 0.3g and 60 Hz for one hour per day (6 d with one rest day). There were no changes to cell proliferation, ALP activity or mRNA levels for a variety of osteogenic genes (ALP, col-I, OPN, BSP, RUNX-2, OCN). The group hypothesise that, rather than having a direct effect on MSC differentiation, LMHFV may have anabolic effects on bone by causing osteocyte signalling, which in turn affects MSC differentiation.

However, many of the *in vivo* studies which have looked at differentiation of MSCs following LMHFV have found increased osteogenic behaviour. This may be due to the differences in cellular environment and vibration transmission between 2D cultures and cells in the 3D *in vivo* environment.

C2C12 murine myoblasts were found to undergo changes following short periods of LMHFV stimulation (5, 8 or 10 Hz, 0.4 mm amplitude, 10 min/d)^[98]. After 3 d, expression of collagen I and decorin were increased for the 8 and 10 Hz vibration conditions. MyoD and myogenin expression was seen to increase in a time- and dose-dependent manner. There were no changes observed in the cell cycle, but 8 and 10 Hz vibration increase myotube number and length (6 and 9 d of vibration). Vibration also increased the fusion of myocytes in a dose dependant manner. This suggests that the cells are promoting vascularisation, which may aid tissue regeneration *in vivo*.

Much of the *in vitro* work discussed above shows beneficial effects on osteogenic cells, which may lead to the beneficial effects seen in some animal models *in vivo*. The lack of interaction between different cell types however means that it is difficult to know whether the effects seen *in vitro* can help direct therapies *in vivo*. Transmission of LMHFV through muscle, fat and bone may alter the effects on MSCs and differentiated cells, leading to reductions in the efficacy of treatment compared to cell signalling responses and differentiation markers seen in cultured cells.

HUMAN CELLS *IN VITRO*

Reported effects of vibration on human cells are summarised in Table 1. As well as the large number of studies on animal cells and cell lines, there is a growing body of work using human cells and cell lines to investigate the possible mechanisms of action of LMHFV. SAOS-2 cells, a human osteosarcoma cell line, were subjected to 30 Hz vibrations (11 mm displacement) once per day for 4 d^[99]. The cells displayed decreased proliferation and increasing mRNA levels for ALP, collagens I and III, osteonectin and fibronectin, suggesting more osteogenic behaviour. Further work by the group looked at the osteoblastic differentiation of human adipose derived MSCs^[100]. They applied LMHFV at 30 Hz to cells for 28 d and found evidence of improved matrix calcification at day 21. They also noted strong increased in the expression of collagen I and osteopontin on day 14 in the vibrated samples when cultured in osteogenic medium, which was not maintained at 21 d. Slightly higher total protein levels were found in vibrated samples compared to non at 21 d, but this effect was lost by 28 d. As many *in vivo* studies do not show any benefits of LMHFV in healthy patients, it is possible that the therapy affects disrupted bone balance, but does not provide any further benefit when normal

Table 1 Summary of effects of low magnitude, high frequency vibration on human cells *in vitro*

Prè <i>et al</i> ^[100]	Adipose derived MSCs	30 Hz, 45 min/d, 28 d	Increase in col-I and osteonectin expression in vibrated group on day 14, lost by day 21. Increased mineralisation in vibrated group at 21 d, lost by day 28
Kim <i>et al</i> ^[102]	MSCs (2 donors)	0.3g, 30 Hz for main study. Applied for 10 min/d, 5 d a week for 21 d	Vibration increased ALP activity (days 7, 14 and 21), mRNA (ALP, osteopontin and VEGF) upregulated at day 7. No changes (bone sialoprotein or osteonectin mRNA). In osteogenic media, LMHFV increased the calcium deposition in cultures
Uzer <i>et al</i> ^[103]	Adipose derived MSCs	30 and 100 Hz, 0.15, 1 and 2g. 30 min per day for 3-14 d	Cell number increased over 3 d for all conditions. Mineralisation at 14 d increased for 100 Hz and 30 Hz (2g). Prevented cytoskeletal remodelling to negate the effects
Zhang <i>et al</i> ^[104]	Peridontal ligament stem cells	0.3g (10, 20, 30, 40, 50, 60, 90 and 120, 150 and 180 Hz), 20 min/d to 5 d	LMHFV decreased proliferation, ALP activity highest at 30 Hz. Osteocalcin expression was highest at 40, 50, 60, 90 and 120 Hz. Levels of col-I, RUNX-2 and osterix increased at 40 and 50 Hz
Wolchok <i>et al</i> ^[105]	laryngeal fibroblasts	100 Hz and 3.4g (1 s vibration followed by 2 s pause), 6 h followed by an 18 h rest for 21 d	Enhanced secretion of TGFB-1, fibronectin, collagen, increased construct stiffness. Increased gene expression of range of matrix molecules
Cho <i>et al</i> ^[106]	Umbilical cord stem cells	10, 20, 30 and 40 Hz, continuous for 5 d	Increased mRNA and protein levels (MAP2, NF-L and NeuroD1). Time dependant increases in O4 and ERK (12 h at 40 Hz)
Choi <i>et al</i> ^[107]	Adipose derived MSCs	10, 20, 30 and 40 Hz, continuous for 4 d	30 Hz increased astrocyte, oligodendrocyte and neuronal markers, inhibited adipogenesis. Increasing frequency reduced cell proliferation. Time dependant changes in ERK phosphorylation
Edwards and Reilly ^[101]	hES-MP 002.5 cells	0.3g and 15, 30, 45, 60 Hz, one bout 4 min	Increased ALP activity at 60 Hz 48 h post vibration

LMHFV: Low magnitude, high frequency vibration; VEGF: Vascular endothelial growth factor; ALP: Alkaline phosphatase; ERK: Extracellular regulated protein kinases.

bone formation is achieved. The ability to accelerate the initial healing or rebalance of bone formation still has the potential to be a useful therapy.

Our own research with LMHFV and a mesenchymal progenitor cell line, hES-MP 002.5 (Cellartis) has shown promising effects on osteogenic differentiation in 2D^[101]. We found evidence that LMHFV at 0.3g was able to affect the cell number and osteogenic activity of these cells in 2D culture. Forty-eight hours following a single 45 min bout of vibration, ALP activity was significantly increased for vibration at 60 Hz. This was not seen at lower frequencies tested or 24 h post vibration.

Kim *et al*^[102] investigated the use of LMHFV for osteogenic differentiation of hMSCs from two different donors. They applied vibration for 10 min per day (5 d per week to 21 d) at a variety of frequencies and accelerations (10, 20, 30 and 40 Hz and 0.1-0.6g in 0.1g increments) before choosing 0.3g and 30 Hz for the main experiments. Cell proliferation was affected by vibration in a dose dependant manner, with increases in cell number at 30 and 40 Hz conditions on day 2. This increase was not maintained at 5 d however. ALP activity was increased in vibrated samples on days 7, 14 and 21, with mRNA for ALP, osteopontin and VEGF also upregulated at day 7. There were no changes to bone sialoprotein or osteonectin mRNA at this timepoint. When cells were cultured in osteogenic media, LMHFV increased the calcium deposition in cultures, suggesting the vibration has increased osteogenesis.

With LMHFV *in vitro*, there is the likelihood of shear stress occurring in the culture plate due to the movement of media within wells. Uzer *et al*^[103] used LMHFV to apply shear stress to human adipose derived stem cells for 30 min a day (3-14 d). They looked at several vibration conditions (0.15, 1 and 2g, frequencies

of 30 and 100 Hz) and characterised the resulting shear stress (0.04-5 Pa). Over the first 3 d of culture, cell number increased for all conditions, with the greatest increases seen for the conditions with lowest induced fluid shear. After 14 d, mineralisation was increased for all accelerations at 100 Hz and for the 2g acceleration at 30 Hz. By disrupting cytoskeletal remodelling, they demonstrated that MSC differentiation was not only regulated by shear stress (as occurred in low stress conditions), but is influenced by cytoskeletal remodelling.

Work by Zhang *et al*^[104] on human peridontal ligament cells studied a large range of frequencies (10, 20, 30, 40, 50, 60, 90 and 120, 150 and 180 Hz) at 0.3g acceleration. They applied the vibration stimulus for 20 min per day for up to 5 d. The study found decreased proliferation and a frequency dependant increase in osteogenic markers. ALP activity was highest at 30 Hz, while osteocalcin expression was higher at 40, 50, 60, 90 and 120 Hz. Levels of col-I, RUNX-2 and osterix increased at 40 and 50 Hz.

Other research studying horizontal vibration investigated the effects of paused vibration on human laryngeal fibroblasts^[105]. This study involved vibration at 100 Hz and 3.4g (1 s vibration followed by 2 s pause) for up to 21 d. Vibration was applied for 6 h followed by an 18 h rest and was shown to increase gene expression for matrix and matrix related molecules. These included TIMP1, TIMP3, col1 and col IX, lysyl oxidase, TGFB1, syndecan, laminin, connective tissue growth factor and PDGF. In addition, there was enhanced secretion of TGFB-1, fibronectin, collagen and an increase in construct stiffness for low values of strain.

In one study^[106], LMHFV (termed sub sonic vibration) was applied at a variety of frequencies (10, 20, 30 and 40 Hz) to human umbilical cord MSCs continuously for

5 d. In this case, the group was interested in neuronal differentiation and found increased in both mRNA and protein levels for several factors including MAP2, NF-L and NeuroD1. They also found time dependant increases in O4 and ERK for 12 h of LMHFV at 40 Hz. A second study^[107] assessed the neuronal differentiation of human adipose-derived MSCs, applying LMHFV at the same frequencies over 4 d. RT-PCR was used to investigate both neuronal and adipogenic markers with upregulation at 30 Hz and found that astrocyte, oligodendrocyte, and neuronal markers were significantly increased while inhibiting adipogenesis. Increasing frequency was shown to reduce the cell proliferation. They also noted time dependant changes in ERK phosphorylation, concluding that the LMHFV promoted differentiation to astrocytes and oligodendrocytes through activation of the ERK pathway.

CONCLUSION

The appeal of low magnitude, high frequency vibrations to act as a mechanical stimulus and improve a range of functions within the MSK system has led to an ever growing body of research. The technique is relatively simple to apply, often needs only short periods of stimulation to show some effect and is suitable for those with fragile bones or limited motion. The response varies between different studies, patients and conditions, however, making it problematic to define a predictable set of responses. For healthy subjects, there are often no apparent benefits to the treatment, which suggests that the LMHFV stimulus provides similar mechanical cues to normal daily activities in healthy patients and therefore provide no benefit to bone mass or structure unless there is an underlying condition. There are many studies showing benefits of LMHFV using an acceleration of 0.3g or 45 Hz vibration, suggesting these conditions may be particularly effective for the MSK system. Higher frequency vibration, such as 90 Hz, has also show some efficacy at low accelerations (0.2g). Evidence is building to suggest that accelerations higher than that due to gravity (> 1.0g) may be undesirable, causing some negative effects on bone and mild discomfort in human patients.

In vitro investigation of LMHFV has also produced a wide variety of data, with new research being published at an increasing rate. As with the *in vivo* studies, the range of conditions tested, outcomes measured and goals of the research is very varied, making it difficult to identify common trends. There is increasing evidence that LMHFV has the power to affect the differentiation of stem cells, particularly MSCs. When more of this data is made available, it may be possible to understand how LMHFV might improve the MSK system and aid in the repair of other tissues in the body. Work with primary human cells from multiple donors may provide insights into which patients may be most likely to benefit from

LMHFV treatments when conducted alongside *in vivo* investigations. There is also a need for more human trials based directly on small animal studies, using the same outcome measures where possible, to further our understanding of the applicability of these animal models and their relevance to human treatment with LMHFV. Both types of work may help stratification of the therapy to target patients who are likely benefit from the treatment. In general, the use of LMHFV to improve the condition of the MSK system in patients who are not able to maintain high levels of activity or exercise therapy shows promise, but there is still much work to be done on understanding the mechanisms, side effects and benefits to patients.

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Role of *Hox* genes in stem cell differentiation

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of any *Hox* gene expression, but these transcription factors are activated in varying spatial and temporal patterns defining the development of various body regions. In the adult body, *Hox* genes are among others responsible for driving the differentiation of tissue stem cells towards their respective lineages in order to repair and maintain the correct function of tissues and organs. Due to their involvement in the embryonic and adult body, they have been suggested to be useable for improving stem cell differentiations *in vitro* and *in vivo*. In many studies *Hox* genes have been found as driving factors in stem cell differentiation towards adipogenesis, in lineages involved in bone and joint formation, mainly chondrogenesis and osteogenesis, in cardiovascular lineages including endothelial and smooth muscle cell differentiations, and in neurogenesis. As life expectancy is rising, the demand for tissue reconstruction continues to increase. Stem cells have become an increasingly popular choice for creating therapies in regenerative medicine due to their self-renewal and differentiation potential. Especially mesenchymal stem cells are used more and more frequently due to their easy handling and accessibility, combined with a low tumorigenicity and little ethical concerns. This review therefore intends to summarize to date known correlations between natural *Hox* gene expression patterns in body tissues and during the differentiation of various stem cells towards their respective lineages with a major focus on mesenchymal stem cell differentiations. This overview shall help to understand the complex interactions of *Hox* genes and differentiation processes all over the body as well as *in vitro* for further improvement of stem cell treatments in future regenerative medicine approaches.

Key words: Genes; Homeobox; Stem cells; Asymmetric cell division; Mesenchymal stromal cells; Growth; Development; Regeneration; Patterning; Cell lineage

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Abstract

Hox genes are an evolutionary highly conserved gene family. They determine the anterior-posterior body axis in bilateral organisms and influence the developmental fate of cells. Embryonic stem cells are usually devoid

Core tip: *Hox* genes are involved in embryonic development as well as in repair mechanisms in the adult body, thus

regulating cell fate. These genes have also been found to be driving factors in various stem cell differentiations *in vitro* and *in vivo* which makes them interesting tools for future improvements in stem cell therapies. Therefore, this review outlines the involvement of *Hox* genes in various stem cell differentiations with a major emphasis on mesenchymal stem cell differentiations.

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INTRODUCTION

The life expectancy of the overall population has increased in most countries, accompanied by an increase in injuries, diseases, and age related degenerative diseases. Thus, reconstructions of hard and soft tissues are an increasing challenge in regenerative medicine^[1]. Stem cells could help to overcome these restrictions using their self-renewal and differentiation properties, with or without biomaterials or artificial scaffolds^[1-3]. Numerous sources are known from which stem cells can be isolated, often with a varying differentiation potential^[4]. Additionally, the commitment might depend on the body site from which the stem cells were isolated^[4,5]. *In vivo*, stem cell fate is determined according to tissues and thus extracellular but also intracellular factors, among them p53, purinergic receptors, collagens, parathyroid hormone-related proteins, or homeobox (*Hox*) genes^[6-9]. The later are the central players in determining regional specificity in the developing embryo and not astonishingly also in numerous differentiations^[10].

The *Hox* gene family consists of 39 members, which are clustered in four paralogous groups and are numbered from 1-13 within the groups. During embryogenesis, *Hox* genes show an expression pattern which is specific for each body region, commonly referred to as the *HOX* code. They are an evolutionary highly conserved collection of genes binding to various DNA recognition sites and thus influencing a cell's developmental fate in a huge variety of species, while determining the anterior-posterior body axis^[11] (Figure 1). The precise function of these genes is achieved by their specific temporal and spatial activation over the course of life. Due to this role in embryonic and adult developmental stages, *Hox* genes are expected to also have fundamental influences on the differentiation of various stem cells *in vivo* and *in vitro* towards their respective lineages. There have been studies which directly identified *Hox* gene involvement in stem cell differentiations, and others which suggest their role based on observed effects of *Hox* genes in *in vivo* or *in vitro* tissue development. It has been speculated that the code may contribute to maintaining the stem cell

character in certain niches, since it is also regulated on an epigenetic level^[12].

This review will outline the *Hox* profile of various stem cells, and discuss the wide range of findings concerning *Hox* genes in stem cell differentiations gathered over the last decade. The nomenclature used for *Hox* genes in this study is according to the current regulations, meaning that *Hox* genes related to findings in mice and rats are written with the first letter capitalized, whereas the following are in small capitals (e.g., *Hoxc8*). For findings in human and chicken, all letters are capitalized (e.g., *HOXC8*). Understanding the principle behind the role of *Hox* genes in differentiations and development may provide improved possibilities to alter stem cell fate *in vitro* and *in vivo*, and therefore might result in new or improved approaches in regenerative medicine.

Therapeutic approaches are more and more based on the use of mesenchymal stem cells (MSCs), due to their easy accessibility and handling compared to other kinds of stem cells. Hence, we put specific emphasis on the differentiation lineages of MSCs within this review.

HOX TRANSCRIPTION FACTORS IN STEM CELLS

Embryonic stem cells

Embryonic stem cells (ESCs) can be gained from the inner cell mass of the blastocyst and are therefore derived from an early stage embryo^[13,14]. They are characterized by various markers, having the most significant surface markers SSEA-1, SSEA-3, SSEA-4 as well as TRA-1-60 and TRA-1-81^[15]. Additionally ESCs are positive for the transcription factors NANOG and OCT3/4^[16,17]. Their pluripotency, the potential to differentiate into each cell lineage and all types of cells of the body, and the proliferation rate causes huge interest in this stem cell type^[18,19]. An active repression of *Hox* clusters has been demonstrated in various studies. On the other hand *Hox* genes are activated when differentiation processes take place^[20]. However, it is not known which *Hox* code exactly commits these cells into the different lineages, yet^[20].

Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are the best characterized type of adult stem cells^[21]. They can be obtained from bone marrow, cytokine-mobilized peripheral blood, and cord blood^[22]. HSCs are characterized with the antigen cell-surface markers CD34, C-KIT, TIE, and CD133/AC133 and they lack CD38, LIN, and CD45RA^[23]. These stem cells differentiate into all kinds of blood cell lineages serving the reconstruction of the lymphohematopoietic system^[24]. In general, the *HOXA* cluster plays a role in hematopoiesis^[25], but the expression of *HOXB4* which directs transition from the embryonic to the adult hematopoietic program is a major factor as

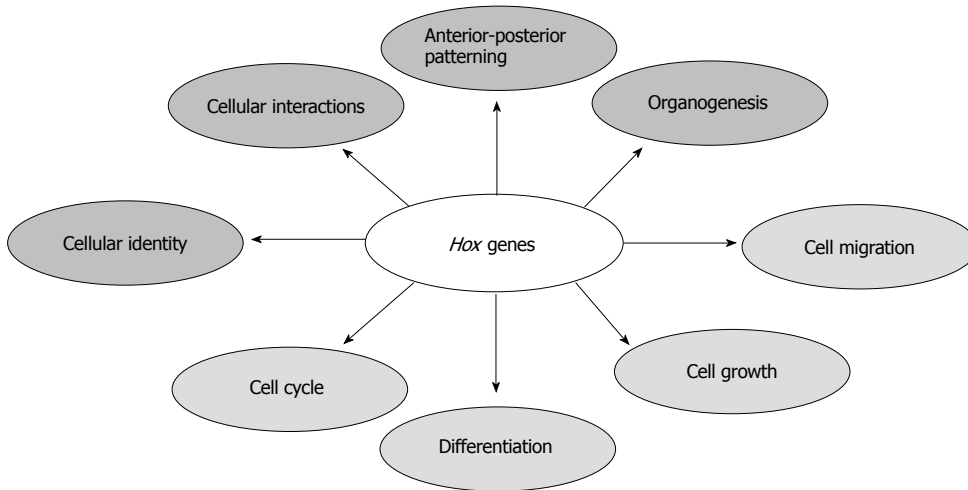


Figure 1 Roles of *Hox* genes in the organisms and the cell^[135-140].

well^[21].

Neural crest cells

Neural crest cells (NCCs) are multipotent cells exclusively present in vertebrates^[26]. These cells are not only found in the early embryonic development but are also present in various neural crest-derived tissues in the fetal and adult organism^[27]. NCCs are located in between the neural epithelium and epidermis in the neurulating vertebrate embryo^[26]. After they have undergone epithelial-mesenchymal transition the NCCs migrate within the embryonic tissue to the respective location and undergo differentiation^[28]. This stem cell type commits towards facial cartilage, bone and connective tissue as well as neural and glial cells of the peripheral nervous system. Additionally, they are able to differentiate into skin pigment cells, mesenchyme and in restricted locations into smooth muscle cells^[29]. NCCs are characterized with the markers p75NTR, AP-2 α and the transcription factors MSX1, MSCX2 and SLUG in the pre-migratory state^[30]. In post-migratory state, embryonic and adult cell markers can be distinguished as follows: in the embryo, adult gut and bone marrow p75 is present, whereas SOX10 is found in the dorsal root ganglia of the embryo and in the adult bone marrow. As an additional marker NESTIN is suggested because it is present in the adult heart and embryonic boundary cap cells. Other markers are MUSASHI in the adult heart, SCA1 and CD34 in the adult cornea as well as SLUG and SNAIL in the adult bone marrow^[26]. Usually there are no *Hox* genes expressed in NCCs^[31]. However data has been found where *Hoxa2* and *Hoxd9* can be induced in mouse trunk neural crest cells by retinoic acid, a major factor triggering differentiation^[32]. Additionally, *Hoxd3* has been shown to have an impact on the formation of somitic mesoderm and neural crest-derived structures during mouse development^[33].

MSCs

MSCs are multipotent adult stem cells which can be *in*

vitro differentiated towards lineages of mesenchymal tissues^[7,34,35], which is in line with their natural repair function in the body where they regenerate mesenchymal tissues like bone, cartilage, muscle ligament, tendon and adipose tissues^[7,36]. In addition it has been described that these stem cells can also differentiate towards lineages of the other two germ layers, such as towards endothelial cells, hepatocytes^[37], or neurons of the ectoderm^[38]. MSCs are found in the stroma of various tissues or organs such as bone marrow, umbilical cord, wisdom teeth and adipose tissues^[5,7,39]. Defined characteristics of these stem cells are the plastic adherence under standard culture conditions, the presence of the antigen surface markers CD73, CD90 and 105 and the absence of CD34, CD45, CD14 or CD11b, CD79a or CD19 and HLA-DR^[40]. Since *Hox* genes are involved in organ formation and regeneration, it is not astonishing that the degree of *Hox* gene expression in various adult stem cell populations differs which enables the discrimination of adult stem cells by the respective *Hox* expression pattern. Cord blood-derived MSCs (CB-MSCs) and unrestricted somatic stem cells (USSCs) are gained from cord blood. Whereas CB-MSCs are positive for *HOXA9*, *HOXB7*, *HOXC10* and *HOXD6*, USSCs resembling the profile of typical ESCs are negative for *Hox* genes. In CB-MSCs and USSCs it has been demonstrated that this expression code is in line with the differentiation potential of stem cells: CB-MSCs can be differentiated towards the adipogenic lineage whereas USSCs generally can be committed towards all three germ layers and show nevertheless no adipogenic differentiation. *HOXB7* and *HOXD6* expression has also been shown in bone marrow-derived MSCs that generally have a similar expression profile as MSCs derived from adipose tissue. Amnion- and decidua-derived MSCs can be distinguished using *HOXC10* expression^[41]. Taken together, a distinct expression of *HOXA5* and *A10*, *HOXB6*, *B7*, *HOXC4*, *C6*, *C8*, *C9* and *C10* as well as *HOXD3* and *D8* is observed in MSCs derived from different human sources^[42]. In murine MSCs the expression of *Hoxb2*, *b5*, *b7* and *Hoxc4* has

Table 1 *Hox* gene expression in various kinds of stem cells

Type of stem cell	<i>Hox</i> expression	Role of <i>Hox</i>
HSC	<i>HOXA</i> cluster <i>HOXB4</i>	Hematopoiesis and its transition from embryonic to adult program
MSC	<i>HOXA5</i> , 10 <i>HOXB6</i> , 7 <i>HOXC4</i> , 6, 8, 9, 10 <i>HOXD3</i> and <i>D8</i>	Degree of <i>Hox</i> expression pattern differs in the different sources and determines differentiation potential
ESC	None	Active repression of <i>Hox</i> genes until commitment starts
CSC	Various	Overexpression of <i>Hox</i> genes present in undifferentiated cells and down-regulation <i>Hox</i> genes present in differentiated cells
	<i>HOXA9</i>	Hematopoiesis
	<i>HOXB7</i> , <i>C6</i> , <i>C8</i>	Vascularization
	<i>HOXA4</i> , <i>D10</i>	Colon carcinoma
NCC	<i>HOXD3</i>	Formation of somitic mesoderm- and neural crest-derived structures
iPS	None	Active repression of <i>Hox</i> genes until commitment starts

HSC: Hematopoietic stem cells; MSC: Mesenchymal stem cells; ESC: Embryonic stem cells; CSC: Cancer stem cells; NCC: Neural crest cells; iPS: Induced pluripotent stem cells.

been shown, which regulate self-renewal and influence the differentiation of other stem cells^[43].

Cancer stem cells

Cancer stem cells (CSCs) are cancer cells with the capability of both, self-renewal and differentiation, which are the typical characteristics of any stem cell^[44]. These stem cells are multipotent, clonogenic, have the capability to induce new tumors and eventually create heterogeneous cell lineages in tumors^[45]. Accordingly they are thought to be responsible for progression of cancer, cancer metastasis, and therapy resistance of cancers^[46]. In addition, the recurrence and spreading of cancer might be due to CSCs that remain in residual tumors after surgery and treatment. It was suggested that a tumor contains different stem cell like populations due to reduced cell differentiation within a tumor and this heterogeneous population also includes CSCs^[44,45]. DNA damages may render tissue stem cells to become CSCs on their way from a stem cell to a fully differentiated cell. However, instead of dying at the different levels of differentiation like normal tissue stem cells do these cancer precursors do not differentiate normally but accumulate forming tumors^[47]. Conclusively, this population differs in marker expression and growth capacities^[45]. In general CSCs can be characterized by the presence of the markers CD24, CD29, CD44, CD90, CD133 and CD166^[44,45]. In some cancers it has been observed that *Hox* genes are upregulated which are normally restricted to undifferentiated or proliferative cells, and *vice versa* a down-regulation has been shown of those *Hox* genes which are normally present in fully differentiated cells^[48]. For instance, *HOXA9* seems to play a role in acute myelogenous leukemia^[49]. *HOXB7*, *HOXC6* and *C9* transcription factors function in intima formation and vascularization in vascular-wall multipotent stem cells, which is in line with vascularization being important for cancer metastasis^[50]. Further, colon carcinomas

show an overexpression of *HOXA4* and *HOXD10* which distinguishes colonic stem cells from colon carcinoma cells^[51].

Induced pluripotent stem cells

Induced pluripotent stem cells (iPS) have similar characteristics as ESCs including pluripotency and immortality^[52]. As can be seen in Table 1, both stem cell types are generally negative for *Hox* gene expression. iPS cells are generated by introducing four defined transcription factors into somatic cells, which are OCT3/4, SOX2, KLF4, c-Myc or NANOG, and Lim2b replacing the later two^[53,54]. In the meantime, several somatic cells can be reprogrammed such as fibroblasts, hepatocytes, keratinocytes and circulating T-cells, also with a reduced set of transcription factors, ranging from four factors to one factor with respect of the target cell^[55]. Both, ESCs and iPS cells are characterized by the pluripotency markers NANOG, OCT3/4, ERAS, ESG1 and ECAT as well as TRA-1-60^[53,56]. As iPS cells can differentiate into the same lineages as ESCs, which is the differentiation into all cell types of the three germ layers^[57], the *Hox* gene expression is similar as well. It is repressed as they are quiescent and up-regulated when the differentiation processes start^[58].

HOX TRANSCRIPTION FACTORS IN VARIOUS DIFFERENTIATIONS

Hox expression in adipogenic differentiation

The understanding of adipose tissue as an organ with a detrimental effect on the whole organism, due to its secreted adipokines, led to increased studies during the last decades, since the number of obesity patients rises all over the world. A dysfunction of adipocytes in obese patients can, *e.g.*, lead to diabetes type II and cardiovascular diseases^[59], the leading cause of death in industrialized countries. Thus understanding the

molecular mechanisms in adipogenesis has therefore gained much importance.

Hox gene expression in developing adipocytes has been studied in the pre-adipogenic committed murine cell line 3T3-L1, where *Hoxa4*, *a7*, and *Hoxd4* are involved in the differentiation towards adipocytes^[60]. In human adipose tissue-derived stem cells (hAT-SC), *HOXC8* expression is drastically down-regulated during adipogenesis and almost vanishes in mature adipocytes. However, the down-regulation does not arise from a change of RNA expression, but is rather due to post-translational modifications by miRNAs. Forced expression of *HOXC8* in hAT-SC inhibits adipogenesis to a certain extent, indicating that this *Hox* gene has a functional role during the differentiation of these stem cells towards adipocytes^[61].

There is also evidence that *HOXA1*, *A4* and *HOXC4* are directly involved in the differentiation and further generation of human white and brown adipose tissue^[62]. Additionally, the *Hox* gene expression pattern is varying in adipocytes derived from different fat deposits within the human body. This pattern is also reflected in the *in vitro* differentiation of human primary pre-adipocytes isolated from the respective region. For instance pre-adipocytes isolated from the abdominal fat depot show an increased expression of *HOXA5* and *HOXB8* during differentiation towards adipocytes, as compared to gluteal pre-adipocytes, where *HOXA10* expression is higher, and *HOXC13* expression is exclusively restricted to gluteal pre-adipocytes^[63]. Due to this variation in the expression pattern, a general statement about the *Hox* gene expression in differentiating adipocytes would not be sensible.

Understanding the principles of *Hox* signaling in white and brown adipose tissue formation might help prevent health damage in obese patients, such as the development of diabetes and consecutive the metabolic syndrome.

***Hox* expression in chondro-osteogenesis**

Understanding the development of joints and bones is of interest in many fields of research, including regenerative medicine. However, the complete formation of limbs and other hard tissues involves various types of cells and a complex interplay between them. Three main tissue types can be attributed to bone and joint formation, namely tendons, cartilage and bone. Chondral tissue is the first to develop in limb formation, and its condensation is needed for correct bone development. Of great importance in tissue engineering are the underlying signaling pathways and proteins involved^[64]. By investigating the *Hox* gene expression in chondrocytes and osteoblasts, both, *in vitro* and *in vivo*, bone formation and regeneration might be improved in the future, since in both cell types *Hoxa2* and *Hoxd9* have been shown to be significantly regulated. *Hoxa2* is up-regulated in bone regeneration of rats after post-fracture day 10, whereas *Hoxd9* is down-regulated

after this time period^[65]. In mice, *Hoxa2* impairs chondrogenesis in the mesenchymal-to-chondrocyte differentiation step leading to a proportionate short stature. Additionally, in a *Hoxa2* gain-of-function mice model molecular regulators for an endochondral ossification are decreased^[66]. In normal mouse development, *Hoxa2* inhibits chondrogenesis and intramembranous ossification as well as bone formation when forcedly expressed in osteoprecursors^[67]. Forced *Hoxa2* expression in cells committed to undergo chondrogenesis also leads to chondrodysplasia and delayed cartilage mineralization and ossification, but does not affect proliferation of the chondrocytes^[68]. Interestingly, *Cbfa1* induction might inhibit *Hoxa2* caused impairment of osteogenic differentiation *in vivo*^[67]. In addition, overexpression of *HOXA2*, and expression of *HOXA3* and *HOXB4*, abolishes the ability of neural crest cells in the rostral domain to differentiate into skeletal structures in chicken^[69]. The expression of certain *Hox* genes seems to inhibit skeletogenic differentiation, particularly jaw formation. In cranial neural crest cells there are specific regions where *Hox* expression or suppression, respectively is essential for appropriate development. In detail *Hoxa2* inhibits *Sox9*, resulting in an impeded development of neural crest cells into ectomesenchymal stem cells and thus no osteochondral progenitors can form in mice. The absence of *Hoxa2* leads to a migration of *Sox9* into areas where *Hoxa2* is normally expressed which induces ectopic chondrogenesis^[70]. In addition, *Hox* genes suppress osteochondrogenesis in more posterior neural crest cells *via* epigenetic mechanisms^[32]. *Hoxa2* exerts an inhibitory effect in differentiation of chondrocytes, but is needed during bone formation. This effect is not related to the patterning function of *Hoxa2*, since it occurs in various tissues independent of their embryonic origin. Moreover, the ossification process itself is not affected by the *Hoxa2* misexpression^[68].

Hoxc8 as well as *Hoxd4* regulate chondrogenesis in a dose dependent manner in mice. *Hoxc8* does not block chondrogenesis, but alters the progression of chondrocytes and their differentiation pathway, leading to an accumulation of proliferating precursor cells, which negatively interfere with the progression of differentiation. A transgenic model for *Hoxc8* and *Hoxd4* signaling in chondrogenesis has been suggested, but the proposed effects have not yet been proven in *in vitro* culture systems with postnatal derived chondrocytes^[71,72].

In *Hoxa11* and *Hoxd11* double mutant mice, chondrogenesis is initiated normally. Pre-chondrocytes express *Sox9* and type II collagen necessary for further differentiation. However, mesenchymal condensations, which later give rise to the radius and ulna, differ in shape and size from non-mutants^[73]. Prehypertrophic cells within these condensations do no longer undergo their differentiation pathway towards hypertrophic chondrocytes. *Hoxa11* and *Hoxd11* double deficient

mice have shorter limb formation and development postnatal, together with delayed ossification, suggesting that these two *Hox* genes are important regulators in mammalian chondrogenesis^[73]. This is in line with the finding that *HOXA11* is expressed only in tissues which do not undergo chondrogenesis in chicken^[74].

Also, *Hox* genes generate a positional memory in skeletal stem cells, influencing their differentiation potential. Murine skeletal progenitors derived from the mesoderm maintain *Hoxa11* expression, whereas mandibular bone progenitors derived from the neural crest are negative for *Hox* expression. Thus an appropriate expression of *Hox* genes provides instructions for site-specific differentiation and function of stem cells *in vivo* and *in vitro*^[75].

Similar as already discussed for the adipogenic lineage commitment, the expression pattern of *Hox* genes in stem cell differentiations towards the osteogenic lineage varies according to the extraction site of the adipose tissue-derived stem cells. *Hoxa4* and *a5* expression is decreased in flank-derived MSCs differentiated towards osteoblasts, compared to the level in abdomen-derived stem cells, but *Hoxa4* is dominantly expressed in arm-derived stem cells. *Hoxc8* and *Hoxd4* on the other hand show a similar expression pattern in stem cells derived from the abdomen, arm, flank or thigh^[76].

Genes within the D family of the *Hox* cluster are suggested to play a role in the condensation of MSCs and the further development into chondrocytes. In detail, *HOXD9* and *HOXD13* were found to be distinctly expressed in developing chondrocytes within MSCs from the chicken limb bud. The inhibition of *HOXD10*, *D11*, and *D13*, respectively, inhibits chondrogenesis^[77]. This is in line with the finding that *HOXD11* was found to act on initial cartilage condensation phases and later growth phases within the bone of chicken, whereas *HOXD13* acts at later stages of bone development. Correspondingly, misexpression of *HOXD13* is associated with a shortening of long bones^[78]. This is supported by data derived from mice where *Hoxd13* and to a lesser extent *Hoxd12* were found to be essential for bone formation. A defect in one of these *Hox* genes leads to limb malformations and shorter bones^[79]. Especially *Hoxd13* seems to be responsible for the genetic switch from long bone to short bone formation^[80]. But also *Hoxd10* affects mouse limb development by impairing proper growth of skeletal elements^[81]. Large fractures heal by processes very similar to bone formation during development, apart from remodeling and inflammatory responses^[5].

The strict *Hox* gene patterning that could be observed in *Drosophila melanogaster* is even more complex during bone formation in vertebrae. In *D. melanogaster* genes closer to the DNA 5'-end are expressed strictly posterior and the more 3' located *Hox* genes are strictly anterior. In agreement with that a knockout of *Hoxd9* has revealed a more posterior impaired growth

in the spine of mice than a knockout of *Hoxd13*^[82].

As already mentioned *Hoxd13* is more essential for proper bone formation than *Hoxd12*, but defects in either of these *Hox* genes lead to a shortening of bones and limb malformations^[79,81]. However, functions missing in knockout mice lacking a certain *Hox* gene may be compensated by other *Hox* genes. Hence, the severity when lacking either one of two *Hox* genes may be far less than if both *Hox* genes are knocked-out^[82,83]. Cases where a *Hox* gene on both alleles is dysfunctional always show more severe bone malformations^[79,81]. Also, stem cells from various sources show an origin-specific *Hox* expression profile. Therefore, it is of great importance to match the *Hox* code of the cells subject to transplantation to the tissue of interest. It has been shown that USSCs, which are *in vivo* *Hox* negative and lack the potential to differentiate into the adipogenic lineage, gain this potency after these cells have been altered in their *Hox* expression profile in a co-culture model with CB-MSCs or bone marrow-derived stem cells. This however also affects the chondrogenic and osteogenic differentiation potential of USSCs^[84]. The positional memory may hence be altered under the influence of *Hox* genes, but side effects have to be taken into consideration.

An indirect link between *Hox* expression and bone formation was examined recently. BMP4 (bone morphogenic protein) as well as BMP7 elevates the expression of KDM6B, which then causes a removal of H3K27me3 that normally inhibits *Hox* expression. Even though BMP is among other tasks directly responsible for bone formation this secondary function might further aid in the development^[85].

A well understood example for how *Hox* genes are involved in bone formation is *HOXA10*. Again a BMP, here BMP2 induces *HOXA10* which subsequently interacts directly with RUNX2, a main and early regulator of osteogenesis-specific gene expression^[86,87]. This is supported by the finding of Gordon and colleagues^[88] that Pbx1 is able to repress this *Hoxa10*-mediated osteogenic differentiation by blocking recruitment of chromatin remodeling factors and that overexpression of *Hoxa10* increased the expression of osteoblast-related genes, osteoblast differentiation and mineralization^[89].

Once bone and cartilage have been formed, they are connected to the musculature *via* tendons. Influencing the proper development and regeneration of tendons and striated muscle is therefore of great medical importance. However, little is known to date about the involvement of *Hox* genes in their developmental processes. The first and only data are about the autopodal *Hox* genes *HOXA13* and *HOXD13* inducing *Six2* expression, which is a transcription factor present in developing tendon precursors, even in areas where it normally would not occur, such as the zeugopods of a chicken limb-bud^[90]. Further investigations are therefore required to be able to improve overall limb regeneration with the help of stem cells under the influence of *Hox* genes.

Hox expression in cardiovascular lineages

Regeneration and repair of the cardiovascular system is a major field of research in the scientific community, since it is the main cause of death with up to 50% in industrialized countries^[91]. After transplantation of human bone marrow-derived stem cells into a murine adult heart, a limited number of cells survived and over time showed morphological and molecular changes, resembling the phenotype of host cardiomyocytes^[92]. This has been reproduced in various studies and with a variety of donor tissues and hosts *in vivo* and *in vitro*^[93,94]. Thus MSCs hence provide a valuable source for repairing injured myocardium in theory, but the underlying mechanism of differentiations towards myocytes and the cells of the blood vessels, *e.g.*, endothelial and smooth muscle cells, has to be understood thoroughly to improve the outcome of such treatments. Knowing about changes in *Hox* gene expression in stem cell types and animal models may in the future help improve the differentiation of MSCs into cardiomyocytes for the transplantation into patients.

HOXA1 deficiency can cause cardiovascular diseases in mouse models as well as in human patients, which suggests its role in cardiomyocyte differentiations^[75,94-98]. TCDD can inhibit mouse embryonic stem cell differentiation towards cardiomyocytes. During this inhibition *Hoxa10*, *a13*, *a7*, *Hoxb9*, *Hoxc8*, *c9*, *Hoxd1*, *d4*, *d9*, *d13* are upregulated, whereas *Hoxb1* is down-regulated. In detail, *Hoxb4* and *Hoxd3* are upregulated with a lower dosage of 10 pmol/L TCDD and down-regulated at a dosage of 100 pmol/L TCDD. *Hoxa9*, *Hoxb3*, *b8*, *Hoxc6* are down-regulated at a dosage of 10 pmol/L TCDD whereas upregulated at a dosage of 100 pmol/L TCDD^[99]. In accordance to these findings, *Hoxa10* has been found to impair murine cardiac differentiation *in vivo*^[100].

In the vasculature, *HOXA11* and *A13* impact myogenic differentiation, as they repress MyoD expression and therefore initiation of myogenic differentiation in chicken limb muscle precursors transfected with these *Hox* genes^[101]. Retention of *Hox* gene expression profiles and positional identity has been found in muscle cells and MSCs *ex vivo*^[75,95-98].

The genes of the paralog groups *Hox6-10* show a higher expression pattern in smooth muscle cells (SMCs) of the athero-resistant thoracic aorta compared to SMCs of the athero-susceptible aortic arch in mice, rat, and pork. This pattern is consistent in human ESCs which were differentiated into precursors of the thoracic aorta and the aortic arch, respectively, which also suggests a possible involvement of the above mentioned *Hox* genes in specific SMC differentiation processes of ESCs^[102]. In vascular wall-resident multipotent stem cells (VW-MPSCs), *HOXB7*, *HOXC6* and *C8* are expressed in high levels compared to human pluripotent ESCs, mature aortic smooth muscle cells and human umbilical cord endothelial cells^[50]. Silencing of these *Hox* genes in VW-MPSCs leads to a

reduction in their in-gel (Matrigel) sprouting capacity, hence suggesting a decreased differentiation potential without affecting proliferation^[50]. Finally it has been found that in myogenic cells *Hox* gene expression is down-regulated *via* hypermethylation^[103].

Changes in *Hox* gene expression over the differentiation time course can also be observed in the stem cell differentiation towards endothelial cells. When mouse ESCs are differentiated towards endothelial cells, *Hoxa3* and *Hoxd3*, which drive angiogenesis and endothelial cell sprouting in adults, show a peak expression at day three of differentiation. Their expression declines over the following days of differentiation, whereas *Hoxa5* and *Hoxd10* arise from a low expression profile at day three of differentiation to a high expression profile over the course of time. In addition, these two *Hox* genes are involved in maintaining a mature quiescent endothelial cell phenotype^[22]. This suggests an involvement of *Hoxa5* and *Hoxd10* in the differentiation of ESCs towards endothelial cells, whereas *Hoxa3* and *Hoxd3* rather mark an immature angiogenic endothelium and/or the onset of angiogenesis^[22]. This has been confirmed by others who found that *Hoxd10* suppresses angiogenesis, while *Hoxd3* and *Hoxb3* promote angiogenesis^[104,105].

Interestingly, the expression profile of *Hox* genes in human ESCs from leukemia prone infants is changed which might account for the impairment of hematopoietic cell differentiation in favor of endothelial cell fate. In these cells, *HOXA9*, *A13*, *HOXB2*, *B3*, *B4*, *B5*, and *B6* are strongly up-regulated^[106]. Next to this, *HOXA9* is down-regulated in endothelial cells derived from endothelial progenitor cells from systemic sclerosis patients, suggesting an involvement of this *Hox* gene in endothelial cell differentiation or mal-differentiation^[107].

During the differentiation of human bone marrow-derived MSCs towards endothelial cells four *Hox* genes were found to be significantly altered in their expression profile. *HOXA7* and *HOXB3* expression is increased, whereas the expression of *HOXA3* and *HOXB13* ceased^[108]. Also, *HOXB5* up-regulates vascular endothelial growth factor receptor-2 which leads to an augmented endothelial cell precursor differentiation. Consequently, *HOXB5* has also been proven to enhance endothelial cell sprouting and coordinated vascular growth in chicken^[109]. A variety of *Hox* genes have already been found to influence cell fate in favor of endothelial and smooth muscle cell lineages, but the exact mechanisms need to be made clearer in order to use them as transcription factors in regenerative therapies.

Hox expression in neurogenesis

Neurodegenerative diseases are also becoming more and more prominent within an aging population. Repairing the neurological systems within the body has been difficult ever since, due to its complexity. A detailed understanding of molecular processes involved in the development and differentiation of neuron precursors is of crucial

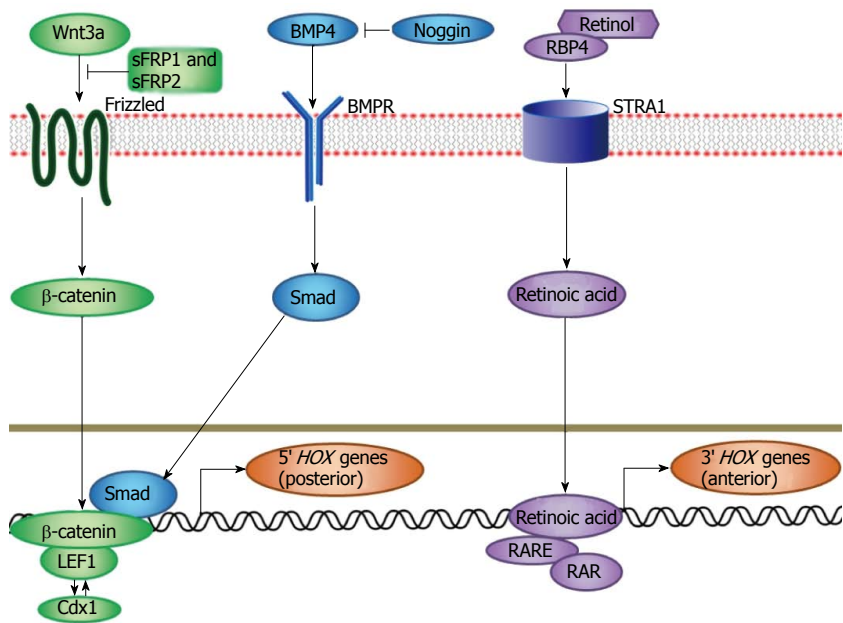


Figure 2 Major signaling pathways involved in *Hox* gene expression. Predominantly posterior 5' *Hox* genes are activated by BMP and Wnt signaling. *Hox* genes closer to the 3' end of a chromosome, which are mainly expressed anteriorly, are activated by retinoic acid pathways^[120,141-150]. BMP: Bone morphogenic protein; RARE: Retinoic acid response element.

Table 2 <i>Hox</i> genes play a role in various differentiations		
Differentiation	<i>Hox</i> gene expression	Ref.
Adipogenesis	<i>a4, a7, d4, A1, A4, A5, A10, B8, C4, C8</i> ↓, <i>C13</i>	[60-63]
Chondrogenesis	<i>a2</i> ↓, <i>a11, c8</i> ↓, <i>d4</i> ↓, <i>d11, D9</i> ↑, <i>D11, D13</i> ↑	[65,66,70-73,77,78]
Osteogenesis	<i>a2</i> ↑, <i>a10, d9</i> ↓, <i>d12, d13, A10</i>	[82,86,87,89]
Tendon differentiation	<i>A13, D13</i>	[90]
Cardiomyocyte differentiation	<i>a10, A1</i>	[75,95-98,100]
Smooth muscle cell differentiation	<i>a6-10, b6-10, c6-10, d6-10, A11, A13, B7, C6, C8</i>	[50,101,102]
Endothelial cell differentiation	<i>a3</i> ↓, <i>a5</i> ↑, <i>d3</i> ↓, <i>d10</i> ↑, <i>A7</i> ↑, <i>A9</i> ↑, <i>A13</i> ↑, <i>B2</i> ↑, <i>B3</i> ↑, <i>B4</i> ↑, <i>B5</i> ↑, <i>B6</i> ↑, <i>B13</i> ↑	[20,106-108]
Neurogenesis	<i>a2, b1, B4</i>	[110-112,114-116]

Small capitals: Murine; Large capitals: Human and chicken; ↑: Up-regulation; ↓: Down-regulation.

importance. Since *Hox* genes control the anterior-posterior (AP) segmentation, the 3' genes in the *Hox* cluster are more prone to play a role in neurogenesis, and the paralog groups 1-4 have restricted expression borders in the hindbrain. The remaining paralog groups 5-13 reveal an anterior expression border in the spinal cord. This AP segmentation translates to the migration of neural crest cells^[12]. In mouse models, *Hoxb1* is essential for the correct development of rhombomeres at early embryonic stages, since it naturally induces neural stem cells towards a hindbrain identity, and has also been shown to be involved in the differentiation, maturation and maintenance of facial nerves^[110,111]. Murine *Hoxa2* and *Hoxb1* can individually and as the sole additional factor induce motor neuron development, even in sections where they are usually not present^[112]. In mice deficient for *Hoxb1* expression, facial neurons do not properly form^[113]. Also, *HOXB4* has been found to be a crucial factor in driving neuronal differentiations in the neural tube^[114]. Further, since the

disruption of *Hoxa1* in mice leads to malformations of the hindbrain, a role for this *Hox* gene in murine neurogenesis has been suggested^[115,116]. Retinoic acid (RA) has been established as an essential factor for adult neurogenesis. Embryonic stem cells treated with RA almost exclusively differentiate into neurons and develop a *Hox* expression profile^[117]. Many *Hox* genes contain retinoic acid response elements (RAREs) which supports the finding that they are involved in RA-induced differentiation of ESCs^[118]. RAREs have been found in *Hoxa1, a4, Hoxb1, b4*, and *Hoxd4*^[12,119-128]. In fact, several *Hox* genes have been found to be strongly up-regulated in differentiations where RA was present^[118]. The interaction between RA, *Hox* genes, and other signaling molecules has been visualized in Figure 2. During embryonic stem cell differentiation, highly conserved elements in the *Hox* gene clusters are epigenetically modified, so that adult spinal cord progenitors express a distinct *Hox* expression profile^[129-131]. The further investigation of *Hox* genes in neurogenesis therefore promises a better insight into the possible

therapies to treat neurodegenerative diseases and should be pursued.

CONCLUSION

Degenerative diseases are one of the major problems arising within a population with high average age. The complex biological systems in the human body have made the development of regenerative therapies a challenging project. Much effort has been put into using stem cells and various signaling molecules for this purpose over the last decades. In this review, we have discussed the expression of *Hox* transcription factors in various types of stem cells and their role in differentiation. Interestingly already the stem cell source has an impact on the *Hox* gene expression in the respective stem cell type. *Hox* genes provide valuable possibilities to influence stem cell differentiations. Either single *Hox* genes or small groups have been found to directly influence the course of several differentiations, but detailed molecular changes under this influence are mostly not yet known. Additionally, many *Hox* genes have only revealed an indirect effect, or have been investigated in *in vivo* development but not in stem cell differentiations. Future studies need to be performed to investigate whether the suggested *Hox* genes, as summarized in Table 2 for mesenchymal stem cell lineages, actually have a functional role in the *in vitro* differentiation of stem cells. Additionally, the effects must be tested *in vivo* to evaluate their therapeutic potential in tissue repair and regeneration^[132]. As a consequence of the observed results it is suggested that cells which have their *HOX* code deleted regain plasticity and can be reprogrammed, similar as it has already been shown in iPS cells^[133,134]. In addition to this and similar to iPS cells, also the epigenetic profile must be considered. We summarized that next to the histological compatibility of donor and host tissues the *HOX* code must be taken into consideration in regenerative therapies. In conclusion, the further investigation of *Hox* genes in stem cell differentiations is an interesting research field in regenerative medicine to improve tissue repair in injury, disease and aging.

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Use of human pluripotent stem cells to study and treat retinopathies

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Abstract

Human cell types affected by retinal diseases (such as age-related macular degeneration or retinitis pigmentosa) are limited in cell number and of reduced accessibility. As a consequence, their isolation for *in vitro* studies of disease mechanisms or for drug screening efforts is fastidious. Human pluripotent stem cells (hPSCs), either of embryonic origin or through reprogramming of adult somatic cells,

represent a new promising way to generate models of human retinopathies, explore the physiopathological mechanisms and develop novel therapeutic strategies. Disease-specific human embryonic stem cells were the first source of material to be used to study certain disease states. The recent demonstration that human somatic cells, such as fibroblasts or blood cells, can be genetically converted to induce pluripotent stem cells together with the continuous improvement of methods to differentiate these cells into disease-affected cellular subtypes opens new perspectives to model and understand a large number of human pathologies, including retinopathies. This review focuses on the added value of hPSCs for the disease modeling of human retinopathies and the study of their molecular pathological mechanisms. We also discuss the recent use of these cells for establishing the validation studies for therapeutic intervention and for the screening of large compound libraries to identify candidate drugs.

Key words: Drug screening; Human pluripotent stem cells; Disease modeling; Retinitis pigmentosa

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Core tip: Human pluripotent stem cells (hPSCs) are usually evoked for their potential for regenerative medicine. However, beside these aspects, novel interests raise from the potential of hPSCs to model human diseases including retinopathies. In this review, we describe how these cells allow the study of the molecular mechanisms leading to some form of retinopathies through the development of retinal specific cell type derived from the differentiation of hPSCs. We also discuss the use of hPSCs cellular models for the validation of gene therapy and for drug screening purpose.

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INTRODUCTION

Retinal diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are the leading causes of blindness in the developed world, collectively affecting to different degree as many as one-third of all people over the age of 75^[1-3]. This burden is predicted to increase with the ageing of the population in Western countries.

RP is one of the most common inherited diseases of the retina (retinopathies). It is estimated to affect 1 in 3500 to 1 in 4000 people in the United States and Europe. Genes involved in RP are numerous (<https://sph.uth.edu/retnet/disease.htm>) and can affect specifically retinal pigment epithelium (RPE) or photoreceptors or both. Forms of RP and related diseases include Usher syndrome, Leber's congenital amaurosis, rod-cone disease, Bardet-Biedl syndrome, and Refsum disease, among others^[4]. RP is traduced clinically with a gradual decline in vision as a consequence of photoreceptor cells (rods and cones) dying either from cell autonomous origin or secondary from a defect of RPE cells, which support their survival.

Up to now, there is no curative treatment for these pathologies. Among others (for review, see^[5]), the more promising areas of research for RP therapies include gene therapy aimed at restoring defective genes, and cell transplantation to replace defective or dead cells.

Gene therapy has been shown to improve visual function in inherited retinal diseases^[6,7]. Most gene therapies involved integration of vector DNA into the specific cells of retina^[8,9] and even combine RNA interference (RNAi)-based gene silencing with gene replacement in RP^[10,11]. While promising, gene therapy aiming at restoring gene defects in the RPE (like *RPE65*) shows limitations. Indeed, the prerequisite to this type of gene therapy is to know precisely the defective gene (need the genotyping of each patient) and a second limitation is to develop a gene delivery candidate drug for each one of the defective genes (cost-intensive). Moreover, recent data from preclinical and clinical studies show that, despite stabilization of vision during the follow-up period, patients treated by *RPE65* gene therapy still undergo photoreceptor degeneration^[6].

The other strategy consists on the replacement of died or defective cells by transplantation procedures. The eye has numerous advantages for developing cell therapies as all tissues of the eye are surgically accessible, transplanted cells can be monitored by microscopic analysis and the inherent amplification of the visual system means that relatively small number of rescued cells or transplanted cells could

have a detectable effect on vision. Moreover the ocular immune privilege might greatly simplify immunosuppressive treatment after transplantation. Initial subretinal transplant studies employed tissue-specific stem cells obtained from human fetuses, known as retinal progenitors^[12]. Extension of this work revealed that post-mitotic photoreceptor precursor cells are optimal for retinal integration and restoration of retinal structures^[13-16]. Unfortunately, in human embryonic development, both retinal progenitor and photoreceptor are generated relatively late (*i.e.*, 14 and 24 wk of gestation respectively^[17-20]). The serious ethical implication of this fact, coupled with the practical reality of the relatively small number of photoreceptor precursor cells that can be obtained from a pair of fetal donor eye make it quite unlikely that this cell will be used to any meaningful extent in the treatment of human retinal disease.

In that context, scientists developed new strategies to overcome these constraints. Moreover, a better comprehension of the different molecular mechanisms leading ultimately to RP or AMD is crucial in order to find new treatments. Current animal models of RP are not available for the hundreds of causal mutations. That's why human pluripotent stem cells (hPSCs) lines are rapidly changing the strategies scientists can implement to understand pathological mechanisms and cure genetic diseases.

In this review, we focus on the different opportunities concerning the use of human pluripotent stem cells for the disease modeling as well as the future challenges of such research. We also develop the recent improvements in the genetic manipulation of human pluripotent stem cells and the consequences of these on disease modeling and drug screening for retinal diseases. Finally the availability of such human cellular models allowing the validation of therapeutical strategies as unexpected as gene therapy will be discussed.

hPSCs

Two main sources of hPSCs are described: human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). hESCs are pluripotent cells harvested from the inner cell mass of day-5 human blastocysts that are leftover from *in vitro* fertilizations^[21]. hiPSCs are reprogrammed somatic cells that share many similarities with hESCs^[22,23]. Pluripotency is key to the derivation of cell phenotypes that are relevant for disease, including those that are essentially inaccessible in any other way (*i.e.*, post-mitotic retinal cells). Unlimited self-renewal provides easy access to the biological resource of interest^[24].

iPSCs have been generated from a range of primary cell types, including fibroblasts, keratinocytes, lymphoblasts, chord blood cells, mesenchymal stem cells and even RPE cells (Figure 1). Each cell type has its own characteristics in terms of the number of factors needed for reprogramming

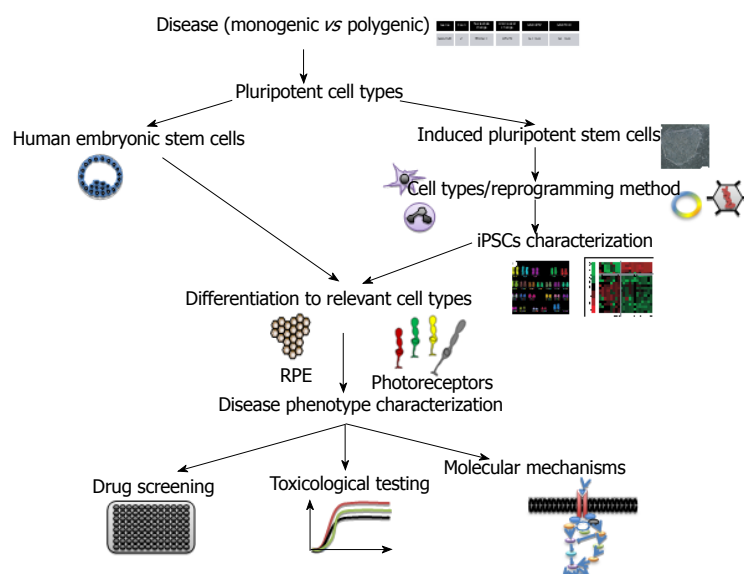


Figure 1 Schematic diagram summarizing the issues to consider when modeling retinal diseases with human pluripotent stem cells. Choice of disease (monogenic vs polygenic); Type of pluripotent stem cells (hESCs and/or iPSCs); for iPSCs: selection of cell type to reprogram (fibroblasts, keratinocytes, blood cells), selection of reprogramming method (retrovirus mediated, non-integrative); choice of quality control for iPSCs characterization (genetic analysis, pluripotency, teratoma assay); differentiation to relevant cell types (photoreceptors, RPE); disease phenotype characterization (molecular characterization, expression studies, genetic rescue); applications (drug screening, toxicological testing, new molecular mechanisms). hESCs: Human embryonic stem cells; iPSCs: Induced pluripotent stem cells; RPE: Retinal pigment epithelium.

and the dynamics of derivation (for review^[25,26]). The generation of iPSCs from older individuals is more difficult and less efficient than the generation of iPSCs from young individuals^[27]. To help overcome this limitation, a variety of different reprogramming factors, reprogramming enhancers, and cell types have been evaluated^[27-39] (Figure 1). Of the accessible cell types used for iPSC generation, the reprogramming of keratinocytes has been shown to be as much as 100-fold more efficient and at least twofold faster than the reprogramming of dermal fibroblasts^[40]. Moreover, the keratinocytes-derived iPSCs are more similar to embryonic stem cells than those generated from fibroblasts^[41]. Finally, for disease modeling, non-integrative methods should be preferable to integrative ones even if less mandatory than for cell therapy programs.

hPSC-derived retinal cells are valuable new tools for investigators seeking to understand and treat degenerative retinal diseases. These cells will allow scientists to explore the pathophysiology of human diseases in ways that were previously possible only in some animal models. The need for better models, given our poor understanding of the pathogenesis of complex diseases and the dismal predictive record of animal models and tumor-derived cell lines push towards the use of hPSCs for modeling diseases in a dish.

DISEASE MODELING OF RP

Any disorder confirmed or suspected to have genetic basis can be modeled, but the choice of which disease to model depends on a number of considerations^[42]. Not all diseases will be equally easy to model. For example, one should consider modeling monogenic and early-onset diseases than polygenic and late-onset diseases, as it would reproduce better aspects of the diseases (Figure 1). Monogenic diseases modeling will allow for a more carefully controlled comparison with genetically corrected cells and demonstration that the disease

phenotypes observed are really caused by the original mutation. Commercial and academic repositories offer primary cell lines or immortalized cell lines for use in research. Most, if not all, disease-causing mutations will be specific to the cell context and affect some cell types more than others. For certain purposes, working with cell lines could be difficult even impossible. This is the case for most retinopathies affecting cells that are not available for biopsies. Genetic disorders of the eye are so numerous that multiple examples exist of conditions that primarily affect photoreceptor cells, ganglion cells, RPE cells, retinal vasculature, choroidal vasculature and eye development. hPSCs systems can offer the possibility to model disease that show a non-cell-autonomous component as both component (*i.e.*, RPE and photoreceptors) can be differentiated from hPSCs and then eventually co-cultured.

Several groups attempted to reconstitute retinal tissue *in vitro* using mice or human cells. A variety of different protocols, utilizing both two- and three-dimensional culture systems, have succeeded in deriving photoreceptor precursor cells from less differentiated precursors^[43-60]. *In vitro* generated organs from hPSCs constitute powerful systems to model human diseases and perform large-scale drug screening.

Within the spectrum of primary retinal disorders, genetic diseases of the RPE are perhaps the most promising candidates for hiPSCs and re-seeded onto a variety of substrates^[61]. RPE cultured from multiple sources, including hPSCs, has been shown to adopt a mature phenotype, and exhibit key physiological functions *in vitro*^[47,62-64].

EXPLORING MOLECULAR MECHANISMS OF RP

Several studies have been published describing disease modeling using hPSCs. Singh and collaborators recently developed a hiPSC-RPE "model in a dish" of Best

vitelliform macular dystrophy (BVMD)^[65]. BVMD is caused by a defect in the RPE gene *BEST1*, which results in the subretinal accumulation of photoreceptor waste products, *i.e.*, lipofuscin, and fluid, leading to secondary photoreceptor death and central vision loss. Using physiological stressors, they reproduced some of the cellular defects observed in the disease and suggested a role of intracellular calcium regulation and oxidative stress in the mechanism of disease^[65]. Similar conclusions were made with other RP models^[66] involving endoplasmic reticulum stress. In this study, several hiPSCs lines were created from 5 patients carrying mutations in different genes involved in RP (RP1, PRPH2, RHO, RP9). After 120-150 d of culture, mature photoreceptors (Rhodopsin positive) derived from all cell lines died, reproducing features observed in the diseases. One line, RP9, showed a much lower level of rhodopsin expression and has higher levels of oxidative stress. Using a slightly different differentiation protocol and using integration-free hiPSC, this research team and others confirm that patient-specific rod cells may recapitulate RP features *in vitro* and could be appropriate model to study retinal degenerative diseases^[67,68]. hiPSC-derived retinal precursors were also used to identify a likely disease-causing homozygous mutation in a gene that had not been previously reported to be associated with disease^[50]. In a more recent study, the same group combined Next-Generation and Sanger sequencing to identify disease-causing USH2A (responsible for Usher syndrome type I) mutations in an adult patient with autosomal recessive RP^[51]. Moreover, by grafting the mutated photoreceptors into mice model of the disease, they showed that the mutation might act *via* post-developmental photoreceptor degeneration rather than during development.

Finally, hPSCs could model systems to study retinal development and implication of specific transcription factors. Phillips *et al.*^[69] (2014) generate hiPSC from a patient with a mutation in the transcription factor visual systems homeobox 2 (VSX2 also known as CHX10) homeodomain. They showed that, using differentiation protocols in order to obtain optical vesicles (OV), mutated hiPSC-OV failed to produce bipolar cells and demonstrated delayed photoreceptor maturation^[69].

All these studies highlight the potential of retinal cells derived from hPSCs to help to identify pathophysiological pathway for targeted development of therapies and decipher key factors in retinal development.

GENE EDITING AS A NEW TOOL TO ACCESS TO HUMAN DISEASE CELLULAR MODELS

The identification and functional validation of sequence variants affecting diverse human traits, including disease susceptibility, is essential for the understanding of human biology and disease mechanisms. Considering the limited number of individual lines often used to model disease mechanisms and the high incidence of line-to-

line variations, the possibility of wrongly attributing to a disease-causing mutation a phenotype that is in fact the result of the uneven distribution of such alterations in control and diseased groups needs to be considered seriously. When disease-specific hPSCs are used to identify novel aspects of disease mechanisms, rather than simply to replicate already known pathological features, internal (isogenic) control experiments such as genetic correction or more classical gain- and loss-of-function experiments are extremely valuable^[24]. While classical gene-targeting technology *via* homologous recombination in mouse embryonic stem cells has proven a powerful tool to dissect gene function^[70-72], this approach has been quite inefficient when applied to hPSCs^[73]. The development of site-specific TALEN- or CRISPR-based genome editing approaches is proving to have great utility for endogenous gene correction in many cell lines^[74-76], including hPSCs^[77-79]. A major advantage of these approaches is that unlike exogenous gene addition in which promoter strength and multiplicity of infection may require patient-specific adjustment, TALEN- and CRISPR-mediated corrections have the advantage that the gene remains under the control of the endogenous promoter. Acting as "DNA scissors", they induce double strand breaks (DSB) at desired genomic loci, triggering the endogenous DNA repair machinery. Processing of DSB by the error-prone nonhomologous end-joining pathway leads to small insertions and deletions (Indels) useful for generating loss-of-function mutations, whereas error-free homology directed repair enables targeted integration of exogenously provided DNA sequences for introducing precise nucleotide alterations or knocking reporters.

Unlike other editing tools, the guide RNAs used for CRISPR/Cas9-based genome editing can be generated relatively easily. The guide RNAs used in this system requires the presence of an adjacent motif, downstream of a 17-20-nucleotide DNA target, termed the protospacer^[80-84]. Complementary guide oligonucleotides can be synthesized, annealed and subsequently ligated into a bicistronic vector-expressing scaffold RNA and a modified Cas9 nuclease optimized for efficient targeting of human cells^[85,86]. Recent study has shown that by combining these gene editing tools (*i.e.*, TALEN and CRISPR) they have developed a genome-engineering platform, named iCRISPR, that enable rapid and highly efficient biallelic knockout hPSCs for loss-of-function studies, as well as homozygous knocking hPSCs with specific nucleotide alterations^[87].

Another strategy could be to use adenovirus in order to replace the mutated gene in hiPSC derived from patients. This was done by Yoshida *et al.*^[68], who generated hiPSC from somatic cell of an RP patient carrying a heterozygous mutation in the rhodopsin gene^[88]. They observed that rod cells had reduced survival rate. To confirm that the phenotype was due to the expression of mutant rhodopsin and not by genetic polymorphism, they used helper-dependent adenoviral vector to replace the mutated gene and

reverted the phenotype observed. Moreover, replacing the wild-type gene reconstructed the pathological condition^[68].

HPSCS FOR THE VALIDATION OF THERAPEUTIC INTERVENTIONS

A number of potential therapeutic interventions are strictly specific to human cells or tissues, such as biotherapies based upon targeting human gene/mutation with RNA interference, exon skipping, and therapeutic antibodies. With these approaches, the uses of classic animal models of RP for experimental or preclinical validation are often not enough informative. Obtaining sufficient cellular material to conduct a wide range of tests, from the initial assessment of therapeutic claim to the final quality of assurance process before clinical batch release, can be challenging.

Some diseases are so rare that it is not economically possible to bring a treatment through the regulatory gauntlet and into clinical availability. One possibility to overcome this issue would be to use cells or tissues created from a patient's own cells to test the molecular efficacy of a viral-mediated gene therapy, including the optimal level of expression, and then deliver that therapy to one eye in a compassionate use manner. Moreover, some genes have a very narrow range of therapeutic dose such that overexpression could be as harmful as under expression^[89]. The first human gene therapy trial for a retinal disease was performed in 2008 in patients with Leber congenital amaurosis (LCA), a congenital blindness resulting from the breakdown of an important metabolic pathway in the RPE but with long-lasting preservation of the photoreceptors. LCA arises due to mutations in the RPE-specific gene, *RPE65*^[90]. Prior to clinical trials, preclinical trials are usually performed on animal models. Many mouse models have been generated that are defective for a gene causing a specific retinal dystrophy, however, certain of these models have proven asymptomatic^[91] or lethal^[92]. Moreover, in general, the mouse eye poorly mimics the situation in the human eye due to its small size and structure. Larger animal models are more appropriate for testing the biodistribution of a gene therapy vector and for ascertaining long-term efficiency and safety. The dog eye is particularly suitable as it resembles the human eye, except for the absence of a macula and foveal pit, in size, and many retinal diseases in man have canine counterparts^[93]. However, for certain diseases, the identification of a corresponding canine model has proven elusive. Thus, taken together, for a growing number of diseases (and this holds true for disorders affecting other tissues), human cell culture is becoming an essential complement to animal disease research^[94]. The results obtained from gene therapy studies in a human system, coupled to *in vivo* studies using animal models, could lead more rapidly to preclinical gene therapy studies indeed a phase I clinical trial.

For pigmentary retinopathies for which an appropriate animal model does not exist, like choroideremia^[95] evaluating the biochemical restoration of a normal cellular phenotype after gene therapy could provide complementary approaches to *in vivo* animal models that investigate systemic effects that are essentially beyond the reach of *in vitro* studies^[96].

hPSCs FOR DRUG DISCOVERY

Many drugs that work in animal models have not performed well in human clinical trials^[97,98]. This discrepancy could be attributable among other proposed reasons to species variation or incomplete phenotype recapitulation. Numerous studies have now showed that retinal cells derived from hPSCs are closely enough representatives of their *in vivo* counterparts^[60,99] that they could be used for identifying and evaluate pharmacological agents capable of mitigating degenerative retinal diseases. High-throughput drug screens of differentiated hPSCs-derived cells have been suggested as a means for drug discovery and personalized medicine^[100-102]. To date, there have been few publications looking at using hPSCs for drug discovery in retinal diseases. Meyer *et al.*^[47] reported the restoration of OAT (Ornithine Amino Transferase) enzyme activity in gyrate atrophy hiPSC-RPE following vitamin B6 treatment. In their study, Jin *et al.*^[66] showed that treatment of the rhodopsin-positive cells (photoreceptors) with α -tocopherol led to a significant preservation of these cells in RP9 cell line. This study provides proof of principle that hPSC technology is useful in screening for drug responses across numerous related, yet genetically distinct retinal diseases. Even if, major advances have been made to differentiate retinal progenitors from hPSCs, the differentiation effect could vary from various hPSC lines. In a very elegant study, Ferrer *et al.*^[103] developed highly efficient methods to differentiate, expand and authenticated iPSC-RPE in parallel to their use in high-throughput assay. The RPE cells were derived from iPSCs and grown in 96- and 384-well plates. They demonstrate differential expression of eight genes in iPSCs, iPSC-derived RPE at two differentiation stages, and primary human RPE using this multiplex assay. This assay provides the basis to screen for compounds that improve RPE function and/or maturation and target disease pathways^[103].

CONCLUSION

Regenerative medicine based on stem cells is likely to make important contributions to the cure of human blindness. This is the main focus of the public creating both fears and hopes. However, it remains only the visible part of the iceberg. Indeed, as described in this review, hPSCs are essential to model disease specific conditions closed to the patient context. They contribute to the development of pharmaceutical compounds that will be identified for efficacy in disease

specific cellular of hPSCs derivatives and for which toxicity will be tested in the same cells. Moreover, hPSCs provides valuable complement to animal models by either highlighting species-specific differences or further validating the observations already made in rodents or larger animals.

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Role of nitric oxide in the maintenance of pluripotency and regulation of the hypoxia response in stem cells

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Abstract

Stem cell pluripotency and differentiation are global processes regulated by several pathways that have been studied intensively over recent years. Nitric oxide (NO) is an important molecule that affects gene expression at the level of transcription and translation and regulates cell survival and proliferation in diverse cell types. In embryonic stem cells NO has a dual role, controlling differentiation and survival, but the molecular mechanisms by which it modulates these functions are not completely defined. NO is a physiological regulator of cell respiration through the inhibition of cytochrome c oxidase. Many researchers have been examining the role that NO plays in other aspects of metabolism such as the cellular bioenergetics state, the hypoxia response and the relationship of these areas to stem cell stemness.

Key words: Pluripotency; Differentiation; Nitric oxide; Metabolism; Hypoxia; Stem cell

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Core tip: Increasing research interest has focused on the role of nitric oxide (NO) in regulating many physiological functions such as metabolism, the hypoxia response, pluripotency, and stem cell differentiation. NO has been proven to act as a powerful agent for promoting the maintenance of cell pluripotency and survival, thus

explaining how it can act as an alternative factor for the maintenance of certain cultured cell lines.

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INTRODUCTION

Nitric oxide (NO) is a short-lived free radical synthesised *via* the L-arginine to L-citrulline pathway, which is mediated by nitric oxide synthase, NOS1 (neuronal NOS), NOS2 (inducible NOS), and NOS3 (endothelial NOS)^[1], in most animal cells^[2]. NO reacts with molecules such as oxygen, superoxide or metals, nucleic acids, and proteins. NO is quickly oxidised into nitrate and nitrite, which are now considered as non-inert products because they are a source of NO through their reduction by reductase enzymes when the endogenous L-arginine/NOS pathway is dysfunctional^[3]. In addition to serving as a germicide in the immune system as part of the inflammatory response and as a neurotransmitter in the central nervous system, NO acts as a second messenger and has multiple biological effects implicated in a variety of physiological functions in mammals, such as the regulation of blood pressure *via* smooth muscle relaxation and inhibition of platelet aggregation^[4-7]. Moreover, it has been shown that it affects gene expression at the level of transcription and translation and regulates cell survival and proliferation in diverse cell types^[8,9]. Furthermore, NO plays a role in growth, survival, proliferation, differentiation, as well as in the pathology of illnesses such as cancer, diabetes, and neurodegenerative diseases^[10,11]. In addition, it has been reported that NO is involved in the control of heart functions and cardiac development^[12,13].

The presence and concentration of other free radicals are critical factors that influence the effects of NO on cellular processes. For example, it has been described that low concentrations of NO inhibit cytochrome c oxidase (CcO), which catalyses the final step of the mitochondrial transport chain, competing with O₂ in a reversible manner^[14,15]. Nevertheless, high levels of NO may cause nitrosylation of protein thiols and perhaps the removal of iron from iron-sulphur centres^[16,17]. CcO, as catalyst of the central step of oxidative phosphorylation and adenosinetriphosphate (ATP) generation, regulates cellular oxygen consumption. The physiological concentration of NO modulates CcO activity, depending on the concentration of intracellular oxygen and the redox state of CcO. This interaction between CcO and NO allows the detection of changes in oxygen concentration and the initiation of adaptive responses. This indicates that NO

might be a physiological regulator of cellular respiration and metabolism. Furthermore, NO has been described to have an important role in regulating the hypoxia response^[15,18,19].

On the other hand, it has been reported that low concentrations of NO have a direct effect on processes such as cell proliferation and survival^[8]. In RINm5F cells homeostatic concentrations of NO (1-10 µmol/L) can initiate signalling pathways implicated in survival actions^[20]. Moreover, higher NO concentrations, induced by the inflammatory response, can cause oxidative and nitrosative stress, and apoptosis. These actions are partly responsible for cell death in chronic and degenerative diseases. Pharmacological treatment with high NO concentrations promote embryonic stem cell (ESC) differentiation^[9,21-23]. However, the functional significance of high NO concentrations on differentiation *in vivo* has not yet been demonstrated.

It has been clearly shown that NO has an important role as regulator of many physiological functions, and has thus become a target of interest in the fields of metabolism, the hypoxia response, pluripotency, and stem cell differentiation. This review aims to describe the progress on understanding the role of NO in these interrelated biological processes.

MECHANISM OF NO ACTION IN STEM CELL BIOLOGY

The downstream effects of NO can be mediated in cyclic guanosine monophosphate (cGMP) dependent or independent ways^[24-26]. When acting independently of cGMP, it has been shown that NO interacts with metal complexes, oxygen (O₂), super-oxide anion (O₂⁻) and CcO^[15]. These interactions have different effects depending on the amount of NO present. Protein nitrosylation and nitration can occur when NO interacts with oxygen species, which happens more frequently at high levels of NO^[27]. On the other hand, cGMP-dependent effects are mediated by the NO receptor, soluble guanylylcyclase (sGC). sGC is a heterodimeric hemoprotein composed of alpha (sGCα) and beta (sGCβ) subunits^[28]. NO activates sGC by interacting with its hemo group and catalysing the conversion of GTP into cGMP. cGMP controls a variety of physiological effects in several tissues by interacting with downstream effectors such as a family of cGMP-dependent protein kinases (PKG), cGMP-dependent phosphodiesterases, and cyclic nucleotide gated channels^[24]. In addition, triple NOS knockout mice, where NO production is abolished, have been shown to have a reduced survival rate and lower numbers of offspring^[29]. Thus, it was proposed that NO/cGMP signalling plays a significant role in embryonic development and cell differentiation. This hypothesis has gained additional support from studies that show differential expression and function of various NO signalling components in ESCs and differentiated cells^[25,26,30]. Although it remains unclear whether the

action of NO on stem cell biology is mediated *via* the cGMP pathway, it has been shown that the effects of NO on bone marrow stem cell potency and differentiation are independent of the sGC-cGMP pathway^[31,32]. Recent studies have also shown that NO can regulate protein function *via* the nitrosylation of sGC cysteine residues^[33]. Indeed, NO has been reported to modulate transcription factor function *via* Cys-nitrosylation^[34]. It was concluded that the effects of NO on stem cell pluripotency and differentiation are independent of the sGC-cGMP pathway^[5]. The mechanisms by which NO modulates the differentiation of ESCs remain unclear. Intriguingly, NO appears to act independently of the LIF/Stat3 pathway, as NO does not induce Stat3 phosphorylation in ESCs^[8]. Moreover, the expression of Nanog is LIF/Stat3 independent, and Nanog over-expression in the absence of LIF is able to maintain ESC pluripotency^[35].

NO REGULATES STEM CELL PLURIPOTENCY AND DIFFERENTIATION

The study of stem cells has gained considerable interest in recent years because they potentially offer an unlimited source of cells for therapeutic purposes. Many research groups have participated in efforts to expand and characterise populations of ESCs and induced-pluripotent stem cells (iPSCs), and to identify ways of directing their differentiation towards particular tissue types. In this context, the identification of small molecules that act on specific cell signalling pathways involved in embryonic development might be instrumental in the design of protocols that can efficiently control cell "stemness" and stem cell differentiation.

NO is, potentially, one such small molecule, with evidence supporting a dual role for it in the control of ESC differentiation and tissue morphogenesis. It is important to note that the effects of NO on differentiation and the maintenance of pluripotency are dose dependent. Moreover, while it is generally considered to be an inducer of apoptosis^[36,37], it has also been shown to protect certain cell types from programmed cell death^[38].

NO has been demonstrated to have the potential to influence the proliferation and differentiation cascades of certain cell types but its effect varies widely from cell to cell. For example, the proliferation and differentiation of human skin cells is modulated by NO^[39], a finding that helps explain the pathophysiology of human skin diseases. NO causes growth inhibition of immortalised human oral keratinocytes and primary oral cancer cells (HN4) mainly *via* apoptotic cell death induced indirectly by stimulating the differentiation of both immortalised and malignant oral keratinocytes^[2]. This suggests that the generation of NO in human skin diseases is not directly associated with local cell destruction, in contrast to observations in several other human diseases^[39].

Our group has reported that the exposure of ESCs to

low concentrations of the NO donor, diethylenetriamine NO adduct (DETA-NO), prevents the loss of expression of self-renewal genes and blocks mouse and human ESC differentiation. Similarly, constitutive NOS3 over-expression in cells grown under LIF withdrawal conditions maintained the expression of pluripotency markers^[8]. On the other hand, exposure to high concentrations of DETA-NO promotes the differentiation of mouse ESCs (mESCs) by down-regulating *Nanog* and *Oct4*, the two master genes that control the pluripotent state^[9].

Low concentrations of NO modulate pluripotency gene expression

It has been shown that low amounts of DETA-NO (2-20 μ mol/L) delay the differentiation of human embryonic stem cells (hESCs), since the addition of NO induces an increase in the expression of *Nanog*, *Oct4* and *Sox2* to levels even higher than when cells are cultured with basic fibroblast growth factor (bFGF). Moreover, the cell surface antigen SSEA-4, which disappears following five passages under bFGF withdrawal, is retained when the growth medium is supplemented with NO. In addition, NO represses some differentiation markers (*Brachyury*, *Gata4*, *Gata6*, *Fgf5* and *Fgf8*), which are present in the absence of bFGF. Similar observations were also seen in mESCs grown in the absence of LIF and supplemented with NO, which also induced *Oct4* and *Nanog* protein expression, together with a decrease in the expression of the aforementioned early differentiation genes (Figure 1). Constitutive over-expression of NOS3 in cells cultured in the absence of LIF protected them from apoptosis and promoting cell survival^[8].

NO has also been shown to play an important role in other cell types, such as multipotent resident cardiac stem cells and adult bone marrow cells (BMCs)^[40,41]. NOS3-generated NO plays an important role in cardiomyocyte proliferation and maturation during early neonatal heart development^[42]. Furthermore, a recent study using a porcine model has shown that activation of the NO pathway directs BMCs toward a preferential cardiomyogenic phenotype and also stimulates cell proliferation^[43].

Genomic studies carried out by our group indicate that low concentrations of NO in mESCs regulate apoptosis, survival and the hypoxia response, areas that will be looked at more closely in the remainder of this review.

Regulation of differentiation by high concentrations of NO

NO plays an important role in development: It has been reported that NO may act as an essential negative regulator of cell proliferation during tissue differentiation and organ development in *Xenopus* and *Drosophila*. In addition, NO production is crucial for the establishment of ordered neuronal connections in the fly's visual system, indicating that NO affects the

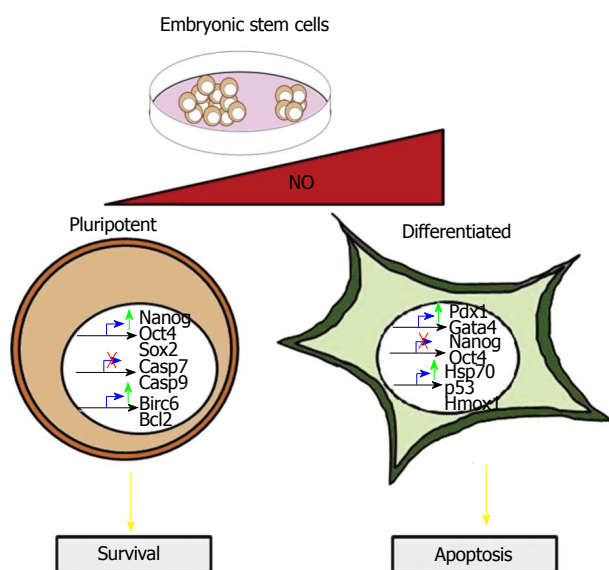


Figure 1 The dual roles of nitric oxide in stem cell pluripotency and apoptosis. Low amounts of nitric oxide (NO) induce the expression of Nanog, Oct4 and Sox2. This confers protection from apoptosis by down-regulating proapoptotic genes including Casp7, Casp9, as well as up-regulating the anti-apoptotic genes Bcl-2 and Birc6. High NO concentrations promote apoptosis markers, such as Poly (ADP-ribose) polymerase degradation, cleaved caspase-3, and increased p53 protein levels. Cells expressing *Hsp70*, a cyto-protective gene, are resistant to oxidative stress and enter into a differentiation program. Differentiation events are initiated by the down-regulation of Nanog and Oct4 and the expression of definitive endoderm markers, such as *Pdx1* and *Gata4*.

acquisition of differentiated neural tissue^[7]. Moreover, blocking NO production in neural precursor cells (NPCs) isolated from the ventricular zone of postnatal mice, resulted in increased proliferation^[44]. In ESCs, it has been demonstrated that NO generation is required for cardiomyogenesis, since NOS inhibitors prevent the maturation of terminally-differentiated cardiomyocytes (CMs). Moreover, it has been shown that the differentiation of mESCs toward a cardiac phenotype is arrested by NO synthase inhibitors, which can in turn be readily rescued by NO donors^[13]. NOS2 and NOS3 isoforms are prominently expressed during the early stages of cardiomyogenesis until NOS expression starts to decline around E14.5^[13]. In the same context, undifferentiated ESCs are known to express high levels of NOS3. During differentiation, NOS3 levels decrease, whereas NOS2, NOS1, sGC α 1, sGC β 1, and PKG levels significantly increase, suggesting that NO could be involved in the early differentiation events or physiological processes of ES cells or ES derived-cell lineages^[25].

On the other hand, high DETA-NO concentrations (0.25-1.0 mmol/L) promote mESC differentiation by down-regulating Nanog and Oct4 expression. The repression of Nanog by NO is dependent on p53 activation associated with covalent modifications, such as Ser315 phosphorylation and Lys379 acetylation. In addition, exposure to high DETA-NO concentrations (0.5 mmol/L) increases the expression of definitive endoderm markers, such as FoxA2, Gata4, Hfn1- β and

Sox 17 (Figure 1)^[9].

The use of NO in differentiation protocols for the generation of different germinal lines: The first report of an efficient differentiation protocol using mESCs treated with NO was published by Kanno *et al.*^[45] in 2004. They demonstrated that NO promoted differentiation in part through its effects on cell survival, leading to the differentiation of mESCs into CMs^[45]. Other groups have identified the role of cGMP signalling components and NO in cardiac development, as well as molecules that may regulate early cardiomyogenesis in ESCs *via* NO-dependent pathways, such as polyphenol curcumin and ascorbic acid^[25,46-48]. In addition, it has been shown that other signalling pathways besides NO are involved in oxytocin-stimulated cardiomyogenesis^[49].

It has been reported that NO is involved in vascular tissue differentiation. Because of the pivotal role played by NO in the biology of endothelial cells (ECs)^[50] and its role in angiogenesis^[51], it was hypothesised that it may also play a role in endothelial development. This was tested by studying the effect of differential expression of NO signalling components, or conversely of NOS inhibition, on endothelial differentiation. These studies indicate that NO is important for later endothelial development and function, although other signalling pathways appear to play a greater role in early development^[52]. Although differentiation protocols for generating ECs from ESCs do not include NO-like active small molecules, the expression of endothelial NO synthase in these cell types is considered to be a characteristic of mature ECs^[53,54]. Moreover, it has been demonstrated that NO/cGMP signalling molecules regulate neural lineage commitment and govern neural precursor differentiation, suggesting that NO/cGMP signalling contributes to the development of neural precursors and enhances the differentiation of precursors toward functional neurons^[55,56]. Furthermore, it was reported that NO exposure to NPCs leads to a decrease in neuronal differentiation paralleled by an increase in astroglial differentiation. Neuron restrictive silencing factor1/repressor element-1 silencing transcription factor has been shown to play an essential role in the regulation of neurogenesis and is required for NO-mediated neural to glial fate induction^[57].

It has been reported that NO modulates the growth and differentiation of human erythroid and myeloid cells from CD34⁺ BMCs *in vivo*^[58], promotes bone and chondrocyte terminal differentiation^[59], stimulates pre-adipocyte differentiation in rat^[60] and mediates osteoblastic differentiation^[61].

Bone marrow multipotent adult progenitor cells (MAPCs) can be purified and cultured from humans, rats and mice. MAPCs express Oct4 and are able to differentiate into multiple cell lineages including endothelial cells and neurons^[62,63]. Importantly, NO appears to play a key role in both the maintenance of MAPC multipotency as well as in promoting their endothelial differentiation. In fact, it has been shown that NO inhibits the proliferation of MAPCs when treated with high concentrations of different

NO donors. In addition, NO promotes the endothelial differentiation of MAPCs through a cGMP-independent-mechanism^[5].

A protocol for inducing the differentiation of ESCs into definitive endoderm has been developed in our laboratory. Exposure of mESC to 0.5 mmol/L DETA-NO induces early differentiation events with the acquisition of an epithelial morphology and expression of definitive endoderm markers, such as FoxA2, Gata4, Hfn1- β and Sox 17. This phenotype was increased when cells were also treated with valproic acid for 10 d^[9].

CONTROL OF APOPTOSIS AND SURVIVAL BY NO

Many reports have indicated that NO down-regulates pro-apoptotic response in mESCs and up-regulates anti-apoptotic responses by regulating Bcl2 family proteins^[64]. mESCs over-expressing Bcl2 are characterised by long-term maintenance of an undifferentiated state and pluripotency. In addition, these cells maintain their potential to differentiate into mature cell types^[65]. This role of NO depends on its local concentration. At low non-toxic levels, NO induces resistance to tumour necrosis factor- α (TNF α)-induced hepatotoxicity^[66], inhibits Fas-induced apoptosis in B lymphocytes^[67], and modulates CD95-induced apoptosis in T-lymphocytes^[68]. Moreover, in carcinogenesis, low NO levels can promote the development and growth of tumour cells, while high levels may be toxic to them^[2]. It has been proved that low concentrations of NO donors can protect murine bone marrow stromal cells against spontaneous apoptosis^[32].

We have reported that NO generation by NOS3 overexpression and low levels of DETA-NO contribute to the survival of pancreatic beta cells through the activation of IGF-1 and insulin-induced survival pathways^[23]. Our group has also reported that low level exposure (2-20 μ mol/L) to DETA-NO protects ESCs from apoptosis. These NO actions involve: (1) decreased caspase-3 activity, combined with the degradation of Poly (ADP-ribose) polymerase; (2) decreased expression of pro-apoptotic genes, such as *Casp7*, *Casp9*, *Bax* and *Bak1*; and (3) increased expression of the anti-apoptotic genes, *Bcl-2* and *Birc6* (Figure 1). Similarly, constitutive NOS3 over-expression in cells deprived of LIF protects them against apoptosis^[8].

On the other hand, several studies show that NO induces apoptosis in different cell types, including pancreatic beta cells^[69,70], thymocytes^[71], hepatocytes^[72], among others^[73,74]. It has been reported in ESCs that the nitrosative stress induced by exposure to high levels of DETA-NO, induces apoptotic events in part of the ESC population. Other parts of the ESC population are resistant to nitrosative stress and express the cytoprotective genes, heme-oxygenase-1 and Hsp70. Moreover, the resistant cells enter into a differentiation program (Figure 1)^[9].

NITRIC OXIDE, HYPOXIA AND STEM CELL METABOLISM

As we have seen in the previous sections, there is very clear evidence indicating that NO can regulate cell pluripotency and differentiation, but, as mentioned at the beginning of this review, the molecular mechanisms behind these functions are still unclear^[8]. The metabolic profile of pluripotent stem cells (PSCs) undergoes substantial changes during differentiation. Indeed, there is a shift from glycolytic to oxidative metabolism^[75]. On the other hand, cellular response to hypoxia is also involved in the control of pluripotency. Hypoxia affects many physiological processes during the early stages of mammalian ontogeny, as it is a key feature of this stem cell niche. Moreover, it has been reported that low oxygen tension and low NO concentrations prevent the differentiation of hESC colonies and are required to maintain the majority of cells within a colony in a fully pluripotent state^[8,76]. Therefore, in this review we will address the relationship between NO and the regulation of cellular respiration, as well as how they combine to effect cellular responses to hypoxia, energy metabolism and pluripotency.

PSC metabolism

PSCs have a short G₁ cell cycle phase, which acts to limit the growth and differentiation potential of PSCs^[77]. To facilitate rapid cell duplication, PSCs must balance energetic and biosynthetic demands, which is similar to what happens in cancer cells. Several studies have shown that mESCs, hESCs and iPSCs have an elevated dependence on glycolysis under aerobic conditions compared to differentiated cell types^[78-81]. In cancer cells and human PSCs, a high glycolytic flux provides sufficient ATP and anabolic precursors for rapid proliferation, with the pentose-phosphate pathway generating ribose-5-phosphate for nucleotides and NADPH-reducing power for nucleotide and lipid biosynthesis^[82,83]. Thus, aerobic glycolysis is a common feature of PSC and cancer cell metabolism in culture^[84,85].

During PSC differentiation, energy production is mainly obtained from oxidative phosphorylation. Studies on mitochondrial morphology and mitochondrial DNA have shown that ESCs contain fewer mitochondria, in a less mature state than in differentiated cells^[83]. The maximum human PSC respiration capacity is limited and it has been suggested that low expression of electron transport chain complex IV cytochrome c oxidase subunits, which donate electrons to oxygen, may be the cause of this decreased level of respiratory activity^[86].

Efforts are being made to understand the molecular mechanisms that regulate energy metabolism in PSCs and the changes that occur during differentiation or reprogramming events. Glycolysis-regulating enzymes, including hexokinase and lactate dehydrogenase, are highly expressed in PSCs and maybe controlled by the mammalian target of rapamycin and phosphoinositide

3-kinase (PI3K) signalling pathways, which regulate glycolytic genes in other cell contexts. Glycolytic and Mitochondrial Oxidative Phosphorylation (OXPHOS) pathway gene expression and DNA methylation patterns also change during pluripotency reprogramming^[80]. A number of possible mechanisms for the low levels of respiration and ATP production in PSCs have been reported, including limited pyruvate access to mitochondria due to an inactive pyruvate dehydrogenase (PDH) complex and the expression of uncoupling protein 2 (UCP2)^[78,81]. The same mechanisms have also been found in cancer cells. The PDH-mediated conversion of pyruvate to acetyl-CoA and its entry into mitochondria is blocked in hypoxic cancer cells by hypoxia inducible factor-1 α (HIF1 α), which induces pyruvate dehydrogenase kinase 1 (PDK1) expression and inactivates PDH phosphorylation^[87]. Also, several types of cancer overexpress UCP2^[87]. In addition to these examples, other mechanisms have also been proposed for the hypoxia response of PSCs but all with the same end result, *i.e.*, reduced energy production by OXPHOS and increased flux through the PSC glycolytic pathway.

HIF regulates pluripotency

PSCs have distinct metabolic requirements and cell reprogramming requires a shift from oxidative to glycolytic metabolism^[78-81]. However, it is still unknown how stem cells activate mitochondrial oxidative phosphorylation pathways during differentiation.

Human iPSCs are usually reprogrammed from somatic cells and are metabolically similar to hESCs^[78,80]. Therefore, a metabolic switch from an oxidative to a highly glycolytic state ought to take place during iPSC formation. The dependency of stem cells on glycolysis to produce ATP could be an adaptation to low oxygen tensions *in vivo*, given that hypoxia appears to be a key feature of "stemness"^[88,89]. Supporting this idea, low oxygen levels have been shown to be beneficial for hESCs, adult stem cells^[76,90-92] and cancer cells^[93,94].

HIF-1 α and HIF-2 α have essential roles during development. Increasing evidence suggests that HIFs can activate factors involved in pluripotency and regulate stem-cell properties in both cancerous and normal cells. For example, it has been suggested that HIF-2 α activates one of the core pluripotency master genes such as *Oct4*^[76,93-98]. This and other evidence indicate that HIF is a regulator of stem cell properties^[88,99-102]. Because of this, there is now a high degree of interest in understanding the hypoxia mechanism involved in cell reprogramming and the maintenance of pluripotent states.

NO regulates mitochondrial respiration and modulates metabolic changes

It has been reported that NO interacts with oxygen bound to CcO, the last enzyme of the electron transport chain^[15]. CcO is located on the inner mitochondrial membrane and contains two heme and two copper

centres. The cytochrome's heme iron and copper molecules (in their reduced form) constitute the binding site for oxygen as it catalyses the reduction of oxygen to water. This process is related to the transport of protons into the mitochondrial inter-membrane space. In this last step, water is generated from oxygen and protons to drive the ATPase^[103]. During normoxia the enzyme is predominantly in an oxidised state, while during hypoxia the reduced state predominates. CcO has a greater affinity for NO than for oxygen, suggesting that this interaction may be significant under certain physiological conditions. If true, it would make NO a physiological regulator that acts directly on the mitochondrial respiratory chain^[14,15,104]. This reaction and its biological consequences are dependent on the redox state and turnover of CcO, the *in situ* oxygen concentration and the activity of NO synthase^[105].

At high oxygen and NO concentrations, CcO is in its oxidised state and NO is metabolised into nitrite and nitrate; whereas at lower oxygen concentrations, CcO is mainly reduced and NO is not metabolised. Thus, under lower oxygen concentrations, NO accumulates in the intracellular microenvironment, with important implications for the balance between glycolysis and OXPHOS. Interaction between NO and CcO changes the availability of intracellular oxygen and the generation of reactive oxygen species (ROS). This results in changes to cell signalling pathways, especially oxygen-sensitive ones, which determine the nature of cellular hypoxia responses^[106].

At low oxygen concentrations mitochondrial ATP generation decreases due to reduced respiratory activity. This results in increased levels of its precursor, AMP, and activation of AMP-activated kinase (AMPK), which is a critical regulator of cellular energy homeostasis and crucial for adapting to low oxygen (hypoxic) conditions^[107]. The activation of AMPK promotes catabolic pathways, including glucose transport, gluconeogenesis, respiration, and the use of oxygen-independent energy sources. Moreover, it acts to down-regulate anabolic pathways.

NO inhibits mitochondrial respiration and increases glycolysis, thus maintaining normal cellular ATP levels (Figure 2). It has been reported that astrocyte energy production is maintained following NO-mediated inhibition of CcO by up-regulating glycolysis^[19]. After the NO-induced inhibition of respiration, there is a cGMP-independent increase in the activity of 6-phosphofructo-1-kinase, a master regulator of glycolysis^[108], and an increase in the concentration of its most powerful positive allosteric activator^[109], fructose-2,6-bisphosphate (F2,6P2). Also, it has been shown that NO-induced glycolysis activation is dependent on the phosphorylation of AMPK resulting in increased 6-phosphofructo-2-kinase activity and protection against apoptosis^[19]. On the other hand, it has also been described that NO regulates HIF-1 α ^[18]. This activity of NO is an important additional mechanism by which NO might modulate cellular

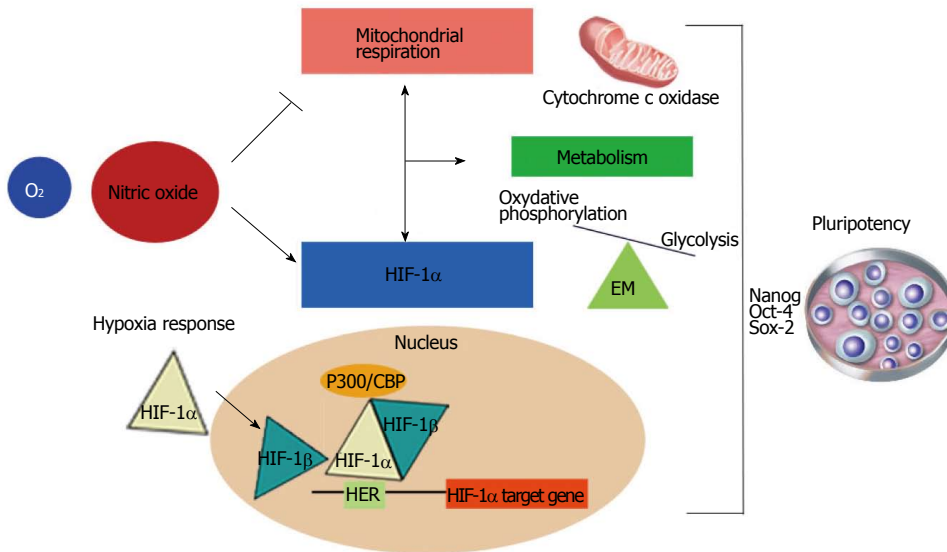


Figure 2 The role of nitric oxide as a regulator of mitochondrial function, metabolism and the hypoxia response. The wide range of functions controlled by nitric oxide (NO) is a consequence of its dose-dependent dual roles. Physiological concentrations of NO inhibit cytochrome c oxidase in a reversible manner in competition with O₂. This interaction depends on NO and oxygen concentrations and the redox state of the enzyme. Because of this, it has been reported that NO might be a regulator of cell respiration, modulating metabolic changes responsible for an increase in glycolytic pathway flux to maintain energy homeostasis. On the other hand, NO can also regulate the hypoxia response through hypoxia inducible factor-1α (HIF-1α). In a normoxic microenvironment, NO induces HIF-1α accumulation in a mitochondrial-dependent and -independent manner. Hypoxia conditions promote delayed cell differentiation. These facts indicate that NO might maintain pluripotency *via* regulation of metabolism and the hypoxia response. EM: Energy metabolism.

responses to hypoxia in mammalian cells (Figure 2).

NO and the hypoxia response

Most cells respond to exposure to low oxygen concentrations through the activation of hypoxia-responsive genes such as erythropoietin, NOS2 and glycolytic enzymes. This adaptive response to hypoxia is mediated by HIF-1α, which regulates the expression of a large number of genes that control cellular responses to reduced oxygen availability^[110].

HIF is composed of two subunits, the constitutively-expressed beta-subunit (HIF-1β), and the alpha subunit (HIF-1α), whose expression is highly sensitive to oxygen concentration and accumulates rapidly under hypoxic conditions^[111,112]. HIF-1α protein degradation is tightly regulated by oxygen levels through two mechanisms, both involving HIF-1α hydroxylation. The first consists of the prolyl hydroxylation of two HIF-1α proline residues, targeting the protein for E3-ubiquitin-ligase mediated ubiquitination. This complex contains the von-hippel-lindau tumour suppressor protein (VHL), which binds to hydroxylated HIF-1α. The second mechanism is mediated by asparagine hydroxylation of HIF-1α residues located in a protein domain that is involved in interaction with the p-300 transcription activator. The result of this hydroxylation is the inhibition of target genes. Under hypoxic conditions, the hydroxylation of HIF-1α does not occur. This allows HIF-1α to dimerise with HIF-1β and translocate to the nucleus. Once in the nucleus, the dimer recruits the p300 and CBP co-activators, which induces the expression of its transcriptional targets, *via* binding to hypoxia-responsive elements (HERs), located in the

promoter region of target genes^[113,114].

Mitochondria play an important role in sensing cellular oxygen concentrations. Studies using pharmacological inhibitors have shown that the hypoxic regulation of HIF-1α is dependent on mitochondrial function through inhibition of the respiratory chain^[115,116]. As NO inhibits CcO, it is possible that NO modulates the hypoxia response through HIF-1α.

NO regulates the accumulation of HIF-1α

Numerous studies have shown that NO affects HIF-1α accumulation. However, there is some controversy as to whether it promotes HIF-1α stabilisation^[117-119] or HIF-1α destabilisation^[120-122]. Moncada *et al.*^[6] demonstrated that NO-induced HIF-1α regulation occurs *via* both mitochondria-dependent and -independent pathways (Figure 2). This work was performed using the HEK-293 cell culture model where NO was generated inside cells in a finely controlled manner using a tetracycline-regulated inducible NOS2 expression system. They demonstrated that NO concentrations of 400 nmol/L prevented the accumulation of HIF-1α under hypoxic conditions, in a mitochondria-dependent manner. This study, also showed that intracellular high NO levels (> 1 μmol/L) always resulted in HIF-1α stabilization, under both hypoxic and non-hypoxic conditions. They found that activation of NOS2 sufficient to yield approximately 1 μmol/L NO, at elevated oxygen concentrations, resulted in significant HIF-1α accumulation, even when there was sufficient oxygen for prolyl hydroxylation-mediated HIF-1α degradation. This observation and those described by other authors^[117-119,123], strongly suggest that HIF-1α accumulation by NO is independent of oxygen

concentration. Moreover, this activity was shown to be independent of any action on the respiratory chain, since it was not affected by compounds that inhibit mitochondrial respiration at different stages and also occurred in cells lacking a functional respiratory chain. In this case, it is possible that the stabilisation of HIF-1 α was the result of S-nitrosylation of thiol groups of the HIF-1 α protein^[119,123] or by the direct inactivation of prolyl hydroxylases (PHD) by NO as suggested by Metzen *et al.*^[124].

NO inhibits HIF-1 α accumulation in a concentration-dependent manner in cells subject to a low oxygen concentration (3%O₂) which would normally allow HIF-1 α levels to increase^[18]. Under hypoxia, endogenous NO (400 nmol/L) destabilises HIF-1 α by inhibiting mitochondrial respiration, thus increasing the cytosolic oxygen concentration where PHD2 is mainly located^[125,126].

Unlike the destabilising effect of NO on HIF-1 α , its effect promoting HIF-1 α accumulation was correlated with inhibition of cell respiration and could be mimicked by all mitochondrial respiration inhibitors, indicating that it is dependent on the respiratory chain. However, under normoxia (21%O₂) and 1 mmol/L of DETA-NO, HIF-1 α accumulates in a way that mimics the stabilising effect observed at high NO concentrations under hypoxia. Interestingly, this explains why, although all other inhibitors completely destabilise HIF-1 α , NO does not, since its stabilising effect is apparent even at intermediate NO concentrations, something not seen for the other inhibitors. It has been proposed that the destabilising effect of NO on HIF-1 α may be due to the inhibition of Complex I-dependent generation of superoxide anion (O₂^{•-}), and consequently, peroxynitrite (ONOO⁻)^[122]. In conclusion, at low concentrations, NO has the ability to destabilise HIF-1 α under hypoxia, an effect that is mitochondria-dependent, while at high concentrations, it can stabilise HIF-1 α in a mitochondria-independent manner. It is also possible that the effect of low NO concentrations is part of its physiological regulatory mechanism related to the inhibition of CcO. The stabilisation of HIF-1 α by high NO concentrations and its synergy with the hypoxic stabilisation of HIF-1 α may play a role in pathological conditions such as inflammation, degeneration and cancer, in which high concentrations of NO, hypoxia and HIF-1 α stabilisation have been described^[18,125,126].

There are various potential mechanisms by which NO regulates HIF-1 α activity. First, NO donors, as well as other growth factors^[127,128], stimulate HIF-1 α translation by activating the PI3K/Akt pathway^[129]. Second, the NO donors S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpenicillamine (SNAP) increase HIF-1 α accumulation by inhibiting the interaction between VHL and hydroxylated HIF-1 α , without blocking PHD2-mediated hydroxylation^[124,130,131]. Third, biotin switch assays have revealed that endogenous and exogenous NO produce the S-nitrosylation of the HIF-1 α trans-activation domain (amino acids 727-826)^[132,133] and the oxygen-dependent degradation domain^[123,134]. Fourth,

direct inhibition of HIF-1 α asparagine hydroxylation activity by SNAP permits interaction between HIF-1 α and p300/CBP^[130]. Fifth, the dependence of VHL recruitment on proline hydroxylation^[124] is controversial, as some authors reported that GSNO and SNAP inhibit it, while others have shown that SNP, PAPA NONOate, and MAHMA NONOate increase it^[135]. However, using a specific antibody, Li *et al.*^[134] were able to detect proline-hydroxylated HIF-1 α . It was also reported that mouse HIF-1 α Cys533 is nitrosylated and that this S-nitrosylation does not inhibit proline-hydroxylation^[134].

Park *et al.*^[130] concluded that SNAP blocks VHL recruitment but not HIF-1 α proline hydroxylation. This inhibitory effect is reversed by reducing agents such as vitamin C and Fe (II)^[130]. They proposed that NO increases p300/CBP recruitment, not by HIF-1 α Cys800 nitrosylation, but by inhibiting HIF-1 α *via* asparagine hydroxylases. They also suggested that SNAP inhibited this enzyme by oxidation of the Fe (II) in its catalytic core or by nitrosylation of some cysteine residues^[136].

CONCLUSION

In this review we have studied in detail the actions of NO on specific cell signalling pathways involved in embryonic development, placing emphasis on the regulation of the hypoxia response. We have extensively analysed the dual role of NO. Low NO concentrations delay stem cell differentiation, inducing the expression of Nanog, Oct4 and Sox2, and the activation of survival pathways. At high concentrations, NO induces differentiation events with the expression of early differentiation markers as well as promoting apoptosis.

Due to these activities, NO represents a promising tool for culturing pluripotent cell lines in an undifferentiated state, as it has been proved that it consistently promotes the maintenance of pluripotency and survival. Thus, NO could enhance the pluripotency-promoting activities of factors such as LIF or bFGF. Moreover, studies based in the generation of chemically-defined xeno-free media using NO to maintain pluripotency are being carried out by our group, which would not only reduce costs, but also would avoid animal contamination issues.

Stem cell culture under hypoxic conditions helps prevent spontaneous differentiation. This is relevant for the design of better strategies for directing differentiation towards specific cell types. The generation of hypoxic cell culture microenvironments can be costly, such as maintaining bioreactors with hypoxic atmospheres. Knowing how NO regulates cellular respiration opens up the possibility of using exposure to low NO levels to mimic the hypoxia response under aerobic conditions by inducing HIF-1 α . Activation of this master hypoxia response regulator would up-regulate glycolytic genes and help maintain energy homeostasis.

On the other hand, high NO levels can be used to develop differentiation protocols for differentiating pluripotent cells into the three germinal lines (ectoderm, mesoderm and endoderm). In this context, NO has been

used to differentiate cells to cardiomyocytes, vascular and endothelial tissues, neurons, astrocytes, erythroid and myeloid cells, bone, chondrocytes and definitive endoderm. To sum up, the studies reviewed here underline the diverse range of potential applications for NO in stem cell biotechnology.

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Stem cell therapy in intracerebral hemorrhage rat model

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and anti-inflammatory drugs, to this moment only symptomatic treatments are available, motivating the search for new alternatives. In this context, stem cell therapy emerged as a promising tool. However, more than a decade has passed, and there is still much to be learned not only about stem cells, but also about ICH itself, and how these two pieces come together. To date, rats have been the most widely used animal model in this research field, and there is much more to be learned from and about them. In this review, we first summarize ICH epidemiology, risk factors, and pathophysiology. We then present different methods utilized to induce ICH in rats, and examine how accurately they represent the human disease. Next, we discuss the different types of stem cells used in previous ICH studies, also taking into account the tested transplantation sites. Finally, we summarize what has been achieved in assays with stem cells in rat models of ICH, and point out some relevant issues where attention must be given in future efforts.

Key words: Cell therapy; Intracerebral hemorrhage; Preclinical assays; Rat; Stem cells

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Core tip: In this review, we first summarize intracerebral hemorrhage (ICH) epidemiology, risk factors, and pathophysiology. We then present different methods utilized to induce ICH in rats, and examine how accurately they represent the human disease. Next, we discuss the different types of stem cells used in previous ICH studies, also taking into account the tested transplantation sites. Finally, we summarize what has been achieved in assays with stem cells in rat models of ICH, and point out some relevant issues where attention must be given in future efforts.

Abstract

Intracerebral hemorrhage (ICH) is a very complex pathology, with many different not fully elucidated etiologies and prognostics. It is the most severe subtype of stroke, with high mortality and morbidity rates. Unfortunately, despite the numerous promising preclinical assays including neuroprotective, anti-hypertensive,

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INTRODUCTION

Marginal or no success was achieved from decades of research for therapeutic alternatives for intracerebral hemorrhage (ICH)^[1,2]. Despite the numerous promising preclinical assays including neuroprotective, anti-hypertensive, and anti-inflammatory drugs, only symptomatic treatments are currently available^[2,3], which motivates the search for new alternatives. In this context, cell therapy emerged as a promising tool^[4-6]. However, more than a decade has passed, and there is still much to be learned not only about stem cells, but also about ICH itself, and how these two pieces come together. It is clear that achieving a more detailed knowledge of each involved element is crucial to obtain better results.

INTRACEREBRAL HEMORRHAGE: A SEVERE AND HETEROGENEOUS PATHOLOGY

Epidemiology

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide. In 2008, nearly 17.3 million people lost their lives because of CVDs (30% of all deaths); of these, 6.2 million (35.8%) were to cerebrovascular accidents^[7], alternatively referred as stroke. Among CVDs, coronary artery disease is responsible for the highest mortality rate on a worldwide basis, followed by stroke^[7]. However, stroke mortality rates surpass those of the coronary artery disease in some world regions, like East Asia, Africa, and South America^[8].

There are two types of stroke: ischemic and hemorrhagic. This review will focus on nontraumatic ICH, which corresponds to spontaneous leakage of blood within the brain parenchyma or ventricular spaces^[1]. ICH is the most lethal subtype of stroke, often causing immediate death^[9]. Secondary brain injuries may lead to delayed fatality when death does not occur shortly after the onset. One-year and five-year survival are estimated to be around merely 46% and 29%, respectively^[10]. Beyond that, surviving patients are usually left with many limitations in motor and cognitive functions. Overall, ICH is less frequent than its ischemic counterpart, but the proportion fluctuates depending on the ethnic and racial group. Among Chinese people, for example, ICH accounts for about 33% of stroke cases, vs 12% in white populations^[11]. On the same hand, global incidence of ICH per 100000 person-years is estimated to be around 24.6, reaching 51.8 in Asian populations^[12].

Risk factors

High blood pressure is the major risk factor for CVDs^[13], particularly ICH^[14,15]. As a matter of fact, it is estimated that about 50% of ICH cases are caused by chronic

hypertension^[1]. There is a well established liaison between regular consumption of alcoholic beverages and high blood pressure, being this effect independent of age, body mass, smoking habits and physical activity^[16-18]. Hypertension and ICH incidence are also linked to other lifestyle-related risk factors, such as smoking^[19,20], physical inactivity^[21], and high dietary sodium and/or fat consumption^[22,23]. About 20% of cases are due to amyloid angiopathy, being this the second cause of ICH^[24]. Amyloid deposition incidence is primarily associated with increasing age^[25-27], but genetic factors also play an important role^[28]. Oral anticoagulant intake is other important risk factor, which not only increases ICH risk, but also has higher intrinsic death rates^[29,30]. Other relevant risk factors are obesity, diabetes mellitus, high blood cholesterol and other lipids, brain tumors, aneurysms, cerebrovascular malformations, cavernous angiomas, and arteriovenous fistulae^[23,31].

Pathophysiology

As previously stated, hemorrhagic events in brain parenchyma or ventricular spaces are included in ICH. Bleeding associated with chronic hypertension usually originates from microaneurysms near or at bifurcations of penetrating brain arterioles which emanate from basilar arteries or anterior, middle, or posterior cerebral arteries^[1,32,33]. In most cases, intraventricular hemorrhage is a consequence of ICH, resulting from hematoma expansion to the ventricular space^[1]. This phenomenon increases mortality in nearly five times^[34]. In more rare occasions, hematoma may extend to the subarachnoid space^[31]. Amyloid angiopathy associated ICH is commonly lobar, often extending into the subarachnoid space and ventricles^[35]. Size and location of hematoma are determinant factors in the disease outcome^[36]. For example, even though patients commonly experience continued bleeding or rebleeding^[37], the latter phenomenon is more recurrent in amyloid angiopathy-derived ICH^[38].

Initial injuries are consequent of mechanical disruption of adjacent brain tissue by physical compression due to hematoma formation^[1,31,39]. Most hematoma enlargement occurs in the first hours following ictus, but this growth can extend for many hours^[40,41]. Disruption of the blood-brain barrier implies in the formation of plasma-derived perihematomal edema^[42], which grows rapidly in the first two or three days and reaches full extension about fourteen days after ICH onset^[43,44]. Edema evolution can be described in three phases^[45]. In the first few hours, hematoma expands and retracts as a result of coagulation, depositing serum molecules in the surrounding tissue. In two to three days, inflammatory mediators come off from circulation. In the third and last phase, erythrocytes suffer degradation, which culminates in hemoglobin toxicity. Thrombin and iron from erythrocytes play major roles in secondary injuries^[9,46]. In fact, iron overload seems to be related to the formation

of the perihematoma zone^[47]. This area surrounds the edema and includes viable but very vulnerable tissue^[45].

CELL THERAPY IN INTRACEREBRAL HEMORRHAGE ANIMAL MODELS

Rat: The animal model of choice

Most of the present ICH pathophysiology knowledge derives from studies in animal models. After all, animals assays offer many research advantages, such as the possibility to work with homogeneous groups by the control of variables concerning the subjects (*e.g.*, age, weight, feeding, activity, genetics, *etc.*), and the ICH itself (*e.g.*, time and site of the injury onset and its intensity). Moreover, this kind of study permits various types of biological assays from surviving subjects at any stage of the pathology.

By far, the majority of ICH studies on animals are performed on rodents, mostly on rats. This choice is understandable, once rats are cheap and convenient to house, easy to manipulate, have a plethora of related products for the most diverse purposes, and have a well described anatomophysiology, which, overall, shares considerable similarities with humans in cerebrovascular parameters^[48]. However, there are some important differences that should not be underestimated or neglected.

White matter paucity in rat brains limits the similarities with the human condition^[32]. Human brain proportion of white/gray matter shares more similitude with that of porcines and primates, but both are incomparably more expensive and complicated to handle if compared to rats^[49]. In addition, if compared to humans, rats also have anatomical disparities in brain perforating arteries, as well as anatomical and biochemical differences in the basal ganglia^[50]. It is important to notice that both structures have fundamental involvement in ICH model, especially considering that the commonest experimentally induced ICH is intrastriatal. Actually, this fact is probably included among the reasons why ICH prognostic differs so much in some parameters between rats and humans (more on these matters in the next section). Thus, even though rodents provide advantages as experimental models, multiple species should be used for more trustworthy results^[51]. Additionally, rats and mice are also different in many anatomophysiological aspects^[52], and tests should not be limited to either of the two.

Intracerebral hemorrhage replication

Bacterial collagenase injection is the most widespread method to induce ICH in rats, followed by autologous blood injection (ABI). The latter is one of the earliest methods developed to replicate ICH in rats. In this model, blood is collected from a superficial vessel and is injected directly into the brain, usually in the striatum^[53]. The application of this protocol should be limited to

blood toxicity and edema formation studies, once very important aspects of ICH pathology are absent, like small vessel rupture and hematoma expansion^[54]. Moreover, especially in rats, ABI implies in exaggerated inflammatory response as a consequence of hemoglobin crystallization^[55].

Collagenases are enzymes that break collagen peptide bonds^[56]. Consequently, intracerebral injection of bacterial collagenase leads to the breakdown of the basal lamina of blood vessels, establishing the leakage of blood within the brain^[57]. Through this method, rebleeding is present and functional impairments are more long lasting than that of ABI model^[57]. Also, application is simpler^[49]. However, dissolution of endothelial basal lamina causes ICH in an unnatural manner^[32].

Even though collagenase injection model mimics ICH with more success than ABI, both show relevant limitations^[54]. As previously stated, the standard collagenase and blood site of injection is the striatum, which differs importantly from the human counterpart in biochemical and anatomical features^[50]. In top of that, ICH is often not restricted to single anatomical regions in humans, while this is what is achieved with both models^[58]. Lastly, but certainly not least, both models comprise intracerebral injections. Therefore, some significant ICH unrelated injuries must be done in order to access the target structures: animal scalp and periosteum must be cut open, a hole must be drilled in the skull, and most importantly, a needle must perforate all brain superjacent structures^[59].

As tacit in the Pathophysiology section of this review, ICH is a disease with multiple etiologies, which directly affect the prognostic. More than that, the role played by the ICH causing factors is probably unrestricted to the way the pathology is going to have a start, but is also determinant how it is going to evolve^[49]. For example, it is deductible that ICH disrupted by anticoagulant intake develops differently, as hematoma might probably include uncoagulated blood^[60].

Even though ICH affects mostly humans with chronic hypertension and advanced age, preclinical studies are predominantly conducted using young and healthy animals. The absence of comorbidities presumably implies in unrealistic outcomes and weaker translational power. Aware of this limitation, many efforts have been made on the last years to develop and use models with characteristic risk factors. ICH related functional outcomes are known to be more severe and long lasting in older^[61-63] and chronic hypertensive^[64] rats. The latter develop spontaneous ICH with location and distribution consistent to what is observed in hypertensive humans^[32]. However, spontaneous chronic hypertension-derived ICH is hard to model, once animals tend to develop ischemic stroke instead of ICH^[65].

Prognostic analysis of experimentally induced ICH

ICH in rats and humans have remarkably different prognostics. Human ICH is generally followed by

important long lasting or permanent cognitive and motor impairments. In contrast, rats subjected to ICH exhibit no (or at least not long lasting) cognitive deficits^[58,66]. Brain structural disparities might play a crucial role in this matter, implying in different affected structures^[58]. Motor function impairments in rats are present and well described, but undergo notable recovery in few weeks^[67]. Moreover, in rats, edema reaches full extension three to four days after ICH onset^[68], whereas in humans it takes weeks^[69]. Although there is much to be discovered in this matter, rat superior neurogenesis and/or neuroplasticity may explain these dissimilarities^[70].

Many behavioral tests are used to assess ICH outcome. Among the most used are the modified limb placement test (mLPT)^[71] and the modified neurological severity score (mNSS)^[72]. In both tests, rats are subjected to a set of simple sensorimotor tasks that evaluate different aspects of neurological function. A score is attributed to the performance in each task, resulting in a final sum that ranges from 0 to 14. Both mLPT and mNSS are theoretically consistent, simple to perform, and cost-free, dismissing the need for complex training and special equipments. However, both tests should be always applied by blinded investigators, preferably the same ones for every session. If such attention is present, it is commonly unknown, as it is not always mentioned. Evaluating rat behaviors through mLPT and mNSS can be considerably subjective and interpretative tasks, relying strongly on the observer sensitivity and judging parameters. Importantly, the small score amplitude (0-2 in mLPT and 0-1 in mNSS) attributed to each parameter may lead to significant variations in the final result. Thus, expectations of any nature and level should be absent in the assigned investigator for trustworthy results. Additionally, more than one single test should be applied for more solid assertions.

Last but not least, pathophysiological features of ischemic and hemorrhagic stroke diverge in many ways^[73]. Thus, great caution should be taken when comparing findings from each disease.

Stem cell types and clinical applications: An ever-emerging science

Stem cells are characterized by ability of self-renewal (production of identical copies of themselves) and capability to differentiate into distinct functional cells. Cells with capability to differentiate into any body cell are called totipotent. These cells are only found in the earliest stages following fertilization. Embryonic stem cells (ESCs) are mostly pluripotent, meaning that they are able to mature into cells of the three germ layers (endoderm, mesoderm, and ectoderm). Unfortunately, ESCs are very difficult to isolate, and their use is very limited and even avoided due to ethical, social and religious concerns^[74]. Induced pluripotent stem cells (iPSCs) were developed to work around ESCs major issues. iPSCs are generated from somatic cells reprogrammed by the introduction

of few defined factors^[75]. However, as embryonic stem cells, iPSCs can potentially generate teratomas after transplantation^[76-78]. It happens as a result of their propensity for uncontrolled self-renewal and triploblastic differentiation^[79].

Somatic stem cells are multipotent, thus possessing the ability to differentiate into only certain cell types. As they can be found in adult tissues, they are sometimes referred as "adult stem cells". This term, however, is misleading and should be avoided, once the embryo starts containing somatic stem cells shortly after implantation^[80]. Mesenchymal stem cells (MSCs) are somatic stem cells present in virtually all organs^[81]. They are able to differentiate into mesodermal cell lineages, like osteoblasts, chondrocytes, adipocytes, and myoblasts^[82,83]. Some groups, however, showed that MSCs can be induced to differentiate into neurons, but is debatable if they are functional^[84]. Bone marrow and adipose tissue have been the preferred sources for MSCs, and cells from each origin are biologically different^[82,85]. Bone marrow-derived stem cells (BMSCs) were the first to be discovered, and are the most tested in preclinical assays. However, if compared to BMSCs, adipose-derived stem cells (ASCs) are simpler and less invasive to harvest, as large amounts of these cells can be obtained *via* liposuction with local anesthesia only^[86,87]. If ASC allogeneic applications become someday proven to be safe, fat from liposuctions can be utilized for stem cell cultures instead of being discarded. Moreover, adipose tissue yields more stem cells than any other source^[88]. For example, ASCs are more frequent in adipose tissue than BMSCs are in bone marrow in a 100 to 500 fold difference^[86]. Although considered very promising^[89,90], ASC use is relatively recent if compared to BMSCs. Thus, more studies are needed in order to effectively evaluate advantages of one over another on particular purposes, concerning efficacy, safety, *etc.* Neural stem cells (NSCs) are multipotent and can differentiate into neurons, astrocytes, and oligodendrocytes. Even though NSCs might initially sound as the most appropriate for nervous system diseases like ICH, the isolation of these cells is a complicated and delicate procedure^[91].

Umbilical cord-derived stem cells (UCSCs) are the most used cells from perinatal tissues. Controversy still exists if UCSCs are MSCs or MSC precursors, as they differ in morphological and behavioral aspects^[83]. UCSCs are harvested from umbilical cord or umbilical cord blood (UCB) from newborns^[92,93], which are presently treated as medical waste. Thus, the isolation method completely avoids donor morbidity^[94]. UCSCs are very abundant and show high proliferative capacity^[94,95]. UCB contains mononuclear cells that are mostly leucocytes, but it also contains UCSCs. Importantly, 0.6% of umbilical cord blood mononuclear cells are multipotent stress-enduring (Muse) cells. Muse cells are a non teratogenic pluripotent cell type that was recently isolated not only from UCB, but also from dermal fibroblasts, bone marrow, and adipose tissue^[79,96,97].

Autologous stem cell transplantation guarantees the absence of immune rejection. However, depending on the circumstances, this might not be feasible. For instance, somatic stem cells require several weeks in culture to achieve sufficient proliferation^[91]. Additionally, the harvested cells themselves may not be appropriate in number and/or quality for therapeutic purposes (e.g., in case of elder patients). More than that, harvesting might be a painful and complicated procedure to the patient (e.g., BMSCs). Cells from perinatal tissues could be used for autologous administration, but this practice would be very resource demanding, comprising long term storage of the isolated cells in freezers until they are eventually needed^[98].

It is not consensual if allogeneic delivery of MSCs would lead to immune rejection. Actually, many findings with somatic stem cells showed that they possess immunosuppressive action, not being rejected by the host organism^[99-102]. If lack of immune rejection becomes confirmed for allogeneic somatic stem cell transplants, this would certainly represent the preferred option, making it possible that cultured cells are readily available for the patients. ESCs, by the other hand, are known to trigger immune responses following allogeneic transplantation. Taking that into consideration, research is being conducted in order to inhibit donor-host immune reactions^[103].

Stem cell therapeutic effects and underlying mechanisms

It was in the beginning of the last decade that stem cells started to be considered as a treatment for neurological disorders. Back then, the expected therapeutic mechanism was very simple: stem cells were expected to repopulate the damaged tissue, differentiating into functional neurons and glial cells^[104,105]. Actually, a considerable number of earlier studies have mistakenly concluded that MSCs and UCBCs could in fact differentiate into functional mature neurons and astrocytes, but these findings were contested^[84,106]. NSCs are the only type of somatic stem cell capable to differentiate into functional mature nervous cells. But again, probably it is not the main action underlying NSC therapeutic effects. The underlying mechanisms are presently recognized to be far more complex, and are still not fully understood.

Evidence show that therapeutic effects primarily emerge from the release of trophic factors, cytokines, and microRNAs that stimulate endogenous mechanisms of repair^[106-109]. In consonance with this, in a recent work, Jeon *et al*^[110] reported a modest, yet significant recovery in rats subjected to ICH treated only with ASC extract. Additionally, different research groups already reported that endogenous NSCs are stimulated and recruited to the injury site in response to exogenous stem cell injection^[111,112]. In fact, the injury itself seems to stimulate stem cells, exogenous and endogenous, to migrate to the perihematoma perimeter^[113-117] and to promote proliferation and plasticity^[108,111,118,119].

These restorative reactions are probably consequent of the secretion of trophic factors, which is the most likely mechanism underlying apoptosis prevention as well^[111,113,119-121]. The same is true to the intensified angiogenesis, presumably due to elevated VEGF release^[66,113,122,123]. Hematoma area reduction is also often observed^[66,115,119,121,124], but not always^[112-114,117]. Likewise, decrement of brain edema volume is a commonly stated benefice^[113,125,126]. Stem cells are also seen to exert important anti-inflammatory effects in the injury site^[66,113,125,126], being known to modulate the action of dendritic cells, and B and T lymphocytes^[127-129]. Stem cells were linked to astrocyte and microglia response modulation as well^[130]. In fact, recent findings showed that some glial cells within the oligodendrocytic and astrocytic lineages present progenitor and neural stem cell functions, with active response to brain injury^[131,132].

Preclinical studies: an heterogeneous approach for an heterogeneous disease

Present available data concerning stem cell therapy for ICH derives from investigations conducted with notably different approaches. Many stem cell administration routes have been tested, but it remains unclear which is the best option. Findings in ICH model support that cervical vein delivery is inefficient^[133], whereas good outcomes were already observed following stem cell delivery in brain parenchyma^[66,111,113-116,121,126,134-137], lateral ventricle^[120,133], tail vein^[117,119,122,124-126,138,139], and carotid artery^[133,140,141]. However, each one of these alternatives has drawbacks. Intracerebral and intracerebroventricular routes are very invasive alternatives, hardly representing the best clinical options. In this sense, intravascular injections are incomparably more feasible and less troublesome, and allows injections with higher cell doses^[142]. However, due to their size, stem cells were reported to get trapped in the lungs, liver and spleen and to cause embolisms, following tail vein and intracarotid injections, respectively^[143-145]. Importantly, the latter adversity can be surpassed with slower injection rates^[143], a generally overlooked variable. It was reported that carotid artery delivery favors cell migration to the brain if compared to tail vein delivery route^[146]. However, no differences in this aspect were seen when comparing carotid artery and jugular vein stem cell injections^[147]. Additionally, concerning intravascular transplantation, the potential of other systemic interactions is of unknown impact, being the safety of this route debatable.

Recently, efforts have been devoted to the development of new cell administration routes. Among these, intranasal cell delivery represents a promising alternative for the treatment of neurological disorders. Not only intranasal administration is simple and noninvasive, but also avoids potentially dangerous systemic interactions^[148-151]. Positive results were reported following the delivery of growth factors^[152-154] and MSCs^[155-158] following experimentally-induced ischemic stroke in rodents. To our knowledge to

Table 1 Methodologies used in preclinical trials of stem cell therapy for intracerebral hemorrhage

Ref.	ICH model	Cells	Time after ICH	Amount	Vol (μL)	Delivery site
Bao <i>et al</i> ^[113]	Col VII	hBMSC (Flk-1+)	24 h	2×10^5	15	BP
Chen <i>et al</i> ^[120]	Col IV	ASC	48 h	3×10^6	10	LV
Fatar <i>et al</i> ^[112]	Col IV	hASC	24 h	3×10^6	500	TV
Jeon <i>et al</i> ^[110]	Col VII	ASC extract	1 h	-	-	IP
Jeong <i>et al</i> ^[138]	Col IV	hNSC	24 h	5×10^6	500	TV
Kim <i>et al</i> ^[125]	Col VII	hASC	24 h	3×10^6	2000	TV
Lee <i>et al</i> ^[126]	Col VII	hNSC	2 h; 24 h	5×10^6 (TV); 1×10^6 (IC)	500 (TV); 2 (BP)	TV; BP
Li <i>et al</i> ^[139]	Col VII	NSC	2 h; 7 h; 14 h; 21 h; 27 h	4×10^6	400	CA
Liang <i>et al</i> ^[114]	Col IV	BMSC	24 h	1×10^6	10	BP
Liao <i>et al</i> ^[66]	Col VII	hUCSC	24 h	2×10^5	10	BP
Nan <i>et al</i> ^[158]	Col VII	hUCB	24 h	2.4×10^6 - 3.2×10^6	500	SV
Otero <i>et al</i> ^[134]	Col IV	BMSC	72 h	2×10^6	10	BP
Otero <i>et al</i> ^[115]	Col IV	BMSC	2 m	5×10^6	15	BP
Otero <i>et al</i> ^[111]	Col IV	BMSC	2 h	2×10^6	15	BP
Qin <i>et al</i> ^[116]	Col VII	iPSC	24 h	2×10^6	10	BP
Qin <i>et al</i> ^[135]	Col VII	iPSC	24 h	1×10^6	10	BP
Seghatoleslam <i>et al</i> ^[124]	Col IV	hUCBMC	24 h	4×10^6 ; 8×10^6 ; 16×10^6	1000	TV
Seyfried <i>et al</i> ^[140]	ABI	BMSC	24 h	1×10^6	100	CA
Seyfried <i>et al</i> ^[122]	ABI	BMSC	24 h	5×10^5 ; 1×10^6	1000; 2000	TV
Vaquero <i>et al</i> ^[136]	Col IV	hBMSC ¹	2 m	5×10^6	30	BP
Wang <i>et al</i> ^[119]	Col VII	BMSC	1 h	1×10^6	1000	TV
Wang <i>et al</i> ^[137]	Col VII	NSC	72 h	1×10^6	500 (TV); 2 (BP)	TV; BP
Yang <i>et al</i> ^[121]	Col I	BMSC (overexpressing GDNF)	72 h	5×10^5	20	BP
Yang <i>et al</i> ^[117]	ABI	hUCSC	24 h; 72 h; 7 d	3×10^6	2000	TV
Yang <i>et al</i> ^[159]	Col VII	hASC	24 h	1×10^6	200	FV
Zhang <i>et al</i> ^[133]	Col VII	BMSC	24 h; 72 h; 5 d; 7 d	2×10^6	20	CA; CV; LV

¹Delivered in platelet-rich plasma scaffolds. Intracerebral hemorrhage (ICH) model: autologous blood injection (ABI) and collagenase (Col). Cell type: ASC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; GDNF: Glial-derived neurotrophic factor; iPSC: Induced pluripotent stem cell; UCB: Umbilical cord blood; UCBMC: Umbilical cord blood mononuclear cell; UCSC: Umbilical cord-derived stem cell; NSC: Neural stem cell. Delivery site: BP: Brain parenchyma; CA: Carotid artery; CV: Cervical vein; FV: Femoral vein; LV: Lateral ventricle; SV: Saphenous vein; TV: Tail vein.

date, no intranasal delivery trials were conducted on the treatment of ICH using MSCs.

Some comparative studies using different routes have been made on ischemic stroke models. Findings comparing cell migration and possible systemic adversities resulting from different stem cell injection routes represent potential lessons to apply in future ICH model investigations.

Stem cells have been delivered at many different times after experimental ICH onset, ranging from one hour to two months following ictus (Table 1). Apparently, earlier NSC transplantation (acute phase) is particularly important in intravenous delivery^[126]. Interestingly, in the same study, time matter seemed to be indifferent when stem cell injection was intracerebral. Also working with NSCs, other group tested the effects of different carotid artery injection times. Acute phase delivery, in this case, returned the worst therapeutic effects^[140]. In late transplantation studies, intracerebral stem cell implantation was performed only two months after ictus. Even so, good functional and morphological outcomes could be observed^[111,136]. It is also important to keep in mind that ICH dynamics in rats and humans, as previously discussed, are clearly different in many aspects, including temporality. Thus, time windows in these different organisms are proportionally divergent, e.g., 24 h after ICH onset, rodents and humans suffer

from different pathological actions, due to the different paces in the development of the pathology.

The ideal amount of cells and vehicle to be injected is another unanswered question. The only studies performed with variations in that parameters used the tail vein route^[122,122,124]. BMSC injection of 1 million cells was more therapeutically effective than half of it^[122,122]. UCMCs were also more effective in the highest tested amount (16 million cells), reducing injury area with more success^[124]. However, apart from this studies, the most common quantity injected in the tail vein is of 3 million cells, suspended in 1 or 2 mL of saline solution^[117,119,124,125]. The amount of intracerebrally grafted cells varied up to 25 fold among studies^[66,113,115,121,136]. Interestingly, in general, the vehicle volume is not necessarily proportional with the number of cells in suspension (Table 1).

Most of the aforementioned findings, however, are very hard to counterweigh and take as conclusive. Not only the time window after ICH varied between studies, but also the cell type, amount, and injection site. Moreover, it must be never forgotten that rats and humans are different organisms, and studies in more models must be performed before calculating the extrapolation to human applications. As stated in previous sections, different stem cell types have different attributes, and knowledge is in its youth about how all these divergences may affect ICH outcome.

CONCLUSION

In this review, we highlighted that there is still much to be learned about ICH and stem cells, both individually as interacting with each other. The approaches adopted by different groups were diverse, concerning not only the outcome evaluation, but also ICH induction and stem cell treatment protocols. Although it is important to perform tests under diverse conditions, more comparative studies should be motivated, including different times for interventions, stem cell types, quantities, and delivery sites. Homogeneity of all implicated variables except the one to be tested is imperative for the improvement of our understanding, preventing waste of hopes and resources in forthcoming human ICH trials.

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Recent advances in bone regeneration using adult stem cells

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vessels and bone cells. Therefore, cells that participate in vasculogenesis and osteogenesis play a pivotal role in bone formation during prenatal and postnatal periods. Nevertheless, spontaneous healing of bone fracture is occasionally impaired due to insufficient blood and cellular supply to the site of injury. In these cases, bone regeneration process is interrupted, which might result in delayed union or even nonunion of the fracture. Nonunion fracture is difficult to treat and have a high financial impact. In the last decade, numerous technological advancements in bone tissue engineering and cell-therapy opened new horizon in the field of bone regeneration. This review starts with presentation of the biological processes involved in bone development, bone remodeling, fracture healing process and the microenvironment at bone healing sites. Then, we discuss the rationale for using adult stem cells and listed the characteristics of the available cells for bone regeneration. The mechanism of action and epigenetic regulations for osteogenic differentiation are also described. Finally, we review the literature for translational and clinical trials that investigated the use of adult stem cells (mesenchymal stem cells, endothelial progenitor cells and CD34⁺ blood progenitors) for bone regeneration.

Key words: Bone tissue; Mesenchymal stem cells; Tissue engineering; Osteogenesis; Angiogenesis; Endothelial progenitor cells

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Core tip: Mesenchymal stem cells and blood derived progenitor cells have the potential to open new horizon for the treatment of bone defects. Numerous preclinical and clinical studies demonstrated angiogenic and osteogenic potential of these cells. Additional research is required in order to improve isolation and expansion techniques, as well as long term studies to follow the safety of this cellular approach.

Abstract

Bone is a highly vascularized tissue reliant on the close spatial and temporal association between blood

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INTRODUCTION

Incidence of bone fractures that require hospitalization is estimated to be 1 million in the United States alone. About 5%-10% of these fractures present delayed healing or nonunion and represent a major treatment challenge in orthopedic reconstructive surgery^[1]. Current gold standard surgical technique for reconstruction of large bony defects involves autologous bone blocks harvested from the fibula, iliac crest, scapula, and radius^[2-5]. These procedures require extended hospitalization and a secondary donor site with associated morbidity and complications. In addition to their surgical complications, these techniques are unpredictable and associated with significant graft resorption^[6,7]. As an alternative to autologous bone block, recent developments in bone tissue engineering, gene and stem cell therapy are currently examined. A tissue engineering approach offers several potential benefits, including the lack of donor site morbidity, decrease in technical sensitivity of the repair, and most importantly, the ability to closely mimic the *in vivo* microenvironment in an attempt to recapitulate normal bone development^[8].

BONE COMPOSITION

The bone comprises of inorganic and organic components. Two thirds of the bone volume consists of inorganic mineral salts: calcium, phosphate, carbonate, citrate, hydroxyl and ions (magnesium, sodium and fluoride) in the form of hydroxyapatite crystals^[9]. The organic component is composed of 99% collagen type-1^[10] as well as several growth factors osteocalcin, osteonectin, phosphoproteins, proteoglycans and bone morphogenic proteins (BMPs)^[11].

In addition, the bone consists of cellular components: pre-osteoblasts, osteoblasts, osteocytes and osteoclasts. Osteoblasts originate from pluripotent mesenchymal cells, characterized as mononucleated, cuboidal cells located at bone margins. Their life span is between 3 d (in young rabbits) and 8 wk (in humans). Osteoblasts produce the inorganic bone matrix (un-mineralized osteoid) and supervise the mineralization process by secretion of alkaline phosphatase. At the end of the process several osteoblasts remain entrapped in the matrix that they have secreted, and develop into osteocytes^[12]. These cells are considered mature osteoblasts. Only 10%-20% of the osteoblasts develop into osteocytes. Osteocytes are smaller than osteoblasts, have a larger nucleus to cytoplasm ratio and have a large number of

processes which provide intercellular communication. Osteocytes are thought to be the cells responsible for bone regeneration, thus their presence is crucial^[13,14]. The process of bone formation is followed by bone resorption. This process is carried out by osteoclasts that secrete enzymes and reduce the pH at the resorption site. Osteoclasts are large, multinucleated, polarized cells with a life span of 15-20 d. These cells originate from hematopoietic precursors of monocytes in the bone marrow, thus comprise phagocytic properties^[15].

EMBRYONIC BONE DEVELOPMENT

The development of bone tissue is controlled by tightly regulated programs of cell proliferation, differentiation, survival, and organization. The bone tissue derives from mesenchymal condensations in which osteochondral progenitors give rise to either chondrocytes to form the cartilage or osteoblasts to form the bone^[16]. Within the mesenchymal condensations, cells differentiate into chondrocytes or osteoblasts, and form cartilage and bone under the control of various transcription factors. Differentiation of mesenchymal cells into chondrocytes leads to the production of cartilage structures of the future bones of all the skeleton, except for the craniofacial skeleton and the clavicle. Through the process of endochondral ossification, the cartilage structures are subsequently replaced by bone and bone marrow. In the craniofacial skeleton and the clavicle, differentiation of mesenchymal cells into osteoblasts produces intramembranous bones directly from the mesenchymal condensations through the process of intramembraneous ossification^[17]. It is well known that bone mineralization during endochondral and intramembraneous ossification is tightly coupled with angiogenesis^[18].

BONE REMODELING

Bone remodeling occurs throughout life. During this process small amounts of bone are resorbed by the activity of osteoclasts, followed by the recruitment of osteoblast precursors that differentiate and replace the amount of the removed bone^[19]. The remodeling process takes place at anatomically distinct sites, termed basic multicellular units, that are dispersed throughout the skeleton^[20]. An exact coupling between bone resorption and bone replacement is essential for successful bone remodeling. Thus, bone resorption by osteoclasts has to be matched with the generation of osteoblasts from precursor cells in order to replace the resorbed bone^[21]. In the coupling between bone resorption and bone formation several factors are involved, such as growth factors released from the matrix, secreted and membrane-bound factors of osteoclasts, signals from osteocytes, signals from immune cells and signaling taking place within the osteoblast lineage^[22].

FRACTURE HEALING AND TREATMENT OF NONUNION

The biologic process of fracture healing is complex and impacted by various factors including the recruitment of stem/progenitor cells from the bone marrow to the fracture site. The healing occurs *via* rapid bone regeneration followed by osteoclast-mediated remodeling, resulting in new bone with structural integrity and a geometrical configuration similar to that prior to the injury^[2,23]. In most cases, healing is uneventful, but in some cases delayed or impaired healing occurs. The absence of proper blood supply, improper stability, soft tissue damage and different host features such as age, osteoporosis and smoking have been found to contribute to failure in achieving complete restoration. Nonunion fracture is a dramatic example of poor healing and is defined as non-consolidation at the fracture site within 6 mo^[4,5,24]. Autologous bone graft is the gold standard treatment for nonunion fractures. Bone block may be harvested from the iliac crest, fibula or calvaria and transplanted at the recipient site. Bone block stimulates bone regeneration by serving as a scaffold for new bone growth (osteoconduction); moreover it contains cells [osteogenesis, *e.g.*, mesenchymal stem cells (MSCs) and osteoblasts] and growth factors (osteinduction). However, this technique has several disadvantage such as donor-site morbidity, pain and prolonged hospitalization^[2-5].

MICROENVIRONMENT AT OSSEOUS TISSUE HEALING SITE

Healing of small bone defects and bone fractures involves complex and well coordinated interactions between cells, cytokines, and a mechanically stable environment with adequate blood supply^[25] to generate new bone instead of a fibrous scar^[26]. This complex dynamic process begins with secretion of pro-inflammatory cytokines from aggregated platelets, such as interleukin-1 (IL-1), IL-6 or tumor necrosis factor- α , which have chemotactic activity towards endothelial cells, fibroblasts, lymphocytes and monocytes-macrophages^[27]. Transforming growth factor β 1 (TGF β 1) plays a key role in this process due to its potent chemotactic and proliferative effect on mesenchymal stem cells, therefore modulate the recruitment of bone cells to the healing site^[28]. In addition, TGF β 1 induces the production of extracellular bone matrix proteins such as collagen, osteopontin, and alkaline phosphatase from MSCs and osteoblasts^[29]. BMPs and vascular endothelial growth factor (VEGF) are additional growth factors that induce osteogenesis and angiogenesis^[30,31]. BMPs belong to the TGF- β superfamily and induce the formation of both bone and cartilage by stimulating the cellular events of mesenchymal progenitor cells. VEGF induces angiogenesis that is crucial for delivering

oxygen and progenitor cells to the site^[26].

RATIONALE FOR USING ADULT STEM/PROGENITOR CELLS FOR BONE REGENERATION

In the process of bone development and bone healing MSCs and adequate vasculature play a central role. Therefore, cell-based therapy strategies to enhance bone regeneration utilize MSCs and endothelial progenitor cells (EPCs).

MSCs contribute to the maintenance of various tissues, especially bone, in adults^[32]. In 2006, the International Society for Cellular Therapy proposed that cells with the following characteristics should be considered as MSCs: (1) cells adherent to plastic in culture; (2) presence of CD105, CD73 and CD90 but absence of CD34, CD45, CD14 or CD11b, CD79 α or CD19, and HLA-DR molecules; and (3) cells with the capacity to differentiate into osteoblasts, chondrocytes and adipocytes^[33]. MSCs can be isolated from the bone marrow, placenta, umbilical cord blood, adipose tissue, muscle^[34], brain^[35], kidney, heart and many others^[36]. While these cells share common characteristics, they are distinctive, and reflect aspects of their tissue of origin^[37]. In animal models, transplantation of MSCs formed ectopic bone and improved healing of bone defects^[38,39]. In clinical trials, MSCs regenerate bone in non-union long bone defects^[40] in children with osteogenesis imperfecta^[41] and in maxillofacial surgeries^[42,43]. However, several difficulties concerning the use of MSCs still exist: (1) their aspiration involves an invasive procedure and morbidity^[44]; (2) age-dependent decline exists in their proliferation and osteogenic differentiation potentials^[45]; and (3) inadequate vascularization to MSC composite graft that causes malfunction and death of the transplanted cells^[46].

In 2009, a novel study presented a successful use of blood derived endothelial progenitor cells to heal critical size bone defects^[47]. Circulating EPCs were discovered in 1997 by Asahara *et al.*^[48] and Murohara *et al.*^[49]. EPCs participate in neovascularization^[50], angiogenesis, vascular repair, blood-flow recovery after tissue ischemia, distraction osteogenesis^[51,52], fracture healing^[53], bone regeneration^[54], and have osteogenic potential^[55]. EPCs are mainly located in bone marrow and mobilize into the circulation under the guidance of signals such as granulocyte colony-stimulating factor (G-CSF), VEGF, basic fibroblast growth factor, placental growth factor, erythropoietin, or stromal-derived factor-1 (SDF-1) in order to home to ischemic sites^[56,57]. EPCs can be isolated from peripheral blood, cord blood or bone marrow^[58] and classically described as cells expressing a combination of an endothelial marker (VEGF receptor-2) and hematopoietic progenitor markers (CD34/CD133). However, this definition is under debate, since none of these markers are fully specific^[59,60].

In vitro culture of mononuclear cell fraction in specific

conditions consistently produces two distinct EPC subtypes: early EPCs and outgrowth endothelial cells^[61] (that are also known as endothelial colony-forming cells^[62] or late EPCs because of their late appearance in culture). Early EPCs live < 7 d in culture and have a spindle shape morphology and limited proliferation potential. These cells are CD133, CD31, CD45, VEGFR-2 and CD14 positive^[63]. Late outgrowth EPCs emerge 2-3 wk after seeding from the nonmyeloid cell population (CD14 and CD45 negative), present a cobblestone morphology, have a high proliferation potential, express CD34, CD144, and VEGF-R2, but are negative for CD133, CD45 and CD14^[63].

Both populations are capable of inducing neovascularization, but with a different mode of action. Whereas early EPCs have limited capacity for population doubling and induce only transient angiogenesis, late outgrowth EPCs can expand to more than 100 population doublings^[64]. Early EPCs exert an angiogenic effect mainly by paracrine effect (secretion of proangiogenic growth factors), whereas late outgrowth cells were thought to produce the effect by direct engraftment and may differentiate to osteoblasts in a proper environment^[55].

G-CSF-mobilized CD34 positive cells

The use of G-CSF was suggested as a favorable method to harvest abundant CD34⁺ cells from adult peripheral blood^[65]. This alternative approach to isolate EPC was introduced in order to avoid EPC culture and to overcome the scarcity of these cells in the circulation (0.0001% of total mononuclear cells in peripheral blood of human adults)^[66]. These cells are also known as endothelial and hematopoietic progenitor cell-rich population and present phenotypic characterization that includes endothelial and hematopoietic markers: positive for CD133, CD31, c-Kit, and CD45 but negative for kinase insert domain protein receptor and CD14. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of these cell revealed weak expression of CD31 and osteocalcin^[65]. *In vitro* and *in vivo* experiments using these enriched CD34⁺ cells demonstrated angiogenic and osteogenic properties similar to expanded EPCs^[67]. Peripheral blood and cord blood CD34⁺ cells were used to enhance tissue neovascularization in several preclinical models: stroke induction in immuno-compromised mice, wound healing in diabetic mice, peripheral nerve injuries and spinal cord injuries^[68-71]. Based on the belief that improved neovascularization may be an attractive strategy for bone healing, several studies were conducted to investigate the efficacy of circulating CD34⁺ cells for bone fracture healing and found promising results^[67,72,73].

MECHANISMS OF ACTION IN BONE REGENERATION

The idea of stem cell therapy relies on two possible

mechanisms of action: (1) Direct pathway - this process starts with homing of the transplanted cells to ischemic or damaged site. This is followed by integration of the cells into the site and differentiation into cells specific to the tissue^[74]; and (2) Indirect, paracrine pathway-transplanted cells secrete peptides (growth factors, interleukins, *etc.*) that modify the environment and recruit resident cells (MSCs and EPCs) to the regenerating site^[75].

Homing of transplanted cells into ischemic or injured site allows systemic application of the cells. This includes the recruitment of the cells along a gradient of chemokines and their receptors. The CXCR4-CXCL12 and CX3CR1-CX3CL1 systems are reported to be involved in MSC migration, and among them, SDF-1 alpha and CXCR4 (the SDF-1 receptor) are strong mediators of MSCs and EPCs^[76-80].

Following integration into the damaged site, the fate of the cells will be determined by environmental cues and allow osteogenic differentiation of the cells. The osteoblastic differentiation of MSCs was demonstrated *in vitro* by culturing the cells in the presence of ascorbic acid, inorganic phosphate (beta glycerol phosphate), and dexamethasone. *In vivo* studies have suggested that transplantation of adult stem cells derived from one tissue are able to engraft into developmentally unrelated tissues and, in certain cases, contribute to their regeneration^[81]. Nevertheless, since *in vivo* transdifferentiation of the cells is more difficult to prove, it was suggested that the supposed differentiation was actually a result of fusion between donor and host cells^[82,83]. The second mechanism by which MSCs contribute to bone formation is through indirect paracrine effect. MSCs secrete trophic agents and pro-angiogenic factors [angiopoietin (Ang)-1, -2, Ang-like-1, -2, -3, -4, VEGF, and fibroblast growth factor-2] that recruit resident MSCs to the site of injury and promote angiogenesis that is indispensable for tissue reconstruction^[84].

There is only little data regarding the role of EPCs in bone healing. Early outgrowth EPCs exert an angiogenic effect mainly by product secretion, whereas late outgrowth cells were thought to produce the effect by direct engraftment. Direct incorporation of transplanted EPCs into intima layer of blood vessels was demonstrated in several *in vivo* models^[48,54,85]. The indirect role of late EPCs to sustain MSC survival and function was demonstrated in an ectopic subcutaneous model^[46]. According to this study, EPC-derived paracrine factors *via* platelet-derived growth factor BB/platelet-derived growth factor receptor- β signaling regulate MSC engraftment^[46]. The mechanism of action of CD34⁺ cells was suggested by Kuroda *et al.*^[86] and includes differentiation of the cells into osteoblasts or endothelial cells and engraftment in the fracture healing site. To confirm this hypothesis, transplanted human cells were labeled in the fracture site using human specific antibodies (immunohistochemistry). Furthermore, human specific endothelial and osteoblastic markers

were identified in the fracture site using RT-PCR. Additional mechanism of CD34⁺ cells in bone healing can be attributed to their paracrine effect, especially VEGF secretion. Recruitment of resident rat endothelial cells and osteoblasts to the fracture site was demonstrated following human CD34⁺ cell transplantation. Moreover, injection of VEGF antagonist reduced the vasculogenic and osteogenic effect that was observed following transplantation of CD34⁺ cells into fracture healing sites^[86].

EPIGENETIC REGULATION IN BONE REGENERATION

Epigenetic mechanisms play essential roles in stem cell maintenance and differentiation during bone regeneration processes^[87,88]. During the differentiation of stem cells into osteogenic cells specific genes are up-regulated, while others are repressed. Epigenetic regulation is a crucial mechanism involved in the control of the complex, dynamic gene expression pattern during stem cell differentiation. Epigenetic modifications lead to functional relevant alterations in the genome without any changes in the nucleotide sequence^[89]. Well known examples for epigenetic modifications are DNA methylation and histone modifications^[90]. During DNA methylation, through the enzymatic activity of DNA methyl-transferases (Dnmts), a methyl group is covalently bound to the carbon 5 of the cytosine in the CpG sites of the genome^[91]. Methylation of cytosine results in the down-regulation of gene expression^[90]. In contrast, the absence of DNA methylation is crucial for gene expression^[92]. During bone regeneration DNA methylation is essential. As such it has been documented that CpG methylation of the osteocalcin promoter significantly decreases during *in vitro* osteoblast differentiation of MSCs and thus leads to the up-regulation of osteocalcin expression^[87]. In addition, hyper-methylation of the LIN28 promoter (a gene responsible for the maintenance of stemness) reduces LIN28 expression during osteoblast differentiation and hence facilitates osteogenesis^[93].

Gene transcription is also regulated by histone modifications^[90]. Histones are positively charged proteins that tightly bind to the negatively charged phosphate-sugar backbone of the double stranded DNA. Histone binding to the DNA modulates the accessibility of the DNA for transcription factors^[94]. The most investigated histone modifications are acetylation and methylation. Acetylation, mediated by histone acetylases, of the histone's lysine residue reduces the positive charge of the histone and thus decreases histone binding to the DNA. This in turn increases DNA accessibility for transcription factors and hence leads to increased gene expression. On the other hand, histone deacetylases remove acetyl from histones and therefore enhance histone binding to the DNA. This leads to a closed chromatin structure, reduced DNA accessibility for transcription factors and

thus decreased gene expression^[95]. Histone acetylation plays an important role during bone regeneration. It has been shown that during osteoblast differentiation both the promoter and the coding region of osteocalcin contain high levels of acetylated H4 and H3 histones, pointing to increased transcriptional accessibility to these sites. Furthermore, it has been demonstrated that down-regulation of histone deacetylase 1 is an important process for osteogenesis^[96].

Histone methylation often occurs on lysine and arginine residues. Histone methylation is tightly controlled by the balanced activity between methyl transferases and demethylases^[95]. Histone methylation is involved in osteogenic differentiation of MSCs. It has been demonstrated that the activation of CDK1 promotes osteogenic differentiation of MSCs through phosphorylation of EZH2, the catalytic subunit of PRC2, that in turn catalyzes the methylation of histone H3 on lysine 27 (H3K27)^[97].

The above mentioned examples are only a few describing the complex involvement of epigenetic regulation in bone regeneration. The importance for the understanding of epigenetics in osteogenic differentiation of stem cells gains growing attendance among scientists coping with the development of new technologies for bone regeneration.

TRANSLATIONAL STUDIES

Cell-based pioneer studies in the field of bone regeneration used non-expanded bone marrow derived MSCs. Autologous bone marrow MSCs were used to treat patients with genetic bone metabolism diseases^[41] as well as nonunion fractures^[40]. However, due to the limited number of MSCs in the bone marrow (0.01% of bone marrow cell population) the results following non-expanded MSC transplantation are unpredictable^[98]. Few years later, technological developments in MSC culture allowed expansion and characterization of these cells. The osteogenic potential of expanded, purified and identified MSCs was extensively studied, with conflicting results^[42,99]. These inconsistent results can be related to several factors that influence MSC function: donor related differences (species, age, systemic disorders), insufficient vasculature to the grafted area that caused malfunction and apoptosis of the cells^[46] and inadequate osteoblastic differentiation of the transplanted cells *in situ*. To overcome these disadvantages, attempts were made to improve osteogenic differentiation of MSCs by genetic modification^[100] and to enhance vascularization of the graft^[101,102]. MSCs that were engineered to express an osteogenic growth factor such as BMP2 and implanted *in vivo* were shown to differentiate and contribute to the process of bone formation^[103-106]. According to their results MSC-BMP2 induced new bone formation in orthotopic (bone defect) and ectopic (intramuscular) models. Histological analysis revealed that MSCs-BMP2 differentiated into osteoblasts and became integrated

in newly formed bone alongside recipient cells that also had differentiated into osteoblasts. In contrast, MSCs that were not genetically transformed survived in the damaged tissue as undifferentiated or fibroblastic cells^[98]. Since early vascularization of the graft is crucial for successful bone regeneration, Kumar *et al.*^[101] examined the vasculogenic and osteogenic potential of MSC expressing BMP2 and VEGF (alone or in combination). In the dual therapy group, improved vasculogenesis preceded increased bone healing, suggesting that increased vascularity during the bone-healing process allows continuous flow of all the necessary nutrients and cells needed for bone regeneration. Another approach to improve vascularization of MSC-composite graft was presented by Koob *et al.*^[102] who used co-culture of human umbilical vein endothelial cells (HUVECs) and MSCs. HUVECs and MSCs were seeded alone or in combination into xenograft scaffolds and implanted into calvarial critical-sized defects. Neo-vessel formation was considerably higher in the co-implantation group, suggesting that implanted MSCs supported HUVEC-triggered neovascularization. In addition, implanted MSCs effectively supported bone formation in calvarial defects. However, the human HUVEC-derived neo-vasculature did not improve MSC-triggered bone regeneration in this orthotopic critical-sized defect model^[102].

With the novel discovery of circulating EPCs^[48], and the detection of cells expressing endothelial markers in newly regenerating bone tissue^[107], a new idea to treat bone defects using EPCs was considered. Transplantation of autologous late outgrowth EPCs into critical size gap in sheep tibiae revealed full bridging at 3 mo in 6 out of 7 EPC-transplanted defects while non or minimal new bone formation was observed radiographically in 8 sham-operated defects^[47]. These authors also suggested that the effect of EPC is not limited to vasculogenesis. When sheep EPCs were sub-cultured under osteogenic conditions, they changed their morphology and formed nodular aggregates (1-2 mm diameter) following 1-2 wk incubation and stained positively for markers of osteoblastic differentiation^[55]. These results were confirmed by other research groups that showed promising results with late EPCs^[108,109]. However, none of them utilized expanded human late EPCs. This could be related to difficulties in human late EPC culturing that demands further improvement. The negligible concentration of circulating EPCs in humans is probably the main obstacle in this process; however, their concentration increases following trauma, bone fractures, malignancy, tissue ischemia, physical activity and GM-CSF administration^[110]. Contrary to the difficulties arising with human late EPC culture, early human EPCs (that have only limited proliferation capacity) are more accessible, therefore can alternatively be used. Seebach *et al.*^[54] compared healing of critical size defect in athymic rats following transplantation of human early EPCs/bone marrow MSCs/and coculture of EPCs + MSCs. Early vascularization was significantly improved

in the EPC + MSC group or EPC group. Bone formation increased significantly when treated with co-culture of MSCs + EPCs and to a lesser extent in the MSC group. Surprisingly, transplantation of early EPCs that increased early vascularization failed to enhance bone formation. Therefore, it is suggested that there is a synergistic effect and that the initial stage of neovascularization by EPCs is considered to be crucial for complete bone regeneration by MSCs in the late phase^[54].

EARLY CLINICAL FINDINGS - BONE REGENERATION USING AUTOLOGOUS STEM/PROGENITOR CELLS

One of the leading research groups in the field of bone regeneration recently published a randomized controlled clinical trial in which human autologous cells were transplanted into bone defects with promising results. Bone repair cells were produced from bone marrow aspirates, isolated, cultured and characterized. Flow cytometry demonstrated that these cells were enriched for mesenchymal and vascular phenotypes. Cell suspension was absorbed onto a gelatin sponge that was transplanted into the extraction site and covered by a bio-absorbable collagen barrier membrane. Six weeks later, a biopsy of the regenerated site revealed highly vascularized, mineralized bone tissue formation^[111]. Promising results were also reported following transplantation of human autologous bone marrow derived MSCs to augment cleft palate and maxillary sinus^[112,113]. Orthopedic use of bone marrow MSCs to treat long bone fractures was also recently reported^[114]. Autologous osteoblasts, isolated from bone marrow and cultured in osteogenic conditioned medium, were transplanted into the fracture two weeks post internal fixation. They showed statistically significant acceleration of fracture healing in the experimental group that was treated with cells compared with the nontransplanted group. Importantly, none of these studies reported on significant inflammatory reaction or serious adverse effects.

From a clinical point of view peripheral blood progenitor cells are more accessible compared with bone marrow derived MSCs. Therefore attempts are made to isolate, culture and transplant blood derived progenitor cells into bone defects. The first clinical case of tibial nonunion treated with peripheral blood progenitors (G-CSF-mobilized CD34⁺ cells) was published by Kuroda *et al.*^[115]. In this case report, delayed healing and nonunion were observed following primary operation that included transplantation of autologous iliac bone block. Nine months later, mobilized CD34⁺ cells were transplanted into the site and resulted in bone union without any symptoms including pain and gait disturbance^[115]. Encouraged by these results, the same research group began a phase I /II a clinical trial: Autologous local transplantation of G-CSF-mobilized

peripheral blood CD34⁺ cells for patients with tibial or femoral nonunion^[110]. Five days following G-CSF injection, each patient's cells were magnet sorted to separate the CD34⁺ cells. Treatment included autologous transplantation of G-CSF-mobilized peripheral blood CD34⁺ cells suspended in atherocollagen gel. Radiological fracture healing at 12 wk was achieved in five of seven (71.4%) patients, which was greater than the threshold (18.1%) predefined by the historical outcome of standard care.

SAFETY OF ADULT STEM CELL TRANSPLANTATION

Clinical trials that assessed the safety of MSC transplantation for the treatment of graft-vs-host disease, ischemic heart disease, spinal cord injury, and systemic lupus erythematosus, did not find any significant adverse effect^[116-120]. Nevertheless, studies that investigated the role of MSCs in tumor-genesis found that MSCs have the ability to interact with tumor cells, support tumor angiogenesis and tumor development by providing the matrix required for new vessels and tumor cell scaffolding^[121,122]. Therefore, the potential of MSCs to facilitate growth of preexisting occult tumors raised concerns^[123,124]. *In vitro* transdifferentiation of MSCs was also reported but may be related to contamination with cancer cells^[125,126].

EPCs initiate vasculogenesis, as such, these cells play a critical role in pathological vasculature in tumor growth^[121] and metastasis^[127]. Recent clinical studies demonstrated an increase in the amount of circulating EPCs in patients suffering from malignant tumors and in cases of invasive or recurrence of the disease^[128,129]. These observations underline the need for careful phenotypic, functional, and genetic characterization of EPCs/MSCs before cell administration or therapy. Also, the question of whether patients should be treated with autologous, or donor-derived progenitor-cells remains to be addressed.

PROSPECTIVE

There is a significant clinical need to develop innovative approaches for the treatment of large bone defects, nonunion fractures and alveolar bone atrophy. In recent years, many efforts are being made to regenerate bone using stem cells and progenitor cells. While animal studies and limited human studies have shown efficacy of bone marrow derived MSCs and circulating EPCs, the mechanisms by which these cells improve bone regeneration are still unclear. Numerous factors restrict the clinical use of stem cells: immune rejection of cells that are not of autologous origin; limited amount of available cells for transplantation that requires *in vitro* cell expansion with xenograft materials; donor related differences (*e.g.*, age and systemic conditions) that affect cell function and the absence of potency test

that can predict the *in vivo* function of cells before transplantation. Moreover, safety and regulation of these procedures have to be performed before clinical trials. To overcome these drawbacks, new technologies that will allow increasing the amount of cells available for transplantation and standardization of growth conditions need to be developed. In addition, understanding the mechanisms of action will enable a breakthrough in bone regeneration induced by cells.

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Current focus of stem cell application in retinal repair

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research. Embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adipose derived mesenchymal stem cells (ADMSCs) are the focus in current endeavors as a source of different retinal cells, such as photoreceptors and retinal pigment epithelial cells. The aim is to apply them for cell replacement as an option for treating retinal diseases which so far are untreatable in their advanced stage. ESCs, despite the great potential for differentiation, have the dangerous risk of teratoma formation as well as ethical issues, which must be resolved before starting a clinical trial. iPSCs, like ESCs, are able to differentiate in to several types of retinal cells. However, the process to get them for personalized cell therapy has a high cost in terms of time and money. Researchers are working to resolve this since iPSCs seem to be a realistic option for treating retinal diseases. ADMSCs have the advantage that the procedures to obtain them are easier. Despite advancements in stem cell application, there are still several challenges that need to be overcome before transferring the research results to clinical application. This paper reviews recent research achievements of the applications of these three types of stem cells as well as clinical trials currently based on them.

Key words: Retina; Adipose derived mesenchymal stem cells; Embryonic stem cells; Induced pluripotent stem cells; Cell therapy

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Core tip: Several retinal diseases are based on different retinal cell layer degenerations. Some of them have no treatment to date. Advances in stem cell therapy are considered a realistic option. In contrast to the early stage, the late stage of these diseases requires treatment techniques based on cell replacement. Currently, a few are in clinical trials using the sources of embryonic stem cells, induced pluripotent stem cells and adipose derived mesenchymal stem cells. The focus is mainly to enhance the output of these techniques, securing faster implementation and patient safety. Nevertheless, there

Abstract

The relevance of retinal diseases, both in society's economy and in the quality of people's life who suffer with them, has made stem cell therapy an interesting topic for

are many challenges still to resolve.

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INTRODUCTION

The retina is a very complex peculiar tissue which has ten different layers, namely retinal pigment epithelium (RPE), photoreceptor outer segments, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and inner limiting membrane. The cells and membranes of these layers are interconnected and in constant physiochemical communication to maintain retinal homeostasis, anatomy and functional integrity. An alteration or damage in any of these layers can impair these communications, thereby affecting the normal homeostasis, anatomy and functions of the retina^[1]. It can result in an onset of retinal diseases which can lead into visual impairment or blindness.

An example of this is the dry form of age-related macular degeneration (AMD) in which the damage affects four interconnected layers: the RPE, photoreceptor, Bruch's membrane and the choriocapillaris^[2]. Post stimulation of damage events, the RPE degenerates finally and consequently the photoreceptor also degenerates, leading to blindness^[2]. In addition to AMD^[2-4], currently there are many other retinal diseases, such as retinitis pigmentosa^[4,5] and diabetic retinopathy^[4,6], and eye diseases, such as glaucoma and non-arteritic anterior ischemic optic neuropathy, in which damage occurs on different retinal layers (Table 1). Thereby, it provokes retinal cell degeneration that results in vision loss in patients with the disease. Remarkably, some retinal diseases have no treatment yet although their impacts on daily life and social and economic consequences are very high.

All the retinal diseases mentioned already cause degeneration and loss of the retinal cells. It is logical to think that the replacement of damaged retinal cells with healthy ones could be a therapeutic option but it is necessary to have a suitable source of the cells. Stem cells are very prominent and promising for treatment development because they have shown very positive results so far. They have the advantage over the patient's adult retinal cells, solving the various issues of treatment development, including the gene related issues. The impact of retinal diseases on daily life and the social and financial consequences enforce the need for research of stem cells-derived therapies applied to human trials as fast as possible without forgetting issues of the safety and ethics^[7]. Because of this, there are several ongoing research studies about this topic.

This review focuses on recent achievements of the applications of embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adipose derived mesenchymal stem cells (ADMSCs) in retinal repair. It also describes the clinical trials based on the use of these three types of stem cells and deals with the disadvantages of the use of each of these stem cells.

Stem cells have also shown neuroprotective effects on degenerating retinal cells which could be applied to delay or stop the cellular damage events; thus, it could be useful to treat retinal diseases in their early stage. However, this review mainly focuses on cell replacement therapy which is applicable in the late stage of retinal degeneration diseases. This review article is based on the approaches used in published articles. It does not describe the biological mechanisms involved in each approach in detail but only describes recent advances in research that seem interesting for performing an *in vivo* test, thus transferring the results to the clinic in a short period.

ESCs

ESCs are pluripotent stem cells which means they can differentiate into ectoderm, mesoderm and endoderm cells. However, some ESC cells lines show more differential potential towards a particular lineage than others do. For example, it has been found that human ESC H7 has good differentiation potential into beating cardiomyocytes but is weak into ectodermal lineages^[8], whereas human ESC Regea 08/023^[9] has better ectodermal than beating cardiomyocytes differentiation potential^[8]. Which lineage of the ESC and protocol will be used in the experiments to achieve the research objective have to be considered in the decision making.

The ESC pluripotent characteristics have allowed several studies to be performed in which differentiating these cells into both neural^[10-12] and epithelial retinal cells^[13-15] has been achieved. A very crucial step is to successfully achieve establishing a method of ESC differentiation to obtain a good number of healthy differentiated retinal cells with no gene related issues because, as described above, different retinal layers are affected with executed cellular degeneration events. A specific type of cells to replace a retinal layer is needed. On the other hand, ESCs have also been used to obtain neural^[16,17] stem cells, multipotent cells which can differentiate into limited cells types including neuroretinal cells.

ESC differentiated cells could be used to replace the damaged cells of the retina. There are many published methods for this purpose. However, it is very important that the cells can be grown and differentiated in standard culture conditions. The viral-free standard culture conditions are preferable to minimize the risks associated with a virus in cellular transplantation. ESCs have been used to obtain neural stem cells by using small molecules such as CHIR99021 and SB431542. It

Table 1 Summary of retinal diseases and the type of cells affected in each case

Retinal disease	Type of cells affected
Age-related macular degeneration	Photoreceptors and retinal pigment epithelial cells
Glaucoma	Retinal ganglion cells and their axons
Non-arteritic anterior ischemic optic neuropathy	Retinal ganglion cells
Retinitis pigmentosa	Photoreceptors
Diabetic retinopathy	Neural retinal cells

has been found that they protect photoreceptors and visual function. Hence, obtaining this type of cell by using small molecules has the advantage that they are obtained efficiently and without the use of virus which could produce a risk^[4]. Thus, they could be used as a potent cell source for treating degenerative retinal diseases^[16].

ESCs have been also used to obtain progenitor retinal cells which can be differentiated to specific retinal cells such as photoreceptors. There are many published methods for this purpose but identifying an effective and efficient method is crucial if performing a human clinical trial. Amirpour *et al.*^[17] compared three methods of obtaining photoreceptors using co-culture of the RPE with retinal progenitor cells differentiated from ESCs. In two of them, the RPE were indirectly co-cultured on filters for 1 and 2 wk respectively, with retinal progenitors cells differentiated from ESCs. The filter blocked the two layers coming in to physical contact, although they were in chemical communication using the different cell secreted biomolecules. The third one was a direct co-culture of both types of cells for 2 wk. They concluded that direct co-culture with the RPE improved the expression of late photoreceptor markers such as arrestin. In another method, Decembrini *et al.*^[10] produced a transgenic mouse ESC line following a three-dimensional (3D) culture method. The combination of these transgenic cells coupled with a 3D culture system allowed achieving the production of a secure and renewable source of photoreceptors compatible with transplantation^[10]. Previously, it was demonstrated that ESC-derived photoreceptor precursor cells were able to integrate when transplanted into degenerated adult mouse retina. In addition, the transplanted cells matured toward outer segments and formed synaptic connections^[11]. In this study, the 3D neuroepithelial cyst culture system was used to achieve the differentiation of ESCs into photoreceptor precursors. In addition, current investigations showed that hypoxia seems to play an important role in making differentiation of mouse ESCs towards the photoreceptors more effective and efficient. Thus, the study of using mouse ESCs culturing under hypoxic conditions provides an important method that can be used to improve modeling of retinogenesis *in vitro*^[12]. Thus, to obtain photoreceptors and in all studies in general, the method used to culture the cells is crucial for the results obtained. Comparing the results of different previous studies for planning new strategies of a method development should be considered.

These studies and their produced results are essential for advances in photoreceptor replacement therapy. As stated above, ESCs are a source of neural stem cells^[16,17] and photoreceptors^[10-12] but they are also able to differentiate into RPE cells^[13-15] which play an important role in the pathogenesis of many retinal diseases, including AMD. Diniz *et al.*^[13] studied the survival of human ESC-derived RPE transplanted in the subretinal space of immunocompromised nude rats. The results of this study^[13] showed that these cells could survive for several months and the obtained results were enhanced when cells were transplanted as a polarized monolayer of the hESC-RPE rather than as a cell suspension. Nevertheless, RPE is a layer of polarized cells which execute different functions at the apical and basal portion.

Most standard culture conditions of stem cell differentiation methods contain animal generated products such bovine serum, fetal calf serum, *etc.*, as nutrient supplements. However, when the cells are transplanted in humans, animal serum could result in host rejection^[18-20]. Therefore, serum-free medium was used in many studies. During the growing and differentiation of ESCs into RPE under serum-free conditions, the cells were found to express several genes which encode a family of proteins known as aquaporins. These proteins are responsible for allowing passive water transport through the RPE^[14]. Thus, this study added a property to ESC differentiated RPE which is essential for retinal homeostasis and functional integrity. Cell replacement therapy is very effective when it repairs the damaged functional properties of a cell layer as well as acquiring the cell phenotype. Hence, this is an important area of study in which the search for effective methods are currently focused^[3,15].

Recently it was reported, as stated above, that it is possible to generate RPE from ESCs using a 3D neuroepithelial cyst culture of these stem cells. This method could also be used to produce other types of neuronal cells in addition to RPE^[15]. It is also very favorable to minimize the host rejection issues if a cell culture method can produce different types of cells.

Despite all the advantages of ESCs, tumor formation is considered an important risk for the clinical application of these stem cells^[21]. Hence, teratoma formation must be studied and controlled for each method before successfully carrying out a clinical trial^[22]. It has been proved that the use of a method which achieves differentiation of ESCs and thus secures the loss of its pluripotency is related to less tumor growth when

the cells were transplanted in animals^[12,13]. The study showed a lack of tumors in immunocompromised nude rats transplanted with the well differentiated ESC-derived RPE. It supports the theory that the risk of this side effect could be minimized in immunocompetent recipients^[13]. It found that WNT signaling cascade plays an important role in tumor formation and therapeutic functionality of ESC-derived retinal progenitor cells^[21]. Such studies exploring signaling cascade roles could support developing an effective cell replacement therapy. Nevertheless, ethical concerns, limited cell sources and the related high incidence of malignant transformation restrict the wide application of ESCs in retinal repair.

Currently, there are some registered clinical trials using ESCs to treat retinal diseases (<http://www.clinicaltrials.gov/>): (1) (NCT01469832) Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (hESC-RPE) Cells in Patients with Stargardt's Macular Dystrophy (SMD); (2) (NCT01691261) A Study of Implantation of Human Embryonic Stem Cell Derived Retinal Pigment Epithelium in Subjects with Acute Wet Age Related Macular Degeneration and Recent Rapid Vision Decline; (3) (NCT01674829) A Phase I / II a, Open-Label, Single-Center, Prospective Study to Determine the Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (MA09-hRPE) Cells in Patients With Advanced Dry Age-related Macular Degeneration (AMD); (4) (NCT02122159) Research With Retinal Cells Derived From Stem Cells for Myopic Macular Degeneration; (5) (NCT01344993) Safety and Tolerability of Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients with Advanced Dry Age Related Macular Degeneration (Dry AMD); (6) (NCT01345006) Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients with Stargardt's Macular Dystrophy; and (7) (NCT01625559) Safety and Tolerability of MA09-hRPE Cells in Patients with SMD.

INDUCED PLURIPOTENT STEM CELLS

Human induced pluripotent stem cells (hiPSCs), generated by somatic cells reprogramming, have been a strong attractive focus of researchers in recent years. This is due to its feasible application in transplantation for cell replacement therapies for retinal diseases. However, it needs intensive investigation in the laboratory and subsequently in early-phase human clinical trial to improve the differentiation, transplantation and integration of the cells, with the goal of obtaining maximal therapeutic efficacy^[7].

Like ESCs, iPSCs are able to differentiate in several retinal cells, such as the photoreceptors^[23], RPE^[24-29], retinal ganglion like cells^[30,31] and retinal progenitor cells^[27,32]. In addition, these stem cells can form optic vesicle-like structures that could be used as a model

system to study the development of the retina^[33].

Using iPSCs of swine, their differentiation into photoreceptors in cell culture has been achieved. In addition, when these cells were transplanted into the subretinal space of pigs, it was found that they integrated in the retina previously damaged with iodoacetic acid^[23]. On the other hand, this study used the pig as an animal model for a retinal stem cell transplantation study, which could be taken into consideration for future transplantation studies. The pig and human eyes show some similarities in anatomical and histological structure and thus provide the opportunity to translate the research results to human clinical use in a short period.

Regarding iPSC derived RPE, the sheets of the differentiated RPE cells have been found to be very similar in morphology and physiology to the native RPE, which is not the case for the RPE cells in suspension. Furthermore, they have other advantages, such as traceability due to the autologous property, the strong coverage of the lesion when they are transplanted and reduction of the risk of graft rejection, mainly in diseases with a disrupted blood-retinal barrier^[24]. Despite the mentioned advantages, the surgical method of sheet transplantation is more complicated and invasive in comparison with cell suspension transplantation^[24]. This important issue must be resolved before planning a clinical trial with any constructed cell sheet.

The RPE plays an important role in the retina, regulating many functions including its participation in the visual cycle. Thus, it is a crucial layer for maintaining vision function. Human iPSC derived RPE in cell culture express proteins related to visual cycle. In blind mice, these RPE cells with a functional visual cycle were transplanted and it was found that they reestablished in part the visual function of mice. This result supports the possibility of the use of these cells for treatment of retinal degenerative diseases^[25]. Generating a functional monolayer of RPE cells showing native RPE characteristics, such as pigmentation, morphology, specific marker expression, polarized membrane, vascular endothelial growth factor secretion and phagocytic activity, in large amounts from human iPSCs by using simple methods has been achieved. The modifications done in this simple method with respect to the standard method were the amplification of human iPSC by clonal propagation. Furthermore, enrichment of the RPE cells has been achieved by serial passages as an alternative to mechanical picking^[26]. In another study, developing an easy method to differentiate iPSCs into the RPE and also into retinal progenitor cells has been achieved. Thus, it provides useful data for reducing the time necessary for translating the results to the clinic. This method consists of culture of confluent human iPSCs without using Matrigel or serum in a proneural medium^[27]. However, it is necessary to control cell passaging during their culture for maintaining the features of human iPSC derived RPE. It was found that in fourth passage of culture the morphological and functional characteristics of the cells were changed^[29].

Thus, these studies provide enough advances for exploring the feasible application of human iPSC derived RPE.

Like ESCs, iPSCs were also used to obtain neural progenitors. In both *in vitro* and *in vivo* studies, neural progenitors derived from human iPSCs showed their differentiation potential into retinal cells. In a study^[32], these cells were transplanted into rats with injured optic nerves and the results showed that there was a rescue of injured optic nerves due to the stem cell-mediated neuroprotective effect on the retinal ganglion cells. This confirmed that iPSCs maintain a stem cell neuroprotective property that could be applicable in developing neuroprotective property based treatments for retinal diseases. However, as mentioned earlier, this review focuses only on cell replacement therapy for retinal diseases.

In addition to these three types of cells, it is also possible to produce retinal ganglion like cells from iPSCs. Applying methods for induction of the adenoviral genes, retinal ganglion like cells were produced faster than what could be produced using lentiviruses. This was due to a more efficient reprogramming process executed inside the cell when mediated by adenoviruses. There was no viral genomic integrations if adenoviruses were used in the study, *i.e.*, these cells were free of exogenous gene integration. In this study, the resulting cells were derived from the mouse fibroblast and they showed electrophysiological characteristics^[30]. The fibroblasts were obtained more easily in sufficient amounts for a patient than the ESCs and their successful reprogramming resolves to an extent the issues related to finding an appropriate cell source and the ethics compared to ESCs. Recently it was reported that the ciliary body derived cells showed higher reprogramming efficiency than the fibroblasts for producing iPSCs; thus, it could be also overcome the issues related to the ocular origin^[34]. However, the concerns related to the autologous cell nature and the surgical complications of obtaining the cells from a patient could discourage the use of ciliary body derived cells.

Furthermore, it has been hypothesized that iPSCs play a role in retinal ischemia and reperfusion (I/R) injury. In a rat model of this retinal disease, iPSCs without c-Myc (oncogene that contributes significantly in tumor formation) were injected subretinally^[35]. Results of this study showed that transplanted non-c-MYC iPSCs rescued I/R retinal damage induced in rats and reduced the tumorigenicity. Thus, it confirms the suitability of iPSCs for treating this disease. Besides this disease, transplantation studies in animals are continuously showing positive results for the feasibility of iPSC application in cell replacement therapy for retinal diseases.

iPSCs are an important renewable source of cells, with all that it means for both medicine and basic research^[36]. Despite numerous challenges that remain, iPSCs could be a realistic option for cell replacement treatment in the future for patients with retinal diseases

that are still untreatable and need replacement of the damaged cells. Thus, it could play a significant role in research strategies to slow down or stop the disease progression or could completely treat the disease and probably enhance visual function^[37].

As stated above, there are still challenges for the application of iPSCs^[37] which need to be overcome before translating the advances to the clinic, such as the risk of leading to malignant tumors^[36]. Unlike ESCs, iPSCs have the advantage of being used for autologous transplantation. Consequently there will be fewer immune rejections, although on the other hand, personalized cell therapy has a high cost in terms of both economics and time^[38]. Therefore, several researchers have a common goal^[26,27] to develop an easy method that could reduce these disadvantages.

Currently there are some registered clinical trials using iPSCs to treat retinal diseases (<http://www.clinicaltrials.gov/>): (1) (NCT02162953) Development of Induced Pluripotent Stem Cells from Patients with Best Disease and Other Inherited Retinal Degenerative Diseases; and (2) (NCT01432847) Generation of induced pluripotent stem (iPS) Cell Lines from Somatic Cells of Participants with Eye Diseases and from Somatic Cells of Matched Controls.

ADMSCs

ADMSCs are a source of adult stem cells which can be obtained by a minimally invasive procedure, liposuction, followed by adequate processing to achieve a population of cells with the desired characteristics^[4]. This is an easier, more accessible and safer source of mesenchymal stem cells than the bone marrow^[6]. In addition, they can be easily expanded under standard culture conditions without progressive culture and enrichment and with a lower risk of contamination^[6,39]. Thus, this is a very important source for obtaining mesenchymal stem cells (MSCs) in large numbers. MSCs are highly significant for patients with retinal diseases, including diabetic retinopathy (DR), AMD and retinitis pigmentosa (RP)^[4].

DR is a common ocular complication in diabetic patients which involves microvascular and neurodegenerative diseases^[6]. ADMSCs could be an effective part of the therapy for the DR since it has been found that these cells homed in to the injured retina and differentiated to form astrocytes and photoreceptor-like cells when injected intravenously in streptozotocin diabetic rats. Besides, ADMSCs were able to reduce blood glucose levels and repair the blood-retina barrier breakdown that occurs in this disease^[6].

AMD is a neurodegenerative disease associated with aging which progressively leads to loss of the central vision. Damage is produced at the RPE, from the posterior extended to the photoreceptor. The RP involve a set of hereditary diseases characterized by degeneration of both types of photoreceptor cells, rods and cones. Patients with RP progressively lose vision, night vision in adolescence, side vision in young adulthood and central vision in later

Table 2 Summary of the routes of administration of stem cells

Type of stem cells	Route of administration	Species used	Ref.
ESC-neural stem cell	Subretinal and intravitreal	Rat	Lu <i>et al</i> ^[16]
ESC- retinal progenitor cells	Subretinal space	Mouse	Cui <i>et al</i> ^[21]
ESC- photoreceptors	Subretinal space	Mouse	Decembrini <i>et al</i> ^[10] Gonzalez-Cordero <i>et al</i> ^[11]
ESC-RPE	Subretinal space	Rat	Diniz <i>et al</i> ^[13]
iPSC	Subretinal space	Rat	Fang <i>et al</i> ^[35]
hiPSC-RPE	Subretinal space	Rat	Kamao <i>et al</i> ^[24]
		Mouse	Maeda <i>et al</i> ^[25]
iPSC-photoreceptors	Subretinal space	Pig	Zhou <i>et al</i> ^[23]
hiPSC-neural progenitors	Intravitreal	Rat	Satarian <i>et al</i> ^[32]
ADMSC	Intravitreal	Rat	Haddad-Mashadrizheh <i>et al</i> ^[43]
ADMSC	Intravenous	Rat	Yang <i>et al</i> ^[6]

ESC: Embryonic stem cells; iPSC: Induced pluripotent stem cells; ADMSC: Adipose derived mesenchymal stem cells.

life^[5]. Both diseases involve photoreceptor cell death. Therefore, studies of MSCs mainly target two aims: cell replacement and neuroprotection. These stem cells have been differentiated into many cell types, including retinal progenitor cells^[40] and RPE^[41], which confirms their application in cell replacement therapy for retinal diseases. In addition, these cells have been found to exert paracrine effects showing neuroprotective effects on RPE damage *in vitro*^[42]. These properties of these cells were confirmed in animal transplantation studies. Hence, ADMSCs are a potent source of cells with properties that could be applicable in developing treatment.

In a transplantation study, when human ADMSCs were injected in the vitreous cavity, they survived and integrated in the rat eye but were found to cross the blood-retina barrier and migrate to non-targeted organs. Thus, it could be a risk for transplantation in patients. This must be taken into consideration in the strategies of further investigations and before performing a clinical trial^[43]. These MSCs show many advantages in terms of a good source of cells with minimum complexity to obtain in sufficient amounts and develop treatments based on the application of their properties. Nevertheless, there are still several questions about these cells that need to be answered. Therefore, further research is needed to understand the action mechanisms of these cells and when they could be transplanted^[39].

Currently there are some registered clinical trials using ADMSC to treat retinal diseases (<http://www.clinicaltrials.gov/>): (1) (NCT02024269) Study to Assess the Safety and Effects of Cells Injected Intravitreally in Dry Macular Degeneration; and (2) (NCT02144103) Effectiveness and Safety of Adipose-Derived Regenerative Cells for Treatment of Glaucomatous Neurodegeneration.

ROUTES OF ADMINISTRATION OF ESCs, iPSCs AND ADMSCs

The present review describes both *in vitro* and *in vivo* studies. In the latter, researchers have used different routes of administration of the stem cells for evaluating

the safety and efficacy of their approaches. The subretinal space^[10,11,13,16,21,23-25,35] is a place most frequently used to inject the stem cells, but intravitreal^[16,32,43] and intravenous^[6] administration have also been used. Table 2 presents a summary of the routes of administration of each type of stem cells discussed in this review.

CONCLUSION

In the last few years, there have been important advances in the area of stem cells which have made their application in cell replacement feasible. It could be used as a cell replacement therapy for untreatable retinal diseases where cells cannot be recovered due to cell damage. A type of stem cells, the MSCs, are useful as neuroprotective agents, but also, the use of stem cells would not prevent apoptotic progression (the main cause of these diseases) and this is an important and still unresolved problem. The advances in both approaches should be taken into consideration as realistic therapeutic options for developing treatments for untreatable retinal diseases. However, before applying these advances to clinical therapy, it is necessary to perform extensive investigations to improve the processes of obtaining, differentiation and implantation of these cells to overcome the challenges which are still present.

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Search for naïve human pluripotent stem cells

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implantation embryonic cells, and thus were called embryonic stem cells (ESCs). More than a decade later, pluripotent cells were isolated from the ICM of human blastocysts. Despite being called human ESCs, these cells differ significantly from mouse ESCs, including different morphology and mechanisms of control of pluripotency, suggesting distinct embryonic origins of ESCs from the two species. Subsequently, mouse pluripotent stem cells were established from the ICM-derived epiblast of post-implantation embryos. These mouse epiblast stem cells (EpiSCs) are morphological and epigenetically more similar to human ESCs. This raised the question of whether cells from the human ICM are in a more advanced differentiation stage than their murine counterpart, or whether the available culture conditions were not adequate to maintain those human cells in their *in vivo* state, leading to a transition into EpiSC-like cells *in vitro*. More recently, novel culture conditions allowed the conversion of human ESCs into mouse ESC-like cells called naïve (or ground state) human ESCs, and the derivation of naïve human ESCs from blastocysts. Here we will review the characteristics of each type of pluripotent stem cells, how (and whether) these relate to different stages of embryonic development, and discuss the potential implications of naïve human ESCs in research and therapy.

Key words: Naïve pluripotent stem cells; Epiblast stem cells; X chromosome inactivation; Human embryonic stem cells

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Core tip: Mouse embryonic stem cells (ESCs) are the *in vitro* equivalent of the epiblast of preimplantation embryos. Human ESCs on the other hand, although also pluripotent, appear to be in a more advanced developmental stage than their murine counterpart. Recently, several reports described culture conditions able to support mouse ESC-like human ESCs, called naïve human ESCs. Here we will review the search for human naïve ESCs, discussing their biological and

Abstract

Normal mouse pluripotent stem cells were originally derived from the inner cell mass (ICM) of blastocysts and shown to be the *in vitro* equivalent of those pre-

practical relevance.

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FROM THE MOUSE EMBRYO TO EMBRYONIC STEM CELLS

The mammalian preimplantation development is a process of cell multiplication and differentiation from the zygote to the totipotent blastomeres at the cleavage stage, and then to the pluripotent cells at the blastocyst stage. During preimplantation development, cell lineage specification and spatial segregation result in two cell fate decisions, which are believed to be sequential. The first segregation is into the trophectoderm, involved in the formation of the fetal portion of the placenta, and the inner cell mass (ICM) at the early blastocyst stage. The second segregation divides the ICM into the primitive endoderm, that origins parietal and visceral endoderm of the yolk sac^[1], and the epiblast, which will give rise to the embryo proper^[2] (Figure 1).

Mouse embryonic stem cells (ESCs) are derived from the ICM of blastocysts and represent the epiblast lineage *in vitro*^[3,4]. They are able to maintain an undifferentiated state (self-renewal) and to differentiate into tissues from all three germ layers *in vitro* and *in vivo*. In addition, when reintroduced in preimplantation embryos, mouse ESCs are able to get incorporated and give rise to all tissues of the resulting chimera.

In the pluripotent state, mouse ESCs are characterized by the presence of transcription factors such as OCT3/4^[5] and Nanog^[6], expression of alkaline phosphatase and specific cell surface glycoproteins such as stage-specific embryonic antigen 1^[7], and high telomerase activity^[8]. In addition, like the epiblast lineage *in vivo*, female mouse ESCs have not undergone random X chromosome inactivation (XCI), and will do so upon differentiation *in vitro*^[9].

Maintenance of the undifferentiated state of ESC *in vitro* is greatly dependent on exogenous factors. The first mESC derived were maintained in medium conditioned by teratocarcinoma cells^[4], or co-cultured with irradiated mouse embryonic fibroblasts (MEF) in an enriched culture medium which guaranteed self-renewal^[3,10]. Later, maintenance of pluripotency was shown to be dependent on myeloid leukemia inhibitory factor (LIF), a molecule which induces differentiation of M1 myeloid leukemia cells^[11]. LIF belongs to the interleukin-6 cytokine family and binds to a heterodimeric receptor consisting of the LIF-receptor and gp130^[12]. LIF acts on mESC self-renewal mainly by activating Stat3 (signal transducer and activator of transcription 3) through the LIF/Stat3

pathway^[13]. In addition, different matrices and scaffolds are also important for maintenance and/or determination of cell fate^[14].

HUMAN ESCS

The first lines of human ESCs were established in 1998 from the ICM of human blastocysts, in culture conditions equivalent to those of mouse ESCs^[15,16]. Subsequently, it was shown that instead of LIF, bFGF was required for the prolonged culture of human ESCs in the pluripotent state^[17].

Despite being derived from preimplantation embryos in apparently the same stage of development, human ESCs differ from their murine counterparts in many ways, including morphology (flat vs domed shaped colonies), passage in culture (clusters vs single cell passage), growth factors dependence (bFGF and TGFβ/ACTIVIN/NODAL vs LIF/STAT3 signaling)^[16], genomic stability^[18], and the epigenetic state of the X chromosome^[19,20]. Furthermore, control of induced specific differentiation of mouse ESCs was shown to differ from human ESCs in many cases^[21], where protocols established with mouse ESCs do not necessarily yield the same differentiated cell population from their human counterpart. This suggests distinct epigenetic states and/or developmental pathways between ESCs from the two species.

Regarding the X chromosome, although female human ESCs in a pre-XCI status equivalent to that of mouse ESCs have been described^[22], this appears to be an unstable epigenetic state, which is lost with time in culture leading to pluripotent cells with one active and one inactive X chromosome^[19,23,24]. This suggested that human ESCs may be in a different developmental stage than mouse ESCs. However, the kinetics of XCI during human pre-implantation development is still not clear, and thus it is not known whether human ESCs are indeed the *in vitro* equivalent of the epiblast lineage, or whether they are altered during establishment/culture by the current *in vitro* conditions^[25,26].

INDUCED PLURIPOTENT STEM CELLS

A novel source of pluripotent stem cells was established in 2006: those derived through nuclear reprogramming of somatic cells by exogenous expression of Oct4, Klf4, c-Myc and Sox2^[27]. These induced pluripotent stem cells (iPSCs) were first established from mouse fibroblasts, and the same strategy was able to convert human fibroblasts also into a pluripotent state^[28].

Human and mouse iPSCs are equivalent to their embryo-derived counterparts human and mouse ESCs, respectively^[27,28], regarding morphology, growth factor dependence and XCI state. Thus, human and mouse pluripotent stem cells derived from developmentally equivalent cells (adult fibroblasts) are still distinct in several important aspects. This may suggest that

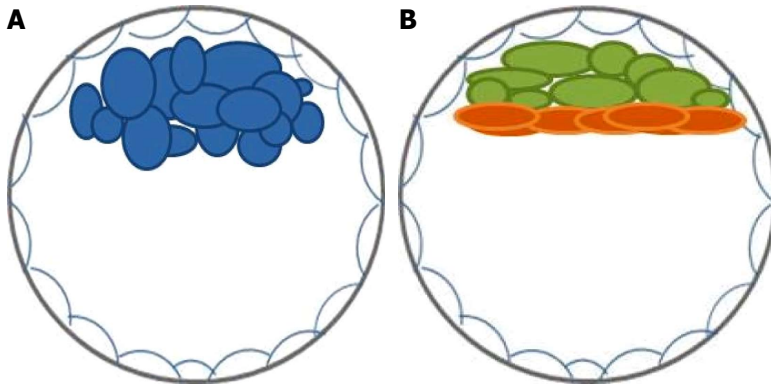


Figure 1 First two cell segregations during preimplantation embryonic development. A: In blue, cells from the inner cell mass (ICM) in the early blastocyst, and white, cells from the trophoblast; B: At the late blastocyst stage, cells from the ICM segregate into cells from the epiblast (green) and the primitive endoderm (orange).

the differences between pluripotent stem cells from the two species are not due to distinct origins of the starting embryonic cell, but rather to distinct pathways defining and controlling pluripotency.

MOUSE ESCS

Indirect evidence that human ESCs are not the developmental equivalent of mouse ESCs came from the establishment of pluripotent SCs derived from the epiblast of post-implantation mouse embryo-epiblast stem cells (EpiSCs)^[29,30]. Although these cells form teratomas when injected in SCID mice, when injected into preimplantation embryos EpiSCs do not integrate to form chimeras like mouse ESCs do. EpiSCs keep their pluripotency *in vitro* when cultured under human ESCs' conditions, present the same flat colony-morphology of the human cells, and are in a post-XCI state. In addition, EpiSC and human ESCs present expression profiles characteristic of the epiblast, suggesting that indeed the human pluripotent cells have embryonic origin distinct of mouse ESCs, and similar to mouse EpiSC.

Therefore, two distinct classes of pluripotent SCs were defined: ground state, or naïve-pluripotent SCs, like mouse ESCs/iPSCs; and primed-pluripotent SC, like human ESCs/iPSCs and mouse EpiSCs, which are in a latter developmental stage^[31]. And thus, the question was rephrased: can human naïve ESCs be established?

In 2009, Bao *et al.*^[32] demonstrated that mouse EpiSC were able to revert to the more primitive epigenetic state of ESCs just by culture in LIF/MEFs. After few passages, ESC-like cells started appearing among EpiSCs, and eventually a sub-line of reprogrammed epiblast ESC-like cells was derived. As part of the reprogramming, the cells reactivated the inactive X chromosome, changing into a pre-XCI state like mouse ESCs and iPSCs. Thus, if mouse EpiSCs could be reverted into ESC-like cells, could human ESCs also be reverted into a naïve pluripotent state by simply changing media conditions?

CONTROL OF PLURIPOTENCY BY SMALL MOLECULES

One important improvement in the dissection of the

pluripotent state was the demonstration that it was not dependent on external stimuli like serum and MEFs, but instead that pluripotency could be maintained by inhibition of endogenous signaling by mitogen-activated protein kinase (MEK), fibroblast growth factor (FGF) receptor tyrosine kinase and glycogen synthase kinase 3 (GSK3). This led to the development of the 3i culture condition, which involves small molecules-inhibition of GSK3, MEK and FGF receptor tyrosine kinase signaling by small molecules CHIR99021, PD18352 and SU5402, respectively^[33]. While inhibition of GSK3 promotes cell growth and viability, inhibition of FGF receptor tyrosine kinase blocks phosphorylation of MEK, thus hampering its action in promoting cell commitment (Figure 2). In comparison, in the original culture conditions of mouse ESCs, LIF acts only in blocking cell commitment, which would be one step later that ERK phosphorylation, and other factors present in serum and secreted from MEFs promote cell growth and viability^[13,34].

Subsequently it was found that inhibition of MEK by PD0325901 was more potent and could substitute both PD18352 and SU5402, in what was called 2i media (CHIR99021 + PD0325901)^[33,35]. The 3i/2i culture conditions allowed the derivation of germ line competent pluripotent stem cells from other species like rat^[36,37] and pigs^[38-43].

Therefore, could this media support derivation of human naïve ESCs? Although one report described the generation of apparently naïve hiPSCs in 3i media^[44], those results were not replicated by any other group, nor were the human cell lines derived ever used in other reports. Thus, by the lack of publications at that time on the derivation of human naïve ESCs, it became obvious that those relatively simple conditions that worked for many mammalian species were not enough for human cells.

Nevertheless, small molecules took the spot light in pluripotent stem cell research, including epi-drugs like inhibitors of DNA methyltransferases and histone deacetylases, shown to have important roles on cell reprogramming and maintenance of pluripotency^[45].

HUMAN NAÏVE ESCS

Indeed, it was subsequently shown that naïve human

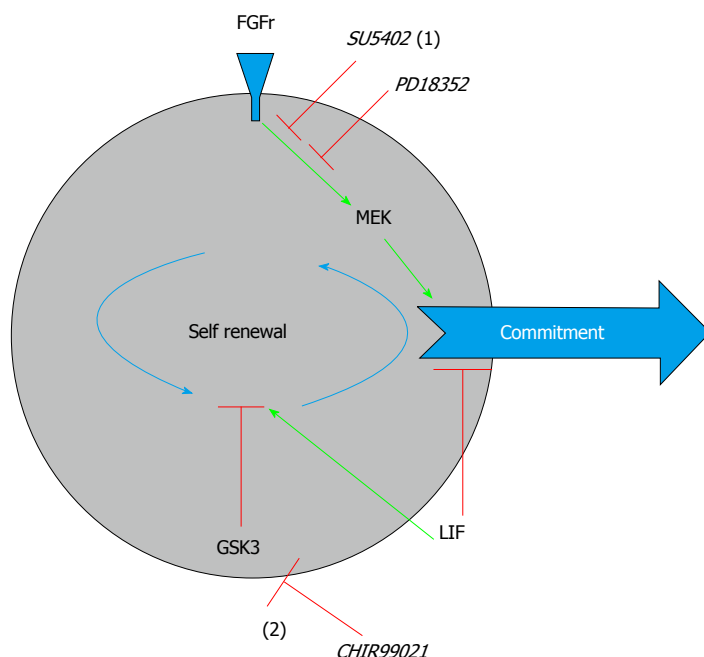


Figure 2 Maintenance of pluripotency by leukemia inhibitory factor and small molecules. Green arrows indicate activation, red blunted lines indicate inhibition. The pluripotent cell must self-renew without committing to any specific cell type. This may be achieved by promoting self-renewal and inhibiting commitment by LIF; or by small molecules (in *italics*) which inhibit (1) signaling through the FGF receptor tyrosine kinase, thus inhibiting commitment; and (2) GSK3, promoting self-renewal. Adapted from^[53]. LIF: Leukemia inhibitory factor; FGFr: Fibroblast growth factor receptor-specific tyrosine kinase; GSK3: Glycogen synthase kinase 3; MEK: Mitogen-activated protein kinase.

pluripotent SCs could only be obtained in 2i when expression of exogenous reprogramming factors was maintained in the cells^[46,47]. Generation of hiPSCs with constant ectopic expression of Oct4, Sox2, Klf4, c-Myc, and Nanog in the presence of LIF lead to human pluripotent SCs with behavior in culture and ease to be genetically manipulated equivalent to those of mouse ESCs^[46]. Similarly, ectopic induction of Oct4, Klf4 and Klf2 in human ESCs in 2i/LIF media reprogrammed the cells into a naïve state transcriptionally similar to that of mouse ESCs, including the reactivation of the inactive X^[47]. Nevertheless, both strategies required continuous expression of the exogenous factors for maintenance of the naïve state.

The next year, Wang *et al.*^[48] reported the efficient generation of exogenous factor-independent naïve hiPSC by transient expression of *Oct4*, *c-Myc*, *Klf4*, *Sox2*, *Rarg* and *Lrh-1* in adult fibroblasts, demonstrating a pivotal role of retinoic acid receptor signaling in reprogramming. However, to our knowledge their results were not replicated by any other group.

After a hiatus of almost two years on reports of naïve human PSCs, within 3 mo four articles described the identification of culture conditions adequate for culture and/or derivation of human naïve ESCs and iPSCs without expression of external factors^[49-52] (Table 1). The first report performed a functional screen of 16 small molecules able to support *OCT4* expression in hiPSCs in LIF/2i. This led to the identification of a combination of 7 factors (including bFGF) able to maintain hiPSCs in a naïve state and to support derivation of naïve human ESCs from blastocysts^[49]. When introduced to mouse embryos, the naïve cells were able to contribute to the formation of E10.5 chimeras, a hallmark of mouse ESCs and not EpiSCs.

In another report, based on the finding that *NANOG* expression is higher in the epiblast of human pre-

implantation epiblast than in human ESCs^[53], Chan *et al.*^[50] tested 11 small molecules that would increase *NANOG* expression in hESCs. They identified a combination of 3 factors that rewired human ESCs into a naïve state responsive to LIF, which was required for self-renewal of the cells (Table 1). In addition, the authors showed that the naïve cells had an expression profile more similar to that of human blastocyst cells than conventional (or primed) human ESCs.

A third report of conditions for naïve human PSCs derivation and culture described a simpler strategy, where human ESCs were exposed to histone deacetylase inhibitors for a few passages before being transitioned to 2i media with bFGF^[51] (Table 1). The 2i + bFGF also supported the derivation of a new line of naïve human ESC that could then be transitioned in to 3i media with LIF. Nevertheless, the efficiency of derivation was very low, and cells in 3i + LIF showed significantly higher rates of differentiation than those kept in 2i + bFGF. Furthermore, although cloning efficiency of the naïve human ESC line established was twice of the primed cell line, it was still relatively low (20%) when compared to other reports, whereas there was no improvement of cloning efficiency of naïve H1 compared to the original primed H1. Thus, the strategy appeared to generate cells in an intermediate naïve state.

Another alternative media for maintenance of transgene-expression free naïve hiPSCs was described by Valamehr *et al.*^[52] (Table 1). While developing culture conditions for high throughput generation of hiPSCs, the group developed a media containing inhibitors of ROCK, GSK3 and MEK pathways, LIF and bFGF that allowed long term culturing of naïve hiPSCs derived in a previously described cocktail of small molecules^[54]. Although those naïve hiPSCs did not have mouse ESC-like morphology, they showed high survival rates in single-cell dissociation, and appeared to be in a pre-

Table 1 Comparison between different protocols to convert/derive human pluripotent stem cell into naïve stem cell

Ref.	Compounds (inhibitor of)	Conversion	Derivation	XCI status
Gafni <i>et al</i> ^[49]	PD0325901 (MEKi) ¹ , CHIR99021 (GSKi) ¹ , SP600125 (JNKi), SB203580 (MAPKi), LIF ¹ , IGF1, FGF2, TGFB1	hESC/hiPSC	hESC/hiPSC	XIST RNA (-) H3K27me3 (-) XaXa nt
Chan <i>et al</i> ^[50] Ware <i>et al</i> ^[51]	PD0325901 (MEKi) ¹ , BIO (GSKi) ¹ , Dorsomorphin (BMPi), LIF ^{1,2} PD0325901 (MEKi) ¹ , CHIR99021 (GSKi) ¹ , FGF2, LIF ¹ , Activin A, NSC74859 (STAT3i), SU5402 (FGFRi)	hESC/hiPSCs hESC/hiPSC	hiPSCs hESC	XIST RNA (-) XaXa
Valamehr <i>et al</i> ^[52]	PD0325901 (MEKi) ¹ , CHIR99021 (GSKi) ¹ , Thiazovivin (ROCKi), FGF2, LIF ¹ , SB431542 (ALKi/TGFB1R)	-	hiPSC	XIST RNA (downreg.) H3K27me3 (< 10%) XaXa
Theunissen <i>et al</i> ^[55]	PD0325901 (MEKi) ¹ , CHIR99021 (GSKi) ¹ , SB590885 (BRAFi), Y27632 (Rocki), WH-4-023 (pan-RTKi), LIF ¹ , FGF, Activin A ²	hESC	hESC/hiPSC	XIST RNA (+) XaXi

¹Compounds used in most works; ²Addition of MEFs. nt: Not tested; ALKi: Inhibitor of transforming growth factor-beta superfamily type I activating; TGFB1: Receptor-like kinase (ALK) receptors; BMPi: Inhibitor of bone morphogenic protein; BRAFi: Serine/threonine-specific protein kinase participating of RAS-RAF-MEK-ERK signaling transduction cascade; FGF: Fibroblast growth factor; FGF2: Fibroblast growth factor 2; FGFRi: Fibroblast growth factor receptor (FGFR)-specific tyrosine kinase inhibitor; GSKi: Glycogen synthase kinase inhibitor; H3K27me3: Tri-methylation of lysine 27 on histone H3; hESC: Human embryonic stem cells; hiPSC: Human induced pluripotent stem cells; IGF1: Insulin-like growth factor 1; JNKi: Inhibitor of c-Jun N-terminal kinase; LIF: Leukemia Inhibitory Factor; MAPKi: p38 mitogen-activated protein kinase inhibitor; MEKi: Mitogen-activated protein kinase inhibitor; Pan-RTKi: Receptor tyrosine kinase (RTK) Inhibitor; ROCKi: Inhibitor of Rho-associated coiled-coil containing protein kinase; STAT3i: Signal transducer and activator of transcription 3 inhibitor; TGFB1: Transforming growth factor beta 1; Xa: Active X chromosome; Xi: Inactive X chromosome; XIST RNA: RNA FISH signals from X-inactive specific transcript - XIST.

XCI state.

More recently, Theunissen *et al*^[55] performed an alternative functional screen of small molecules able to support growth of human naïve ESCs based on the finding that naïve pluripotent stem cells use the distal while primed cells used the proximal enhancer of *OCT4*. Using a GFP-reporter gene under the control of the distal enhancer of *OCT4* in human ESCs, the authors screened a kinase inhibitor library in the presence of 2i/LIF, identifying a combination of 5 compounds which, together with Activin A and FGF, supports conversion of primed into naïve human ESCs and the derivation of new naïve human ESCs and iPSCs (Table 1). It is important to note that, although these converted or newly established naïve cells had the expected dome-shaped morphology and expression profile more similar to mouse ESCs than to primed human ESCs, they presented a few undesired characteristics, namely, (1) high frequency of aneuploidies, and (2) XCI. The authors suggest that, rather than limitations of their “naïve” cells, these may represent differences between naïve human and murine ESCs.

HUMAN NAÏVE ESCS: FACT OR ARTIFACT?

Which brings us to a crucial point: what is a human naïve ESC? When searching for the human naïve pluripotent state, are we looking for the *in vitro* equivalent of the human epiblast, or for a human pluripotent stem cell as amenable to manipulation in culture as mouse ESCs? Depending on the goal, the characteristics of the resulting naïve human cells can be very different.

The methods for culturing human primed ESCs

have significantly improved in the last 10 years with the development of defined and feeder-free culture conditions and of solutions to substitute manual passage. Nevertheless, these cells are still harder to culture and to genetically manipulate than their mouse counterpart. Thus, the development of culture conditions that make human ESCs behave like mouse ESCs may facilitate their use in research and therapy, regardless of whether this naïve state has any relevance as a model for human development. In that case, we know that these human naïve ESCs should have enhanced proliferation, be passaged as single cells (high cloning efficiency), be genetically stable and amenable to efficient genetic manipulation *in vitro*. In addition, being in a pre-XCI state and undergoing random XCI upon differentiation would be desirable for the generation of naïve hiPSCs to model X-linked diseases^[56].

On the other hand, if the goal is to obtain cells that model the pluripotent epiblast from human blastocysts, then we must step back and look at what we know about these cells *in vivo*. Despite the difficulties in studying the human embryo, enough has been done to know that there are significant differences between human and mouse blastocysts so that one cannot completely extrapolate data from one species to the other^[57]. For instance, at the blastocyst stage, while *Oct4* expression is restricted to epiblast cells in mouse^[58], it is also found in the human trophectoderm^[59]. Thus, the organization and/or the markers of pluripotent cells in the human blastocyst may differ from mouse.

Recent advances in single-cell RNA sequencing (RNAseq) have allowed the study of expression profiles of individual cells during human pre-implantation development^[53,60]. Analysis of RNAseq data from 30

single-cells derived from human blastocysts identified three distinct clusters corresponding to cells from the epiblast, primitive endoderm and throphectoderm. Furthermore, comparison of the expression profile of human epiblast cells with human ESCs at different stages of derivation showed that, although similar, there are significant changes in gene expression in the embryo-derived cells at the initial primary outgrow of hESCs^[53]. That is not surprising given that the cells are adapting to the *in vitro* conditions, but how distinct can they be while still modeling the epiblast *in vitro*? It will be interesting to do the same comparison in mice in order to evaluate whether these changes between murine epiblast and ES cells are less accentuated. Nevertheless, it would be reasonable to use the single-cell RNASeq data from human blastocysts as a signature of “true” naïve human ESCs. Indeed, Chan *et al.*^[50] showed that the expression profile of their naïve human ESCs was more similar to those of single cells from human blastocysts. The same analysis should be performed with the other reported naïve human ESCs for comparison.

Regarding XCI, the kinetics of the process is very well characterized during mouse preimplantation development, starting at the two-cell stage with the imprinted inactivation of the paternal X (pX) in all cells of the embryo. Then, at the blastocyst stage, cells of the ICM reactivate the pX, and go through a second wave of XCI, where each cell will randomly choose the Xi. In contrast, much less is known about the XCI status of the human preimplantation embryo, and results are conflicting^[23]. It would be extremely valuable if “true” naïve human ESCs were in a pre-XCI state like their murine counterpart in order to study the mechanisms of this epigenetic phenomenon *in vitro*. However, we still don't know whether this would represent the *in vivo* situation, and thus, whether the pre-XCI state is a prerequisite for being a “true” naïve human ESC.

While this manuscript was in revision, Wang *et al.*^[61] showed that small populations of naïve human pluripotent SCs exist within cultures of primed human ESCs and iPSCs. These naïve cells can be purified and maintained in 2i/LIF media. In addition, the authors identified increased expression of the primate-specific endogenous retrovirus HERVH as a genetic signature of naïve human pluripotent SCs, which can be used for the development of culture conditions to support human naïve PSC culture. More importantly, the study of HERVH-associated transcription during human pre-implantation embryonic development may provide more insights into this still much-unknown process.

In conclusion, in the last year different culture conditions were developed that support the derivation and growth of human ESCs more similar to mouse ESCs. Whether the distinct methods generate similar or distinct epigenetic states of human pluripotent SCs, and more importantly, which of these potentially naïve human pluripotent SCs are more similar to the cells of

the human epiblast remain to be determined. These studies will have great impact on the establishment of a good *in vitro* model for human embryonic development.

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Smart scaffolds in bone tissue engineering: A systematic review of literature

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PubMed from January 2004 to December 2013. Studies which performed smart modifications on conventional bone scaffold materials were included. Scaffolds with controlled release or encapsulation of bioactive molecules were not included. Experiments which did not investigate response of cells toward the scaffold (cell attachment, proliferation or osteoblastic differentiation) were excluded.

RESULTS: Among 1458 studies, 38 met the inclusion and exclusion criteria. The main scaffold varied extensively among the included studies. Smart modifications included addition of growth factors (group I -11 studies), extracellular matrix-like molecules (group II -13 studies) and nanoparticles (nano-HA) (group III -17 studies). In all groups, surface coating was the most commonly applied approach for smart modification of scaffolds. In group I, bone morphogenetic proteins were mainly used as growth factor stabilized on polycaprolactone (PCL). In group II, collagen 1 in combination with PCL, hydroxyapatite (HA) and tricalcium phosphate were the most frequent scaffolds used. In the third group, nano-HA with PCL and chitosan were used the most. As variable methods were used, a thorough and comprehensible compare between the results and approaches was unattainable.

CONCLUSION: Regarding the variability in methodology of these *in vitro* studies it was demonstrated that smart modification of scaffolds can improve tissue properties.

Key words: Bone tissue engineering; Scaffold; Growth factor; Nanoparticle; Extracellular matrix

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Abstract

AIM: To improve osteogenic differentiation and attachment of cells.

METHODS: An electronic search was conducted in

Core tip: Currently, special attention has been directed to the design of new scaffolds by adding bioactive molecules and nanoparticles. "Smart scaffolds" in bone tissue engineering not only act as cell delivery materials, but they are also responsive to their environment and therefore

stem cells are more likely to attach, proliferate and differentiate on them. These scaffolds can be fabricated by adding either of growth factors, extracellular matrix proteins or nanoparticles to the bone substitutes using various techniques. These modifications can enhance the *in vitro* response of bone scaffolds toward cells.

Motamedian SR, Hosseinpour S, Ahsaie MG, Khojasteh A. Smart scaffolds in bone tissue engineering: A systematic review of literature. *World J Stem Cells* 2015; 7(3): 657-668 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i3/657.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i3.657>

INTRODUCTION

Tissue engineering is a helpful alternative strategy for conventional treatments in medicine. It was defined by Langer and Vacant^[1] as “an interdisciplinary field of research that applies the principals of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ”. Bone tissue engineering uses life sciences and engineering to aid the function of injured bone tissue *via* a triad of artificial extracellular matrix (ECM) scaffold, stem cells that can become osteoblasts and growth factors^[2,3]. The efficacy of this combination has been studied in animals^[4-6] and humans^[7-9]. Mesenchymal stem cells, the most common source of osteoprogenitor cells, can be derived from bone marrow^[10-13], adipose tissue^[14-16], and dental and periodontal tissues^[17-19]. Growth factors, such as bone morphogenic proteins (BMPs), have the capability to modulate stem cell activity towards bone regeneration^[20-24].

Scaffolds play a key role in bone tissue engineering providing a 3-dimensional environment for cell seeding and proliferation as well as filling bone defects while providing mechanical competence during bone regeneration^[25-27]. Osteoconductivity, porosity and biodegradation are the required properties for a scaffold to be successful in bone tissue engineering, enhancing bone formation and angiogenesis and supporting attachment and proliferation of osteoblasts^[28]. In this context, various scaffolds such as hydroxyapatite HA, tricalcium phosphate (TCP), collagen, chitosan, polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLGA), have been used^[2,3].

Recently, tissue engineers have focused on structural design and surface properties of scaffolds. Various modifications such as addition of bioactive molecules or nanoparticles can enhance attachment and proliferation of stem cells on the scaffold^[29-31]. These “smart scaffolds” improve osteogenic differentiation of stem cells leading to a more responsive reaction to changes in their surrounding environment^[32]. The current study reviews newly developed scaffolds and their smart modifications.

MATERIALS AND METHODS

Study selection

Papers relevant to *in-vitro* experiments on newly fabricated smart scaffolds were reviewed. Three modifications were considered as smart namely: (1) addition of nanoparticles; (2) addition of ECM-like molecules; and (3) addition of growth factors. Studies which performed these smart modifications on conventional bone scaffold materials were included. Scaffolds with controlled release or encapsulation of bioactive molecules were not included. Experiments which did not investigate response of cells toward the scaffold (cell attachment, proliferation or osteoblastic differentiation) were also excluded.

Search strategy

An electronic search was conducted in PubMed limited to English language, relevant publications from January 2004 to December 2013 with available full texts. Published papers on smart scaffolds were found using the following keywords alone or in combination: bone tissue engineering, smart scaffold, xenograft, xenogenous bone, allograft, allogeneous bone, bone substitute, bovine bone matrix, allogenic bone, xenogenic bone, coat*, surface modification, surface enhancement, biofunctional*, bioact*, biomimet*, nano*, extracellular matrix, collagen and growth factor.

Initial paper selection was done by reviewing titles and abstracts of all selected papers. The full texts of potentially suitable articles were obtained for final assessment according to the exclusion and inclusion criteria. The reports of the most relevant data were included and analyzed in a qualitative manner.

Statistical analysis

This study was a literature review and no statistical method was used in this study.

RESULTS

Figure 1 demonstrates the design of the current review. The initial search resulted in 1458 articles. Following the screening of titles, abstracts and full texts, 38 papers had the criteria for review.

Growth factors

Eleven experiments were studied (Table 1) for scaffold modification; in all these studies, surface coating *via* growth factors to increase scaffold activity was used (Figure 2A). In addition, modification of architecture and spatial conformation of the main scaffold was performed^[38]. The main scaffold differed in each study. The most commonly used scaffold was PCL, followed by TCP, PLGA, Bio-Oss, Calcium/magnesium-doped mesoporous silica, calcium phosphate cement, collagen, carbon nanotubes, TolAIII fusion-osteopontin-switch tag and glutaraldehyde cross-linked gelatin (GTG). The most

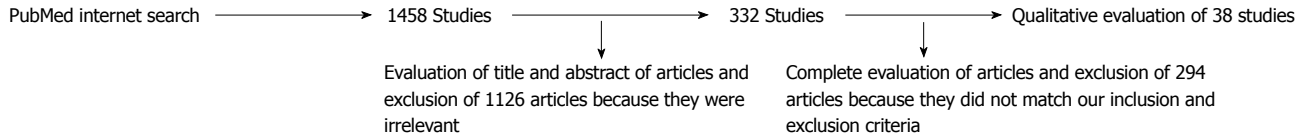


Figure 1 Study design.

Table 1 Description of included studies which modified the scaffolds by addition of growth factors

Ref.	Scaffold	Fabrication	Type of modification	Cell type	Tests and results
Yang <i>et al</i> ^[33] , 2005	GTG + BMP-4	-	Surface modification	NRCOCs	ALP activity: ALP levels in GTG + BMP-4 samples higher than GTG samples H and E staining: Greater numbers of attached cells and richer matrix deposits in the GTG + BMP-4 samples VK staining: Larger mineralizing nodules, in greater numbers
Turhani <i>et al</i> ^[34] , 2007	TCP + rhBMP-2	-	Surface coating	SaOS-2Cs	Cell viability and ALP activity: TCP + BMP-2 > TCP (control) OC secretion: TCP + BMP-2 = TCP
Abarrategi <i>et al</i> ^[29] , 2008	β-TCP + rhBMP-2	Homogenized + demineralized + heterogeneous deacetylation	Surface coating	C2C12Cs	No alteration in biocompatibility <i>In vivo</i> : New bone formation 3 wk after surgery, much shorter time than control β-TCP ceramics
Fei <i>et al</i> ^[35] , 2008	PLGA/CPC + rhBMP-2	Solvent-extraction technique	Surface coating	BMMSCs	ELISA: OC: PLGA/CPC + rhBMP-2: 1.2 ng/mL rhBMP-2/CPC: 0.4 ng/mL ALP activity: PLGA/CPC + rhBMP-2: 0.12 µg/h rhBMP-2/CPC: 0.06 µg/h
Yilgor <i>et al</i> ^[36] , 2010	PCL + BMP-2/BMP-7	Plotting procedure Bioplotter's CAD/CAM software + wet spinning	Scaffold architecture + surface coating	BMMSCs	ALP activity: BMP-2/BMP-7 + PCL: 1.2 nmol/min
Zhang <i>et al</i> ^[37] , 2010	PCL + BMP-2	Crosslinking conjugation method	Surface coating	BMMSCs	RT-PCR: (relative) p-smad: PCL + BMP-2 conjugated five times higher than adsorption and control Col 1: PCL + BMP-2 conjugated: 3 PCL + BMP-2 adsorption: 1.5 Control (PCL): 1 ALP activity: 2 times higher than adsorption and control
Mitchell <i>et al</i> ^[38] , 2010	Tol-OPN-ST+BMP2	-	Spatial and conformational display + surface coating	BMMSCs	Cell attachment: Tol-OPN-ST + BMP2: 175 mm ³ Tol-OPN-ST: 60 mm ³ Luciferase activity: Tol-OPN-ST + BMP2: 8000 ability unit Tol-BMP-ST: 6000 ability unit
Huh <i>et al</i> ^[39] , 2011	Bio-Oss® + rhBMP-2 + iH	Deep and dry methods	Surface coating	MG63Cs	Cytotoxicity and proliferation: No difference compared to control (Bio-Oss®) ALP activity: 0.2 µmol/min per µg higher than control
Dai <i>et al</i> ^[40] , 2011	CMMS + rhBMP-2	Polymeric sponge method	New composition + surface coating	BMMSCs	MTT assay: More viable cells on CMMS + rhBMP-2 compared to CMMS RT-PCR: (relative) RunX2: CMMS + rhBMP-2: 32 Control (CMMS): 4 OPN: CMMS + rhBMP-2: 38 Control: 3 <i>In vivo</i> : Induced the ectopic bone formation in the thigh muscle pouches of mice
Lu <i>et al</i> ^[41] , 2012	Col/PLGA + CBD-BMP4	Forming collagen microsponges	Surface coating	BMMSCs	ALP activity: No differences compared to control group Scaffold supports cell adhesion and proliferation
Li <i>et al</i> ^[42] , 2013	SWNTs-COOH/ SWNTs-CH3 + BMP-2	Organic phase/aqueous phase replacement approach + sonication	Surface coating	C2C12Cs	ALP activity: (relative) SWNTs-ch3 + BMP-2: 150% SWNTs-cooh + BMP-2: 120%

TCP: Tricalcium phosphate; PCL: Poly(ϵ -caprolactone); CMMS: Calcium/magnesium-doped mesoporous silica; CPC: Calcium phosphate cement; GTG: Glutaraldehydecrosslinked gelatin; PLGA: Poly(lactic-co-glycolic acid); col: Collagen; SWNTs-COOH and SWNTs-CH₃: Hydrophilic COOH- and hydrophobic CH₃-terminated single-walled carbon nanotubes; Tol-OPN-ST: TolAIII fusion-osteopontin-switch tag; rh: Recombinant human; BMP: Bone morphogenetic protein; CBD: Collagen-binding domain; BMMSCs: Bone marrow mesenchymal stem cells; NRCOCs: Neonatal rat calvaria osteoblast cells; MG63Cs: Human osteosarcoma cells; SaOS-2 Cs: Sarcoma osteogenic cells; C2C12C: Immortalized mouse myoblast cells; ALP: Alkaline phosphatase; H and E: Hematoxylin and eosin; VK: Von Kossa; RT-PCR: Real time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; XTT: Sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt; RunX2: Runt-related transcription factor 2; OPN: Osteopontin.

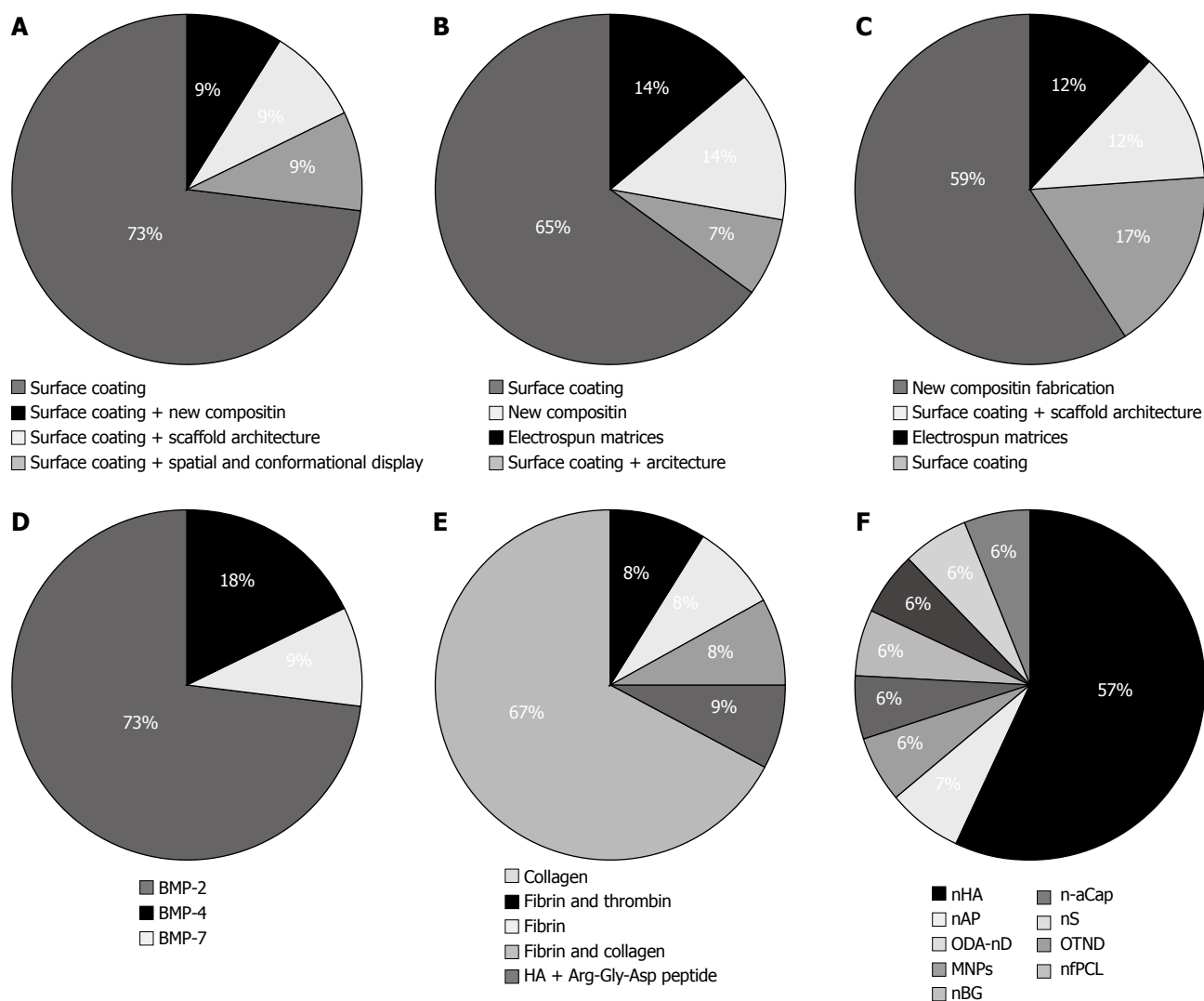


Figure 2 Type of modification method and smart materials in each category. A: Methods for growth factor addition; B: Methods for ECM molecules addition; C: Methods for nanoparticles addition; D: Growth factors added; E: ECM molecules added; F: Nanoparticles added. ECM: Extracellular matrix.

commonly applied growth factor was BMPs including BMP-2, BMP-4 and BMP-7 (Figure 2D). Different methods were used to add BMPs to the main scaffold: attachment of BMP-4 to scaffolds by cross linking conjugating techniques, deep and dry methods for addition of BMP-2 to Bio-Oss and using a set of nanoparticles providing the release of BMP-2. Scaffold fabrication methods varied vastly among studies.

Regarding the study methods, bone marrow mesenchymal stem cells (BMMSCs) were used in 6 experiments, while different type of cells such as neonatal rat calvarial osteoblast cells, Human osteosarcoma cells (MG63Cs), sarcoma osteogenic cells (SaOS-2Cs) and immortalized mouse myoblast cells (C2C12Cs) were used in other studies. In almost all studies, cell viability was assessed with alkaline phosphatase (ALP) activity assay and the best results were obtained using new compositions compared to control groups. In just one study, no meaningful changes were observed^[41]. One study used luciferase activity as their major assay^[38]. In 2 studies, real time

polymerase reaction assay was used with different biomarkers such as: BMP-2, Runt-related transcription factor 2 (Runx-2), Osteopontin, P-smad and collagen 1.

ECM-like molecules

Among 67 articles, which used ECM-like molecules to enhance scaffold properties, 13 experiments were studied (Table 2). Type of scaffold modification in 10 studies was surface coating. In 1 study, architectural and spatial conformation was performed on the scaffolds^[48], and in 2 experiments collagen fibers were electrospun in conjugation with polymer and ceramic components to form a fibrous scaffold (Figure 2B)^[45,52]. Xu *et al.*^[30] produced a new composite scaffold by combining collagen, bioglass, hyaluronic acid and phosphatidylserine. In a study by Lechner *et al.*^[44], fibrinogen was added to bone tissue core. In their study, CD31 was assessed before and after treatment with newly developed scaffold by fluorescent-activated cell scan techniques and their results showed 100-fold increase in expression of CD31 marker^[44]. The most

Table 2 Description of included studies which modified the scaffolds by addition of extracellular matrix molecules

Ref.	Scaffold	Fabrication	Type of modification	Cell type	Tests and results
Kim <i>et al</i> ^[43] , 2006	BGNF + Col 1	Electrospinning process	Surface coating	MG63Cs	ALP activity: BGNF + Col 1 > Col 1
Lechner <i>et al</i> ^[44] , 2006	β-TCP + F + T	-	New composition	BMMSCs	ALP activity: Increased during 28 d of culture FACS: CD 31: Increased expression by 100 folds
Turhani <i>et al</i> ^[34] , 2007	HA + Col 1 + rhBMP-2	-	Surface coating	SaOS-2Cs	XTT proliferation assay: (relative) HA + col 1 + rhBMP-2: 1.5 Control: 0.5 ALP activity: HA + col 1 + rhBMP-2: 50 U/μg Control: 25 U/μg
Srouji <i>et al</i> ^[45] , 2008	PCL + Col	Electrospun meshed scaffold	Electrospun nanofiber membrane	BMMSCs	Alamar Blue assay: PCL + Col = PCL [Data as mean ± SD (<i>P</i> < 0.05)] <i>In vivo</i> : Good integration after subcutaneous implantation
Hao <i>et al</i> ^[46] , 2010	A: ADSCs-Col/ PLGA-β-TCP B: Acellular Col/ PLGA-β-TCP	Low- temperature deposition manufacturing (LDM) based on the layer-by-layer manufacturing principle of solid free-form fabrication	Surface coating	ADSCs	ALP activity: ECM mineralization in group A > group B Evident calcification level in group A, no apparent calcification in group B <i>In vivo</i> : Woven bone with a trabecular structure in group A No bone formation in group B
Xu <i>et al</i> ^[30] , 2009	BG + Col-HYA-PS	Sol-gel method	New composite fabrication	BMMSCs	Cell attachment: Number of attached cells on BG + Col - HYA - PS was the highest Cell proliferation: BG + Col - HYA - PS > BG + Col and BG + Col - HYA > BG ALP activity: BG + Col - HYA - PS > BG + Col and BG + Col - HYA > BG
Kawai <i>et al</i> ^[47] , 2009	OCP + Col	Mixing + lyophilization	Surface coating	MSST-2 Cs	Proliferation and attachment : OCP + Col > OCP (control) <i>In vivo</i> : OCP + Col: Enhanced bone regeneration ratio 83:17 generated maximum repair level of approximately 64% of the defect at 12 wk
Zhang <i>et al</i> ^[48] , 2010	β-TCP + Col	Electrospun + impregnating methods	Architecture + surface coating	MG63Cs	MTT assay: (relative) β-TCP + Col: 0.30 Control (β-TCP): 0.25
Qu <i>et al</i> ^[49] , 2010	C + HA + RGD peptide	<i>In situ</i> compositing hybridization + lyophilization	Surface coating	BMMSCs	Fluorescence microscopy: Cell adhesion rate: CS + HA: 54.7% C + HA + RGD peptide: 71.6% and 80.7% ALP activity: C + HA + RGD peptide: 0.00596 ± 0.00081 U/L per ng C + HA: 0.00283 ± 0.00025U/L per ng
Kang <i>et al</i> ^[50] , 2011	Fi + HAH	The multi-head deposition system	Surface coating	ADSCs	ALP activity: Single day treatment: 0.5 mmol/mg No treatment: 0.5 mmol/mg Daily treatment: 3 mmol/mg
Marelli <i>et al</i> ^[51] , 2011	nBG + DC	Plastic compression technique	Surface coating	MC3T3-E1 CS	Confocal microscopy of fluorescently: Attachment no difference ALP activity: nBG + DC: 3.5 × 10 ⁵ Control (nBG): 2.5 × 10 ⁵ Alamar blue assay: nBG + DC: 6.5 × 10 ⁵ Control: 8 × 10 ⁵
Phipps <i>et al</i> ^[52] , 2011	PCL + Col I + nHA	Electrospun	Bone-mimetic electrospun matrices	BMMSCs	Activation of focal adhesion kinase: Cells seeded onto PCL/Col/nHA scaffolds were better spread, and exhibited greater amounts MTS assay: (relative) PCL + Col + nHA: 5 PCL + nHA: 2.5 PCL: 1
Weeks <i>et al</i> ^[53] , 2012	PLLA + CXCL12, 13 + F + Col IV	-	Surface coating	BMMSCs	Antibody-blocking studies: Anti-α5β1 inhibits MSC attachment: PLLA + CXCL12, 13 + F + Col IV: 500 cells/mm ² PLLA + F + Col IV: 400 cells/mm ²

BG: Bioglass; PLLA: Poly(l-lactic acid); Col: Collagen; C: Chitosan; PLGA: Poly(D,L-lactic-co-glycolic acid); PCL: Poly(ε-caprolactone); HA: Hydroxy apatite; TCP: Tricalcium phosphate; HAH: Hyaluronic acid hydrogel; nBG: Nano-sized bioactive glass; BGNF: Bioactive glass nanofiber; OCP: Octacalcium phosphate; F: Fibronectin; Fi: Fibrin; nHA: Hydroxy apatite nano particles; CXCL: Chemokine ligand; T: Thrombin; rh: Recombinant human; BMP: Bone morphogenetic protein; HYA: Hyaluronic acid; PS: Phosphatidylserine; DC: Dense collagen; iH: Immobilized heparin; RGD: Arg-Gly-Asp; BMMSCs: Bone marrow mesenchymal stem cells; MG63Cs: Human osteosarcoma cells; SaOS-2 Cs: Sarcoma osteogenic cells; MC3T3-E1: Osteoblast precursor cells; ADSCs: Adipose-derived stem cells; MSST-2 Cs: Mouse bone marrow cells; ALP: Alkaline phosphatase; FACS: Fluorescent activated cell scan; XTT: Sodium 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt; MTT: 2-(4,5-dimethyl-2 thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; MTS: 5-[3-(carboxymethoxy)phenyl]-3-(4,5 dimethyl-2-thiazolyl)-2-(4-sulphophenyl)- 2H-tetrazolium inner salt; CD: Cluster of differentiation.

commonly used ECM-like molecule was collagen 1. Other molecules were fibrin, collagen IV, and hyaluronic acid (Figure 2E). The main scaffolds used were TCP, HA, PCL, bioactive glass nanofiber, octacalcium phosphate, Bio-Oss and Bioglass. Scaffold fabrication methods varied vastly among included studies.

Regarding the study method, in the majority of studies cell differentiation was assessed with ALP activity assay and higher activity was reported for experimental scaffolds compared to control groups. In addition, various methods were applied to assess cell attachment, proliferation and differentiation. In 6 studies, the cell type was BMMSCs; whereas in other studies MG63Cs, SaOS-2Cs, osteoblast precursor cells (MC3T3-E1Cs), adipose-derived stem cells (ADSCs), and mouse bone marrow cells (MSST-2 Cs) were used.

Nano particles

Among 85 articles, which used nano particles to enhance scaffold properties, 17 were studied (Table 3). Type of modification was surface modification and scaffold architecture in 5 studies; in another 6 experiments, spatial conformation was performed. Others fabricated new scaffolds by adding nanoparticles to the scaffold composition (Figure 2C). The main scaffold on which modifications were made varied in the studies. These scaffolds included PCL, chitosan, PLGA, gelatin, TCP, poly L-lactic acid (PLLA), single-walled carbon nanotubes, polyamide and HA. Nanoparticles of HA were the most commonly added particles followed by nano fibrous PCL, nano diamond, amorphous calcium phosphate nanoparticles, nano silica, nano apatite, magnetic nanoparticles and nano-sized bioactive glass (Figure 2F). Different fabrication techniques were applied: flame spray pyrolysis, sonication, thermally induced phase, electrospun, blunt-end needle tip and gamma high voltage. Scaffold fabrication method varied vastly among the included studies.

Regarding the study method, various tests were performed to assess cell viability, attachment, proliferation and differentiation. ALP activity assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay, bicinchoninic acid assay, bone Gla protein activity and Alamar blue assay were among these tests. In 5 studies, PCR test was used to assess level of gene expression. Various biomarkers were used in each study such as ALP, OCN, Runx-2, bone sialoprotein, TGF- β 1, BMP-2, collagen 1 and integrin subunits together with myosins. In 10 cases, cell type used in the study was BMMSCs. Other cell types used in the studies were: Mouse bone marrow cells (7F2Cs), MC3T3-E1Cs, osteoblastic cells (ROS 17/1.8 Cs), human airway fibroblast cells, ADSCs, MG63Cs, primary human osteoblasts-like cells and rat calvarial osteoblast cells.

bone regeneration. The most relevant applied methods to design smart scaffolds are surface modification by adding nano particles such as nHA and adding an ECM-like molecule such as collagen or growth factor like BMP-2 to the scaffold. Factors such as scaffold material, fabrication method and type of modification defining the physical, chemical and mechanical properties of scaffolds need assessment. Due to the variability of influencing factors including methodology and scaffold properties, the results were not comparable to determine the most successful design. *In vitro* tests for analyzing the behavior of the fabricated scaffolds vary among the studies. The source of cells also influences the results, and qualitative report of cell migration, attachment, infiltration and differentiation must be similar to compare the results. The results of included *in vitro* experiments demonstrated that smart design of scaffolds supported cell attachment, proliferation and differentiation.

Modification

Surface modification of scaffold materials was the most commonly used design. However, when implanting the graft materials, their surfaces are covered with ECM proteins and molecules and thermodynamic forces cause surface absorption. This issue is commonly overlooked. Alteration of surface properties influences cell adhesion. In addition, surface plays a role in cell migration towards the scaffold and stem cell differentiation. Addition of either HA or collagen to the outer surface of polyethylene terephthalate (PET) caused greater attachment of rat bone marrow cells to the surface and deposition of an HA layer on the surface^[69]. The morphology of attached cells mimics that of more differentiated cells. Although chemical modification of scaffold surface affects cell to scaffold response, effect of material design on cell behavior should also be considered^[70]. Hatano *et al.*^[71] demonstrated that cell differentiation is affected by surface roughness. In the cited study, rougher surfaces caused expression of osteoblastic markers.

Growth factors and bioactive molecules

Understanding the physiological processes of bone regeneration and the involved regulating molecules is necessary to enhance bone repair. Several attempts at adding these molecules to scaffolds were demonstrated (Tables 1 and 2). Regulating molecules include growth factors and other proteins found in bone ECM.

In the reviewed studies, BMPs seemed to be more frequently used in the scaffolds compared to other growth factors. BMPs induce osteogenic differentiation of stem cells and osteoprogenitor cells, and their efficient and safe use in human was licensed in 2001. In addition, growth factors like BMPs and vascular endothelial growth factors can accelerate healing processes^[72,73]. Chemically conjugated BMP-2 on PCL scaffolds caused significantly greater ALP gene expression compared to un-treated PCL scaffolds^[37]. In a study by Lu *et*

DISCUSSION

This study reviewed current trends in smart scaffolds for

Table 3 Description of included studies which modified the scaffolds by addition of nanoparticles

Ref.	Scaffold	Fabrication	Type of modification	Cell type	Tests and results
Kim <i>et al</i> ^[54] , 2006	PLGA + nHA	SC/PL and GF/PL	New composition fabrication	RCOCs	Average cell density: GF/PL = 2.4×10^6 cells/scaffold (86.5% increase) SC/PL = 2.1×10^6 cells/scaffold (69.7% increase) ALP activity: GF/PL = 0.6 mol/min per 10^6 SC/PL = 0.5 mol/min per 10^6
Wang <i>et al</i> ^[55] , 2007	PA + nHA	Thermally induced phase inversion	Surface coating	BMMSCs	MTT assay and ALP activity: No negative effects on the BMMSCs <i>in vitro</i> <i>In vivo</i> : Good biocompatibility and extensive osteoconductivity with host bone <i>in vivo</i>
Lv <i>et al</i> ^[56] , 2009	PLGA + nHA	Microsphere sintering method (modification of the emulsion and solvent evaporation method)	New composition fabrication	BMMSCs	MTS assay: (cell number) PLGA + nHA : 1.2 million/mm ² PLGA: 0.06 million/mm ² ALP activity: PLGA + nHA: 0.10 mL/ μ g PLGA: 0 mL/ μ g
Roohani-Esfahani <i>et al</i> ^[57] , 2010	BCP/PCL + nHA	Sonication	Surface coating	PHOLCs	ALP activity: BCP/PCL + nHA: 2 mmol/h per mg BCP: 0.5 mmol/h per mg RT-PCR: (relative) BSP: BCP/PCL + nHA: 0.4, PCL: 0 Runx2: BCP/PCL + nHA: 8, PCL: 5 OCN: BCP/PCL + nHA: 1.2, PCL: 0.8 Col I: BCP/PCL + nHA: 4.5, PCL: 2.5
Ye <i>et al</i> ^[58] , 2010	PCL + nAP	Freeze-dried	Scaffold architecture	MG63Cs	Cell proliferation on composite scaffolds porosity of 76% > porosity of 53%
Phipps <i>et al</i> ^[52] , 2011	PCL + Col I + nHA	Electrospun	Bone-mimetic electrospun matrices	BMMSCs	Activation of focal adhesion kinase: Cells seeded onto PCL + col I + nHA scaffolds were better spread, and exhibited greater amounts MTS assay: (relative) PCL + Col + nHA: 5 PCL + nHA: 2.5 PCL: 1
Zhang <i>et al</i> ^[59] , 2011	PLLA + ODA-ND	Sonication	Surface coating	7F2CS	Alamar Blue assay: A slight reduction in cell viability compared to control RT-PCR: (relative) ALP: PLLA + ND-ODA: 1, PLLA: 1 OCN: PLLA + ND-ODA: 3, PLLA: 2.8
Zeng <i>et al</i> ^[31] , 2012	HA + MNPs	Tuning	New composition fabrication	MC3T3-E1Cs ROS 17/1.8Cs	MTT assay: HA + MNPs: MC3T3-E1: 0.2 OD value, ROS 17/1.8: 1.8 OD value HA: MC3T3-E1: 0.9, ROS 17/1.8: 0.25 ALP activity: HA + MNPs: MC3T3-E1: 0.75 U/mg, ROS 17/1.8: 4.5 U/mg HA: MC3T3-E1: 0.5 U/mg, ROS 17/1.8: 3.5 U/mg BGP activity: HA + MNPs: MC3T3-E1: 350 ng/L, ROS 17/1.8: 4000 ng/L HA: MC3T3-E1: 300 ng/L, ROS 17/1.8: 3500 ng/L
Hafezi <i>et al</i> ^[60] , 2012	G + nBG	Homogenization through stirring	New composition fabrication	hAFCs	MTT assay: No difference compared to control <i>In vivo</i> : Radiographic evaluation: Improved the speed of the bone healing process
Buschmann <i>et al</i> ^[61] , 2012	PLGA + n-aCaP	Electrospun	Electrospun PLGA/a-CaP scaffold architecture	ADSCs	MGTS: Extracellular matrix production was significantly higher FACS: CD13, CD29, CD44 and CD105 were expressed on PLGA + n-aCaP
Ganesh <i>et al</i> ^[62] , 2012	nfPCL + nS	Electrospun	New composition fabrication	BMMSCs	FACS: CD29 = 3.3%, CD44 = 77.1%, CD73 = 94%, CD31, 34, 45 = 0% Cell viability: No difference compared to control BCA assay: NS + PCLN: 250 Ug/mg, PCLN: 100
Im <i>et al</i> ^[63] , 2012	SWCNT + C + nHA	Lyophilization procedure	Scaffold architecture + new composition	BMMSCs	Cell adhesion and proliferation: SWCNT + C + nHA > SWCNT + C
Rodrigues <i>et al</i> ^[64] , 2012	TCP + nfPCL	Electrospun + dynamic culturing environment	Scaffold architecture + new composition	BMMSCs	ELISA: TCP + nfPCL: 0.8 μ g/mL TCP: 0.2 μ g/mL ALP assay: TCP + nfPCL: 100 mol/h TCP: 20 mol/h
Panzavolta <i>et al</i> ^[65] , 2013	G + nHA	Foaming + freeze-drying method	Surface coating + scaffold architecture	BMMSCs	ALP activity: No difference compared to control RT-PCR: (Relative) ALP: G + nHA: 3.2, G: 1.8 Col I: G + nHA: 0.25, G: 0.25 Runx2: G + nHA: 0.5, G: 0.4 TGF- β 1: G + nHA: 0.6, G: 0.55

Xing <i>et al</i> ^[66] , 2013	PLLA + OTND	Manual perfusion technique under pressure	New composition fabrication	BMMSCs	BCA assay: No difference compared to control RT-PCR: (relative) OPN: PLLA + OTND: 3.5, PLL: 1 BSP: PLLA + OTND: 3, PLL: 1 BMP-2: PLLA + OTND: 4, PLL: 1 <i>In vivo</i> : New bone formation: PLLA + OTND: 50%, PLL: 10%
Liu <i>et al</i> ^[67] , 2013	C + nHA	Electrospun	New composition fabrication	BMMSCs	Cell attachment: C + nHA: 1100 μm^2 C: 250 RT-PCR: (Relative) BMP-2: C + nHA: 1.5, C: 1 BMP-4: C + nHA: 25, C: 0 Smad1: C + nHA: 9, C: 1 ALP: C + nHA: 1.2, C: 1 Runx2: C + nHA: 22, C: 1 Itga1: C + nHA: 17, C: 1 Itgb1: C + nHA: 7, C: 1 Itgb3: C + nHA: 8, C: 1 Myh9: C + nHA: 7, C: 1 Myh10: C + nHA: 3.5, C: 1 Col 1: C + nHA: 4.5, C: 1 <i>In vivo</i> : Superior ability of bone reconstruction
Wang <i>et al</i> ^[68] , 2014	C + nHA	Lyophilization procedure + cold atmospheric plasma (CAP) treatment	Scaffold architecture + surface coating	BMMSCs	SEM: MSCs adhesion and infiltration were enhanced ELISA: (Relative) Fibronectin: C + nHA 0.8 compared to control (C) Vitronectin: C + nHA 1.1 compared to control

TCP: Tricalcium phosphate; PLLA: Poly(L-lactic acid); Col: Collagen; C: Chitosan; PCL: Poly(ϵ -caprolactone); HA: Hydroxy apatite; G: Gelatin; PLGA: Poly(D,L-lactic-co-glycolic acid); GF/PL: Gas forming and particulate leaching; SC/PL: Solvent casting and particulate leaching; BCP: Biphasic calcium phosphate; SWNT: Single-walled carbon nanotubes; PA: Polyamide; nf: Nano fibrous; nHA: Hydroxy apatite nano particles; nAP: Nano apatite; OTND: Oxygen-terminated nanodiamond particles; nS: Nanoparticles of silica; n-aCaP: Amorphous calcium phosphate nanoparticles; MNPs: Magnetic nanoparticles; ODA-ND: Octadecylamine-functionalized nanodiamond; nBG: Nano-sized bioactive glass; BMMSCs: Bone marrow mesenchymal stem cells; MG63Cs: Human osteosarcoma cell line; MC3T3-E1: Osteoblast precursor cells; ADSCs: Adipose-derived stem cells; RCOCs: Rat calvarial osteoblasts cells; hAFCs: Human airway fibroblast cells; ROS 17/1.8 Cs: Osteoblastic cells; 7F2CS: Mouse bone marrow cells; PHOLCs: Primary human osteoblasts-like cells; ALP: Alkaline phosphatase; MTT: 2-(4,5-dimethyl-2 thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; MTS: 5-[3-(carboxymethoxy)phenyl]-3-(4,5 dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; FACS: Fluorescent activated cell scan; RT-PCR: Reverse transcriptase polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; SEM: Scanning electron microscope; BGP: Bone Gla protein; BCA: Bicinchoninic acid; MGTS: Masson Goldner Trichrome staining; CD: Cluster of differentiation; Itga1: Integrin α 1; Itgb1: Integrin β 1; Itgb3: Integrin β 3; Myh9: Non-muscle myosin 9; Myh10: Myosin 10.

al^[41], results indicated that spatial immobilization of BMP-4 in a collagen-PLGA hybrid scaffold caused high expression of osteogenic genes and supported cell adhesion and proliferation^[41]. In 2005, Yang *et al*^[33] evaluated the effect of BMP-4 immobilization on GTG scaffolds on NRCOC activity. A significant effect of BMP-4 was reported after 4 wk. The cited study also showed greater cell attachment and osteoblast differentiation, higher Gla-type osteocalcin (Gla-OC) activity and larger mineralizing nodules following co-culture of osteoblasts with BMP-4 immobilized scaffold. The same results were achieved using BMP-2. Dai *et al*^[40] reported that rhBMP-2 significantly promoted the osteogenic differentiation of BMMSCs, by enhanced expression of Runx-2, osteopontin, osteocalcin and bone sialoprotein. On the other hand, combination of BMP-4 and PLGA scaffold resulted in a reduction in ALP activity^[41]. Although BMPs seem to have positive effects on the function of scaffolds and cells, their regularity and efficacy constraints limit their clinical application.

Surface of scaffolds can be modified by proteins and plasma treatment. Immobilization of proteins like integrin and laminin on the scaffold surface not only facilitates cell adhesion but also increases the surface

wettability^[74]. To add a mixture of fibrin and hyaluronic acid, multihead deposition system and fibrin-thrombin shell formation were used^[50]. Using immobilized RGD peptide (Arg-Gly-Asp) improved cell adhesion from 71.6% to 80.7% and increased ALP activity^[49]. Similarly, high cell proliferation, differentiation and viability were reported following addition of other ECM molecules to scaffold surfaces^[34,47].

On the other hand, although biomolecules like growth factors and ECM proteins can improve scaffold properties, these molecules do not tolerate severe chemical conditions or high temperature and only soft fabrication methods like sol-gel, which does not allow interconnected porous scaffold synthesis, are applicable^[75].

Nano particles

Nano particle addition to the scaffold is another way to improve scaffold properties. The most commonly applied nanoparticle was nano-HA, which provides osteoconductivity while the main scaffold provides the porosity. This combination resembles organic/inorganic nature of the bone matrix and mimics the nano-sized characteristics of natural bone^[26]. Addition of nano-

HA to scaffold doubled the expression of osteogenic genes^[67] and increased osteoblastic attachment^[63]; whereas osteocalcin gene expression level in PLLA-nano diamond composites was 3 times less than in the PLLA scaffold^[59]. Ye *et al.*^[58] produced a composite scaffold containing nano non-stoichiometric apatite and poly-epsilon-caprolactone, which had well-interconnected pores encouraging cell proliferation, migration and stimulation.

Future prospects

In vitro studies are generally considered as primary steps for evaluating newly developed materials. These experiments evaluate cell viability, attachment, proliferation and differentiation on new scaffolds by various methods. Although some factors like the effect of recipient bed, body fluids and interaction between different cell types cannot be evaluated in *in-vitro* experiments, they are useful to analyze the behavior of materials toward cells omitting individual characteristics of animal or human models. However, animal and clinical studies are necessary to assess the regenerative ability of materials *in-vivo*. A systematic review on *in-vivo* bone tissue engineering studies by Khojasteh *et al.*^[2] demonstrated that only few animal experiments used smart modification of scaffolds; most of which, were addition of BMP-2. It seemed that the application of BMP-2 with stem cells such as ADSCs and BMSC in TCP scaffolds significantly enhanced osteogenesis^[2]. Use of growth factors such as BMP-2 in combination with scaffold and stem cells could complete the classic tissue engineering triangle. However, the results of *in vitro* studies showed that addition of ECM-like molecules and nano-particles also induced osteoblastic differentiation. The most common composite used in bone tissue engineering was found to be polymer-ceramic and use of smartly modified scaffolds in animal models has been relatively fewer^[76]. Compared to the large number of *in-vitro* studies assessing the properties of newly developed materials, *in-vivo* application of these smart scaffolds and the effect of structural features and surface modification on *in-vivo* bone regeneration have yet to be studied.

In conclusion, comparing smart scaffolds to other presently available conventional materials shows great advantages, as they have the ability of *in-situ* osteoblastic differentiation induction and interesting biological functions. The more we understand the fundamentals of cell proliferation, differentiation and mechanisms of osteoblast adhesion, the better we can design smart materials. The researches now aim to design and synthesize composite materials made from fusion of different kinds of synthetic and natural materials. Surface treatment for facilitating cell attachment and differentiation and fabrication of strong scaffolds that can tolerate physiological forces are the main challenges in this regard. *In-vitro* studies should be performed primarily using standard methods

followed by *in-vivo* investigations. Combination of stem cells and smart scaffolds with a modulus of elasticity of the same magnitude as that of natural bone, and incorporating growth factors may complete the bone tissue engineering triad. Several factors including material, fabrication and modification methods as well as physical, chemical and mechanical properties may determine bone scaffold features. Considering the variability of *in-vitro* methodology of researches and variety of scaffolds, a thorough and comprehensible comparison between the results and approaches of these *in vitro* experiments was unattainable. However, the results of the included *in-vitro* studies indicate that incorporation of growth factors and other bioactive molecules as well as nano-particles as smart modifications can enhance cell differentiation, proliferation and attachment and therefore may improve new bone formation. The current study presented various types of smart modifications, which may provide an acceptable design to approach *in-vivo* and *in-vitro* hard tissue engineering.

COMMENTS

Background

Scaffold, cells and growth factors are the three main parts of bone tissue engineering. Currently, special attention has been directed to the design of new scaffolds by adding bioactive molecules and nanoparticles. Hydroxyapatite-based scaffolds carry cells to bone defects and provide an extracellular matrix (ECM). By changing the physical and chemical properties of the bone substitutes, smart interaction with seeded cells such as acceleration of differentiation, increasing in proliferation and attachment of the cells has been reported. It seems that these "smart modifications", can improve osteogenic differentiation and attachment of cells; therefore, they can better respond to their surrounding environment.

Research frontiers

Surface modification of scaffold materials was the most commonly used design. However, it is not clear how surface would influence bone regeneration *in vivo*. This study reviewed current trends in smart scaffolds for bone regeneration. Adding nano particles such as nHA and adding an ECM-like molecule such as collagen or growth factor like bone morphogenetic protein (BMP)-2 to the scaffold increase bioactivity of synthetic materials.

Innovations and breakthroughs

Several factors such as scaffold material, fabrication method and type of modification defining the physical, chemical and mechanical properties could affect *in vitro* experiments. Due to the variability of influencing factors including methodology and scaffold properties, the results were not comparable to determine the most successful design. Overall conclusion shows that in most studies smart design of scaffolds supported more cell attachment, proliferation and differentiation.

Applications

Although use of growth factors such as BMP-2 in combination with scaffold and stem cells could complete the classic tissue engineering triangle and result in bone regeneration *in vivo*, *in vitro* studies suggest that addition of ECM-like molecules and nano-particles also might have same results as BMP-2. The efficacy of these modifications should be assessed *in vivo*.

Peer-review

The matter of the review is interesting.

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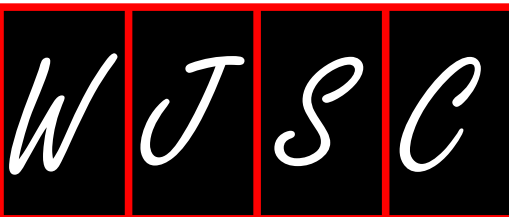
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**EDITORIAL**

- 669 Spermatogonial stem cells: Current biotechnological advances in reproduction and regenerative medicine
Aponte PM

REVIEW

- 681 Osteogenic differentiation of amniotic fluid mesenchymal stromal cells and their bone regeneration potential
Pipino C, Pandolfi A
- 691 Stem cell therapy in the management of shoulder rotator cuff disorders
Valencia Mora M, Ruiz Ibán MA, Díaz Heredia J, Barco Laakso R, Cuéllar R, García Arranz M
- 700 Epigenetic regulation of stemness maintenance in the neurogenic niches
Montalbán-Loro R, Domingo-Muelas A, Bizy A, Ferrón SR
- 711 Epidermal stem cells and skin tissue engineering in hair follicle regeneration
Balaña ME, Charreau HE, Leirós GJ
- 728 Structural properties of scaffolds: Crucial parameters towards stem cells differentiation
Ghasemi-Mobarakeh L, Prabhakaran MP, Tian L, Shamirzaei-Jeshvaghani E, Dehghani L, Ramakrishna S

MINIREVIEWS

- 745 Systems biology approach to developing S²RM-based "systems therapeutics" and naturally induced pluripotent stem cells
Maguire G, Friedman P
- 757 Ovarian stem cells: From basic to clinical applications
Ozakpınar OB, Maurer AM, Ozsavci D
- 769 Placental-derived stem cells: Culture, differentiation and challenges
Oliveira MS, Barreto-Filho JB

ORIGINAL ARTICLE**Basic Study**

- 776 Fetal kidney stem cells ameliorate cisplatin induced acute renal failure and promote renal angiogenesis
Gupta AK, Jadhav SH, Tripathy NK, Nityanand S

Contents

World Journal of Stem Cells
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Spermatogonial stem cells: Current biotechnological advances in reproduction and regenerative medicine

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Abstract

Spermatogonial stem cells (SSCs) are the germ stem cells of the seminiferous epithelium in the testis. Through the process of spermatogenesis, they produce sperm while concomitantly keeping their cellular pool constant through self-renewal. SSC biology offers important applications for animal reproduction and overcoming human disease through regenerative therapies. To this end, several techniques involving SSCs have been developed and will be covered in this article. SSCs convey genetic information to the next generation, a property that can be exploited for

gene targeting. Additionally, SSCs can be induced to become embryonic stem cell-like pluripotent cells *in vitro*. Updates on SSC transplantation techniques with related applications, such as fertility restoration and preservation of endangered species, are also covered on this article. SSC suspensions can be transplanted to the testis of an animal and this has given the basis for SSC functional assays. This procedure has proven technically demanding in large animals and men. In parallel, testis tissue xenografting, another transplantation technique, was developed and resulted in sperm production in testis explants grafted into ectopical locations in foreign species. Since SSC culture holds a pivotal role in SSC biotechnologies, current advances are overviewed. Finally, spermatogenesis *in vitro*, already demonstrated in mice, offers great promises to cope with reproductive issues in the farm animal industry and human clinical applications.

Key words: Cell culture; Spermatogonial stem cells; Spermatogenesis; Pluripotency; Transplantation; Fertility

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Core tip: This article reviews the current body of knowledge on biotechnological applications of spermatogonial stem cells (SSCs). SSCs are the founding adult germ stem cells of the sperm producing process spermatogenesis. SSCs belong to the male germline and can be expanded *in vitro* in several species. Through mechanisms not fully understood they can derive pluripotent stem cells *in vitro*. Thus, they can be genetically modified with some advantages over embryonic stem cell-based technologies. SSCs can be transplanted to homotopical or ectopical locations, offering great potentials in fertility related issues and regenerative clinical applications in domestic or wild animals and men.

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INTRODUCTION

Spermatogonial stem cells (SSCs), the germ stem cells of the seminiferous epithelium in the testis are the founder cells of a sperm producing stem cell system called spermatogenesis. SSCs were initially regarded as unipotential cells. Nowadays, however, current knowledge about SSCs has expanded to new limits and the general concept of SSCs as cells just supporting the production of the terminally differentiated sperm has rather radically changed. As a result of continuous and elaborate SSC biology research, the manipulation of this special cell type offers novel biotechnological solutions to contemporary human problems. SSCs are the only adult stem cells capable of transmitting the genome of a given species from one generation to the next, while at the same time having the capacity to convert into pluripotent stem cells^[1-3]. They can also give rise to cells from the basic three embryonic layers *in vitro*, which opened new opportunities for regenerative medicine^[1]. Since SSCs are adult cells, they do not bring about ethical concerns as occurs with the use of embryonic stem (ES) cells. However, the use of SSCs is not without risk, since they show some issues regarding epigenetic inheritance, as such a topic will be covered later on this article.

The faculty of SSCs to deliver genes over generations makes them a valuable vehicle for transgenesis techniques. This is particularly important for gene targeting in domestic animals, in which, contrary to laboratory rodent species, the techniques involving SSCs are still not feasible. In this process, SSC culture plays a central role in the exploitation of the unique properties of SSCs for biotechnological applications. Historically, the characterization of the SSC niche *in vivo* gave the basis for SSC culture conditions, especially SSC population expansion *via* self-renewal in several species, included men. A stem cell niche consists of a microenvironment with supporting cells and a corresponding signal exchange that regulates self-renewal and differentiation of the stem cells. Accordingly, SSCs are located in the seminiferous epithelium, near the basement membrane and the niche environment normally contains factors produced by neighboring somatic cells (sertoli cells, leydig cells, and peritubular myoid cells), the basement membrane and in particular the vascular network, near where SSCs are preferentially located^[4]. This microenvironment in the testis provides signals to control stem cell self-renewal, differentiation, or survival, guaranteeing a normal cell kinetics in spermatogenesis^[5,6].

SSC behavior *in vitro* could be further investigated thanks to the development of germ cell transplantation

assays, which allow functional verification of the presence of SSCs (see the topic Germ cell transplantation). The addition of certain growth factors to the culture media, in particular glial cell line-derived neurotrophic factor (GDNF), and the availability of a functional SSC assay, allows SSC populations to be expanded during long term culture, an advance first made in rodent models.

SSC biotechnologies are being aimed to solve fertility related issues (*i.e.*, fertility restoration in oncological patients, infertility treatments and reproduction of endangered species). They also provide important developments in germplasm preservation and the emerging technology of spermatogenesis *in vitro*. Finally, several of these applications require species-specific knowledge of SSC culture conditions as well as transplantation techniques, two aspects that will be covered in this article (for a summary of the biotechnological approaches that involve the use of SSCs, see Figure 1).

SSC ISOLATION AND CULTURE

SSC isolation procedures have been described for several domestic species and humans^[7-14]. SSCs are isolated by enzymatic digestion, usually involving two steps of various combinations of proteolytic enzymes (commonly, collagenases I or IV, trypsin, hyaluronidase and DNase I). From this enzymatic digestion, a germ cell suspension is obtained that now has to go through at least one type of purification methods, but more often a combination of them (Zheng *et al*^[15], 2014 summarize the current enriching methods for SSC isolation). There are two groups of purification methods, one group based on the SSC phenotype, like fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS), and the other utilizes physical methods, like differential plating, extracellular matrix selection and density gradient sedimentation^[15]. Simple models for SSC isolation include the use of young animals^[16], cryptorchid animals^[17], vitamin A deficient animals^[18], but SSC isolation becomes more complex when their source is the adult testis with full spermatogenesis. In this scenario, all differentiating germ cell types are present and SSCs represent a smaller proportion (see the topic SSC markers). Most recent articles in which SSC isolation is carried on, include one phenotype-based method and one or more physical methods. Most researchers do not use a functional assay (germ cell transplantation) to unambiguously identify the isolated germ cells as SSCs. They instead characterize the germ cell colonies arising during culture by means of immunohistochemistry or RT-PCR, aiming to identify gene products or RNA expression respectively (see the topic SSC markers). MACS is the most widely used technique nowadays. It is simple and inexpensive, but relies on specific SSC markers usually present in subsets of the SSCs, therefore sub-sampling the

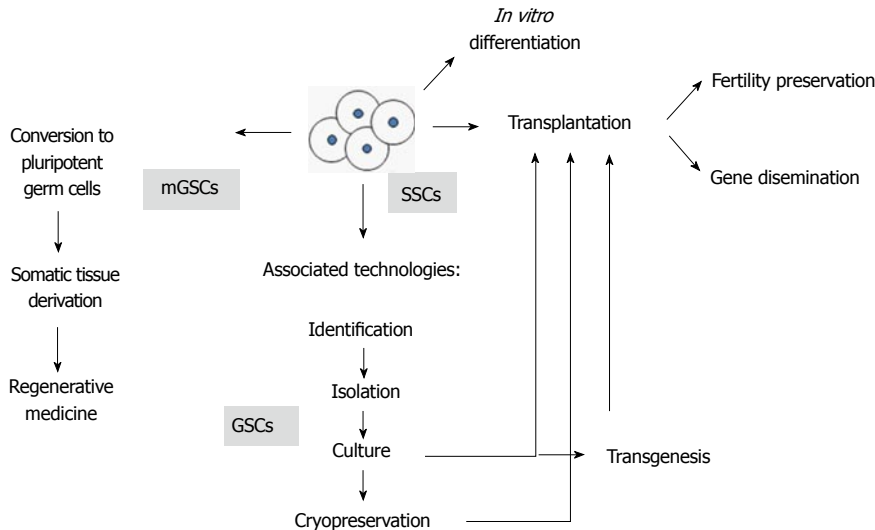


Figure 1 Overview of biotechnologies involving spermatogonial stem cells. Fertility preservation includes fertility restoration in oncological patients after therapeutic agents are administered that produce germ cell damage, strategies for infertile male patients and reproduction of endangered species. Gene dissemination represents the possibility to use several low-genetics animals to disseminate elite genes in animal production operations. Culture involves maintenance, survival, expansion and possibly genetic manipulations of spermatogonial stem cells (SSCs). Germ stem cells (GSCs), that is, SSCs that self-renew *in vitro*, therefore proliferating and expanding. Multipotent germ stem cells (mGSCs), which spontaneously appear in culture from within the SSC population, showing pluripotency properties^[2].

general SSC population. Interestingly, one group, in a recent work, produced an enriched human SSC suspension by using MACS and the marker ITGA6 (alpha 6 integrin) together with a proper authentication of SSCs through the germ cell transplantation assay^[11]. A recent novel approach for SSC isolation involved the combination of traditional isolation methods with testicular tissue grafting (see the topic Germ cell transplantation). In this work, adult mouse testis tissue was grafted into the dorsal skin of immunodeficient mice and after 2-4 wk the researchers observed the depletion of advanced differentiating germ cells of the seminiferous epithelium. Afterwards, the tissues left only with undifferentiated type A spermatogonia (a population including the SSCs) were recovered. The SSCs were isolated from the tissues, which were not only able to proliferate *in vitro* but also to recapitulate spermatogenesis in receptor testes devoid of endogenous spermatogenesis and furthermore to produce offspring through the original donor derived sperm^[19]. This method, although perhaps not as practical as MACS, results in a larger true SSC population since the whole array of markers confirm the phenotypic profiles of SSCs.

Culture conditions for SSC propagation *in vitro* were first developed in mice^[16,20]. This was the base for the development of domestic animals SSC culture systems^[7-10,21-25]. Most of these systems have in common the use of GDNF and serum. Common growth factors, besides GDNF, include LIF, FGF2 and EGF^[26], however important responses of SSCs regarding maintenance, survival and growth *in vitro* are dependent on age, species and even the strain of the donor animals^[27]. Very recently, a feeder/serum-free culture was established for mice SSCs^[28] and this method was improved for maintenance of SSCs^[29].

This represents a big advance because serum, when present, introduces unknown variable factors into the culture systems^[27] besides promoting the growing of contaminating somatic cells^[2]. Current efforts are being made to set up functional human SSC culture systems^[11,30,31].

SSC DIFFERENTIATION *IN VITRO*

Normally, SSCs self-renew during the steady state cell kinetics of spermatogenesis. They do so to maintain the undifferentiated pool while at the same time producing daughter cells that enter the differentiation path, ultimately generating sperm^[32]. Thus, the production of sperm *in vitro* requires culture conditions favoring the pathways of sperm production and not self-renewal. Going through the meiosis step of germ cell differentiation *in vitro* was one of the most difficult steps for many research groups. This fact hindered the progress in this particular research area for many years. An spermatogenesis *in vitro* system going beyond that developmental stage and traversing spermiogenesis was achieved and mouse sperm could be generated when testicular tissue was set on the liquid/gas interphase^[33]. This system included a very simple culture medium and testicular tissues came from young animals^[34]. One of the main features of this culture system is the preservation of the cytoarchitecture of the testis, that is, maintaining the structure of SSC niches. The reconstitution of spermatogenesis *in vitro* from SSC suspensions has proven more challenging. Some groups have obtained sperm *in vitro* by using testicular cell suspensions from prepubertal animals but three dimensional support matrices are necessary^[35,36]. Although these successful systems have been developed for sperm production

in vitro with rodent species, it is not yet foreseeable that these techniques can be translated to a clinical setting in the near future. Human SSC differentiation in culture systems is still under investigation at the present time.

The exciting possibility of producing sperm *in vitro* has large implications for farm animal industries, human fertility restoration, preservation of endangered species and many other associated technologies related to mammalian reproduction. For instance, in the cattle industry, keeping animals in large facilities would be a thing of the past when renewable SSC pools from elite bulls produce high numbers of sperm in Petri dishes at small biotechnological facilities. In many cases of human infertility it would just be enough to have one healthy SSC derived (and possibly engineered) sperm to produce offspring through assisted reproduction technologies (ARTs). Autologous SSC culture systems could be set for individuals before oncological treatments and sperm generated *in vitro* could be cryopreserved for later use to fertilize an egg and produce offspring from these patients. This way, the danger of reintroducing malignant cells back into the patient could be avoided. SSC banking could then become possible, as another mean for germplasm preservation in many species. Moreover, the cumbersome step of transplanting genetically modified SSCs to a receptor male in order to recapitulate spermatogenesis and thus obtain transgenic sperm could then be omitted. In addition, these techniques could also provide means for preserving endangered species, for instance, animal populations with very few individuals that at the moment of dying could have their SSCs collected for sperm to be produced in the laboratory.

SSC MARKERS

The characterization of SSCs is an important tool to study their presence on culture systems and during other techniques. SSCs, representing only the 0.03% of germ cells in the testis^[37], and the more numerous type A spermatogonia committed to differentiate, are morphologically undistinguishable. Furthermore, there is no general consensus on the phenotypic profile of SSC as this may vary among different species^[38] and there seems to be no unique SSC marker. In general, some phenotypic characteristics of SSCs have been advanced thanks to the molecular dissection of the SSC niche in the testis. Within the testis, Sertoli cells are the main cell type involved in the SSC niche^[39]. The key factor GDNF, secreted by Sertoli cells, promotes SSC proliferation^[40,41]. Any receptors expressed in SSCs that bind self-renewal promoting ligand molecules from the niche define their stem cell phenotype and constitute potential SSC markers^[42]. That is the case of the widely accepted marker GFR α 1, the receptor for GDNF in SSCs^[43]. In domestic animals, besides GFR α 1, another important marker for both gonocytes (perinatal germline stem cells) and SSCs is THY1^[44-46].

In monkeys, SSCs express the phenotype (CD90+ DC49f⁺ CD117⁺), SSEA-4⁺ PLZF⁺^[47]. Apparently, SSEA-4 seems to be conserved in humans^[48]. SSCs from humans also express GPR125^[49], besides GFR α 1^[50]. Another human SSC marker that adds to the known panel is ITGA6^[51]. Recently, a new SSC gene product has been reported: PAX7, that might represent a definite common SSC marker for several species including mice, cats, dogs, pigs, bulls, monkeys and humans^[52].

SSC PLURIPOTENCY

Pluripotent stem cells so far considered for regenerative medicine strategies include inner cell mass derived ES cells^[53,54], somatic derived induced pluripotent stem cells (iPS cells)^[55], fetal derived embryonic germ (EG) cells^[56] and SSC derived multipotent germ stem (mGS) cells^[57,58]. All of these cells form teratomas when injected to seminiferous tubules or subcutaneous tissue of immunocompromised mice and contribute to chimera formation when injected to a blastocyst. When SSCs are placed in culture they proliferate in the presence of GDNF. This growth factor is produced by Sertoli cells in the testis, stimulating SSCs to self-renew^[41]. SSCs proliferating in culture are designated germ stem cells (GS) cells^[16]. They are unipotent, genetically and epigenetically stable, do not produce teratomas and do not contribute to chimera^[59]. Extraordinarily, while in culture, they can spontaneously derive multipotent germ stem cells (designated mGS cells) at a low frequency (1 out of 30 testes)^[58]. They represent SSCs with pluripotency, sharing not only ES cell traits such as self-renewal properties, tumorigenicity and chimera production but also genomic and epigenetic abnormalities^[2]. Pluripotent germ cells from the testis can circumvent some of the typical objections or technical limitations of other pluripotent cells (*i.e.*, ES cells and iPS cells) for their use in therapeutic purposes. These limitations include issues in cell proliferation, cell differentiation, genetic stability, allogenicity and ethical/religious issues (reviewed in^[60]). Remarkably and unlike other pluripotent cells, the conversion of GS cells into mGS cells occurs on a dedifferentiation process not dependent on genetic manipulations, which opens important theoretical possibilities for these technologies to be translated into clinical applications in regenerative medicine. Nevertheless, despite all the promising traits of mGS cells, caution is required on the grounds of their genetic and epigenetic behavior. Although GS cells maintain a normal karyotype, genomic imprinting, and SSC activity during long-term culture^[59], mGS cells are unstable and tend to accumulate genomic and epigenomic abnormalities^[2,61]. Also, GS-like cells derived from mouse fetal germ cells had SSC activity but some of the produced offspring had abnormal genomic imprinting patterns, which were also transmitted to future generations^[56]. SSC-derived pluripotent cells

are able to generate very diverse tissues while in cell culture^[1,3]. For instance, differentiated tissues appear in culture from a specific subpopulation of mouse mGS cells that show a pluripotent cell phenotype (POU5F1⁺) while at the same time expressing SSC proteins (c-Kit⁺)^[62].

All in all, SSCs show excellent perspectives for applications in regenerative medicine. According to the "organ niche" theory, developmental failure in organogenesis caused by defective genes, can be compensated by xenogeneic pluripotent stem cell transplantation into genetically affected blastocysts^[63]. The pluripotential cells would then integrate into the embryo, resulting in chimera formation with colonization of stem cells of xenogeneic origin in the organ forming niches during development. This has been demonstrated through animal models such as mouse and pigs to successfully generate functional kidneys^[64] and pancreas^[63,65]. SSC-derived pluripotent stem cells, with all their inherent advantages, could be used for these purposes.

Although the current advances in the generation of testicular pluripotent cells in rodents are unquestionable, similar results in the humans have been controversial. Although one work demonstrated teratoma formation and pluripotency markers expression^[66] on these cells, tumors showed a relatively small volume^[67]. Several other groups have produced allegedly human mGS cells as well, but these cells, although expressing several pluripotency markers, do not produce teratomas when transplanted into the skin of immunocompromised mice^[68-70]. In fact, one group recently found that human testicular embryonic-like cells (allegedly mGS pluripotent cells) do not possess a transcriptome similar to that of human ES cells but rather have the phenotype of mesenchymal stem cells (MSCs), therefore, concluding that these cells are not pluripotent and most likely not of germ cell origin but instead mesenchymal^[71]. Current knowledge of human SSC culture conditions probably is insufficient and do not emulate the mouse SSC culture environment that has allowed the generation of mGS cells in this species. For further progress to be made, the exact mechanisms of SSC reprogramming need to be addressed. For instance, we know that primordial germ cells from the fetal period convert to ES-like pluripotent cells^[72] via the AKT signaling pathway^[73]. A very recent study found that spontaneous SSC reprogramming would be caused by unstable DNA methylation with involvement of the Dmrt1-Sox2 signaling pathway^[74]. However, more research is required to successfully obtain human mGS cells. This will require very specific culture conditions and the molecular dissection of SSC pluripotency, all of which should continue to be investigated.

GENE TECHNOLOGIES AND SSC

ES cell-based transgenesis technologies are well

developed and routinely used in the context of mouse species. However, SSCs can be used for a more straightforward route to transgenesis. ES cells, after genetically intervened, are injected into blastocysts with the hope of successful integration and contribution to the germline, which not always can be guaranteed. By contrast, SSCs are already constituents of the germline. Some other important differences with ES cells make SSCs best suited for mutagenesis (Table 1), especially in species for which ES technologies are not feasible or physiological limitations exist like in farm animals. Additionally, unlike mice, some farm animals, primates and humans are uniparous species as a consequence of not producing large numbers of oocytes under normal physiological conditions. In addition, these species go through lengthy periods of time to attain puberty and sexual maturity. SSC genetic and epigenetic stability keep these cells committed to the germline phenotype so that they do not tend to differentiate into other lineages^[59]. This trait makes their potential role for gene therapies clinically safer than other pluripotent stem cells. The use of SSCs for transgenesis in farm animals in comparison to ES cell-based technologies in mice is depicted in Figure 2.

Another alternative for knockout (KO) animal production is nuclear transfer of genetically modified somatic cells, but these cells have limited proliferation capacities, the technique shows inefficient offspring production and postnatal degenerative problems. For these reasons, cloning does not efficiently compete with ES cells or SSC technologies for gene targeting.

Due to their potentials, SSCs have been used to generate KO animals in several species. Transgenic mice generation by using SSCs was first accomplished with the use of retroviral vectors, however with low efficiency^[75]. Later on, with the advent of GS cells (SSCs that proliferate *in vitro*) this cell type could be expanded *in vitro* and genetically modified through vectors (viruses or plasmids) with drug resistant genes to allow for the detection of SSCs carrying the transgene. These SSCs were transplanted to recipient mice and took over the spermatogenetic functions in the new environment to ultimately produce transgenic sperm. Transgenic heterozygous males with transgene carrying sperm were mated with females and progeny was produced, near 50% of which carried the transgene^[76]. This illustrates the importance of an optimal culture system in order to expand SSC populations and thus increase the efficiency of the transgene incorporation. Soon after, sophisticated techniques added to the gene function modification toolbox such as the CRE/LoxP system for conditional gene targeting, was successfully adapted to the use with SSCs^[77]. Interestingly, SSC mediated rat transgenesis was achieved in 2007^[78] while parallel efforts with ES cells on this species were only successful until 2010^[79]. Just recently, ES cells from domestic animals could be obtained for gene targeting goals^[80]. Advances with farm animal SSCs continued in parallel. For instance, goat transgenic

Table 1 Summary comparison of characteristics of embryonic stem cells and spermatogonial stem cells in the context of transgenesis techniques

Trait	Embryonic stem cells	SSCs
Mutagenesis technical status	Available for mice. Many genes mutated	Experimental level in mouse ^[75] , rats ^[78] , goats ^[81] , pigs ^[82]
Source	Embryo	Adult testis
Age of derivation	Embryonic period	Postnatal period
Ethical/legal concerns	Yes	No
Efficiency		5 × to 10 × higher than with ES cells
Cell differentiation state	Undifferentiated - pluripotent	Differentiated up to germ-line
Tumorigenesis	Produce teratomas	Do not produce teratomas
Chimera formation	(+)	(-)
Germline gene transmission	Do not transmit genes from one generation to the next	Transmit genes from one generation to the next
<i>In vitro</i> phenotype	Colonies with tightly attached cells	GS cells loosely attached (easily dissociated with trypsin) ^[2]
Speed of growth	Faster	Slower
Requires other non-transfected SSCs during culture	No	Yes ²
Karyotype	Variable - unstable ¹	Normal - stable
Epigenetics	Variable - unstable DNA methylation pattern	Normal - stable DNA methylation pattern
Offspring	Abnormal	Normal

Otherwise specified, information presented is based on^[2]: ¹Often lose their germ cell potential due to trisomy^[16]; ²Spermatogonial stem cells (SSCs) that proliferate in culture (GS cells) require specific non-transfected SSC densities during the process of drug selection^[118]. ES: Embryonic stem.

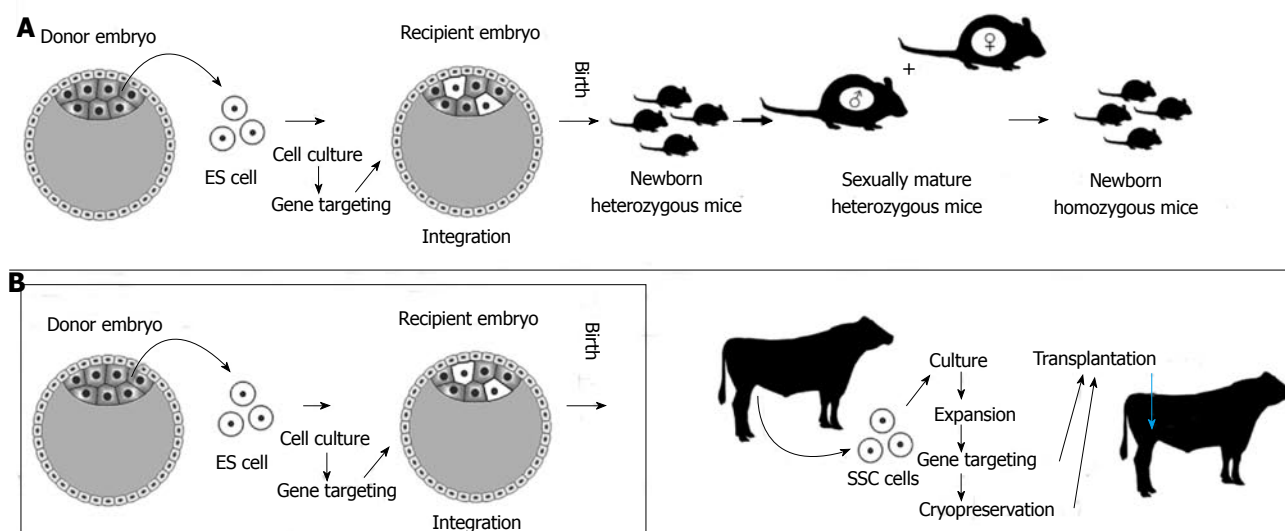


Figure 2 Alternative ways to exploit stem cells to produce transgenic animals. A: Standard method (well developed in the mouse): Embryonic stem cells (ES cells) are obtained from a donor mouse blastocyst. These cells are cultured and genetically modified (usually, DNA sequences are inserted on a gene of interest to disrupt its expression or gene knockout). ES cells are injected into another mouse blastocyst so that they can integrate into the embryo (chimera formation) with the hope that some of them will contribute to the male germline. In this case, donor germline cells will undergo homologous recombination (meiosis) in the testis after animals are born and develop towards puberty. The male animals will be heterozygous for the mutated gene and when crossed with their (also heterozygous) sisters, offspring will be obtained homozygous for the transgene; B: Transgenesis technique involving spermatogonial stem cells (SSCs), particularly useful for farm animals, for which ES cell technologies are not feasible. SSCs from postnatal animals can be expanded in culture, subject to gene targeting and transplanted to a recipient foreign testis. This way, steps used with ES cell technologies become redundant (Figure 2B, inside the box). With the advent of spermatogenesis *in vitro*, even the SSC transplantation procedure will not be necessary. Cryopreservation techniques for SSCs are already available.

sperm for human growth hormone was derived from male goats that had been transplanted with SSCs previously manipulated *in vitro*^[81]. In a report from the same group, pig embryos, transgenic for a reporter gene (enhanced green fluorescent protein), could be obtained after oocytes were fertilized with transgenic sperm from culture transduced and later transplanted SSCs^[82]. Very recently, bovine gonocytes were transduced through a lentivirus vector and the reporter transgene product proved the success of the

procedure^[83]. In dogs, a species with a strong potential to provide new models for human genetic diseases, SSCs have been characterized, cultured, genetically modified and allogeneically transplanted with eventual collection of transgenic sperm^[84].

GERM CELL TRANSPLANTATION

SSC transplantation is an important tool for several other biotechnological applications such as gene

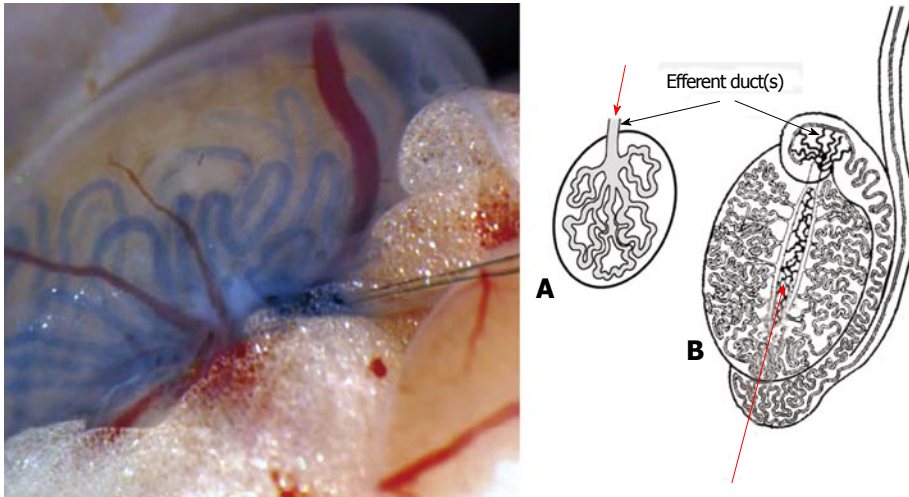


Figure 3 Species differences on anatomical points for spermatogonial stem cell injection for transplantation. The transplantation procedure for spermatogonial stem cells (SSCs) is simple in the mouse. In this species, (A) there is a single efferent duct emerging from the rete testis, which is easy to cannulate to inject SSCs (arrow). In most farm animals and men (B) several efferent ducts emerge from the testis, a reason why transplantation of SSCs is often done by injection of SSCs into the rete testis (arrow) with ultrasound guidance.

therapy or fertility restoration. The first SSC successful transplantation was reported in 1994 in the pioneering work of the Brinster's group^[85]. Inspired on original hemopoietic fundamental transplantation work^[86], this research group isolated germ cells from a donor mice that were later unequivocally identified as SSCs on a recipient mouse testis after injection into the seminiferous tubule system, based on their property to regenerate spermatogenesis in the foreign testicular niche (for a comprehensive review on this technique see^[87]). This allogeneic type of transplantation is presently the basis for the functional assay to detect true SSCs in experimental models. The technique could be developed due to the presence of a single, easy to cannulate efferent duct in mouse species and to the fact that the mice strain used was immunocompromised. Unfortunately, this SSC transplantation procedure is technically demanding in species with several efferent ducts because, in these species they tend to be short, thin and highly convoluted (Figure 3). This anatomical condition corresponds to most farm animals^[88], primates^[89] and humans^[90].

Xenogeneic transplantation of SSC cell suspensions into the mouse testis has been attempted by several groups^[91]. For the first time, SSCs from a foreign species (rat) not only were able to colonize mouse seminiferous tubules but also to differentiate and produce normal rat sperm^[92]. However, attempts with other SSC donor species showed that the farther apart in phylogenetical distance is the donor species from the mouse (recipient species), the lower level of progress of differentiation in spermatogenesis from the transplanted SSCs will be achieved within the foreign seminiferous epithelium environment (for details on SSC transplantation into the mouse testis, involving different donor species, see^[91]). Accordingly, domestic animals and human beings as SSC donors are among

those species in which this technique is not yet successful. Thus, on these and many other species, functional assays to unequivocally detect the presence of their SSCs are very necessary.

Allogeneic SSC transplantation (SSCs transplanted from a donor animal to a recipient testis of another animal, both from the same species) has proven difficult in species other than rodents, particularly in large animals. Besides anatomical difficulties, each species seems to require specific technical protocols. Allogeneic transplantation has been attempted in several farm animals, including pigs, goats and bulls^[93-95]. For instance, transgenic sperm from a donor goat could be unequivocally identified in a recipient buck semen, meaning that transplanted SSCs could colonize and regenerate the spermatogenetic process in the unrelated testis^[96]. When the limitations of these techniques are overcome they will facilitate several of the biotechnological applications described on this article. One aspect that still needs to be addressed is the method for depletion of endogenous spermatogenesis in recipient animals. This is typically performed by using either chemical compounds like the alkylating agent busulfan or alternatively testis irradiation. However, protocols need to be species optimized, which requires further research^[97,98]. SSC transplantation could also be used for gene delivering purposes in order to increase the frequency of certain genes in animal populations. This would be particularly useful in extensive systems of livestock production, in which animals reproduce through natural service. Surrogate males could be transplanted with SSCs that over-express certain genes linked to increased production traits or from elite bulls in order to disseminate the desired genes in a population. Endangered species could also be benefited with such technologies. For instance, SSCs from the wild species to be preserved could be transferred to a

phylogenetically related domestic animal embryo and this way become incorporated into the germline to eventually produce exotic sperm^[99,100], this way saving the endangered germplasm.

As an alternative to SSC transplantation into testicular environments, a new technique was developed in which whole testicular tissue explants (containing SSCs attached to their niche) from the desired species are placed in ectopical places such as the back skin (subcutaneous tissue) of immunocompromised mice. This approach was first successfully tried with mouse testis tissue (donor) grafted into mouse skin (recipient)^[101,102]. This technique was designated testis tissue xenografting. When xenotransplanting testicular tissues from prepubertal animals (with only undifferentiated germ cells, including SSCs), complete spermatogenesis developed within the transplanted testicular tissues under the sponsoring of the host (mouse) vascular environment and intrinsic steroidogenesis of the donor tissue. This technique has been so far successful with a wide range of donor species including hamsters, rabbits, dogs, cats, sheep, goats, horses, bovines, alpacas and monkeys^[103]. Work has also shown that testicular tissue pieces can be cryopreserved and later used with this technique in various species^[104,105].

Tissue xenografting has represented a step forward in SSC biology that circumvents many of the current limitations the transplantation of germ cell suspensions into the seminiferous tubules of foreign recipient animals. This technique has been privileged by researchers during the recent years because of the positive outcomes in a range of very dissimilar species. Recently, a related technique was developed in which no whole tissue pieces are transplanted into the mouse skin but instead an assorted cell suspension composed of germ cells and testicular somatic cells, including Sertoli cells. Interestingly, all these cells reassemble under the mouse skin and testicular tissue becomes organized in a process that has been called *de novo* testicular morphogenesis^[106,107]. This novel technique has been tested with testicular cell suspensions from rats^[108], mice^[109], pigs^[110], sheep^[111] and recently from a wild pig, the peccary^[112]. Testis xenografts have produced so far fertile sperm in pigs and the related peccaries^[112-114] demonstrating that SSCs present in the testicular tissues show a correct functioning that leads to the production of viable normal sperm within the body of a foreign species.

FERTILITY RESTORATION

Infertility is one of the critical side-effects of oncological treatments in humans and particularly in children, an effect which is caused either by chemotherapeutic agents or irradiation. A proposed solution for this problem is the restoration of fertility *via* SSC transplantation. Since many of the patients in this situation are prepubertal (*i.e.*, do not yet produce

sperm), a current approach consists on banking testicular tissue or cell suspensions prior to the oncological treatment and submitting the patient to an autologous transplantation of the preserved material if fertility problems arise later in life^[115] (for recent advances in the methods for preservation of fertility on young male human oncological patients see the comprehensive review from Goossens *et al*^[116], 2013). A word of caution is valid regarding safety issues when malignant diseases are managed with transplantation procedures. Invariably, cells suspensions including SSCs to be transplanted should be devoid of cancer cells. In experiments with rats, only as few as 20 leukemia cancer cells were enough to produce the disease when transplanted into healthy rat testis^[117]. It is unknown to what extent this is reproducible in humans, but as an obvious measure, cell suspensions should be screened and positively selected for SSCs and/or negatively selected for cancer cells, through approaches such as FACS or special culture conditions^[116]. An alternative approach would be to use testis xenografting or allografting with the hope to restore the spermatogenic process ectopically to a degree just enough to produce a few sperm to be later used with ARTs. Nevertheless, although already developed for several animal species (see the topic on Germ cell transplantation above) testicular xenografting requires further investigations to be implemented in humans. In general, more research in monkeys and humans is still needed, particularly on safety issues.

FINAL REMARKS

The tremendous progress in SSC research during the last decade is undeniable. Appropriate conditions for SSC long-term culture are already widely known for the most important experimental model: the mouse, but research efforts are still being made to develop culture systems suitable for humans and other species, altogether with the study of the very specific SSC nutrient requirements. Advances such as SSC *in vitro* mutagenesis are already possible in the mouse. The imminent unraveling of the molecular mechanisms of SSC pluripotency will bring about many other interesting biotechnological possibilities. The long-awaited accomplishment of SSC transplantation techniques for farm animals and humans is under current development, despite the widespread use of testis xenografting in many species with high rates of success. Finally, spermatogenesis *in vitro* is already possible for laboratory species, so important applications in regenerative medicine and animal reproduction are expected.

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Osteogenic differentiation of amniotic fluid mesenchymal stromal cells and their bone regeneration potential

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Abstract

In orthopedics, tissue engineering approach using stem cells is a valid line of treatment for patients with bone defects. In this context, mesenchymal stromal cells of various origins have been extensively studied and continue to be a matter of debate. Although mesenchymal stromal cells from bone marrow are already clinically applied, recent evidence suggests that one may use mesenchymal stromal cells from extra-embryonic tissues, such as amniotic fluid, as an innovative and

advantageous resource for bone regeneration. The use of cells from amniotic fluid does not raise ethical problems and provides a sufficient number of cells without invasive procedures. Furthermore, they do not develop into teratomas when transplanted, a consequence observed with pluripotent stem cells. In addition, their multipotent differentiation ability, low immunogenicity, and anti-inflammatory properties make them ideal candidates for bone regenerative medicine. We here present an overview of the features of amniotic fluid mesenchymal stromal cells and their potential in the osteogenic differentiation process. We have examined the papers actually available on this regard, with particular interest in the strategies applied to improve *in vitro* osteogenesis. Importantly, a detailed understanding of the behavior of amniotic fluid mesenchymal stromal cells and their osteogenic ability is desirable considering a feasible application in bone regenerative medicine.

Key words: Mesenchymal stromal cells; Amniotic fluid; Amniotic fluid mesenchymal stromal cells; Amniotic fluid stem cells; Osteogenesis; Bone regeneration

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Core tip: Several papers regarding the osteogenic differentiation potential of cells isolated from amniotic fluid have been published so far with particular attention to various feasible approaches to improving differentiation both *in vitro* and *in vivo*. Hence, an overview is necessary on the data reported up to now in order to understand the potential of amniotic fluid-derived cells in bone regenerative medicine. This review takes a general look at the current state-of-the-art of the osteogenic ability of amniotic fluid-derived cells and the different strategies available to improve bone regeneration.

Pipino C, Pandolfi A. Osteogenic differentiation of amniotic fluid mesenchymal stromal cells and their bone regeneration potential.

INTRODUCTION

In the last few decades degenerative lesions of the musculoskeletal system have become increasingly common, leading to social and economic problems, and intensifying the demand on medical services^[1]. Common examples are temporary and permanent disability due to osteoarthritis or fractures caused by loss of bone mass such as osteoporosis or osteopenia, particularly in the elderly. In the younger population widespread active lifestyles including sport activities have increased the possibility of bone and cartilage damage. Moreover, bone defects mostly emerge from trauma, tumor resection and congenital malformation. So far, the most effective clinical method for the reconstruction of large bone defects is the use of autogenous bone grafts, commonly harvested from the posterior iliac crest and transplanted into a local bone defect^[2]. The weakness of this method is the surgical stress the patient undergoes in extracting bone with possible subsequent inflammation and donor site morbidity. Furthermore, the quantity of extractable bone is limited.

Recently, advances in the field of stem cells have come to represent a valid alternative to this method^[3]. Nowadays there is growing interest in therapies based on mesenchymal stromal cells (MSCs) as a potential effective treatment for bone defects. MSCs are multipotent cells with the potential to engender a range of specialized cell types, such as osteoblasts, chondrocytes and adipocytes^[4]. The mesenchymal progenitor cells in the bone marrow are able to differentiate into osteoblasts following the influence of multiple osteogenic signals^[5,6]. In particular, after fracture MSCs of bone marrow are transferred to the site of bone injury through peripheral blood, thus enhancing the healing potential of local MSCs^[7]. In this condition, bone morphogenic proteins (BMPs) play an important role. Indeed, osteoblastic differentiation begins when the BMPs bind their receptors activating the transcription factors runt-related transcription factor 2 (Runx2) and Osterix, and subsequent downstream osteoblast specific genes, through activation of a Wnt/LRP5 cascade, which is crucial in bone mass modeling^[5,8,9].

Regarding the principal signaling pathways involved in MSC differentiation, various evidences have suggested that during cultivation *in vitro*, an inverse relationship exists between the commitment of MSCs toward osteogenic and adipogenic lineages^[10]. Several cell signaling cascades are involved in this cell fate decision between osteo- and adipogenesis^[11]. The master regulator of osteogenesis is Runx2, the gene target of many signaling pathways, including but not limited to transforming growth factor-beta 1 (TGF- β 1), BMP^[12],

Wingless type (Wnt)^[13], Hedgehog (HH)^[14], and (NEL)-like protein type 1 (NELL-1)^[15].

Thus, considering the crucial role of MSCs in bone healing, the strategy of using the osteogenic potential of such cells transplanted into the bone defect seems promising^[7,16].

Mesenchymal stromal cells derived from bone marrow (BM-MSCs) have been used in clinical trials for the treatment of bone defects^[17]. However, bone marrow aspiration is a difficult, invasive and painful procedure for the donor. The amount of BM-MSCs is usually between 0.001% and 0.01% of the total population and, in addition, the BM-MSC number and differentiation potential may be affected by increasing donor age. This implies an extensive *in vitro* expansion of such cells before transplantation, thus increasing the risk of possible differentiation induction and epigenetic modifications^[18]. On the other hand, the use of allogeneic BM-MSCs for bone repair is unsuitable due to immune rejection^[19].

As an alternative, mesenchymal stromal cells can be found in various adult organs and tissues^[20], including blood^[21], adipose tissue^[22] and dermis^[23]. Although sharing similar properties, these MSCs from various sources have different gene expression profile and differentiation ability^[24].

Mesenchymal stem-like cells have also been recognized in fetal blood, liver and bone marrow^[25]. In this regard, Guillot and colleagues have shown that first trimester fetal blood, liver, and bone marrow MSCs possess greater osteogenic differentiation potential than adult BM-MSCs^[26]. They found through quantitative real-time RT-PCR that 16 osteogenic specific genes (*OC*, *ON*, *BSP*, *OP*, *Col1*, *PCE*, *Met2A*, *OPG*, *PHOS1*, *SORT*, *ALP*, *BMP2*, *CBFA1*, *OSX*, *NOG*, *IGF1*) were more expressed in fetal MSCs under basal conditions and were up-regulated during osteogenic differentiation both *in vitro* and *in vivo*^[26]. These cells are more primitive than adult MSCs^[25], therefore potentially promising for therapeutic use in regenerative medicine, even if isolating them is subject to considerable public unease.

An alternative approach could result from the use of MSCs derived from extra-embryonic tissues, which possess the advantage of being isolated from tissues normally discarded after birth, hence exempt from ethical concern, such as amniotic fluid, umbilical cord and placenta^[27-29].

This review will focus on the biological properties of MSCs isolated from amniotic fluid (AF-MSCs) with particular attention to their *in vitro* osteogenic differentiation potential with a view to possible final application in bone regenerative medicine (Figure 1). Of note, these cells possess a greater proliferative capacity, lower immunological reactivity and lower risk of graft-versus-host disease than those derived from adult bone marrow^[30]. Importantly, compared with other MSC sources, such as umbilical cord blood-derived MSCs, AF-MSCs are more easily isolated and show better proliferation ability^[31].

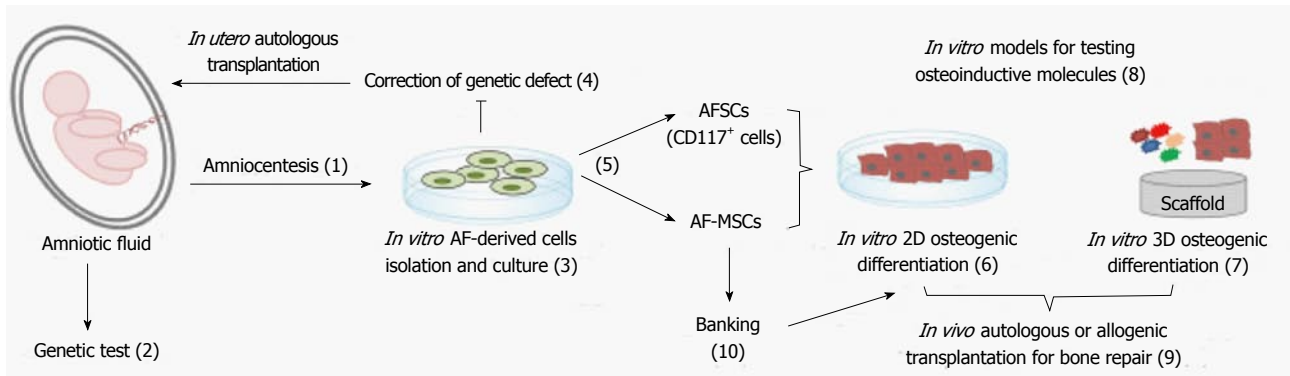


Figure 1 *In vitro* and *in vivo* application of amniotic fluid-derived cells. Amniotic fluid-derived cells isolated from amniotic fluid samples obtained following ultrasound-guided amniocentesis (1) for genetic test (2), could be *in vitro* expanded (3). These cells may be genetically manipulated and transplanted back into the same fetus (4). AF-derived cells can be used unselected (AF-MSCs) or selected for CD117 (AFSCs) (5). Both cellular populations can be *in vitro* differentiated in 2D (6) or 3D (7) culture conditions. They represent an ideal *in vitro* model for testing osteoinductive molecules (8) and for *in vivo* autologous or allogeneic transplantation for bone repair (9). Alternatively, they could also be banked (10) and adopted post-thawing.

In particular, AF-MSCs are autologous to the foetus representing an attractive source for the treatment of perinatal disorders such as congenital malformations^[32,33]. Hence, affected children could benefit from their own cells which could be banked, expanded in culture or properly engineered and implanted in the neonatal period. In addition, AF-MSCs are semi-allogeneic to each parent, therefore potentially useful for the other members of the family^[34].

Moreover, the beneficial effect of AF-MSCs observed in preclinical studies, such as lung injury^[35], ischemic heart^[36], acute bladder injury^[37], neovascularization^[38], encourages their future application in regenerative medicine tissue engineering.

AMNIOTIC FLUID-DERIVED CELLS

Human amniotic fluid, contained within the amniotic cavity, begins to appear at the second week of gestation. It surrounds the growing fetus, protects from outside injury and acts as the vehicle with the mother^[30]. It contains a variety of cells arising from all three germ layers (ectoderm, mesoderm and endoderm) routinely obtained for prenatal diagnosis of fetal abnormalities during second trimester pregnancy, through a minimally invasive technique. Amniotic fluid cellular composition substantially depends on the gestational age^[27]. Most of the cells present in the amniotic fluid are terminally differentiated and have low proliferative capacity^[32]. Three major cellular types can be classified based on morphological, biochemical and growth characteristics: epithelioid, amniotic fluid and fibroblastic cells^[39].

Interestingly, a population that expresses the surface antigen c-kit (CD117), stem cell factor receptor, can be isolated from amniotic fluid. These cells, named amniotic fluid stem cells (AFSCs), represent about 1% of the total cells^[40]. It should be noted that, mesenchymal stromal cells (AF-MSCs) with a multilineage differentiation potential are present in the amniotic fluid^[41,42].

AF-MSCs are highly proliferative with a normal karyotype after long-term *in vitro* culture and do not form teratomas when transplanted *in vivo*^[43]. They do not display any hematopoietic molecular markers (CD14, CD31 and CD45), while they express the intracellular stemness markers TERT, SOX2 and Nanog and the surface adhesion molecules CD29, CD58, CD166 and CD90^[44].

The absence of HLA-DR and presence of HLA-ABC suggest that these cells may be applicable in immune-mediated disorders as well as in the treatment of graft-versus-host disease^[43]. It is also important that AF-MSCs display a broad differentiation potential toward multiple lineages (*i.e.*, adipogenic, chondrogenic, myogenic and osteogenic)^[34]. They possess an intermediate differentiation potential between embryonic (pluripotent) and adult cells with advantages over both. Compared to adult cells, AF-MSCs possess greater differentiation potential and more primitive properties with fewer accumulated mutations^[45]. Respect to embryonic stem cells (ESCs), AF-MSCs possess the advantage to do not form teratomas when transplanted *in vivo*. Indeed, although ESCs are pluripotent cells which maintain high plasticity and extensive self-renewal capacity, possible host immune rejection after allotransplantation and the formation of tumors when injected undifferentiated or partially differentiated *in vivo*, raise safety concerns^[46-48]. Furthermore, their clinical use is limited by ethical issues due to the need to isolate them from the inner cell mass of a blastocyst^[49].

In the last few years methods able to generate patient-specific pluripotent stem cells (iPSCs) from adult cells have been developed to overcome the limitations associated with ESCs^[50,51]. Such iPSCs, obtained through the ectopic expression of defined transcription factors (Oct4, Sox2, Klf-4, c-Myc), are molecularly and functionally similar to ESCs. They show similar morphology and growth properties, express pluripotency markers, are able to generate germline-competent chimeras and form tumors when injected into immune-compromised mice^[51]. Although iPSCs

allow one to overcome both the ethical and the tissue compatibility problems of ESCs, current studies are still investigating the safety profile of these cells for therapeutic application^[52]. The main limitation related to iPSC generation is the use of retroviruses or lentiviruses, which could cause mutagenesis leading to a risk for teratogenesis.

Therefore, given the easier accessibility and the faster availability of a great number of AF-MSCs in culture in comparison to iPSCs, AF-MSCs may hold great promise in regenerative medicine.

However, since iPSCs seem one of the most promising future sources of stem cells for tissue regeneration, research is going ahead to find the best cell source to reprogram and develop alternative methods for generating pluripotent cells using non integrating systems. In this context, recent evidences has suggested that cells derived from amniotic fluid are more easily and rapidly reprogrammed than adult cells^[53-55]. More recently, iPSCs have been developed from amniotic fluid cells without ectopic factors by culture in an appropriate medium^[56], thus reinforcing their potential application in cellular replacement therapies.

These important features, together with the high proliferation rate, ease of retrieval and more stable profile, provide a convincing proof-of-principle for potential autologous application of AF-MSCs for bone regeneration in perinatal applications^[57]. Furthermore, a bank of such cells is achievable and may in the future provide a plentiful source for autologous therapy in adulthood as well as for transplantation into HLA-matched recipients.

IN VITRO OSTEOGENIC DIFFERENTIATION

The osteogenic differentiation capacity of AF-derived cells obtained from various sources (human, sheep, mouse and rat) has been broadly documented^[39,40,58,59]. Some studies have reported the great potential of c-kit selected amniotic fluid cells prior to osteogenic differentiation^[40], while others have demonstrated that unselected cells are also able to properly differentiate into osteo-specific cells^[13,41,44,60]. In this review we mainly discuss the *in vitro* osteogenic differentiation of AF-MSCs with occasional mention of papers in which authors use c-kit selected cells, named AFSCs (Table 1). It should be pointed out that, compared to BM-MSCs, AF-MSCs have higher self-renewal capacity and are more potent for lineage-specific differentiation^[40]. Therefore, considering also their more easily isolation and the great number of available cells, AF-MSCs may represent excellent candidates for cell replacement therapies^[61]. Of note, even if there are other MSC sources easily available, like adipose-derived MSCs, the osteogenic capacity of such cells compared to BM-MSCs is now still debated^[62].

The conventional protocol applied to induce osteogenic differentiation of AF-MSCs consists of basal medium, such as Dulbecco's modified Eagle's medium, supplemented with 10% Fetal Bovine Serum, β -glycerolphosphate, ascorbic acid and dexamethasone (standard osteogenic medium). For the purpose of assessing *in vitro* differentiation, protein and gene expression of specific osteogenic markers [e.g., alkaline phosphates (ALP), Collagen type I, bone sialoprotein, osteocalcin, RUNX2] are usually evaluated in addition to specific colorimetric assays, such as ALP, Alizarin Red S and Von Kossa staining.

However, various approaches have been applied to improve and accelerate differentiation of amniotic fluid-derived cells into osteogenic cells. Attempts have been made to produce osteoblastic cells from amniotic fluid samples in a very short time, using a single step culture procedure, which allows a 20-d reduction in culture time^[60].

One notes that several studies have demonstrated how such osteoinductive molecules are able to improve the osteogenic differentiation process^[44,63-68].

In detail, then, AF-MSCs transfected with a defective adenoviral vector expressing human lim mineralization protein 3, an intracellular positive regulator of osteoblast differentiation, showed downregulation of stemness kruppel-like factor-4 and then increased osteogenic differentiation^[63].

Again, Simvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor able to act on cholesterol endogenous synthesis, has been shown to be efficient in stimulating new bone formation. When added to osteogenic differentiation medium, Simvastatin was able to induce massive osteogenic differentiation of AF-MSCs, as observed by Alizarin Red S staining, and increased expression of typical osteogenic genes, such as osteopontin (OPN) and osteocalcin^[64].

Herbal medicines have also been used to improve the osteogenic differentiation of AF-MSCs. Naringin, the main active compound of Chinese herbal medicine (*Rhizoma drynariae*), proved able to enhance osteogenic differentiation, increasing ALP activity and the expression of the osteogenic specific markers OPN, Collagen I, bone morphogenetic protein 4 (BMP4), RUNX2, β -catenin and cyclin D1^[66].

Another traditional Chinese herbal medicine commonly used in treating orthopedic disorders, Curculigoside, was found to have a positive effect on the osteogenic differentiation of human AFSCs^[65].

Moreover, during osteogenic differentiation of both amniotic fluid and dental pulp stem cells, the *in vitro* addition of Ferutinin, a phytoestrogen able to prevent osteoporosis caused by ovariectomy-induced estrogen deficiency, was efficacious in enhancing the production of a calcified matrix^[67].

It has been noted that, in co-culture with osteoblasts obtained from the differentiation of dental pulp stem cells AF-MSCs prove able to generate osteoblasts. The same effect was observed when AF-MSCs were

Table 1 Studies describing *in vitro* modification of standard culture condition to induce osteogenic differentiation of AF-derived cells

Cell source	Methods to induce osteogenic differentiation	Ref.
hAF-MSCs	Culture in standard osteogenic medium on sandblasted and acid etching titanium (SLA titanium)	[60]
hAF-MSCs	Transfection with a defective adenoviral vector expressing human LMP3	[63]
hAFSCs	Standard osteogenic medium plus curculigoside	[65]
hAFSCs	Standard osteogenic medium plus naringin	[66]
hAFSCs	100 μ mol/L 2P-ascorbic acid, 100 nmol/L dexamethasone, supplemented with different concentrations of Ferutinin	[67]
hAF-MSCs	Induction of miR-21	[71]
hAFSCs	Medium containing 50 nmol/L rhBMP-7, 50 mg/mL ascorbic acid, and 10 mmol/L β -glycerophosphate on nanofibrous or solid walled scaffolds	[86]
hAFSCs	10 nmol/L dexamethasone, 6 mmol/L β -glycerol phosphate, 50 mg/mL ascorbic acid 2-phosphate, 50 ng/mL L-thyroxine on electrospun nanofiber meshes	[88]
hAFSCs	Standard osteogenic medium on: (1) Fibroin scaffold; (2) Collagen scaffold; and (3) Poly-D,L-lactic acid scaffold;	[89]
hAFSCs	Standard osteogenic medium on microfibrillar starch and poly(ϵ -caprolactone) scaffold	[90]
hAFSCs	Medical-grade poly- ϵ -caprolactone scaffold	[91]
hAFSCs	Standard osteogenic medium on collagen matrix derived from porcine bladder submucosa matrix and poly (lactide-co-glycolide)	[92]
hAFSCs	Standard osteogenic medium on construct composed of collagen type I	[93]
hAFSCs	α -MEM plus 17% FBS, 1 μ mol/L dexamethasone, 6 mmol/L of β -glycerol phosphate, 50 μ g/mL ascorbic acid 2-phosphate, and 50 ng/mL thyroxine on biomaterial named Skelite	[38]

MSCs: Mesenchymal stromal cells; AF-MSCs: MSCs isolated from amniotic fluid; AFSCs: Amniotic fluid stem cells; LMP3: Lim mineralization protein 3.

cultured in the conditioned medium of osteoblasts-differentiated dental pulp stem cells^[69].

Moreover, some findings show that microRNAs (miRNAs) are involved in determining the fate of stem cells^[70]. For example, a recent paper has shown the role of miR-21 in accelerating osteogenesis of AF-MSCs^[71]. In that work two cellular populations were isolated from amniotic fluid: spindle-shaped (SS) and round-shaped (RS). Interestingly, induction of miR-21 was found to accelerate osteogenesis more in the SS population than in RS cells^[71].

Finally, human AF-MSCs analyzed by an atomic force microscope during osteogenic differentiation showed a decrease in cell elasticity, which is typical of mature osteoblasts; thus the mechanical properties of AF-MSCs again add to the interest in applying them in bone regenerative medicine^[72].

Up to now, little is known about the cues regulating the AF-MSCs' ability to differentiate to osteoblasts. In depth study of the mechanism involved in the osteogenesis of AF-MSCs may hence help to develop standard protocols for clinical application in bone regenerative medicine. In this context, there is some evidence to suggest a role by the canonical Wnt signal pathway in bone formation as activation of this pathway stimulates osteoprogenitor proliferation and osteogenesis of human MSCs^[73,74]. Wnt signaling is also involved in AF-MSC commitment toward osteogenesis^[13].

Recently, we identified for the first time, the presence of Calcium Sensing Receptor (CaSR) in ovine and human AF-MSCs^[44,68]. CaSR, originally cloned from parathyroid glands, acts by controlling the secretion of parathyroid hormone in response to changes in extracellular calcium levels^[75]. However, it is well known that CaSR plays an important role in controlling osteoblasts as well as in osteoclast

recruitment, differentiation and survival *via* multiple intracellular signals^[76]. Interestingly, we observed that CaSR expression in both ovine and human AF-MSCs increased at the membrane when cells were treated with calcimimetic R-568, a molecule able to modulate bone cell metabolism *via* CaSR^[77]. This effect was abolished by CaSR allosteric inhibitor Calhex-231 and by selective inhibitor NPS-2143. Importantly, downregulation of CaSR by a gene-silencing approach confirmed the crucial role of CaSR in supporting osteogenic differentiation^[44].

These findings support the role of calcimimetics in the osteogenic differentiation of AF-MSCs, and suggest a strategy to develop therapy against bone injury.

Although the aforementioned studies have shown that AF-MSCs can be made to differentiate into osteoblasts *in vitro*^[40,41] and some pathways have been investigated, more studies and clinical trials are needed before AF-MSCs can be applied clinically for *in vivo* bone regeneration.

TISSUE ENGINEERING APPROACHES FOR *IN VIVO* BONE REGENERATION

For a long period time autogenous bone grafting was considered the gold standard for *in vivo* bone regeneration^[78]. Because of its limited availability and potential donor site morbidity, several bone substitutes have been successfully tried as an alternative, combining principles of biology and engineering. *In vivo* bone regeneration based on tissue engineering using scaffolds offers a plausible way of creating a favorable microenvironment for cells^[79].

The choice of an appropriate scaffold along with selection of the best suited cell source is currently

Table 2 Studies describing *in vivo* osteogenic differentiation of AF-derived cells

Cell source	Scaffold	Animal model	Ref.
oAF-MSCs	Magnesium-enriched hydroxyapatite/collagen-based scaffold	Sheep model of sinus augmentation	[85]
hAFSCs	Nanofibrous or solid walled scaffolds	Midsagittal incision made on the dorsa and two subcutaneous pockets created using blunt dissection on male outbred thymic nude mouse model (nu/nu)	[86]
hAFSCs	Fibroin scaffolds compared to collagen and poly-D,L-lactic acid scaffolds	Dorsal mid-sagittal incision made on the dorsa and two subcutaneous pockets were created using blunt dissection male outbred rats	[89]
hAFSCs	Microfibrous starch and poly(ϵ -caprolactone) scaffold	Subcutaneous implantation into the dorsal side of athymic female nude rats	[91]
hAFSCs	Construct composed of collagen type I	Critical-sized rat calvarial defect after oral administration of phytoestrogen ferutinin	[93]
hAFSCs	Biomaterial Skelite	Subcutaneous implantation in the back of CD-1 nu/nu mice	[38]

MSCs: Mesenchymal stromal cells; AF-MSCs: MSCs isolated from amniotic fluid; AFSCs: Amniotic fluid stem cells.

being widely studied.

As discussed above, the advantages of using amniotic fluid-derived cells are well established. Their high proliferation and osteogenic differentiation ability, together with the possibility of autologous or non-immunogenic transplantation and the absence of teratoma formation, makes possible a three-dimensional application of amniotic fluid cells in bone regeneration.

In general, a scaffold is a three-dimensional construct able to support adhesion, proliferation, and function of appropriate cells^[78]. Stimuli mimicking the *in vivo* bone environment are needed for tissue-engineered constructs. It is well known that the bone regeneration ability of MSCs is mainly due to their paracrine effects^[80]. They secrete bioactive substances that are able to enhance migration, proliferation, and differentiation of the neighboring resident cells^[81,82]. Thus, what is required is the right combination of cells and the most appropriate support and factors.

Various synthetic and non-synthetic scaffolds have been employed to support osteogenic differentiation of AF-MSCs. One commonly used non-synthetic scaffold is collagens, which are present in the bone tissue, where they stimulate MSCs to differentiate into osteoblasts, initiating new bone formation^[78]. This natural scaffolds are frequently used for their high availability, biological plasticity, biocompatibility, biodegradability and non-toxicity^[83,84].

It has been found that osteoblasts obtained from AF-MSCs were able to adhere and grow well on SLA (Sandblasted and Acid Etching) titanium surfaces, materials commonly utilized in dental implantology, as revealed by electron microscopy observation^[60].

Berardinelli *et al.*^[85] have demonstrated that a biomimetic commercial scaffold (magnesium-enriched hydroxyapatite (MgHA)/collagen-based scaffold) engineered with ovine AF-MSCs improves bone regeneration in a sheep model of sinus augmentation. Of note, the surface of this commercial scaffold was able to entrap a very high concentration of cells (1×10^7 cells/cm²) under dynamic cultural conditions. The osteoinductive properties of this scaffold, together with

the potential of ovine AF-MSCs clearly accelerated the formation of new bone.

Human AFSCs seeded on nanofibrous (NF) or solid walled (SW) scaffolds were induced in rhBMP-7-containing medium for 7 d and implanted into male outbred thymic nude mice (nu/nu). Six weeks after implantation, bone formation was found on scaffolds as noted by von Kossa staining with greater mineralization on NF than SW scaffolds^[86].

Synthetic NF scaffolds were developed with a morphology similar to that of natural collagen fibers, the aim being to mimic the morphological function of collagen fibers^[87].

Human AFSCs were differentiated on electrospun nanofiber meshes and compared to BM-MSCs. In these experimental conditions, the cells displayed a delay in alkaline phosphatase activity, but elevated mineral deposition after 4 wk in culture, compared to BM-MSCs^[88]. The nanofiber mesh scaffold possesses high porosity, large surface-area-to-volume ratios and size scale similar to extracellular matrix components. It allows the attachment of the cells and act as an efficient vehicle to deliver them to a defective site. A study by Maraldi *et al.*^[89] demonstrated that mineralization of hAFSCs tended to be enhanced on fibroin scaffolds, better than collagen and poly-D,L-lactic acid scaffolds. The cells were pre-differentiated on scaffolds for one week and then bone formation *in vivo* was determined after subcutaneous implantation into immune-suppressed rats^[89].

Again, human AFSCs were able to proliferate and differentiate into the osteogenic phenotype, producing mineralized extracellular matrix similar to BM-MSCs in 2D culture conditions^[90]. Moreover, when seeded on microfibrous starch and a poly(ϵ -caprolactone) scaffold, AFSCs successfully differentiated into osteogenic cells. The high porosity of the scaffold influenced the sequential development of osteoblastic cells and improved the osteogenic differentiation process. Some differences from BM-MSCs were observed in the expression of RunX-2, collagen I and ALP activity, denoting that cells from different origins may express different osteogenic markers at different times^[90].

In addition, AFSCs were also able to produce mineralized matrix within porous medical-grade poly-ε-caprolactone (mPCL) scaffold. The latter has good mechanical stability, large surface area and high porosity, allowing the attachment of AFSCs and extracellular matrix deposition. The construct, composed of an mPCL scaffold and AFSCs pre-differentiated for 28 d, was succeeded in producing seven times more mineralized matrix when implanted subcutaneously on the dorsal side of athymic female nude rats^[91].

The same cells also proved able to adhere to a composite scaffold formed of a collagen matrix derived from porcine bladder submucosa matrix and poly(lactide-co-glycolide). In this condition, the osteogenic differentiation of AFSCs was improved, as revealed by increased mineralization and upregulation of osteogenic genes^[92]. Indeed, the combination of natural scaffold with synthetic polymers was able to provide a microenvironment to facilitate the osteogenic differentiation.

More recently, a construct composed of collagen type I and AFSCs was used to evaluate bone regeneration in critical-sized rat calvarial defect after oral administration of phytoestrogen ferutinin^[93]. Interestingly, 4 wk after implantation, the construct had reconstructed almost 70% of the critical-size defect^[93]. Collagen type I, the major component of the extracellular matrix, facilitated cell adhesion and bone-forming cells in the defect site.

Another interesting study, by Mirabella *et al.*^[38] demonstrated that AFSCs loaded onto a biomaterial named Skelite and subcutaneously implanted in the back of CD-1 nu/nu mice gave rise to mineralized bone. Skelite disk is composed of silicon stabilized tricalcium phosphate biomaterial with a porosity of approximately 60% and an open structure similar to bone. However, they found that AFSCs did not have a direct role in new bone formation, but contributed to host progenitor recruitment and vessel formation in the engineered bone.

The unique features of AF-derived cells combined with the interesting results obtained in animal models of skeletal damage (Table 2) make them promising for the regeneration of bone tissue. Thanks to their immunosuppressive properties, allogeneic transplantation of AF-MSCs may be applied in various orthopedic conditions, and cell banks may be set up for regenerative medicine (Figure 1). However, more accurate understanding of the behavior and homing of AF-MSCs *in vivo* is necessary before they can be clinically applied. Moreover, additional studies are required to improve implanted cell survival and to ascertain the best biomaterial and the optimal combination of cytokines and growth factors.

CONCLUSION

One purpose of bone regeneration is to find the most appropriate source of stem cells for clinical application together with the best bio-compatible support.

Bone marrow harvesting requires invasive procedures while the MSC number and differentiation potential decline with increasing age of the donor^[94].

MSCs from extra-embryonic tissues are easily accessible. Moreover, their osteogenic potential and their safety in humans have been already tested. What is more, considering the high number of MSCs that can be isolated from amniotic fluid, a possibility of banking these cells adds greater promise of their clinical application. Furthermore, the osteogenic commitment of AF-MSCs could be enhanced by using appropriate osteoconductive scaffolds and osteoinductive growth factors^[90].

Although these cells are very promising, therefore, in order to apply them for bone regeneration further investigations are needed to select the safest and most efficient cell-based approach. Certainly the results obtained so far are most promising: preclinical and clinical studies should be continued, thus opening new insights in the foreseeable future.

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Stem cell therapy in the management of shoulder rotator cuff disorders

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Abstract

Rotator cuff tears are frequent shoulder problems that are usually dealt with surgical repair. Despite improved surgical techniques, the tendon-to-bone healing rate is unsatisfactory due to difficulties in restoring the delicate transitional tissue between bone and tendon. It is essential to understand the molecular mechanisms that determine this failure. The study of the molecular environment during embryogenesis and during normal healing after injury is key in devising strategies to get a successful repair. Mesenchymal stem cells (MSC) can differentiate into different mesodermal tissues and have a strong paracrine, anti-inflammatory, immunoregulatory and angiogenic potential. Stem cell therapy is thus a potentially effective therapy to enhance rotator cuff healing. Promising results have been reported with the use of autologous MSC of different origins in animal studies: they have shown to have better healing properties, increasing the amount of fibrocartilage formation and improving the orientation of fibrocartilage fibers with less immunologic response and reduced lymphocyte infiltration. All these changes lead to an increase in biomechanical strength. However, animal research is still inconclusive and more experimental studies are needed before human application. Future directions include expanded stem cell therapy in combination with growth factors or different scaffolds as well as new stem cell types and gene therapy.

Key words: Rotator cuff; Enthesis; Biologic; Stem cells

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Core tip: Current surgical techniques in rotator cuff repair do not achieve good tendon-to-bone healing. The use of stem cells to improve healing is a promising alternative. Different *in vivo* animal studies have shown

good results in achieving restoration of the native enthesis. However, human studies are scarce so the use of stem cell therapy in rotator cuff repair should still be considered and experimental technique. Further basic and clinical research is needed.

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INTRODUCTION

The rotator cuff is a structure formed by the tendinous insertions of a group of muscles that dynamically stabilize the glenohumeral joint. Rotator cuff disease is the most common condition of the shoulder for which patients seek treatment and can be found in 30% to 50% of the population aged older than 50 years^[1,2]. However, it also affects athletes and active individuals regardless of age and activity level.

Rotator cuff tears often require surgical treatment in order to increase function and decrease pain^[3,4]. The objective of the treatment is the repair of the damaged tendons. Whether or not healing of the tear is a prognostic factor on function and pain after rotator cuff repair has been controversial. However, most of the authors have found that tear recurrence determines lower functional scores and a decrease in patient satisfaction^[5-7]. In an attempt to improve the strength of the surgical repair, new materials and surgical techniques that aim to reproduce the anatomical footprint of the rotator cuff have been proposed^[8,9]. Despite these significant technical advances, several studies have shown a persistently high failure rate of tendon to bone rotator cuff repair that ranges from 30% to 94%^[6,10,11].

The main problem with failure in rotator cuff repair is probably biologic, as it is well known that the delicate and highly specialized fibro-cartilaginous transition zone between the rotator cuff and the bone does not regenerate after repair^[12,13]. Standard tendon to bone repair techniques attain only a fibro-vascular scar tissue that has relatively poor mechanical properties^[14]. Thus, the focus in research has changed from mechanical improvement of the repair techniques to finding ways to improve the biological environment around that repair^[15-22]. This would include growth factors (GF), bone morphogenetic proteins (BMPs) as well as stem cells. The hypothesis is that biological therapies might facilitate the regeneration of the normal tendon-to-bone insertion microarchitecture and limit the amount of scar tissue. In this direction, isolated GF or platelet rich plasma has been recently used with variable results but stems cell are a more

Table 1 Main biochemical and histological characteristics of the four areas of the enthesis^[28]

Zone	Histological characteristics	Collagen type	Extracellular matrix composition
Zone 1	Tendon	I	Decorin
Zone 2	Non-mineralized fibrocartilage	II and III (small amounts of I, IX and X)	Aggrecan and decorin
Zone 3	Mineralized fibrocartilage	II (small amounts of X)	Aggrecan and mineral component
Zone 4	Bone	I	Mineral component

promising alternative^[23-25].

Stem cells have demonstrated great potential in enhancing the biologic healing process based on their influence in angiogenesis and the inflammatory pattern^[26]. However, several questions still remain before they can be used clinically for augmenting tendon to bone healing. The purpose of this paper is to outline the current knowledge on the role stem cell therapy might have in dealing with rotator cuff tears and the future implications of the ongoing research's results.

ENTHESIS: TENDON TO BONE HEALING IN THE ROTATOR CUFF

Tissue regeneration in the tendon-to-bone interphase is a complex process. The stiffness difference between tendon and bone is responsible for significant mechanical stress in the regeneration zone^[27]. The enthesis represents a transitional tissue that allows for efficient energy transmission due to the gradual changes that occur in its microstructure, its histological characteristics and its biomechanical behaviour.

The enthesis has been divided into four zones: tendon, non-mineralized fibrocartilage, mineralized fibrocartilage and bone^[28] (Figure 1 and Table 1). In the tendon area (zone 1) there is a predominance of type I collagen fibres together with a small amount of decorin which is a small cellular or pericellular matrix proteoglycan; In the non-mineralized fibrocartilage area (zone 2), type II and III collagen fibres are predominant and small amounts of type I, IX and X collagen fibres have also been detected. Aggregans and decorine are also present. Zone 3 is constituted by the mineralized fibrocartilage, with a highly specialized mineralized content and type I collagen fibres. Lastly, zone 4 is characterized for a bone-alike composition, as it corresponds to the bony insertional area. As previously mentioned, it has been demonstrated that this specialized tissue does not regenerate after injury and repair. The fibro vascular tissue that substitutes the native enthesis is characterized by a predominance of type III collagen due to the excessive formation of scar tissue and the absence of fibrocartilage.

The reparative process can be divided into 3 phases (inflammatory, reparative and remodelling) and numerous cells and cytokines have been

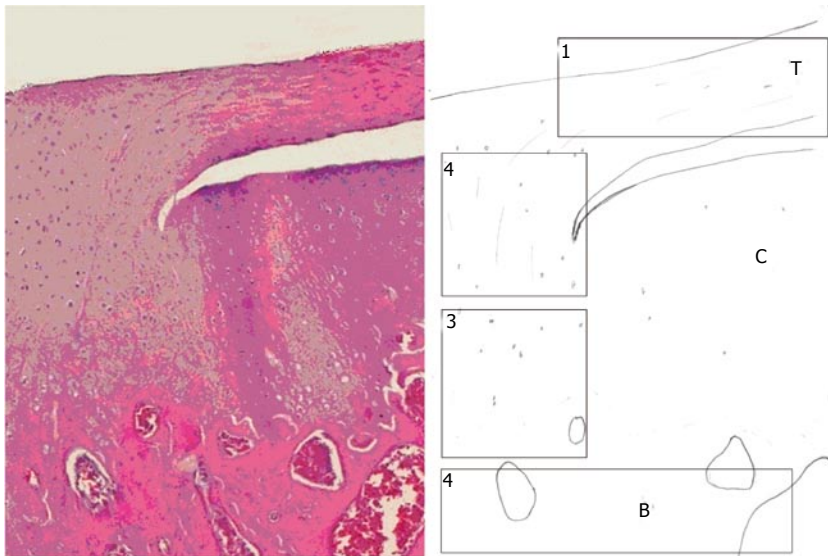


Figure 1 The normal enthesis (longitudinal image and diagram of the bone-tendon junction of the supraspinatus tendon of a rat; hematoxylin-eosine, $\times 10$): the supraspinatus tendon (T) approaches the humeral bone (B) immediately adjacent to the normal cartilage (C). The normal tendon (zone 1) gradually transforms into a fibrocartilaginous tissue with large mononucleated cells (zone 2). As the fibers progress into the bone the extracellular matrix is progressively calcified (zone 3) until it turns into normal bone (zone 4). Further explanation of the biochemical environment of these zones is shown in Table 1.

implicated^[13,29,30]. Diaz-Heredia *et al.*^[30] have studied the gradual variation of vascular endothelial growth factor (VEGF), interleukin-1 (IL-1) and transforming growth factor- β 1 (TGF- β 1) in an animal model of rotator cuff tears in rats. Some authors have pointed out that the inability to regenerate the native enthesis could be caused by the incomplete expression of the genes implicated in its formation^[28]. During embryogenic development, healing occurs without expression of TGF- β 1 but with expression of TGF- β 3, which determines an absence of scar tissue. On the contrary, during postnatal life, TGF- β 1 is active during the three phases of the healing process^[13,30,31]. Another important group of factors widely studied are BMP-12, 13 and 14 as well as fibroblast growth factor- β (FGF- β) and insulin like growth factor-1 (IGF-1). Matrix metalloproteinases are multi-domain proteinases regulated by tissue inhibitors of metalloproteinases (TIMPs) and play a determinant role in the remodelling phase.

The enthesis structure is developed successfully during embryogenic period so knowledge of the biological mechanism of its development could help in pinpointing which factors are relevant in trying to regenerate the native transitional tissue^[28]. Galatz *et al.*^[32] found that the mature fibrocartilage does not appear until 21 d after birth. Supraspinatus fibroblasts expressed type I collagen during all the process. Type II collagen was expressed firstly in the non-mineralized fibrocartilage and at 7 d in the mineralized fibrocartilage, where it persisted until 56 d. Type X collagen was initially seen in mineralized collagen at 14 d and it persisted until 56 d. There was a change in the presence from TGF- β 3 to TGF- β 1 at 15 d. The gradual expression of different factors present in the development of the physal plate as (sex determining region Y)-box 9, Scleraxis, Patched 1, Parathyroid hormone-related protein (PTHrP) and Indian Heddegog (Ihh) has also been studied^[32-34]. It has been proposed that the stratification in the structure and composition along the different zones of the enthesis could be a

consequence of the gradual expression of these and other factors. For example, the amount of mineral deposit in the mature enthesis could be determined by the presence of osteogenic factors such as runt-related transcription factor 2 (Runx2) and bone morphogenetic protein-2 (BMP2). On the other hand, the formation of fibrocartilage could be related to a greater expression of PTHrP, Ihh and Sox9. Lastly, tendon development would be conditioned by the expression of BMP-12, tenomodulin and scleraxis^[32]. Scleraxis is a protein member of the basic helix-loop-helix superfamily of transcription factors.

BIOLOGIC APPROACH

In the past decades, as mentioned before, numerous biology-based strategies have been developed in order to improve the rate and quality of healing in rotator cuff models. The main areas of research, apart from stem cells, are matrix metalloproteinase (MMP) inhibitors and GF.

MMP inhibitors

MMP expression is increased in degenerative rotator cuff tissue and it is known to cause progressive weakness in extracellular matrix. They are involved in tumoral growth, aneurysmatic disease and post-surgical tissue remodelling in the rotator cuff^[35-37]. Tissue metalloproteinase inhibitors are thus, potential biological tools. In particular, inhibition of MMP-13, a MMP that is increased in degenerative rotator cuff tears, allows for higher amount of fibrocartilage formation, better collagen fiber organization and higher load to ultimate failure in the enthesis^[36,38].

GF

GF factors are key in the development of the different enthesis zones. The regeneration of the most specialized zone, the mineralized fibrocartilage, can be stimulated by osteoinductive factors^[39]. The GF are

usually delivered with a vehicle, such as augmented sutures, fibrin gels or collagen sponges^[16,40,41]. Rodeo *et al.*^[16] developed an animal model of supraspinatus repair in sheep in which they used BMP-2 to 7, TGFβ1, TGFβ2, TGF-β3 and FGF. They detected better histologic and biomechanical properties^[16]. Other investigators have obtained similar results with BMP-12^[42], BMP-13^[17], BMP-14^[43], FGF^[40,44], IGF-1^[45] and PDGF-b^[15]. Some of these factors seem to play different roles depending in which zone of the enthesis they act or the timing of their effects.

The most widespread treatment, however, is platelet rich plasma (PRP) obtained from autologous blood^[46]. It has been proposed that PRP facilitates coagulation and homeostasis, stimulates wound closure, restores intraarticular hyaluronic acid, equilibrates angiogenesis, promotes glucosamine synthesis and serves as a cellular support for migration and differentiation^[23]. Despite the variable results obtained, it has been used for muscular, ligamentous, tendinous or cartilaginous injuries^[47-50]. With regards to its application in rotator cuff tears, the results have also been controversial. Neither Sánchez Márquez *et al.*^[24] or Ruiz-Moneo *et al.*^[51] found any relevant clinical improvement with the use of PRP to augment suture in massive tears. However, other investigators support the use of PRP in selected cases^[52]. For example, Randelli *et al.*^[25] in a prospective randomized clinical trial, found less postoperative pain and accelerated healing rate in patients with non-massive rotator cuff tears but there were no differences in functional scores and re-rupture rate. Due to the chronic nature of these injuries, it has been suggested that PRP application should be serial in order to enhance its benefits^[53]. Another explanation for this fact is that the expression of growth factors is ephemeral. In this context, stem cell and gene therapies could be a more definitive and long-lasting treatment.

STEM CELL THERAPY: ANIMAL STUDIES

The use of stem cell therapy in the regeneration of musculoskeletal tissue is a very dynamic field. MSCs of different origins, with their innate ability to differentiate into several mesenchymal tissues including bone, fat, muscle and tendon have been used extensively in tissue repair. Applications in which its usefulness has been confirmed are: treatment of bone defects, cartilage regeneration, meniscal regeneration and healing, management of tendinopathies and management of muscle lesions^[54-56]. Investigators usually prefer adult MSCs over embryonic or fetal stem cells as the former are usually locally available and easier to obtain for the treatment of these non-life-threatening problems. Furthermore, the low immunogenicity of MSCs allows for the use of allogenic strains^[57].

Some authors have also performed extensive research in animal bone-to-tendon healing models. Until recently the most widespread model reproduced

the integration of anterior cruciate ligament tendinous grafts in a bone tunnel. In this animal model, the hamstring grafts are introduced into bony tunnels in both femoral and tibiae bones and pull out strength is tested. Lim *et al.*^[58] have used MSCs in this model in rabbits and found a significant increase in maximum load to failure.

It was not until 2009 that MSC therapy was applied to a rotator cuff model, since then the available literature has grown consistently. MSCs of different origins have been used for rotator cuff repair. Different tissue sources have been identified: bone marrow, adipose tissue, muscle, synovia, periosteum, tendon, dermis and umbilical cord or peripheral blood, have all been evaluated as sources of multipotent and pluripotent cell^[26]. Although generally speaking MSCs of different origins have similar biological potential, there is increasing knowledge that certain MSC populations are better than others for specific tissue regeneration^[59-61]. Table 2 shows the main animal investigations performed on rotator cuff repair.

Bone marrow MSCs

The principal source for stem cell-enhanced healing of the rotator cuff has been autologous bone marrow (BM-MSCs). Gulotta *et al.*^[20] performed an experimental unilateral detachment of supraspinatus tendon and a transosseous repair in rats. BM-MSCs were harvested by performing lavage of intramedullary canals of long bones with Hank's Balanced Salt Solution (Gibco, Gaithersburg, MD). They showed that MSCs were present at the repair site and that they were metabolically active. Although they did not find significant differences in between the treated and untreated groups, at 4 wk, there was a higher amount of fibrocartilage formation and better orientation of fibrocartilage fibers.

In order to reproduce rotator cuff surgery, Kida *et al.*^[62] designed a study in which they performed additional drilling to the greater tuberosity to release bone marrow and allow bone marrow cells to migrate into the suture zone. They tested chimeric rats that expressed green fluorescent protein in the bone marrow cells and looked for the expression of this protein after a period of 2, 4 and 8 wk. It seems that drilling and the subsequent migration of stem cells might improve maximum load to failure at 4 and 8 wk.

More recently, Gulotta *et al.*^[63-65] have used genetically modified MSCs in order to express scleraxis and produce MIT1 and BMP-13 with promising results. MSCs genetically modified to over-express MT1-MMP might be useful for augmenting suture as it has demonstrated improved biomechanical strength at 4 wk based on a higher presence of fibrocartilage^[63]. Results of studies with application of MSCs genetically modified to overexpress BMP-13^[64] were not that successful. On the contrary, MSCs genetically modified with Scx demonstrated to promote better biomechanical

Table 2 Rotator cuff repair animal models using mesenchymal stem cells

Ref.	Animal	Type of cells	Tendon repair model	Method of delivery	Results
Gulotta <i>et al</i> ^[20]	Rat	Allogenic BM-MSC	Supraspinatus tendon Acute repair	Fibrin glue carrier	No differences in structure, composition or strength at the repair site
Gulotta <i>et al</i> ^[63]	Rat	Allogenic BM-MSCs transduced with MT1-MMP	Supraspinatus tendon Acute repair	Fibrin glue carrier	Improved fibrocartilage Improved biomechanical strength
Gulotta <i>et al</i> ^[64]	Rat	Allogenic BM-MSCs transduced with human BMP-13	Supraspinatus tendon Acute repair	Fibrin glue carrier	No differences in structure, composition or strength at the repair site
Gulotta <i>et al</i> ^[65]	Rat	Allogenic BM-MSCs transduced with scleraxis	Supraspinatus tendon Acute repair	Fibrin glue carrier	Improved fibrocartilage Improved mechanical resistance and stiffness
Shen <i>et al</i> ^[74]	Rabbit	Allogenic T-MSCs	Supraspinatus tendon Acute repair	Seeded scaffold (silk-collagen)	T-MSCs differentiated into tenocytes Improved collagen content Improved biological environment Less inflammation
Kida <i>et al</i> ^[62]	Rat	Autologous BM-MSC	Supraspinatus tendon Acute repair	Transosseous drilling	BM-MSCs infiltrated the repaired tendon Improved mechanical resistance
Oh <i>et al</i> ^[71]	Rabbit	Allogenic A-MSCs	Subscapularis tendon Chronic repair	Injection	Improved muscle function Improved tendon healing Decreased fatty infiltration

Different types of cells have been used: Bone marrow derived MSCs (BM-MSCs), Tendon derived MSCs (T-MSCs) or Adipose derived MSCs (A-MSCs). MT1-MMP: Metalloproteinase inhibitor-1; BMP-13: Bone morphogenetic protein-13; MSCs: Mesenchymal stem cells.

characteristics at 2 wk^[65].

Adipose tissue MSC

Adipose Tissue derived stem cells (AMSC) have also shown multipotentiality *in vitro*^[66]. Due to its mesodermal origin, they can differentiate into adipose lineage cells^[67], osteogenic cells^[68], chondrogenic cells^[59] and myogenic cells^[69]. *In vivo*, they have also demonstrated their capacity to differentiate into adipose tissue using different scaffolds as polyglycolic acid, collagen sponges or fibrin gel^[54,70,71].

Recently, Oh *et al*^[71] have published the first study in a rotator cuff model using AMSCs. Four groups were compared for a suture of the subscapularis tendon in rabbit using saline, saline and AMSCs, only AMSCs and only suture. They found better healing properties and a capacity of regeneration after fatty infiltration of the muscle.

MSCs of other origins (non-hematopoietic)

Muscle-derived stem cells (M-MSCs) have been isolated using a modification of a method known as the preplate technique^[72]. Pelinkovic *et al*^[73] have shown that the injection of M-MSCs into the supraspinatus tendon of athymic rats resulted in the engraftment of transplanted cells in a pattern with a morphology comparable to resident tendon fibers. The authors suggest that more studies are necessary before assuming that M-MSCs can improve rotator cuff healing.

Lastly, Shen *et al*^[74] performed a study using tenocyte-derived stem cells (T-MSCs) proliferated *in vitro* and obtained from human fetal Achilles tendon samples. Implantation of this type of cells in the rabbit rotator cuff defect did not elicit an immunologic

response but increased fibroblastic cell ingrowth and reduced infiltration of lymphocytes.

Choice of scaffold for MSCs deployment

Cell adhesion to the scaffold depends on the interaction that is established in between the scaffold microstructure and the cell surface receptors denominated integrins. Transmembrane contacts are key factor for MSC survival, proliferation and differentiation^[75]. Numerous studies have investigated the behaviour of stem cells in different scaffolds and have demonstrated that the scaffold can determine the differentiation capacity into one or other lineages^[75]. Two different types of interactions have been described: physical and biochemical. Vehicles that maintain the rounded shape of the cells and avoid contact in between them, promote the chondrogenic differentiation and avoid expression of type I collagen. Porous gelatine vehicles or those that use fibrin favour a fibro cartilaginous phenotype due to the expression of collagen types I and II^[76].

STEM CELL THERAPY: HUMAN STUDIES

Although there is a lack of consensus on whether the application of stem cells to enhance the rotator cuff healing is effective or not, some authors have started developing different strategies for the clinical application of the experimental findings.

Beitzel *et al*^[77] studied the quantity and characteristics of BM-MSCs obtained from proximal humerus and distal femur bone marrow aspiration and found them comparable, supporting the previous experimental research by Kida *et al*^[62]. Rotator cuff derived MSCs have been isolated and compared to BM-derived stem

cells. It seems that the myogenic potential of MSCs derived from rotator cuff cells is higher than for BM-MSCs^[78]. Randelli *et al.*^[79] could isolate tenocyte-derived stem cells from supraspinatus tendon and long head of biceps tendon. Utsunomiya *et al.*^[80] also studied the subacromial bursa as a potential source for MSCs and found that the synovial cells found in the bursa were a good cell source.

Ellera Gomes *et al.*^[81] published their work in 14 patients with a complete tear of the rotator cuff that was repaired in a trans osseous fashion through a mini-incision augmenting the suture with mononuclear stem cells from iliac crest bone marrow aspirate. At 12 mo, 12 of the 14 tears had healed according to clinical and magnetic resonance imaging results^[81]. This is the only published investigation on clinical application of stem cells in rotator cuff tears.

Lastly, Beitzel *et al.*^[75] have also focused their attention in how different scaffolds behave in humans in order to extrapolate results obtained from experimental research. MSCs adhesion, proliferation, and scaffold morphology were evaluated by histologic analysis and electron microscopy. According to their findings, significant differences existed: non cross-linked porcine collagen scaffolds showed superior results for cell adhesion and proliferation, as well as on histologic evaluation.

FUTURE ALTERNATIVES

Advanced stem cell therapy and gene therapy represent the most feasible option in order to improve rotator cuff healing^[21]. A better knowledge of the molecular phases of embryogenesis of the enthesis as well as the injury and healing patterns have allowed to identify the growth factors and proteins to target^[13,28].

A combination of stem cells, modified before implantation, using exposure to different growth factors or modifications to the culture conditions to generate a desired phenotype is one of the most investigated pathways^[26]. Moreover, the newly recognized anti-inflammatory and antiapoptotic impact of MSCs on tissue healing may provide a great potential for functional restoration^[76,82].

On the other hand, specific growth factor supplementation, in the form of transgenic therapy may allow longer-term tendon repair and potential return to function. Fetal-derived embryonic stem cell-like cells have recently been evaluated for tendon and ligament repair. More recently, induced pluripotent stem cells, developed by genetically reprogramming adult-sourced cells, may be particularly beneficial in the challenging environment of rotator cuff injury. Generation of iPS cells can use viral or, more recently, nonviral vector delivery of reprogramming genes. However, these transgenic therapies lack safety clearance when it comes to oncologic and teratogenic risks^[26].

Lastly, stem cells associated to bio or nanotechnology can control the proliferation and differentiation

into complex, viable 3D tissues. So we might be able to use biodegradable polymer scaffolds to promote cell growth and differentiation and formation of 3D structures. This could be useful in order to avoid scarring during the healing process.

CONCLUSION

Current literature regarding the clinical use of stem cells in rotator cuff tears is limited. Although in vivo animal studies have shown promising results to enhance tendon-to-bone healing, the use of stem cell therapy in rotator cuff should still be considered an experimental technique. Further basic and clinical research is needed.

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Epigenetic regulation of stemness maintenance in the neurogenic niches

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is established and maintained by the coordinated interaction between transcription factors and epigenetic regulators which control stem cell fate. Epigenetic mechanisms are heritable alterations in genome function that do not involve changes in DNA sequence itself but that modulate gene expression, acting as mediators between the environment and the genome. At the molecular level, those epigenetic mechanisms comprise chemical modifications of DNA such as methylation, hydroxymethylation and histone modifications needed for the maintenance of NSC identity. Genomic imprinting is another normal epigenetic process leading to parental-specific expression of a gene, known to be implicated in the control of gene dosage in the neurogenic niches. The generation of induced pluripotent stem cells from NSCs by expression of defined transcription factors, provide key insights into fundamental principles of stem cell biology. Epigenetic modifications can also occur during reprogramming of NSCs to pluripotency and a better understanding of this process will help to elucidate the mechanisms required for stem cell maintenance. This review takes advantage of recent studies from the epigenetic field to report knowledge regarding the mechanisms of stemness maintenance of neural stem cells in the neurogenic niches.

Key words: Neurogenesis; Neural stem cell; Epigenetics; Gene expression regulation; Chromatin modifications; DNA methylation

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Abstract

In the adult mouse brain, the subventricular zone lining the lateral ventricles and the subgranular zone in the dentate gyrus of the hippocampus are two zones that contain neural stem cells (NSCs) with the capacity to give rise to neurons and glia during the entire life of the animal. Spatial and temporal regulation of gene expression in the NSCs population

Core tip: Neural stem cells (NSCs) are capable of extensive self-renewal while preserving the ability to generate cell progeny that can differentiate into different cell types from the nervous system. Intrinsic mediators as well as extrinsic cues provided by the neurogenic niche (microenvironment where NSCs reside *in vivo*) are important for stem cell self-renewal and differentiation. Epigenetic changes, including alterations in DNA methylation, histone modifications and imprinting alter

the way a gene interacts with the cell transcribing machinery, turning genes “on” or “off”. These heritable changes must be reversible and context-dependent being responsible of stem cell plasticity.

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NEURAL STEM CELLS AND THE NEUROGENIC NICHES

Adult stem cells have the ability to divide, self-renew and generate functional differentiated cells that replace lost cells throughout an organism's lifetime. The existence of adult stem cells was first described in tissues with high proliferation rates, such as the hematopoietic system and the intestine. Since then, stem cells have been found in almost all adult tissues including the nervous system^[1]. In the adult mouse brain two main regions continue to generate new neurons throughout adulthood: the subventricular zone (SVZ) in the walls of the lateral ventricles^[2] (Figure 1A-C) and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus^[3] (Figure 1D-F). Adult neurogenesis is supported by multipotent neural stem cells (NSCs) deriving from embryonic radial-glia and thus expressing astroglial characteristics^[4,5]. Astrocytic-like stem cells are relatively quiescent and can be identified by the expression of the glial fibrillary acidic protein (GFAP), the stemness-related transcription factor Sox2 [Sex determining region Y (SRY)-box 2], and the neural progenitor marker Nestin^[2,6,7]. Moreover, their slow division rate can be detected by the label retention of thymidine analogs incorporated during DNA replication^[6,8,9]. NSCs can also be isolated from their natural niche and cultured *in vitro* in the presence of the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) mitogens. In culture, NSCs form free-floating aggregates called “neurospheres” (Figure 1C). Self-renewal and multipotency characteristics of NSCs are assessed *in vitro* by clonal analysis in which single cells give rise to neurospheres^[10,11] (Figure 1C).

SVZ and the olfactory bulb system

The SVZ is located lining the walls of the lateral ventricles and constitutes a complex microenvironment or niche in which proliferation and self-renewal of NSCs are strongly regulated by multiple extracellular factors such as EGF, bFGF, bone morphogenetic protein and pigment epithelium derived factor^[12-14]. This significant extrinsic signaling is possible because of the special cytoarchitecture of the niche that allows NSCs to be

in direct contact with the cerebrospinal fluid (CSF) produced by the choroid plexus in the ventricles, with the vasculature and with other cells from the niche like astrocytes or microglia^[15,16]. Subventricular NSCs (also known as type B1 cells) present a radial glia-like morphology, with an apical primary cilium contacting the ventricular lumen and a basal process reaching the basal lamina and the vascular structures^[17,18] (Figure 1A). The walls of the lateral ventricles show a typical organization where the small apical process of one or more type B1 cells are surrounded by a rosette of epithelial ependymal cells that form structures known as pinwheels at the surface^[19]. There is another astrocyte-like type B cell that is more frequently located close to the underlying striatal parenchyma known as type B2 cells^[20]. When activated, these slowly dividing NSCs give rise to fast cycling cells called transit-amplifying progenitors (TAP or type C cells). TAP cells contribute to reducing the number of cell division rounds that NSCs have to undergo to preserve their genome integrity. Mash1-positive type C cells generate neuroblasts (type A cells) that migrate along the rostral migratory stream (RMS) into the olfactory bulb (OB) where they differentiate and integrate into interneurons (Figure 1B). These chains of polysialylated neural cell adhesion molecule (PSA-NCAM) and DCX (doublecortin) positive neuroblasts reach the core of the OB, where they detach from the RMS and migrate radially into the granular and periglomerular layers^[21-23]. These immature neurons then integrate and differentiate into inhibitory interneurons, playing an important role in rodent olfaction. In addition of being a neurogenic region, the SVZ can serve as a niche of oligodendrocytes although generated in much lower numbers than neuroblasts. Thereby, Olig2-positive transit amplifying cells give rise to oligodendroblasts that migrate to the corpus callosum and striatum while tightly associated with blood vessels^[24], where they differentiate into myelinating and nonmyelinating oligodendrocytes^[25].

SGZ of the hippocampus

Along with the SVZ, the subgranular zone in the dentate gyrus of the hippocampus constitutes the other main neurogenic niche in the adult mouse brain^[26-28]. The SGZ is also a complex microenvironment in which the vasculature plays an important role. Dividing stem cells in the SGZ are in close proximity to an extensive network of interconnected blood vessels and parenchymal astrocytes that can regulate their proliferation and differentiation *via* paracrine signaling^[29]. The SGZ is located between the granular layer and the hilus of the DG and the SGZ NSCs constitute a subpopulation of GFAP-positive cells that are analogous to subventricular type B1 cells^[30]. In this region, two types of neural progenitors can be identified according to different expression of molecular markers and their morphologies^[23] (Figure 1D and E). Type I progenitors exhibit a radial process spanning the granule cell layer and arborizing profusely in

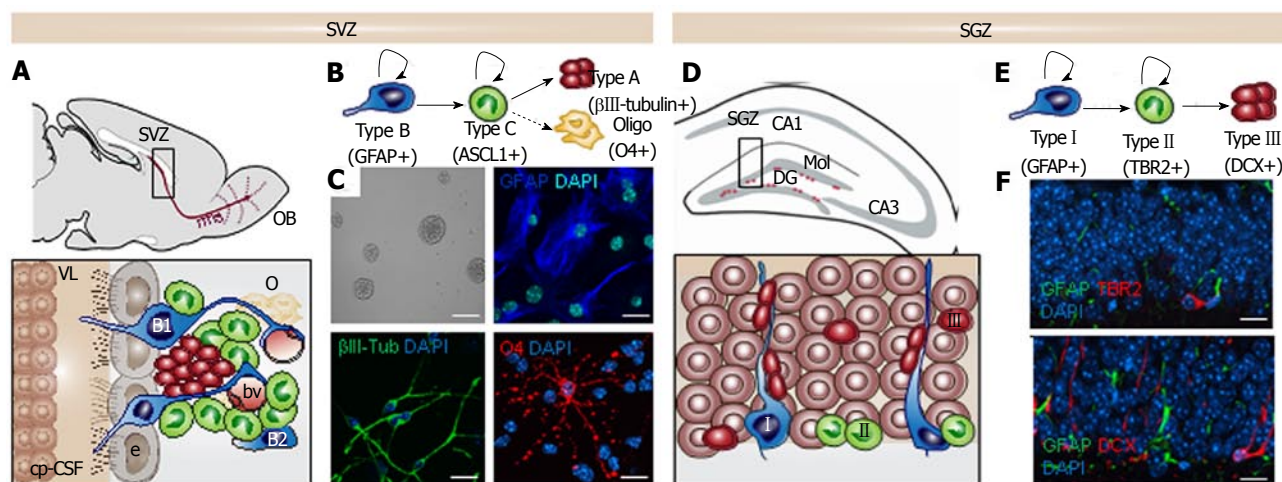


Figure 1 The neurogenic niches in the adult murine mammalian brain. A: Sagittal view showing the adult mouse subventricular zone (SVZ) and the migrating neuroblasts (red) reaching the olfactory bulb (OB) through the rostral migratory stream (rms). Enlarged view of SVZ: type B1 stem cells (blue) express the astrocyte marker glial fibrillary acidic protein (GFAP) and contact the ventricle with a thin process extended between the ependymal cells (e; gray); type B2 stem cells (blue) contacting the brain parenchyma; transit amplifying progenitors (TAP) or type C cells (green) express the achaete-scute homolog 1 (ASCL1) transcription factor and give rise to type A cells (red) that migrate through the rostral migratory stream (rms). Dividing stem cells and their TAP progeny are tightly opposed to blood vessels (bv); B: Schematic drawing showing the lineage progression in the SVZ; C: SVZ neural stem cell (NSC) cultures in self-renewal (neurosphere formation) and differentiation. The astrocyte marker GFAP in blue, the neuronal marker β III-tubulin in green and the oligodendrocyte marker O4 in red; The Choroid plexus-cerebrospinal fluid system (cp-CSF) is shown. D: Coronal view showing the adult mouse subgranular zone (SGZ) and the newborn neurons (red) being integrated in the granular cell layer (gr). Enlarged view of the dentate gyrus (DG): Type I stem cells (blue) are GFAP+ and show a radial single prolongation through the granular layer; Type II precursors give rise to neuronal lineage-restricted progenitor cells type III cells (red) that differentiate into neurons in the granular layer; E: Schematic drawing showing the lineage progression in the SGZ; F: Confocal images showing immunostaining in the DG for the astrocyte marker GFAP in green, for the progenitor precursor marker T-box brain protein 2 (TBR2) in red and for the neuronal precursor marker Doublecortin (DCX) in red. DAPI is used to stain DNA. Scale bar in C: Top left panel 100 μ m, rest 10 μ m; In f: 10 μ m.

the molecular layer^[27]. These cells express nestin, GFAP, and Sox2^[31]. Type II hippocampal progenitors have short processes and contrary to type I cells, express TRB2 but not GFAP (Figure 1F). There is evidence suggesting that type II cells may derive from type I cells but a lineage relationship study is still lacking^[31]. In the adult SGZ, precursors give rise by asymmetrical divisions to intermediate neuronal lineage-restricted progenitor cells and in a minor number, to glial lineage-restricted progenitor cells (both of them are GFAP-negative cells). Compared to the SVZ, few oligodendrocytes are generated in the SGZ. Type II cells generate in turn type III cells, which are neuronal precursors that express markers of immature migrating neurons, such DCX and PSA-NCAM (Figure 1F). These differentiated cells integrate neuronal circuits into the hippocampal CA3 region forming dendrites and spreading their axons^[22]. In addition to the production of granular neurons, a low percentage of activated NSCs divide asymmetrically to give rise to astrocytes. The latter migrate into the hilus and the molecular layer where they lose their stem cell identity and cause the depletion of the pool of NSCs^[32,33] thus explaining the possible decrease in hippocampal neurogenesis associated with ageing.

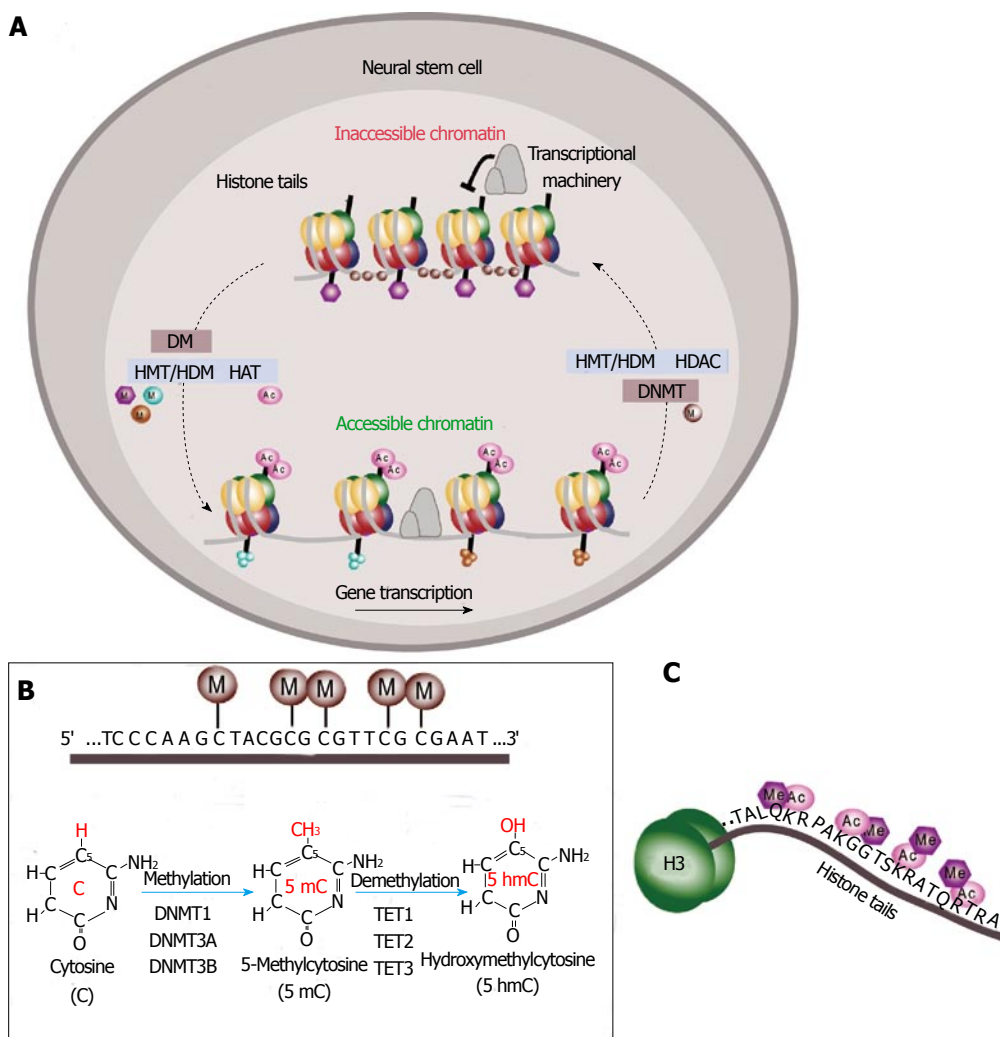
EPIGENETIC REGULATION OF GENE EXPRESSION IN THE NEUROGENIC NICHES

Epigenetic is defined as the study of heritable altera-

tions in genome function that do not involve changes in DNA sequence itself^[34,35]. These epigenetic marks modulate gene expression either by directly altering the chromatin structure or by creating bindings sites for chromatin and transcription regulatory subunits. Two general classes of epigenetic regulation can be defined: covalent modifications to DNA and post-translational covalent modifications to the histones (H) around which the DNA is bound, influencing whether DNA is accessible or not for gene transcription^[36,37] (Figure 2A). Moreover, the three-dimensional structure and arrangement of chromatin within the nucleus are both regulated by and contribute to the establishment and maintenance of epigenetic states^[34]. These different classes of epigenetic modifications are intimately related, resulting in multiple layers of control allowing cells to maintain their identity over time^[34,38]. Dysregulation of these mechanisms leads to new cellular phenotypes by causing altered gene expression without a change in genotype. In the neurogenic niches, epigenetic regulators and the associated transcription factors play an important role in the control and maintenance of NSC stemness.

DNA methylation

DNA methylation involves the addition of a methyl group to the fifth carbon in the cytosine pyrimidine ring (Figure 2B). In most mammalian genes, CpG dinucleotides are methylated and concentrated in clusters called "CpG islands" which often have regulatory functions and tend to be found in the promoter and first exon regions of genes^[39] where it promotes a closed chromatin structure and aids the prevention



of expression^[34,40]. DNA methylation marks repress gene expression either by attracting DNA methyl-binding domain proteins (MBDs) such as methyl-CpG binding protein 2 (MeCP2) which recruit repressors and chromatin remodeling molecules to generate an inactive chromatin environment or by directly inhibiting transcription factor binding^[41-43]. MBD proteins have been suggested to play a role in neurogenesis. For example, mice deficient in MBD1 show decreased neurogenesis and hippocampus-related behaviour defects. Indeed, *Mbd1* deficient NSCs generate a reduced number of neurons when compared to wild-type cells, suggesting a role for MBD1 in neuronal fate commitment^[44].

DNMT3b, important for normal embryogenesis and development and responsible for the establishment of methylation patterns. The other type is maintenance methylation mediated by DNMT1 that effectively maintains CpG methylation upon DNA replication and provides the heritable “memory” of the methylation state of the parent cell^[45,46]. DNMT1 is highly expressed in the embryonic, perinatal and adult CNS in both dividing neural progenitors and mature neurons where it maintains DNA methylation^[47-49]. A lack of DNMT1 alters neuronal excitability and increases apoptosis in post-mitotic cortical neurons^[50]. In support of this, mice deficient for *Dnmt1* specifically in neural progenitors at embryonic stages exhibit deficits in neuronal function and die postnatally, suggesting a requirement for methylation in brain development^[51]. DNMT3a and DNMT3b are highly expressed in postnatal

NSCs and are required for neurogenesis and neuronal maturation^[48,49,52,53]. Loss of DNMT3a results in gene silencing^[53] and depletion of DNMT3b leads to deficient neuronal differentiation *in vitro*^[54].

DNA is hypomethylated in neural progenitor cells and methylation is progressively increased during lineage commitment^[55]. The suppression of astrogliogenesis during neuronal specification is also associated with changes in DNA methylation^[56,57]. This silencing is attenuated later in development resulting in the generation of astrocytes which correlates with the suppression of neurogenesis. Demethylation and expression of the genes coding for the astrocytic markers *Gfap* and the calcium binding protein *S100β* during astrocytic maturation, correlates with methylation and downregulation of neurogenic genes such as Neurogenin 1^[58-60]. Activation of the *Gfap* promoter requires binding of the signal transducer and activator of transcription 3 (STAT3) to a consensus sequence. Early progenitors are refractory to astrocyte differentiation due to methylation of the STAT3 binding site. At later development stages, loss of STAT3-binding element methylation is associated with *Gfap* promoter activation^[60,61]. A similar alteration in methylation pattern occurs at another STAT3 binding site in the *S100β* promoter^[58].

Hydroxymethylation

DNA methylation marks are reversible through both passive replication-dependent demethylation and active demethylation which probably involve the recently characterized 5-hydroxymethyl (5hmC) intermediate^[62] (Figure 2B). In mammals, three members of the ten-eleven translocation (TET) family of enzymes have been identified: TET1, TET2 and TET3^[63,64]. TET hydroxylases may catalyze active DNA demethylation by oxidation of 5mC to 5hmC^[65-67] (Figure 2B). 5hmC is relatively abundant in mouse embryonic stem cells (ESCs), the early embryo and in adult brain^[68,69]. In the brain, 5hmC is enriched at active genes, associated with the strong depletion of 5mC from these regions^[70]. It has been proposed that TET enzymes in the blastocyst and ESCs are involved in pluripotency by maintaining the hypomethylated state of key regulatory regions^[69,71]. Recent studies have also shown that TET1 is involved in the epigenetic regulation of neural progenitor cell proliferation in the adult hippocampus^[72-74]. Mice lacking *Tet1* exhibit impaired hippocampal neurogenesis accompanied by poor learning and memory^[72-74]. However, the full role and importance of hydroxymethylation in the brain remains to be elucidated.

Histone modifications as regulators of adult neurogenesis

In eukaryotic cells, a histone octamer including two H2A-H2B dimers and a H3-H4 tetramer acts as a scaffold around which DNA is wrapped to form a nucleosome^[75,76]. The interaction between histones and DNA is mediated by an N-terminal tail of histone

proteins available for post-translational modifications that control the chromatin structure^[75] (Figure 2C). These covalent modifications in the histone tails alter the interaction between adjacent nucleosomes and/or between histones and the DNA, changing the three-dimensional chromatin structure. Modifications in the body of histones have also been shown to alter chromatin structure influencing gene expression^[77]. Histone modifications are divided into repressive and active marks according to how they correlate with levels of transcriptional activity. For example, histone acetylation of lysine residues of histones, catalyzed by histone acetyltransferases enhances the recruitment and activation of the transcriptional machinery and is generally associated with areas of active gene transcription^[78]. However, histone deacetylases (HDACs) remove acetyl groups promoting the condensation of chromatin^[79] (Figure 2A). HDAC1 is expressed by GFAP-positive cells within the SVZ whereas HDAC2 is found in migrating neuroblasts and in TAP cells within the SVZ^[80]. Deletion of HDAC2 in the SVZ results in a defective neurogenesis to the OB^[81] and neurospheres treated with class I and II HDAC inhibitors promotes neuronal differentiation^[82] suggesting a role for this enzyme in neuronal fate determination. Furthermore, oligodendrocyte fate commitment is accompanied by a decrease in histone deacetylation at transcriptional repressors of oligodendrocytic differentiation such as *Sox11*^[83] and at neuronal genes such as *Sox2*^[84].

Histone methylation is associated with both active and silent chromatin and is catalyzed by histone methyltransferases (HMTs). Trimethylation of lysine (K)-27 and lysine 9 of histone H3 (H3K27me³ and H3K9me³) tends to associate with regions of inactive gene transcription, whereas H3K4, H3K36 and H3K79 methylations are associated with active transcription^[85]. Histone demethylases (HDMs) also have a key role in regulating neural development^[86]. During neural stem cell commitment, H3K27 methylation decreases in key developmental genes following downregulation of the HMT *Enhancer of Zest homolog 2* (EZH2) and upregulation of the HDM Jumonji domain containing-3 (JMJD3). Indeed, deletion of *Ezh2* in SGZ progenitor cells results in cell proliferation restriction leading to a reduced number of neurons that ultimately leads to impairment in spatial learning and memory^[87]. Additionally, JMJD3 is upregulated in neuroblasts, and *Jmjd3* deletion targeted to SVZ NSCs in both developing and adult mice impairs neuronal differentiation. JMJD3 regulates neurogenic gene expression *via* interaction at not only promoter regions but also neurogenic enhancer elements such as *Dlx2*^[88]. Moreover, H3K9me³ is enriched in the adult murine SVZ and it has been recently shown that its repression in undifferentiated cells is engaged in the maintenance of cell type integrity in this neurogenic niche^[89]. MLL1, another HMT that methylates H3K4, has been associated with the trithorax group of transcription factors. In mice where *MLL1* is knocked out in NSCs, neurogenesis is impaired.

Mll1 is associated with the promoter of the homeobox transcription factor *Dlx2* and although loss of *Mll1* does not affect the methylation of H3K4, it does increase H3K27me³ on the promoter indicating that *Mll1* is recruiting a H3K27 demethylase^[90]. In summary, the above studies indicate that different chromatin modifiers have a critical role in adult neurogenesis^[91].

Genomic imprinting and control of gene dosage

Imprinted genes are expressed predominantly from one chromosome in a parental-origin dependent manner. While most genes are expressed from both alleles, imprinted genes are functionally monoallelic and are expressed from either the maternally or the paternally inherited chromosome^[92]. In mammals, this affects around 100 genes that are found in clusters. Imprinting control regions (ICRs) regulate the parental allele-specific pattern of gene expression and have differentially methylated regions (DMRs) on the two parental chromosomes. ICRs can be divided into those which are methylated on the paternally inherited copy and those with maternally inherited methylation^[93]. DMRs are also characterized by the asymmetrical accumulation of different histone modifications on the two parental chromosomes and the recent identification of a "tri-mark", comprising the trimethylation of H3K4 and H3K9 and the trimethylation of H4K20 at all known ICRs^[94]. The majority of imprinted genes are expressed in the brain and several exhibit brain-specific imprinting. Their monoallelic expression makes these loci very vulnerable as mutation of the expressed allele can compromise expression and lead to severe developmental defects. For example, human congenital imprinting syndromes including Angelman syndrome and Prader-Willi syndrome are all characterized by neurological and behavioral impairments and learning difficulties^[95]. Evidence is suggesting that selective regulation of imprinting is a normal mechanism of modulating gene dosage and is associated with the control of stem cell potential in the neurogenic niche. For instance, relaxation of imprinting of the gene for the atypical NOTCH ligand delta-like homologue 1 (*Dlk1*) usually expressed from the paternally inherited chromosome has been shown in the neural stem cells and niche astrocytes within the SVZ^[96]. Notably, this selective absence of *Dlk1* imprinting is associated with acquisition of DNA methylation at the germline-derived imprinting control region^[96]. *Igf2* is also an imprinted gene expressed only from the paternally-inherited allele although it is specifically biallelically expressed in postnatal human and mouse choroid plexus epithelium and leptomeninges^[97,98]. Thus, CSF produced from the choroid plexus and blood vessels is a biallelic source of neurogenesis-promoting IGF2^[99].

Epigenetic changes during NSCs reprogramming to induced pluripotent stem cells

Epigenetic reprogramming consists in the transition from one cell type to another, permitted by the loss

of the molecular characteristics of the cell of origin and the acquisition of an entirely new molecular identity without changing the genomic sequence^[100]. Reprogramming involves changes in the transcriptome and chromatin state of the reprogrammed cell type to that of a pluripotent cell^[101-103]. This implicates different levels of changes in DNA factor binding, transcription and chromatin state^[103]. Since the discovery by Takahashi and Yamanaka in 2006 that the introduction of four transcription factors, *Oct3/4*, *Klf4*, *c-Myc*, *Sox2* (known as OKMS) could reprogram mouse embryonic and adult fibroblasts into induced pluripotent stem cells (iPSCs)^[104], the field of reprogramming has considerably evolved and several studies have reported the use of sets of these transcription factors in various combinations to reprogram mouse and human somatic cells^[105-108]. More recently, murine B lymphocytes, liver, stomach and pancreatic β -cells were showed to reprogram into iPSCs using the combination of factors OKMS^[109-111]. In 2008, Eminli *et al.*^[112] reported the generation of iPSCs from murine NSCs by retroviral infection of the same combination of factors. Since neurosphere cultures express *Sox2* and *c-myc*, a considerable advance consisted in showing that they could be reprogrammed only with *Oct4* and *Klf4* at similar efficiency to the reprogramming rate of murine fibroblasts with the original four factors^[112-114]. Finally, the forced expression of *Oct4* alone was shown sufficient to reprogram murine NSCs, albeit with a ten-fold lower efficiency than with two factors^[113]. Because NSCs are originally closer to the pluripotency state than somatic cells and require fewer factors to be reprogrammed, they constitute a more simple and attractive system to study epigenetic mechanisms occurring during the acquisition of pluripotency. Importantly, iPSCs derived from human and murine NSCs exhibited markers of ESCs, showed demethylation of pluripotency genes, formed teratomas, and contributed to viable chimeras^[112-114].

Reprogramming factors and epigenetic mechanisms

Reprogramming of somatic cells is a stochastic event^[115]. However, in NSCs, *Oct4* only seems sufficient to repress genes responsible for NSCs molecular identity and activate the pluripotency genes, suggesting that epigenetic of NSCs renders them easier to reprogram and that the combination of factors necessary for reprogramming is dependent on cellular context^[112]. iPSCs have lower levels of methylation than somatic cells, suggesting that demethylation is an important chromatin feature to achieve pluripotency^[116]. During reprogramming, it is stipulated that reprogramming factors interfere with methylation of the newly synthesized DNA by binding to specific promoters or enhancer regions leading to demethylation and activation of the pluripotency genes. In addition, active DNA demethylation mechanisms could be required for the reactivation of pluripotency genes^[117]. Recent studies in NSCs have shown the importance of methy-

lation level in the context of reprogramming. Undifferentiated neurospheres highly express DNMT1 and contain methylated chromatin suggesting the role of methylation for the maintenance of the quiescent or undifferentiated state of NSCs^[118]. It is then probable that NSC chromatin is dynamically remodelled and that DNA methylation modification is essential for reprogramming to a pluripotent state. For instance, histone methyltransferase G9a is responsible for the downregulation of Oct4 during NSC differentiation and its inhibition results in iPSC formation after overexpression of exogenous Klf4 and c-myc only^[119]. In addition, interference with DNMT1 promotes iPSC formation, also supporting that DNA methylation is a feature limiting reprogramming to pluripotency^[101]. All reprogramming techniques involve demethylation of the genome thus appearing as a crucial process for successfully achieving pluripotency^[120,121].

Loss of epigenetic memory

During reprogramming, NSCs downregulate specific genes like *Nestin* and progressively express the markers of pluripotency *Oct4*, *Nanog*, *Fgf4*, *Zfp42*^[113,114]. In addition, efficiency and timing of reprogramming highly depends on the differentiation state of the initial cell type. Importantly, comparative studies with ESCs reported that efficiently reprogrammed iPSCs show transcriptional pattern and epigenetic marks highly similar to ESCs. For instance, Oct4 and Nanog promoters are demethylated and histones H3 lysine 4 (K4) and lysine 27 mostly exhibit patterns of trimethylation^[101,106,122]. However, reprogramming of NSCs into iPSCs is often incomplete and leaves epigenetic marks including DNA methylation, chromatin modification and transcriptional regulation in the resulting iPSC genome^[123,124] known as epigenetic "memory". Partially reprogrammed cell lines are characterized by an absence of complete downregulation of the exogenous reprogramming factors and partial demethylation and reactivation of pluripotency genes^[101,104]. During reprogramming, somatic markers get progressively downregulated demonstrating the importance of silencing its differentiation program as a step towards pluripotency. Treatment of partially reprogrammed iPSCs with inhibitors of ERK1/2 and GSK3b signaling^[125], induced genome demethylation of 30% explained by decreased levels of DNMT3a/b and their targeting factor DNMT3L^[126-128]. The two inhibitors repress DNMT3A/B expression inducing demethylation of certain genomic regions in ESCs. Thus, DNA demethylation of the reprogrammed cell type as a way to remove epigenetic marks is important for complete reprogramming into iPSCs. Reprogrammed iPSCs often present the limitation of not being fully reprogrammed thus keeping epigenetic traces of the tissue of origin. Future generation of iPSCs without epigenetic memory is an important challenge in the field to ensure that differentiation decisions are not affected by events from the past^[116].

FUTURE PERSPECTIVES

Determining the mechanisms by which neural stem cells maintain self-renewal capacity and at the same time generate differentiated progeny is a central challenge in stem cell biology. Several recent studies have demonstrated that epigenetic gene regulation plays a crucial role in the control of stem cell behaviour. Epigenetic mechanisms include changes in chromatin structure that provides a way for coordinately activating or repressing genes during proliferation and differentiation. Extracellular signaling from the micro-environment or niche in which NSCs reside *in vivo* interacts with these diverse epigenetic mechanisms, thus regulating transcription factors and intracellular pathways. These changes in gene expression are often heritable and reversible, features that support stem cell plasticity such as the ability to dedifferentiate or become reprogrammed under certain conditions. Finally, aberrant epigenetic mechanisms are known to be involved in the development of many neurological diseases. Characterizing epigenetic changes associated with a particular neural pathology may be used as biomarkers of disease and the manipulation of those epigenetic mechanisms holds great promise as a potential therapeutic strategy.

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Epidermal stem cells and skin tissue engineering in hair follicle regeneration

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pending in tissue engineering. The loss of hair follicles caused by injuries or pathologies such as alopecia not only affects the patients' psychological well-being, but also endangers certain inherent functions of the skin. It is then of great interest to find different strategies aiming to regenerate or neogenerate the hair follicle under conditions proper of an adult individual. Based upon current knowledge on the epithelial and dermal cells and their interactions during the embryonic hair generation and adult hair cycling, many researchers have tried to obtain mature hair follicles using different strategies and approaches depending on the causes of hair loss. This review summarizes current advances in the different experimental strategies to regenerate or neogenerate hair follicles, with emphasis on those involving neogenesis of hair follicles in adult individuals using isolated cells and tissue engineering. Most of these experiments were performed using rodent cells, particularly from embryonic or newborn origin. However, no successful strategy to generate human hair follicles from adult cells has yet been reported. This review identifies several issues that should be considered to achieve this objective. Perhaps the most important challenge is to provide three-dimensional culture conditions mimicking the structure of living tissue. Improving culture conditions that allow the expansion of specific cells while protecting their inductive properties, as well as methods for selecting populations of epithelial stem cells, should give us the necessary tools to overcome the difficulties that constrain human hair follicle neogenesis. An analysis of patent trends shows that the number of patent applications aimed at hair follicle regeneration and neogenesis has been increasing during the last decade. This field is attractive not only to academic researchers but also to the companies that own almost half of the patents in this field.

Key words: Adult stem cells; Skin grafts; Epidermis; Multipotential differentiation; Tissue regeneration; Dermal papilla; Epithelial-mesenchymal interactions

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Abstract

The reconstitution of a fully organized and functional hair follicle from dissociated cells propagated under defined tissue culture conditions is a challenge still

Core tip: Loss of hair follicles caused by injuries or pathologies affects the patients' psychological well-being and endangers inherent functions of the skin. Different experimental strategies and approaches to obtain mature hair follicles have been designed based upon current knowledge of the epithelial and dermal cells involved in embryonic hair generation and adult hair cycling, and in the epithelial-mesenchymal interactions among them. This review summarizes the current advances in hair follicle neogenesis and regeneration, with emphasis on those involving neogenesis of hair follicles in adults from isolated cells and tissue engineering as well as an analysis on patent trends in this field.

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INTRODUCTION

Regenerative medicine aims to create living, functional tissues that repair or replace lost or damaged organ function resulting from disease, injury, congenital defects or aging.

The main challenge of tissue engineering is the reconstitution of fully organized and functional organ systems from dissociated cells that have been propagated under defined tissue culture conditions.

Skin is the largest organ in the human body, acting as a barrier with protective, immunologic and sensorial functions. Deep skin injuries produce a complete destruction of skin regenerative elements. These wounds heal by contraction, with epithelization from the edges only and extensive scarring, resulting in reduced joint movements and cosmetic defects^[1]. Moreover, if these lesions are too extensive, the healing process is unsuccessful and they become life-threatening for the patient. Tissue engineering has emerged as a new interdisciplinary field combining scaffolds, cells and biomolecular signals towards the treatment of skin lesions. This useful strategy may contribute not only to the treatment of deep skin injuries but also to the understanding of skin regeneration.

The main goal of tissue-engineered skin grafts is to restore lost barrier function. Many dermal-epidermal composites or skin equivalents have been described for use in the clinic^[2] but the inability of these skin constructs to regenerate skin appendages as hair follicles has limited their use. Although the ability to reconstitute adult skin with functional skin appendages has long been a major clinical objective, the regeneration of epidermal appendages, such as hair follicles, and sebaceous and sweat glands, is a challenge that is still pending.

Recent studies clearly demonstrate that there are

multipotent stem cells with the capability to regenerate hair follicles and sebaceous glands in adult mammalian skin and this multipotency can be maintained in cell culture^[3,4]. The hair-differentiation potentiality of epidermal stem cells can be activated by inductive dermal cells. As will be extensively reviewed in this article, co-grafting of those cellular components from mice allows complete hair reconstitution^[5]. The successful regeneration of hair follicles in immunodeficient mice suggests that creating human hair follicles in tissue-engineered skin grafts is feasible. However, regeneration of human hair in a similar manner has not been reported.

Skin appendages cannot be restored in healed wounds by current tissue-engineered skin grafts^[6] mainly because of their limited self-regeneration capability in adults^[7], the lack of appendage structures in the human skin grafts and probably an improper molecular microenvironment. Many strategies still need to be explored, particularly enriching isolated cells with trichogenic capability, maintaining this capability during processing, and providing the cells with proper environmental signals.

The lack of skin appendages by skin wounds and by pathologies such as different kinds of alopecia not only affects the patients' psychological well-being^[8], but also endangers the inherent functions of the skin.

Skin appendages cannot regenerate on their own after damage^[7]. For the fully functional regeneration of ectodermal organs, it has been proposed that a bioengineered organ could be developed by reproducing the embryonic processes of organogenesis.

This review summarizes current advances in the different experimental strategies to regenerate or neogenerate hair follicles, *in vitro* and *in vivo*, with emphasis on approaches that include the neogenesis of hair follicles in adults from isolated cells and by tissue engineering as well as an analysis on patent trends in this field.

ANATOMY AND BIOLOGY OF HAIR FOLLICLES

The skin consists of three layers: epidermis, dermis, and hypodermis. The epidermis is in fact a multi-layered epithelium extending from the basement membrane that separates it from the dermis to the air. The dermis is located below the epidermis and is a connective tissue comprised of extracellular matrix, fibroblasts, vascular endothelial cells, and skin appendages.

Skin appendages, including hair follicles, sebaceous glands and sweat glands, are linked to the epidermis but project deep into the dermal layer. A human has around five million hair follicles with two types of hair, terminal hair (long, thick pigmented) and vellus hair (thin, unpigmented)^[9].

Hair follicles are composed of an outer root sheath

(ORS) that is contiguous with the epidermis, an inner root sheath (IRS) and the hair shaft (HS). The matrix surrounding the dermal papilla (DP), in the hair root, contains actively dividing and relatively undifferentiated cells that are essential for follicle formation.

In embryogenesis, the skin begins as a single layer of epidermal stem cells. Then, mesenchymal cells populate the skin to form the underlying collagenous dermis^[10]. Human hair follicles start to develop through complex morphogenetic processes resulting from highly coordinated series of bidirectional epithelial-mesenchymal interactions^[11]. Hair follicle development is initiated by the appearance of a thickening in the embryonic ectoderm called placode resulting from the condensation of the underlying mesoderm that will form the DP^[12]. The DP becomes a permanent part of the follicle base^[13] enveloped by the hair bulb. It is considered the commander of the hair follicle determining the hair thickness, length, and life cycle^[8]. Signals from the condensed mesoderm induce the proliferation of the placode which forms mature hair follicles by a systematic series of differentiation and proliferation processes of epithelial cells. The hair follicle becomes fully mature when its bulb nears the bottom of the dermis. At this point, the proliferative cells (matrix) at the follicle base continue to divide, producing progeny cells that terminally differentiate to form the growing hair that emerges from the skin surface. The inner layers begin to differentiate into concentric cylinders to form the central HS and the surrounding channel, the IRS.

These tissues undergo continual rejuvenation and, in response to injury, they must be prepared for wound repair. The capability of the skin for maintaining tissue homeostasis, regenerating hair, and repairing the epidermis after injury resides in its stem cells.

HAIR FOLLICLE STEM CELLS AND THE HAIR CYCLE

Stem cells are undifferentiated cells that are ultimately responsible for the constant renewal of the skin due to their distinguishing properties of self-renewal for the entire life span of an organism and their ability to differentiate into a variety of specialized cells.

A pool of progenitor cells is located on the basement membrane of the skin. These cells contribute to epidermal homeostasis undergoing continuous self-renewal and differentiation to keratinocytes that migrate towards the surface of the skin where they undergo terminal differentiation and maturation providing the skin's barrier properties.

Another reservoir of slow-cycling multipotent stem cells that gives rise to a range of differentiated cell types in skin is located in a specialized region of the ORS in the hair follicle, known as the bulge^[3,4,14-16]. Early work showed that these slow cycling multipotent stem cells not only contribute to the growth of hair

follicles themselves and of the sebaceous glands^[3] but also can be activated and migrate out of hair follicles in order to repair the damaged epithelium^[17]; however, they contribute little to the turnover of the intact epidermis. In the absence of these hair follicle stem cells (HFSC), hair follicle and sebaceous gland morphogenesis is blocked, and epidermal wound repair is compromised^[18]. The epithelial-mesenchymal interactions with the underlying DP play a pivotal role in embryonic hair genesis^[12], in the regulation of post-natal hair follicle cyclical activity, and in the repair of wounded skin^[19-22].

Unlike most organs, hair follicles do not reach homeostasis once they mature. Each mature hair follicle is a regenerating system, which physiologically undergoes cycles of growth (anagen), regression (catagen), and rest (telogen) numerous times in adult life^[23]. In catagen, hair follicle stem cells are maintained in the bulge. Then, the resting follicle re-enters anagen (regeneration) when proper molecular signals are provided. During late telogen to early anagen transition, signals from the DP stimulate the hair germ and quiescent bulge stem cells to become activated^[24]. Many paracrine factors are involved in this crosstalk at different hair cycle stages and some signaling pathways have been implicated^[3,19,25]. In anagen, stem cells in the bulge give rise to hair germs, then the transient amplifying cells in the matrix of the new follicle proliferate rapidly to form a new hair filament^[26].

Even if it is generally believed that hair follicles do not form after birth in humans^[27], the formation of vellus hair follicles from the reconstituted epidermis of an abraded area of facial skin was described in 1956^[28]. However, the great challenge of skin tissue engineering and skin wound regeneration remains to be its inability to reliably reconstitute skin appendages, most notably hair follicles and sweat glands. The information about the mechanisms that generate and maintain skin appendages provided by recent studies with skin-derived progenitor cells, may be the basis of new therapies that could help to overcome these limitations.

CAUSES OF HAIR LOSS AND REGENERATION OF THE HAIR FOLLICLE

Hair loss responds to different causes ranging from mild traumas, such as hair fiber plucking, to severe traumas such as partial- or full-thickness skin lesions. Pathological processes, such as cicatricial or non cicatricial alopecia, can also be responsible for hair loss. After plucking, if HFSC and DP remain, a new hair filament would be spontaneously regenerated. Some label retaining studies have shown that hair follicle stem cells in the bulge remain intact after hair plucking^[29,30] whereas others reported bulge stem cells suffering apoptosis after plucking which are replaced by label retaining cells coming from hair germ^[31].

It was also observed that the slow-cycling bulge cells (CD34 positive cells in mouse) are involved in normal hair homeostasis and wound healing, whereas regeneration after hair plucking involved actively cycling cells from the lower ORS^[26]. However, the cell dynamics in this process is less clear than in the physiological renewal and further studies are required to understand this process.

However, when the cellular niches are completely lost, it is necessary to generate a completely new hair follicle in a process called hair follicle neogenesis.

Based on the knowledge on the epithelial and dermal cells, and their interactions, during the embryonic hair generation and adult hair cycling, different experimental approaches have been designed to regenerate hair follicles or generate new ones by the neogenesis process.

These hair regeneration and neogenesis attempts are recapitulated in this article, and can be classified into 4 categories: (1) reversion of pathological intra- and/or extra-follicular environment, for instance androgenetic alopecia (AGA); (2) regeneration of complete hair follicles from the recombination of hair follicle parts; (3) neogenesis of hair follicles from isolated cells; and (4) neogenesis of hair follicles by tissue engineering.

Reversion of pathological intra- and/or extra-follicular environment

Hair follicle cycle and growth can be affected and deregulated by paracrine factors from the follicle itself and/or from the surrounding dermal tissue, or by endocrine factors^[32,33] leading to hair loss. Many factors have been described as molecular mediators of hair follicle growth^[11]. Some of them, such as PDGF^[33] and Wnt proteins^[34], are positive regulators while others, such as BMPs^[35], suppress hair growth. Many paracrine factors involved in the epithelial-mesenchymal crosstalk at different stages of the hair cycle, and some implicated signaling pathways, have also been studied^[3,19,25].

In addition, endocrine factors, such as sexual hormones, have both physiological and pathological effects on hair growth as in human male beard, and androgenetic alopecia respectively. Androgenetic alopecia^[36] is a common, progressive disorder in which large, terminal scalp hairs are gradually replaced by smaller hairs following a defined pattern. The DP is the site through which androgens act on follicle cells by altering the regulatory paracrine factors involved in the differentiation and proliferation of HF stem cells. DPs from balding scalp have higher levels of androgen receptor and 5- α reductase. Dihydrotestosterone can induce DP to secrete factors including, TGF- β 1 and DKK-1 which down regulate keratinocytes growth^[37,38].

A functional crosstalk between the androgen receptor and Wnt signaling pathways in DPCs has been described in target tissues^[39]. Moreover, our group has demonstrated that androgens regulate secreted factors involved in normal HF stem cell differentiation via the

inhibition of the canonical Wnt signaling system in androgen sensitive DPCs. We provided evidence that androgen activation of GSK-3 β would be responsible for the inhibition of Wnt/ β -catenin signaling^[40]. The current pharmacological treatment for AGA includes androgen metabolism modulators, such as the 5- α reductase inhibitor finasteride and the antiandrogen agent flutamide. Nevertheless, the results of these treatments are not only variable and patient-dependent, but can also cause undesirable side effects. Therefore, therapies acting on specific molecular targets are necessary to treat this pathology downstream from the actual antiandrogenetic pharmaceutical modulators. The crosstalk between the androgen receptor (AR) and Wnt/ β -catenin signaling pathways could be one alternative therapeutic target, among many others recently reviewed^[41].

In these experimental approaches, modulation of the intra-follicular microenvironment and the extra-follicular macroenvironment could contribute to promote growth of the existing hair follicles but not to increase their number by the formation of new ones.

Regeneration of hair follicles by recombination of hair-follicle parts

Taking into account the inductive ability of DP and the current knowledge on epithelial-mesenchymal interactions, different recombination of dermal and epidermal tissue have been employed for hair follicle regeneration. In the early 1960s, researchers showed that whole follicle end bulbs and isolated whole whisker papillae (containing dermal and epidermal components) remain viable and may produce whisker hair follicles in a new site^[42]. The most surprising finding was that isolated DPs (without epidermal components) were capable of inducing the generation of new hair follicles recruiting epithelial cells from the implantation sites. The same results were obtained when epidermis from different origins were transplanted together with whisker DP^[43]. Regeneration of hair follicles was also observed in both mice^[43-45] and humans^[46] when dermal sheath tissue was used, which was sufficient to regenerate also the DP structure. After implantation, the whisker DP was capable of inducing hair follicle regeneration retaining the information to determine hair fiber type and follicle size^[47].

Neogenesis of hair follicles in adult individuals from isolated cells

Grafting of dermal-inductive tissue is limited by the fact that it is not possible to generate more hair follicles than the obtained from the donor tissues. To overcome this limitation different approaches and experimental models using freshly or cultured isolated cells from both dermal and dermal/epidermal origin were tested. Most of them involved neonatal and embryonic murine cells. One of the first models used was the chamber assay (Figure 1) in which dermal and epithelial cells are seeded inside a chamber consisting of a cylinder

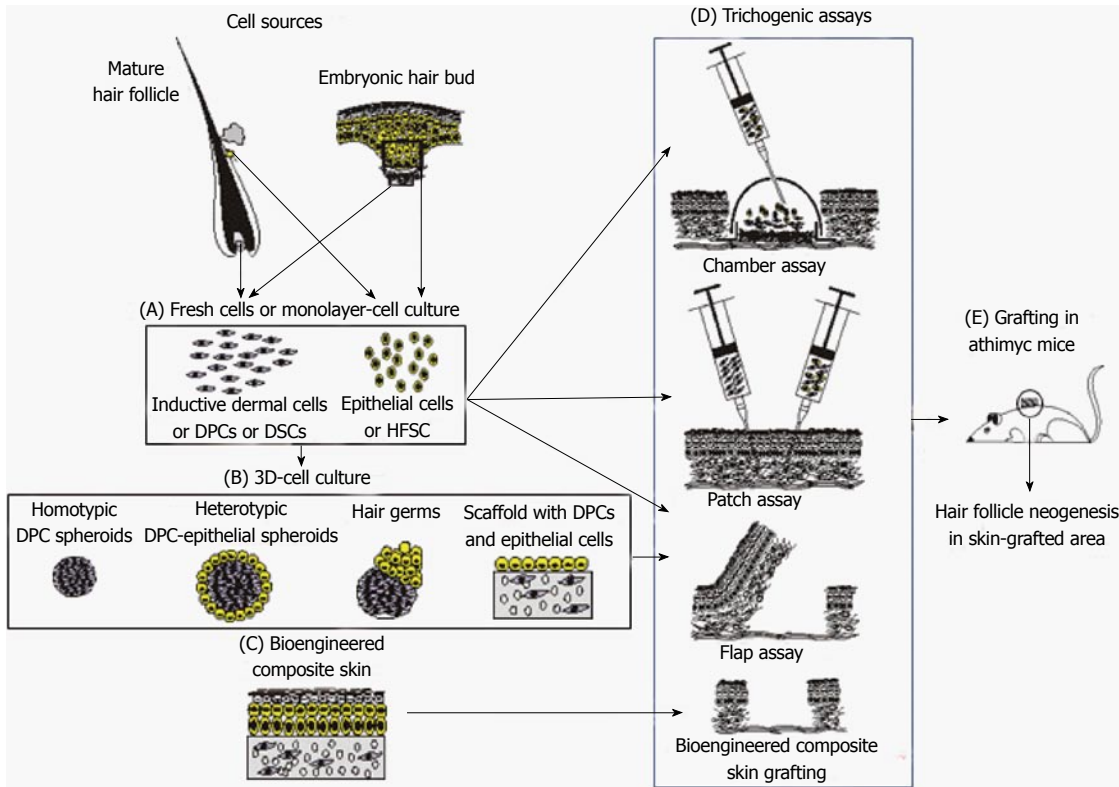


Figure 1 Hair follicle neogenesis using isolated epithelial and dermal cells. Inductive-dermal and Epithelial Cells can be obtained from adult tissue or embryonic sources. Isolated cells by enzymatic digestion of living tissues, or monolayer cell cultures (A) can be used in the trichogenic assays (D): Chamber assay; Patch assay; and flap assay. Moreover, isolated cells can be cultured in 3D-conditions, allowing them to create their own extracellular matrix as in the case of homotypic and heterotypic spheroids and hair germs (B). Also, cells can be seeded on a precast scaffold used for cellular support or used to produce a bioengineered composite skin (C). Any of these constructs can be evaluated by the trichogenic assays (D): Flap assay; and Bioengineered composite skin grafting. All these trichogenic assays are mainly performed in the back-skin of athymic mice in order to avoid immune rejections and to take advantage of its bare-skin (E). DPCs: Dermal papilla cells; DSCs: Dermal sheath cells; HFSC: Hair follicle stem cells.

inserted through a full-thickness skin lesion in mouse dorsal skin, covered by a dome. In former studies using this device in nude mice^[48], it was showed that hair buds obtained from neonatal mice combined with fresh neonatal dermal cells or with immortalized clones from vibrissae rat DPCs produced mature and cycling hair follicles. Using the same chamber assay, hair bud preparations without any additional inductive dermal component were unable to form hair follicles resulting in a scarring reparative tissue, a thin epidermis and a dermis without appendages^[49]. On the other hand, the combination of hair buds with fresh dermal cell preparations or early passages of cloned vibrissae cell lines resulted in skin with normal epidermal and dermal layers.

Although these studies confirmed the trichogenic potential of dissociated dermal cells, particularly DPCs, the conditions of the chamber assay correspond to a wound healing environment and do not represent the physiological status. Other approaches allow studying the trichogenic potential of isolated cells in a more physiological environment, as is the case of the patch assay (Figure 1). This assay consists in injecting dissociated murine neonatal epidermal and dermal cells hypodermically into adult mouse skin. This system, originally described by Zheng

et al^[50], showed the formation of cell clusters that resulted in "infundibular cysts" which generated hair germs, followed by pegs that grew to differentiate into cycling mature pilosebaceous units. Surprisingly, whereas patch hair follicles were in anagen phase, the host skin hair follicles were in telogen and vice versa, suggesting that follicle cycling depends on the trichogenic cells and not on the host skin hair cycling. Even when the shaft grows inside an intradermal cyst that tends to extinction by inflammatory processes, none of the other already described systems at that moment showed to be so efficient to evaluate the trichogenic potential of isolated cells. A variation of this method is called tracheal grafting method. It consists in inoculating rat tracheas with fresh or cultured keratinocytes from newborn mice together with newborn fibroblasts or cultured DPCs and implanting them subcutaneously in athymic mice for four weeks. In this assay hair follicles and sebaceous glands were generated using cultured keratinocytes from newborn mice and fresh newborn fibroblasts^[51]. Although adult mice keratinocytes successfully produced a two layered epithelium, they failed to generate hair follicles or sebaceous glands^[51].

Other strategy to evaluate the hair inductive capability of dermal cells is the "flap assay" (Figure 1). This

approach originally described by Qiao *et al.*^[52] consists in: (1) generating a skin flap in the dorsal skin of nude mice; (2) locating an embryonic epidermis supported by a silicone sheet in direct contact with the muscle with the basal side of the embryonic epidermis facing upwards; (3) seeding embryonic murine dermal cells or cultured DPCs onto the basal face of the graft-epidermis; and (4) pulling over the graft and suturing the flap edges. In this configuration the graft-dermal cells were layered between the graft-epidermis and the host connective tissue. When fresh embryonic dermal cells were used, abundant hair was developed four weeks after grafting. The induced hairs were oriented vertically down towards the silicone sheet. After surgical flap inversion, the formed hairs were exposed in the host skin surface and survived for more than 12 mo.

Neogenesis of hair follicles by tissue engineering

Although the assays described above provided much information respect to the hair neogenesis process and the cells involved in it, the high number of cells required, the need of *in vivo* animal models and the poor results obtained with human cellular sources make these procedures not applicable for clinical uses in the near future. The development of new strategies has become necessary and begun to emerge. They are mainly focused on tissue engineering-based follicle neogenesis, including three-dimensional (3D) cell culture conditions generated by the cells themselves or by the use of biocompatible scaffolds.

Lee *et al.*^[53] developed a simplified procedure to reconstitute hair-producing skin. They obtained both epidermal and dermal cells from newborn mice, and mixed them in different ratios. A high density cellular suspension was prepared in drops of minimal volume on tissue culture inserts or wells. They allowed the cells to settle until gel consistency was obtained. Alternatively, they seeded the cells on the collagen-side of the IntegraTM matrix (porous matrix of cross-linked bovine tendon collagen and glycosaminoglycan and a silicone layer). Both constructs were grafted in full thickness skin wounds generated on the back of athymic mice. After grafting, the epidermal cells formed a basal layer and some epidermal microcysts could be observed. Dermal cells started to form dermal condensations adjacent to the epidermal layer or cysts. Eight days after grafting hair germ started to appear and progressed to hair peg, observing complete hair follicles four days later that kept the ability to continuously cycle for at least one year. This method would allow preparing constructs with specific sizes and shapes useful to treat alopecia by tissue implants.

Two separate reports^[54,55] showed hair follicle neogenesis using hair germs obtained by the previously described "organ germ method"^[56]. One of them^[54] generated bioengineered hair germs mixing epithelial and mesenchymal cells derived from mouse embryos within a collagen gel drop. In these culture conditions, the two cellular types generated a structure with

two cellular layers separated by a translucent region they called hair germ. When these hair germs were transplanted ectopically into subrenal capsules, the presence of mature hair follicles was observed. These bioengineered hair follicles had a normal histological structure with the concentric epithelial layers of ORS and IRS, DP, hair matrix, and sebaceous glands. These follicular units transplanted intracutaneously in nude mice (Figure 1), were connected to the arrector pili muscle and nerve fibers and were able to produce hair shafts and cycling.

In addition, a fully functional orthotopic hair regeneration was demonstrated via intracutaneous transplantation of bioengineered hair follicle germs generated by epithelial and mesenchymal cells derived from mouse embryos^[55] (Figure 1). These bioengineered hairs responded to hair cycles and had the correct structure of natural hair follicles and shafts, and the proper connections with surrounding host tissues. Qiao *et al.*^[57], using embryonic dermal cells and hair-peg-derived keratinocytes, generated cellular aggregates by hanging drop methods. These aggregates cultured in methylcellulose coated wells elongated, and after seven days, acquired hair follicle features. These structures termed "proto hairs", showed a morphological structure more developed respect the previously described "hair germs"^[54,55]. They presented hair-like characteristics as an inner mass of cells similar to the DP structure surrounded by matrix-like keratinocytes and a partially keratinized substance that could represent intent to produce a hair shaft. After implantation into shallow incisions in nude mice (Figure 1), black-pigmented hair fibers were observed emerging from epidermis. These hairs persisted throughout the experimental period (6 mo) and were shown to regrow after plucking.

Although embryonic tissues are used as cellular sources, these studies showed the potentiality of dissociated cells to form hair follicle germs or proto-hairs able to generate mature hair follicles both by ectopic and orthotopic implantation. Collectively, these studies provide a proof of concept for the use of Follicular Cell Implantation to restore hair in patients suffering from hair loss.

As it was already mentioned, dermal cellular components from the skin, such as DPCs or dermal sheath cells (DSCs), have the potential to induce hair formation in *in vivo* assays and also induce *in vitro* differentiation of hair follicle stem cells to hair lineage^[25,40]. Even if it is possible to isolate and culture DPCs, the inductive ability tends to fade with time in 2D cultures^[6,58]. Considering that the hair follicle neogenesis requires a large number of DPCs, it is necessary to look for new strategies to maintain their inductive potential in culture in order to use them in skin bioengineering and hair follicle neogenesis.

The niche generated by the extracellular matrix (ECM) of DP probably plays a key role in keeping DPC inductive activity. Accordingly, the loss of ECM throughout culture time could be responsible for the

fading of DPC inductive properties.

DPCs are naturally aggregated at the hair-bulb base. In proper culture conditions, these cells tend to aggregate^[13,59] and their inductive activity is preserved after they are grafted in this state. It has been demonstrated that it is possible to generate dermal papilla spheroidal microtissues using different methods including rotation, two-step rotation and flotation, and hanging drop^[60-62]. Osada *et al.*^[63] artificially prepared DPC spheres by aggregation of mouse vibrissae follicle DPCs in a round-bottom 96-well low-binding plate. These spheres expressed higher amounts of versican, an indicator of inductive capability, than DPCs in monolayer cultures. In a "patch assay" (Figure 1), together with embryonic-epidermal cells, they induced hair follicle neogenesis, keeping their inductive capability for at least twenty-six passages.

However, these methods are labor intensive and the microtissues obtained have a very variable size and cell number content, which reduces reproducibility. DPCs can self-assemble into dense spheroids when seeded on controlled biomaterial surfaces such as poly (ethylene-co-vinyl alcohol) (EVAL)^[64] or polyvinyl alcohol (PVA) membranes^[65]. The DPC microtissues generated on EVAL surface^[64] expressed DPC-markers such as neural cell adhesion molecule (NCAM)^[66] and α -smooth muscle actin^[67]. Alkaline phosphatase activity, which correlates with hair follicle inductive ability of DPC^[68], was higher in spheroid microtissues than in monolayer cultured cells. Finally, the spheroids mixed with newborn mouse epidermal cells and injected into the hypodermis of nude mice in a "patch assay" (Figure 1) were able to generate new hair follicles.

The use of PVA as substratum material enhanced DPC aggregation^[65], preventing cellular attachment and spreading. Using this material and reducing culture surface it is possible to control the number, size and compaction of spheroidal microtissues. DPCs quickly aggregated into a single spheroid whose diameter decreased progressively due to tissue compaction. The expression of DPC-inductive capability markers, versican^[69] and alkaline phosphatase^[68], were present in spheroids of various sizes. All these experiments were made both with adult DPCs from rat vibrissae, and human scalp hairs, giving similar results. The hypodermal injection in nude mice of eighty spheroids altogether with keratinocytes from newborn mice induced HF neogenesis. These results are very promising given that transplantation of dispersed cultured human DPCs had shown very low efficiency in inducing new hair follicles^[70]. Using the same approach, heterotypic folliculoid microtissues or hair follicle-like organ germs were produced using dissociated adult epithelial and mesenchymal cells by self-assembly on EVAL coated wells^[71]. Keratinocytes and DPCs seeded together grew into floating or loosely attached multicellular spheroids. Histological analysis showed that the spheroids are composed by

a core of DPCs surrounded by keratinocytes. These keratinocytes expressed keratin 6, a cytokeratin preferentially expressed by ORS *in vivo*^[72], and DPCs expressed versican, a marker associated to HF-inductive ability. Using "patch assays" (Figure 1) disperse DPCs and keratinocytes did not form new hair follicles, whereas heterotypic folliculoid microtissues did. These last experiments would contribute to the development of new strategies for scalable production of organoid microtissues of epithelial organs for hair follicle neogenesis by bioengineering approaches.

Altogether, these results strongly indicate that the hair-inductive capability of DPCs can be restored by three-dimensional spheroid cultures.

Hair follicle neogenesis using human cells

During embryogenesis, dermal mesenchymal cells drive HF development and a condensate of these cells remains during adult life forming hair follicle DP. These cells retain the capability to induce hair follicle regeneration and neogenesis^[21,73] as reviewed by Ohyama *et al.*^[5] and Yang *et al.*^[6]. However, most of these experiments were performed using murine and embryonic cells, whereas DPC from human origin showed many difficulties to maintain inductive capability in culture^[74].

As discussed above, rat cultured DPCs self-aggregate within the dermis forming a papilla-like structure that synthesizes its own extracellular matrix^[13]. However, such behavior was not observed with cultured human DPCs, highlighting specie-specific differences. Human keratinocytes from ORS and DPCs cultured in Matrigel™ (3D network of extracellular matrix proteins and collagen) have shown to form tubule-like structures in this skin-equivalent *in vitro*^[75] and organize themselves into epidermoid cyst-like spheroids^[72,76], but did not form complete hair follicles. Similarly, Sriwiriyanont *et al.*^[77], observed neofollicle formation in nude mice grafted with engineered skin substitutes containing murine DPC and human keratinocytes in a collagen-glycosaminoglycan matrix, but not in those containing human DPC and human keratinocytes.

Ehama *et al.*^[78] reported the formation of hair follicle-like structures using human primary cultures of foreskin- or adult-derived epidermal cells co-grafted with murine DPCs by a chamber assay (Figure 1). The innermost regions were similar to the hair cortex and medulla of mature human follicles. Hair shaft-like fibers occasionally emerged at the skin surface, and structures, reminiscent of the DP, at the follicle bottom were also observed. However, these structures showed no bulge region, nor all the follicular epithelial layers, and the DP-like tissue failed to express the anagen DP marker versican. These hair follicle-like structures correspond to the initial follicle formation suggesting that the differentiation process was altered.

Using the "flap assay"^[52] (Figure 1), Qiao *et al.*^[79] showed that DPCs from human scalp combined with epidermis from mouse embryo produced mature

hair follicles that persisted and grew. The authors reported that the inductive potential of DPCs could be maintained using keratinocyte-conditioned medium. These DPCs were able to form DP and dermal sheath *in vivo*.

Spheroids microtissues were also obtaining by culturing human DPCs in a 96-well low-binding plate, and implanted intradermally into nude mice using the "patch assay" (Figure 1)^[80]. This combination of human DPC spheres with murine neonatal epidermal cells generated new hair follicles. In contrast, hair follicles were never observed when monolayer DPC cultures were used.

Cultured human DPCs do not induce hair neogenesis unless changes in the culture conditions are made. In this sense, the formation of DPC-microsphere tissues has shown to improve hair-inductive properties of rodent cells as well as the expression of cell-markers associated with these properties^[63-65]. Therefore, all these observations encouraged investigators to look for new strategies intended to achieve the first step toward human hair follicle neogenesis that is, providing culture conditions that confer hair-inductive ability to human DPCs. Higgins *et al.*^[74] evaluated the human DPC-transcriptome observing that monolayer DPC cultures showed the most important changes immediately after early outgrowths from DP explants. These changes involved 3729 transcripts, including several involved in hair follicle development. This large number of differentially expressed transcripts shows the physiological changes that 2D culture conditions do on the molecular signature of DPCs. The generation of DPC spheroids by the hanging drop method partially restores the intact DP signature. These human-DPC spheres induced hair follicle neogenesis when placed in between foreskin epidermis and dermis and grafted onto nude mice (Figure 1). These hair follicles showed a DP and dermal sheath highly positive for alkaline phosphatase, hair specific keratin markers disposed in concentric layers and blood vessel formation around follicles. Nevertheless, neither sebaceous glands nor hair fibers emerging from the skin surface were seen. These results suggested that human DPC spheroids would be able to initiate hair follicle morphogenesis but the production of a complete hair follicle requires additional signals.

Recently, Miao *et al.*^[81] produced DPC-spheroids from cultured DPCs on a MatrigelTM scaffold. As reported by Higgins *et al.*^[74], the DPC spheres were shown to restore DP-signature gene expression of NCAM, versican and α -smooth muscle actin (α -SMA), lost during monolayer culture. The authors also claimed that these DPC-spheres, combined with hair germinal matrix cells, onto Matrigel-coated plates produced colorless fiber-like structures *in vitro*.

Other alternative to culture dermal mesenchymal cells in 3D conditions is the organotypic method consisting in culturing cells inside a scaffold. Using this method, Wu *et al.*^[82] prepared a collagen gel with

human DPCs or dermal sheath cells (DSCs). This gel was seeded with keratinocytes from interfollicular skin, superior ORS or inferior ORS obtaining *in vitro* a bilayered skin. Nonetheless, only the constructs containing superior ORS keratinocytes showed hair follicle-like structures. That is, an outer root sheath, nearly ten layers of concentric epithelial cells, a middle layer IRS-like and some keratinoid substances in the center, similar to a hair fiber. When these constructs were transplanted into nude mice (Figure 1), only the organotypic cultured tissue containing superior ORS keratinocytes and DPCs exhibited hair follicle structures.

In a recent study^[83], composite skin substitutes were generated by seeding human neonatal foreskin keratinocytes onto a dermal equivalent structure cultured in air-liquid interface condition. This dermal equivalent was composed of DPCs from human scalp contained in a collagen-I gel. Eight weeks after grafted onto nude mice (Figure 1), these constructs presented hair follicles showing bulb, dermal sheath, hair matrix and cortex. Histological analysis showed concentric layers of IRS and ORS, sebaceous glands and hair shaft. Immuno-histochemistry assays revealed that both epithelial and dermal cells from neo-follicles were from human origin, and that DPCs and DSCs expressed human nestin and versican.

The analysis of all these reports leads to the conclusion that hair follicle neogenesis using human epithelial and dermal cells is a very difficult task that requires special culture conditions, somehow recreating the normal or embryonic skin environment, and the use of embryonic or neonatal cells.

Very recently, we prepared in our laboratory a dermal-epidermal skin substitute by seeding an acellular dermal matrix with cultured hair follicle epithelial stem cells and DPCs, both obtained from adult human scalp^[84]. These constructs were grafted into a full-thickness wound generated on nude mice skin (Figure 1). In only fourteen days, histological structures reminiscent of many different stages of embryonic hair follicle development were observed in the grafted area. These structures showed concentric cellular layers of human origin, and expressed k6hf, a keratin present in epithelial cells of the companion layer. Although the presence of fully mature hair follicles was not observed, these results represent, up to our knowledge, the first report showing that both epithelial and dermal cultured cells from adult human scalp in a dermal scaffold were able to produce *in vivo* structures that recapitulate embryonic hair development.

ANALYSIS OF PATENTS TRENDS IN HAIR FOLLICLE REGENERATION AND NEOGENESIS

So far, we have made a concise review of the scientific

literature describing the attempts to regenerate the hair follicle. We have also mentioned a number of reviews on academic articles dealing with hair regeneration or wound induced follicle neogenesis that have been published in recent years^[85]. However, reviews on tissue engineering and hair regeneration patents are less common. Many of these strategies have been combined in different methods intended for use in active applications. In this section, we review and analyze published patents on hair regeneration^[86-150], as a measure of the interest in the industry for this area of research, identifying the currently available technical developments, favorite research strategies and main points of interest. Parameters analyzed include not only chronological patent publishing trends, but also the most cited patents, top patent owners (assignees), most cited documents, and the classification of patents among technology areas according to the International Patent Classification (IPC). Moreover, hair regeneration applications that have been patented throughout the years are screened (Figure 2).

LITERATURE STUDY

Patent search was performed using the Thomson Innovation Database, a collaboration platform for searching and analyzing global patents, integrated with analytics and workflow tools which allow access to more than 40 databases from different countries. For each particular group of hair regeneration, specific keywords (descriptors) were selected. Descriptors were chosen based on the synergy of two approaches: the search of specific keywords and concepts used in academic publications, and the selection of additional descriptors from specific patent vocabulary. Therefore, the keywords are: Stem cell; progenitor cell; precursor cell; repair; growth; wound; regeneration; neogenesis; skin; tissue engineering; dermis; epidermis; dermal; hair follicle; sebaceous gland and sebaceous unit. The presence of the chosen descriptors was checked in the title, abstract and claims of existing patents, considering documents published between years 1990 and 2014. More than forty technology patent and patent application databases were used for patent search, including the Spanish OEPM, the American USPTO, the worldwide WIPO, the European EPO, the patent offices of France, Germany, Great Britain and the Far-Eastern patent offices of Japan, China and South Korea. Besides the keywords, the search process was completed with the use of the IPC codes under which the patents of interest could be framed. The combination of the search approaches previously mentioned offered a universe of about 475 patent families, which were manually reviewed and filtered, setting aside those directed to gene therapies, mesenchymal, embryonic stem cell, osteoblast, neuron and hematopoietic cell and others. The result of this process was the identification of 65 patent families^[86-150]. The selection of the document to be considered in patent trends was the first published.

It is important to note that patent applications can be published in different countries under different national numbers. This is called the family of the patent. In the search performed for the present study, care was taken to include just one patent per family.

Tables showing patents that are most frequently cited take into account all the patents in the family, since it may occur that the earliest document is not necessarily the most cited one.

Competitive dynamics

The competitive dynamics indicator shows the distribution of patents among the different organizations, providing a general picture of the tissue repair regeneration industry and their relative positioning. On the basis of this information we can identify the most prolific applicants for hair regeneration technology patents in the world, by establishing the number of applications filed by each company.

Table 1 lists the applicants that have filed more than one application, which will be our "Reference Group". Most of the members of this group are manufacturing companies, indicating that this technology belongs to the industrial sector. The highest percentile is shared by a small number of applicants with five patents each, while a second group includes a wide range of companies with no clear technological leadership.

The group of companies of the first percentile shown in Table 1, are the leading hair regeneration manufacturers Shiseido Co Ltd and Aderans Research Institute, which own more than five published patents each, and which own almost 20% of all filed applications in this field.

The second percentile of institutions having one or two applications each could offer a favorable scenario for technology transfer.

It should be highlighted that there are over 20 institutions or about 40% patent applications from non-profit institutions including universities and foundations. The most prolific institutions are: Jilin University, University of Pennsylvania, University of Southern California and National Taiwan University, which gives an idea that this field is very prolific in academic institutions.

Technology trends

The patent technology trends indicator introduces the serial number of patent applications showing how technology has evolved over time as well as its appropriation by companies and non-profit institutions in the field of hair regeneration. This technological area has experienced constant growth. In the 90's an average of 2.5 patent applications were filed per year, and in the first decade of the 21st century the average increased to 6 patent applications per year (Figure 2).

The years 2011 and 2012 stand out as the ones with the most patent applications, 10 and 16 respectively.

Table 1 Owners of hair regeneration patents and patent applications matching all the descriptors found in academic literature

Assignee	No of patent applications	Percentage
Shiseido Co Ltd	5	7.69%
Aderans Research Institute Inc	5	7.69%
Hospital for Sick Children	2	3.08%
Biointegrence Inc, phoenixbio Co Ltd	2	3.08%
Follica Inc	2	3.08%
Organ technologies Inc	2	3.08%
University of Jilin	2	3.08%
University of Southern California	2	3.08%
National University of Taiwan	2	3.08%
Aderans Research Institute Inc, Bioamide Inc	1	1.54%
Agency for science, technology and research	1	1.54%
Alvi Armani Genomics Inc	1	1.54%
Anticancer Inc, Li L, Yang M	1	1.54%
Beijing Yonghe Hair Transplant Technology	1	1.54%
Biomaster KK	1	1.54%
Chen B, Gao Q, Zeng Q	1	1.54%
Chinese Academy of Science Institute of Zoology	1	1.54%
Total No of patent applications	65	100.00%
No of Entities	50	

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Aderans Corp Company filed most patent applications from 2005 to 2008. Shiseido and Follica, as well as the University of Pennsylvania have filed patent applications in recent years.

From this general perspective, it can be inferred that the technological field is, at present time, in a developmental stage, with an annual growth rate of 10%, based on the number of patent documents filed since 2010.

From another perspective, Figure 3 shows the cumulative patent families published in the last 20 years. As it can be seen, since 2005 there has been a remarkable increase of attraction in the area: while 50% of the patents required 15 years to be generated, the remaining 50% has been filed over the past five years.

The analysis of patent application-trends shows that, at the present time, the development of hair regeneration technology is evolving positively, making this technological field attractive to companies that start producing and selling products as a result of the development of hair regeneration technology.

Technological leadership indicator

The technology leadership indicator identifies specific companies with the most consolidated and developed technologies in this specific field. The Patents which have received the highest number of citations are considered to have the highest degree of technological progress or impact. The analysis of patent citations allows the identification of patents which have been most useful for later researchers and for the development of newer technologies and may illustrate the diffusion of technical knowledge and its different practical applications throughout the years.

Just as those patents that have received most

citations are not always the most relevant ones, and those most relevant are not always the most cited ones; a number of empirical studies demonstrate a strong correlation between those variables. In Table 2 the 10 most cited institutions as references are listed.

Up to the present, New York University and Pennsylvania State University have received the highest number of citations with 29 citations which reflects the importance of the works of Lavker *et al*^[86] previously described. Following these institutions, companies like Biointegrence Co and Phoenixbio Co Inc, and Anticancer Inc have 17 citations. The Hospital for Sick Children, Aderans Research Institute Inc, Sci Torico Innovations Inc and Trichoscience Innovations Inc are among the most innovative companies.

Skin substitutes were among the earliest products to be developed using principles of tissue engineering, and their success is evident in the clinical use of several commercially available products. However, skin substitutes capable of performing all the functions of normal skin are not currently available, which limits their use in patients. Hair follicle neogenesis is not observed using current skin substitutes. Thus, the efforts in hair and skin bioengineering continue to be in a leading place in regenerative medicine. Furthermore, hair restoration is one of the fastest growing areas of cosmetic therapies for both men and women.

Hair follicles are important not only for appearance, skin hydration, barrier formation, and protection against pathogens but also in wound healing. As hair follicles store epidermal stem cells, skin with hair follicles heals faster. In addition, bulge stem cells are less susceptible to loss through minor trauma and damage through ultraviolet light. Thus, treatments that involve neogenesis of normal hair follicles would find much wider application for restoring normal skin

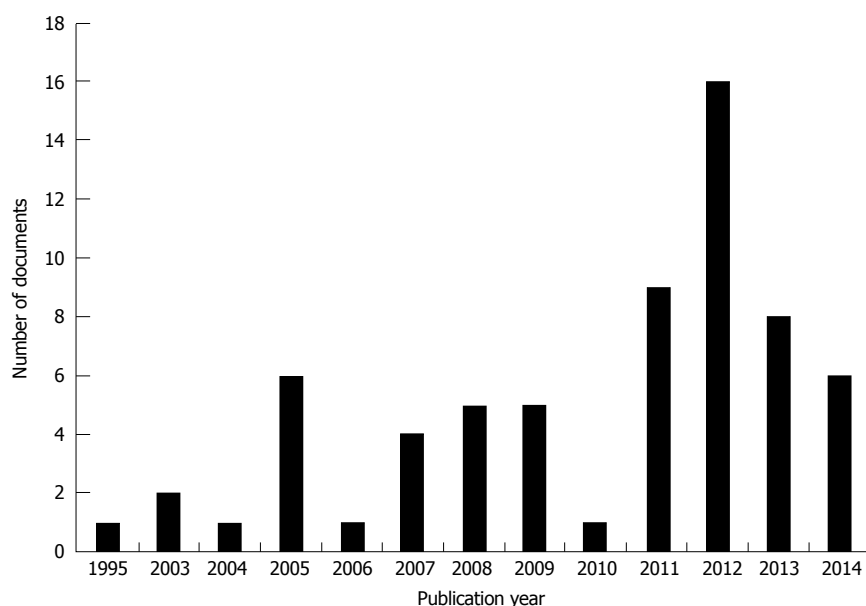


Figure 2 Evolution of the number of patent applications related to hair regeneration (time frame 1990-2014). Own investigation using patent data base Thomson Innovation.

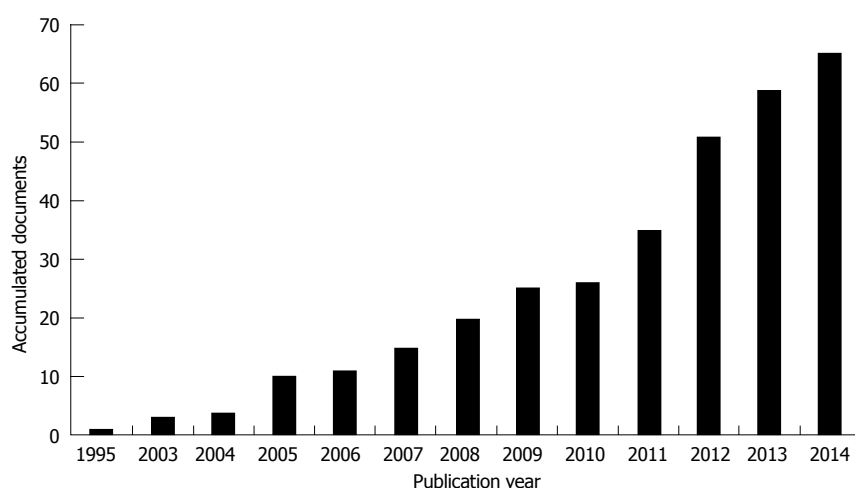


Figure 3 Evolution of accumulated patent families. Tendency line. Own generation based on patent databases.

function and appearance.

Methods and compositions capable of generating morphologically-correct, fully-developed human hair follicles, useful for treating conditions such as full- or partial-thickness skin loss, wounds, burns, scars, and hair loss have been developed in order to fulfill this necessity. In this analysis, only patents aiming to hair follicle regeneration or neogenesis have been selected. As reviewed, it has been determined that both epithelial cells and mesenchymal cells are essential for hair follicle regeneration. The regeneration of chimeric hair follicles comprised of mouse DPC and human epithelial cells has been shown to be possible, however it is still not possible to regenerate completely human hair follicles. One of the reasons for this is the difficulty to obtain an adequate number of human DPCs having the ability to induce hair follicle formation to be used

for transplantation. Consequently, most of the patents filed in this field provide cellular compositions capable of hair neogenesis and regeneration or methods for isolating progenitor skin cells as well as the culture conditions that will allow them to keep their inductive properties to promote hair neogenesis or regeneration.

The first most cited patent listed in Table 2 (WO1995001423), published in 1995, described a method of culturing and modulating subpopulations of follicular keratinocytes from upper portions of hair follicle to be used for identifying agents which stimulate hair growth or prevent hair loss and useful for follicular reconstruction and transplantation as well as wound coverage in burns and skin ulcer^[86].

In 2005 a patent application (WO2005053763A1) developed a method for transplanting the dermal papilla or dermal papilla cells and outer skin epidermis

Table 2 Most cited patents from 1990 to present

Publication number	Title	Assignee	Inventor	Publication year	Count of citing patents	Ref.
WO1995001423A1	Methods of culturing and modulating the growth of hair follicular stem cells	New York State Univ, Pennsylvania Univ	Lavker RM, Sun T, Yang J	1995	29	[86]
WO2005053763A1	Hair growth method	Biointegrence Inc, Phoenixbio Co Ltd	Matsunaga M, Matsunaga MC, Shimada T, Shimada TC, Toyoshima K, Toyoshima KC, Yoshizato K	2005	17	[94]
WO2003024406A2	Nestin-expressing hair follicle stem cells	Anticancer Inc	Li L, Lingna L, Meng Y, Ri R, Yang M	2003	17	[87]
WO2005071063A1	Methods of making and using skin-derived stem cells	Hospital For Sick Children	Biernaskie J, Fernandes K, Fernandez K, Mckenzie I, Miller F, Miller FD	2005	16	[91]
WO2002060396A2	Hair follicle neogenesis by injection of follicle progenitor cells	Aderans Res Inst Inc, Bioamide Inc	Barrows TH	2002	16	[88]
US20050214344A1	Tissue engineered biomimetic hair follicle graft	Aderans Res Inst Inc	Barrows T, Barrows TH, Cochran S, Cochran SA, Marshall B	2005	15	[90]
US20070092496A1	Method of delivering cells to the skin	Aderans Res Inst Inc	Du X, Stenn K, Stenn KS, Washenik K, Washenik KJ, Zhang Y, Zheng Y	2007	10	[99]
WO2003104443A2	Hair follicle mesenchymal stem cells and use thereof	Torico Sci Innovations Inc, Trichoscience Innovations Inc	Hoffmann R, Mcelwee KJ	2003	9	[89]
US20070122387A1	Hair grafts derived from plucked hair	Aderans Res Inst Inc	Barrows TH, Cochran SA, Marshall B, Schlicher R, Su Y	2007	9	[105]
WO2007062386A2	Hair follicle graft from tissue engineered skin	Aderans Res Inst Inc	Barrows TH, Macintyre P, Washenik KJ	2007	8	[98]

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tissue or epidermal cells to hairless scalp to regenerate hair^[94].

Different tissue engineered-hair follicle grafts were also patented; a scaffold constructed to mimic the architecture of the native hair follicle and for percutaneous implantation facilitating the follicle neogenesis process^[90] (US20050214344), a hair graft comprising plucked hairs having adhered epidermal stem cells and follicular dermal cells for implantation into wounded skin^[105] (US20070122387A1) and a hair graft comprising a scaffold, and tissue engineered epidermal and dermal layer with hair follicle progenitor cells (WO2007062386A2)^[98].

Several methods for isolating skin-derived precursor stem cells from hair follicles or dermal papillae as well as the culture conditions or the delivery into the skin of these cells in order to induce hair growth are also among the most cited patents (WO2002060396A, WO2005071063A1 and US20070092496A1)^[88,91,99].

Finally, among the most cited patents are those describing methods for isolating hair follicle mesenchymal stem cells useful for treating alopecia and gene therapy (WO2003104443A2)^[89] or useful for hair loss, burns or skin replacement as well as for treating neurological or degenerative disorders (WO2003-024406A2)^[87].

The rest of the patents analyzed whose content is not mentioned, can be found in the References section^[86-150] at the end of this review.

CONCLUSION

Although a successful strategy to generate human hair follicles from adult cells has not yet been reported, the results presented in this review suggest the issues that need to be addressed before success can be achieved. Perhaps the most important of those issues is to provide cells with a three-dimensional structure that simulates the natural scaffold. Herein the cells should develop and interact among themselves or with other cell types in the epithelial-mesenchymal interactions. Improving culture conditions that allow the expansion of these cells without losing their natural properties, as well as selecting the appropriate epithelial stem cells, should give us the tools needed to face the challenge of regenerating human hair follicles.

These efforts in hair and skin bioengineering are also visible in the growing number of patent applications filed during the last decade, indicating that this technological field is not only attractive to academic research but also to the companies that own almost half of these patents.

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Structural properties of scaffolds: Crucial parameters towards stem cells differentiation

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for regeneration of damaged tissues. Stem cells have attracted much interest in tissue engineering as a cell source due to their ability to proliferate in an undifferentiated state for prolonged time and capability of differentiating to different cell types after induction. Scaffolds play an important role in tissue engineering as a substrate that can mimic the native extracellular matrix and the properties of scaffolds have been shown to affect the cell behavior such as the cell attachment, proliferation and differentiation. Here, we focus on the recent reports that investigated the various aspects of scaffolds including the materials used for scaffold fabrication, surface modification of scaffolds, topography and mechanical properties of scaffolds towards stem cells differentiation effect. We will present a more detailed overview on the effect of mechanical properties of scaffolds on stem cells fate.

Key words: Stem cells; Tissue engineering; Differentiation; Mechanical properties; Surface modification; Topography

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Core tip: Application of stem cells in tissue engineering and regenerative medicine has attracted many researchers and it is considered as a hot topic of research. The research was mostly aimed towards the fabrication of a suitable scaffold with appropriate physical, chemical, biological and mechanical properties to guide the differentiation of stem cells to specific lineages of interest. This paper reviews the previous researches on effect of chemical structure, surface modification, topography and mechanical properties of scaffolds on stem cells behavior. Fabrication of scaffolds with desirable properties can open new strategies for cell therapy using tissue engineering technique.

Abstract

Tissue engineering is a multidisciplinary field that applies the principles of engineering and life-sciences

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INTRODUCTION

Autologous cells obtained from adult patients might deliver a less difficult route to regenerative-cell therapies. In the past, many tissues were assumed incapable of self-regeneration upon its damage, mainly because they do not possess any endogenous stem cells. However, recent discoveries show that more adult tissues harbor cells with capacity for regenerative repair^[1]. Tissue engineering comprises of suitable cells, scaffolds and appropriate biochemical/physical factors, such that it function as a strategy towards the engineering of tissues and cellular delivery. Encapsulating stem cells within biocompatible scaffolds provide a matrix which result in cell adhesion, migration and differentiation suitable for transplantation and tissue regeneration^[2-4].

Stem cells have uncovered a new perspective as therapeutic tools in regenerative medicine. These cells with the ability to proliferate indefinitely (*in vitro*) and the capacity to differentiate into any somatic cell type are a potential cell source. Stem cells are originated in the early embryo, the fetus, placenta, umbilical cord, and it occurs in many different tissues of the body. Also they have been engineered from somatic cells, termed as induced pluripotent stem cells (iPSCs). iPSCs give rise to different cell types including the neural cell types (neurons, oligodendrocytes, and glia), cardiomyocytes, osteoblasts, hepatocytes, and haemopoietic progenitors cells^[5-8]. The two main stem cell types are the embryonic stem cells (ESCs) and the adult stem cells. ESCs are derived from fetal tissue or from the inner cell mass of the blastocytes with ability for unlimited growth in culture that could be associated to a high risk of teratoma formation. Several researches have been done on ESCs pluripotency to obtain specific cell lineages for tissue engineering and regenerative medicine, as well as for therapeutic treatments^[8-13]. Adult stem cells are specialized cells including the mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and neural stem cells (NSCs)^[9]. MSCs are self-replicating cells that are capable of differentiating in multidirectional pathways, resulting in cell lineages such as the osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, adipocytes, neural cells, oligodendrocytes and haemopoietic cells^[9,14,15]. These cells have been used for the engineering of different tissues such as the cardiac^[16], tendon^[17], cartilage^[18-20], vascular^[11,21] Meniscus^[22], bone^[23], Ligament^[24], myocardia^[25], fat^[26], and neural tissues^[5,16,17,27].

While a routine source of human MSCs is the bone marrow, they have been also derived from multiple adult tissues comprising of adipose tissue (ADSCs), umbilical cord blood, placenta, thymus, and dental pulp^[28]. In comparison to MSCs derived from the bone marrow^[29], ADSCs have several advantages such that they are easy to obtain after minor donor site morbidity, possess high proliferating capacity and can be preserved for longer period of time in cell banks^[30]. Under appropriate culture conditions, ADSCs can differentiate to classical mesenchymal lineages, which include adipogenesis, chondrogenesis, osteogenesis, and myogenesis^[31,32]. Moreover, the ability of ADSCs differentiation to numerous lineages when seeded into polymeric scaffolds has been well documented in several literatures^[33,34].

The sources of HSCs are bone marrow, peripheral blood, umbilical cord blood, fetal hematopoietic system^[35], ESCs and embryonic germ cells^[11]. These cells are also a suitable cell source for blood vessel engineering^[12]. Avoidance of anaesthesia without the need for hospitalization or blood transfusion, and low risk of serious adverse events are the major advantages of the peripheral stem cells, which make them the favourable source of stem cells worldwide. On the other hand, NSCs are defined as self-renewing multipotent progenitors existing in the developing and adult CNS. Generally, they are considered by their capacity to symmetrically self-renew and their ability to discriminate into neurons, oligodendrocytes, and astrocytes through asymmetrical fate-committed division^[27]. Neural stem or progenitor cells can be obtained in different approaches such as direct isolation of these cells from embryonic or adult brain tissue^[36,37]. NSCs have been used for tissue engineering of different tissues such as multiple cell types of the adult nervous system^[5,38]. It was shown in several studies that stem cells behavior such as their adherence, proliferation and differentiation on scaffolds, depends on the material itself. Additionally, the surface chemistry, surface geometry/micro architecture of scaffolds, and the mechanical properties of scaffolds^[39-42] play a significant role in determining the cellular responses after culture of stem cells on them^[43].

Therefore, recent studies have focused on the selection of an appropriate biomaterial, whit focusing on the surface modification of the scaffolds, creating appropriate microstructures and controlling the mechanical properties of scaffolds to recapitulate the *in vivo* microenvironment suitable for regenerating tissues or for the differentiation stem cells to specific cell lineages^[44].

This review will discuss on the influence of different aspects of scaffolds including scaffold composition, surface modification, micro-nano architecture of scaffolds and mechanical properties of scaffolds pertaining to stem cells differentiation. An emphasis is also given to the effect of mechanical properties of

scaffolds towards stem cells differentiation.

EFFECT OF SCAFFOLD COMPOSITION ON STEM CELLS DIFFERENTIATION

The interaction of stem cells with their surrounding microenvironment is fundamental to multiple processes such as cell migration, proliferation, lineage specificity, and tissue morphogenesis^[45]. Biomaterials play an important role in directing tissue growth and chemical properties of the scaffolds have been shown to influence the behavior of stem cells whereas the scaffold composition has an significant role in stem cells differentiation towards preferred lineages^[42,43,45]. The ability to selectively guide stem cells differentiation by merely changing the properties of an underlying biomaterial scaffold is a smart approach in tissue engineering, which can help compliment or potentially eliminate the use of exogenous differentiation inducers like the viral gene vectors and small molecule drugs^[46].

Natural polymers such as the collagen, fibrinogen, hyaluronic acid, glycosaminoglycans, cellulose, chitosan, silk fibroin, *etc.*, consist of components found in the native extracellular matrix, such that they can assist cells to attach to cell surface receptors and provide a physical environment to control cell function compared to synthetic materials^[40,47]. However these materials suffer from fast degradation rates, difficulty in sterilization and purification, high variability, high contamination potential, and induce immune response upon implantation, while synthetic materials offer the potential for improved control, repeatability and safety^[40,47]. Scaffolds fabricated by synthetic materials can be chemically modified by natural materials to carefully regulate the differentiation of stem cells^[47]. Immune response is an important concern in synthetic biomaterials used for scaffold fabrication and it can be reduced by selection of materials which are inert inherently or by modification of scaffolds to avoid detection by the immune system. Hydrophobic materials usually tend to increase monocyte adhesion compared to hydrophilic materials leading to a local immune reaction at the scaffold site^[48].

Awad *et al.*^[34] compared the chondrogenic differentiation of human adipose-derived adult stem cells seeded in alginate, agarose and gelatin scaffolds and their findings revealed that the growth and differentiation potential of adult stem cells seeded on the scaffolds varied and the cells on gelatin scaffolds showed better proliferation and differentiation. Collagen has attracted much interest as a biomaterial for fabrication of scaffolds in tissue engineering as a key structural protein found in the extracellular matrix of many connective tissues^[49,50]. In spite of the several advantages of collagen as a biodegradable, biocompatible, safe and multifunctional material, its potential to evoke immune response has raised concerns as an animal-derived biomaterial^[51].

Meinel *et al.*^[52] investigated the osteogenic differentiation of human MSCs on different protein substrates including unmodified collagen with fast degradation rate, cross-linked collagen with slow degradation rate and silk; in both static and dynamic culture conditions. Under dynamic condition, MSCs on cross-linked collagen and silk scaffolds deposited more calcium and had a higher alkaline phosphatase activity than MSCs on unmodified collagen scaffolds indicating the importance of scaffold properties in causing osteogenesis of the cultured MSCs^[52].

Jäger *et al.*^[39] cultivated MSCs on the D,D,L,L-poly lactide (PLLA), collagen I/III, and polygalactin-910/polydioxanone (PGPD) scaffolds and their results showed significant differences in the proliferation, differentiation, and Ca^{2+} accumulation of MSCs on different scaffolds. Cell adhesion on PGPD was lower compared to the cell adhesion on PLLA and collagen I/III scaffolds, which was related to the significant biodegradation rate of PGPD scaffolds compared to other scaffolds. They found significantly higher cell numbers on collagen I/III membrane compared to that for PLLA scaffolds after 4 h cell culture while the differences were less significant after 28 d of cell seeding. Lower cell number on PLLA scaffolds compared to collagen I/III after 4 h could be attributed to the hydrophobic nature of PLLA while an increasing deposition of serum proteins from culture media on the PLLA scaffold may reduce the effect of its hydrophobic nature and decreased the quantitative differences in cell attachment between collagen I/III and PLLA scaffolds after 28 d cell seeding^[39].

PEG is a relatively inert, biocompatible and hydrophilic polymer^[53]. Hwang *et al.*^[41] encapsulated MSCs in PEG based hydrogels containing type I collagen, type II collagen and hyaluronic acids (HA) which are the main components of musculoskeletal tissue matrix. Chondrogenic differentiation of MSCs was slightly enhanced in collagen-matrix-based hydrogels, whereas osteogenic differentiation was induced in HA-containing hydrogels suggesting the potential of ECM components to modulate the fate of MSCs differentiation^[41].

Ravichandran *et al.*^[54] fabricated a composite scaffold containing PLLA and Poly-benzyl-L-glutamate (PBLG), a polymer of glutamic acid in which the carboxyl groups have been benzoylated, by electrospinning. Nanohydroxyapatite (n-HA) was also deposited on the surface of the nanofibers by calcium-phosphate dipping method. Their results proved that the incorporation of PBLG along with n-HA deposition on the surface of scaffolds promoted greater osteogenic differentiation of ADSCs in the absence of an induction medium highlighting the chemical composition of the scaffold as a determinant factor towards ADSCs differentiation^[54].

Shah *et al.*^[46] reported selective differentiation of NSCs into oligodendrocytes by seeding NSCs in PCL nanofibrous scaffolds coated by graphene oxide (GO).

Higher concentration of GO was found to promote NSCs differentiation into mature oligodendrocytes without introducing differentiation inducers in the culture media. Their results showed that the presence of GO in the structure of nanofibrous scaffolds was a crucial factor that determined the stem cell-scaffold interactions in directing NSCs towards oligodendrocyte differentiation^[46].

One of the major challenges in cartilage and disc-tissue engineering using MSCs is the rapid expression of type X collagen as a marker of chondrocyte hypertrophy associated with endochondral ossification. Nelea *et al.*^[55] used polypropylene and nylon-6 polymers for the fabrication of scaffolds and modified the surface of scaffolds by glow discharge plasma treatment in ammonia gas to investigate the potential of each polymer in its ability to inhibit the expression of type X collagen. They cultured MSCs on modified and unmodified polypropylene and nylon-6 and evaluated the chondrogenic differentiation pathway using aggrecan and types I, II, and X collagens. Their results showed that MSCs did not adhere to unmodified PP while they observed cell adhesion on modified PP, nylon 6 and unmodified nylon 6 and concluded that the nature of the surface should be vital for the interaction of MSCs. Both modified and un-modified nylon-6 suppressed the expression of type X collagen, while the modified polypropylene scaffold almost completely inhibited its expression indicating the impact of polymer type and surface characteristics on the fate of stem cells^[55].

Hosseinkhani *et al.*^[56] encapsulated DNA nanoparticles into collagen sponge reinforced with poly (glycolic acid) fiber for bone tissue engineering. More osteogenic differentiation of MSCs and formation of homogeneous bone upon implantation of scaffolds containing DNA nanoparticles was observed by these researchers^[56].

In yet in another study, semi-interpenetrating polymer networks made of collagen type I fibers combined with fibronectin or laminin were fabricated to investigate the effect of matrix composition towards ESCs differentiation. Results showed that scaffold composition has an important role in ESCs development and differentiation whereas the presence of fibronectin in collagen scaffolds strongly induces endothelial cell differentiation and vascularization. On the contrary, laminin stimulated ESCs to differentiate into beating cardiomyocytes^[57]. Costa-Pinto *et al.*^[58] prepared chitosan-poly (butylene succinate) scaffolds in different weight ratios of chitosan (50%, 25%, and 0%) and compared the potential of osteogenic differentiation of human bone marrow stromal cells on these scaffolds. Their results revealed higher cell viability, adhesion, proliferation, and osteogenic differentiation on scaffolds with more ratios of chitosan^[58]. Overall, it was clear that the scaffold material and its properties played a critical role in directing the stem cells towards differentiation, with proteins and peptides had a positive influence towards this effect.

EFFECT OF SURFACE MODIFICATION OF SCAFFOLDS ON STEM CELLS DIFFERENTIATION

Cell-biomaterial interactions has a significant role on the growth and differentiation of stem cells within scaffolds^[34]. It is well known that the surface properties of scaffolds have an important role in stem cells-biomaterials interactions, ultimately controlling the stem cell adhesion, proliferation, and differentiation after its attachment on the surface^[59,60]. Stem cells differentiation can be regulated by presenting suitable biological or chemical signals within the structure of the scaffolds^[47]. Therefore the chemical and biological modification of the scaffolds can affect stem cells behavior which is also defined by the substrate properties and their degradation rate, eventually manipulating the signal transduction pathways in stem cells^[47]. It can be concluded that the surface chemistry of materials can affect protein adsorption and the binding of different integrins and influence the cell behavior^[59]. Surface modification of scaffolds is therefore an important issue in tissue engineering in order to control the cellular behavior^[59]. More recently different methods have been established for the modification of scaffolds thorough incorporation of biomolecules within the scaffold structure by method like simple physical adsorption or by covalent conjugation^[47,61].

Ayala *et al.*^[45] demonstrated the influence of interfacial matrix hydrophobicity on stem cells behavior. They fabricated tunable synthetic scaffolds with control over the hydrophobicity of the scaffolds, by copolymerizing acrylamide with acryloyl amino acids and their results showed that the small changes in matrix hydrophobicity can dramatically alter the cell-matrix interaction and can influence various cellular behaviors^[45].

Chua *et al.*^[62] investigated the effect of surface-functionalized polyethersulfone (PES) scaffolds including the hydroxylated, carboxylated and aminated PES nanofibers and films and their results showed that the surface-bound amino groups highly influenced the proliferation and differentiation of hematopoietic stem/progenitor cells. The effect of coating of polycaprolactone-co-lactide scaffolds with collagen I (coll I) and coll I/chondroitin sulfate (CS) on osteogenic differentiation potential of ovine bone marrow MSCs has been investigated. The results showed surface modification of scaffolds using coll I/CS induce osteogenic differentiation of cells, without using any differentiation supplements such as dexamethasone, revealing the osteoinductive characteristics of the modified scaffolds^[63].

Rim *et al.*^[64] fabricated electrospun PLLA nanofibrous scaffolds and coated scaffolds with polydopamine through immersion in an aqueous solution of dopamine with mild shaking for 1 h. Their results showed more

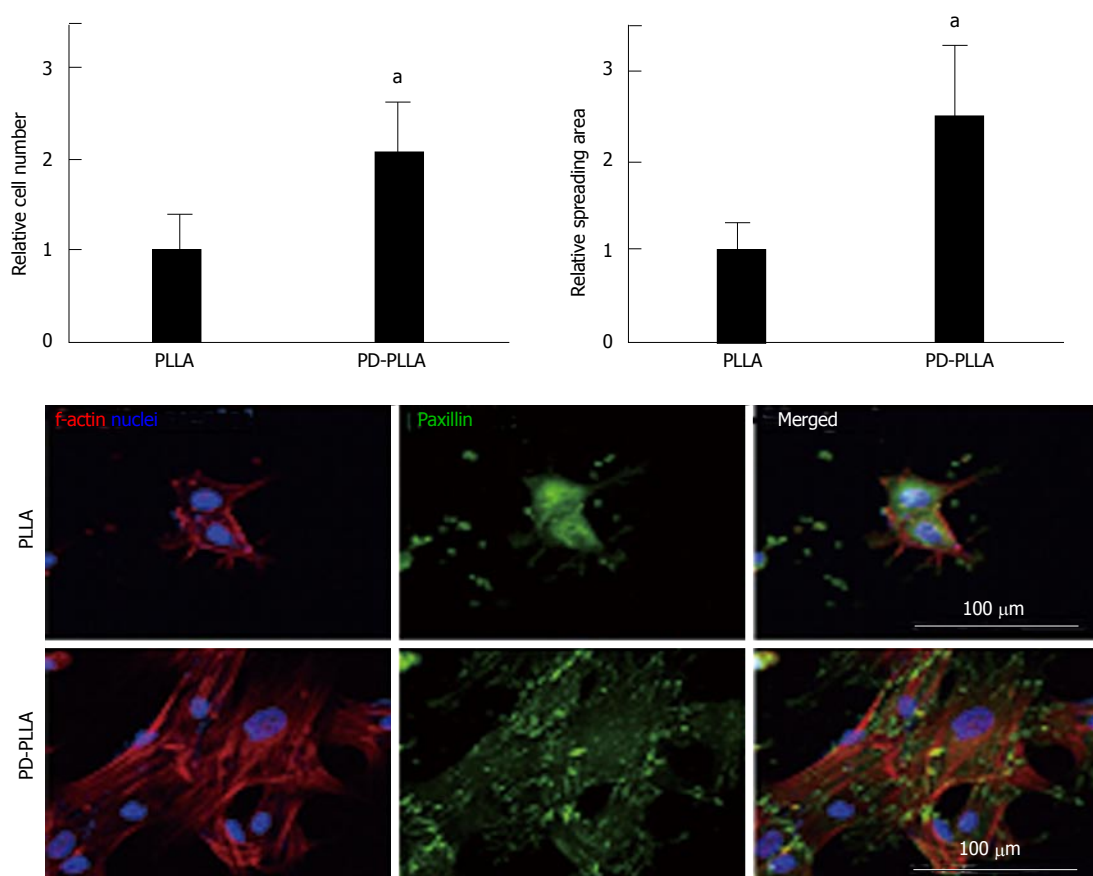


Figure 1 Quantitative analysis of initial cell adhesion from human mesenchymal stem cells cultured on the fibers. Relative adherent cell numbers and spreading area of human mesenchymal stem cells (hMSCs) cultured on PLLA and PD-PLLA fibers were analyzed after 12 h of culture. ^a $P < 0.05$, PD-PLLA vs PLLA group. Adherent morphology of hMSCs on PLLA and PD-PLLA fibers was observed by confocal microscopy. Scale bars represent 100 μm. Reproduced with permission from Rim *et al*.^[64] PLLA: Poly(L-lactide); PD-PLLA: Poly(L-lactide) (PLLA) fibers coated with polydopamine.

adhesion and proliferation of MSCs cultured on surface modified nanofibrous scaffolds with dopamine relative to cells seeded on unmodified scaffolds (Figure 1). More osteogenic differentiation as well as angiogenesis was also observed for MSCs seeded on modified scaffolds compared to unmodified scaffolds^[64].

In yet another study, electrospun PCL nanofibers were coated using polydopamine by simple immersion of substrates in an alkaline dopamine solution. RE-1 silencing transcription factor (REST) was then absorbed onto PCL nanofibers coated with polydopamine to induce scaffold-mediated gene knockdown for enhanced neuronal differentiation of neural stem/progenitor cells. The results showed significant enhanced neuronal commitment and decreased glial cells differentiation due to presence of the silencing of REST^[44]. Controlled release of insulin-like growth factor I (IGF-I) from silk fibroin scaffolds for chondrogenic differentiation of human MSCs has been studied by Uebersax *et al*.^[65] and the results showed that IGF-I loaded silk fibroin scaffolds have the potential to provide chondrogenic stimuli to human MSCs, thus beneficial for cartilage repair.

In yet another study, copolyester of 3-hydroxy-butyrate-cohydroxyhexanoate (PHBHHx) scaffolds were fabricated and coated with PHA granule-

associated proteins (PhaP) and PHA granule-associated proteins PhaP fused with RGD peptide (PhaP-RGD) for investigating the effect of surface modification towards chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs). Their results showed that the surface modification of scaffolds with PhaP-RGD promote chondrogenic differentiation of hBMSCs compared to PhaP coated or uncoated scaffolds even without the presence of chondrogenic induction medium (Figure 2). Hyaline cartilage regeneration, and inhibited fibrocartilage formation in hBMSCs derived chondrocytes was also observed on PhaP-RGD coated PHBHHx scaffolds indicating suitability of this substrate for cartilage tissue engineering compared to the uncoated ones^[61].

In yet another study, poly (ethylene glycol) (PEG) hydrogels were functionalized with heparin and osteogenic differentiation of human MSCs was evaluated. The results through increased ALP production and gene expression of osteopontin and collagen type I showed that functionalization of hydrogel with heparin induce osteogenic differentiation which is likely due to improvement of cell-scaffold interactions due to the presence of heparin^[66]. Surface modification of poly-(lactic-co-glycolic acid) (PLGA) (75:25) scaffolds with RGD peptides also enhanced the human

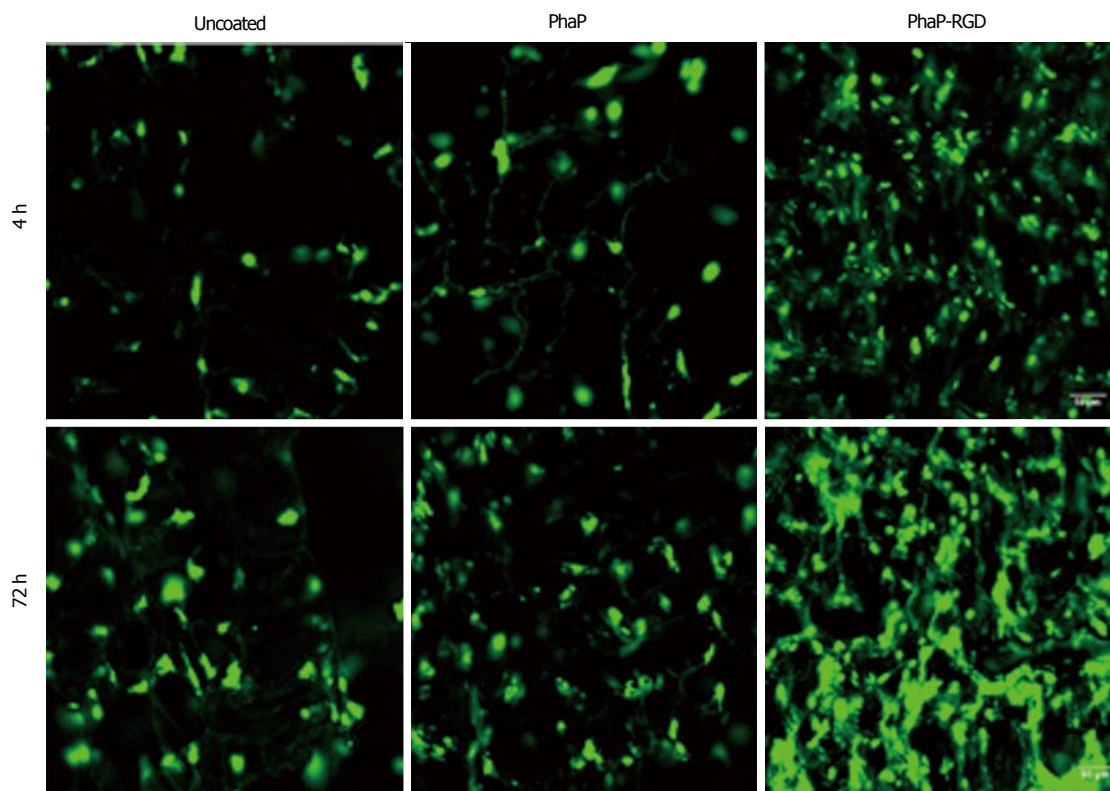


Figure 2 Confocal microscopic imaging of human bone marrow mesenchymal stem cells grown in uncoated, PhaP and PhaP-RGD coated 3-hydroxybutyrate-co-hydroxyhexanoate scaffolds after 4 or 72 h of incubation, respectively. Phalloidin-fluorescein isothiocyanate was used to F-actin of cells grown in the scaffolds (Green). Reproduced with permission from You *et al*^[61]. PhaP-RGD: PhaP binding protein fused with arginyl-glycyl-aspartic acid.

osteoprogenitor differentiation and osteogenesis^[67]. While RGD peptide immobilized macroporous alginate scaffolds showed enhanced chondrogenesis properties of human MSCs compared to the un-modified ones^[68].

In vitro osteogenic differentiation of human MSCs was also observed on RGD peptide functionalized PLLA nanofibers by Paletta *et al*^[69]. Their results showed more osteogenic differentiation of human MSCs on modified scaffolds revealing the osteoinductive effect of the scaffolds functionalized with RGD^[69].

Kuo *et al*^[70] fabricated PLGA /chitosan scaffolds and functionalized it with type I collagen, whereby these researchers were able to improve the cell adhesion and viability on PLGA/chitosan/collagen scaffolds. Moreover, MSCs differentiated towards osteoblasts in the modified scaffolds without induction procedures, while neural differentiation was observed on the scaffolds by the induction MSCs with neuron growth factor (NGF)^[70].

In another study Yang *et al*^[71] fabricated porous poly L-lactide-co- ϵ -caprolactone (PLCL) and did surface modification *via* crosslinking of chitosan on the surface of scaffold. Their finding showed elongated morphology of MSCs on modified scaffolds while cells on unmodified scaffolds showed more spherical morphology with lower spreading. Moreover, the surface modified scaffolds provide surfaces for early differentiation of MSCs with more *in vitro* cartilage tissue formation revealing more chondrogenic differ-

entiation of MSCs on modified scaffolds compared to the unmodified PLCL scaffold^[71].

In yet another study, Budiraharjo *et al*^[72] obtained carboxymethyl chitosan scaffolds (CMCS), coated with hydroxyapatite and compared the behavior of osteoblasts and human MSCs on both modified and un-modified scaffolds. Coating the scaffold with HA substantially enhanced the osteogenic differentiation of the human MSCs.

Surface modification using plasma has been frequently used to improve surface properties of scaffolds fabricated with synthetic polymeric materials through formation of functional groups on the surface^[73]. Lin *et al*^[74] investigated the effect of modification of PLLA scaffolds by gas plasma towards the differentiation of ADSCs and their results showed that the cells seeded on modified scaffolds displayed significantly increased differentiation into endothelial cells^[74].

Wang *et al*^[60] fabricated porous nanocrystalline HA/chitosan scaffolds using a lyophilization technique, further treated them with cold atmospheric plasma as a simple, quick, and inexpensive method to modify the surface of scaffolds. Their results showed enhanced stem cell growth and osteogenic differentiation on modified scaffolds, which might be due to higher amounts of protein adsorption on the scaffold surfaces due to improves surface roughness and wettability after its modification^[60].

An ideal bone implant should recruit osteoblasts or

progenitor cells from the adjacent native tissue and induce cell proliferation and osteogenic differentiation for new bone formation. Hu *et al.*^[75] modified Ti6Al4V (TC4) implants using biofunctional multilayer coating of gelatin, chitosan, gelatin and human recombinant BMP2. *In vitro* results showed that such modifications of the implant surface stimulated the adhesion and osteogenic differentiation of MSCs and *in vivo* results showed improved induction of new bone formation at the interface of bone and implant and the integration of the implant within the surrounding living tissues^[75].

EFFECT OF SCAFFOLD TOPOGRAPHY ON STEM CELLS DIFFERENTIATION

The topography of the extracellular matrix is known to influence cell attachment, morphology, proliferation and differentiation^[76]. Topography can also enhance differentiation of progenitor cells into their programmed pathways^[77].

Topographic surfaces with micro and nano scale structures have been shown to stimulate the cell alignment, polarization, elongation, migration, proliferation, gene expression, *etc.*, while the mammalian cells have been shown to react to nanoscale structures on a synthetic surface^[77,78]. Nano scale topographies are obtained by different fabrication methods such as electrospinning, polymer phase separation, photolithography, chemical vapor deposition, electron beam lithography, *etc.*^[2]. The similarity of electrospun nanofibers to the morphology of native extracellular matrix has attracted interest in the application of these scaffolds as a substrate for stem cell attachment and differentiation^[2].

Smith *et al.*^[79] compared the *in vitro* osteogenic differentiation of ESCs on nanofibrous scaffolds and traditional scaffolds with two and three dimensional structure without the nanofibrous features. The results showed that the nanofibrous features enhanced the osteogenic differentiation and mineralization of ESCs compared to traditional substrates. Moreover, the osteogenic differentiation was observed on nanofibrous scaffolds without osteogenic supplements, while cells on other scaffolds required osteogenic supplements and growth factors for osteogenic differentiation^[79].

Ragety *et al.*^[80] evaluated the chondrogenic differentiation of MSCs on chitosan microfibrillar scaffolds and chitosan sponges. More chondrogenesis differentiation was observed on chitosan microfibers compared to that on sponges indicating the effect of scaffold microstructure towards the cell behavior^[80].

Christopherson *et al.*^[81] evaluated the effect of topographical features of nanofibrous scaffolds in view point of fiber diameter on rat adult neural stem/progenitor cell (rNSC) differentiation and proliferation. They fabricated different nanofibrous scaffolds with average fiber diameters of 283 ± 45 nm, 749 ± 153 nm and 1452 ± 312 nm and their findings showed that the fiber diameter of PES nanofibrous scaffolds significantly

influenced the differentiation of rNSCs, whereas by decreasing of fiber diameter, more cell spreading and proliferation was observed. Additionally, the differentiation of stem cells towards oligodendrocytes was observed on nanofibrous scaffolds with fiber diameter of 283 ± 45 nm while morphology similar to neuronal progenitors was observed on nanofibers with fiber diameter of 749 ± 153 nm. Although, the number of cells on nanofibers with fiber diameter of 1452 ± 312 nm was found to be low which may be due to the difficulty of adhesion and migration of rNSCs on fibers with large diameter. However, the cells present within these nanofibrous scaffolds showed the morphology of neurons or neuronal progenitors^[81].

In yet another study, aligned PLLA nanofibrous scaffolds with a mean diameter ranging from 307 to 917 nm and random PLLA nanofibrous scaffolds with a mean diameter ranging from 327 to 1150 nm were fabricated and the behavior of NSCs were evaluated on these scaffolds. The results showed that the depending on the fiber diameter and pattern, NSCs behaved differently revealing that the fiber alignment and diameter had significant effects on cellular behavior^[82].

Yin *et al.*^[76] examined the effect of alignment of PLLA nanofibrous scaffolds on differentiation of stem cells from human tendon and the differentiation of the cells seeded on aligned nanofibrous scaffolds was found to be higher with either normal or osteogenic media than on the randomly-oriented nanofibers^[76].

Subramony *et al.*^[83] also evaluated the effect of PLGA nanofiber alignment and mechanical stimulation towards MSCs differentiation. Their results showed fibroblastic differentiation of MSCs in the absence of chemical induction factors on aligned nanofibers together with mechanical stimulation under tensile loading. No fibroblastic differentiation was observed on the non-aligned scaffolds even after the application of mechanical stimulation, indicating the effect of topography of scaffold towards stem cells differentiation^[83].

Bakeine *et al.*^[84] investigated the effect of nanoscale surface topography on adhesion, proliferation and neural differentiation of mouse ESCs by fabrication of thin films of gold with different root mean square (RMS) surface roughness of 10, 21, 30 nm and 50 nm. Their results showed that surface topography influenced the neural differentiation whereas highest differentiation was observed on gold films with RMS surface roughness of 21 nm after five days of cell seeding without addition of the traditional soluble neurotrophic factors^[84].

The effect of topography with different sizes (groove width: 350 nm/ $2 \mu\text{m}$ / $5 \mu\text{m}$, depth = 300 nm) on the differentiation of human induced pluripotent stem cells (hiPSCs) towards neuronal lineage was studied by Pan *et al.*^[78]. Their results showed the obvious effect of topography on directing differentiation of hiPSCs towards neuronal lineage with noticeable up-regulation of neuronal marker expression on surfaces with nanostructured topography^[78].

Yim *et al.*^[77] studied the proliferation and differentiation

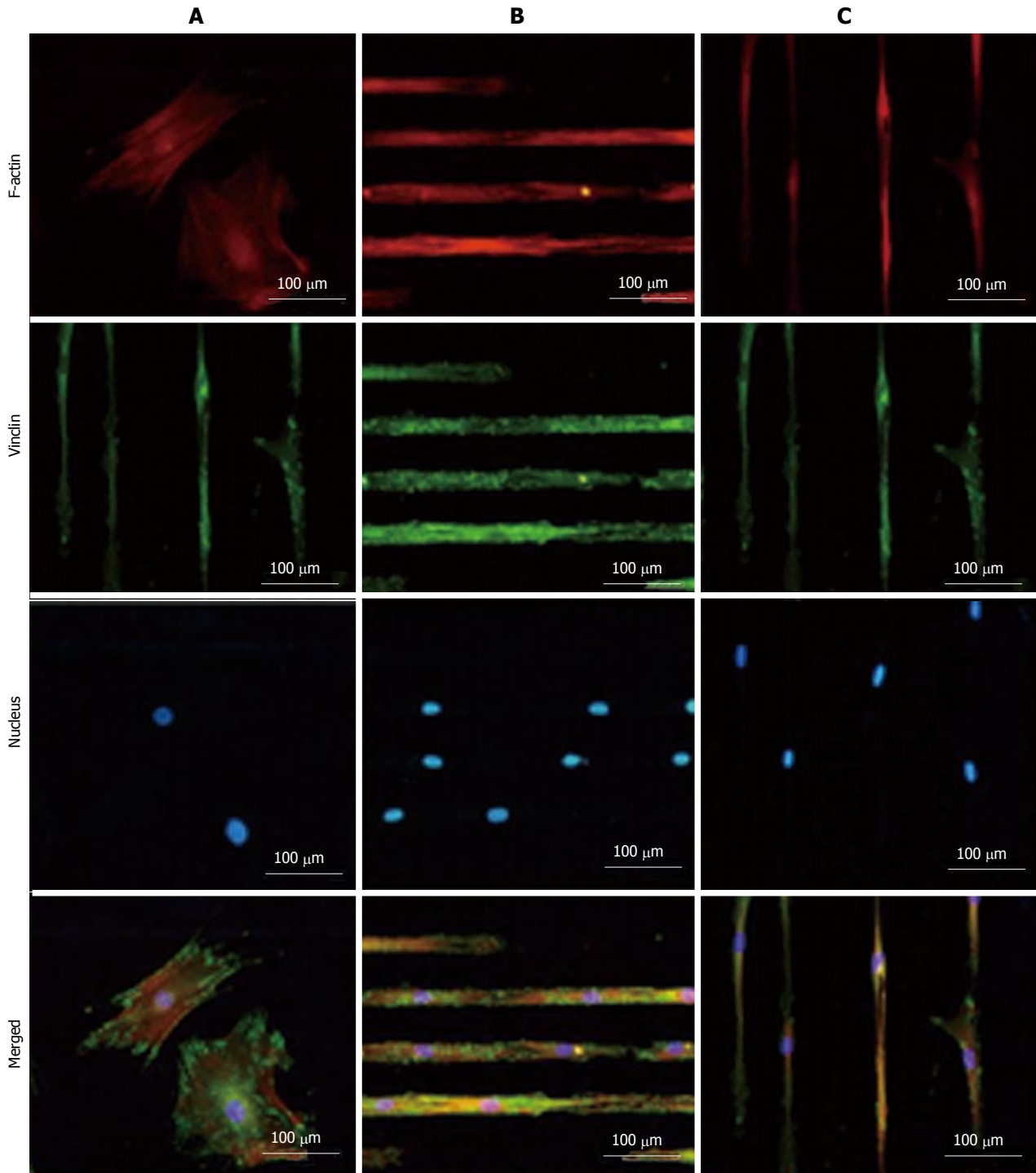


Figure 3 TRITC-Phalloidin labeled F-actin (red), AlexaFluor 488 labeled vinculin (green), diamidino-2-phenylindole nuclear staining (blue) and overlaid fluorescent image of immuno-stained cellular components (merged) for the unpatterned (A) and patterned human mesenchymal stem cells (B and C). Samples were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution for 4 d before they were fixed and stained. All images were taken with a 20 × objective lens. (Scale bar = 100 μm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). Reproduced with permission from Tay *et al.*^[86].

rentiation of human MSCs on nano-gratings of 1 μm, 10 μm, 350 nm width and their results showed the alignment of cytoskeleton and nuclei of human MSCs along the nano-gratings. Significant up-regulation of neuronal markers was also observed on patterned surface compared to un-patterned and micro-patterned controls^[77].

Chaubey *et al.*^[85] evaluated the effect of the surface topography on the differentiation of multipotent mouse bone marrow stromal precursors (D1 cells) to adipocytes. They compared the differentiation potential of D1 cells on patterned PLLA films, plain PLLA films and tissue culture-plate and their results showed the impact of patterned surfaces on cell differentiation^[85].

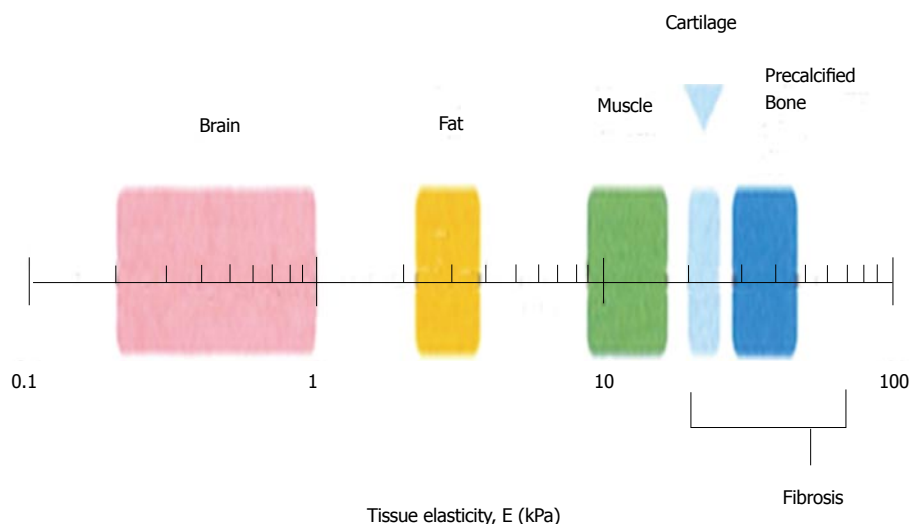


Figure 4 Schematic depicting the normal variation in elasticity of the indicated tissue^[92].

Tay *et al.*^[86] printed fibronectin on PLGA thin films and investigated the differentiation of human MSCs on patterned and un-patterned scaffolds, where they revealed the differentiation of human mesenchymal stem cells (hMSCs) into myocyte like cells on the micropatterned films (Figure 3).

Shi *et al.*^[87] fabricated two nano-grating substrates with a period of 250 and 500 nm and with a depth of 120 nm on quartz, and investigated the effect of substrate nanotopography on differentiation of ADSCs into endothelial cells which play an important role in vascularization. Decreased cell proliferation along with enhanced endothelial gene expression of the ADSCs and *in vitro* angiogenesis was observed on nanograting suggested the role of nanotopography on ADSCs differentiation to endothelial cells^[87]. Oh *et al.*^[88] fabricated PCL cylindrical scaffolds with different pore size (90-400 μm range) along the longitudinal direction; and investigated the effect of pore size on the chondrogenic differentiation of ADSCs. Their results showed that the pore size of 370-400 μm provided a more suitable environment for chondrogenic differentiation than other pore size groups^[88].

Teo *et al.*^[89] applied nanotopography technique along with NGF controlled release from polydimethylsiloxane substrate for neuronal tissue engineering and their results showed enhanced expression of neuronal genes on nanopatterned substrate combined with NGF delivery^[89].

The influence on nanotopography, whether in the form of fibers, porous microstructures or pits and grates, was studied extensively by various researchers. However, comparison of the results between such studies was difficult due to the differences in various factors involved in each study, where some researchers used mechanical stimulation, while others used a specific biomolecule for the differentiation to take place. A systematic evaluation is therefore necessary, to clearly define the fiber length or the pore size that

might specifically cause the differentiation of stem cells to nerve, osteolineages or endothelial cells.

EFFECT OF CULTURE ENVIRONMENT ON STEM CELLS DIFFERENTIATION

Bioreactors have been used in tissue engineering to overcome the drawbacks of static culturing conditions and to obtain uniform distribution of cells within the scaffolds and provide sufficient levels of oxygen, nutrients, cytokines, and growth factors, and to expose the cultured cells to mechanical stimuli^[90]. Dynamic condition of cell culture has also been shown to influence stem cells differentiation.

Gomes *et al.*^[90] evaluated the effect of static and dynamic culturing conditions on the proliferation and osteogenic differentiation of rat bone marrow stromal cells seeded on two starch-based scaffolds. Calcium content analysis, VonKossa-stained sections, tetracycline fluorescence and histological analysis revealed the enhancement of osteogenic differentiation under dynamic condition created by flow perfusion bioreactor^[90]. Mauney *et al.*^[91] also showed increased levels of alkaline phosphatase activity, bone-specific protein transcript levels, and mineralized matrix production by human bone marrow stromal cells under dynamic culture condition indicating increase in osteogenic differentiation of human BMSCs using mechanical stimulation applied by bioreactor. However, the influence of mechanical stimulation towards stem cells differentiation is not studied highly and more investigations are required.

EFFECT MATRIX MECHANICAL PROPERTIES TOWARDS STEM CELL DIFFERENTIATION

Matrix stiffness varies with respect to different organs

present in the human body, and Figure 4 shows the difference in matrix stiffness of a few organs, as depicted by Vincent *et al.*^[92]. It explains how the matrix stiffness varies throughout the body: the brain is the softest tissue with an elasticity ranging between 0.2 to 1 kPa, while the precalcified bone is the hardest tissue with an elasticity value of more than 30 kPa^[87], explaining the differences in the tissue matrix stiffness. Cells of different lineages survive in distinct natural environment, and they possess different stiffness. This is also the main reason why we believe that the stiffness have a higher influence towards the differentiation of stem cells. For tissue engineering, the most considered factors are the structure and the components used for scaffold fabrication, which determine the physical, chemical and biological properties of the scaffolds, and these properties will furthermore affect the differentiation of stem cells.

Bone

Various strategies has been acknowledged and selectively chosen to alter the biomaterial surfaces or to create micro-environment to establish both mechanical and biochemical cues for the differentiation of stem cells, especially for application in regenerative medicine. Chemical factors have been individually studied, suggesting the ability of BMP-2 to promote pro-osteogenesis or IGF to promote tenogenic phenotype^[93,94]. However, aligning the mechanical properties of the scaffolds with the specific tissue of interest was an interesting strategy applied to hydrogels, nanofibers and ceramics, in order to advance the field of stem cell differentiation on bioengineered substrates. Among these, injectable biodegradable hydrogels eliminate the need for surgical interventions or they allow for minimally invasive procedures causing minimal harm to the patients. However, physically crosslinked hydrogels lack strength and stability, while the chemically cross-linked hydrogels are more appreciated due to their high mechanical strength and stiffness. Hydrogels are useful substrates that assist to determine the interplay between physical cues/stiffness and their relationship with the fate of stem cell differentiation. A thiolene chemistry was utilized by Gandavarapu *et al.*^[95] to functionalize poly(ethylene glycol) hydrogels with a pendant peptide moiety, c(RRETAWA) and gels with Young's Modulus (YM) of 2 kPa (soft) and 25 kPa (Stiff) were prepared. The stiffer substrate was found to give 3.5 fold higher hMSCs attachment. The ALP activity of hMSCs cultured on stiff gels containing 0.1 mmol/L and 1 mmol/L c(RRETAWA) increased by 2.5 and 3.5 fold, respectively after 14 d of cell culture. At the same time, the expression of osteopontin and collagen-1a for cells cultured on stiffer gels were higher, suggesting the application of high substrate stiffness gels for osteogenic differentiation of MSCs. In order to adjust the matrix stiffness, crosslinking of the thiol functionalized hyaluronic acid (HA-SH) and thiol functionalized recombinant human gelatin (Gtn-

SH), were performed by using varied amounts of poly (ethylene glycol) tetra-acrylate (PEGTA)^[96]. Further hMSCs were seeded on the hydrogels to assess their potential towards osteo-lineage differentiation. MSCs grown on gels with 1.5 kPa stiffness, was found to express the early bone protein, namely osteopontin, while the cells on stiffer (4 kPa) gel expressed late osteogenic gene or the bone sialoprotein (BSP). This highlights the potential of higher osteogenic differentiation on stiffer hydrogels, mainly because the focal adhesion structures might have increased and F-actin might have got organized on such substrates, supporting the differentiation of MSCs to osteo lineages.

In order to study the role of substrate stiffness towards cell differentiation, Sun *et al.*^[97] prepared crosslinked gelatin scaffolds, where by the influence of scaffold strength was demonstrated *in vitro* and *in vivo* during this study. Crosslinking of gelatin scaffolds was performed by these researchers, using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) to prepare three dimension gelatin scaffolds with high stiffness properties. The elastic modulus of the EDC crosslinked scaffolds (2.5 kPa) was significantly higher than those of the non-crosslinked scaffolds (0.6 kPa). High mechanical strength of cross-linked scaffolds was suggested to promote stem cell mediated bone regeneration *via* endochondral ossification process, than the non-crosslinked scaffolds. *In vivo* studies using Inbred C57BJL/6 mice, showed significantly high trabecular bone formation for the crosslinked gelatin implanted animals, by micro-computed tomography and histology analysis^[92]. Wang *et al.*^[98] prepared gelatin-hydroxyphenylpropionic acid-tyramine (Gtn-HPA-Tyr) conjugates to simulate osteogenic differentiation of hMSCs, by conjugation of Tyr to Gtn-HPA conjugate by means of carbodiimide/active ester mediated coupling reaction. Oxidative coupling of phenol moieties were further carried out by hydrogen peroxide (H₂O₂) and horseradish peroxidase (HRP), whereby the concentration of H₂O₂ was varied producing Gtn-HPA-Tyr hydrogels of varying storage modulus (G₀). Hydrogels with G₀ > 20000 was found suitable for the osteo-specific differentiation of MSCs, with osteocalcin upregulation and high calcium accumulation was observed on these gels compared to hydrogels with G₀ of 13500. Stiffer hydrogels were suggested beneficial for the repair of bone defects. In yet another attempt, the ability of cells to differentiate to osteogenic cells were studied on transglutaminase cross-linked gelatin (TG-Gel) of varying rigidity and these researchers found that the softer matrix promote cell proliferation, while stiffer matrix promote osteogenesis^[99]. Osteogenic differentiation was found to occur in 6% and 9% TG-Gels with rigidity of 13.51-2.13 kPa and 32.32-1.9 kPa, respectively than using 3% TG-Gels. However, little or no effect of BMP-2 was found in the case of stiffer (6% and 9%) TG-Gel towards MSC differentiation. The 9% TG-Gels with and without BMP-2 demonstrated high

calcium deposition with expression of osteogenic markers during *in vitro* studies. On the other hand, the ALP activity and early markers of osteogenic differentiation were observed in 6% TG-Gel and these cells differentiated to osteoblast-like cells at a later time point. Stiffer substrates are also demonstrated to direct the osteogenic differentiation of ADSCs, even in the absence of any growth factors^[100]. PDGF and BMP-2 were covalently immobilized to collagen-Glycosaminoglycan (CG) membrane in defined patterns, by combined photolithography-carbodiimide crosslinking strategy and the elastic modulus (stiffness) of the matrix was orthogonally manipulated. These researchers explained that by increasing the ratios of EDC:NHS:COOH, the stiffness of the CG got increased. Photoimmobilized BMP-2 was demonstrated to have a little effect on lineage-specific gene expression of ADSCs during this study, while the matrix stiffness had a higher impact. In yet another study, using BMP-2 modified poly(acrylamide-co-acrylic acid) hydrogels, BMP-2 was found to have little or no effect towards stem cell differentiation if grafted on softer gels^[101]. Hence the mechanical properties of the substrate dictated the potential for stem cell differentiation than the biochemical reagents, explaining the diversity of stem cell behaviors. Efforts to leverage approaches to explore a wider range of combinatorial environments and biochemical pathways, in a systematic manner is necessary, such that the results obtained by different kinds of material systems can be compared. In yet another study, CG scaffolds were prepared by cross-linking treatment *via* dehydrothermal and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide methods, with varying stiffness of 0.5 to 1.5 kPa^[102]. MSCs differentiation studies on these scaffolds, showed higher level of RUNX2 expression on the stiffer scaffolds (1.5 kPa) indicating MSCs differentiation to osteogenic lineage. Chondroitin sulphate and hyaluronic acid was found to have a positive influence towards the MSCs differentiation towards osteogenic and chondrogenic lineages, respectively.

Actin filaments of cells are structures for force transmission in response to the substrate with which it comes in contact^[103,104]. Increased cell spreading in response to geometric features of the substrate, could result in enhanced actomyosin contractility associated with osteogenic differentiation^[103]. The expression of osteogenic markers was demonstrated as stiffness dependent, with maximum osteogenesis observed for fibronectin coated micropatterned polyacrylamide gels with stiffness of 30 kPa.

Studies on the rate of proliferation of mouse ESCs suggest higher proliferation on stiffer substrates of polyacrylamide hydrogels compared to the proliferation on soft substrates, favoring mESC colonization with high levels of Oct3/4 and the Nanog biomarker production^[105]. In maintaining the stemness of mESCs on soft hydrogels with varying topography, no significant advantages were observed, suggesting the

insensitiveness of topography effect towards mESC stemness. However, surface topography might have a role on substrates with hexagonal or pillar shapes topology or even on stiffer surfaces. It is also believed that either stiffness or topography might play an independent decision factor towards colony formation on 3D substrates. Geometric control of osteogenesis was also studied on polyacrylamide gels prepared by microcontact printing of adhesion proteins where cell spreading and cell geometry are suggested to have some influence towards MSCs differentiation^[106]. Stiffer matrices can assist to increase cell spreading, thus promote osteogenesis through enhanced actomyosin contractility and this was observed up to a stiffness of 30 kPa. However, the density of seeded cells were described to over-ride the effect of gel stiffness to cause osteogenic differentiation, according to Xue *et al.*^[107]. However, this effect was not pronounced on soft gel, where chondrogenic differentiation was induced. Overall, their study highlights the importance of cell-matrix and cell-cell interactions in causing MSCs differentiation. Substrate stiffness was also found to play a predominant role in regulating the growth of rat BM-MSCs, while substrate topography manipulate the cell morphology and spreading, with spherically shaped cells were observed on pillar substrates but not on grooved substrate^[108].

In general, studies using mechano-variant polyacrylamide substrates showed that the osteogenic differentiation reduced on substrates with low stiffness and ligand density. However, compared to fibronectin, BMSCs were demonstrated to have a higher binding affinity towards an equivalent bulk of collagen (Figure 5). Such comparisons were further delineated by Sharma *et al.*^[109], and they found the occurrence of osteogenic differentiation on fibronectin substrates, while tenoblast gene expression was only found on collagen substrates. Two entirely different microsystems namely, sponges and hydrogels were compared by Zhang *et al.*^[110] where they studied the behavior of MSCs and they found fast cell proliferation in sponges than in hydrogels. Sponges might have encouraged cell-cell and cell-matrix interaction and upon inducing them with media containing TGF- β 3, the chondrogenic differentiation of MSCs were better compared to those in hydrogels.

Nerve

From Figure 4 and the abovementioned researches, it can be indicated that for neural differentiation, scaffolds with relatively small stiffness is required. The fate of MSCs towards neural or glial lineages could be manipulated by adjusting the substrate stiffness. Three dimensional collagen-HA scaffolds (fabricated by lyophilization) with higher elasticity (E approximately 1 kPa) induced MSCs into neuronal differentiation, while those with less elasticity (E approximately 10 kPa) induced MSCs into glial differentiation. The elasticity was adjusted by using different concentration

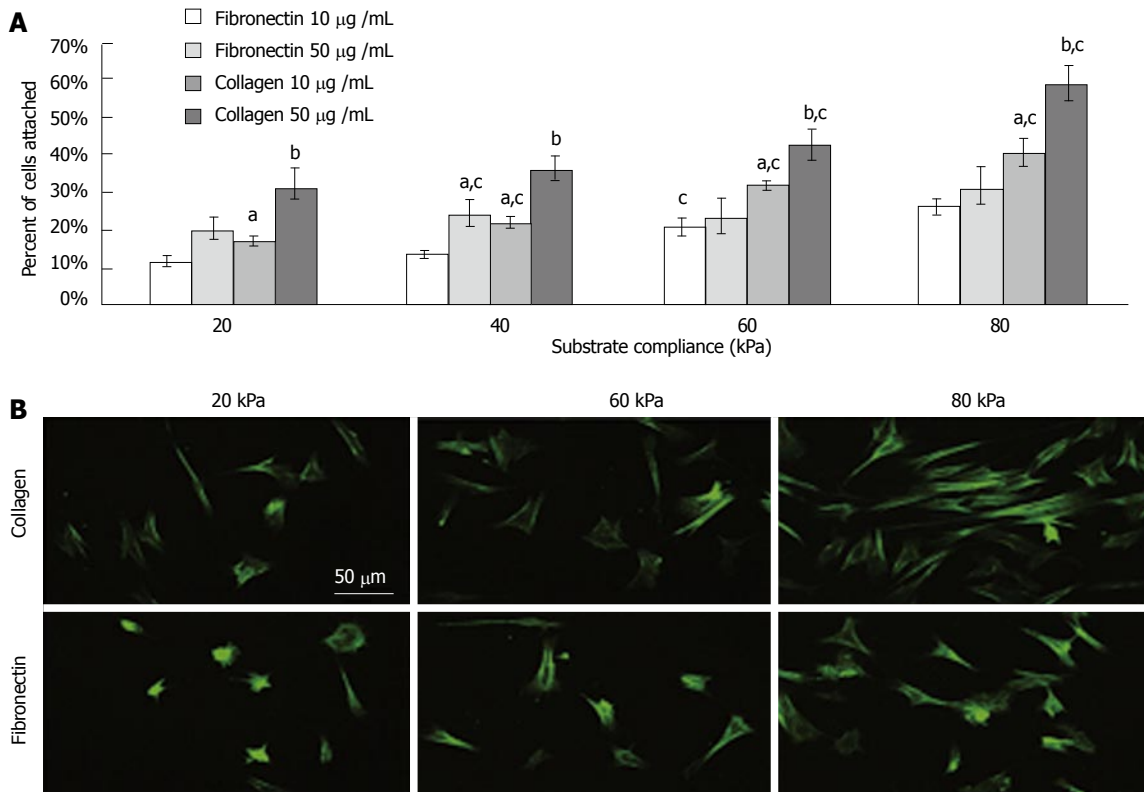


Figure 5 (A) As the rigidity increased, so did cell attachment. Collagen elicited a greater degree of cell attachment, and increased bulk density of the ligand further enhanced this (B) Morphology on functionalized substrates at 8000 cells/cm² for 24 h. ^astatistical difference compared to fibronectin at 10 mg/mL, ^bdifference compared to collagen at 10 mg/mL, ^cdifference compared to preceding substrate compliance. Cells were stained with FITC-phalloidin. (Scale bar represents 50 µm). Reproduced with permission from Sharma *et al*^[109].

of the crosslinking agent, namely 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC)^[111]. Tuning the modulus of hydrogels affects neurite outgrowth and ESCs differentiation; with higher modulus gels offered better substrate for neural differentiation and neurite outgrowth, while less modulus enhanced astrocyte formation and cell migration. Thus, ESCs differentiation into neural and glial lineages is influenced by the type, composition, architecture and stiffness of the matrix and the type and concentration of signaling molecules comprehensively^[112]. Neuronal differentiation was favored on softer surfaces such as the photopolymerizable methacrylamide chitosan hydrogel with Young's modulus less than 1000 Pa as confirmed by both immunohistochemistry and qRT-PCR. Oligodendrocyte differentiation was favored on stiffer scaffolds with Young's modulus more than 7000 Pa. However, myelin oligodendrocyte glycoprotein gene expression suggested that oligodendrocyte maturation and myelination was best on scaffolds with modulus less than 1000 Pa where more mature neurons were present. Astrocyte differentiation was only observed on scaffolds with modulus less than 1000 Pa and 3500 Pa and represented less than 2% of the total cell population^[113]. Elastic moduli, even in the range of neural tissue, would have a great impact on adult neural stem cell differentiation. The adult NSCs expressed a high level of the neuronal marker,

β -tubulin III, on variable moduli interpenetrating polymer networks of 500 Pa, which is similar to the physiological stiffness of brain tissue. Furthermore, under mixed differentiation conditions with serum, softer gels (approximately 100-500 Pa) greatly favored neurons, whereas harder gels (approximately 1000-10000 Pa) promoted glial cultures^[114].

To achieve the scaffold with favorable stiffness for nerve tissue regeneration, the fabrication method and the materials should be carefully considered. Hydrogels possess low modulus, and they are suitable as substrates for neuronal differentiation of stem cells. Alternatively, polymers with low modulus might be appropriate for this purpose. Within the category of the neuronal tissue structures, glial differentiation of stem cells might be gained on stiffer scaffolds, and neural differentiation can be achieved on softer scaffolds. By fine tuning the stiffness of the matrix, there is a great possibility to control the differentiation of stem cells into appropriate nerve cells.

Cardio-vascular

Lineage commitment of MSCs towards specific vascular cells (MSCs to ECs or MSCs to SMCs) on electrospun scaffolds could be manipulated by controlling the substrate modulus, from 2 to 15 kPa. Around 95% of MSCs seeded on soft scaffolds (3 kPa) showed Flk-1 endothelial markers within 24 h, while only 20% of

MSCs seeded on the rigid scaffolds (> 8 kPa) showed Flk-1 marker. In contrast, about 80% of MSCs seeded on rigid scaffolds (> 8 kPa) showed smooth muscle α -actin marker within 24 h, while fewer than 10% of MSCs seeded on soft scaffold (< 5 kPa) showed α -actin markers^[115]. Besides, the synergistic effect of scaffold elasticity and growth factors has been studied for the differentiation of MSCs. In one such study, MSCs were seeded on soft nanofibrous matrices with or without VEGF, and in Petri dishes with or without VEGF, where these researchers found that MSCs in soft matrices with VEGF showed significant increase in the expression of endothelial markers (vWF, eNOS, Flt-1, and Flk-1), with faster up-regulation of the endothelial markers. The results indicate that it is critical to control both mechanical factors and biochemical factors to regulate vascular endothelial regeneration of MSCs^[116]. MSCs gained very low rate of cardiogenic differentiation after transplantation to infarcted heart, partly because stiffer scar tissue lack the capacity to support cardiogenic differentiation. Thermosensitive and injectable hydrogels with different moduli (16, 45 and 65 kPa) were achieved by controlling the concentration of the hydrogel solution. After 14 d, more than 76% MSCs differentiated into cardiac cells in gels with 45 and 65 kPa, while MSCs in the 65 kPa gel had the highest differentiation efficiency^[117]. But the effect of stiffness towards the differentiation of stem cells might be time-dependent. Human embryonic stem cells are sensitive to substrate stiffness during early mesodermal specification, but not late fate choices during cardiac differentiation, when they are seeded on polyacrylamide hydrogels. An intermediate stiffness was most beneficial for cardiomyogenic differentiation than soft or stiff substrates^[118].

CONCLUSION

Selection of appropriate biomaterials for scaffold fabrication and their modification by incorporation of biological constituents, or by patterning, thus altering the topography and mechanical properties of the scaffolds, might have a significant effect on stem cells behavior. Major literature review shows that MSCs are in favor of soft substrate to differentiate to neuronal cells, with moderately stiffer materials promote myogenic differentiation and more stiffer substrates support osteogenic differentiation, suggesting the effect of substrate mechanical properties in influencing the differentiation of stem cells toward specific lineages. The stiffness of mature tendon range between 500-1000 MPa and bone falls in the range of 20 GPa and this might be a reason for such specific behaviors of stem cells on substrates with proportionate mechanical properties. Prioritizing the stiffness of the substrate alone might not serve as a smart approach, but the presence of specific ligands along with the matrix mechanical compliance, might assist for more specific stem cell differentiations. There exists great

difficulty in comparing the results obtained by different research groups, mainly because the parameters such as the ligand type, their concentrations, surface topology or matrix type and their mechanics, and even the cell numbers differ between studies. Hence more uniform and systematically organized studies, are required for a thorough understanding of the stem cell differentiation effect. The extent of differentiation or the % of differentiated cells is another parameter that deserves great attention, such that the cell-substrate can be used for its maximum potential.

The cells on a 2-D platform might be less sensitive to mechanical properties than when they are in 3-D environment. The 3-D structures might provide an additional dimension to mechanical properties, such that the cell related factors such as the integrin ligation, cell contraction, and associated intracellular signaling might be influenced.

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Systems biology approach to developing S²RM-based “systems therapeutics” and naturally induced pluripotent stem cells

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biology has been successfully used to analyze multiple pathways, the approach is often used to develop a small molecule interacting at only one pathway in the system. A new model is emerging in biology where systems biology is used to develop a new technology acting at multiple pathways called “systems therapeutics”. A natural set of healing pathways in the human that uses SRM is instructive and of practical use in developing systems therapeutics. Endogenous SRM processes in the human body use a combination of SRM from two or more stem cell types, designated as S²RM, doing so under various state dependent conditions for each cell type. Here we describe our approach in using state-dependent SRM from two or more stem cell types, S²RM technology, to develop a new class of therapeutics called “systems therapeutics.” Given the ubiquitous and powerful nature of innate S²RM-based healing in the human body, this “systems therapeutic” approach using S²RM technology will be important for the development of anti-cancer therapeutics, antimicrobials, wound care products and procedures, and a number of other therapeutics for many indications.

Key words: Stem cell; Paracrine; Growth factors; Pluripotency; Wound healing; Cancer

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Abstract

The degree to, and the mechanisms through, which stem cells are able to build, maintain, and heal the body have only recently begun to be understood. Much of the stem cell's power resides in the release of a multitude of molecules, called stem cell released molecules (SRM). A fundamentally new type of therapeutic, namely “systems therapeutic”, can be realized by reverse engineering the mechanisms of the SRM processes. Recent data demonstrates that the composition of the SRM is different for each type of stem cell, as well as for different states of each cell type. Although systems

Core tip: A fundamentally new type of therapeutic, namely “systems therapeutic,” can be realized by reverse engineering the mechanisms of the stem cell released molecules (SRM) processes. Recent data demonstrates that the composition of the SRM is different for each type of stem cell, as well as for different states of each cell type. Although systems biology has been successfully used to analyze multiple pathways, the approach is often used to develop a small molecule interacting at only one pathway in the system. A new model is emerging in biology where systems biology is used to develop a new technology acting at

multiple pathways called “systems therapeutics”.

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INTRODUCTION

The initial few days following fertilization of the human egg, all stem cells in the developing egg are able to create any tissue in the human body, *i.e.*, the stem cells are totipotent. However, about four days following the fertilization of a human egg, the stem cells in the blastocyst begin to differentiate and become pluripotent instead of totipotent, thus being able to differentiate into a more limited set of adult cell types^[1]. At this point in time, many different stem cell types are beginning to form that will have unique function related to the development, maintenance, and healing of various tissues throughout the body. The degree to which stem cells differentiate into specific adult phenotypes is only recently beginning to be understood. For example, cell types, such as the progenitor cell preadipocyte and adipose-derived mesenchymal stem cells, each of which was previously classified as one cell type, have now been shown to have phenotypic differences depending on the location of the preadipocyte niches or mesenchymal stem cell niches^[2,3]. The signaling factors controlling the development and function of the stem cell types, and indeed the signaling factors that each stem cell type releases, are relatively unknown, but progress is being made. For example, we know that adult stem cells release hundreds of types of proteins within the molecular pool^[4], called the stem cell released molecules (SRM), and that each cell type will release a unique pool of molecules^[5,6]. These molecules in the SRM will prove to be important for developing many types of therapeutics, including, for example, immunoregulators for organ transplantation^[7].

The more differentiated the stem cell, the more specialized the SRM will become. Further, that unique pool of molecules from one stem cell type can change in composition, including the types of molecular species, depending on intrinsic and extrinsic regulatory factors. For example, intrinsic factors related to simple passage number of a stem cell will change the composition of the SRM^[8,9] and mesenchymal stem cells (MSCs) in different parts of the body will secrete unique pools of SRM^[10]. Telomere dysfunction, whether the cause is intrinsic or extrinsic, will change the nature of the SRM^[11]. Likewise, when MSCs derived from fat tissue are conditioned with TNF- α , a significant effect on the SRM is observed with an increased release of factors

such as Cathepsin L, interleukin (IL)-6, IL-8, monocyte chemotactic protein-1, matrix metalloproteinase (MMPs), and pentraxin-related protein 3^[12]. Further, signaling conditions during the immune modulating responses of human MSCs through Toll-like receptors (TLRs) on the MSCs leads to two basic phenotypic changes of the cells (MSC1 and MSC2) and a consequent dramatic difference in their SRM^[13]. Phenotypic changes in the MSC are consequent to: (1) low-level exposure of TLR4 agonists that drives hMSCs toward a pro-inflammatory MSC1 phenotype important for early injury responses; and (2) the TLR3 agonist exposure of hMSCs driving the phenotype to an immunosuppressive MSC2 phenotype that is important to later anti-inflammatory responses that help repair the wound. Culture conditions can also have dramatic effects on SRM. A significant increase in SRM [Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), phosphatidylinositol-glycan biosynthesis class F protein, and TGF- β] was observed after subjecting hMSCs to 72 h hypoxia compared with normoxic conditions^[14]. Serum deprivation is another model for ischemia, and was shown to increase the secretion of angiogenic factors released by hMSCs, although the results could have been attributed in full, or part, to differences in cell proliferation rates^[15]. Glucose levels have been shown to differentially affect the phenotype of endothelial progenitor cells and mesenchymal progenitor cells^[16]. Indeed, subtle variations in cell culture conditions can have significant consequences to the phenotype of stem cells^[17].

The state of the extracellular matrix in the stem cell niche is also an important regulator of stem cell phenotype, where, for example, the absence of the SPARC protein in the extracellular matrix (ECM) can drive hematopoietic stem cells into a state of quiescence^[18]. Antioxidants and FGF-2 were shown to cause rapid proliferation and a retention of stem cell properties in MSCs, and even enhanced their adipogenic and osteogenic potentials^[19]. Interestingly, new studies suggest that adult stem cells, and even somatic cells, may exist in a state of dynamic transition between different levels of potency^[20,21] that is dependent on many factors, including paracrine and autocrine factors in the SRM from surrounding stem cells in the same niche, and through the physical state of the surrounding stem cell niche^[22]. The state of the oxidative stress in the stem cell may be a contributing factor in phenotype, including the state of pluripotency where the antioxidants curcumin and sesamin were shown to decrease oxidative stress and increase pluripotency^[23]. Stem cell derived control factors for determining the fate of stem cells and the potency of cells, including the dedifferentiation of somatic cells, their proliferation, and subsequent differentiation, may include growth differentiation factor 11, a protein secreted by bone marrow mesenchymal stem cells^[24] that has been shown to be involved in stem cell fate

and proliferation^[25], and has recently been shown to induce a number of regenerative effects, including neurogenesis^[26]. Recent studies also demonstrate that NANOG, a pluripotency transcription factor in embryonic stem cells, is also present in at least some adult tissues further raising the possibility that a dynamic state of pluripotency is a naturally occurring process in adults^[27]. Again, these shifts in the state of pluripotency will have concomitant shifts in the composition of the SRM released from the stem cell.

Given the differentiation of stem cells into distinct phenotypes, each of which releases a distinct pool of molecules with each distinct pool of molecules inducing a specific set of functions, a knowledge base of the secreted factors (SRM) from each stem cell type and the resulting actions from each pool of molecules will be instructive in the development of therapeutics. The resulting therapeutics that can be developed using the combination of many types of molecules has been termed "systems therapeutics"^[28]. The "systems therapeutic" approach, where multiple molecule types target multiple pathways, is in contradistinction to the more traditional approach of small molecule development for perturbation of one pathway.

Moreover, as the stem cell types are cultured *ex vivo* in the laboratory and driven to state-dependent specific phenotypes through genetic, epigenetic, and other state-dependent variables, the concentration and composition of the SRM as a result can be experimentally manipulated for the purposes of therapeutic development. In addition, distinct pools of SRM from two or more stem cell types cultured in state-dependent conditions can be combined into a collective pool of molecules called S²RM, mimicking the collective actions of multiple stem cell types in their native state in the human body.

TWO OR MORE TYPES OF STEM CELL INDUCE HEALING

There are two basic forms of cell replacement and regeneration: (1) a maintenance function where renewal of damaged cells during tissue homeostasis (homeostatic growth) are restored; and (2) a response to external injury, such as traumatic wounding, burn, ulceration, or surgery. A given healing response will require many processes acting through a well-orchestrated concert of mechanisms and molecules in the given tissue, and the results of these processes depend on many factors, including the developmental age of the organism. Fundamental factors, such as caspases released from cells undergoing apoptosis, will activate both stem cells and progenitor cells in the wound healing process^[29], where apoptosis may be a key factor in cell proliferation during tissue regeneration^[30]. Wounds occurring in early to mid-gestational fetal skin have been shown to heal through regeneration without the formation of a scar^[31],

whereas adult wounds heal by a fibroproliferative response that emphasizes repair over regeneration. The complexity of this process, where fetal wounds differ from adult wounds in inflammatory responses, ECM components, growth factor expression and responses, and profiles of gene expression is exemplified by the observation that adult skin in a fetal environment will still exhibit scar formation^[32]. The state dependency of stem cells is so critical as shown in diabetes where the adipose stem cell niche *in situ* is altered, and the stem cells in the diabetic state are compromised in their ability to establish a vascular network both *in vitro* and *in vivo*^[33] where glucose itself has profound direct effects on stem cells^[34].

Following injury, wound healing begins rapidly and involves resident and migratory stem cell types, ECM, and soluble factors, including SRM. Stem cells and progenitor cells resident in the skin are certainly involved, but recruitment of stem cells from other sources, including bone marrow, is thought to be important^[35]. The mechanisms underlying wound healing include: (1) a rapid release of pro-inflammatory mediators; (2) cell to cell, and cell to extracellular matrix interactions that help mediate cell proliferation, migration, and differentiation; (3) a cascade of events including epithelialization, fibroplasia and angiogenesis^[36]; (4) contraction of the wound; and (5) remodeling of the tissue. These events begin at the time of physical injury and proceed continuously throughout the process of tissue repair. Although the processes of repair begin immediately after an injury in all tissues, and all wounds proceed with a similar cascade of healing, some tissues, for example, liver, skeletal tissue, and the eye have different forms of regeneration and repair with variations on the underlying mechanisms^[37]. Severe injury has been shown to increase the number of circulating stem cells^[38,39] and that these stem cells will participate in the wound healing process^[6].

At the onset of trauma bone marrow stem cells will sense histamine released from platelets at H1 receptors and change their phenotype to one of releasing more IL-6 and more IL-8. The increased IL-8 will attract polymorphonuclear neutrophil cells, and the increased IL-6 will facilitate their survival through antiapoptotic functions^[40]. When the trauma inducing the injury has stopped, and hemostasis is achieved with an immune response activated, the tissue repair phase will then begin^[41]. On the third day after wounding the proliferative phase starts and continues for two or more weeks thereafter. Proliferation begins with fibroblast migration and deposition of newly synthesized ECM, elaborating the initial network of tissue built by fibrin and fibronectin. This phase of wound healing can be clinically observed as an abundant formation of granulation tissue. The complex nature of the proliferative phase is briefly described below^[42].

First, fibroblasts and myofibroblasts in the tissue surrounding the wound are stimulated to proliferate for 3 d^[43]. The fibroblasts and myofibroblasts then migrate from the surrounding tissue into the wound, attracted by soluble factors TGF- β and platelet-derived growth factor (PDGF) that are released by platelets and inflammatory cells^[44]. Appearing in the wound on the third day after injury, the accumulation of fibroblasts in the wound requires their phenotypic modulation. Within the wound, the fibroblasts greatly proliferate, producing and locally releasing the matrix proteins hyaluronan, fibronectin, proteoglycans, and type 1 and type 3 procollagen^[45]. Abundant new ECM has accumulated at the end of the first week, further supporting cell migration that is essential for the repair process. Fibroblasts then change to a myofibroblast phenotype. The myofibroblast phenotype contains thick actin bundles that extend below the plasma membrane with pseudopodia attaching to fibronectin and collagen in the ECM. Wound contraction, critical to the reparative process by closing the wound margins, then takes place as the actin bundles begin to retract. Any overabundance of unneeded fibroblasts are then eliminated by apoptosis^[44].

The three phases of wound healing involve MSCs to varying degrees, whereby, for example, they recruit macrophages to the wound site^[46], induce the fibroblast response to injury^[47], and remodel the wound site^[48], including a preferential release of collagen type III at the site^[35]. While the MSCs contribute directly to wound repair by releasing molecules such as collagen to the wound^[35], the MSCs also act indirectly by releasing an instruction set to other cells thus initiating, for example, progenitor cell migration to the wound^[49]. The MSCs are key to the wound's ability to proceed beyond the inflammatory phase and not maintain a chronic wound state. A significant early component of the mechanism of action of MSCs is their attenuation of the inflammatory response. The addition of MSCs to an active immune response decreases secretion of the proinflammatory cytokines TNF- α and interferon- γ (IFN- γ) while simultaneously increasing the production of anti-inflammatory cytokines IL-10 and IL-4. These anti-inflammatory properties of MSCs impart a particular benefit to chronic wound treatment through SRM, given the SRM can restart healing in chronic wounds by advancing the wound past a chronic inflammatory state into the next stage of healing.

Many studies have shown that MSCs possess antimicrobial activity, critical for clearance of infection in the wound. The antimicrobial activity of MSCs is mediated by two mechanisms: (1) direct secretion of antimicrobial factors such as LL-37^[50]; and (2) indirect, by secreting immune-modulating SRM that will upregulate the bacterial killing and phagocytosis of immune cells^[51]. Further, the phenotype of macrophages can be regulated by MSCs into various M1 and M2 classes directed to either antimicrobial, phagocytic

activity, or one of controlling inflammation^[52].

Endogenous MSCs migrate to sites of injury in response to chemotactic signals where they can then modulate inflammation, repair damaged tissue, and facilitate tissue regeneration. Furthermore, bone marrow stem cells home to the injury where cells in the wounded area secrete a protease that interacts with collagen matrix to produce a homing agent^[53]. Differentiation and paracrine signaling are two key mechanisms used by MSCs for tissue maintenance and repair. While differentiation of MSCs contributes by directly regenerating damaged tissue, the paracrine signaling by MSCs regulates the local cellular responses to injury, including the differentiation process itself. However, studies of exogenous MSCs show that the contribution of differentiation of these stem cells is limited due to poor engraftment and survival of MSCs at the site of injury, whereas the activation of endogenous stem cells by SRM may provide better results for the differentiation pathway^[54]. Paracrine signaling by MSCs appears to be the primary mechanism for the beneficial effects of MSCs in wound healing, including the reduction of inflammation, enhanced angiogenesis, and induction of cell migration and proliferation^[55].

An analyses of the conditioned medium indicate that MSCs secrete many known SRM mediators of tissue repair including growth factors, cytokines, and chemokines, including VEGF, PDGF, bFGF, EGF, keratinocyte growth factor (KGF), and TGF- β . Stem cells are also known to release exosomes^[56], and exosomes from mesenchymal stem cells have been shown to contain factors, including miRNA, that switch cancer stem cells into a dormant state^[57]. Such a mechanism is important to dampen the cells in a wound from moving into a state of cancer^[58]. Many cell types, including epithelial cells, endothelial cells, fibroblasts, and keratinocytes are responsive to MSC paracrine signaling, where a number of different cellular responses including cell survival, proliferation, migration, and gene expression are regulated. The SRM from MSCs acts as a chemoattractant for dermal fibroblasts, macrophages, endothelial cells, and epidermal keratinocytes, *in vitro*. The presence of either MSCs or the SRM from MSCs have been shown to promote wound closure through the activation of dermal fibroblasts. MSCs also secrete mitogens, leading to the proliferation of keratinocytes, dermal fibroblasts, and endothelial cells *in vitro*. Further, dermal fibroblasts secrete increased amounts of collagen type I and alter gene expression in response to either MSCs in co-culture or the SRM from MSCs. These data suggest that SRM from MSCs stimulate proliferation and migration of the key cell types in the wound. In addition, the SRM of MSCs imparts anti-scarring properties to wound healing through the secretion of VEGF and hepatocyte growth factor (HGF), and through maintaining a normal balance between

TGF- β 1 and TGF- β 3. The pathways underlying MSC processes in wound healing are complex, and further details of these processes can be found in recent reviews. Stem cell niches in other regions of the body, including the hematopoietic stem cell niche, appear to be equally complicated as the skin stem cell niche with a rich interaction amongst many cell types, including a number of stem cell types and their respective SRM^[59].

NATURALLY INDUCED PLURIPOTENT STEM CELL WITHIN THE STATE DEPENDENT STEM CELL NICHE

Naturally occurring endogenous iPSCs, or naturally induced pluripotent stem cells (NiPSCs) occur within the state dependent stem cell niche. While induction to a totipotent state has not been realized, dedifferentiation seems to be an important adaptive mechanism in both the animal^[60] and plant kingdoms^[61] where cells can be induced to become pluripotent. In addition to the therapeutic development of embryonic stem cells and iPSCs, the use of adult stem cells and the molecules that they release have been intensively investigated and have current therapeutic applications. Further, SRM from stem cells or other molecules from neighboring cells, such as ciliary neurotrophic factor (CNTF), have been shown to dedifferentiate myoblasts into multipotent progenitor cells. The dedifferentiated myoblasts were then able to differentiate into several new phenotypes^[62].

The endogenous mechanisms of adult stem cells, and possibly somatic cells in the stem cell niche, seem to include the ability to reprogram themselves into more primordial states that are pluripotent. That is, the adult stem cell, and even somatic cells, may exist in a state of dynamic transition between different levels of potency that is dependent on many factors, including paracrine and autocrine factors in the SRM from surrounding cells in the stem cell niche, and by the physical, chemical, and electrical state of the stem cell niche^[63-65]. Recently, treatment with reversine, a type of purine, transformed 3T3-L1 preadipocytes into MSC-like cells, as evidenced by the expression of MSCs marker genes. The transform allowed differentiation of lineage-committed 3T3-L1 preadipocytes to osteoblasts under the osteogenic condition *in vitro*^[66]. Beyond transcription factors contained in the SRM, physical manipulation through the cytoskeleton is known to transmit signals to the chromatin^[67] and reprogram cells^[68], and may represent an additional biophysical, in addition to biochemical means, for driving cells to varying levels of potency. Reprogramming of differentiated cells to stem-like cells has been described in several tissues and is well studied in the epithelial-mesenchymal transition where a differentiated epithelial cell transforms to a mesenchymal cell with a stem cell-like phenotype. Thus, by understanding adult stem cell

function, we may develop the means to use these cells in many ways to maintain and heal the body, including a means of controlling naturally occurring iPSCs (NiPSCs).

The physical, chemical, and electrical state of the stem cell niche will have profound influences on stem cell function. Alterations of the stem cell niche in diseases such as diabetes will decrease the ability of endogenous stem cells, or autologous administered stem cells, to increase neovascularization and promote wound healing^[33].

In Figure 1, we see levels of interactions that may control the natural iPSC state. Considering wound healing as described in the aforementioned section, many factors, such as histamine, an important regulator of cell fate, including neurons^[69,70] are released at the site of injury. As an example of the actions of these factors, histamine will activate TRPM4 calcium channels in the mesenchymal stem cells and bias the dynamic transition of the stem cells toward differentiation into the needed mature cells types at the injury site^[71], including osteoclastogenesis^[72]. Similarly, exposure to sunlight will stimulate vitamin D3 levels and induce differentiation of stem cells, doing so through a downstream pathway that includes histamine^[73].

Reprogramming of cells to push the dynamic transition towards more potency has been specifically shown in mammalian cells whereby muscle cells^[74] and pancreas cells^[75] will dedifferentiate into a more pluripotent state following injury, and where fibroblasts were incubated in the SRM of adipose-derived stem cells. The fibroblasts displayed gene expression that was indicative of pluripotency in which repressive histone modifications were reduced, and increased global demethylation was present. The *Col1a1* and *Col1a2* genes, typically found in differentiated cells only, demonstrated reduced expression, and also demonstrated increased methylation in the 5'-flanking regulatory regions^[76]. Of the many factors released by mesenchymal stem cells, microRNA is one of the factors that have been shown to induce pluripotency in mouse and human somatic cells^[77]. In general, stress is a key factor that can naturally induce pluripotency. For example, simple isolation of mammalian cells from contact with other cells and their normal niche, originally exhibiting a limited differentiation potential, may become multipotent^[78]. Pluripotent cells can reside in the naïve state or the primed state where the naïve state is more potent than the primed state^[79]. Dedifferentiation under hypoxic conditions can drive committed cells beyond the primed state fully back to the naïve state of potency where the pluripotent cells are then capable of forming teratomas^[80].

Cancer cells and pluripotent stem cells follow certain common rules. Both cell types, when placed in a dysregulated extracellular matrix, will exhibit an increased state of potency. Cancer cells, when returned to a regulated ECM, will revert to a normal phenotype^[58,81]. Likewise, dedifferentiation of cells into

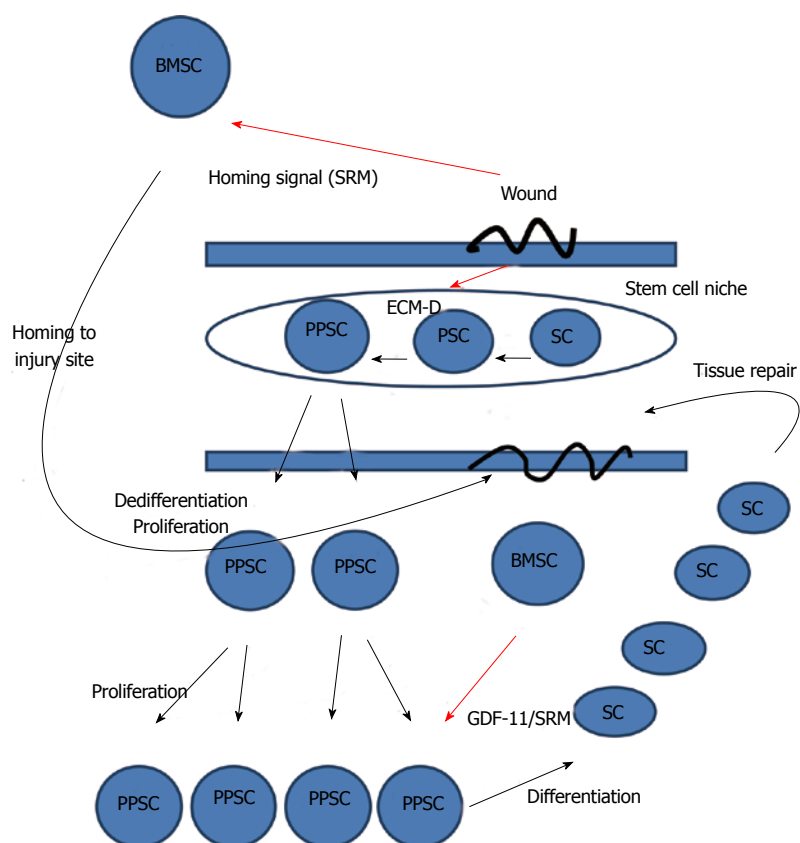


Figure 1 General model of wound healing. The wounded state sends a homing signal to bone marrow stem cells and disrupts the ECM. Disruption of the ECM will shift the dynamic transition of potency towards dedifferentiation and the more pluripotent state. The more pluripotent state will cause the cells to proliferate. After proliferation, the migration of bone marrow stem cells to the wound site will release stem cell released molecules, including GDF-11, that stops proliferation and induces differentiation allowing newly differentiated somatic cells to repair the tissue. Thus, in our model, GDF11 is released from BMSCs and is a master regulator of stem cell transcription that inhibits cell proliferation and migration by down-regulating the expression of numerous genes involved in both these processes^[93]. ECM-D: Extracellular matrix disruption; SC: Somatic cell; PPSC: Pluripotent stem cell; BMSC: Bone marrow stem cell; PSC: Potent stem cell.

a pluripotent state can occur when the cell is isolated and loses connections with other cells and the ECM^[78], and stem cells that have differentiated can revert to a more pluripotent state by changes in the concentration of the ECM associated protein, L-proline^[82]. Thus, induction of pluripotent stem cells is a naturally occurring phenomenon that can be controlled *in vivo* for therapeutic effect by manipulating the state of the stem cell niche.

CONCEPTS OF A SYSTEMS THERAPEUTIC

An abnormality in one pathway, or even at one level of the organism, such as at the level of genes, does not explain a disease. Rather, disease reflects the perturbations of the complex system of biological pathways acted on by a complex set of environmental regulators. Most previous work to understand disease and drug response traits have focused on single dimensions, and even single pathways, of the system. Achieving a more comprehensive and predictive understanding of disease and drug response requires examining living systems in multiple dimensions and

at multiple scales. Although biological engineering principles are necessary, with the requirement to remove superfluous complexity for the development of a particular therapeutic, the individual components of complex systems are highly coupled such that the individual components cannot be analyzed in isolation. This predicament in biology, such as the desire to place the sequencing of the genome as the singular predictor of disease, is similar to that dictum in physics where electrodynamics was broken down into the misbegotten particles and fields theorem by Bohr and his Copenhagen interpretation^[83]. Biological complexity is an extreme example of complexity, arising from a biological system that includes active, plastic components, nested feedback loops, flexible design principles, component multi-functionality, and multiple layers of system dynamics developed through evolutionary processes that are, at least partially, driven through the downward causation of environmental regulators. The power of the dynamic biological system has been recognized in engineering where, for example, neuromorphic engineering^[84] has become an important player in the development of new computer chip technologies such as TrueNorth^[85].

Despite the use of systems analysis in the fields

of biology and therapeutic development, therapeutic development has often remained as one using systems biology techniques for finding the one pathway, or the one target, that is best perturbed to develop the therapeutic. "Finding the magic bullet" is a common phrase that describes this common problem. A shift in mindset to one of finding the minimum set of pathways, or the minimum set of targets, using the "minimum molecule set" to perturb these targets in order to best develop a therapeutic is now needed. Thus, biological function results from a system, and a particular disease state is the result of multiple perturbations in that system, not just one perturbation. Therefore, through an understanding of complex pathways in normal and disease states, and using computationally intensive biological design-build-test-analyze cycles, with therapeutic molecule production batches based on this process, we can hope to develop safe and efficacious therapeutics. This will occur through a multi-targeted, "systems therapeutic" approach. The approach then is to use a system of molecules, the minimum molecule set, that is not overly reductionist so as to be ineffective, but instead use the optimal number of molecule species that are sufficient to realize a safe and efficacious therapeutic. Recognizing that diseases are the result of complex interactions among many networks has significant implications for drug discovery, leading to the design of combinations of molecular species that impact entire network states, rather than designing reductionist drugs that target specific genes that are associated, often weakly, with disease.

DEVELOPMENT OF SYSTEMS ANTIMICROBIALS

The attempt to develop animal-derived antimicrobials is not new. For example, in the 1990s great hope, and many dollars spent, was placed on the development of a small peptide from frog (*Xenopus laevis*) skin as an antibiotic^[86]. The observation that frog skin heals itself, despite the frog living in a very septic environment, led to the formation of Magainin Pharmaceuticals. After years, and millions of dollars, spent on development and Phase II clinical trials, today Magainin's assets are the auction block (Magainin changed names to Genera and then liquidated: <http://www.fiercebiotech.com/press-releases/genaera-corporation-announces-approval-plan-liquidation-and-dissolution-board-direct-0>). Why? Because the frog's skin does not heal itself through a reductionist approach with only one molecule (a peptide), and Magainin didn't fully learn the frog's lesson. The lesson not learned was that Magainin developed their antibiotic based on one peptide, a reductionist approach, instead of a mix of antimicrobial factors, a systems antimicrobial approach.

Lipids were first demonstrated by Koch^[87] to have antibiotic activity, and exists in human skin, for example, as a wide range of molecule types comprising

a significant part of the innate immune system^[88]. Like Magainin, a similar reductionist approach was used in the development of squalamine, a lipid compound (aminosterol) derived from the dogfish shark (*Squalus acanthias*). Squalamine was initially discovered on the basis of its anti-bacterial activity, and has broad spectrum antimicrobial activity against fungi, protozoa, and many viruses^[89]. Sadly, isolated squalamine was never approved for antimicrobial use and is now sold as a nutritional product by a number of companies in capsule form. Once again, the "Copenhagen reductionist" approach to therapeutic development has failed us. Here again, instead, an approach to developing antimicrobials using a collection of molecules, including peptides and lipids, is in development.

DEVELOPMENT OF CANCER SYSTEMS THERAPEUTIC

Cancer is strongly associated with a deregulated ECM^[58,90]. While cancer and stem cells are regulated by many factors, both cancer cells and pluripotent stem cells follow certain common rules such as regulation by the ECM. Both cell types, when placed in a dysregulated extracellular matrix, will exhibit an increased state of potency. Cancer cells, when returned to a regulated ECM, will revert to a normal phenotype^[58,81]. As beautifully explained by Mina Bissell at University of California Berkeley, during development, cells can spatially arrange themselves, differentiate, and change their SRM composition in response to a variety of signals in the microenvironment, including morphogens, biophysical manipulation, juxtacrine signals, and the ECM. All of these components in the microenvironment are sensitive to signals from other tissues and organs of the developing embryo as well as through downward causation from the macroenvironment. However, following organ formation, the microenvironment/ECM integrates and constrains the organ architecture and function, thus ensuring structural and functional homeostasis and therefore, a normal organ phenotype. However, when the organ architecture in adults is insulted by mutations and/or changes in the microenvironment such as ECM dysregulation and/or inflammation, that organ is transformed by the initiation of developmental and embryonic circuits. However, in the adult, the microenvironment is no longer embryonic in nature, and the ECM dysregulation and inflammation leads to a pluripotent state, *i.e.*, the cancerous state. Bissell argues that tumors become new evolutionary organs searching for homeostasis^[58]. Recent work fits the paradigm of Bissell, such as that of Liou *et al.*^[91] who describes the detailed steps that Kras-mutated acinar cells follow as they change into duct-like cells with a more potent state. They observed that Kras proteins in the acinar cells switched on

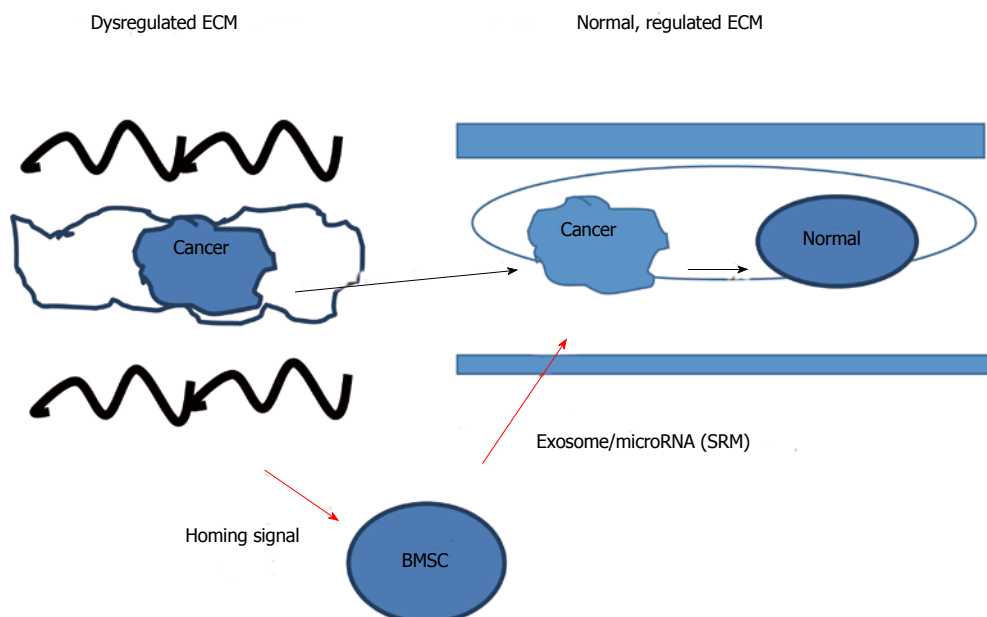


Figure 2 Regulation of the cancer/pluripotent phenotype by stem cells and extracellular matrix. The cancer/pluripotent cell phenotype can be regulated by the extracellular matrix (ECM) and stem cells, where cancer cells can be removed from a dysregulated ECM and placed into a normal ECM and the cancer/pluripotent phenotype will revert to a normal, somatic cell phenotype. Likewise, if a dysregulated ECM is reconstructed into a normal state, the cancer/pluripotent phenotype will revert to the normal somatic cell phenotype. Further regulation of the cancer/pluripotent phenotype can be regulated by a number of factors, including microRNA contained within exosomes that were released from mesenchymal stem cells serving to change the state of the cancer cell into one of quiescence. MBSC: BM-stroma cell.

intercellular adhesion molecule-1. This in turn attracts macrophages. The macrophages then release a variety of proteins, including MMPs that degrade ECM. Following dysregulation of ECM, the acinar cells then transform into the stem cell-like phenotype. Thus, the direct link between Kras mutations, the inflammatory environment, and dysregulated ECM that drive the initiation of pancreatic cancer is demonstrated.

Similar to the cancer state, dedifferentiation of cells into a pluripotent state can occur when the cell is isolated and loses connections with other cells and the ECM^[78], and stem cells that have differentiated can revert to a more pluripotent state by changes in the concentration of the ECM associated protein, L-proline^[82]. Given that that the ECM can act through mechanical and biochemical mechanisms to regulate the cancer phenotype, one important means to revert the cancer phenotype to the normal somatic cell phenotype is to use S²RM technology to reestablish a normal ECM microenvironment for the cancer cell. That is, using one progenitor cell type to release the building blocks of the ECM, such as collagen, and using another stem cell type to release other building blocks and the instruction sets to build the architecture of the ECM, the normal state of the ECM can be rebuilt and lead to the reversion of the cancer cell phenotype to a more normal somatic cell phenotype as depicted in Figure 2. Thus building on the model developed by Mina Bissell, our model suggests that that the microenvironment/ECM, which is largely comprised of, and developed by S²RM, is key to the regulation of the initiation and degree of pluripotency of cells, controls the "stop" signals for driving potency and then

initiates differentiation of the pluripotent stem cells. The S²RM thus controls homeostasis whereby the state of dynamic transition between different levels of potency^[92] is set to a proper level in which to produce enough new cells to maintain and heal tissue, but not too much so as to allow uncontrolled, cancerous growth of the tissue.

In summary, the S²RM technology provides a natural means for mimicking and stimulating the healing properties of the human body. Instead of using foreign molecules, natural molecules are used that will induce the initiation of natural processes with little or no side-effects. Further, instead of using a small molecule approach where one molecule interacts at one pathway underlying a multi-pathway disease is used, here the S²RM approach uses multiple molecules to perturb multiple pathways underlying the disease, thus yielding a more efficacious result than the one molecule-one pathway reductionist approach.

The S²RM approach will introduce all of the needed molecules to the tissue to induce a full wound healing cascade of events, unlike an approach using the molecules from one stem cell type that will introduce only a portion of the needed molecules and thus provide a fraction of the efficacy that the S²RM provides. And, S²RM uses the particular molecules from the particular stem cells types relevant to the particular tissue to be healed. This is distinct from the "one size fits all" approach where one stem cell type is used to develop therapeutics for the whole body. Therefore, S²RM provides all of the building blocks, such as the different collagen types, to rebuild the tissue, and also provides the instruction set molecules, such as microRNA, that

will deliver the needed architectural commands that will lay the building blocks in their proper places for that particular tissue. During this rebuilding process, the immune response will also be modulated by S²RM, so that inflammation is quelled, allowing the rebuilding to proceed within a normalized framework that is not swollen. The S²RM rebuilding process institutes two fundamental stem cell healing processes: (1) Mimicking the actions of multiple stem cell types and the molecules that they release in the relevant tissue, and (2) reconditioning the endogenous stem cell niche itself and driving the niche to a more primordial, potent state, allowing endogenous stem cell processes to better induce a healing response. Thus, a systems therapeutic approach using multiple molecules from multiple stem cell types called S²RM is used to develop a safer, more natural, and more efficacious therapeutic that both mimics and facilitates the natural adult stem cell healing processes of our body.

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Ovarian stem cells: From basic to clinical applications

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successfully isolated and characterized ovarian GSCs in postnatal mice. The ovarian GSCs are located in the ovarian surface epithelium and express markers of undifferentiated GSCs. When transplanted into mouse ovaries, mouse ovarian GSCs could differentiate and produce embryos and offspring. Similarly, in a recent study, ovarian GSCs were found to be present in the ovaries of women of reproductive age. Conversely, there is increasing evidence that stem cells responsible for maintaining a healthy state in normal tissue may be a source of some cancers, including ovarian cancer. Cancer stem cells (CSCs) have been found in many tissues, including ovaries. Some researchers have suggested that ovarian cancer may be a result of the transformation and dysfunction of ovarian GSCs with self-renewal properties. Drug resistant and metastasis-generating CSCs are responsible for many important problems affecting ovarian cancer patients. Therefore, the identification of CSCs will provide opportunities for the development of new therapeutic strategies for treatments for infertility and ovarian cancer. In this article, we summarize the current understanding of ovarian GSCs in adult mammals, and we also discuss whether there is a relationship between GSCs and CSCs.

Key words: Cancer; Cancer stem cell; Germline stem cell; Ovary; Reproductivity

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Abstract

The field of reproductive biology has undergone significant developments in the last decade. The notion that there is a fixed reserve pool of oocytes before birth was established by Zuckerman in 1951. However, in 2004, an article published in nature challenged this central dogma of mammalian reproductive biology. Tilly's group reported the existence of ovarian germline stem cells (GSCs) in postnatal ovaries of mice and suggested that the bone marrow could be an extragonadal source of ovarian GSCs. These findings were strongly criticized; however, several independent groups have since

Core tip: This review provides an overview on postnatal ovarian germinal stem cells (GSC) of mammals. The characteristics of these cells and the last developments in the field of oogenesis have been presented. We also discuss the relationship between ovarian GSCs and ovarian cancer stem cells. The identification and characterization of these two types of cells are essential for a better understanding of tumor initiation, progression and treatment.

Ozakpinar OB, Maurer AM, Ozsavci D. Ovarian stem cells: From basic to clinical applications. *World J Stem Cells* 2015;

INTRODUCTION

The field of the reproductive biology was revolutionized ten years ago when Johnson *et al.*^[1] published a study in *Nature* that challenged the long-held dogma established by Zuckerman^[2] in 1951. Since that time, it has been generally believed that the ovaries of mammals do not possess renewable stem cells but instead contain a finite reserve of oocytes that diminishes through postnatal life. Although the existence of ovarian germline stem cells (GSCs) has been obviously demonstrated and fully accepted for adult females of non-mammalian species^[3-5] and for adult males of a majority of species^[6], the existence of ovarian GSCs in adult female mammals is still a subject of intense debate. In 2004, Johnson *et al.*^[1] demonstrated the existence of proliferative GSCs in the ovaries of adult mice, and recently, ovarian GSCs have been isolated and characterized in the ovaries of postnatal mice and reproductive-age women^[7].

Amid the controversy created by Johnson *et al.*^[1], the presence of cancer stem cells (CSC) in ovarian cancer was established by Bapat *et al.*^[8], and accumulating data have provided substantial evidence for the involvement of CSCs in ovarian cancer^[9-13]. Ovarian cancer (OC) is associated with enhanced tumor aggressiveness and metastasis, as well as drug resistance. The heterogeneous populations of cancer cells within an ovarian tumor tend to be more resistant to chemotherapeutic agents. In this context, the identification and characterization of CSCs in ovarian cancer is essential for a better understanding of the signaling pathways involved in tumor development and progression. In this review, we will focus on the latest developments in the field of oogenesis in the postnatal mammalian ovary. We will also discuss whether there is a link between ovarian GSCs and CSCs.

OVARIAN GSCS IN ADULT MAMMALS

Existence of ovarian GSCs

In 2004, Johnson *et al.*^[1] published a study that challenged the dogma established by Zuckerman^[2] in 1951. The authors demonstrated that ovarian GSCs are present in the adult mouse ovary, contrary to the principle established more than 60 years ago (Table 1). In a first series of studies, Johnson *et al.*^[1] counted the numbers of healthy (non-atretic) and degenerating (atretic) follicles in ovaries of mice to study germ cell dynamics in female mammals. The numbers of non-atretic quiescent (primordial) and early growing (primary, preantral) follicles in ovary was higher than expected and their rate of clearance in the immature ovary (day 1-day 4) was less than expected. According

to their experiments on the clearance of degenerative oocytes contained within immature follicles, from 1% to 33% of the immature follicle pool was atretic at any given time. The authors considered that the degeneration of this cell would deplete the primordial follicle reserve by young adulthood and that ovarian GSCs represent the source of oocytes produced *de novo*^[1].

By using unbiased assumption-free stereological methods to count follicles in the mouse ovary, Kerr *et al.*^[14] demonstrated the presence of actively dividing surface epithelial cells in prepubertal mouse ovaries and supported the concept of follicle renewal in postnatal and adult ovaries in mice. This study, published in 2006, confirmed the data from Johnson *et al.*^[1] (Table 1).

Johnson *et al.*^[1] reported the presence of large ovoid cells in the surface epithelium of juvenile and young adult mouse ovaries by histological analysis. These cells seemed similar to the germline cells of fetal mouse ovaries. Immunohistochemical staining for Mouse Vasa Homologue (MVH, also called Deadbox 4 or Ddx4), a gene expressed exclusively in the germ cells in both vertebrate and invertebrate species^[15], demonstrated that these large cells were of a germline lineage. Incorporation of 5-bromodeoxyuridine (BrdU) into the DNA of the MVH-positive cells demonstrated the proliferative capacity of these double-positive cells, which were localized to the ovarian surface epithelium. Moreover, postnatal ovarian expression of genes involved in the initiation of meiosis was determined by RT-PCR. Protein synaptonemal complex protein SCP3, the endonuclease Spo11, and the recombinase Dmc1 (required for the initiation of meiosis in mammals) were all detected at the mRNA level^[1]. These results confirmed the presence of proliferative germ cells in postnatal mouse ovaries. Supplemental evidence of continuous folliculogenesis during post natal life was provided by grafting experiments. Ovarian fragments from wild-type mice were grafted onto hemi-ovaries of transgenic mice with ubiquitous expression of green fluorescent protein (GFP). After 3-4 wk, follicles containing GFP-positive oocytes were detected in the wild-type ovarian fragments surrounded by unlabeled granulosa cells. These results suggested that transgenic germ cells had migrated into the grafted ovarian fragment and formed new follicles in adult mice. Collectively, these findings demonstrated that proliferative GSCs exist in the postnatal mammalian ovary. The GSCs support oocyte and follicle production in the postnatal mammalian ovary. This new concept challenged the dogma of a fixed reserve of oocytes.

Following the publication of this landmark article, Johnson *et al.*^[1] were confronted by criticism and skepticism^[16-20]. The main criticisms were related to the subjectivity of the scoring, the tissue fixation protocol, the mathematical model used to calculate the follicular dynamics, the toxicity of busulfan to follicles, the capacity of BrdU-MVH-double-positive cells for oocyte

Table 1 Articles in the field of ovarian germline stem cells and oogenesis

Ref.	Main findings
Johnson <i>et al</i> ^[1]	Ovarian GSCs are observed within the ovarian surface epithelium and provide the adult mouse ovary with oocytes
Johnson <i>et al</i> ^[25]	GSCs are detected in mouse bone marrow and provide the postnatal mouse ovary with oocytes
Kerr <i>et al</i> ^[14]	The follicle numbers remain constant in ovaries of juvenile and early adult mice. Follicle renewal in postnatal and adult mouse ovaries is suggested
Zou <i>et al</i> ^[29]	Mouse ovarian GCS are isolated by immunoselection and characterized. Ovarian GCSs transplanted into ovaries of infertile mice undergo oogenesis and produce offspring
Pacchiarotti <i>et al</i> ^[30]	The adult mouse ovarian GSCs are isolated by FACS. Long-term expanded mouse ovarian GSCs maintain their characteristics, telomerase activity and express germ cell and stem cell markers
White <i>et al</i> ^[7]	The ovarian GSCs are isolated by FACS. Xenotransplantation of human GCSs into NOD-SCID mice leads to the formation of follicles containing oocytes
Zhang <i>et al</i> ^[32]	No mitotically active female GSCs exist in postnatal mouse ovaries
Lei <i>et al</i> ^[33]	The adult female mouse ovary does not contain active ovarian GSCs. The number of follicles produced during fetal development is sufficient to provide ovaries with oocytes in adult life
Park <i>et al</i> ^[39]	Existence of mitotically active germ cells in the postnatal mouse ovary demonstrated by a genetic approach coupled with a GSCs selection strategy

GSCs: Germline stem cells; FACS: Fluorescence-activated cell sorting.

renewal, and the migration of GSCs to form new follicles in wild-type ovaries in the grafting studies.

Several groups criticized the methods used by Johnson *et al*^[1]. Moreover, some of them proposed alternative explanations for Johnson's findings. First, the numbers of preantral and antral immature follicles reported as atretic by Johnson *et al*^[1] were very high and in contrast to previous studies^[16]. Scoring atretic follicles represents a subjective and not reliable method for estimating the rates of follicular atresia^[16,17]. Moreover, the morphological appearance of the follicles can be modified by the use of a harsh fixative^[16,17]. Consequently, healthy and atretic immature follicles may have been misclassified in Johnson *et al*'s study, leading to an overestimation of the number of atretic immature follicles^[17]. The mathematical model of the dynamics of follicle progression was also questioned by several authors. Johnson *et al*^[1] were criticized for applying the rate of follicle disappearance from the CBA/Ca mouse strain, reported as a fast-depleting strain, in comparison to the C57Bl/6 strain^[16]. To verify Johnson *et al*'s results, Bristol-Gould *et al*^[18] proposed examining whether the initial population of follicles was sufficient to support fertility in adulthood by using a mathematical model of the dynamics of follicle progression. These authors counted the follicles in each stage from postnatal day 6 through to 12 mo in mice. The dynamics of the follicle population were simulated according to two distinct models: a fixed pool model and a stem cell model in the mouse ovary. The fixed pool model accurately reflected the experimental decrease in follicle numbers and allowed the authors to refute the concept of GSC replenishment in the adult ovary^[18]. Johnson's assumption that busulfan was only toxic to GSCs and does not kill primordial follicles was questioned by two groups^[16,17]. The loss of primordial follicles after busulfan treatment was considered to be a consequence of busulfan toxicity to primordial follicles and not due to the depletion of GSCs^[17]. The evidence that BrdU and MVH double-

positive cells in the epithelial layer of juvenile and young adult mouse ovaries represented functional GSCs was also challenged^[16-20]. The critics of Johnson *et al*^[1] findings suggested some alternative explanations for the detection of these cells in the ovarian surface epithelium. They asserted that these putative GSCs could be germ cells migrating out of the ovary, as had been reported for these cells in the past^[16,21]. Alternatively, these double-labeled cells could be oocytes from primordial follicles, released by the ovary^[16,20]. The grafting experiments were also subject to reinterpretation by opponents^[16,20] of the findings from Johnson *et al*^[1]. The plasticity of the mouse ovary has recently been demonstrated in experiments with chimeric ovaries^[22]. In addition, the mouse ovarian tissue has been shown to reaggregate after injury^[23]. The chimerism observed in the wild-type transplanted ovaries could result from the subsequent ovarian repair after the tissue trauma due to the transplantation rather than the generation of follicles *de novo*^[16,20].

In response to the critics, in a new study, Tilly and his group counted the numbers of atretic and non-atretic follicles in wild-type (WT) female mice and caspase-6 knockout female mice^[24]. They observed that the number of primordial follicles in caspase-6 deficient young adult females was nearly double the number of primordial follicles in WT females. As no decrease in the number of atretic follicles was detected, the authors interpreted the increase in the primordial follicle pool between birth and adulthood in caspase-6 mutant females as a consequence of neo-folliculogenesis.

Following this study, Skaznik-Wikiel *et al*^[24]s reinforced the validity of their findings and the concept of postnatal oocyte and follicle production in mammals.

Bone marrow as a source of GSCs

In 2005, Johnson *et al*^[25] proposed a new concept that further challenged the dogma of a fixed reserve of oocytes in the ovaries of postnatal females. They suggested that the bone marrow (BM) could be a

source of germ cells for the postnatal mouse ovary. The authors demonstrated that BM cells isolated from adult female mice expressed octamer-binding transcription factor-4 (Oct-4), MVH, deleted in azoospermia-like, a germ cell-specific RNA-binding protein (Dazl), Stella (a maternal factor) and Fragilis (Ifitm3, interferon-induced transmembrane protein 3), which are specific germline markers in mice. Transplantation of BM from adult wild-type female mice was performed with adult female mice treated with cyclophosphamide and busulfan to destroy the existing germ cell pools, and also into ataxia telangiectasia-mutated (Atm)-deficient female mice, which are unable to produce mature cells. After transplantation, oocyte-containing follicles at all stages of maturational development were detected in the ovaries of the chemotherapy-sterilized wild-type and Atm-mutant recipient mice. Moreover, oocytes and follicles were detected in the ovaries more than 11 mo after the initial BM transplantation. Johnson *et al.*^[25] and his group concluded that the putative BM-derived germline cells were able to support long-term oocyte production.

Consequently, they hypothesized that the BM-derived germ cells could be transported by the peripheral blood (PB) to replenish the ovaries. Transplantation of PB mononuclear cells from transgenic female mice with GFP expression driven by an Oct-4 promoter was performed with chemotherapy-sterilized wild-type and atm-mutant female mice. GFP-positive oocytes were observed in the ovaries of the chemoablated adult wild-type and atm-mutant female mice. Therefore, PB was capable of generating oocytes. GSCs derived from the BM could circulate in the PB to colonize the ovaries. Johnson *et al.*^[25] concluded that the BM could be considered as a potential source of germ cells that could support oocyte production in adulthood.

The criticism of this new concept developed by Johnson *et al.*^[25]'s group was again severe^[26,27]. A group of 16 authors published a short correspondence in which they questioned Johnson *et al.*^[25] findings. Their main criticisms concerned the expression of germ cell markers on BM and PB cells, the lack of a reciprocal experiment showing that mononuclear PB cells from wild-type mice could produce oocytes in Atm-Oct4/GFP mutant mouse ovaries, the capacity of generated oocytes to sustain fertilization and subsequent embryo development, and the recurrent question of ovarian failure in women treated by chemotherapy and subsequent BM or PB transplantation.

Subsequently, Johnson *et al.*^[28] published documented responses to the critics to clarify the misinterpretations of their studies. Among the different points discussed by Telfer *et al.*^[26], they defended the germline specificity of the markers used in their studies. They also clearly explained that GFP could not have been absorbed by oocytes from the blood because blood cells from GFP-transgenic males did not induce the generation of GFP-expressing oocytes in wild-type female recipients. The authors reported some cases of

fertility restoration in women treated with high-dose chemotherapy followed by BM or PB transplantation. They also suggested that the BM-derived stem cells involved in blood cell regeneration might be different from those that are able to promote de novo oogenesis, noting that the protocols are designed for the recovery of hematopoiesis and not oogenesis.

After the Johnson *et al.*^[25] publication, Eggan *et al.*^[27] performed parabiosis experiments with adult female mice to test the physiological relevance of BM-derived germ cells to contribute to oogenesis. To evaluate the capacity of circulating BM-derived GSCs to colonize ovaries, they examined ovulated oocytes from wild-type and ubiquitously-expressing GFP female mice that were surgically joined in parabiotic pairs at the age of 4-8 wk. The parabiotic pairs showed a high level peripheral blood chimerism after 6-8 mo (65% GFP-positive blood leukocytes in both mice).

After superovulation, no chimerism of the oocytes was observed in the parabiotic mice. Moreover, occasional GFP-positive cells associated with wild-type oocytes in the cumulus masses were positive for CD45, a pan-hematopoietic marker, demonstrating that these cells were circulating blood cells. The authors concluded that although these circulating cells could associate with ovulated oocytes in the ovary, they were not involved in the production of oocytes; the authors suggested that these hematopoietic cells may play an immune role in ovaries.

In subsequent studies, the investigators treated wild-type mice with cyclophosphamide and busulfan 1 d before joining them to GFP-expressing partners for a duration of 2 wk or 2 mo. In the treated and untreated control parabionts, PB and BM exhibited extensive leukocyte chimerism whereas no chimerism was observed in the oocytes. As indicated above, it was also shown that all GFP-positive cells associated with the ovulated cells were CD45 positive in both the treated and treated wild-type parabiotic mice. In addition, 2 mo after the chemoablative treatment, the ovaries still contained oocytes, revealing that the chemotherapy did not induce a complete depletion of oocytes in the treated parabionts.

The authors then suggested that a direct intravenous transplantation of bone marrow cells might introduce cells into the circulation that might be able to contribute to oogenesis. To investigate this possibility, wild-type mice were treated by chemotherapy (cyclophosphamide and busulfan) or sterilized by low-dose total body irradiation. The mice were injected or transplanted with GFP-transgenic BM cells and were superovulated after 2 mo. Chimerism was observed in hematopoietic stem cells in both chemotherapy-treated and irradiated recipient mice. However, no GFP-positive oocytes were detected. Furthermore, a small number of oocytes was ovulated in chemotherapy-treated mice. No oocytes were detected in irradiated mice.

In conclusion, these findings demonstrated that PB and BM cells did not directly contribute to the

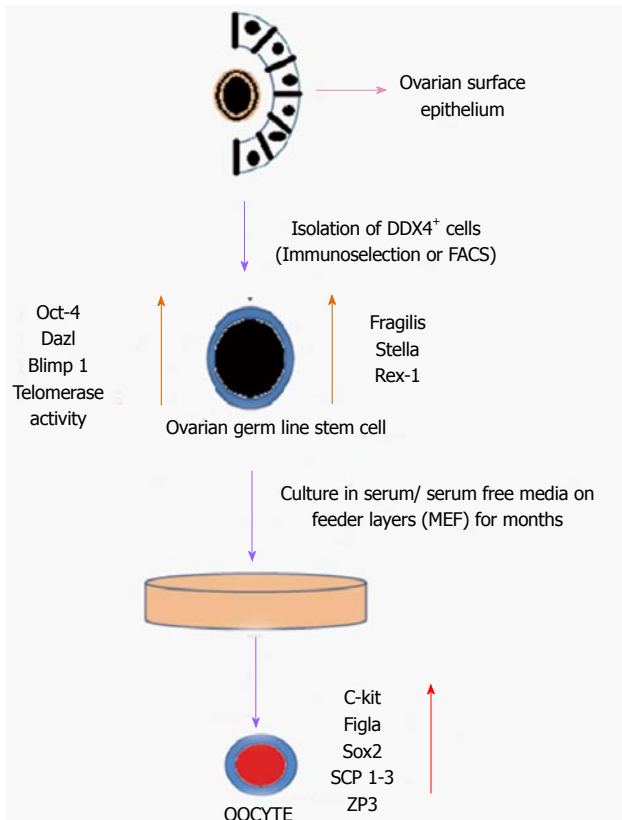


Figure 1 Schematic diagram of isolation and differentiation of ovarian germ stem cells. DDX4: DEAD box polypeptide 4; FACS: Fluorescence activated cell sorting; MEF: Mouse embryonic fibroblast; SCP 1-3: Synaptonemal complex protein 1,2,3; ZP3: Zona pellucida protein 3.

formation of ovulated oocytes or their recovery after chemotherapy. Additionally, the BM-derived cells combined with oocytes belonged to the hematopoietic lineage, and oocytes in the adult mammalian ovaries could not be regenerated by circulating germ cell progenitors.

Isolation and characterization of GSCs from mouse and human ovarian tissues

Despite the controversies, the existence of GSCs was finally reported in 2009 by Zou *et al.*^[29]. They developed a strategy to isolate GSCs from neonatal and young adult mouse ovaries by immunoselection with the antigen MVH (Figure 1). The isolated MVH-positive cells were morphologically similar to type A spermatogonial cells (SSCs). They possessed large cell bodies with little cytoplasm and large nuclei. These MVH-positive cells were present in the ovarian surface epithelium. The isolated MVH-positive cells were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) cell feeders. The MVH-positive cells incorporated BrdU in culture, and the BrdU-MVH double-positive cells were thus considered to be ovarian GSCs. After several passages, the MVH-positive cells formed compact clusters with blurred cell boundaries that did not resemble embryonic stem (ES) cell colonies. The nGSCs from neonatal mice

could be maintained in culture for more than 15 mo (68 passages) and maintained a morphology similar to that of freshly isolated nGSCs. GSC from adult (aGSCs) mice had been passaged in culture for more than 6 mo (more than 25 times). The cultured nGSCs and aGSCs expressed Oct-4, MVH, Dazl, Blimp-1 (a transcriptional repressor), Fragilis, Stella, and Rex-1 (zfp-42, zinc-finger protein 42), which are markers of undifferentiated GSCs (Figure 1). The cells did not express c-Kit (stem cell factor receptor), Figla (a meiosis-specific marker), Sox-2 (a key transcription factor that regulates stemness), Nanog (a pluripotency sustaining factor), synaptonemal complex protein 1,2,3 (Scp1-3), or zona pellucida protein 3 (ZP3), which are established markers of differentiated oocytes. This expression profile revealed that the cultured cells possessed undifferentiated GSC phenotypes. Moreover, the cultured cells showed high telomerase activity. After the transplantation of GFP-expressing GSCs into the ovaries of chemotherapy-ablated wild-type mice, the ovaries contained many GFP-positive oocytes at all stages of development. Moreover, the transplanted mice produced offspring after mating with a wild-type male. Eighty-two percent of nGSCs and 80% of aGSCs produced F1 offspring. The offspring were fertile and could also generate F2 GFP-transgenic progeny (Figure 1).

Furthermore, Pacchiarotti *et al.*^[30] used fluorescence-activated cell sorting (FACS) to isolate GSCs from the ovaries of a transgenic mouse model. In this model, GFP was expressed under the control of the Oct-4 promoter. The GFP-Oct-4-positive isolated cells were expanded on mouse MEFs for more than 1 year. GFP-Oct-4-positive cells formed round, flat colonies. These colonies maintained their telomerase activity and normal karyotype after 20 passages. The cells expressed germ cell and stem cell markers, such as germ cell nuclear antigen, c-kit, Oct-4, Nanog and GFR- α 1 (the receptor for GDNF), and they possessed a normal karyotype. In the presence of growth factors, these GSC cells formed embryoid bodies (EB) and expressed specific markers for the three germ layers. In the absence of growth factors, EBs produced oocyte-like cells. The authors' results thereby indicated the existence of GSCs in the postnatal mouse ovary.

In a recent study published in *Nature Medicine*, White *et al.*^[7] reported the isolation and characterization of ovarian germ stem cells from mouse and human ovarian tissue. GSCs were isolated and purified by FACS using the Ddx4 protein. Isolated mouse and human OSCs express high levels of Prdm1 (PR domain containing 1 with ZNF domain, referred to as Blimp1), Dppa3 (developmental pluripotency-associated 3, referred to as Stella), and Ifitm3, which identify primitive germ cell lines in mammals. Moreover, mouse and human OSCs express high levels of the catalytic subunit of telomerase (Tert), a feature of pluripotent stem cells and germ cells. The Ddx4-positive mouse and human ovarian GSCs were maintained on MEF-

free cultures. After 10-12 and 4-8 wk of culture respectively, the mouse and human ovarian GSCs formed actively dividing germ cell colonies. Gene expression analysis of cultured cells confirmed the maintenance of early germ line markers. Moreover, mouse and human ovarian GSC cells cultured *in vitro* spontaneously underwent oogenesis 24-48 and 72 h after each passage, respectively. Ploidy analysis of the cultured mouse and human GSCs detected 4n, 2n, and 1n populations of cells. These 1n cells were purported to be haploid germ cells.

Transplantation of GFP-expressing mouse ovarian GSCs into ovaries of non-chemotherapy-conditioned wild-type mice resulted in the formation of developing follicles containing GFP-positive oocytes. *In vitro* fertilization of the GFP-expressing oocytes led to the formation of embryos expressing GFP. In co-cultures of human GFP-transduced ovarian GSCs with adult ovarian cortical tissue, GFP-positive oocytes were found to be enclosed by GFP-negative somatic granulosa cells; these were present in tightly compact structures that resembled follicles within 24 h after seeding. GFP-expressing human GSCs injected into fragments of human adult ovarian cortical tissues were also transplanted into NOD-SCID female mice. Human ovarian grafts contained primordial and primary follicles with GFP-negative oocytes and also immature follicles containing GFP-positive oocytes.

After the publication of this new study, Oatley and Hunt published a brief commentary to raise some questions regarding these latest findings^[31]. They questioned whether the purported GSCs represented true stem cells. Oatley and Hunt also questioned the increase in haploid cells, indicating that oocytes completed both meiotic divisions *in vitro* and proposed that the oocytes underwent an abnormal maturation that does not occur *in vivo*. They also criticized the lack of studies on the normalcy of embryos derived from GFP-positive GSCs, arguing that the developmental potential and the genetic quality of the embryos needed to be tested.

Latest developments in the area of GSCs

After the publication of the above-mentioned studies, two different groups presented contradictory results^[32,33]. These investigators refuted the existence of GSCs in the adult ovary and provided evidence supporting the dogma of a fixed pool of oocytes after birth.

In 2012, Zhang *et al.*^[32] claimed that no mitotically active female germline progenitors existed in postnatal mouse ovaries. In their study, they challenged the existence of ovarian GSCs using a genetic approach. They developed a multiple fluorescent Rosa26rbw/+;Ddx4-Cre germline reporter mouse model to trace the development of Ddx4-expressing ovarian cells *in vitro* and *in vivo*. This model expresses GFP ubiquitously. The Ddx4-expressing germline cells

and the non-expressing Ddx4 somatic cells can be recognized by a difference in color. The authors tested the ability of adult mouse ovaries to support the formation of new follicles when provided with female primordial germ cells. Transplantation of enhanced GFP (EGFP)-expressing fetal ovarian cells into adult wild-type female mice led to the formation of fluorescent follicles at different stages of development in the cortex and the medulla of the recipient ovaries. Consequently, the adult mouse ovaries supported *de novo* follicular formation if progenitor cells that were able to differentiate into oocytes were provided. In addition, the adult mouse ovary cells did not transform into oocytes or granulosa cells, and no chimeric follicles were observed. Moreover, when the EGFP-expressing fetal ovarian cells were transplanted into the ovaries of chemotherapy-sterilized WT adult female mice, only newly formed EGFP-positive follicles were detected in the ovaries, which suggests that postnatal mouse ovaries are able to sustain *de novo* follicle formation when provided with exogenous germline progenitors.

The ovaries of Rosa26rbw/+;Ddx4 Cre postnatal mice were dissociated, and single cells were cultured to determine the proliferation capacity of Ddx4-expressing cells (Red FP positive cells, RFP).

None of the RFP-positive ovarian cells underwent any cell divisions, and they rapidly died off. In long-term cultures on feeders, RFP-positive ovarian cells did not form any colonies. Some Ddx4-negative ovarian cells formed colonies of cells that were passaged stably, but the cells did not express germ cell markers. These cells did not participate in follicle formation after injection into adult wild-type ovaries. The authors concluded that these clonal cells presented a stem cell-like morphology, but they were not functional female germline progenitors.

Recently, another group published a study supporting the dogma of a fixed pool of oocytes after birth^[33]. By using a sensitive lineage-labeling system created in transgenic mice (Rosa26-YFP mice), Lei and Spradling affirmed that the primordial follicles generated during fetal life in mice are sufficient to support adult oogenesis. Moreover, female mouse ovaries do not contain active GSCs, and they do not produce new oocytes *in vivo*^[33].

In 2013, Woods *et al.*^[34] published a review on ovarian GSCs that took into consideration two commentaries^[31,35] released after the publication of the studies by White *et al.*^[7] and by Zhang *et al.*^[32]. Woods *et al.*^[34] responded to the comments, analyzed the findings of the Zhang *et al.*^[32] study, and, more importantly, defended their point of view on the existence of ovarian GSCs and oogenesis in postnatal life in mammals.

Woods *et al.*^[34] considered their strategy for the purification of mammalian GSCs from the postnatal ovary to be a validated and reliable method. The protocol using an antibody against the extracellular

epitope of the Ddx4 protein exposed on the surface of the plasma membrane of GSCs was also confirmed to isolate these cells from oocytes, which possess only cytoplasmic Ddx4. To respond to Oatley *et al.*^[31] about their doubts about the validity of the stem cell properties of the ovarian GSCs, Woods *et al.*^[34] explained that the GSCs expressed the same markers as primitive germ cells before and after culture, as described in their publication^[7]. In cultures lacking somatic granulosa cells, GSCs-derived oocytes can exit meiotic arrest and complete both meiotic divisions; thereafter, they exhibit a haploid status, similar to spontaneous oocyte activation *in vivo* when the regulation by somatic granulosa cells is disrupted by ovulation^[36]. With the support of data obtained from several groups^[29,30] Woods *et al.*^[34] claimed that GSCs have the capacity to differentiate into fully mature eggs that can be fertilized to generate normal and viable embryos and offspring in response to the criticisms expressed by Oatley and Hunt.

In the second part of their article, Woods *et al.*^[34] also questioned the results reported by Zhang *et al.*^[32]. They affirmed that the transgenic mouse model used by Zhang *et al.*^[32] possessed a permanent genetic alteration in cells that have activated the *Ddx4* gene promoter region. They stated that the fluorescent recombinant reporter gene would be expressed even if the endogenous *Ddx4* gene is not active anymore. Zhang *et al.*^[32] did not isolate GSCs from the ovaries of their recombinant mice using the methods reported by other groups because they refuted the existence of a *Ddx4* protein exposed on the extracellular surface of the plasma membrane. In experiments involving the transplantation of dissociated fetal ovarian cells into wild-type adult mice, Zhang *et al.*^[32] did not separate the fractions of purified germ line progenitors (primordial germ cells) and fetal follicular somatic cell progenitors to observe neofolliculogenesis in the adult ovary. A fetal somatic cell population or a fetal germ population transplanted separately could stimulate the adult ovary to recruit its own germ cell or somatic cell progenitors to sustain new follicle formation. Morphologic and genetic profiles of the RFP-positive postnatal ovarian observed in cultures were also questioned. The size of the *Ddx4*-expressing cells was significantly larger than that of the GSCs (5-8 mm in diameter). The method of selecting recombinant cells from mouse ovaries was discussed because Zhang *et al.*^[32] recovered the cells by the filtration of crude extracts of mice ovary with a size cutoff of 40 μm . Small immature oocytes present in postnatal mouse ovaries would not have been retained in the filter. The *Ddx4*-expressing cells observed in this study should have been considered as oocytes rather than putative GSCs, explaining their incapacity to proliferate in cultures. Identification of the *Ddx4*-expressing cells needed to be addressed by polymerase chain reaction (PCR)-based analysis of the gene expression patterns of GSCs and oocytes, which would have certainly given positive results for oocyte-

specific genes.

Finally, in the discussion of their article, Woods *et al.*^[34] compared their findings on ovarian stem cells with the observations established for the germinal stem cells in females of non-mammalian species, such as *Drosophila* and in males of mammalian species, such as mice. Although the reproductive period in women has a limited duration of 3 decades, Woods *et al.*^[34] argued that the presence of germinal stem cells in the ovaries should be accepted as it is in *Drosophila*, in which the GSCs persist after the failure of reproductive life^[37], or in male mice, in which the presence of germinal spermatogonial stem cells has been detected after spermatogenic failure^[38]. The authors defended the idea that mammalian females should not be excluded from that standard.

Following the puzzling questions about the Zhang *et al.*^[32] study, Park *et al.*^[39] decided to carry on experiments using transgenic *Ddx4*-Cre mice crossed with mice carrying a *Rosa26*^{tdTm/tdTm} fluorescent gene reporter to identify GSCs in adult mouse ovaries^[39]. In experiments performed according to the protocol used in the study by Zhang *et al.*^[32], Park *et al.*^[39] confirmed that cells collected from crudely dispersed ovaries from recombined mice at postnatal day 8 and selected by filtration were immature oocytes based on PCR analysis of oocyte-specific markers. Moreover, the tdTM/*Ddx4* double-positive cell population purified by FACS from dissociated *Ddx4*-Cre;*Rosa26*^{tdTm/+} ovaries expressed the primitive germ cell markers *Prdm1*, *Dppa3*, *Ifitm3*, *Tert*, and *Dazl* but did not express the oocyte-specific markers newborn ovary homeobox (*Nobox*), *ZP3*, and growth differentiation factor 9 (*Gdf9*). In addition, *Ddx4* mRNA was detected in freshly purified tdTM/*Ddx4* double-positive cells, although it was present at a low level. Cultures of the double-positive cells resulted fluorescent germ cell colonies that were mitotically active. In this study, Park *et al.*^[39] demonstrated that a genetic approach associated with a FACS cell selection based on extracellular *Ddx4* was a reliable and pertinent strategy for the isolation of GSCs from the adult mouse ovary.

From the studies described above, we can infer that the presence of GSCs in the adult ovary remains a subject of intense debate and controversy, and the field of ovarian biology is undergoing profound changes. Progress in ovarian biology has also been made since the discovery of ovarian CSC in 2005^[8]. The characterization of specific ovarian CSCs based on markers of stemness has also generated intense research and discussion. In the following section, we will focus on the characteristics of ovarian CSCs and their relationship with GSCs.

OVARIAN CANCER STEM CELLS: IS THERE ANY RELATIONSHIP BETWEEN GSCS AND CANCER STEM CELLS?

To date, the question concerning the cellular origin of

Table 2 Markers of ovarian cancer stem cells

Marker	Ref.
Side population	Szotek <i>et al</i> ^[9]
CD44/CD117	Zhang <i>et al</i> ^[10]
Natriuretic peptide receptor A	Kong <i>et al</i> ^[11]
CD133	Ferradina <i>et al</i> ^[45]
CD24	Gao <i>et al</i> ^[13]
DEAD box polypeptide 4	Hashimoto <i>et al</i> ^[12]

DEAD: Asp-Glu-Ala-Asp.

cancer is still debated. Two major hypotheses have been proposed regarding the formation of cancer: (1) Clonal evolution, or a stochastic model; and (2) a CSC model^[40]. In the first model, every cell within a tumor mass has the equivalent self-renewal capacity and multilineage potency to drive tumor development. In the CSC model, cancer originates from a small rare population of undifferentiated stem cells with self-renewal capacity. Given this theory, it is not surprising that the CSC hypothesis has gained considerable attention in recent years.

As it is known, CSCs share similar biological properties with normal somatic stem cells, including an extensive self-renewal capacity, expression of specific stem cell surface markers and genes and common signaling pathways. However, CSCs differ significantly from normal stem cells in their drug resistance and metastatic activities. Therefore, recent developments in the field of stem cell biology have led to the reconsideration of the definition of cancer. Many researchers suggest that cancer is a stem cell disease and focus on the CSCs theory to understand which cancer cells are responsible for the development of tumors, chemoresistance and high rates of recurrence.

Epithelial ovarian cancer (EOC) is the most lethal of all gynecological malignancies worldwide and the seventh leading cause of cancer-related death^[41]. It is generally often diagnosed at late stages of the disease. Greater than 90% of EOCs arise from the ovarian surface epithelium (OSC). The molecular and cellular mechanisms underlying EOC remain poorly understood because of the complexity and heterogeneity of the tumor development process^[42].

Accumulating experimental data have demonstrated that EOC is a stem cell-driven disease^[43,44]. Over the last few years, several studies have focused on the isolation, identification, and characterization of stem cells from ovarian cancer cell lines, primary tumor tissues and ascites from cancer patients. Although no universal single marker has been found to isolate ovarian CSCs, several putative markers have been identified by several groups (Table 2). However, the isolation and characterization of CSCs have been the subject of extensive studies and the experimental strategies are still a matter of debate.

The first evidence for the existence of a CSC population in EOC patients was reported by Bapat *et al*^[8]. The

authors isolated a single tumorigenic clone from the ascites fluid of an ovarian cancer patient and defined the characteristics of this cell clone, which was identified as a stem/progenitor cell. They suggested that ovarian cancer may be the result of transformation and dysfunction of stem cells in the ovary.

Subsequently, Szotek *et al*^[9] identified stem cell-like cells, or side population (SP) cells, in the murine transgenic epithelial ovarian cancer cell line MOVCAR-7, the human ovarian cancer cell lines OVCAR 3, SKOV-3 and IGROV-1 and ascites from a small group of ovarian cancer patients using the dye efflux marker SP. Then, they went on to characterize the cell surface markers of these cells by flow cytometry. The authors found that c-kit/117 and CD44 were expressed in the mouse SP cells. However, human SP cells were not further functionally characterized in this study.

In 2008, Zhang *et al*^[10] isolated and identified a self-renewing subpopulation of cancer-initiating cells from human ovarian primary tumor tissues for the first time. They showed that these cells expressed various stem cell markers, including stem cell factor, Nanog, ATP Binding Cassette G-member protein (ABCG2), and Oct-4. These cells also demonstrated chemoresistance to cancer therapeutics and were able to initiate tumors in mice, as determined by evaluating the CD44⁺/CD117⁺ phenotype. Subsequent studies have demonstrated that CD133(+) cells have higher clonogenic and proliferative potential than CD133(-) cells in primary ovarian tumors^[45,46]. This result is not consistent with a recently published study indicating the CD133 expression in tumorigenic ovarian cancer cells is heterogeneous^[47]. Similarly, contradictory results have been obtained using CD24 for the characterization of ovarian CSCs^[13,48].

Although the origin of CSCs has been debated for a long time, a common consensus on this issue has not been reached. While some authors support the hypothesis that CSCs originate from normal stem cells undergoing malignant transformation^[49,50], *in vitro* cancer models have shown that CSCs do not arise from normal stem cells^[51]. Although several putative ovarian CSC markers have been identified, these markers can also be found on normal stem cells^[52]. To understand the relationship between CSCs and normal stem cells, the gene expression profiles in human ovarian stem cells and ovarian cancer was analyzed, and the results showed that the expression of some genes is significantly reduced in malignant ovarian tumors but relatively unchanged in benign tumors compared to normal ovary^[53]. Surprisingly, in a more recent study, a link between normal stem cells and CSCs in ovarian cancer was established^[54]. The authors analyzed the expression patterns of the ovarian GSC marker DDX4 and the ovarian CSC marker CD133 in ovarian cancers using tissue microarrays. They found that the levels of both DDX4 and CD133 were significantly increased in ovarian cancer, and the expression pattern of DDX4 was similar to that of CD133. Even more interesting, they

showed that almost all CD133-positive cancer cells also expressed DDX4 whereas the CD133-negative cells did not.

The genetic program regulating the self-renewal and differentiation of CSCs plays a key role in cancer initiation, invasion and migration. A great body of evidence suggests that epigenetic changes and chromosomal alterations are responsible for tumor development including colorectal^[55], breast^[56], prostate^[57], and hepatocellular carcinoma^[58]. Epigenetic changes include altered DNA methylation, chromatin remodeling and non-coding small RNA (miRNA) expression. Recent developments in the understanding of the role of epigenetic mechanisms have shed new light on carcinogenesis. Taken together, similar to other cancers, determination of the epigenetic mechanisms involved in ovarian cancer opens promising new therapeutic avenues for treatment.

It has been emphasized that DNA methylation might play a seminal role in tumor formation, development and metastasis. DNA methylation of the cytosine residues of CG (also designated as CpG) dinucleotides is the most well-known epigenetic mechanism. Long-term silencing of genes in ES cells is under the control of the polycomb complex of proteins (PcG), and recent findings have shown that stem cell PcG targets are up to 20-fold more likely to have cancer-specific promoter methylation than nontargets, supporting a stem cell origin of cancer, including ovarian cancer^[59,60]. Moreover, Baba *et al.*^[52] demonstrated that CD133 expression, which is a somatic stem cell marker, in ovarian cancer is directly controlled by both histone modifications and promoter methylation. Additionally, specific methylated DNA markers can be detected in the serum, plasma and peritoneal fluid of ovarian cancer patients^[61].

MicroRNAs (miRNAs) are small noncoding RNAs that control gene expression post-transcriptionally and play pivotal roles in stem cell biology. Interestingly, emerging evidence suggests that some miRNAs are differentially expressed in CSCs and somatic cells and even in cancer cells, which indicates that these miRNAs may be involved in the regulation of cancer-related processes^[62-65]. Recently, the involvement of miRNAs in the tumorigenesis, metastasis and drug resistance of EOC has been increasingly reported; therefore, it has been proposed that miRNAs could present a novel therapeutic strategy for the management of ovarian cancer.

Guo *et al.*^[65] showed that miR-9 was downregulated in human ovarian cancer compared to normal ovary, and overexpression of miR-9 repressed cell growth. Li *et al.*^[66] examined the role of miR-27a in the development of drug resistance in ovarian cancer cells, and they found that inhibition of miR-27a decreased the expression levels of the multi drug resistance 1 (MDR1) gene and increased paclitaxel-induced apoptosis. Another recent study reported aberrant miRNA expression in ovarian cancers compared to normal ovary. Iorio *et al.*^[67] reported the overexpression of miR-

200a, miR-141, miR-200c, and miR-200b, as well as the downregulation of miR-199a, miR-140, miR-145, and miR-125b1, in ovarian cancer cell lines.

In light of these findings, it is clear that the CSCs play an important role in the development and maintenance of EOC, and epigenetic changes are responsible for the behavior of cancer progenitor cells and their progeny.

CONCLUSION AND FUTURE PERSPECTIVE

The field of reproductive biology is still divided over the possibility of neo-oogenesis in the mammalian adult ovary between the supporters of this new theory^[1,7,29,30] and the opponents who subscribe to the long-held dogma of a fixed number of follicles at birth^[32,33,68]. In addition, a recurrent question concerns the physiological role of GSCs in the adult mammalian ovary. Nonetheless, the isolation and characterization of GSCs from human ovaries open new perspectives to design protocols for producing mature, competent oocytes *in vitro* for clinical application in assisted reproduction techniques (ART).

During the past years, attempts to generate competent oocytes from ES cells and induced pluripotent stem cells have failed to meet expectations, and fertilization of ESC-derived oocytes remains to be demonstrated^[69,70]. Recently, stem cells derived from newborn mouse skin have been shown to produce oocytes when cultured in an appropriate environment, but these oocytes were not able to undergo complete maturation^[71]. Therefore, as a pure stem cell population that can be easily isolated, female GSCs provide a model to explore postnatal oogenesis in mammals. In addition, these cells may plausibly contribute to the preservation of sterility in women.

Many unanswered questions in the development of oocytes from GSCs remain to be addressed. Cultures of GSCs facilitate the characterization of factors that contribute to the development and regulation of oocytes^[72]. Importantly, the epigenetic program and modifications involved in *de novo* oocyte formation in the adult ovary need to be further established^[73]. A better understanding of these mechanisms of regulation may provide a means to stimulate the generation of oocytes in adult human ovaries *in vivo*.

The question of menopause also remains to be addressed. If ovaries can generate new oocytes during adulthood, it can therefore be suggested that the process of menopause may be the consequence of GSC and ovarian somatic cell aging and not the result of depletion of the oocyte pool. Aging GSCs may arrest to differentiate into oocytes, and/or the environment of the GSC niche may fail to sustain oocyte generation. Therefore, the issue of menopause occurrence is not incompatible with the concept of postnatal neo-oogenesis.

Translational applications of germinal stem cell-

based strategies in the field of infertility treatment in women may be achievable in the near future. Before the use of treatments that negatively impact fertility in women, samples taken from the ovaries could facilitate the isolation of GSCs for cryopreservation and/or for ex vivo expansion and subsequent differentiation into oocytes. Cultured GSCs could be transferred back into the ovaries after treatment to support neofolliculogenesis, or GSC-derived cultured oocytes could be fertilized in a program of *in vitro* fertilization^[74].

Although these potential clinical applications give hope for the future, we are a considerable way from using female GSCs in ART to treat infertility in women.

OC is the most lethal of all gynecological malignancies worldwide. To better understand cancers, researchers are now focusing on identifying the relationship between CSCs and normal stem cells. While both stem cell types have the ability to self-renew, common signaling pathways and similar gene expression profiles, these important properties make it difficult to isolate and distinguish these cells from each other. Additionally, the field faces major challenges, including the absence of specific cell surface markers, inadequate isolation and differentiation protocols, the paucity of genome-wide studies, and also ethical restrictions.

Taken together, it remains uncertain which ovarian cancer phenotype follows the stem cell model due to the complexity and heterogeneity of EOC. On the other hand, the initiation and progression of EOC is characterized by the activation of oncogenes and the switching of tumor suppressor gene expression by epigenetic mechanisms^[75-77]. More importantly, there is evidence on the epigenetic regulation of pluripotency transcription factors, signaling mechanisms and self-renewal pathways that play a critical role in the transformation of GSCs into CSCs in EOC. This knowledge provides us with not only a better understanding of how cancer cells may arise from germinal stem cells, but it also contributes to the development of new technologies or strategies in the management of EOC.

Over the last several years, a great body of evidence supports the idea that ovarian CSCs are responsible for metastasis and drug resistance after chemotherapy in this life-threatening disease. The lack of markers for early detection is the main obstacle to effective treatment strategies. A better understanding of the molecular pathogenesis of EOC is needed to develop new drug therapies and/or diagnostic biomarkers.

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Placental-derived stem cells: Culture, differentiation and challenges

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potentially sources of stem cells. Placenta-derived stem cells (p-SCs) are in between embryonic and mesenchymal stem cells, sharing characteristics with both, such as non-carcinogenic status and property to differentiate in all embryonic germ layers. Moreover, their use is not ethically restricted as fetal membranes are considered medical waste after birth. In this context, the present review will be focused on the biological properties, culture and potential cell therapy uses of placental-derived stem cells. Immunophenotype characterization, mainly for surface marker expression, and basic principles of p-SC isolation and culture (mechanical separation or enzymatic digestion of the tissues, the most used culture media, cell plating conditions) will be presented. In addition, some preclinical studies that were performed in different medical areas will be cited, focusing on neurological, liver, pancreatic, heart, muscle, pulmonary, and bone diseases and also in tissue engineering field. Finally, some challenges for stem cell therapy applications will be highlighted. The understanding of the mechanisms involved in the p-SCs differentiation and the achievement of pure cell populations (after differentiation) are key points that must be clarified before bringing the preclinical studies, performed at the bench, to the medical practice.

Key words: Fetal membrane; Placenta; Embryonic stem cells; Mesenchymal stem cells; Cell therapy

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Core tip: Fetal membranes are a source of stem cells, namely placenta-derived stem cells (p-SCs), which are in between embryonic and mesenchymal stem cells sharing characteristics with both, such as the capacity to differentiate in all germ layers and the dearth of tumorigenicity. Many preclinical studies have been investigated the potential of p-SC use in different medical areas, such as neurology, cardiology,

Abstract

Stem cell therapy is a promising approach to clinical healing in several diseases. A great variety of tissues (bone marrow, adipose tissue, and placenta) are

orthopedics, gastroenterology, and tissue engineering, showing promising results, but also some drawbacks. The present review will focus on different aspects of biological properties and potential clinical uses of p-SCs.

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INTRODUCTION

Stem cell therapy is a promising approach to clinical healing in several diseases. Neurology^[1], orthopedics^[2] and cardiology^[3] are just a few medical branches in which the benefits of stem cell therapy have been mentioned. The prolific capacity of stem cells (either embryonic or mesenchymal), taken together with the ability to generate different cell lineages and its self-renewal property are the major features of these cells that allow their use and manipulation in biology and medicine^[4,5]. A great variety of tissues (bone marrow, adipose tissue, and endometrium) are potentially sources of stem cells and among them the placenta is a transient organ from which it is possible to obtain mesenchymal stem cells^[5].

The term placenta constitutes a very reliable rich source of fetal mesenchymal stem cells^[6-9]. These cells are capable of differentiating into multiple different cell types and have immunological properties that suggest their use in an allogenic transplantation setting^[10-12].

In this respect, it is important to remember that placenta has a fundamental role in maintaining fetomaternal tolerance and, therefore, the immunomodulatory properties of these cells have been investigated with the aim of exploring their applicability in cell therapy-based treatments^[11-13]. Their recovery does not involve any invasive procedures for the donor and their use does not create any ethical issue. In addition, the fact that placenta is generally discarded after birth as medical waste and is available in large supplies, makes placental-derived stem cells (p-SCs) excellent candidates for cell therapy. The current review is focused on the biological properties, culture and potential cell therapy applications of p-SCs.

P-SC

Biological properties

Animal development is initiated by fertilization of the egg with sperm, which is immediately followed by mitotic cell divisions, or cleavages, to generate blastomeres. For eutherians, such as the mouse and human, the first cell differentiation event is the establishment of two distinct cell lineages: the trophoblast (TE) and the inner cell mass (ICM)^[14].

TE engages in implantation by directly interacting with the mother's uterus, and gives rise to tissues in the placenta. It is only after implantation that the three germ layers form from the ICM, which ultimately generates all the tissues in the animal body^[14]. Pluripotency is a characteristic of the ICM and is related to the expression of octamer binding transcription factor 4 (Oct4). Oct4 is essential to prevent ICM from diverting towards the TE lineage. Another important transcription factor, caudal type homeobox 2 (Cdx2), is specifically expressed in TE. It was demonstrated that Cdx2 is necessary to repress the expression of Oct4 in TE^[15]. Moreover, Cdx2 null embryos exhibit high incidence of apoptosis^[16].

Embryonic stem cells (ESCs), which are ICM-derived cell lines, despite of showing pluripotency and self-renewal capacity, have ethical concerns restraints^[4], because of their tumorigenic potential. On the other hand, mesenchymal stem cells (MSCs) are not subject of these ethical restraints, but show multipotency, that means a lower capacity to multiply and originate different cell lineages^[5]. Surrogate systems to study early placental development in the human species have been tried using human and mouse embryonic stem cells under bone morphogenetic protein (BMP) 2/4 stimulus to trophoblast differentiation and spontaneous formation of embryoid bodies^[17,18]. MSCs are found in a lot of tissues, like the dentary tissue, adipocytes and bone marrow which culture provide inadequate cell numbers and have limitations like donor site morbidity and the need of aspiration to collect them, besides being a well-established culture. Attempts to overcome these problems were tried with alternative sources of MSCs and cells isolated from reproductive organs showed to be very useful. Thus, ovaries, uterus (endometrium) and specially the placenta^[19-21], because of its discarded as medical waste after delivery, are potential sources of MSCs that can be used without ethical restrictions.

The p-SCs, which are TE-derived cell lines, gather some features from adult and embryo cells: possible differentiation to originate each of germinative cells and absence of carcinogenicity^[22-27]. In the First International Workshop on p-SCs terminologies standardization have been made^[28], and according to the cell origin we should have amniotic epithelial or mesenchymal stromal cells, chorionic mesenchymal stromal or trophoblastic cells. In the present review it will be considered p-SCs in general, referring to any kind of them.

Cellular characterization

Immunophenotype characterization is the most common procedure used to distinguish different cell clusters, by means of surface marker expression, and some essays employed are immunolabeling^[29] and flow cytometry^[30]. The p-SCs show profiles merged with those found in embryonic and mesenchymal stem cells^[26]. None of these three cell lineages (embryonic,

mesenchymal, and p-SC as well) express the clusters of differentiation (CD) 11b, 34, and 45. Regarding CD expression, p-SCs are positive for 29, 73 and 166, and negative for 11b, 31, 34, 45. In addition, they are positive for human leucocyte antigen (HLA) A, B, C and negative for HLA-DR, DP, DQ^[26,31,32].

The p-SCs fulfill the essential characteristics to categorize MSC^[33] and are capable to originate ectodermal, endodermal, and mesodermal (adipocytes, osteocytes, and chondrocytes) lineages *in vitro*^[25,31]. Moreover, p-SCs express surface cell markers only expressed by embryo-derived cells, such as Oct-4, SRY (sex determining region Y)-box 2 (Sox 2), Nanog, stage-specific embryonic antigen (SSEA)-1, SSEA-4, germ cell tumor marker (GCTM)-2, Tra-1-60, and Tra-1-80^[22-24]. High levels of Oct-4, Sox 2 and Nanog were detected in p-SCs using q-PCR method^[25]. Musashi-1, vimentin, polysialylated-neural cell adhesion molecule, and Nestin were observed in cells from amniotic membrane, unlike other MSCs, when evaluated by immunofluorescence technique^[34].

Cell culture

Many different protocols for isolation of fetal membranes-derived stem cells are found in literature. For human beings and large animals, a separation of amniotic epithelial cells^[23,35], amniotic mesenchymal cells^[7] and chorionic mesenchymal cells^[8] are possible, meanwhile for small animals, such as rodents, such characterization is not possible^[31].

Fetal membranes are submitted to enzymatic disaggregation following different protocols, which differ among themselves particularly regarding enzymatic digestion and duration^[7,8,23,31,35]. In every protocol, the cells are incubated with trypsin-EDTA or other enzymes, at 37 °C, and then, are harvested through physical methods. Then cells are plated onto noncoated tissue culture dishes or flasks. Serum, like fetal bovine serum (FBS) ranging from 10% to 20%, must be added to the media. The most commonly used media are Roswell Park Memorial Institute (RPMI)-1640, Dulbecco's modified eagle medium (DMEM), Minimum essential medium (EMEM), and DMEM/F12. Also, media should be enriched with nonessential amino acids, L-glutamine, β -mercaptoethanol, sodium pyruvate, and antibiotics. Cells are raised at controlled conditions of air atmosphere and temperature. For maintenance of the cell culture, p-SCs must have their medium (such as DMEM or RPMI) changed every other day. To passage the cells, it should be used trypsin, and the cells are generally plated into T75 tissue culture flasks. Such culturing conditions are very similar with MSCs. On the other hand, ESCs need to be plated into mouse embryonic fibroblasts - MEFs - feeder layer or MatrigelTM and the medium (such as DMEM-F12 or mTeSRTM-1) must be changed every single day. To passage the cells, it should be used collagenase type IV or dispase, and the cells are generally plated into 6-well

plates.

Finally, p-SCs may be cryopreserved using different media containing various concentrations of dimethyl sulfoxide, FBS and DMEM. Viability and cell numbers must be evaluated after thawing.

CELL THERAPY USING P-SC

Basic considerations

Three characteristics are important for the clinical application of p-SCs: the lack of ethical restrictions, pluripotency and their low immunogenicity^[12]. An explanation for this is the low expression of MHC class II molecules, allowing these cells to be effectively employed in transplants^[26].

Regarding to cellular therapy we have to consider the existence of cell banking due to the large amount of cells needed. Thus, those cells must have essential properties like to keep stable in subcultures, availability, great prolificity, freezing resistance, and high viability after thawing. The p-SCs fulfill most of these requirements. It has been mentioned that p-SCs even at subculture 30 showed high cellular division rates keeping a steady karyotype^[25]. Different from p-SCs and ESCs, all other MSCs do not neither replicate nor exhibit high levels of cellular survival around subcultures 8 to 10.

Considering the freezing process, p-SCs require ordinary media (like MSCs, unlike ESCs), show intermediate resistance to low temperature (in between ESCs and MSCs) and show great viability after frozen-thawing (like ESCs, unlike MSCs).

Some of the p-SC uses in research are listed in Table 1, at the end of this section.

Neurological diseases

Human p-SC demonstrated neuroprotective effects after stroke in rats. Treatment of stroke with p-SCs (*via* intravenous administration) significantly increased vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and brain derived neurotrophic factor (BDNF) levels in the ischemic brain compared to controls (dextran vehicle or phosphate buffer saline) after middle cerebral artery occlusion^[36].

In Alzheimer's disease, β -amyloid peptide is considered to be its root cause. Also, the neuroinflammatory process mediated by β -amyloid plaque-induced microglial cells and astrocytes contributes to Alzheimer's disease pathogenesis. Thus, it was demonstrated that p-SCs transplanted into an Alzheimer's disease mouse model modulated the properties of microglial cells toward a β -amyloid peptide plaque-reducing anti-inflammatory response. Moreover, p-SCs injected mice, compared to phosphate-buffered saline controls, had higher levels of β -amyloid degrading enzymes, reduced levels of proinflammatory cytokines, increased levels of anti-inflammatory cytokines (TGF- β and IL-10), slower progression of Alzheimer's pathology, and

Table 1 Preclinical studies using placental-derived stem cells for different disease models

Disease	Animal model	Cells	Ref.
Stroke	Rats	p-SC	[36]
Alzheimer	Mouse	p-SC	[37]
Injured liver model	Rat	p-SC	[39]
Myocardial infarction	Rat	amniotic membrane fragment	[43]
Myocardial infarction	Pig	p-SC	[44]
Lung fibrosis model	Mouse	p-SC	[47]
Idiopathic pulmonary fibrosis	Human (trial study)	p-SC	[48]
Osteogenesis imperfecta	Murine	p-SC	[49]
Bone lytic lesions	Mice	p-SC	[50]
Bone disease	rabbit	p-SC + scaffold	[51]

p-SC: Placenta-derived stem cell.

improved memory function^[37].

Liver disease

Cell therapy for liver diseases has also been investigated. Among other sources of MSCs, p-SCs showed the greatest potential for hepatogenic differentiation and proliferation *in vitro*. The p-SCs, mainly those from chorionic plate, expressed higher levels of hepatocyte growth factor after differentiation^[38].

In another study, chorionic-plate derived mesenchymal stem cells isolated from placenta could trigger autophagy to enhance regeneration in carbon tetrachloride injured rat liver model. After p-SC transplantation they observed reduction in apoptosis (caspase activity) and increasing levels for autophagy, survival and regeneration in liver cells^[39].

Pancreatic disease

Human placenta-derived mesenchymal stem cells (p-SCs) have the potential to differentiate into insulin producing cells^[40,41]. Also, p-SCs can form islet-like cell clusters which are capable of restoring normoglycemia when transplanted into streptozotocin-induced diabetic Balb/C mice^[42].

Heart disease

In order to evaluate whether human amniotic membrane could limit postischemic cardiac injury, a fragment was applied onto the left ventricle of rats that had undergone ischemia through left anterior descending coronary artery ligation. The authors observed that the amniotic membrane fragment onto ischemic rat hearts could significantly reduce postischemic cardiac dysfunction once the rats showed higher values of left ventricle ejection fraction, fractional shortening and wall thickening on echocardiographic examinations^[43].

In another study of myocardial infarction, pre-treated (with a hyaluronan mixed ester of butyric and retinoic acid - HBR) p-SCs were intramyocardial injected in a pig model. Treated animals showed

smaller infarct scar size and a significant improvement of the end-systolic wall thickening and circumferential shortening of the infarct border zone evaluated by magnetic resonance imaging^[44].

Muscle disease

First-trimester chorionic-villi-derived cells were evaluated for potential therapeutic use in Duchenne muscular dystrophy, which is a X-linked disorder characterized by the absence of dystrophin at the sarcolemma of muscle fibers. The p-SCs efficiently differentiated into myotubes after myogenic induction, at which point Nanog and Sox2 were down-regulated, whereas MyoD, myogenin, desmin and dystrophin were up-regulated. The p-SCs could be efficiently directed to differentiate *in vitro* into skeletal muscle cells that express dystrophin as the last stage marker of myogenic differentiation^[45].

The myogenic differentiation of fetal stem cells (p-SC, amniotic fluid stem cells, cord blood and wharton's jelly cells), were already reviewed and considerations about limitations and perspectives for therapeutically use were done^[46].

Pulmonary disease

On a mouse model of bleomycin-induced lung fibrosis, cellular therapy using p-SCs promoted a decrease in neutrophil infiltration and a significant reduction in the severity of fibrosis, compared to control^[47].

On a single centre, non-randomized, dose escalation phase 1b trial study, human patients with moderately to severe idiopathic pulmonary fibrosis received p-SCs *via* peripheral vein and they were evaluated follow up 6 mo. The variables analyzed were lung function (forced vital capacity and diffusing capacity for carbon monoxide), 6-min walk distance, gas exchange (assessed by resting PaO₂), and lung fibrosis score (assessed by high-resolution computed tomography chest). Compared to baseline, at 6 mo all parameters were unchanged, with no evidence of worsening fibrosis and no side effects reported in the patients who had received p-SC injections^[48].

Bone disease

Human fetal early chorionic stem cells (p-SCs) treatment was studied in a murine osteogenesis imperfecta model. It was demonstrated that intraperitoneal injection of p-SCs in osteogenesis imperfect neonates reduced fractures, increased bone ductility and bone volume, increased the numbers of hypertrophic chondrocytes, and upregulated endogenous genes involved in endochondral and intramembranous ossification^[49].

Regenerative medicine using p-SC for bone disease (large lytic lesions) secondary to multiple myeloma was investigated. The authors founded p-SCs inhibitory effects on myeloma bone disease and tumor growth were dose-dependent and intrabone engraftment of p-SCs inhibited bone disease and tumor growth in mice severe combined immunodeficiency-rab model.

The p-SCs also promoted apoptosis in osteoclast precursors and inhibited their differentiation, indicating a promising therapeutic approach for myeloma osteolysis using cytotherapy^[50].

Tissue engineering

The utility of p-SCs for bone tissue engineering has been investigated using different biomaterials and showing promising results. Transplantation of human p-SCs grown in a silk fibroin/hydroxyapatite scaffold into injured radius segmental bone in rabbits enhance tissue repair^[51]. A combination of p-SCs and polylactide/poly(ϵ -caprolactone)-poly(ethylene glycol)-poly(ϵ -caprolactone) fibrous scaffolds promoted good cellular response and excellent osteogenic potential *in vitro*^[52].

An elastic scaffold, obtained combining a poly-(ether)urethane-polydimethylsiloxane (PEtU-PDMS) semi-interpenetrating polymeric network (s-IPN) with fibrin, was used as a substrate for *in vitro* studies of human p-SC growth and differentiation. The s-IPN PEtU-PDMS/fibrin combined scaffold allowed a better proliferation and metabolic activity of p-SC cultured up to 14 d, compared to the ones grown on plastic dishes and also sustained the beginning of p-SCs differentiation process towards a cardiomyogenic lineage^[53].

As an alternative to corneal transplantation, researchers evaluated a biomaterial derived from fetal membranes to promote corneal repair. The pericellular matrix of decidua-derived mesenchymal cells was demonstrated to provide a xeno-free substrate for efficient culture of human corneal endothelial cells. The matrix enhanced corneal cell attachment, promoted cell proliferation, and suppressed apoptosis, offering a viable *in vitro* expansion protocol for human corneal endothelial cells^[54].

Cell-based therapy, using p-SC, combined with bioactive materials improved bone regeneration prior to dental implant. Amniotic epithelial cells, loaded on a calcium-phosphate synthetic bone substitute, displayed a reduced fibrotic reaction, a limited inflammatory response and an accelerated process of angiogenesis. In addition, the presence of p-SCs significantly stimulated osteogenesis by enhancing bone deposition, as suggested by the presence of p-SCs entrapped within the newly deposited osteoid matrix and by their ability to switch-on the expression of osteocalcin when transplanted into host tissues^[2].

CHALLENGES

A routine medical practice using stem cells is an exciting promise. And for this reason, any preclinical study regarding cell therapy must be considered cautiously. Besides the rapid evolution in differentiating stem cells into many different cell types, all protocols result in a mix cell preparations and how to get specialized cells in sufficient amount and purity is still a challenge. So, the really understanding of all the

mechanisms from culturing stem cells to transplanting differentiated ones into a patient is of great importance in order to let the dream comes true.

CONCLUSION

In summary, either the fetal or the maternal components of the placenta may be considered potential sources of stem cells once they share features from both cells of embryonic and mesenchymal origin. Phenotypically, these cells show common characteristics of embryo-derived and adult stem cells and do not express hematopoietic cell markers CD11b, CD 34 and CD 45. Different media to culture and stimuli to onward differentiation in p-SCs are under investigation and they have been used on research trials of cell therapy with promising results. Furthermore, it is important to get enough purity cells and to understand the mechanism of differentiation and engraftment of p-SC to improve cell-based therapy.

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Basic Study

Fetal kidney stem cells ameliorate cisplatin induced acute renal failure and promote renal angiogenesis

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by Animal Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India (letter number: PGI/RC/882/2005).

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Data sharing: Technical appendix, statistical code, and dataset available with the corresponding author at soniya_nityanand@yahoo.co.in, who will provide a permanent, citable and open-access home for the dataset. No additional data are available (animal study).

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Abstract

AIM: To investigate whether fetal kidney stem cells (fKSC) ameliorate cisplatin induced acute renal failure (ARF) in rats and promote renal angiogenesis.

METHODS: The fKSC were isolated from rat fetuses of gestation day 16 and expanded *in vitro* up to 3rd passage. They were characterized for the expression of mesenchymal and renal progenitor markers by flow cytometry and immunocytochemistry, respectively. The *in vitro* differentiation of fKSC towards epithelial lineage was evaluated by the treatment with specific induction medium and their angiogenic potential by matrigel induced tube formation assay. To study the effect of fKSC in ARF, fKSC labeled with PKH26 were infused in rats with cisplatin induced ARF and, the blood and renal tissues of the rats were collected at different time points. Blood biochemical parameters were studied to evaluate renal function. Renal tissues were evaluated for renal architecture, renal cell proliferation and angiogenesis by immunohistochemistry, renal cell apoptosis by terminal deoxynucleotidyl transferase nick-end labeling assay and early expression of angiogenic molecules *viz.* vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1 α and endothelial nitric oxide synthase (eNOS) by western blot.

RESULTS: The fKSC expressed mesenchymal markers *viz.* CD29, CD44, CD73, CD90 and CD105 as well as

renal progenitor markers *viz.* Wt1, Pax2 and Six2. They exhibited a potential to form CD31 and Von Willebrand factor expressing capillary-like structures and could be differentiated into cytokeratin (CK)18 and CK19 positive epithelial cells. Administration of fKSC in rats with ARF as compared to administration of saline alone, resulted in a significant improvement in renal function and histology on day 3 (2.33 ± 0.33 vs 3.50 ± 0.34 , $P < 0.05$) and on day 7 (0.83 ± 0.16 vs 2.00 ± 0.25 , $P < 0.05$). The infused PKH26 labeled fKSC were observed to engraft in damaged renal tubules and showed increased proliferation and reduced apoptosis ($P < 0.05$) of renal cells. The kidneys of fKSC as compared to saline treated rats had a higher capillary density on day 3 [13.30 ± 1.54 vs 7.10 ± 1.29 , capillaries/high-power fields (HPF), $P < 0.05$], and on day 7 (21.10 ± 1.46 vs 15.00 ± 1.30 , capillaries/HPF, $P < 0.05$). In addition, kidneys of fKSC treated rats had an up-regulation of angiogenic proteins hypoxia-inducible factor-1 α , VEGF and eNOS on day 3 ($P < 0.05$).

CONCLUSION: Our study shows that fKSC ameliorate cisplatin induced ARF in rats and promote renal angiogenesis, which may be an important therapeutic mechanism of these stem cells in the disease.

Key words: Fetal kidney stem cells; Mesenchymal and renal progenitor markers; Acute renal failure; Stem cell therapy; Angiogenesis

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Core tip: This study provides novel data on the therapeutic effect of culture expanded fetal kidney stem cells (fKSC) in acute renal failure (ARF). The fKSC represent primitive renal stem cells that express mesenchymal and renal progenitor markers. They exhibit *in vitro* angiogenesis and potential to differentiate into renal epithelial cells. On administration in ARF rats, they rapidly improve renal function and histology. The therapeutic effects of fKSC are accompanied with increased capillary density in kidney tissues suggesting that induction of renal angiogenesis may be an important therapeutic mechanism of these stem cells.

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INTRODUCTION

Acute renal failure (ARF) is characterized by rapid loss of renal function due to damage of renal tubular epithelial cells by ischemia, nephrotoxins or other means. Some cases of ARF spontaneously recover from renal injury while in others the recovery process

is either delayed or does not occur at all, leading to chronic kidney disease (CKD), which is associated with high morbidity and mortality^[1,2]. Thus, it is very important to restore normal structure and function of the kidney after ARF using a suitable regenerative therapy in order to prevent its progression into CKD^[3]. Recent data suggest that injury of endothelial cells of small peritubular arterioles and capillaries plays a crucial role in the pathogenesis of ARF by mediating hypoxia and impairment of renal perfusion that eventually results in injury of renal tubular epithelial cells and thereby loss of renal function^[4-6]. Furthermore, reduction in renal capillary density following ARF may perpetuate the tubular epithelial injury leading to CKD^[7,8]. These observations suggest that neovascularization of the kidney and regeneration of damaged renal tubular epithelial cells may be potentially effective in recovery from ARF.

During the past decade, stem cell based regenerative therapy has emerged as a promising therapeutic strategy for ARF and stem cells derived from various sources are being extensively explored for the treatment of ARF^[9,10]. However, a suitable stem cell type for therapeutic application in clinical ARF has not yet been identified. Fetal kidney stem cells (fKSC) represent a novel stem cell type for treatment of ARF because they are multipotent stem cells with an inherent ability to differentiate into cells of renal lineage. In addition, few studies have shown that embryonic or fetal kidney also contains a subpopulation of endothelial and epithelial progenitors^[11-13] and hence fKSC may have an important role in neovascularization and the regeneration of the damaged kidney. However, there is a paucity of data on therapeutic effect of fKSC in ARF and no information is available about angiogenic role of these stem cells in mediating their therapeutic effect in the disease.

Therefore, the aim of the present study was to investigate the therapeutic effect of fKSC in cisplatin induced ARF in rats and to evaluate whether neovascularization in the damaged kidney has a role in the therapeutic efficacy of these stem cells in the disease.

MATERIALS AND METHODS

Animals

Experiments were performed on Sprague Dawley (SD) rats (220-250 g) purchased from Central Drug Research Institute, Lucknow, India. All animal experimental procedures in this study were performed as per guidelines of the Institutional Animal Ethics Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The protocol was approved by the Animal Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

Isolation and culture of fKSC

The fKSC were isolated from SD rat fetuses at gestation

day 16. The kidneys were removed from fetuses of 10 pregnant female rats (8-12 fetuses/animal), minced, and digested with 1 mg/mL collagenase type-IV (Worthington Biochemical, NJ, United States) in serum free α -MEM (Gibco, NY, United States) medium for 40 min at 37 °C with intermittent stirring in water bath. After two washes with α -MEM, the digested tissue was cultured at 37 °C in a 5% CO₂ in 25 cm² tissue culture flasks (BD, New Jersey, United States) in complete culture medium consisting of α -MEM medium, 2 mg/mL of Glutamax (Gibco, NY, United States), 16.5% fetal bovine serum (Gibco, NY, United States) and bacteriostatic level of penicillin-streptomycin (Gibco, NY, United States). After 48 h of seeding of fetal kidney cells, the culture media containing non-adherent cells were replaced. On day 3 adherent cells were harvested by trypsinization with TrypLE Express (Gibco, NY, United States) and further cultured in complete culture media. The cells of 3rd passage were used for this study.

Characterization of fKSC

The fKSC were characterized by studying their expression of mesenchymal and renal progenitor markers. The expression of mesenchymal markers on fKSC was studied by flow cytometry. The fKSC at passage 3 were incubated for 30 min with following antibodies: Fluorescein isothiocyanate (FITC) conjugated CD90 (Abcam, MA, United States), CD44 (BD Biosciences, CA, United States), phycoerythrin conjugated CD45 (Abcam, MA, United States), MHC class II (Abcam, MA, United States) and with unconjugated CD29 (Abcam, MA, United States), CD73 (BD Biosciences, CA, United States), CD105 (Santa cruz biotechnology, inc. CA, United States). The corresponding FITC-conjugated secondary antibodies (Abcam, MA, United States) were added in CD29, CD73 and CD105 tubes. Isotype-identical antibodies (IgG) served as controls.

Expression of renal progenitor markers *viz.* Wilms tumor1 (Wt1), paired box2 (Pax2), SIX homebox2 (Six2) was studied by immunocytochemistry. The cells were fixed with 4% para-formaldehyde for 1 h at room temperature and incubated overnight at 4 °C with following primary antibodies: Wt1, Pax2 (both from Abcam, MA, United States) and Six2 (Santa cruz biotechnology, Inc. CA, United States). After washing with PBS, cells were incubated with corresponding FITC-conjugated secondary antibodies and counter stained with Hoechst dye (Sigma-Aldrich, MO, United States). The fKSC incubated with non-immune serum or IgG in place of primary antibodies were used as negative controls. The images were obtained using Nuance Multispectral Imaging System (CRI Inc., MA, United States).

In vitro differentiation towards epithelial lineage and angiogenic assay

To induce epithelial differentiation, fKSC (2×10^4 cells/well) were cultured on cover slips in 6 well plate

in complete culture medium containing 0.1 μ mol/L Retinoic acid, 10 ng/mL activin-A and 50 ng/mL Bmp7 (all from R and D Systems, MN, United States). After 7 d, cover slips with induced fKSC were fixed in 4% para-formaldehyde for 1 h at room temperature. The fixed cells were incubated at 4 °C overnight with primary antibodies *viz.* cytokeratin (CK)18 and CK19 (Abcam, MA, United States). After washing with PBS, cells were incubated with corresponding FITC-conjugated secondary antibodies for 1 h at room temperature and counter stained with Hoechst dye (Sigma-Aldrich, MO, United States). The fKSC cultured without induction medium were used as negative controls.

The tube-formation assay was performed to assess the angiogenic potential of fKSC. Briefly, fKSC (2×10^4 cells/well) were seeded onto the growth factor reduced matrigel (BD Biosciences, CA, United States) coated cover slips in 6 well plate and incubated overnight in standard culture condition with complete medium. The cells were examined by phase contrast microscopy and immunocytochemistry. For immunocytochemistry, cells were fixed with 4% para-formaldehyde for 1 h at room temperature. The fixed cells were incubated at 4 °C overnight with primary antibodies *viz.* CD31 (AbD serotech, Oxford, United Kingdom) and Von Willebrand factor (vWF) antibody (Abcam, MA, United States). After washing with PBS, cells were incubated with corresponding FITC-conjugated secondary antibodies for 1 h at room temperature and stained with Hoechst dye. The fKSC cultured without growth factor reduced matrigel were used as negative controls.

Development of ARF model and fKSC therapy

ARF was induced in male SD rats ($n = 36$) weighing 200-225g by cisplatin injection as described by Ozyurt *et al.*^[14]. Briefly, the animals were given a single intra-peritoneal injection of cisplatin (0.5 mg in 1 mL of saline, sigma, United States) at a dose of 7 mg/kg of body weight. A significant increase in blood biochemical parameters and renal damage were observed in rats on day 5 of cisplatin injection. Hence at this time point, rats were randomized into two groups *viz.* fKSC treated group ($n = 18$) and saline treated group ($n = 18$) for evaluation of the efficacy of stem cell therapy. In addition, a group of healthy rats ($n = 6$) was also included in the study as healthy controls to compare renal function and histology of fKSC and saline treated groups.

The fKSC were labeled with PKH26, a red fluorescent cell linker (Sigma-Aldrich, MO, United States) according to manufacturer's protocol. Labeling efficiency of PKH26 with fKSC and cell viability of labeled cells were > 95% and > 97% as revealed by fluorescent microscopy and trypan blue exclusion, respectively. On day 5 of cisplatin injection, a total of 2×10^6 PKH26 labeled fKSC in 150 μ L of saline or 150 μ L saline alone were injected intravenously through tail vein in each rat of fKSC treated or saline treated groups, respectively.

Animals were euthanized by barbiturate overdose

(intravenous injection, 150 mg/kg pentobarbital sodium) before fKSC therapy (day 0) and after three days (day 3) and seven days (day 7) of fKSC therapy. Blood samples were collected to determine blood urea nitrogen (BUN) and serum creatinine levels by using commercial Span diagnostic kits with an autoanalyser (BioSystems BTS-330). The kidney tissues were excised to perform the assays described below.

Histopathological and Immunohistochemical analysis

Ten percent of formalin fixed kidney tissues were cut into 5 μ m serial sections and stained with hematoxylin and eosin to evaluate sequential histopathological changes in saline and fKSC treated animals. Quantitative assessment of renal tubular necrosis was performed using the grading scores of Jablonski *et al.*^[15]. For immunohistochemical analysis, 5 μ m thick paraffin sections of kidneys were deparaffinized with xylene and rehydrated in a series of alcohol and water. After rehydration, tissue sections were incubated with primary antibodies *viz.* aquaporin (AQP)1, vWF (both from Abcam, MA, United States) and CD31 (AbD serotech, Oxford, United Kingdom) and proliferating cell nuclear antigen (PCNA; BD Biosciences, CA, United States). After overnight incubation, sections were washed with PBS and incubated with corresponding FITC-conjugated secondary antibodies and then counter stained with Hoechst dye. Images were taken by Nuance Multispectral Imaging System (CRi Inc., MA, United States). To determine the capillary density, CD31 stained capillaries were counted in 10 randomly chosen high-power fields (HPF; 20 μ m) using a fluorescent microscope and expressed as capillaries per HPF. To determine the number of proliferating cells in the kidney, PCNA positive nuclei were counted in 10 HPFs per section (20 μ m) in the cortico-medullary area under a Nuance Multispectral Imaging System (CRi Inc., MA, United States) and the proliferative index was expressed as average number of PCNA⁺ cells /HPF.

dUTP nick-end labeling

Apoptotic scores in kidney tissue sections were measured by the terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) as per manufacturer's instruction. Briefly, kidney sections were deparaffinized, rehydrated, and digested with proteinase K and labeled with TUNEL reaction mixture for 60 min at 37 °C. TUNEL positive nuclei were counted in 10 HPF per section in the cortico-medullary area at 20 μ m magnification under a Nuance Multispectral Imaging System (CRi Inc., MA, United States) and the apoptotic index was expressed as average number of TUNEL⁺ cells /HPF.

Western blot

Expression of angiogenic signaling molecules in the kidneys of rats was evaluated by western blotting on day 3 after fKSC therapy. The kidney tissues of

animals were homogenized in RIPA buffer containing 1 mmol/L phenylmethanesulphonyl fluoride and 1% protease inhibitor cocktail (Sigma-Aldrich, MO, United States). Kidney tissue homogenate was centrifuged at 10000 *g* for 10 min and the supernatant was stored in -80 °C. 40 mg proteins were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA for 1 h at room temperature and then incubated at 4 °C overnight against primary antibodies *viz.* vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1 α and endothelial nitric oxide synthase (eNOS) (all from Abcam, MA, United States). β -Actin antibody (Abcam, MA, United States) was used as a loading control. Primary antibodies were detected by corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies using super signal west pico chemiluminescent substrate (Thermo scientific, IL, United States). The bands were quantified by densitometry using the Quantity One software (Bio-Rad, CA, United States).

Animal care and use

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

Statistical analysis

Values were expressed as mean $\pm$ standard error (SE). The statistically significant differences between groups were analyzed by one way-analysis of variance (ANOVA) with Bonferroni multiple-comparison post hoc test. Statistical analysis was performed using GraphPad Prism Software Version 5 (GraphPad, San Diego, CA) and a *P* value of < 0.05 was considered statistically significant.

RESULTS

Expression of mesenchymal and renal progenitor markers by fKSC

The fKSC exhibited spindle-shaped and polygonal morphology in culture (Figure 1A). Flow cytometry analysis showed that fKSC expressed CD29 (37.59%), CD44 (32.76%), CD73 (51.72%), CD90 (68.71%) and CD105 (37.88%), whereas the expression of CD45 and MHC class II was < 5% (Figure 1B). Immunocytochemistry revealed that fKSC also expressed renal progenitor markers *viz.* Wt1, Pax2 and Six2 (Figure 1C).

In vitro epithelial differentiation and angiogenic potential of fKSC

On treatment with specific induction medium, fKSC

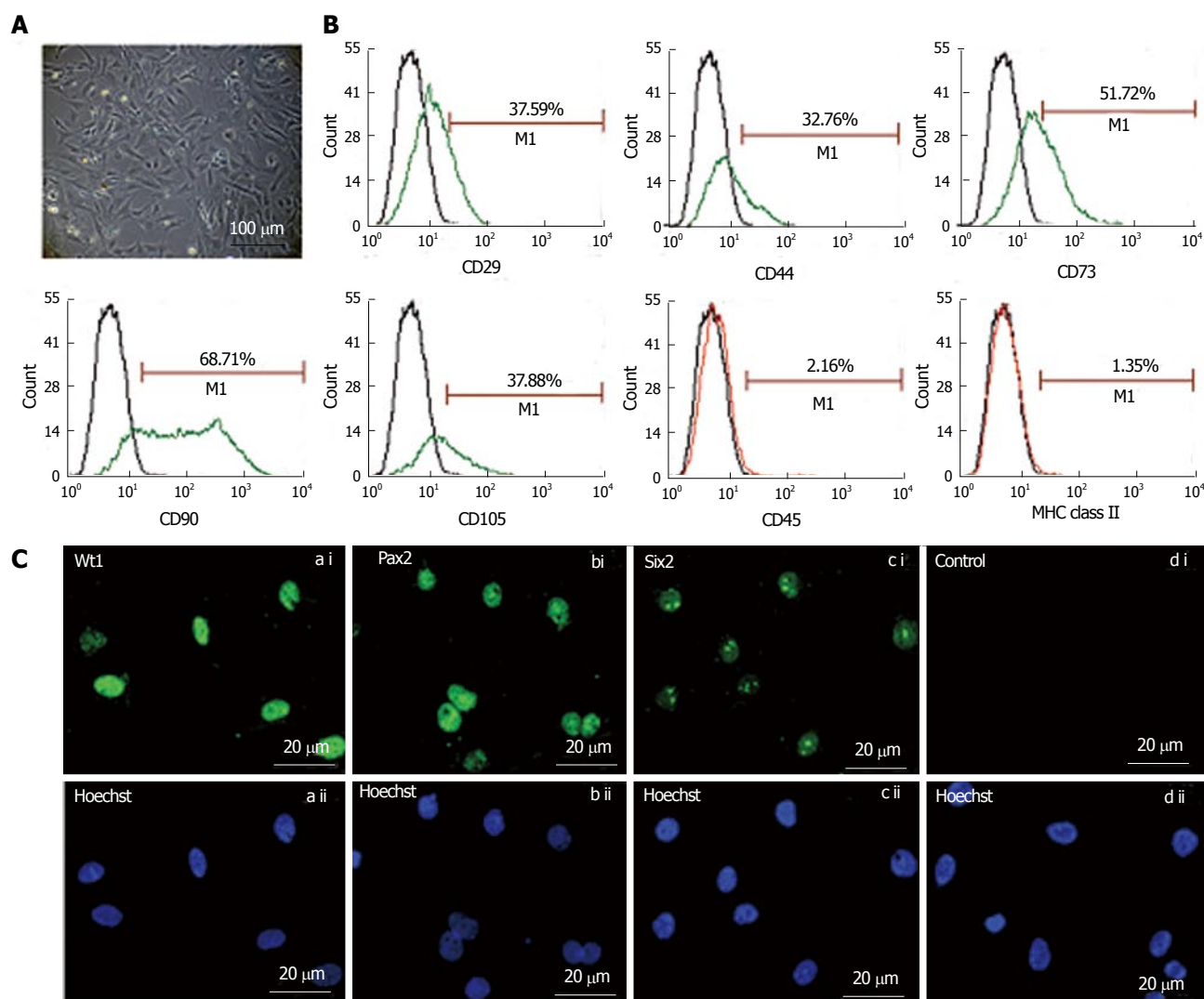


Figure 1 Morphology (A) and characterization of fetal kidney stem cells (B and C). A: Representative photomicrograph (Scale bars indicate 100 μ m) showing spindle-shaped and polygonal morphology of fetal kidney stem cells (fKSC); B: Phenotypic characterization of fKSC by flow cytometry showing expression of CD29, CD44, CD73, CD90, CD105, CD45, and MHC class II (green or red lines, detected with FITC - or phycoerythrin-conjugated antibodies, respectively) with isotype controls (black lines); C: Representative immunofluorescent photomicrographs (Scale bars indicate 20 μ m) showing expression of renal progenitor markers viz. Wt1, Pax2 and Six2 on

exhibited differentiation ability into epithelial cells as demonstrated by expression of CK18 and CK19 by immunocytochemistry (Figures 2A and B). When grown on matrigel, fKSC exhibited capillary-like tubular structures assessed by phase contrast microscopy (Figure 2C) and expressed endothelial markers viz., CD31 and vWF by immunocytochemistry (Figure 2D and E).

Improvement of renal function following fKSC therapy in ARF rats

On the 5th day of cisplatin injection, the rats exhibited a significant increase in levels of both BUN and serum creatinine as compared to healthy controls ($P < 0.05$). The same day (day 0 of therapy), the rats were randomized to receive fKSC therapy or saline alone. On day 3 of therapy, fKSC treated group had significantly lower levels of these blood biochemical parameters as compared to saline treated group ($P <$

0.05), but both groups had significantly higher levels as compared to healthy controls ($P < 0.05$). On day 7 of therapy, the serum creatinine and BUN levels in the fKSC treated group were comparable to healthy controls but they were still significantly higher in the saline treated group in comparison to healthy controls ($P < 0.05$) (Figure 3A and B).

Improvement of renal histology following fKSC therapy in ARF rats

The kidneys of ARF rats on the 5th day of cisplatin injection showed severe tubular necrosis, hyaline cast formation, loss of brush border in the proximal renal tubules and tubular dilatation (Figure 4B). On day 3 of fKSC therapy, the kidneys showed a significant attenuation of tubular injury as compared to saline treated rats (Figure 4C and D) and Jablonski grading score revealed significantly lower necrosis in the kidneys of fKSC treated as compared to saline treated

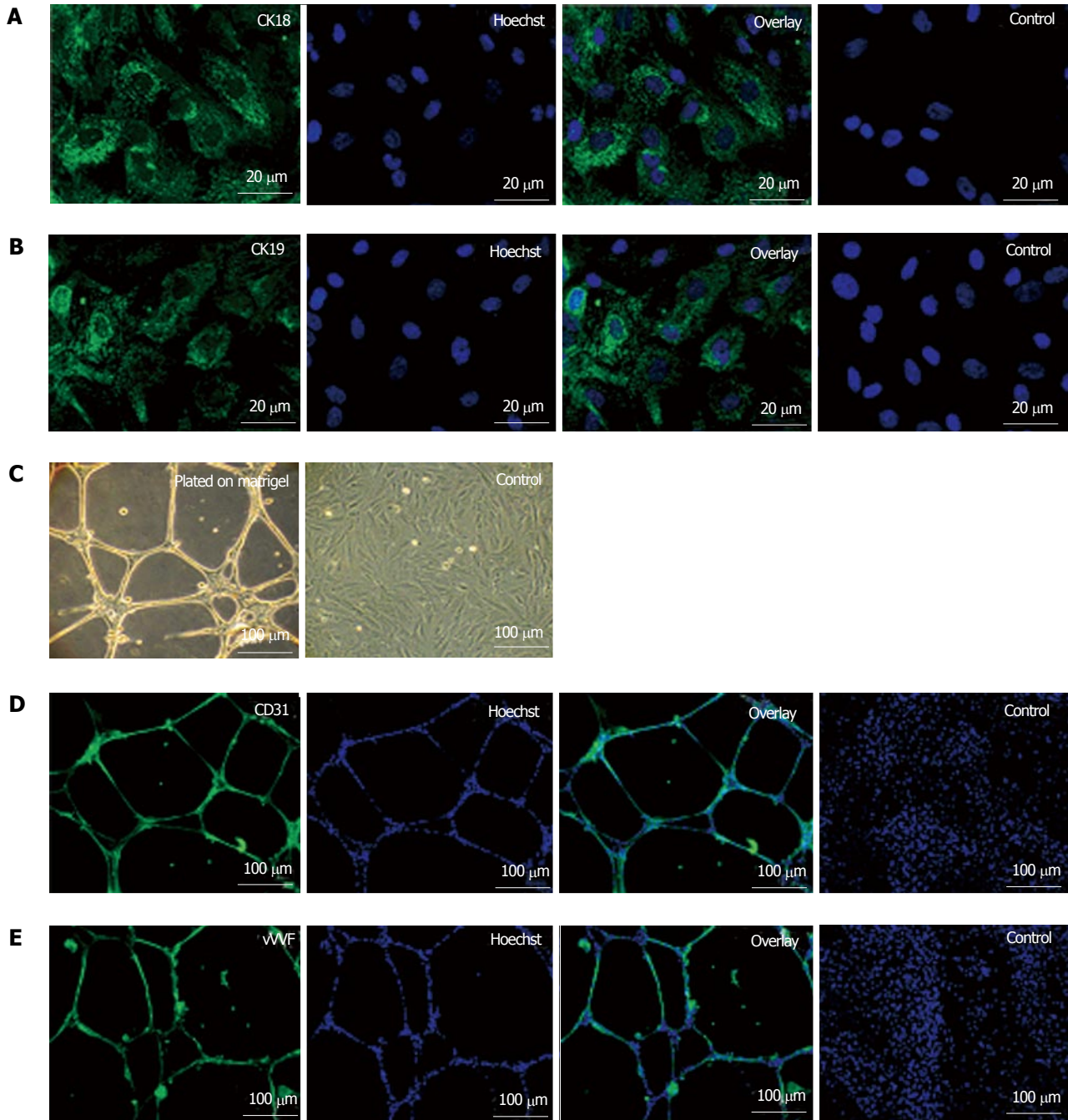


Figure 2 Epithelial differentiation (A, B) and angiogenic potential (C-E) of fetal kidney stem cells. A: Representative photomicrograph (Scale bars indicate 20 μ m) showing CK18 expression, Hoechst, overlay and untreated control cells with only Hoechst; B: Representative photomicrograph (Scale bars indicate 20 μ m) showing CK19 expression, Hoechst, overlay and untreated control cells with only Hoechst; C: Representative photomicrographs (Scale bars indicate 100 μ m) showing tubule like structure formation by fetal kidney stem cells (fkSC) cultured on matrigel and without matrigel as control; D: Representative immunofluorescent photomicrographs (Scale bars indicate 100 μ m) showing CD31 expression, Hoechst, overlay and fkSC cultured without matrigel as controls with only Hoechst; E: Representative immunofluorescent photomicrographs (Scale bars indicate 100 μ m) of fkSC cultured on matrigel showing Von Willebrand factor expression, Hoechst, overlay and fkSC cultured without matrigel as controls with only Hoechst.

rats (2.33 ± 0.33 vs 3.50 ± 0.34 , $P < 0.05$, Figure 4G). On day 7 of therapy, the kidneys of fkSC treated ARF rats showed disappearance of necrotic cells with almost normal architecture of tubules, whereas those of saline treated ARF rats still had necrotic tubular cells and hyaline casts (Figure 4E and F) and there was also a significant difference in Jablonski grading score between the fkSC treated and saline treated groups

(0.83 ± 0.16 vs 2.00 ± 0.25 , $P < 0.05$, Figure 4G).

Engraftment of infused fkSC in renal tubules

To study their engraftment, PKH26 labeled fkSC were evaluated in rat kidneys on day 7 of therapy. The fkSC were observed to engraft preferentially in renal tubules and capillaries stained with tubular epithelial marker AQP1 and endothelial marker vWF, respectively (Figure

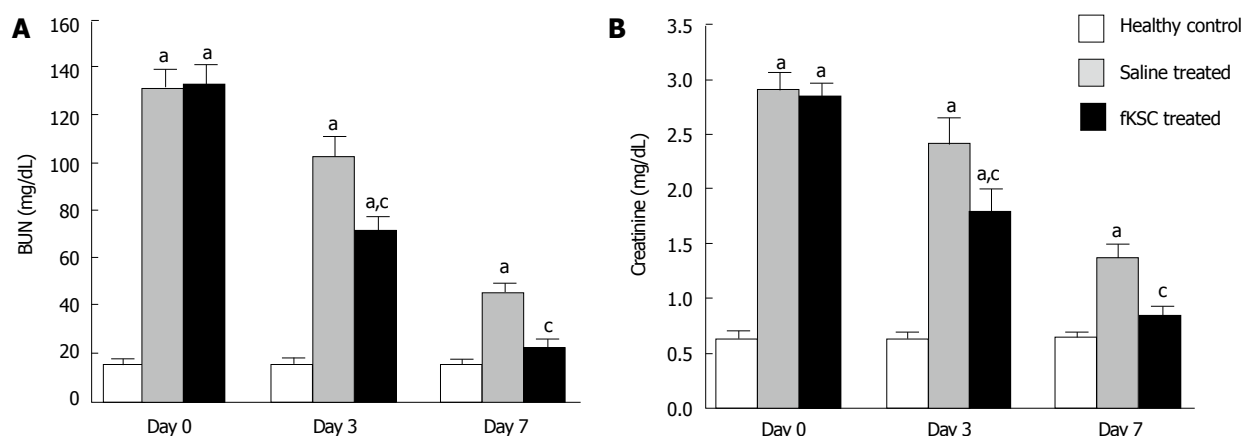


Figure 3 Effect of fetal kidney stem cells therapy on renal function in rats with cisplatin induced acute renal failure. The changes in the levels of (A) blood urea nitrogen (BUN) and (B) serum creatinine in healthy control, saline treated and fetal kidney stem cells (fKSC) treated groups on day 0, 3 and 7 after fKSC therapy. Values expressed as Mean $\pm$ SE. ^a $P < 0.05$ for saline and fKSC treated vs healthy control group; ^c $P < 0.05$ for fKSC vs saline treated group.

5).

Administration of fKSC reduces apoptosis and promotes proliferation of renal tissues

On day 3 of therapy, the TUNEL-positive cells in the kidneys of fKSC treated rats were significantly lower than those in the saline treated rats ($P < 0.05$). On day 7, their numbers further decreased significantly in fKSC treated rats as compared to those in the saline treated rats ($P < 0.05$; Figure 6A). On days 3 and 7 of therapy, the number of PCNA-positive cells in the kidneys of fKSC treated rats were significantly higher as compared to those in the saline treated rats ($P < 0.05$, Figure 6B).

Administration of fKSC promotes renal angiogenesis

On day 3 of therapy, the morphometric analysis of CD31 labeled peritubular capillaries showed a significantly higher capillary density in the kidneys of fKSC treated than that in saline treated rats (13.30 ± 1.54 vs 7.10 ± 1.29 , capillaries/HPF, $P < 0.05$). On day 7 of therapy, the capillary density in the kidneys of fKSC treated rats was further significantly increased than that in saline treated rats (21.10 ± 1.46 vs 15.00 ± 1.30 , capillaries/HPF, $P < 0.05$; Figure 7).

To determine the early angiogenic effect of fKSC, the protein expression of angiogenic signaling molecules were studied on day 3 of fKSC therapy. The expressions of HIF-1 α , VEGF and eNOS in the kidneys of fKSC treated rats were significantly higher as compared to saline treated rats ($P < 0.05$; Figure 8).

DISCUSSION

The present study demonstrates that fKSC expressed mesenchymal as well as renal progenitor markers and exhibited the formation of CD31 and vWF expressing capillary-like structures and differentiation into CK18 and CK19 positive epithelial cells *in vitro*. The administration of fKSC in rats with cisplatin induced ARF resulted in rapid improvement in renal function

and histology. The infused fKSC were observed to engraft in renal tubules and promote proliferation and reduce apoptosis of renal tubular cells. In addition, the kidneys of fKSC treated rats exhibited increased angiogenesis and up-regulation of angiogenic signaling molecules. To our knowledge this is the first study showing therapeutic efficacy of *in vitro* expanded fKSC in cisplatin induced ARF model and role of angiogenesis in renal regeneration by these stem cells.

We have recently observed that fKSC have maximal growth at a seeding density of 1000 cells/cm², population doubling time of approximately 34 h and normal karyotype up to 3rd passage. In addition, these cells successfully differentiated into cells of all the three germ layers (communicated elsewhere). In the present study, we have observed that fKSC express mesenchymal (CD29, CD44, CD73, CD90 and CD105) as well as renal progenitor markers (Wt1, Pax2 and Six2). The Pax2 and Wt1 genes cross-regulate expression of each other during kidney development^[16]. However, we have observed a high expression of these markers in fKSC. A plausible explanation of this could be that a significant proportion of fKSC are a population of stem cells of metanephric mesenchyme which have been shown to express both Pax2 and Wt1 transcription factors^[13,17,18]. Similar to our observation high expression of Pax2 and Wt1 has recently been shown in human fetal kidney progenitors as well as human embryonal cells derived nephron progenitors^[19]. The fKSC could be induced to differentiate *in vitro* into CK18 and CK19 positive epithelial cells. In addition, they exhibited an angiogenic potential as shown by the formation of CD31 and vWF positive capillary-like tubular structures when plated on matrigel. Two previous studies have shown that stem cells derived from whole fetal kidneys express mesenchymal and renal progenitor markers, but they have not studied their *in vitro* angiogenic potential and differentiation into epithelial cells^[20,21]. However, corroborating with our data, one study has shown that human embryonic kidney derived CD24⁺ CD133⁺ stem cells exhibit *in vitro* differentiation into epithelial

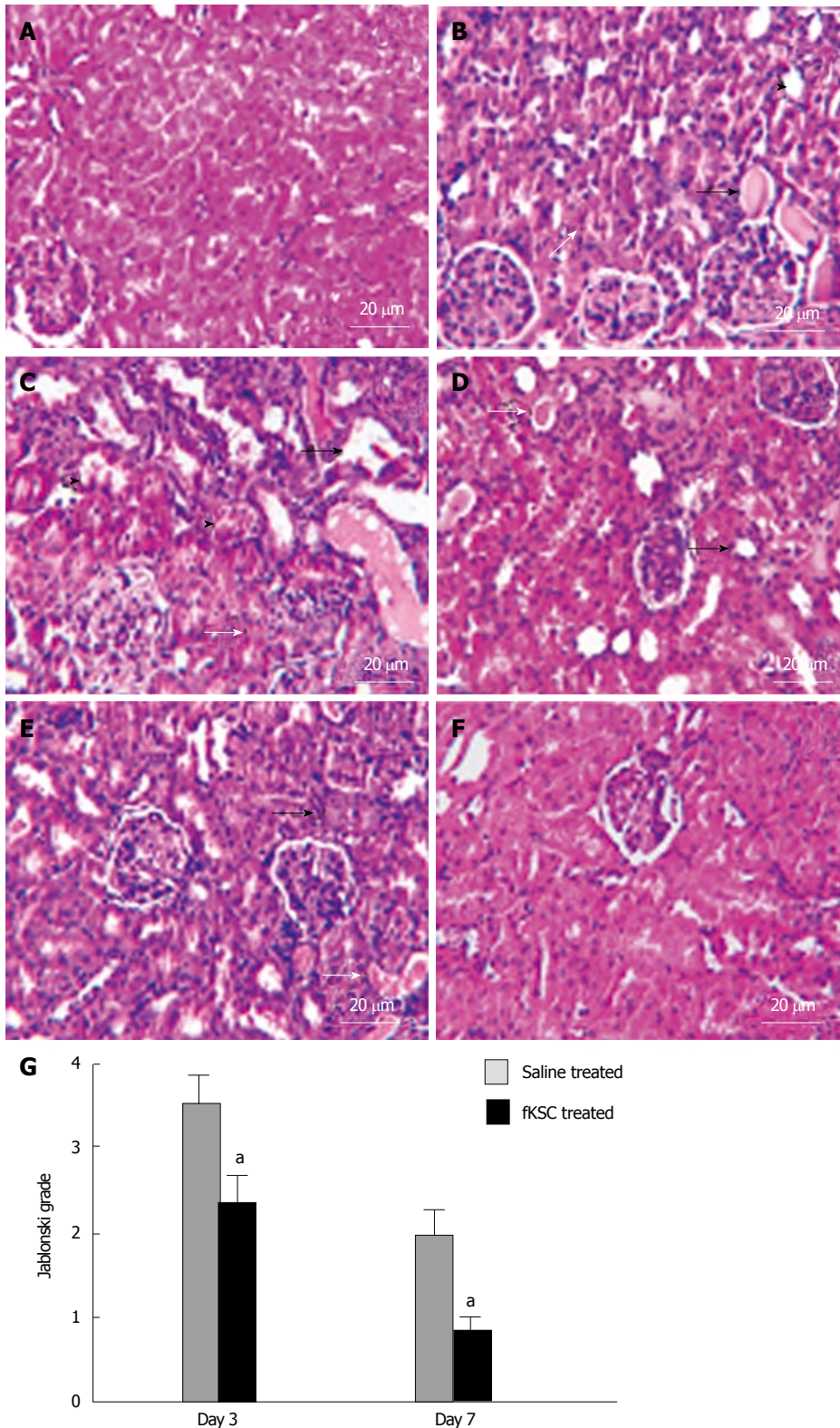


Figure 4 Fetal kidney stem cells attenuate renal structural damage in rats with cisplatin induced acute renal failure (Scale bars indicate 20 µm). A: Kidney section of healthy control rats showing the normal architecture of tubules and glomeruli; B: Kidney section after 5 d of cisplatin treatment showing severe tubular necrosis (white arrow), hyaline cast formation (black arrow) and tubular dilatation (black arrow head); C: Kidney section of saline treated rats on day 3 of therapy showing severe tubular necrosis (white arrow), tubular vacuolization (black arrow) and tubular swelling and obstruction (black arrow head) with cell debris on day 3 after therapy; D: Kidney section of fetal kidney stem cells (fKSC) treated rats on day 3 of therapy showing attenuation of tubular injury with mild tubular dilatation (black arrow) and few hyaline casts inside the lumen (white arrow); E: Kidney section of saline treated rats on day 7 of therapy showing few necrotic tubular cells (black arrow) and luminal hyaline casts (white arrow); F: Kidney section of fKSC treated rats on day 7 of therapy fKSC showing almost normal architecture of tubules; G: Jablonski grading score of histological assessment of tubular necrosis in saline and fKSC treated kidneys on day 3 and day 7. Values expressed as mean $\pm$ SE. ^a $P < 0.05$ for fKSC vs saline treated group.

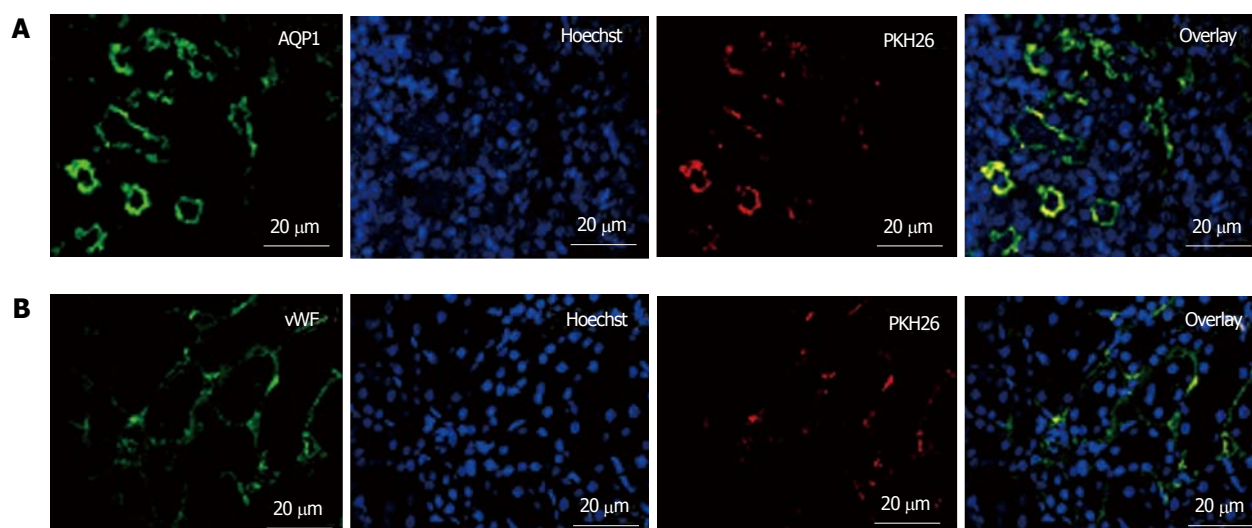


Figure 5 *In vivo* homing and engraftment of administered fetal kidney stem cells in renal tubules. Representative immunofluorescent photomicrographs (Scale bars indicate 20 μ m) showing engraftment of administered fetal kidney stem cells (fKSC) in renal tubules at day 7 of therapy, and expression of tubular epithelial and endothelial markers aquaporin (AQP)1 (A) and Von Willebrand factor (vWF) (B) respectively, with corresponding Hoechst, PKH26 labeled administered fKSC in injured kidney tissue respectively.

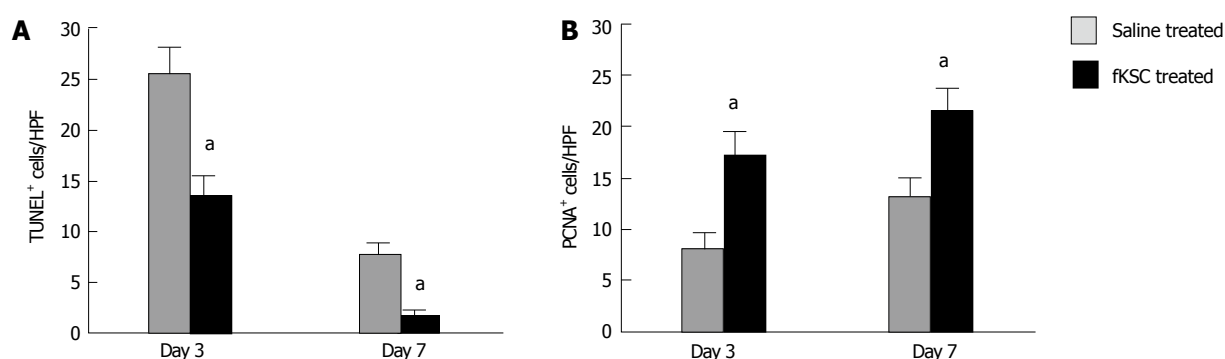


Figure 6 Effects of fetal kidney stem cells on apoptosis and proliferation of tubular cells in cisplatin injured kidney. A: Quantification of dUTP nick-end labeling (TUNEL)-positive cells in kidney sections of saline and fetal kidney stem cells (fKSC) treated groups at day 3 and 7 after fKSC therapy; B: Quantification of proliferating cell nuclear antigen (PCNA)-positive cells in kidney sections of saline and fKSC treated groups at day 3 and 7 after fKSC therapy. Values expressed mean $\pm$ SE. ^a $P < 0.05$ for fKSC vs saline treated group. HPF: High-power field.

and endothelial lineages^[22]. We did not evaluate the markers of other renal progenitors such as endothelial and epithelial ones on fKSC which may be a limitation of the present study.

To investigate their therapeutic effect in ARF, we infused intravenously PKH26 labeled fKSC in cisplatin induced rat model of ARF. On day 3 of therapy, we observed a significant improvement in the renal function and histology and on day 7 these parameters became comparable to healthy control rats indicating almost complete recovery. Similar to our data, culture expanded CD24⁺ CD133⁺ stem cells from human embryonic kidney have been shown to mediate a complete functional and structural recovery of glycerol induced ARF in SCID mice^[22]. We observed that PKH26 labeled fKSC engraft in the damaged areas of the kidney and express vWF and AQP1 showing the integration of infused fKSC into damaged renal vasculature and tubules, respectively. It is difficult to distinguish whether vWF and AQP1 are expressed by endothelial

and epithelial cells differentiated from infused fKSC or these markers represent fusion of administered fKSC with damaged kidney cells in the areas of engraftment. Similar to our observation, administration of human embryonic kidney derived CD24⁺ CD133⁺ stem cells in SCID mice with glycerol induced ARF and embryonic stem cell derived mesenchymal like progenitors in rats with cisplatin induced ARF have been shown to engraft into damaged renal tubules and vasculature of kidney^[22,23].

The kidneys of fKSC treated ARF rats were observed to have a higher capillary density correlating with their structural and functional improvement as compared to saline treated ARF rats. In addition, on day 3 of fKSC therapy, there was an increased expression of HIF-1 α and its downstream signaling molecules VEGF and eNOS in the kidneys of fKSC treated rats in comparison to saline treated rats, showing a role of early angiogenesis in the renal regeneration. It has been reported that HIF-1 α also activates expression of

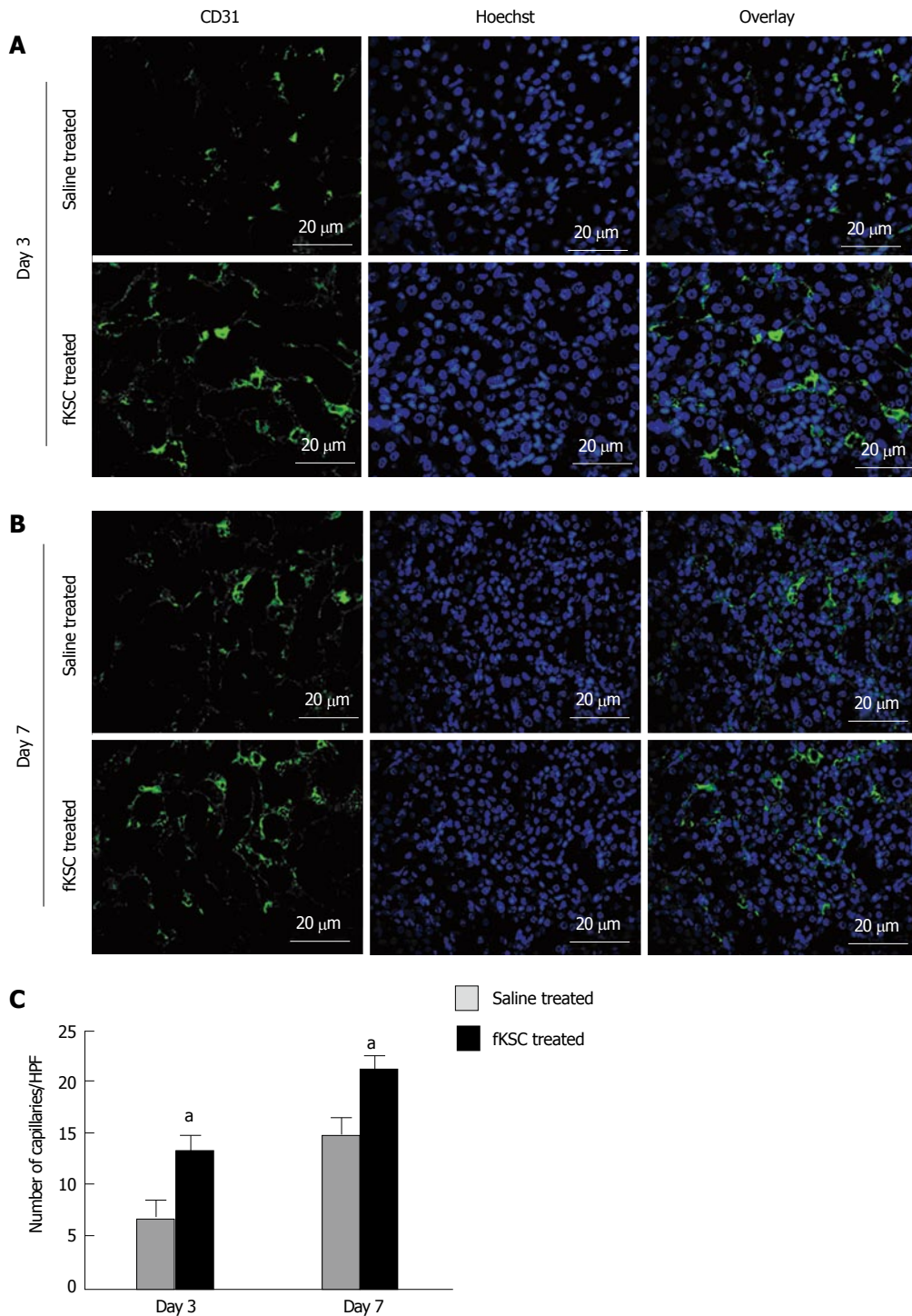


Figure 7 Fetal kidney stem cells accelerate angiogenesis in rats with cisplatin induced acute renal failure. Representative immunofluorescent photomicrographs (Scale bars indicate 20 μ m) showing perivascular capillaries stained with CD31 in saline treated and fetal kidney stem cells (fKSC) treated groups on day 3 (A) and on day 7 (B) after fKSC therapy. C: Quantification of perivascular capillaries stained with CD31, expressed as capillaries per HPF, in saline and fKSC treated groups. Values expressed Mean $\pm$ SE. ^a $P < 0.05$ for fKSC vs saline treated group. HPF: High-power field.

Wt1, which in turn directly up-regulates VEGF and thus acts as an important regulator of angiogenesis^[24-27]. The fKSC that were infused in ARF rats were already expressing Wt1, so we did not study its expression in the kidney tissues but as fKSC therapy up-regulated HIF-1 α , it is likely that kidney tissues also have an up-regulation of Wt1. However, since the angiogenic role of Wt1 has mostly been reported in tumors, it is not

known whether Wt1 also has a similar role in normal tissues like kidney. Corroborating with our data, it has been shown that acetazolamide administration in mice with ischemic kidney results in up-regulation of angiogenic molecules HIF-1 α and eNOS in kidney tissue along with reduction in the apoptosis of renal tubular cells, increase in renal blood flow and improvement in renal function and histology^[28]. Similarly, the

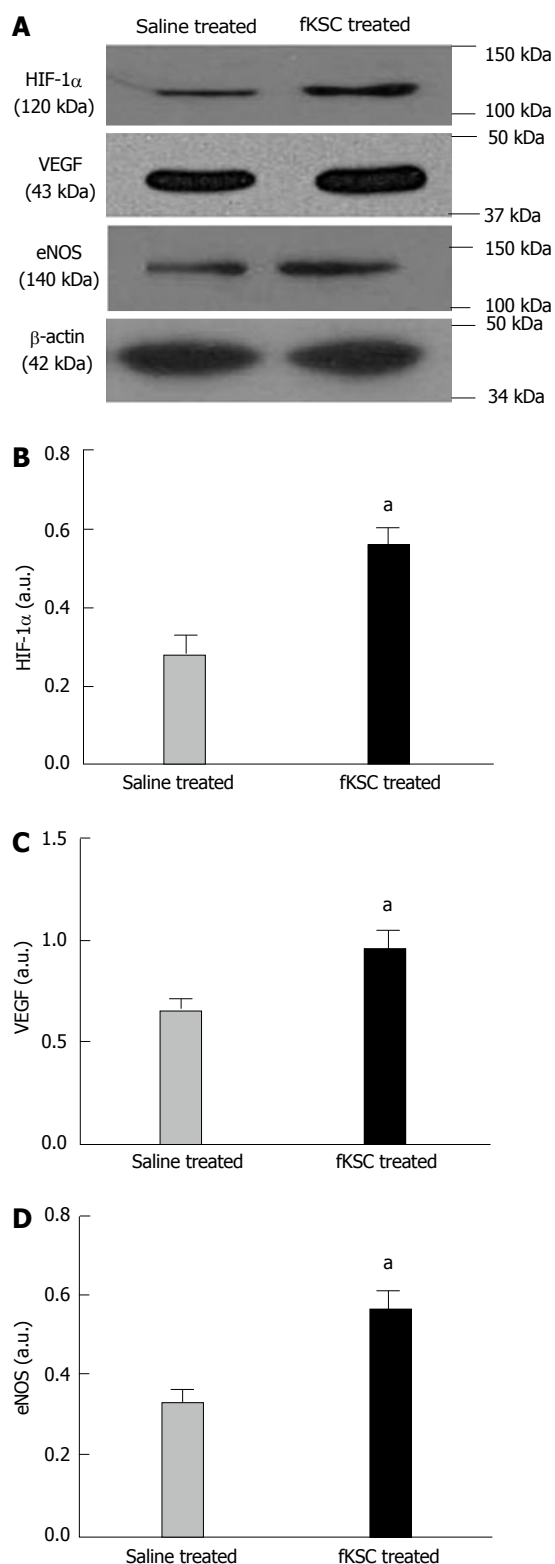


Figure 8 Early angiogenic effect of administered fetal kidney stem cells in cisplatin injured kidney. Representative immunoblots showing the expression of hypoxia-inducible factor (HIF)-1 α , vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in saline and fetal kidney stem cells (fKSC) treated groups on day 3 after fKSC therapy (A-D). Bar diagrams showing densitometric quantification of the expression of HIF-1 α (B), VEGF (C) and eNOS (D). Comparative gene expression ratio was calculated by referring each gene to β -actin as an internal control. Densitometric analysis applied for comparison of relative protein expression and represented in densitometric arbitrary units (a. u.). Values expressed mean $\pm$ SE. ^a $P < 0.05$ for fKSC vs saline treated group.

VEGF has been shown to promote regeneration of cisplatin induced injury of renal tubular epithelial cells *in vitro* by inducing proliferation and reducing their apoptosis. In addition, administration of VEGF modified embryonic mesenchymal stem cells (MSC) in cisplatin induced ARF in rodents has been shown to reduce renal apoptosis and promote microcirculation and cell proliferation in the damaged kidney^[29]. There is no previous study showing angiogenic role of fKSC in ARF but in agreement with our study, it has been shown that a clonal cell line of MSC derived from kidney of adult Tie-2 green fluorescent protein transgenic mice exhibit *in vivo* angiogenesis and tubular regeneration by increasing epithelial proliferation and inhibiting apoptosis when administered in mouse model of renal ischemia^[30]. These studies suggest that the improved capillary density, proliferation and reduced apoptosis in renal tissue observed by us following fKSC therapy may be mediated by the up-regulation of VEGF, HIF-1 α and eNOS, and thus these molecules could play an important role in renal regeneration.

In conclusion, the present study shows that fKSC express mesenchymal and renal progenitor markers, exhibit an *in vitro* angiogenic potential and ability to differentiate into cells of renal epithelial lineage. The administration of fKSC in cisplatin induced ARF results in rapid recovery of renal function and histology, and promotes renal angiogenesis. Further studies on fKSC mediated renal angiogenesis and regeneration may lead to the development of novel pharmacological therapies for ARF.

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COMMENTS

Background

Tissue specific fetal stem cells represent the most suitable stem cell type for tissue regeneration/repair due to their inherent ability of tissue specific differentiation. Thus, stem cells derived from the fetal kidney may be a novel cell type for renal regenerative therapy. The epithelial and microvascular damage are the main events in acute renal failure (ARF) and this led us to evaluate whether fetal kidney stem cells (fKSC) mediate their therapeutic effect by augmenting renal angiogenesis in cisplatin induced ARF in rats.

Research frontiers

The authors had demonstrated in the present study that fKSC differentiate into cells of epithelial lineage and exhibit angiogenic potential *in vitro*. Following infusion in ARF rats, they engraft in renal tubules and rapidly improve renal function and histology along with increased angiogenesis in the kidney tissues. It is thus logical to investigate whether induction of renal angiogenesis by fKSC is an important mechanism of renal regeneration and whether it is mediated by trans-differentiation of infused fKSC or their paracrine effect on resident kidney

stem cells.

Innovations and breakthroughs

To the best of our knowledge, this is the first study showing therapeutic efficacy of *in vitro* expanded fKSC in ARF model and their *in vitro* and *in vivo* angiogenic potential suggesting that induction of renal angiogenesis may be an important therapeutic mechanism of these stem cells.

Applications

The fKSC represent primitive renal progenitors expressing mesenchymal as well as renal progenitor markers and treatment of ARF rats with these stem cells results in rapid recovery in renal function and histology leading to their normalization. Further studies using human fKSC or human embryonic stem cell derived renal progenitors with similar characteristics may lead to the development of clinical therapy for ARF.

Terminology

The fKSC represent primitive renal progenitors and express mesenchymal as well as renal progenitor markers. They can be culture expanded to generate a requisite number of cells for therapeutic dose. They improve renal function and histology and lead to their normalization by inducing renal angiogenesis. It needs to be studied whether the angiogenesis observed in the kidney tissues in the fKSC treated rats is due to trans-differentiation of infused stem cells or by stimulation of resident endothelial cells or both.

Peer-review

It is a very interesting article about a possible protective role of fetal kidney stem cells in experimental model of acute renal failure.

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**EDITORIAL**

- 789 Mesenchymal stem cells: A new diagnostic tool?
Valenti MT, Mori A, Malerba G, Dalle Carbonare L

REVIEW

- 793 Contemporary perspective on endogenous myocardial regeneration
Milasinovic D, Mohl W
- 806 Corneal stem cells and tissue engineering: Current advances and future perspectives
de Araujo AL, Gomes JÁP
- 815 Stem cell applications for pathologies of the urinary bladder
Mousa NA, Abou-Taleb HA, Orabi H
- 823 Modeling diseases of noncoding unstable repeat expansions using mutant pluripotent stem cells
Yanovsky-Dagan S, Mor-Shaked H, Eiges R
- 839 Human dental pulp stem cells: Applications in future regenerative medicine
Potdar PD, Jethmalani YD

MINIREVIEWS

- 852 Histone deacetylases and cardiovascular cell lineage commitment
Yang JY, Wang Q, Wang W, Zeng LF
- 859 Hepatic stem cells: A viable approach for the treatment of liver cirrhosis
Habeeb MA, Vishwakarma SK, Bardia A, Khan AA
- 866 Hair follicle stem cells: *In vitro* and *in vivo* neural differentiation
Najafzadeh N, Esmailzade B, Dastan Imcheh M

ORIGINAL ARTICLE**Basic Study**

- 873 Utility of tricalcium phosphate and osteogenic matrix cell sheet constructs for bone defect reconstruction
Ueha T, Akahane M, Shimizu T, Uchihara Y, Morita Y, Nitta N, Kido A, Inagaki Y, Kawate K, Tanaka Y
- 883 New model for cardiomyocyte sheet transplantation using a virus-cell fusion technique
Takahashi Y, Tomotsune D, Takizawa S, Yue F, Nagai M, Yokoyama T, Hirashima K, Sasaki K

Contents

World Journal of Stem Cells
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Mesenchymal stem cells: A new diagnostic tool?

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Abstract

Mesenchymal stem cells (MSCs) are progenitor cells capable of self-renewal that can differentiate in multiple tissues and, under specific and standardized

culture conditions, expand *in vitro* with little phenotypic alterations. In recent years, preclinical and clinical studies have focused on MSC analysis and understanding the potential use of these cells as a therapy in a wide range of pathologies, and many applications have been tested. Clinical trials using MSCs have been performed (*e.g.*, for cardiac events, stroke, multiple sclerosis, blood diseases, auto-immune disorders, ischemia, and articular cartilage and bone pathologies), and for many genetic diseases, these cells are considered an important resource. Considering of the biology of MSCs, these cells may also be useful tools for understanding the physiopathology of different diseases, and they can be used to develop specific biomarkers for a broad range of diseases. In this editorial, we discuss the literature related to the use of MSCs for diagnostic applications and we suggest new technologies to improve their employment.

Key words: Mesenchymal stem cells; Biomarkers; Next generation sequencing; Diagnostic tool; Tumor-initiating cells

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Core tip: Mesenchymal stem cells have been considered potential tools for therapeutic applications in a wide range of pathologies. However, these cells may be used to develop specific biomarkers in a broad range of diseases and they could be considered useful tools to perform new strategies to get early diagnoses. Rare stem cell populations can be studied by recent technologies such as Next Generation Sequencing in order to develop specific marker for diagnostic and prognostic applications.

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INTRODUCTION

Mesenchymal stem cells (MSCs) reside in many tissues during development, and their differentiation produces specific phenotypes such as osteoblasts, chondrocytes, adipocytes, and myoblasts. In recent years, MSCs have been considered an important source for cell therapy and tissue regeneration in many clinical applications. Tissue damage is followed by an inflammatory response, and pro-inflammatory factors can rescue MSCs and start the repair process. The process of regeneration is quite complex; MSCs interact with stromal and inflammatory cells, and derived factors play an important role in this process^[1]. MSCs are also involved in immunosuppression *via* inhibition of T cells, B cells, dendritic cells and natural killer cells^[2-5], and they may exert immunomodulatory activity during the co-transplantation process^[1].

In addition to cell therapy, MSCs are also a promising choice for other clinical applications because they can be used as diagnostic tools. The involvement of MSCs in many physiological or physiopathological aspects offers the possibility of targeting these cells, and their related molecular products, circulating in peripheral blood to obtain an early diagnosis by a non-invasive approach.

Diagnostic application of MSCs

In recent years, MSCs have been considered important "biomarkers" for a non-invasive prenatal diagnosis^[6]. Accurate prenatal diagnoses without fetal damage are needed to prevent genetic diseases, and MSCs have been identified in fetal blood during the first trimester, albeit at low concentrations. Despite 20 years of research in this area, technical challenges have produced many obstacles to reproducible fetal MSC isolation, and culture strategies have been developed. However, fetal MSCs have been observed in maternal peripheral blood, suggesting that fetal surface antigens could be considered promising biomarkers for a noninvasive prenatal diagnosis^[7], and the analysis of neural and MSCs in the amniotic fluid represents a useful tool for the identification of neural tube defects^[8].

Recruitment of progenitor cells in the blood stream in response to skeletal damage has been reported^[9], and we have shown that circulating MSCs are increased in patients affected by osteoporosis as a consequence of the impairment of osteoblast differentiation^[10]. Furthermore, the molecular analyses of genes involved in the differentiation process have revealed an abnormal level of expression of the transcription factor *runx2*, the master gene of osteogenic differentiation. These data suggest the possibility of using MSC analysis for the evaluation of bone diseases, and the identification of circulating MSCs could be proposed as a noninvasive diagnostic tool.

Circulating stem cells have been observed after

ischemic stroke in patients with myocardial infarction^[11,12], and this finding suggests that stem cells in the peripheral blood may provide a potential cell marker for prognosis and risk evaluation in patients with cardiac diseases.

Kim *et al.*^[13] showed that CD105 MSCs were mobilized in patients with cerebrovascular stroke, and, in particular, the authors demonstrated that the percentage of apoptotic CD105 cells, based on annexin V expression, was higher in patients with cerebral infarcts than that in normal control subjects.

The epithelial mesenchymal transition that occurs during cellular neoplastic transformation makes MSCs an important target for the diagnosis and prognosis of malignant tumors^[14]. Because cancer may originate from the neoplastic transformation of progenitors or related early differentiated cells, stem cell-like markers expressed in tumor cells could be of interest for biomarker identification.

New technologies for stem cells

Recent technologies have made possible the study of the molecular biology of cells at different levels (*i.e.*, the genome, epigenome, transcriptome, small RNAome and metabolome). However, the study of stem cells is still a major challenge due to their low numbers and their potency and plasticity. Currently, most stem cell studies reported in the literature apply microarray technology and focus on the gene expression profile analysis of MSCs^[7,15-19], cancer stem cells^[20-24], tumor-initiating cells^[25] and embryonic stem cells. The study of gene expression at the global level is generally a pitfall and an ordeal due to the presence of low expression levels of many genes, which occurs because stem cells need to be ready to undertake a variety of differentiation programs. Accordingly, the study of different individual gene expression profiles is very difficult. Such issues cause the true gene expression signal to be confounded by background noise. At present, more recent Next Generation Sequencing (NGS) technologies^[26] are entering the stem cell arena. NGS is based on massive sequencing, and it can be applied for several purposes, such as the detection of transcripts and estimation of their levels (transcriptome), the identification of genome sequence variants (exome, genome) or the examination of modified methylated nucleotides (methylome). It is noteworthy that epigenomic NGS applications^[27] can be used to study stem cells because they can be used to investigate gene regulation and genome function during the differentiation and reprogramming processes. Therefore, the Epigenomics Roadmap consortium has been formed by the NIH to create extensive maps of genomic and epigenomic elements in stem cells and *ex vivo* tissue^[28]. NGS methods can be used to sequence single cells (DNA and/or RNA) opening avenues to thoroughly investigate how differential gene expression in individual cells defines cellular differentiation, function and physiology^[22],

making it possible to study rare stem cell populations and to investigate the prevalence and differences of potential stem cell subpopulations in cancers or other types of tissue^[29].

CONCLUSION

Although several molecules such as Stro-1, CD271, SSEA-4 and CD146 are already recognized as markers for MCS cells^[30], it would be useful to have even more specific markers for stem cell potential before MSC cells can serve as reliable tools for standardized diagnostic methods. In conclusion, the analysis of MSCs and their related molecular products supported by new sequencing technologies may lead to the identification of potential biomarkers that could represent useful tools for noninvasive or less invasive diagnostic and prognostic applications for a wide range of diseases. In addition, the individualization of specific markers linked to MSCs could be used to develop new strategies to obtain early diagnoses and start therapeutic approaches for patients as soon as possible.

Certainly, further investigations are necessary to validate and improve the sensitivity of specific biomarkers linked to MSCs, and innovative and costly technologies are required considering the low number and plasticity of MSCs.

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Contemporary perspective on endogenous myocardial regeneration

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Abstract

Considering the complex nature of the adult heart, it is no wonder that innate regenerative processes, while maintaining adequate cardiac function, fall short in myocardial jeopardy. In spite of these enchaining

limitations, cardiac rejuvenation occurs as well as restricted regeneration. In this review, the background as well as potential mechanisms of endogenous myocardial regeneration are summarized. We present and analyze the available evidence in three subsequent steps. First, we examine the experimental research data that provide insights into the mechanisms and origins of the replicating cardiac myocytes, including cell populations referred to as cardiac progenitor cells (*i.e.*, c-kit⁺ cells). Second, we describe the role of clinical settings such as acute or chronic myocardial ischemia, as initiators of pathways of endogenous myocardial regeneration. Third, the hitherto conducted clinical studies that examined different approaches of initiating endogenous myocardial regeneration in failing human hearts are analyzed. In conclusion, we present the evidence in support of the notion that regaining cardiac function beyond cellular replacement of dysfunctional myocardium *via* initiation of innate regenerative pathways could create a new perspective and a paradigm change in heart failure therapeutics. Reinitiating cardiac morphogenesis by reintroducing developmental pathways in the adult failing heart might provide a feasible way of tissue regeneration. Based on our hypothesis "embryonic recall", we present first supporting evidence on regenerative impulses in the myocardium, as induced by developmental processes.

Key words: Cardiac regeneration; Cardiac development; Embryonic recall; Pressure-controlled intermittent coronary sinus occlusion; Heart failure; Myocardial infarction

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Core tip: Unlike in primitive vertebrates, any regenerative effort in adult mammalian hearts after an acute event remains unsatisfactory. Most efforts to repopulate failing hearts with functioning and integrated cardiomyocytes have not achieved clinical importance. In this overview, after describing several options for endogenous myo-

cardial repair, we support the notion of a paradigm change towards inducible developmental processes in regeneration research. Major efforts have been made to convert tissues upstream in the Waddington scheme. Recently, stress transformed acquired pluripotency raised enormous expectations, but results and proof of concept were seriously questioned. We want to introduce pressure-controlled intermittent coronary sinus occlusion as a potential resource to decipher the unsolved equation of re-inducing the developmental processes in the human heart.

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INTRODUCTION

Advances in therapeutic approaches to acute life-threatening cardiovascular conditions, such as early revascularization in patients with acute myocardial infarction (AMI), have contributed to a dramatic decrease in short-term mortality. Hence prolonged life expectancy coupled with the residual impairment of heart function and the progressive nature of cardiovascular diseases^[1] have exponentially increased the number of patients with chronic insufficiency of heart function. While aggressive medical therapy of severe chronic heart failure (HF) may alleviate symptoms, the ultimate treatment option for this growing patient group remains heart transplantation. However, due to infrastructural and technical constraints, heart transplantation could barely meet the needs of the vast population of patients with HF^[2], who have deleterious prognosis comparable to malignant diseases with the mortality rate as high as about 25% within the first year of diagnosis^[3,4]. In the United States the estimated prevalence of HF is > 5 million patients, annual incidence is > 500000 patients and annual mortality with HF as underlying cause is > 50000^[5], which makes paramount the development of a therapy regimen effective beyond the acute phase and available to a broad population of patients.

The pathophysiological substrate of heart failure is the loss of functioning cardiomyocytes during the overall process of remodeling of the heart tissue, which in turn leads to diminishing of the contractile function^[6]. Between the two ends of contemporary clinical reality represented by acute therapies, such as early revascularization, which are available to many, but effective mainly in improving short- and mid-term clinical outcomes, and heart-transplantation as ultimate treatment strategy for a failing heart, but available to only few, there is an unmet need to reverse the process of remodeling and foster myocardial regeneration.

The notion of replenishing lost cardiomyocytes is founded on the triad of rejuvenation, replacement and regeneration^[7] with the common goal of structural and functional heart repair. While the term rejuvenation refers to activation of myocardial self-renewal processes, replacement and regeneration are based on transplantation into the damaged host myocardium, of functional tissues and progenitor cells, respectively^[8].

First studies that tested the effects of transplantation of progenitor cells in patients with AMI or chronic ischemic heart disease while proving safe, showed modest improvement in clinical outcomes^[9,10]. The translation of this luring concept of repopulating heart with transplanted stem cells into every-day clinical practice has been challenged by the following issues: type and dosing of progenitor cells, ex-vivo processing and expansion, timing and route of administration, as well as timing of transplantation respective the stage and level of myocardial ischemia^[11]. Immunological response of the host-microenvironment that impacts long-term prognosis and possible ethical implications of transplanting pluripotent cells of embryonic origin have additionally burdened the research in the field of stem-cell based approaches to heart repair.

Opposite to the transplantation-based regimens, interventions aiming at rejuvenation of damaged heart tissue by activating innate cardiac repair pathways seem to provide a more holistic approach, while avoiding possible pitfalls of cell injection into the microenvironment of an ischemic heart. The development of such therapeutic strategies is supported by the evidence of postnatal formation of new cardiomyocytes, thus indicating the potential for endogenous myocardial regeneration^[12,13]. Since the effects of stem-cell therapies have been attributed not only to engraftment of transplanted cells and generation of new cardiomyocytes, by means of transdifferentiation or cell fusion, but to a considerable degree to paracrine effects^[14], the transplantation of cells could seem redundant in wake of a possible intervention succeeding in activation of endogenous repair pathways.

Our aim was to review the literature on the mechanisms of endogenous myocardial regeneration and to analyze the contemporary therapeutic approaches to activation of these inborn pathways of myocardial repair. We proceed in three subsequent steps by examining (1) the evidence for the self-regenerative capacity of the heart; (2) the initiation of endogenous myocardial repair mechanisms in a clinical setting such as acute or chronic myocardial ischemia; and (3) the results of the hitherto conducted clinical studies that investigated different methods of triggering endogenous myocardial regeneration in failing human hearts.

SELF-REGENERATIVE CAPACITY OF THE HEART

The axiom of regenerative medicine has been that

apart from intestinal epithelium, skin and bone marrow, no other tissue contained stem-cells capable of self-renewal^[15], with the predominant belief that in postnatal life cardiomyocytes respond with hypertrophy when exposed to stress^[16]. According to this view, from the birth on cardiomyocytes could only grow in size without any significant reproduction capacity, which is how > 20 fold increase in heart mass from birth to adulthood has been explained^[15,17-19].

The described axiom that cardiac myocytes could only grow in size, has been challenged by an early work that lists myocyte hyperplasia as mode of adaption to microenvironmental stimuli within the heart^[20]. Several contemporary experiments have contributed evidence in favor of a more dynamic view of myocardial cellular homeostasis, attributing cardiac myocytes the potential to increase in number^[21-24]. Of note, pathologic stimuli like acute myocardial infarction^[21] and chronic ischemia^[23] that lead to destruction and functional deterioration of myocytes, seem to concurrently provide the groundwork for their replenishment. It has been shown that during the initial stages of pressure overload hypertrophy the DNA synthesis in cardiomyocytes takes place^[25]. Cardiomyocyte hypertrophy and hyperplasia appear to be in no way mutually exclusive and both part of heart's defense strategy, with myocyte proliferation commencing with severe functional deterioration of the heart^[26].

In order to assess the clinical relevance of the posited myocardial self-regeneration capacity two issues need to be clarified. First, what is the expected magnitude of cardiomyocyte proliferation? Second, what is the origin of the newly formed cardiomyocytes?

Quantification of cardiomyocyte replication

A landmark study, that investigated cardiomyocyte proliferation based on carbon-14 integration in the DNA, reported that fewer than 50% of cardiomyocytes are renewed during a normal life span^[12]. The annual myocyte turn-over rate decreased from 1% at the age of 25 to 0.45% at the age of 75^[12]. On contrary, another study found aging to be associated with increased rates of cardiomyocyte turnover^[27]. In chronically failing hearts the presence of the proliferating cell nuclear antigen, a marker of DNA synthesis and cell proliferation, was documented in 49% ± 22% of the left ventricular myocytes^[26]. Quantitative analysis from the same study described 11 cardiomyocytes nuclei with mitotic images out of a million^[26]. After myocardial infarction, two out of a million of cells were observed to have images of mitotic divisions, predominantly in the adjacent zones^[26]. Another study documented the mitotic index after acute myocardial infarction of 520 cardiomyocytes out of a million, thus accounting for approximately 2 million cardiac myocytes in the mitosis within the left ventricle wall early after AMI^[24,28]. Further calculation that assumed the existence of 5.2×10^9 cardiac myocytes

in the left ventricle and the mitosis duration of approximately 1 h, showed that the presumed mitotic index of 11 per million^[26] would produce 10% new left ventricular myocytes in a year and would double the number of cardiac myocytes in the left ventricle over a period of 10 years^[29]. Of note, different studies estimated annual rates of cardiomyocyte turnover to be 1%-10%^[12,15,30,31]. The reported variability in estimated rates of cardiomyocyte replication translates into uncertainty regarding the true magnitude of myocardial self-regeneration and fuels the skepticism regarding the clinical relevance of the observed findings. The observed variability may have originated from different morphometric methodologies and different experimental settings (normal vs diseased heart).

Criticism of the theory of cardiomyocyte replication:

Although increased DNA synthesis in cardiomyocytes has been postulated as part of their adaptation to myocardial injury, many researchers remained skeptical of cardiomyocyte replication^[32]. Chien *et al.*^[33] reinforced the old tenet that cardiomyocytes were terminally differentiated and that the response to myocardial damage is mainly hypertrophy. Thereby, the experimentally obtained images of myocytes with clearly replicating DNA were ascribed to the fact that around 20% of heart muscle cells are multinucleated but still do not divide^[24,28].

It is clear that in order to prove the potential for self-regeneration of the myocardium one must show the actual mitosis and quantify the increase in actual cardiomyocyte numbers and not only show DNA synthesis, the more so as the ploidy formation in the hypertrophied heart has already been documented^[34]. Due to the fact that heart harbors a proportion of multinucleated myocytes accounting for 1/4 of the human cardiac myocytes^[29], it is important to mention that aging, cardiac hypertrophy and ischemic cardiomyopathy do not alter the proportion of mononucleated and multinucleated cardiomyocytes^[35]. It may thus be assumed that if the proportion is not changed under stress conditions, then cardiomyocyte hyperplasia could not be excluded. In this respect it has been pointed out^[25] that two studies have documented mitotic images in an experimental model of myocardial injury^[36,37].

Despite presented evidence for cardiomyocyte hyperplasia^[37,38], due to the methodological constraints and the fact that myocardial ischemia is followed by a vast loss of cardiomyocytes, the actual cardiomyocyte turnover may hardly be reliably assessed. Nevertheless, the contention that cardiomyocyte proliferate appears to be further strengthened by the aging-related telomeric shortening^[39]. On the other side, experiments with dog hearts exposed to acute heart failure documented increase in telomerase action^[40], which was interpreted as sign of ongoing regeneration^[41].

Endogenous origin of replicating cardiomyocytes

Similar to the true magnitude of the rate of cardiomyocyte renewal, uncertainty exists regarding the origin of the replicating cardiomyocytes. Besides circulating extracardiac progenitor cells that are homed into myocardium only upon a strong pathologic signal and to a very limited extent, two possible endogenous mechanisms have been postulated: (1) mitotic replication of pre-existing cardiac myocytes; and (2) development of heart tissue cells from a pool of multipotent stem cells residing within the heart^[15].

The long-standing contention that postnatal cardiomyocytes were unable to re-enter cell-cycle and undergo mitosis has been challenged by a study that posited the division of pre-existing cardiomyocytes to be the cornerstone of the observed myocardial renewal^[30]. On contrary, it has been reported that in response to injury, myocardium regenerates on the basis of residing progenitor cells with no significant contribution by division of pre-existing cardiomyocytes^[42]. Another study concluded that both resident stem cells and division of pre-existing cardiac myocytes contribute to myocardial regeneration, albeit the progenitor cells were attributed the leading role^[31]. In wake of the opposing results, it has been argued that different experimental techniques may have caused the controversy, while the most likely source for myocardial repopulation remain resident cardiac stem cells^[15].

Cell types: Endogenous cardiac stem cells appear to be a highly heterogeneous population of cells, each being defined by expression of surface markers. The hitherto described types include c-kit⁺ cardiac stem cells, Isl 1⁺ cardiac progenitors, cardiospheres and cardiosphere-derived cells, cardiac mesoangioblasts, side-population cells and cardiac resident colony-forming unit-fibroblast cells^[15,43,44].

In the heart, c-kit⁺ are located in significantly higher numbers in atria, as compared to left and right ventricles^[45]. Initial experiments showed the ability of c-kit⁺ cardiac stem cells to differentiate into cardiomyocytes, smooth muscle and endothelial cells^[46]. A recent study confirmed the potential of c-kit⁺ lineage to give rise to new cardiomyocytes in response to aging or myocardial injury, albeit not to a significant extent that could have an impact on heart function^[47].

A subgroup of CSCs, Isl 1⁺ progenitor cells have been identified as key factors in cardiac development, particularly concerning atria, the right ventricle and outflow tract^[48]. Despite their ability to differentiate into all three cardiac lineages, the role of Isl 1⁺ cells in myocardial regeneration is limited due to rapid decrease in number with the increasing age, with high levels exclusively during fetal and neonatal life^[49,50].

Cardiospheres are generated from endomyocardial biopsies and represent a heterogeneous population of endogenous cardiac origin^[51]. Primary cardiospheres are enzymatically digested and cultivated on fibronectin-coated dishes, to eventually give rise to cardiosphere-

derived cells (CDCs), which in turn form secondary cardiospheres when treated with epidermal growth factor (EGF)^[43]. The main characteristic of the myocardial tissue-derived cardiospheres is high heterogeneity of cells that mimics cardiac microenvironment, which apart from multipotency of so derived CSCs accounts for paracrine effects leading to generation of new cardiomyocytes^[52]. CDCs were shown to have superior paracrine effects when compared to exogenous stem cell types, such as bone-marrow derived mononuclear or mesenchymal and adipose-tissue derived mesenchymal cells^[53].

Recently, it has been debated that seemingly different types of CSCs essentially belong to a single cell type, based on a study that failed to detect differences between CDCs and c-kit⁺ cells in respect to surface-antigens and gene expression pattern^[54].

In any case, the observed potential to initiate cardiac regeneration seems not to translate into a clinically relevant effect on myocardial repair, due to limited number of residing CSCs^[44].

MYOCARDIAL INJURY AS THE SIGNAL FOR SELF-REGENERATION

Clinical applicability of endogenous myocardial regeneration is founded on the observation that myocardial injury serves as a trigger for activation of CSCs. In this context, progenitor cells within myocardium represent the prime therapeutic target, with an aim of boosting the heart's potential for self-renewal.

Several studies showed that under stress conditions such as ischemia or volume overload, myocardial tissue responds with initiating the pathways of regeneration^[13,21,25,55-57]. The experiments with bromodeoxyuridine (BrdU) labeled rat cardiomyocytes unveiled the tendency of myocytes to remain mitotically inactive once the adult values of the volume and pressure loads on the ventricle were reached, thus 3 or 4 wk after birth^[58]. The pressure and volume burden seemed to be strongly related to the cell cycle of the cardiomyocytes. In relation to this finding, Insulin growth factor (IGF-1) was found to be down-regulated within the postnatal myocardium. Thereafter the hypothesis was introduced that the IGF-1 and IGF-1 receptor (IGF-1R) system plays a crucial role in proliferation but not hypertrophy of cardiomyocytes^[58]. The upregulation of mRNA for IGF-1 and IGF-1R in cardiac myocytes was shown to be the direct consequence of ischemia. Interestingly, it was also documented that after the myocardial infarction the remaining viable myocytes expressed mRNA for IGF-1R. The fact that IGF-1/IGF-1R system ceases to be as abundantly represented within the myocardium as the time progresses and myocytes grow in volume, and the fact that the system seems to be reactivated during myocardial stress inspired the hypothesis of postnatal cardiomyocyte proliferation signaled in part by IGF-1^[25]. Interestingly, an over-

expression of IGF-1 in infarcted mice hearts was shown to attenuate the ventricular dilatation, wall stress and myocardial hypertrophy^[59].

Self-regenerative capacity of myocardium suffering from acute ischemia

Acute myocardial ischemia appears to set a strong proliferative impulse with a around 90% increase in DNA replication 2 d after the infarction and over 300% after 7 d. This marked up-regulation of the DNA synthesis was mainly observed in the myocytes adjacent to the necrotic myocardium^[25]. The authors commented that due to the consequent elevation of myocardial mass (hypertrophy), reduced "loading condition" on the myocardium temporarily takes away the trigger for the myocyte proliferation until the global functional deterioration reaches certain critical point where the DNA replication commences^[25].

The analysis of patients who died shortly after AMI revealed proliferating myocytes that were identified by the presence of Ki-67, a nuclear antigen associated with cell division and detectable during the phases G1, G2 and S of the cell cycle^[21]. The rate of Ki-67 presence was 4% in the border zones and 1% in the remote zones of myocardial infarction^[21]. Subsequent analysis showed that post-infarction myocytes from the border zones that express Ki-67 antigen are 84 times higher in number compared to the healthy myocardium. At the same time, remote myocardial portions contained 28 times more myocytes expressing Ki-67 in their nuclei as compared to the myocytes of a normally perfused heart^[21]. However, the extent of myocardial proliferation remains disputable due to different methodological approaches^[12,24].

Self-regenerative capacity of myocardium suffering from chronic ischemia

An experimental model of ischemic cardiomyopathy, produced by narrowing but not occluding coronary arteries, yielded similar observations as the described experiments with acute ischemia. One week after the intervention, the BrdU-labeled myocyte nuclei expended 7.31-fold, thus indicated an augmentation in DNA synthesis. After 3 mo this increase in DNA synthesis fell back to virtually the same rate as before the intervention^[25].

Cardiomyocyte loss, cellular hypertrophy and ventricular scarring are immediate histological sequelae of chronic myocardial ischemia leading to functional impairment of the left ventricle. Fibrosis is formed where the most severe myocyte loss occurs. Even though end stage ischemic heart failure would imply around 90% decrease in left ventricular cardiomyocytes only 30%-decrease was documented^[56,60]. In order to supply more evidence for the capacity of cardiomyocytes to proliferate, a study^[56] analyzed tissue samples of hearts of patients with chronic myocardial ischemia. Cardiomyocyte analysis of the failing myocardium

revealed higher mitotic indices of roughly 140 myocyte nuclei per million as compared to cardiomyocytes from the healthy myocardium.

Both acute and chronic myocardial injury may provide stimulus for myocardial self-regeneration. However, the magnitude of cardiomyocyte proliferation does not seem to compensate for the structural and functional myocardial damage.

FROM BENCH TO THE BEDSIDE:

RESULTS FROM CLINICAL TRIALS

The inadequacy of myocardial self-regenerative response has led to the development of therapeutic techniques that are based on isolation, *ex vivo* expansion and finally transplantation of endogenous cardiac stem cells.

Hitherto, three clinical trials assessing the effects of transplantation of cardiac-derived stem cells have been conducted (Table 1): the CARDiosphere-Derived aUtologous stem CELls to reverse ventricUlar dysfunction (CADUCEUS) study^[61], cardiac Stem Cell In Patients with Ischemic cardiomyopathy (SCIPIO) trial^[62] and AutoLogous Human cArDiac-Derived Stem Cell To Treat Ischemic cArDiomyopathy (ALCADIA) trial^[63].

CADUCEUS was a two-center randomized trial that assessed the effect of intracoronary infusion of cardiosphere-derived cells on infarct size reduction in patients after myocardial infarction, with left ventricular ejection fraction (LVEF) 25-45%. The autologous cardiac stem cells, cultivated from endomyocardial biopsies, were injected 1.5-3 mo after acute MI in 17 patients (control group consisted of 8 patients). At six months, magnetic resonance imaging (MRI) showed significant reduction in scar mass and increase in viable heart mass and regional contractility^[61].

SCIPIO was an open label trial that randomized post-infarction patients with left ventricular dysfunction who were scheduled for coronary artery bypass grafting (CABG) to either intracoronary infusion of autologous c-kit⁺ cardiac stem cells or placebo. In total, 16 patients received infusion of stem cells while 7 patients were controls. In 14 patients who had undergone follow-up four months after cell infusion there was a significant increase of LVEF (absolute difference vs baseline was about 8%), compared to no change in the control group. At one year the increase in LVEF was sustained (absolute difference vs baseline about 12%)^[62].

ALCADIA is an open label, non-randomized safety/feasibility study that evaluates intramyocardial injection of autologous cardiac-derived progenitor cells in combination with administration of basic fibroblast growth factor (bFGF), in patients with ischemic cardiomyopathy and heart failure. Cardiac progenitor cells were derived from endomyocardial biopsies and expanded for a month, prior to intramyocardial injection

Table 1 Clinical studies with endogenous cardiac stem cells

Study	Design	Population	Intervention	No. of patients receiving treatment	Primary endpoint	Conclusion
CADUCEUS	RCT	Post-MI with LVEF 25%-45%	Intracoronary infusion of CDCs	17	Occurrence of death, new MI, new cardiac tumour on MRI and/or admission for HF	Safety proven; MRI-scar reduction and increase in viable myocardium
SCIPIO	RCT	Post-MI with LVEF ≤ 40%	Intracoronary infusion of autologous c-kit+ CSCs	16	Short-term safety	Safety proven; LVEF improvement, infarct size reduction
ALCADIA	Non-randomized	Ischemic CMP with LVEF 15%-35%	Intramyocardial injection of autologous CSCs with bFGF	6	-	No adverse events; improvement of NYHA class and LVEF

RCT: Randomized controlled trial; CDCs: Cardiosphere-derived stem cells; MRI: Magnetic resonance imaging; HF: Heart failure; CSCs: Cardiac stem cells; bFGF: Basic fibroblast growth factor; CADUCEUS: CARDiosphere-Derived aUTologous stem CELls to reverse ventricUlar dysfunction; SCIPIO: Stem Cell In Patients with Ischemic cardiomyopathy; ALCADIA: AutoLogous Human CARDiac-Derived Stem Cell To Treat Ischemic cARDiomyopathy.

during CABG^[64]. So far 6 patients have been enrolled, one patient was excluded due to graft occlusion and one developed worsening heart failure symptoms.

Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration (ALLSTAR) study is ongoing (ClinicalTrials.gov Identifier NCT01458405) and will randomize 274 patients to intracoronary infusion of either allogeneic cardiosphere-derived cells or placebo, 4-12 wk after anterior MI and successful primary percutaneous intervention for LAD coronary artery, if LVEF ≤ 45%.

The combined enrollment for all three trials has been 39 patients, with no major safety concerns. Common denominator of the three trials was the use of autologous cardiac stem cells that have been cultivated from endomyocardial biopsies. In SCIPIO trial, the so cultivated cells were then filtered to obtain the c-kit⁺ lineage. The number of cardiosphere-derived cells, used in CADUCEUS study, was limited due to their size (about 20 microns, as compared to the size of coronary capillaries of about 7 microns)^[64]. ALCADIA trial evaluated an interesting concept that combined cardiac stem cell transplantation with administration of a growth factor, with the purpose of enhancing the retention of the transplanted cells^[64]. Principal limitations of the hitherto conducted trials are the small number of included patients and the non-standardized cell therapy that varied both in respect to the cell type (cardiosphere-derived cells vs isolated c-kit⁺ cells) and the route of delivery (intracoronary vs intramyocardial). In addition, one study (ALCADIA) used bFGF to boost the retention of the transplanted cells.

The transplantation of cardiac-derived stem cells appears to at least in theory upgrade the hitherto developed cell therapies for treatment of myocardial injury (*i.e.*, bone-marrow derived cells), inasmuch as CSCs resemble the host myocardial microenvironment. However, the endogenous pathways of regeneration still remain largely enigmatic. As has been presented in the above sections of this review, there is still controversial evidence regarding the origin of the

newly formed cardiac myocytes (myocardium-specific progenitor cells vs self-replicating cardiomyocytes). The ambivalence in respect to the key factors behind the process of endogenous myocardial regeneration hampers clinical research, inasmuch as it makes the standardization of the therapeutic approach virtually impossible - the variety of scientific theories that are aiming at deciphering the endogenous signals is mirrored by the variety of clinical interventions and techniques (different cell types, routes of delivery, target populations, *etc.*, Table 1). In addition, there are significant constraints that are intrinsic to any process of cell transplantation, irrespective of how well the transplanted cells may fit to the host microenvironment and that could limit the effects of endogenous regeneration.

ACTIVATION OF MYOCARDIAL SELF-REGENERATION WITHOUT CELL TRANSPLANTATION

The process of cell transplantation presupposes stem cell isolation from the tissue of origin (*i.e.*, myocardium or bone marrow), *ex vivo* expansion and ultimately cell delivery into the host myocardium^[65] (Figure 1). Hence, the use of autologous stem cell renders acute treatment impossible, even though the issue of timing may be the crucial component of a successful myocardial regeneration. It has been shown that mice heart could regenerate if partial surgical resection is performed on day 1 after birth, while the ability of regeneration is lost by day 7^[66]. Genetic fate mapping showed that mainly the replication of preexisting cardiomyocytes was responsible for the myocyte replenishment^[66].

In situ activation of endogenous myocardial repair without cell transplantation (Figure 1) would help to avoid the inherent problematic issues associated with the transplantation of stem cells, such as long time periods and high cost of the preparation of cells, non-standard route of delivery (*i.e.*, intramyocardial

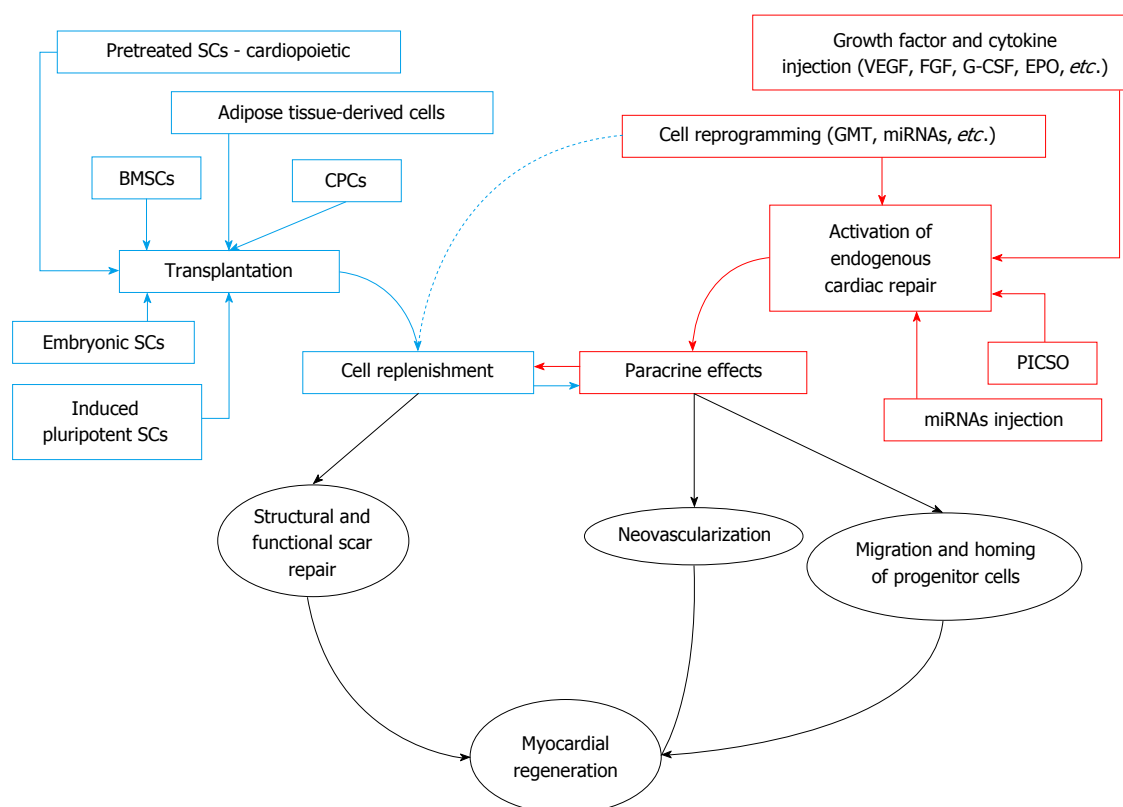


Figure 1 Transplantation of stem cells vs initiation of endogenous myocardial regeneration without cell transplantation. Depicted are two basic approaches to myocardial regeneration: (1) via transplantation of different types of stem cells into the damaged myocardium (on the left, in blue); and (2) by activation of endogenous pathways of cardiac repair, without cell transplantation (on the right, in red). Both sets of techniques are thought to ultimately exert their effects on the paracrine route and by replenishing lost cardiomyocytes. SCs: Stem cells; BMSCs: Bone marrow-derived stem cells; CPCs: Cardiac progenitor cells; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; G-CSF: Granulocyte colony stimulating factor; EPO: Erythropoietin; GMT: Gata4, Mef2c, Tbx5; miRNAs: microRNAs; PICSO: Pressure-controlled intermittent coronary sinus occlusion.

injections) and insufficient regenerative response due to problematic retention of the transplanted cells. Thus, four criteria for successful regenerative therapy have been proposed: (1) available at all times; (2) safe and routine application, compatible with gold standard therapies, such as primary PCI; (3) affordable; and (4) effective in salvaging myocardium (at least 50-60 g of tissue) and thus preventing heart failure^[15].

The existence of endogenous reservoir of stem cells within myocardium warrants the development of therapeutic strategies directed at activating dormant salvage pathways and thus avoiding the costly and laborious process of cell transplantation.

Growth factor injection

The idea behind treating the diseased myocardium with growth factor injection rests on the observations that transplanted CSCs impact the host tissue microenvironment twofold, by differentiating into cardiac lineages and by exerting paracrine effects^[44,67]. Growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), placental growth factor (PIGF),

stem cell factor (SCF) and angiopoietin (Ang) have been postulated as activators of endogenous stem cells within the myocardium and so as initiators of its self-regeneration^[68].

Intracoronary administration of the combination of IGF-1 and HGF in the infarct-related coronary artery has been associated with reduced ventricular remodeling and improved myocardial regeneration^[69]. However, the fact that IGF-1/HGF stimulated cardiomyocyte generation is a slow process may hamper the overall improving of the heart's function and the clinical implementation of this strategy^[15].

Initial trials on the effects of FGF in patients with coronary artery disease (CAD), via intracoronary delivery of adenoviruses, showed potential of this method to improve regional blood flow^[70]. However, further clinical studies failed to produce reliable favorable clinical outcomes in CAD patients, albeit the effect of FGF seemed to have been gender-specific with improvement in outcomes in women^[71].

Experimental studies have demonstrated the potential of G-CSF to improve LV function after MI, mainly by fostering arteriogenesis^[72]. Initial clinical application of G-CSF in MI patients indicated the potential to improve echocardiographic LV functional parameters^[73], but subsequent randomized trials

did not show improvement in clinical outcomes^[68,74]. Similarly, in patients with chronic occlusive CAD, subcutaneous injection of G-CSF improved neither myocardial perfusion nor LV function, albeit it appeared to reduce anginal symptoms^[75].

GM-CSF has been associated with mobilization of endothelial progenitor cells (EPCs) and ultimately neoangiogenesis^[68]. A clinical trial showed enhanced collateral flow following the subcutaneous injection of GM-CSF in CAD patients^[76]. However, the study raised a safety concern due to occurrence of acute coronary syndrome in patients on treatment with GM-CSF^[76].

The use of VEGF as a potential mediator of neovascularization has been extensively studied both experimentally and in clinical trials. Animal models of myocardial ischemia indicated the potential of VEGF to induce angiogenesis^[68]. Subsequent trials showed tendency toward improvement of functional parameters such as regional myocardial perfusion or wall motion, but with no significant effect on clinical outcomes^[77,78].

EPO is secreted in tissues under hypoxia and is posited to foster angiogenesis and progenitor cell development, while inhibiting apoptosis^[68]. Whereas animal studies indicated that EPO administration was associated with the decrease in infarct size^[79], a clinical trial in patients with acute MI did not show improvement of heart's function^[80].

Further growth factors such as PIGF, Ang and the ligand of c-kit, SCF, which have been proposed to induce neovascularization and mobilization of progenitor cells, showed their potential to regenerate damaged myocardium in animal models, but solid clinical evidence is still lacking^[68].

A common characteristic of the described growth factors and cytokines is that their potential to induce myocardial regeneration, mainly *via* paracrine effects, has been established in experimental models, but with no direct translation in the improvement of clinical outcomes.

Cell reprogramming

The idea of cell reprogramming to achieve structural and functional renewal of the damaged myocardium has been substantiated through the concept of induced pluripotency, whereby fibroblasts could be reprogrammed to assume pluripotency and further directed into the state of cardiac lineage cells^[81-83]. However, this process implies transplantation of such induced cells into the damaged myocardium (Figure 1). On the other hand, direct reprogramming of the scarred myocardium involves conversion of fibroblasts into functioning cardiomyocytes by delivery of the following transcription factors into the cells of heart tissue: Gata4, Mef2c, Tbx5 (GMT)^[83,84]. Despite its luring perspective, cardiomyocyte replenishment *via* GMT reprogramming has been hampered by a purported lack of efficiency, relating primarily to

the degree of molecular and electrophysiological development of the newly formed cardiomyocytes^[83,85].

Cell reprogramming has been enhanced by the delivery of microRNAs only^[86] or in combination with transcription factors^[87]. Furthermore, the delivery of sets of microRNAs that have been associated with cardiac developmental pathways may present another option of initiating myocardial regeneration without the need for cell transplantation^[88].

Pressure-controlled intermittent coronary sinus occlusion

Pressure-controlled intermittent coronary sinus occlusion (PICSO) is a technique that is based on repeated cycles of coronary sinus occlusion. PICSO alters the expressional pattern of endo- and perivascular cells by exerting shear stress and cyclic strain on endothelial cells. It has been shown that PICSO up-regulates the expression of VEGF and heme oxygenase-1 (HO-1)^[89]. In an animal model of myocardial infarction PICSO was shown to upregulate VEGF and VEGF-receptor 2 in the capillary endothelial cells of the remote zones of myocardial infarction^[90]. ICSO procedure, which differs from PICSO by not being controlled by coronary sinus pressure, was able to significantly reduce infarct size in experimental trials^[91]. In a clinical trial, PICSO significantly reduced occurrence in MACE in patients with acute MI treated with thrombolysis^[92].

It has been postulated that PICSO has the potential to elicit myocardial regeneration by activating innate regenerative pathways^[93,94]. Intermittent pressure elevation in the venous system of the heart results in shear stress and pulsatile stretch of the vessel wall, which in turn translates into gene expression on the way of mechanotransduction, thereby mimicking the pulsatile signal on the developing heart tube (Figure 2)^[95-98].

Intermittent pressure elevation in the coronary venous system may thus serve as a signal for the activation of endogenous cardiac stem cells *via* paracrine routes and induce dormant pathways of myocardial salvage *via* mechanotransduction, which mimics the structural and functional interplay of the developing heart.

The ease of the application of PICSO without biohazards and instant availability may help to collect clinical data in heart failure patients, thus giving an example that STAP (stress transformed acquired pluripotency) in spite of the unsolved questions in hitherto collected results, might not be an illusion.

CONCLUSION

Recent findings have overturned the classical contention that postnatal myocardium is terminally differentiated by providing evidence for the formation of new cardiomyocytes. The postulated origin of new cardiomyocytes is the population of resident cardiac stem cells that have the ability to differentiate into the

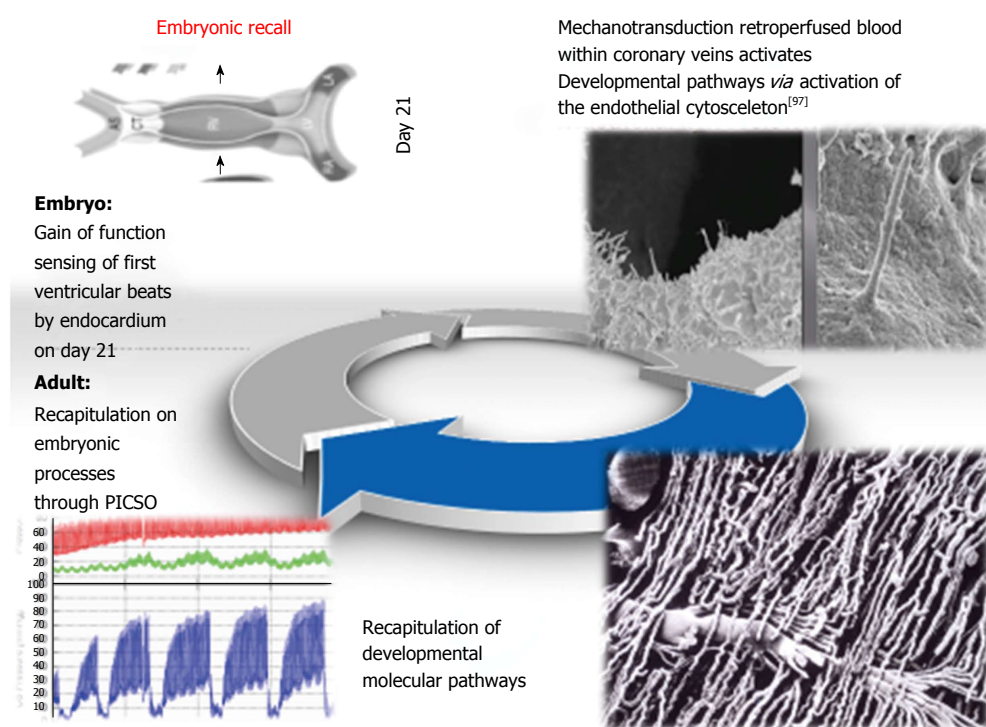


Figure 2 Hypothesis of embryonic recall. In embryos, the first heart beat is sensed by mechanotransduction of the endocardium, leading to a burst of developmental signals thriving cardiac morphogenesis. In analogy, elevated pressures in cardiac veins act *via* stretch and shear stress on perivascular cells, thus reiterating the same mechanotransductory signals in the adult failing heart. Pressure elevation in cardiac veins have to be pressure controlled not to increase arterial resistance to nutritive flow (PISCO).

three cardiac lineages. Myocardial injury provokes the initiation of the innate pathways of regeneration, but the extent remains insufficient to provide significant improvement of heart function. Isolation of cardiac-derived stem cells *via* endomyocardial biopsy and *ex vivo* expansion of stem cells are necessary steps in the process of cell transplantation. However, the transplantation of cardiac stem cells to repair the damaged myocardium is costly and time-consuming and of diminished efficacy due to problematic long-term retention of the transplanted cells. Hence, new therapeutic approaches such as growth factor injection and PISCO are under scrutiny with the proposed goal of activating endogenous progenitor cells and thus initiating myocardial regeneration.

Why does cardiac endogenous regeneration in mammals fail in contrast to reptiles and numerous regenerative signals? One very important aspect is the timing of these signals. Unlike morphogenesis of the developing heart, which is a controlled stepwise approach, regenerative pulses in necro-apoptosis are random in time and region. So one aspect would be to synchronize and amend the signals in order, presuming that we understand the full picture of the molecular signals involved. Therefore reviving a developmental process *via* an epigenetic stimulus like activation of vascular cells by mechanotransduction might initiate the plethora of morphogenetic pulses necessary to enforce and synchronize endogenous repair.

Morphogenesis of the heart seems to be a controlled

stepwise approach in tissue generation, alternating gene transcription and established physical functions which again initiate molecular growth signals. These "epigenetic" signals which could be explained as "morphometric windows" can be used as initiation of endogenous repair.

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Corneal stem cells and tissue engineering: Current advances and future perspectives

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Abstract

Major advances are currently being made in regenerative medicine for cornea. Stem cell-based therapies represent a novel strategy that may substitute conventional corneal transplantation, albeit there are

many challenges ahead given the singularities of each cellular layer of the cornea. This review recapitulates the current data on corneal epithelial stem cells, corneal stromal stem cells and corneal endothelial cell progenitors. Corneal limbal autografts containing epithelial stem cells have been transplanted in humans for more than 20 years with great successful rates, and researchers now focus on *ex vivo* cultures and other cell lineages to transplant to the ocular surface. A small population of cells in the corneal endothelium was recently reported to have self-renewal capacity, although they do not proliferate *in vivo*. Two main obstacles have hindered endothelial cell transplantation to date: culture protocols and cell delivery methods to the posterior cornea *in vivo*. Human corneal stromal stem cells have been identified shortly after the recognition of precursors of endothelial cells. Stromal stem cells may have the potential to provide a direct cell-based therapeutic approach when injected to corneal scars. Furthermore, they exhibit the ability to deposit organized connective tissue *in vitro* and may be useful in corneal stroma engineering in the future. Recent advances and future perspectives in the field are discussed.

Key words: Cornea; Stem cells; Cell-based therapy; Limbal transplantation; Tissue engineering; Corneal endothelium

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Core tip: Corneal stem cell-based therapies represent a novel strategy that may substitute conventional corneal transplantation, albeit there are many challenges ahead given the singularities of each cellular layer of the cornea. To date, the most widespread clinical approach to regenerative medicine in the eye has been the utilization of limbal stem cells for ocular surface diseases. New therapeutic strategies are under development due to the recent identification

of corneal stromal stem cells and corneal endothelial cell progenitors. This review recapitulates the current advances and discusses future perspectives in the field.

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INTRODUCTION

The human cornea consists of 5 recognized layers, 3 of them are cellular (epithelium, stroma and endothelium) and 2 are interface layers (Bowman's layer and Descemet membrane)^[1]. Corneal epithelium is a stratified, non-keratinizing squamous layer characterized by extreme uniformity from limbus to limbus^[2]. Epithelial cells are regularly shed from the surface and replaced by new cells that are ultimately provided by stem cells located at the limbal area of the peripheral cornea^[2]. Corneal stroma provides the bulk of the structural framework of the cornea and comprises approximately 80% to 90% of its thickness. Keratocytes are the major cell type of the stroma and are involved in maintaining its extracellular matrix^[1]. An intricate collagen network in the extracellular matrix accounts for the cornea's shape, stability, mechanical strength and transparency^[3]. Posteriorly, a thin monolayer of polygonal cells constitutes the corneal endothelium. Since it is in contact with the aqueous humor, the endothelium regulates corneal hydration through active cell pumps, thereby being crucial to maintain corneal transparency^[4]. Except for oxygen, all corneal nutrients come from the aqueous humor through endothelial cells^[5].

Regenerative medicine for the cornea represents a novel treatment strategy for patients with corneal diseases. Enormous promise has been ascribed to this modality because it offers a paradigm shift from conventional corneal transplantation to stem cell-based therapeutic strategies^[6]. Cellular therapies are challenging given the singularities of each cellular layer of the cornea. To date, the most widespread clinical approach to regenerative medicine in the eye has been the utilization of limbal stem cells for ocular surface diseases. In 1989, Kenyon and Tseng^[7] reported successful autologous transplantation of limbal tissue from the healthy fellow eye in 26 cases of unilateral limbal stem cell deficiency. Many reports and studies of limbal stem cell transplantation have followed, from heterologous donors to *ex vivo* cellular cultures.

In the present article, we discuss the current status in corneal stem cell research and the perspectives regarding corneal regenerative therapies and tissue engineering.

CORNEAL EPITHELIUM

Corneal epithelial stem cells are located at the corneoscleral limbus and are the ultimate source for regeneration of the entire corneal epithelium under normal and injured states^[2,8,9]. Multiple diseases that destroy the limbal epithelial stem cell, its environment, or a combination of both can lead to a clinical condition known as limbal stem cell deficiency (LSCD)^[10]. The clinical picture of LSCD is a conjunctivalized corneal surface with neovessels and recurrent or persistent epithelial defects^[11]. As a result, patients with LSCD have decreased vision, photophobia, tearing, and recurrent pain episodes^[2,11,12].

The classic limbal stem cell transplantation procedure involves grafting of a healthy limbal biopsy onto the recipient corneal surface. The technique was developed in the late 1980s and early 1990s and proved useful in LSCD^[13]. Limbal grafts are obtained either from an unaffected fellow eye^[7,14], from a live relative^[14,15] or from cadaveric donors^[14,16,17]. For patients inflicted with unilateral total LSCD, where the entire limbal circumference is damaged in only one eye, transplantation of limbal stem cells from the fellow eye has shown success rates greater than 80%^[18,19]. Due to the autologous source of cells, there is no risk of immune rejection in these cases. Concerns regarding irreversible changes that may occur at the limbal site of the donor eye, as reported in rabbits^[20,21], prompted clinicians to consider cadaveric donors. However, in this procedure, there is an increased risk of allograft rejection and therefore systemic immunosuppression is mandatory^[14,16,17]. Although larger grafts can be procured from cadaveric eyes (360 ring grafts), a recent study^[22] found living-related limbal allografts to have better results in terms of vision gain and ocular surface restoration than cadaveric keratolimbal allografts. In a series of 39 eyes that received keratolimbal allografts from cadaveric donors, the overall survival of ambulatory vision, namely 20/200 or better, was 53.6% at 3 years, but a progressive decline of the visual outcome and graft survival became evident with time^[23]. The failure of the graft is related to loss of donor cells overtime, probably due to recipient's preoperative conditions such as dry eye and/or lid abnormalities, persistent inflammation or postoperative asymptomatic and progressive allograft rejection^[23].

Regardless of the source of epithelial stem cells, amniotic membrane transplantation (AMT) has been widely used in an attempt to improve limbal grafts success^[24-26]. Nevertheless, a recent study interestingly showed no differences in the outcomes of chemically injured eyes treated with or without AMT as an adjunctive procedure to limbal transplantation^[27]. Amniotic membrane is a biologic, avascular tissue with anti-angiogenic, anti-scarring and anti-inflammatory characteristics^[28,29]. Since the process of

conjunctivalization in LSCD is invariably associated with destruction of the basement membrane^[10], amniotic membrane transplanted onto the corneal surface facilitates epithelial expansion *in vivo*. Amniotic membrane has a thick basement membrane that serves as a substrate upon which epithelial cells can migrate and regenerate to form new and healthy tissue^[28]. In cases of partial LSCD, AMT has been successfully used alone, without stem cell transplantation^[30]. Amniotic membrane can also be used as a biologic bandage in the acute phase of inflammatory disorders, such as ocular burns^[31,32] and Stevens-Johnson syndrome^[33-36], in order to prevent development of LSCD and other surface complications. Nevertheless, most of the studies are non-comparative, and a clinical trial that addressed the potential benefits of AMT did not find definite advantage of early AMT over conservative medical treatment in preventing scarring sequelae in eyes with chemical burns^[37].

With the development of cell therapy, amniotic membrane began to be employed in *ex vivo* expansion of limbal stem cells or their progeny^[38]. Pellegrini and colleagues published the first clinical report in the field of tissue engineering for the cornea in 1997^[39]. They reported an autologous cultivated epithelial cell transplantation that successfully restored the ocular surface in 2 cases of LSCD due to chemical burns. Other series of cultivated epithelial limbal transplantation (CLET) have followed^[38,40-46] and efforts to culture corneal cells *in vitro* are currently being made in different centers worldwide. Protocols use either an explant or cell suspension for culturing progenitor cells. The benefits of using explants are that they are easy to prepare and there is no danger of damaging the donor's corneal epithelium through enzyme exposure^[40]. However, limbal stem cells may poorly migrate from the limbal explants onto the carrier^[40,47]. Some protocols include co-cultivation with murine 3T3 feeder layers^[48], which are believed to promote niche regulation of limbal stem cells and thus delay epithelial differentiation^[49]. The use of animal-derived products carries a theoretical risk of infection, rejection or acquisition of prion diseases^[50]. By using autologous human serum instead of fetal bovine serum^[51] and eliminating feeder cells, complete xeno-free cultivation for CLET is possible^[52].

A novel possibility for delivering limbal stem cells on the corneal surface is to use contact lens as a carrier and as a substrate for cell expansion. Two studies with animal models of LSCD reported promising results with contact lens delivery, as stem cells retained their phenotype and mitotic activity after being transferred to the ocular surface^[53,54]. Furthermore, the seeded contact lenses were easy to manipulate and its material is nonimmunogenic^[54].

We perform transplantation of other cell lineages onto the ocular surface for LSCD when total LSCD occurs bilaterally and therefore no autologous tissue for CLET is available. Conjunctival epithelial cells are

the most akin biologically to corneal epithelial ones in the human body and our group has successfully cultivated and transplanted them for ocular surface reconstruction^[55]. Use of non-ocular surface epithelial cells was first made with cultivated oral mucosal epithelial transplantation in rabbits^[56] and application of this technique in humans has followed thereafter^[57-65]. Both conjunctival and oral mucosal epithelial cells are not expected to differentiate into corneal epithelial cells, like stem cells would. However, most studies show adequate functionality of the grafts, giving them clinical relevance in tissue engineering.

A promising source of stem cells for ocular therapy is the human deciduous teeth. These undifferentiated cells exhibit all characteristics of pluripotent adult stem cells and continuously express markers of limbal stem cells^[66]. Tissue-engineered sheets of dental-pulp stem cells were effective for cornea reconstruction in a rabbit model of chemical burn^[67]. Other recently proposed sources of stem cells for cornea epithelium are hair follicle^[68], bone marrow^[69,70] and umbilical cord^[71]. It has been demonstrated that adult tissue-specific stem cells have the ability to differentiate into lineages other than the tissue of origin^[72].

CORNEAL STROMA

One of the most challenging steps in engineering human corneal tissue is to produce the corneal stroma, given the difficulty in reproducing its highly ordered hierarchical ultrastructure, which relates to its exceptional biomechanical properties and optical transparency^[73]. Keratocytes are native resident cells of the corneal stroma and are responsible for maintaining the extracellular matrix (ECM)^[1]. The bulk of the stroma comprises approximately 200 orthogonally oriented lamellae, and each of them is made up of long uniform collagen fibrils lying parallel one to another with regular inter-fibril spacing^[74,75]. Under *in vitro* expansion in serum containing media, keratocytes lose their phenotype and soon become fibroblastic. Unlike keratocytes, fibroblasts secrete a disorganized ECM typically found in opaque corneal scars^[76-78]. Even in serum-free conditions, primary keratocytes maintain an *in vivo* phenotype through some population doublings, but lose their morphology, gene expression, and the ability to organize a cornea-like ECM after greater expansion^[79]. A more recent study, however, demonstrated that corneal fibroblasts respond to a specific pattern of topographical cues from the substratum and regain differentiation potential, thus being able to secrete highly organized ECM typical of corneal stroma^[79].

Recently, Du and coworkers identified the first human cells with keratocyte progenitor potential in the corneal stroma^[80]. This small population of cells exhibited properties of stem cells, such as clonal growth *in vitro*, extended lifespan, and the ability to differentiate into several different cells types, including

keratocytes of the cornea. When cultured as floating pellets in the absence of rigid scaffolding or substratum, in a serum-free medium, the human corneal stromal stem cells (hCSCs) differentiate into cells expressing a gene profile similar to that of human keratocytes and deposit tissue-like ECM with a composition and structure similar to that of the corneal stroma^[81]. A comparative study showed that, under the same culture conditions, human corneal fibroblasts tended to differentiate into myofibroblasts and deposited a less-organized collagen-fibrillar construct, while hCSCs successfully differentiated into keratocytes and secreted multilayered lamellae with orthogonally-oriented collagen fibrils, in a pattern mimicking human corneal stromal tissue^[73]. This *in vitro* secretion of stromal-like connective tissue points to a potential use of hCSCs in tissue bioengineering. An interesting finding is that hCSC may exhibit immune privilege: injected into mice corneas with scars, these cells did not elicit immune T-cell response^[82], as it would be expected in a xenotransplantation model. As a matter of fact, a successful restoration of corneal organization and transparency was achieved after the injection^[82]. Therefore, besides the utility in bioengineering stromal tissue, hCSC may have the potential to provide a direct cell-based therapeutic approach^[83].

Other engineering strategies for the corneal stroma include animal sources as a substitute of native tissue. Once rejection is a major obstacle to widespread use of xenotransplantation, fully acellular corneas have been proposed to overcome immune rejection. A decellularized animal tissue provides a three-dimensional ECM structure, favorable biocompatibility, adequate biomechanical tension and high transparency that mimics the native cornea and can be applied with or without the addition of a cellular component^[13]. Most studies use porcine corneas^[84-87], but other sources such as cow^[88] and cat^[89] are amenable for xenotransplantation. Since decellularized corneal stroma retains basement membrane components, it was used as a matrix scaffold for limbal stem cell expansion *in vitro*, and as a carrier for epithelial transplantation in animal models, with good results^[90,91]. Decellularization may be applied not only to xenogeneic, but also to allogeneic cornea tissues for transplantation. Theoretically, this would reduce the complications associated with allogeneic corneal transplants and improve the clinical outcomes.

CORNEAL ENDOTHELIUM

Corneal endothelium is a monolayer of cells that maintains a nonproliferative state, with cell density decreasing steadily under normal circumstances throughout life^[92,93]. It has been for long believed that they do not replace themselves once they are lost in aging or diseases. On the contrary of corneal epithelial cells, which readily and continuously divide from limbal stem cells, corneal endothelial cells fail to expand *in*

vivo, and expand slowly *in vitro*^[94,95]. Accumulating evidence, however, points to the existence of a dormant progenitor cell population located in the posterior limbus, the transitional area from the periphery of the endothelium and Schwalbe's line to the anterior portion of the trabecular meshwork. These putative progenitor cells are likely to be capable of producing both endothelial and trabecular cells^[96]. Identification of a circumferential and discontinuous cord of cells with unusual ultrastructural features along the Schwalbe's line in monkeys dates from 1982^[97]. Attention to these cells was drawn later, when repopulation of trabecular cells after argon laser trabeculoplasty was reported^[98,99]. Possibly, these cells are invigorated and proliferate after laser trabeculoplasty through the release of growth factors and cytokines^[96].

There is substantial evidence that the human corneal endothelium cells hold different characteristics according to their position in the cornea. Stained corneas revealed that cell density at the peripheral inner cornea, up to 1.6 mm from Schwalbe line, is about 30% higher than in the central cornea^[100]. Additionally, specular microscopy documented the uneven cell distribution from center to periphery *in vivo*^[101]. The reason for this difference is not known, albeit it has been suggested that the peripheral cornea may provide a physiologic reserve and storage region of endothelial cells for continuous, lifetime cellular supply for the central cornea^[101]. Bednarz *et al.*^[102] showed that cells isolated from the central part are densely packed and exhibit no mitogenic activity, while cells derived from the peripheral part of the cornea are characterized by mitogenic activity but their cell-to-cell attachment seems to be less tight than *in vivo*. Konomi *et al.*^[103] studied human donor corneas with different donor ages and found that cells from both the central and peripheral areas retain potential proliferative capacity, although the doubling time tended to be higher for cells from the central cornea and from older donors.

Data from molecular marker studies provide support for the existence of a stem-like cell niche at the corneal endothelium. Dissected endothelial tissues from human and rabbits exhibited positive telomerase activity in the peripheral and intermediate sections, but no activity was observed in the central endothelial tissues or the limbus between the trabecular meshwork and Schwalbe's line^[104]. Telomerase activity is a characteristic marker for transient amplifying cells. The same study also tested endothelial cells for alkaline phosphatase-catalyzed bromodeoxyuridine (BrdU) labeling, which demonstrates cell division. In the case of rabbit corneal endothelium, which is known to divide with regularity, BrdU staining was always seen following a mechanical wound, but in the human corneal endothelium, staining only occurred at the wound site in 10% of the corneas tested^[104]. Such results corroborate a typical lack of propensity toward cell division in wounding response in the human

endothelium. Another cellular marker for proliferation is Ki67, which is detected in the nucleus of proliferating cells. Ki67 antibody staining produces characteristic patterns depending on cell position within the cycle, so it makes possible to quantify cell cycle entry and completion *in vitro*^[105]. Stem cell markers that were also identified in the posterior limbus cells are nestin and alkaline phosphatase^[106]. Human corneal endothelial cell precursors have been isolated using the sphere-forming assay and expressed markers of the mesenchymal (α -smooth muscle actin) and neural (β 3-tubulin, glial fibrillary acidic protein) lineages, although none of these are specific markers of endothelial cells or their progenitors^[107]. More recently, two monoclonal antibodies taken together, anti-GPC4 and anti-CD200, were found to stain HCECs with specificity^[108]. The finding is useful for purification of cultures that may be contaminated by stromal fibroblasts. Since the endothelial cells are not expected to proliferate *in vivo*, isolation and stimulation of cell division *in vitro* is an important step towards cell replacement therapy^[109].

Human corneal endothelial cells proliferate and replicate under special conditions. In medium containing fetal bovine serum and fibroblast growth factor, cells from older donors proliferated but responded more slowly and to a lesser extent than cells from younger donors. When epidermal growth factor added to the medium, cells from older donors were stimulated to enter the cell cycle faster and the amount of actively cycling cells and mitotic figures increased^[105]. An important factor to activate endothelial cell cycle is the disruption of cell-cell contact. Zhu and colleagues demonstrated that maturation of intercellular adherent junctions correlates with a rapid decline of the proliferative potential in endothelial cell monolayers during cultivation^[110]. To overcome that, EDTA solution was used in an organ culture model once it has the ability to release endothelial cells from cell-cell contact. After treatment of the intact endothelial monolayer with EDTA, approximately 17% of the cell population entered the cell cycle^[111]. The use of EDTA alone, rather than a trypsin/EDTA solution to digest cells, may afford a greater cell survival rate^[112]. Also, the presence of L-ascorbic acid 2-phosphate (Asc-2P) in the culture was found to increase the proliferation of endothelial cells. Asc-2P is an antioxidant derivative of Asc that upregulates hepatocyte growth factor production *in vitro*^[113].

Further advance is required for the delivering method. Different cell carriers have been tested in animal models, and the main challenges are to achieve biomaterial transparency, structural strength and integration with native tissues^[113,114]. One approach to deliver cultured endothelial cells through direct injection employed magnetic attraction to attach cells to the Descemet's membrane, thus preventing them to disperse in the anterior chamber^[115]. Mimura *et al.*^[115,116] incorporated iron powder spheres in rabbit corneal endothelial cells and a neodymium magnet

was placed anterior to the eyelid to attract the particles after cell suspension injection. They demonstrated that the use of 5-10 micromoles of iron powder solution was associated with good cell survival rate and 12 mo later no noticeable toxicity was present. Since rabbit's endothelial cells are able to regenerate spontaneously, a control group that had been submitted to the same corneal injury did not receive the cell injection. The control group showed signs of endothelial failure. Magnetic field was later used in a human *ex vivo* model. Superpara-magnetic microspheres were incorporated into the cytoplasm of human corneal endothelial cells and facilitated cell migration and attachment without affecting cell viability or light transmittance^[117]. Toxicity to cells and other ocular tissues from magnetite oxide particles is a concern though, and safety remains to be demonstrated. Such compounds are used as magnetic resonance imaging contrast agents, on which safety and efficacy studies have been conducted, but the toxicity profile of this agent is likely to be different when delivered to the anterior segment of the eye.

The literature to date supports that the putative endothelial cells progenitors are precursors, not stem cells *per se*. Common features of precursors and adult stem cells are that both have self-renewal capacity and the ability to differentiate into mature effector cells, but stem cells can renew themselves indefinitely and produce one or more progenies through symmetric and asymmetric division, whereas progenitor cells have limited self-renewal capacity and more restricted differentiation abilities^[96,107].

CONCLUSION

Progress in corneal stem cell research provides an optimistic prospect on their use in regenerative medicine and tissue engineering. In this paper, we presented different limbal transplantation techniques, using either autologous or heterologous source of limbal stem cells, and the epithelial cells cultivated *in vitro* that are being successfully transplanted to the ocular surface. Future perspectives include the use of pluripotent stem cells or other adult stem cells to restore the corneal epithelium. However, a measured scientific approach is required to guarantee safety before transplantation can be done in patients. We discussed the growing data on human stromal stem cells, their immune privilege, and their potential to secrete organized collagen lamella that may eventually be useful in tissue engineering. We also summarized the accumulating evidence for the presence of putative stem cells in the corneal endothelium. These cells require specific conditions to show proliferative activity *in vitro* and researchers have already addressed that. Cell therapies for each corneal layer will target a specific disorder, instead of a full or partial thickness corneal transplant, which is the current treatment. Furthermore, one donor cornea will potentially treat multiple patients if the cells are successfully cultivated,

and certainly this would have a positive impact on the shortage of donor corneas worldwide.

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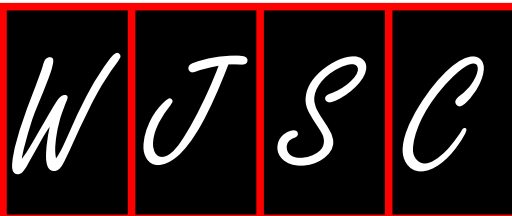
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Stem cell applications for pathologies of the urinary bladder

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Abstract

New stem cell based therapies are undergoing intense research and are widely investigated in clinical fields including the urinary system. The urinary bladder performs critical complex functions that rely on its highly coordinated anatomical composition and multiplex of regulatory mechanisms. Bladder pathologies resulting in severe dysfunction are common clinical encounter and often cause significant impairment of patient's quality of life. Current surgical and medical interventions to correct urinary dysfunction or to replace an absent or defective bladder are sub-optimal and are associated with notable complications. As a result, stem cell based therapies for the urinary bladder are hoped to offer new venues that could make up for limitations of existing therapies. In this article, we review research efforts that describe the use of different types of stem cells in bladder reconstruction, urinary incontinence and retention disorders. In particular, stress urinary incontinence has been a popular target for stem cell based therapies in reported clinical trials. Furthermore, we discuss the relevance of the cancer stem cell hypothesis to the development of bladder cancer. A key subject that should not be overlooked is the safety and quality of stem cell based therapies introduced to human subjects either in a research or a clinical context.

Key words: Stem cells; Urinary bladder; Urethra; Bladder reconstruction; Incontinence; Cancer stem cells; Safety

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Core tip: This article reviews the current status of the stem cell based therapies in the field of treating urinary tract pathologies. We provide a particular focus on bladder reconstruction models that were based on stem cellular therapies. Stress incontinence and voiding dysfunction represent common clinical problems that could benefit from advancing the stem cell field. A

brief highlight is given to bladder cancer stem cells and therapeutic value that could arise from controlling their behavior. We also note the pressing need for more robust regulations and quality guidelines to prevent the transfer of research findings prematurely into routine clinical practice.

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INTRODUCTION

The bladder and urethra compose the lower urinary tract. Pathologies of this part of the urinary system are common and clinically significant. Due to the bladder role as a versatile and dynamic reservoir of urine, it has unique structural and regulatory mechanisms that need to be thoroughly considered when cell and tissues engineering based therapies are thought of. Its complex mucosal-muscular-neural elements work together efficiently to produce a synchronized effortless urinary filling and emptying process. For instance, the bladder mucosa hosts a particular type of epithelium, "the transitional epithelium or urothelium", with cellular, intercellular and architectural features that make it expansible, durable, and resilient to life long persistent irritation by urine^[1]. Additionally, the detrusor muscle that surrounds the mucosa has a peculiar pattern of fibre arrangement to enable instant and complete emptying of the bladder once it is contracted. It can adjust to chronic outflow obstruction by hypertrophy and hyperplasia^[2]. The neurological control of the bladder and its urethral sphincter is compounded by autonomic, sensory and motor neural interaction^[3]. Multiple other hormonal and metabolic factors contribute to the integrity of the bladder function. Disturbance in one or more of these elements results in dysfunction in the vital process of urination which could significantly impair a patient's quality of life and may, in severe cases, cause disability or even death. Surgical removal of the bladder due to cancer or end stage benign disease necessitates the replacement of its function using alternative mechanisms, of which, stem cell based engineered tissue is proposed as a possible future strategy.

CONSIDERATIONS IN THE APPLICABILITY OF STEM CELL THERAPY

Stem cells are classically defined by their self-renewal based long-term survival and their flexible fate, being able to differentiate into other cell types. However, as simple as the concept seems to be, the accurate

identification, isolation and transplantation of stem cells could be more intricate than originally thought. For example, the functional distinction between stem cells and their progenitor cells remains ambiguous^[4,5]. This should be taken in consideration when planning stem cell therapies; a successful therapy may require other progenitor cells to turn on differentiation and proliferation signals of the parent stem cell. As well, the transition of the stem cell between quiescent and active stages is a dynamic process that challenges the accurate prediction of stem cell behaviour in different environments^[6]. The control of stem cell niche and microenvironment appears to be fundamental in the long-term success of stem cell therapy. Therefore, studying the stem cell niche could identify important biochemical and mechanical cues that need to be provided in addition to the cellular component to bridge the variability gap between the donor and host stem cells' niche and allow their effective engraftment and proper function *in vivo*^[7].

BLADDER ADULT STEM CELLS

The ability to use someone's cells to repair another area of one's own body could indeed reduce risks of infectivity and immunogenicity. For that, adult stem cells maintained its key position among other stem cell types^[8]. Tumorigenicity is also of much less concern. However, adult stem cells could be of less potential for long term survival due to inherent cell aging concerns^[9]. Various adult stem cell types have been used in treating bladder dysfunction including muscle; bone marrow and adipose tissue derived stem cells^[10]. In addition, urine stem cells have been isolated and differentiated into specialized cells to offer a readily accessible stem cell source for various applications^[11]. Although a general depot of adult stem cells exists in fat and bone marrow, however, lineage specific stem cells are believed to dominate in specific organs such as skin and cornea^[12,13].

The urothelial adult stem cells are thought to be slow cycling *in vivo* (3-6 mo), clonogenic, highly proliferative and located in protected sites. These cells are commonly identified by their localization in the basal layer of the urothelium, and by being label-retaining cells with high expression of β -4 integrin^[14,15]. The identification and isolation of these cells are important for tissue engineering of urothelium-lined organs including bladder, urethra and ureters.

CLINICAL APPLICATIONS OF STEM CELLS IN BLADDER PATHOLOGIES

Stem cells for urinary bladder replacement

Following cystectomy for benign or malignant bladder pathologies, bladder replacement or reconstruction is a critical step for maintaining patient's life. Whether ureterosigmoidostomy, ileal conduit or orthotopic

neobladder are used for reconstructing a new urinary reservoir, significant morbidity and mortality often occur due to the incorporation of intestinal segment into the urinary tract. This could result in recurrent urinary tract infection, metabolic and electrolyte disturbance, mucous retention and anastomotic site cancer. Moreover, the patient is left to deal with either an external draining bag through an opening on the skin called stoma or self catheterization with no external bag, both of which could interfere with body image and daily activities. Current *in situ* surgical bladder constructs are also unable to contract and squeeze the urine through the urethra since it lacks the muscle layer and the patient needs to adapt to techniques to push urine out such as contracting the abdominal muscles^[16-18]. Therefore, seeking new therapies to provide optimal bladder reconstruction is of utmost clinical importance.

Ideally, a perfect bladder reconstruct should be made of low immunogenic or autologous tissue that contains all anatomical layers of the bladder wall (mucosa, submucosa and muscle layer). It should be designed to mimic the detrusor muscle mechanics and to provide significant dispensability. It has also to provide similar urothelial mucosal barrier and ultimately should be incorporating functioning neuronal elements. Accordingly, advanced and complex tissue engineering and regenerative models are required. Thus far, there has been no such comprehensive efficient model; however preliminary studies are ongoing worldwide to achieve such goals.

Recently, tissue engineering using cell seeded scaffolds has been investigated in urinary bladder bioengineering studies^[19]. This method includes the seeding of a scaffold with autologous bladder muscle and epithelial cells. The use of autologous cells, however, may not be available as in cases of cancer^[20] or benign end-stage bladder diseases^[21]. Alternatively, stem cells can be derived from other sources including adipose tissue, bone marrow or amniotic fluid cells. They can be seeded on scaffolds and transplanted for *in vivo* differentiation. However, current data shows that such differentiation occurs only in a small percentage of the delivered cells^[22]. Another method is to differentiate stem cells into urothelial and smooth muscle cells *in vitro*. Stem cells have shown a good potential for urothelial differentiation. This was achieved by using either conditioned medium^[23,24] with or without growth factors such as all-transretinoic acid^[25]. Both direct culture (seeding stem cells with urothelial cells) and indirect co-culture (using trans-well system) have been attempted with variable outcome, though cell-to-cell contact in direct culture appears to be an enhancing factor for stem cell differentiation^[26,27]. Stem cell differentiation into smooth muscle cells is more feasible and can be achieved either by chemical induction^[28] or co-culture with smooth muscle cells^[22].

Induced pluripotent stem cells (iPS) reprogrammed

from adult tissue such as skin fibroblasts, urinary tract stromal cells and urine-derived cells have been also used and were subsequently differentiated into urothelial and smooth muscle cells^[29-31]. However, urinary tract-derived iPS cells are believed to be of superior differentiation properties to other sources, which should emphasize the epigenetic differences between individual iPS cell lines and stress on the importance of organ-specific iPS cells for tissue-specific studies^[29-31].

Stem cells for voiding dysfunction

Voiding dysfunction (VD) can affect the patient's quality of life and interfere with social activities. It can be manifested clinically as various disorders of urine storage or emptying. Current therapies of VD are generally inadequate and often fail to target the actual pathophysiology of the disease. Stem cell therapies have been also investigated in this field and were shown to cause some positive response either due to differentiation or more likely due to indirect paracrine effect associated with the release of growth factors and cytokines. The later mechanism could lead to modulation of local and systemic inflammatory responses and mobilization, stimulation and differentiation of native stem cells in addition to the enhancement of vascularization of regenerating tissues and reduction of fibrosis^[32]. A variety of stem cell types have been explored for the treatment of VD, including bone marrow, skeletal muscle and adipose derived stem cells^[33-35]. Adipose stem cells was most popular due to their easy harvest, high yield of stem cells and easier smooth muscle differentiation compared to other types^[35]. They could improve VD in animal models of bladder overactivity or hypoactivity associated with different etiologies such as diabetes mellitus, post radiation and hyperlipidemia^[32,35]. Nevertheless, more studies are needed to determine the efficiency of such therapy, the form of transplanted cells (unmodified or differentiated cells), the method of cell delivery (systemic or local injection into the bladder) and the appropriate cellular dose (number of cells per injection and frequency of treatment).

Stem cells for stress incontinence

Stress urinary incontinence (SUI) is a widespread disorder, particularly in women^[36], due to inherent predisposing anatomical and physiological factors specific to the female urethra. In addition, SUI is commonly initiated or aggravated by female specific physiological stages such as pregnancy, vaginal birth and menopause or pathological conditions such as uterine fibroid tumors^[37]. The current treatment of SUI relies on pelvic floor exercises in mild cases and surgical interventions in severe cases. The surgical methods aim to provide support to the urethra using various artificial tapes or autologous tissue slings. Each one of these methods has its adverse effects and post-operative complications^[38,39]. More recently,

Table 1 Examples of clinical studies of stem cell therapy used in the treatment of stress urinary incontinence showing a comparison of stem cell source, the duration of *in vitro* expansion and the follow up of patients post treatment

Origin of stem cells and duration of <i>in vitro</i> expansion	Number of women and route of injection	Follow up (yr)	Success rate (improvement and cure)	Ref.
Autologous muscle derived stem cells (SC), expanded 8-10 d	16 female patients, transurethral approach	2	Up to 75%	[76]
Muscle derived SC	222 male patients, transurethral injection	1	Up to 54%	[77]
Minced autologous muscle cells, no <i>in vitro</i> expansion	35 female patients	1	Up to 63%. Improvement (clinical, diary, and ICIQ-SF scores)	[78]
Muscle derived SC, expansion duration NA	8 female patients, transurethral injection	1	Significant improvement in 5 women (pad-weight, bladder diary and QOL assessment)	[79]
Muscle derived SC, expanded <i>in vitro</i> for 7 wk	20 female patients	2	Significant improvement (clinical, QOL and cystometry). Therapy based on this method is now licensed in Europe	[80]
ASCs combined with bovine collagen	5 female patients	1	2 out of 5 patients were satisfied with treatment with negative cough test	[81]
Autologous ASCs with and without fat	11 male patients with post-prostatectomy incontinence	1	60% improvement in urine leakage, frequency and amount of incontinence in 8 patients with one patient achieved total continence	[82]

the injection of bulking agents around or through the urethra to treat SUI has gained some popularity. Many agents have been used with variable success rates and complications were reported. Collagen, fat, Teflon, silicon or carbon coated beads are common examples of various agents employed^[40]. A recent systematic review in the Cochrane database showed that current data is insufficient to prove benefit of these therapies especially that saline injection was of similar effectiveness to bulking agents, with some reports of serious side effects associated with some of these agents^[41]. Consequently, stem cell therapies appeared as the next generation of therapies in SUI, which gained recent attention. Animal studies showed potential benefit in treating SUI. The animal model of SUI is usually induced by cutting the pudendal nerves. Muscle derived stem cells have been the most widely applied source and are believed to provide stem cells that are able to differentiate into committed striated muscle cells more than other stem cell sources. However, many other sources have been attempted with comparable success. To evaluate the success of stem cell therapy in SUI animal models, multiple outcome measures were used including leak point pressure, intra-bladder pressure, maximum bladder volume, urethral functional length, maximum urethral closure pressure, morphological examination of sphincter muscle and matrix^[42].

Adipose derived stem cells have been injected in rat models of stress incontinence using intravenous or trans-urethral routes, and showed significant improvement in terms of increased elastin content and voiding function measured by cystometry^[43]. Adding nerve growth factor and poly (lactic-co-glycolic) acid to the adipose stem cells when injected in the rat urethral sphincter improved stem cell proliferation *in vivo* and that was increasing in a dose dependant pattern. Such factors appear to improve stem cell survival and

functional performance of the urethra compared to using adipose stem cells alone^[44]. Furthermore, human amniotic fluid stem cells seem to be of potential benefit and favourable safety profile in restoring normal urethral function in the animal models of SUI due to their low immunogenicity and tumorigenicity^[45]. A triple stem cell therapy approach used human amniotic stem cells that were processed to the stage of early differentiation into three lineages *in vitro* (myogenic, neurogenic and endothelial). This approach was able to improve SUI signs in the animal model compared to using only one or two types of differentiated cells^[46]. A combination of gene therapy strategy by inducing urine derived stem cells to over express VEGF showed improvement of the sphincter composition especially the nerve fibres, muscle cells and vascularisation^[47].

The reconstruction of stem cell tissue engineered based slings to support the urethra was also investigated. A silk scaffold covered with bone marrow derived mesenchymal stem cell sheet has been implanted as a sling to support the rat urethra showing a better matrix deposition compared to using a silk sling alone^[48]. Likewise, adipose tissue derived stem cells and silk fibroin microspheres were combined together and they were able to retain improvement in SUI for longer duration than the silk fibroin microspheres alone^[49]. Following animal studies, a number of clinical trials have been attempted; examples are shown in Table 1.

Urinary sources and applications of iPS

iPS can be generated from somatic cells by inducing the expression of different transcription factors, originally Oct4, Sox2, Klf4 and Myc (OSKM). The induced pluripotent stem (iPS) cells have been considered as the stem cell that could offer several advantages over the adult stem cells while maintaining the plasticity of embryonic stem cells^[50]. However,

close examination of their possible adverse outcomes, such as the reported immunogenicity, should be undertaken prior to wide application^[51]. The urinary bladder and prostate cells were also reprogrammed successfully to iPS cells, offering a new method of the urinary system disease modeling^[29]. Likewise, urine cells were reprogrammed into iPS cells that were differentiated successfully into cardiac cells *in vitro*^[52]. Moreover, a model of personalized medicine was introduced by reprogramming urine cells of a haemophilia A patient into liver cells that express the traits of the disease^[53]. Conversely, the urinary bladder cells were obtained from iPS cells *in vitro*^[54]. Therefore, theoretically, the use of urine cells to produce urinary bladder wall cells for bladder reconstruction purposes would be attractive as they are originating in the same system^[55].

Bladder cancer stem cells

On the other end of the stem cell paradigm, adult cancer stem cells (CSC) are implicated in cancer development, progression and recurrence after conventional therapy. CSC are practically identified and confirmed by their ability to initiate tumours in immunocompromised mice and by their *in vitro* clonogenicity. The cancer stem cell theory was a build up on older clinical observations that related cancer to stemness^[56]. Starting with blood cancers that showed strong representation of the cancer stem cell model^[57], most solid cancers were studied for their CSC population^[58-62]. Several groups were able to isolate cells with CSC features from urinary bladder cancers. These cells were identified by several markers as being CD44+, negative for EMA, high expression of 67LR, low expression of CD66C, strong association with ALDH1A1 expression or as a side population based on the efflux of the Hoechst dye^[63,64]. It appears that CD44+ is one of the reproducible markers and its expression on bladder CSC was commonly supported by several groups^[65].

The value of such findings is to tailor specific target based therapies. Unlike traditional bladder cancer therapies, a cancer stem cell targeted therapy is postulated to be able to eradicate the resistant cancer initiating cells and minimize risk of recurrence. It is also important to understand bladder carcinogenesis and recognize how risk factors initiate cancer. For instance, activation of the transcription factor stat3 led to transformation of CK14+ urothelial stem cells into invasive cancer cells in a stat3 transgenic mouse model^[66]. Also, arsenic could transform normal prostate stem cells into cancer stem cells; this may explain its known detrimental effect on the urogenital system^[67].

One of the promising molecular targets include the CD47 which is an integrin associated protein known for its "do not eat me" effect on the cancer cell, leading to less recognition by cell mediated immunity especially

macrophages. The CD47 was found to be significantly more expressed in CSC compared to normal cells^[68]. A CD47 antibody labeled with fluorescence was used to distinguish human bladder cancer cells from normal cells with a specificity of more than 90%^[69]. A number of clinical trials have started to use the antibody against CD47 as a model of immunotherapy in leukemia and solid tumors^[70]. The bladder CSC niche and the microenvironment are also believed to significantly protect CSC from immunity and therapeutic factors and to maintain its carcinogenic behavior. Therefore, understanding the composition of this niche could highlight other potential targets for new therapies^[71].

Safety and quality

It is obvious that the use of stem cells in clinical investigations and therapeutics of bladder and urethral pathologies will be of potential importance in the near future. Nonetheless, the rapidly gained popularity of these interventions should be cautiously perceived and publicized in scientific and non-scientific media. Premature application of such therapies in human subjects without matching scientific evidence as off label treatment in research studies or their unregulated use in clinical practice should be truly concerning. In underdeveloped settings, such unregulated practices are quite common. Stem cell therapy tourism is also expanding and could expose patients to considerable health hazards^[72]. Factors such as sterility and viral screening of the introduced cell therapy, the lack of contaminants and toxic molecules, cell viability and cell karyotyping should be all included in stem cell clinical studies and should be reported clearly by researchers before being accepted in formal scientific publications^[73]. Tumorigenicity of stem cell therapies is another factor that should not be overlooked and available tumorigenicity assays should be routinely used^[74]. Awareness about current good manufacturing practices in stem cell therapy should be widely raised^[75]. Nonetheless, this would not be adequate in the absence of strict regulations enforced on using such therapies in human especially in underdeveloped settings where baseline research ethics and quality control are not well developed or monitored. Wide international collaboration is, therefore, strongly needed to come up with a global consensus and standards that allow the safe use of stem cell therapies in human subjects without ignoring the cost and training involved to implement these standards in poor settings.

CONCLUSION

Stem cell therapies will be in the clinical setting in the near future. At present, their use in bladder dysfunction and bladder reconstruction is under intense investigation. Various sources of stem cells

have been attempted with comparable success. More complex tissue engineered models that mimic the original bladder anatomy are awaited. Bladder cancer stem cells are investigated as a potential target for cancer therapies.

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Modeling diseases of noncoding unstable repeat expansions using mutant pluripotent stem cells

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Abstract

Pathogenic mutations involving DNA repeat expansions are responsible for over 20 different neuronal and neuromuscular diseases. All result from expanded tracts of repetitive DNA sequences (mostly microsatellites) that become unstable beyond a critical length when

transmitted across generations. Nearly all are inherited as autosomal dominant conditions and are typically associated with anticipation. Pathologic unstable repeat expansions can be classified according to their length, repeat sequence, gene location and underlying pathologic mechanisms. This review summarizes the current contribution of mutant pluripotent stem cells (diseased human embryonic stem cells and patient-derived induced pluripotent stem cells) to the research of unstable repeat pathologies by focusing on particularly large unstable noncoding expansions. Among this class of disorders are Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome, myotonic dystrophy type 1 and myotonic dystrophy type 2, Friedreich ataxia and C9 related amyotrophic lateral sclerosis and/or frontotemporal dementia, Facioscapulohumeral Muscular Dystrophy and potentially more. Common features that are typical to this subclass of conditions are RNA toxic gain-of-function, epigenetic loss-of-function, toxic repeat-associated non-ATG translation and somatic instability. For each mechanism we summarize the currently available stem cell based models, highlight how they contributed to better understanding of the related mechanism, and discuss how they may be utilized in future investigations.

Key words: Unstable repeat associated disorders; Human embryonic stem cells; Patient-derived induced pluripotent stem cells; Disease modeling; Epigenetics; repeat-associated non-ATG translation; RNA toxicity; Repeat somatic instability

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Core tip: This review summarizes the current contribution of mutant pluripotent stem cells (diseased HESCs and patient-derived induced pluripotent stem cells) to the research of unstable repeat pathologies by focusing on particularly large unstable noncoding expansions. It demonstrates their importance as an unlimited cell source for generating rarely available impaired cells in culture, and as a model system for

exploring the mechanisms that are involved with this class of mutations. For each mechanism we describe the currently available stem cell based models, highlight how they contributed to better understanding of the related mechanism, and discuss how they may be utilized in future investigations.

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INTRODUCTION

Unstable repeat expansions, termed also dynamic mutations, are the cause of over 20 different neurodevelopmental, neurodegenerative and neuromuscular heritable conditions that result from a change in the number of repeat units in coding genes when ordered in tandem (for comprehensive review see^[1]). Repeat instabilities change across generations, and may vary between cells within affected individuals. Nearly all are inherited as autosomal dominant conditions and are typically associated with anticipation, meaning that the disease symptoms tend to be more severe and occur earlier in age with successive generations. Most unstable repeat associated disorders are caused by the addition of repeat units (mostly tri-nucleotides) that, above a certain threshold, lead to misregulation of genes and/or to their RNA/protein products. Disease associated unstable repeat expansions can be classified into two groups according to the length and gene location of the expansion. One class of disorders results from small repeat copy changes (few to tens of units) that usually reside in exon coding regions of genes. Among them are the ployglutamine CAG expansion disorders like Huntington disease, Huntington's disease like 2 (HDL2), Dentatorubral-pallidoluysian atrophy (DRPLA), Spinal and bulbar muscular atrophy (SBMA) and spinocerebellar ataxia (SCA) 1, 2, 3, 6, 7 and 17, which lead to protein gain-of-function alterations. A second class of unstable repeat expansion disorders results from large expansions, ranging from hundreds to thousands of copies. Large pathogenic repeat expansions are typically located in noncoding regions including promoters, introns and untranslated regions (UTRs) of genes^[2]. Among disorders in this class are Fragile X syndrome (FXS) and [Fragile X-associated tremor/ataxia syndrome (FXTAS)]; both FXS and FXTAS are caused by large expansion of a CGG repeat in the 5'-UTR of the *FMR1* gene^[3-5], [myotonic dystrophy type 1 (DM1); caused by a CTG expansion in the 3' UTR of the *DMPK* gene^[6-8] and type 2 (DM2; caused by a CCTG expansion in intron 1 of the *ZNF9* gene^[9]), [Friedreich ataxia (FRDA); caused by a GAA expansion in intron 1 of the *Frataxin* gene^[10]], C9 related

[amyotrophic lateral sclerosis and/or frontotemporal dementia (ALS-FTD); caused by a GGGGCC^[11] expansion in intron 1 of the *C9orf72* gene^[12]], and [Facioscapulohumeral Muscular Dystrophy (FSHD); caused by a contraction of the D4Z4 macrosatellite repeat in sub-telomeres of chromosome 4q35].

The outcome of the expansion mutation may be different depending on its gene location and length. Unlike small expansions, which commonly result in alterations in protein function, large noncoding expansions introduce further complexity because they can lead to either loss-of-function, RNA gain-of-function, toxic protein gain-of-function, or to a combination of all these pathogenic mechanisms in unison^[13]. In addition and in contrast to small expansions, large noncoding expansions frequently coincide with marked changes in repeat tract length between and within tissues of affected individuals^[8,14,15]. This phenomenon, termed somatic repeat instability, results in mosaicism for expansion size and occasionally correlates with disease age of onset and severity. Modeling dynamic mutations, specifically large expansions, in mice can be particularly challenging due to the difficulty in artificially inducing and stably maintaining very large repeat expansions (especially CG-rich) *in vitro* and *in vivo*. In addition, despite the similarities between mouse and human, there are still major differences between the two species, leading to dissimilarities in biochemical pathways and phenotypes. These crucial discrepancies emphasize the need for complement model systems that will reproducibly copy the underlying mechanisms and clinical phenotypes in human.

Mutant human pluripotent stem cells, embryonic [human embryonic stem cells (HESCs)] or artificially induced [induced pluripotent stem cells (iPS cells)], may provide a great opportunity to study unstable repeat expansions by complementing existing models^[16]. They are expected to be especially useful in the study of heritable conditions where the animal model fails to fully recapitulate the phenotype of the disease in human, or when disease relevant cell cultures from patients are unavailable. The many advantages to the utilization of these cells for biomedical research include the fact that they are human derived, untransformed cells with an unlimited self-renewing capacity. In addition, they have the potential to differentiate into a wide range of cell types in culture, and recapitulate early human embryo development while they differentiate *in vitro*. Moreover, as mutant pluripotent stem cells can be derived directly from genetically affected embryos that are obtained through preimplantation genetic diagnosis (PGD) procedures (HESC)^[17], or by reprogramming somatic cells obtained from patients (iPS)^[18], they can reproduce disease cellular phenotypes as they occur *in vivo* without the need to artificially intervene with their genome through genetic manipulation. In addition, they are human derived and potentially be used to generate large amounts of impaired disease relevant cells in culture. This is particularly beneficial in the

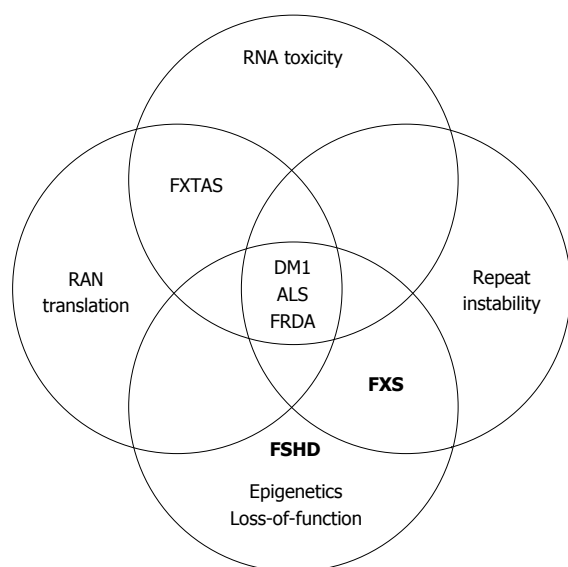


Figure 1 Modeling pathogenic noncoding repeat expansions by the use of mutant pluripotent stem cells. Unstable noncoding repeat expansions have several features in common. They typically display somatic instability, and can lead to either epigenetic loss-of-function, toxic RNA gain-of-function, toxic RAN translation, or to a combination of all these pathogenic mechanisms. Future research might discover more similarities in the pathogenesis of these and additional repeat associated disorders. Disease modeling through the use of mutant HESCs and iPS cells is anticipated to be particularly useful considering their potential to generate impaired cells, and their ability to recapitulate developmentally regulated events upon differentiation. Indeed, using this model system, new mechanistic insights have thus far been gained for diseases marked in bold. FSHD: Facioscapulohumeral Muscular Dystrophy; FXS: Fragile X syndrome; ALS: Amyotrophic lateral sclerosis; FRDA: Friedreich ataxia; FXTAS: Fragile X-associated tremor/ataxia syndrome; DM1: Myotonic dystrophy type 1.

case of unstable repeat pathologies, where studies are frequently limited to postmortem brain samples or to unsuitable cell types obtained from patients such as peripheral blood cells or skin fibroblasts. Furthermore, as these cells can recapitulate early stage embryo development, they may be particularly valuable in modeling disease associated mechanisms that are developmentally regulated such as those that are elicited by differentiation. In terms of applied research, mutant pluripotent stem cells provide a powerful cell culture based system for gene correction. For instance, they may facilitate the efficient induction of irreversible changes in DNA that will correct the disease causing mutation by shortening the repeat tract through genome editing or other gene manipulation approaches^[19]. They can also provide a platform for drug screening and development, conditioned by the accessibility of efficient differentiation protocols and the availability of reliable biomarkers.

In this review we summarize the current contribution of mutant pluripotent stem cells to the research of unstable repeat pathologies by focusing on common mechanisms that are associated with large unstable noncoding expansions (Figure 1). A complete survey of the data regarding the use of mutant pluripotent stem cells for modeling phenotypes of coding unstable repeat

expansions, and the use of these cells as a platform for gene therapy, are beyond the scope of this review and can be found elsewhere^[20-22]. The discussion here will thus be limited to only pathological noncoding repeat expansions. For each mechanism we highlight the currently available pluripotent stem cell models by describing their exclusive utility for investigation of the mechanism, how they contributed to better understanding of the related mechanism, and we raise potential routes for future investigation.

TOXIC RNA GAIN-OF-FUNCTION

Over the past decade it has become evident that RNAs containing abnormally large microsatellite repeat expansions underlie the pathogenesis of at least 9 dominantly inherited human diseases. These are mostly neurological and neuromuscular diseases, including DM1^[23], DM2^[24], FXTAS^[25], C9/ALS-FTD^[26], SCA3^[27], SCA8^[28], SCA10^[29], SCA31^[30] and HDL2^[31]. These diseases arise when the repeat-containing RNA disrupts the function of specific RNA binding proteins (RBPs) *in trans*. The paradigm for such toxic RNA gain-of-function pathology is DM1, where a CUG repeat expansion located in the 3'-UTR of the *DMPK* gene interferes with the cellular function of specific CUG-binding splice-regulating proteins termed MBNL1 and CUG-BP1 (CLEF protein family)^[32,33]. The altered function of these splice regulators hinders the normal processing of transcripts encoding over twenty different muscle genes, resulting in inappropriate expression of embryonic rather than adult splice variants in adult disease manifesting tissues^[34,35]. The general hallmarks of unstable repeat disorders with RNA-mediated toxicity are the accumulation of repeat-containing RNAs in intra-nuclear inclusions along with the sequestration of common RBPs (mainly proteins involved with RNA metabolism) and alternative splicing misregulation^[36]. Co-localization of the large ribonucleoprotein (RNP) aggregates with ubiquitin and proteasome subunits is a common feature^[37]. These striking parallels have led to the identification of the most common (currently known) mechanisms underlying noncoding unstable microsatellite disorders. This subclass of disorders is caused not due to the loss of function of the mutant transcripts and haploinsufficiency, but rather from the loss of function of RBPs that aggregate with the lengthy RNAs, and possibly from the toxic effect of the aggregates themselves. As the RNA seeds accumulation of the proteins it interacts with, it alters their biological function by changing their abundance and/or localization in the cell. Furthermore, since most of these expansions are bi-directionally transcribed, multiple repeat-containing toxic RNAs seem to be involved^[38-40]. The susceptibility of neurons to this process is a common feature, most likely related to the ageing course of this specific cell type. Considering the reversibility of the process^[41-44], it may be possible to develop effective therapeutic strategies to ameliorate

the symptoms of these diseases.

Taking advantage of two DM1 affected pluripotent stem cell lines derived from embryos identified through PGD, Denis *et al.*^[45] produced impaired early progenitor neural cells in culture. To validate the pathological relevance of their differentiated cells, they show that DM1-neural stem cells (NSCs) and derived neurons harbored intranuclear RNP foci, contain the mutant RNA and co-localize with the splice factor MBNL1. Interestingly, functional characterization of DM1 HESC-derived neurons revealed reduced proliferative capacity and increased autophagy linked to the mTOR signaling pathway. In addition, the researchers were able to demonstrate that the loss of function of MBNL1 in wild type (WT) HESCs results in alteration of the mTOR signaling pathway, whereas gain-of-function experiments rescued the phenotype. Collectively, their findings provided a mechanism by which the DM1 mutation might affect a major signaling pathway, and highlighted the pertinence of using pluripotent stem cells to study neuronal defects in general. A complement model system to support the formation of intranuclear RNA foci by *in vitro* derived neurons was developed in iPS cells, validating the potential of pluripotent stem cells to offer an unlimited cell resource for CNS mechanistic studies^[46].

A different disease for which pluripotent stem cells have been employed to further understand the toxic RNA-mediated mechanism underlying unstable repeat expansions is in C9/ALS-FTD. Amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD) is an autosomal dominant neurodegenerative disorder characterized by adult onset of one or both of these conditions in an affected individual^[47-49]. The leading cause for ALS-FTD is a G₄C₂ repeat expansion in the first intron of the *C9orf72* gene, between noncoding exons 1a and 1b. Expansion of G₄C₂ repeat tract beyond a certain threshold, results in RNA gain-of-function resulting from bi-directional transcription^[40,50]. Its low incidence (1:100000) and mainly sporadic occurrence (90%), late onset, and accelerated progression, have thus far facilitated the preparation of iPS cells, but not HESC lines. By establishing multiple iPS clones from skin biopsies of patients' fibroblasts with over 1000 G₄C₂ repeats, researchers were able to generate motor neurons in culture that recapitulate key neuropathological features of ALS-FTD including RNA foci reminiscent of those observed in postmortem brain autopsies from patients^[51]. Interestingly, the RNA foci containing G₄C₂ repeats were present in all iPSC-derived human neurons as well as in undifferentiated iPSCs and primary fibroblasts. This suggests that these foci are not restricted to a specific cell type or developmental stage, as one would predict if functionally significant. Moreover, transcription of the repeat containing *C9orf72* transcripts, led to accumulation of G₄C₂ repeat-containing RNA foci selectively in various C9-ALS iPSC-derived neural cell types^[52]. It was also shown that the pathologic RNA foci co-localize with

the major known RBPs like hnRNP A1, Pur- α ^[52] and ADARB2^[53], suggesting that they may be able to disrupt RNA metabolism. Furthermore, it was found that the *in vitro* derived C9-ALS motor neurons present altered expression of genes involved in membrane excitability, and demonstrated a diminished capacity to fire continuous spikes upon depolarization compared to a WT motor neuron control^[52]. By targeting the RNA foci through antisense oligonucleotide (ASO), either through knockdown (KD) of the full length *C9orf72* transcripts or by differentially targeting the repeat containing mRNA isoforms (V1 and V3), researchers were able to suppress RNA foci formation and reverse gene expression defects in C9-ALS neuronal cells^[52,53], as well as, rescue to some extent disease phenotype (Glutamate toxicity^[53]). These data show that patient-derived motor neurons can be used to elucidate the pathogenic role of the toxic RNA in C9/ALS-FTD. More generally, they neatly illustrate the power of mutant pluripotent cells as a translational platform for therapeutic development.

Many questions regarding this repeat-associated mechanism remain to be addressed. For example, what dictates the cell-type specificity of this process? How is it age related? What is the protein composition of the aggregates in each disorder and, what is the nature of the inclusions? Are they toxic or do they simply represent clearance of toxic RNA? In addition, although animal models have provided firm evidence for RNA gain-of-function as a major contributing mechanism^[54,55], clinical and pathophysiological differences between human and available animals (mouse and *Drosophila*) limit their usefulness in delineating specific mechanisms underlying these toxic RNA-mediated pathologies^[56-61]. Therefore, a complement human-based model system is needed to enable molecular characterization of the onset and course of this class of conditions.

REPEAT-MEDIATED EPIGENETIC MODIFICATIONS

Among the disease associated expansions there are several, counting FXS, FRDA, DM1, and C9/ALS-FTD, that results from particularly large noncoding expansions (hundreds to thousands of copies) that reside within CpG islands (CGIs). CGIs typically remains free of DNA methylation and are rich in histone modifications associated with transcriptionally active chromatin. Yet, expansion of repeat copy number beyond a certain threshold leads to local acquirement of abnormal CpG methylation in addition to loss of active histone modifications and concomitant gain of repressive histone modifications that are typical to densely packed chromatin (like H3K4 demethylation and H3K9 and H3K27 tri-methylation, respectively). These aberrant epigenetic modifications can change local gene transcription. Their effect may vary from one locus to another, depending on the location of

the expansion in the gene. While in some genes it abolishes the activity of a promoter (as in FXS and C9orf72)^[62,63] or an enhancer (as in DM1)^[64], in other genes it interferes with RNA transcription elongation (as in FRDA)^[65] or termination (as in FSHD^[66]). Furthermore, even when hypermethylation leads to the loss of promoter activity, the outcome may be very different. For example, while in FXS transcriptional silencing results in FMRP protein deficiency (loss-of-protein function)^[67], in C9/ALS-FTD epigenetic silencing may potentially lead to increased use of an alternative promoter (RNA gain-of-function)^[39]. In addition, in some disorders hypermethylation seems to be acquired in a developmentally regulated process (FXS)^[17] whereas in others it is more of a tissue or an age-dependent phenomenon (DM1)^[68]. Therefore, it is essential to first understand when, where and how hypermethylation affects local gene expression, in order to examine whether and by what mechanism this contributes to disease pathogenesis. More generally, by unraveling how repeat expansions lead to the loss of CGI identity, we may gain new insights on how CpG islands are normally protected from *de novo* methylation throughout the genome.

The availability of mutant pluripotent stem cell lines carrying pathologic repeat expansions may provide a great model system to address the questions of when, where and how hypermethylation is aberrantly acquired. In particular, such cell lines can be utilized to better determine the timing, intervene with the mechanism, and monitor the effect of epigenetic modifications. In addition, they may provide an exceptional opportunity to rescue the mutant phenotype by resetting the undesired epigenetic modifications that are coupled with the expansion through cell reprogramming.

To date, pluripotent stem cell-based studies related to the epigenetic aspects of unstable repeat expansions have been mainly limited to FXS. In FXS, when the CGGs reach the full mutation range, they lead to the spread of abnormal CpG methylation and repressive histone modifications at the 5' regulatory region, resulting in epigenetic gene silencing and consequently to FMRP protein deficiency in patients^[62]. Initially, when the first FXS XY HESC line was established, it was found to express FMR1 at comparable levels with WT HESCs and to be completely FMR1 unmethylated^[17]. These findings validated a commonly accepted view that FMR1 epigenetic silencing is a developmentally regulated process that is triggered by differentiation. Moreover, it encouraged investigators to propose that since epigenetic FMR1 inactivation is differentiation-dependent (rather than being maternally transmitted in the gamete), it may be possible to rescue the cellular phenotype of FXS neurons simply by removing the epigenetic marks that are erroneously gained, through cell de-differentiation in culture. However, as more FXS HESC lines were available, it became apparent that FMR1 hypermethylation commonly occurs in the undifferentiated state. Taking advantage

of a large set of FXS HESCs (9 cell lines, including the former), Avitzour and colleagues found that FMR1 hypermethylation frequently occurs in these cell lines (six of nine lines, ranging from 24% to 65%)^[69]. This suggests that the wide variability in methylation levels between and within the different FXS HESC lines reflects a widespread event within mutant FMR1 embryos, where methylation state is initially set. It should be interesting to determine whether FXS preimplantation embryos are already FMR1 methylated, and whether methylation at this stage is incomplete. Mechanistically, the researchers tried to address the question of how the CGG expansion at the FMR1 locus leads to *de novo* methylation of the CpG island at the 5'-UTR of FMR1^[69]. They aimed to associate FMR1 hypermethylation with CTCF binding next to the repeats, taking in to consideration the well-known role of CTCF as an insulator binding protein that counteracts heterochromatin spreading^[70,71] and taking in to account previous reports demonstrating CTCF occupancy next to the CGG repeats at the FMR1 locus in differentiated cells. However, since no enrichment for CTCF could be detected, in both WT and affected HESC lines, this study ruled out the possibility that hypermethylation may be attributed by the binding loss of CTCF, as formerly suggested^[38,72]. Employing two other XY FXS HESC lines, Colak and colleagues uncovered an mRNA-mediated mechanism that drives epigenetic FMR1 inactivation in a way that relies on neuronal differentiation^[73]. In this study the researchers showed that the CGG-lengthy FMR1 mRNA hybridizes to the complementary CGG-repeat portion of the *FMR1* gene to form an RNA:DNA duplex. Upon differentiation the RNA:DNA hybrid elicits gene silencing on the protein and mRNA levels. According to their study, disrupting the interaction of the mRNA with the CGG-repeat portion of the *FMR1* gene at a critical time point during cell differentiation prevents promoter silencing. It remains to be determined whether silencing in undifferentiated cells is achieved by the same mechanism or by distinct one, and whether the loss of FMR1 mRNA and protein in the FXS HESC-derived neurons involves DNA hypermethylation.

In line with this study, it has been proposed that it may be possible to reverse the adverse effect of the modifications and correct the mutant phenotype by reprogramming terminally differentiated FXS cells. However, somatic cell reprogramming of FXS patients' fibroblasts by numerous independent studies failed to de-methylate and reactivate the gene simply by de-differentiation^[74], suggesting that once established, methylation is irreversible and/or is acquired prior to blastocyst formation^[75-77]. In line with this view, it should be feasible to obtain FXS-iPS clones that are FMR1 active from somatic cells with a full mutation provided that they carry an unmethylated full expansion at least in some of their cells. On the other hand, perhaps reprogramming may wrongly lead to hypermethylation of this mutation prone site, regardless of its initial

epigenetic state. Indeed, reprogramming fibroblasts from an unusual asymptomatic subject, bearing a full mutation that is entirely unmethylated, resulted in complete silencing, regardless of the reprogramming method used^[78]. Maybe the high levels of DNMT3b, that are induced as part of the reprogramming process^[79], led to this unexpected result. This suggests that FXS iPS cells, and potentially additional iPS models for epigenetically regulated disorders, may not serve as an ideal model for better understanding the mechanism by which hypermethylation is induced. Reprogramming of somatic cells from additional subjects with hypomethylated expansions will be required in order to establish whether this is a widespread event that is irrelevant to the disease. Regardless, it should be possible to use these disease affected iPS cells to explore the consequence of FMRP deficiency or reverse this effect in *in vitro* differentiated neurons, a disease relevant cell type. Indeed, re-activation of FMR1 by the treatment with the de-methylating agent 5-azaC has already been successfully practiced in neuron derived FXS-iPS cells^[80].

Apart from FXS HESCs, there is indirect evidence to suggest that hypermethylation is recapitulated in patient derived iPS cells for other unstable repeat related loci. One example is C9/ALS-FTD iPS cells that carry a G₄C₂ expansion at the *C9orf72* gene. In the *C9orf72* the G₄C₂ repetitive sequence is flanked by two CpG islands (CGIs) that span from the promoter sequence into intron 1 of *C9orf72*. Like many CGIs in the genome, this region typically remains unmethylated and is transcriptionally active. Yet, expansion to more than 90 repeats leads to the formation of one large CGI exclusively in patients, and to local acquirement of abnormal CpG methylation upstream^[11]. Hypermethylation is coupled with the local gain of repressive histone modifications, and is responsible for the inactivation of variant 2 of *C9orf72* that, unlike repeat-containing variants 1 and 3, initiates transcription from exon 1b and harbors the G₄C₂ sequence in its promoter^[26]. Overall, C9/ALS-FTD iPS cells demonstrate altered gene expression. While some reports demonstrate general reduction in the transcription of all variants, others show differential expression favoring variants 1 and 3 over variant 2^[26,52,81]. All together, this suggests that hypermethylation in fact exist in these cells. Nevertheless, the contribution of epigenetic gene silencing to disease pathogenesis is not fully clear. There is conflicting evidence regarding the correlation between hypermethylation and type of pathology (ALS vs FTD), age of onset or severity^[11,82,83]. Experimental evidence in animal models demonstrates inconsistent results^[84,85]. On the other hand, hypermethylation was suggested to have a protective role from the accumulation of pathogenic RNA/protein foci contributed by repeat-containing mRNA variants 1 and 3^[86]. These conflicting studies warrant further investigation regarding the contribution of DNA methylation to ALS-FTD pathogenesis, and may be

considerably advanced by the use of *in vitro* derived neurons from C9/ALS-FTD pluripotent cells.

Another elegant example for the power of pluripotent stem cells in resolving the contribution of epigenetics to disease pathogenesis is in FSHD. FSHD is caused by a contraction of a macrosatellite repeat (D4Z4 repeat, 3.3 kb unit) located in the sub-telomeres of chromosome 4q35. Non-affected individuals carry 11-100 repeat units, while FSHD patients have less than 10 repeats, but must have at least one unit to manifest the disease^[87-89]. Interestingly, the loss of DNA methylation and reduced H3K9me3 that coincide with repeat contraction have been found to be causally related to the up-regulation of a full transcript from the *DUX4* gene, a transcript which is exclusive to the most distal (telomeric) unit of the D4Z4 repeat containing region^[66,80-93]. Expression of the full length *DUX4* transcript, which is exclusive to FSHD myotubes, is responsible for the induction of nuclear foci, impaired myogenesis and ultimately to cell death by apoptosis^[94]. Immediately downstream to the most-telomeric unit there is a polymorphic site that acts as an alternative polyadenylation signal that controls the length and stability of the *DUX4* transcript. While normal alleles produce short transcripts and truncated proteins, contracted FSHD alleles utilize a cryptic splice donor to form a full length toxic protein. As alternative polyadenylation seemed to be a tissue-specific process, it was speculated to be a developmentally regulated event that is mediated by the loss of repressive epigenetic modifications due to D4Z4 repeat contraction. By comparing WT and FSHD iPS cell lines, Snider and colleagues^[94] found that both cell types equally express full length *DUX4*, independent of repeat number. However, upon differentiation, full length expression was switched off and converted back to short transcripts in WT controls while full length expression persisted in the diseased cells. Importantly, the researchers were able to correlate the switch in splice site usage with the enhancement of repressive chromatin modifications (H3K9me3) in the WT differentiated cells. Thus, it appears that H3K9me3 modification (and most likely CpG methylation) mediates the splicing for the truncated protein upon differentiation, and that the contraction of the D4Z4 repeats abolishes this developmentally regulated process. It remains to be determined how D4Z4 contraction interferes with the normal induction of heterochromatin. Nevertheless, the use of FSHD iPS in this particular study, neatly illustrates the power of mutant pluripotent stem cells in resolving the molecular mechanisms contributing to disease pathogenesis.

RAN TRANSLATION

During the past years it has become apparent that a third potential mechanism may act in the context of pathogenic repeat expansions. A novel form of

translation was found by Ranum's group, which results from translation in the absence of an initiating AUG codon next to disease causing unstable microsatellite repeats^[95]. They found that the CAG repeat tract at the DMPK (DM1) and ATXN8 (SCA8) loci undergo noncanonical translation in disease relevant tissues of patients, resulting in the production of homopolymeric proteins in all three frames (CAG, AGC, and GCA). This newly discovered phenomenon was termed RAN translation, abbreviated for Repeat Associated Non-ATG translation.

To date, RAN translation has been shown to occur across four different types of repeat motifs: CAG, CTG, CGG and GGGGCC, in a growing number of conditions, including DM1, SCA8^[95], FXTAS^[96] and C9/ALS-FTD^[39,40,50,97,98]. Among its effects, RAN translation leads to ubiquitin-positive intracellular protein inclusions, which may be toxic to cells^[96]. Furthermore, researchers have shown that when repeats are bi-directionally transcribed to form sense and anti-sense transcripts, six potential homopolymeric proteins can be produced^[48] as each transcript can be RAN translated in three different reading frames. Importantly, they provided evidence that long repeat tracts can express multiple RAN-translated products, simultaneously, in a single cell and suggested that RAN translation may contribute to disease pathogenesis by a protein gain-of-function mechanism^[95]. In addition, as longer expansions are more liable to the accumulation of RAN translated products, it was speculated to be involved with disease anticipation^[99]. Nonetheless, evidence for a role of RAN translation, if any, in disease pathogenesis still awaits further exploration.

Thus far, RAN translation has been documented in pluripotent stem cells only for C9/ALS-FTD. Indeed, postmitotic neurons derived from C9/ALS-FTD iPS cells have already been established, and were shown to present disease-relevant phenotypes that include RAN translation products in the form of cytoplasmic TDP-43 negative/p62 positive protein inclusions^[51]. Immunofluorescent staining of the C9/ALS-FTD iPSNs (induced pluripotent stem cells derived neurons) revealed cytoplasmic poly-(Gly-Pro) inclusions similar to those observed in C9orf72 expansion carriers postmortem affected brains^[53]. In addition, as the appearance of RAN peptide foci was coupled with increased sensitivity to cellular stress induced by autophagy inhibitors^[51,53], it raised the possibility that this mechanism may contribute (directly or indirectly) to impaired autophagy function in ALS-FTD and possibly additional conditions associated with RAN translation^[53].

There is by now enough evidence to indicate that mutant differentiated iPS cells can authentically recapitulate the late onset and tissue-specific cellular phenotypes that are typically seen in human C9orf72 postmortem brain samples. It should be interesting to explore whether similar cellular phenotypes can be reproduced in iPS cells derived from younger, yet

asymptomatic, C9 mutation carriers. In addition, it would be useful to establish additional C9/ALS-FTD iPS cells from patients who carry small, intermediate and large expansions to correlate inclusions with repeat tract size, C9orf72 transcription and disease severity. Such associations are not yet fully understood. Nevertheless, it should be kept in mind that mitigation of RNA foci and RBP aggregation by antisense oligonucleotide (ASO) treatment in affected iPSNs had no effect on the formation of RAN-translation inclusions^[53]. This would suggest that the detected homopolymeric inclusions may not have a major role in disease pathology. However, as only one type of di-peptide repeat was monitored [poly-(Gly-Pro)], it may be possible that in fact the other RAN peptides (such as the ones that are produced by antisense transcription) are crucial for the manifestation of the abovementioned cellular phenotypes. In any case, even if the RAN-translated products observed in iPS derived neurons will be proved to be irrelevant to disease pathology, they may be useful as a biomarker, providing an opportunity to develop a platform to test therapeutic agents for their effect on brain features of the disease. Compounds that will reverse the formation of the inclusions in the diseased cells are expected to offer a starting point for therapeutic development.

Many questions regarding RAN translation arise. For example, what is the clinical significance of RNA translation? Does RAN translation occur across all unstable microsatellite disorders? Is it age dependent? When and what determines its tissue specificity? In addition, it is still not yet clear which RNA structures and flanking sequences are required to facilitate RAN translation? Intrastrand RNA repeat associated hairpins and G-quadruplex structures have been suggested as candidates for RAN translation involvement^[95,99-101]. When more stem cell based disease models (HESC and/or iPS) will become available, it is anticipated that human pluripotent stem cells will be important in clarifying at least some of these fundamental questions.

REPEAT INSTABILITY

Repeat instability is a form of dynamic mutation that results from a change in the number of repeat units when ordered in tandem. Unlike static mutations, which involve permanent change in the DNA, repeat instabilities change across generations, and may vary between and within tissues of affected individuals (for comprehensive reviews see^[102,103]). We refer to germ-line instability when the change in repeat number occurs during transmission from parent to offspring, and somatic instability when the change takes place in the somatic cells of the patient. Germ-line instability, which is frequently biased towards maternal or paternal transmissions, is typically associated with anticipation (reviewed in^[103]). This is because the longer the repeat tract the more likely it will undergo expansion

in the next generation. As a result, disease symptoms worsen from one generation to the next. However, in some pathologies repeat instability extends beyond the germ-line, leading to a high degree of variation in repeat tract length in affected individuals^[14,104,105]. Unlike in germ-line repeat instability, the contribution of somatic instability to disease pathogenesis is not at all times clear. In some conditions there is an obvious correlation between expansion size and cell dysfunction^[106,107], whereas in others this association is not noticeable^[108,109]. Nevertheless, it is imperative to understand the molecular details of this mutation-associated phenomenon, considering that in some cases expansion size is clinically significant and may be used as a prognostic marker^[110].

It is important to note that not all expansions are alike in range and location. While expansions residing in coding regions are usually restricted in length (tens of copies) and limited to germ-line mutations, noncoding expansions are frequently large (hundreds-thousands of copies) and typically coincide with somatic instability. Furthermore, in some disorders somatic instability can be restricted to early embryonic development or to highly replicating cells, whereas in others it may be tissue-specific or age-dependent, and may be limited to particular post-mitotic cell types. Therefore, while the type of mutation is common to all of these heritable and devastating conditions, the marked variation in the pattern and distribution of expansion size among them may stem from assorted mechanisms.

It is generally accepted that repeat instability results from local disruption of DNA replication, repair or recombination (for comprehensive review see^[111]). Regardless of the mechanism, all proposed models for instability are based on the formation of unusual structures in the DNA (like hairpins and G-quadruplexes) that are eventually resolved by the addition (expansions) or deletion (contractions) of repetitive units^[112-120]. It remains to be determined when and how these changes occur. In that sense, expansion carrying pluripotent stem cells provide an exceptional opportunity to gain more insights on the mechanisms that are involved with repeat instability. This is because they recapitulate early molecular events as they happen *in vivo*. They can be induced to differentiate into a wide range of cell types, allowing the monitoring of repeat instability in diseased relevant cells that are otherwise inaccessible for research like heart muscle cells or post-mitotic neurons. In addition, there is no evidence for further repeat amplification beyond the range observed in somatic cell of patients. Thus far, only a limited number of reports dealing with repeat instability in pluripotent cells have been published. All are descriptive in nature. For example, by following CTG expansion size at the *DMPK* gene of DM1-affected HESC lines, it was possible to demonstrate a change in repeat tract heterogeneity along with time in culture^[121,122].

Furthermore, by inducing the cells to differentiate *in vitro*, instability was lost^[122], apparently contradicting observations *in vivo*^[123,124]. Nevertheless, it should be emphasized that tract-length heterogeneity within cell lines does not necessarily indicate that instability in fact exists, but rather proves that instability has occurred sometime before or during cell line growth in culture. This equally holds for CGG expansions at the *FMR1* locus, where repeat length heterogeneity is clearly observed in a large cohort of FXS HESC lines^[17,69]. Interestingly, in this case variations seem to be inversely correlated with CpG hypermethylation, a cis element that was formerly identified to constrain instability in FXS-affected fibroblasts^[125]. Indeed, CGG somatic instability has been shown to be largely restricted to early fetal development and to cease with epigenetic gene silencing of *FMR1*^[15]. In addition, as these cell lines are widely heterogeneous for expansion size and *FMR1* hypermethylation, it may indicate that differentiation by itself is irrelevant to the repression of repeat instability in FXS, as formerly suggested^[17]. It will be essential to generate single cell lineages from those FXS HESCs and monitor them for CGG repeat instability during growth *in vitro*, to unambiguously show that instability is, in fact, active in these cell cultures. Alternatively, it may be particularly useful to use patient iPS cells for these types of studies, considering the clonal origin of iPS cells. Two relevant examples for this application are C9/ALS-FTD iPS cells, where G₄C₂ tract size heterogeneity is evident both in undifferentiated and in *in vitro*-derived neurons^[51], and the GAA expansion at the frataxin gene in FRDA^[126,127]. On the other hand, the iPS based complimentary system should be cautiously tested before being adopted, as reprogramming may incorrectly enhance instability at repetitive loci as reported in FRDA iPS cells^[128].

Apart from monitoring repeat instability along with time or as a consequence of reprogramming/differentiation in culture, diseased pluripotent stem cells (HESCs or iPS cells) may provide an unusual opportunity to gain new insights into the mechanisms underlying this mutational event. For example, it was shown that when the mismatch repair proteins, including MSH2 and MSH6, are downregulated upon differentiation repeat instability is dramatically enhanced in DM1 (CTG), HD (CAG)^[122] and FRDA (GAA)^[126,128] pluripotent stem cells, suggesting that repeat instability is assigned to difficulties in DNA repair at these particular loci. This is in contrast to CGG somatic instability in FXS, where perturbations in DNA replication are assumed to be the underlying mechanism. In line with this perception is the study of Gerhardt and colleagues^[129], who employed the DNA combing methodology to associate CGG expansion with alterations in origin of replication (ORI) usage and rate of progression in undifferentiated FXS HESCs. Using a pair of FXS HESC lines, these researchers reported a difference in ORI usage ex-

clusive to undifferentiated FXS cells, proposing a switch in replication direction relative to the CGG repeats. According to their study, unlike in WT HESCs, replication proceeds predominantly from a downstream ORI in FXS HESCs, resulted in the replication of the CGGs by lagging strand synthesis (rather than by the leading strand) which favors expansions to occur. It remains to be determined whether the change in ORI usage in FXS HESCs is a cause or effect of the expansion, and whether it is mechanistically related to CGG somatic instability. The availability of a large cohort of FXS HESC lines that are diverse in their epigenetic state and CGG instability should help to address the aforementioned questions.

Lastly, it should be mentioned that while all of the aforementioned studies relate to the issue of somatic instability using mutant pluripotent stem cells, it should be most attractive to utilize the same systems in order to recapitulate germ-line instability and better understand why such disease associated repetitive sequences become increasingly unstable during germ-line transmission. This can be accomplished once efficient protocols for inducing HESC/iPS to differentiate into primordial germ cells and fully matured gametes will be available. Indeed, differentiation of ES cells into fully matured oocytes^[130] and the generation of viable offspring from these cells^[131] have already been accomplished in mice, paving the way for such studies to be carried out in human cells in the near future.

CONCLUDING REMARKS

HESCs are undifferentiated pluripotent cell lines with the potential for unrestricted self-renewal as well as the ability to differentiate into a wide range of cell types^[132-134]. They are established from *in vitro* fertilization (IVF)-derived embryos, and can be obtained from genetically affected embryos following PGD procedures. The derivation of HESCs directly from diseased embryos provides a powerful tool for disease modeling^[135,136] by affording the exploration of disease relevant tissues^[137,138] and/or developmental stages^[139] that are otherwise inaccessible for research. The main limitation of this approach is that it relies on the availability of genetically affected embryos, a valuable resource that is accessible to only a small number of stem cell deriving centers worldwide. In such cases, a complement model system may be employed which relies on the derivation of patient-derived iPS cells; ES-like cells that are established by somatic cell reprogramming of patients' cells in culture^[140]. The primary advantage of this alternative system is that it facilitates the establishment of pluripotent cell lines that share many features with HESCs and harbor the disease causing mutation/s without the need for affected embryos. As such, and although artificially induced, it has become the most common alternative for modeling human diseases in culture by many researchers^[16,141-146]. Nevertheless, iPS cells can be

useful only if the disease is not embryonic lethal, and if complete erasure of the epigenetic modifications that are normally gained with cell differentiation do not interfere with investigation of disease pathogenesis. It should be mentioned that both HESC and iPS cells have been reported to carry typical chromosomal aberrations resulting from culture adaptation^[147,148]. For this reason it is very important to perform routine characterization for chromosomal abnormalities in HESC and iPS cells lines.

The main benefits of both mutant HESC- and iPS-based model systems include the fact that they: (1) are human derived; (2) naturally carry disease causing mutations; (3) have the potential to differentiate into a wide range of cell types; (4) can self-renew without limitation while maintaining their undifferentiated and pluripotent potential; and (5) they mimic, to some extent, early embryo developmental stages as they spontaneously differentiate *in vitro* and *in vivo*. For all these reasons, they have become a commonly used model system for studying various disease associated mechanisms, particularly in cases where animal models do not authentically reproduce the affected human phenotype, or when the disease relevant cell types are unavailable for research.

In the context of disease causing unstable noncoding repeat expansions, which account for approximately half a dozen heritable conditions, the availability of mutant pluripotent stem cells is of particular importance. This is because most of these diseases are neuron-associated, restricting human-based research to a limited number of postmortem brain samples or to the use of disease irrelevant cell types. In addition, since noncoding expansions are hard to clone, it has been extremely difficult to artificially produce them in culture or to generate appropriate animal models^[149-153]. In general, pluripotent stem cells seem to reproduce many of the pathological features that are observed in noncoding repeat expansion disorder patient cells^[52,53,127,154-157]. Some of these features include nuclear RNA-protein foci, di-peptide cytoplasmic inclusions and hypermethylation. Therefore, stem cells are useful for basic research studies related to the different mechanisms that are involved with this group of disorders. By enlarging the number and repeat expansion heterogeneity of mutant stem cells cohort, we may gain new insights related to the underlying mechanisms that are associated with these pathologies early during embryo development. Once efficient differentiation protocols will be established, it will also be possible to generate large amounts of cells from affected tissues in culture. Studying cell types that are particularly vulnerable in these diseases will improve our understanding regarding the tissue specificity seen in patients. Moreover, such cells can be utilized for drug screening and development studies. Stem cells provide a convenient platform to genetically intervene with the molecular defect that is inherently found in the cells. Considering the recent advancements in the

Table 1 Modeling unstable noncoding repeat expansions repeat pathologies using pluripotent stem cells

Disease	Molecular mechanism	Ref.
DM1	Toxic RNA gain-of-function	[45,46]
C9/ALS-FTD		[47-49,51-53]
FXS	Reaped-mediated epigenetic modifications	[17,62,69,71-76,78]
C9/ALS-FTD		[52]
FSHD		[92]
C9/ALS-FTD	RAN translation	[51,53]
FXS	Repeat instability	[17,69,127]
C9/ALS-FTD		[51]
FDRA		[124-126]
DM1		[119,120]

DM1: Myotonic dystrophy 1; C9/ALS-FTD: C9 related amyotrophic lateral sclerosis and/or frontotemporal dementia; FXS: Fragile X-syndrome; FSHD: Facioscapulohumeral muscular dystrophy; FDRA: Friedreich ataxia.

field of artificially engineered nucleases (particularly the CRISPR/Cas9 system), it may be possible in the near future to insert, replace or remove repetitive DNA sequences from the genome of HESCs/iPS cells in a fairly uncomplicated procedure. This may hold a great promise for the development of new therapeutic approaches and hopefully will allow the ease, or even cure, pathologies resulting from unstable repeat expansions.

At present, mutant pluripotent stem cell lines are available for FXS, FXTAS, DM1, FRDA, C9/ALS-FTD, FSHD, and FRDA (Table 1). Most of these cell lines have been utilized for generating disease relevant cell types in order to validate the feasibility of the stem cell model system. In a few cases, where disease pathology is developmentally regulated, they have been particularly informative. For example, in FSHD stem cell lines facilitated the discovery of alternative polyadenylation signals responsible for the generation of toxic protein in a differentiation-dependent fashion^[94]. In the case of FXS, an mRNA-mediated mechanism that drives epigenetic *FMR1* gene silencing in affected neurons was uncovered in stem cells as well^[73]. In other cases, particular cellular phenotypes in differentiated stem cell-derived cultures have been monitored by effect of therapeutic agents or by defining proteins that are associated with pathological protein inclusions, as reported in C9/ALS iPS-derived neuronal cells^[52,53].

As a note of caution, it should be understood that the development of efficient stem cell differentiation protocols is not that straightforward. In particular, it can be quite challenging to use stem cells for the derivation of homogeneous cell populations that are functionally impaired. Moreover, molecular phenotypes in mutant pluripotent cells must be validated to see if they reproduce molecular events that are observed in diseased individuals. For example, despite all the benefits of iPS cells, investigators are becoming increasingly more aware of epigenetic instability in these cell types^[158-161]

as many iPSs wrongly acquire hypermethylation as a consequence of reprogramming^[78,162-164]. Accordingly, a comparison between mutant HESCs and patient-derived iPS cells will be essential in future investigations of the epigenetic aspects of repeat expansion diseases especially because many mechanistic questions still remain to be addressed in this area. Unanswered questions in the field include what molecular events lead to hypermethylation of repeat expansions? What pathogenic role do nuclear riboprotein or cytoplasmic di-peptide inclusions have, and what are the proteins that are sequestered into these structures? What are the mechanisms that are responsible for germ-line and somatic repeat instability, and why does somatic instability differ according to patient age and by tissue-specificity among differing genomic loci? Given proper experimental conditions, the answers to these riveting questions may be waiting to be uncovered by stem cell-based model systems.

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Human dental pulp stem cells: Applications in future regenerative medicine

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Abstract

Stem cells are pluripotent cells, having a property of differentiating into various types of cells of human body. Several studies have developed mesenchymal stem cells (MSCs) from various human tissues,

peripheral blood and body fluids. These cells are then characterized by cellular and molecular markers to understand their specific phenotypes. Dental pulp stem cells (DPSCs) are having a MSCs phenotype and they are differentiated into neuron, cardiomyocytes, chondrocytes, osteoblasts, liver cells and β cells of islet of pancreas. Thus, DPSCs have shown great potentiality to use in regenerative medicine for treatment of various human diseases including dental related problems. These cells can also be developed into induced pluripotent stem cells by incorporation of pluripotency markers and use for regenerative therapies of various diseases. The DPSCs are derived from various dental tissues such as human exfoliated deciduous teeth, apical papilla, periodontal ligament and dental follicle tissue. This review will overview the information about isolation, cellular and molecular characterization and differentiation of DPSCs into various types of human cells and thus these cells have important applications in regenerative therapies for various diseases. This review will be most useful for postgraduate dental students as well as scientists working in the field of oral pathology and oral medicine.

Key words: Human dental pulp stem cells; Mesenchymal stem cells; Dentin; Pluripotency; Stem cell therapy; Molecular markers

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Core tip: Human dental pulp stem cells (DPSCs) have shown a potentiality for the treatment of various human diseases including dental related problems. The review will overview the information about DPSCs, their isolation, cellular and molecular characterization, differentiation into various types of cells and their applications in regenerative therapies for various diseases. This review will be most useful for postgraduate dental students as well as the scientists working in the field of oral pathology, oral medicine and regenerative medicine.

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INTRODUCTION

Stem cells are unspecialized cells having a property of self renewal and further differentiate into various types of specialized cells^[1]. Stem Cells are identified in a number of adult tissues including skin, adipose tissues^[2], peripheral blood^[3,4], bone marrow, pancreas, intestine, brain, hair follicles, as well as in the dental pulp cells^[5-7]. Stem cell research has expanded well due to their usefulness in regenerative therapies for improving the life of patients suffering from various genetical and neurological diseases. Studies have shown that the dental pulp tissue can also be used to derive mesenchymal stem cells (MSCs) when tissue is grown in culture^[7]. These MSCs can differentiate into several cell types as shown in Table 1. In year 2012, Shinya Yamanaka and John Gurdon have won Noble prize award for their excellent work on induced pluripotent stem cells (iPSCs) derived from adult somatic cells. This work has resulted into development of innovated technology to make an iPSCs from individual patient who needs treatment for specific disease. It is proposed that dental pulp stem cells (DPSCs) can develop iPSCs which can be used for therapies of various diseases^[8]. This review is mainly highlighting the importance of DPSCs, their isolation, characterization by cellular and molecular markers, differentiation and their applications in treatment of various diseases.

MSCs

MSCs are derived from any part of human body tissues or cells. They have a property of self-renew and differentiation into specific functional cell types as described before^[9]. MSCs are useful cells due to their therapeutic potentiality^[9,10]. Even though they are not immortal, they grow very well in culture and maintain their pluripotency property. MSCs are identified by their positive expression of *CD105* (SH2) and *CD13* (SH3/4) and *CD73* genes and are negative for the hematopoietic markers such as CD34 and CD45. Animal experimentation has shown that MSCs has migratory property and can reach to the site of injury. Light or phase contrast microscopy studies have shown that MSCs are elongated cells and look like fibroblast cells as shown in Figure 1. After subculture, MSCs exhibit a high expansion potential without loosing their normal karyotype and telomerase activity^[10-12].

As MSCs are derived from adult tissues, there is no

ethical concern required to use these cells for human therapies. Similarly, they have low immunogenicity and therefore they are promising candidates for regenerative therapies. *In vitro* differentiation of MSCs is mainly dependent on presence of growth factors and cytokines in growth medium. Bone marrow (BM) has been considered as the main source of MSCs, however, collection of BM from patient is a painful and invasive procedure. Therefore, there is a need to find out other sources of tissues which are non invasive and can give similar types of MSCs. Several investigators are presently testing adipose tissue derived stem cells and DPSCs for this purpose. Now it is already established that both these cells have same renewal and differentiating properties of BM cells and thus, these cells can be used for future regenerative therapies of various diseases^[12].

DPSCs

Dental Pulp tissue is extracted from the teeth recovered during routine dental procedure throughout the life and these teeth are the most convenient and valuable source of DPSCs which are well characterized as a MSCs as shown in Figure 1.

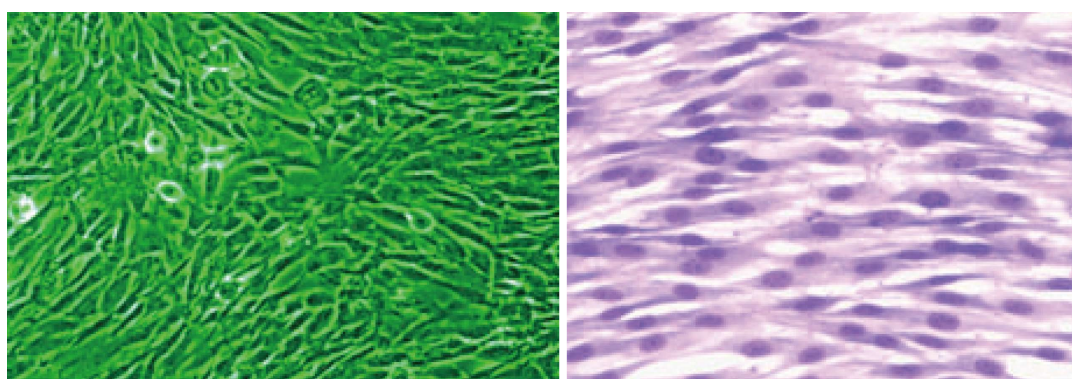
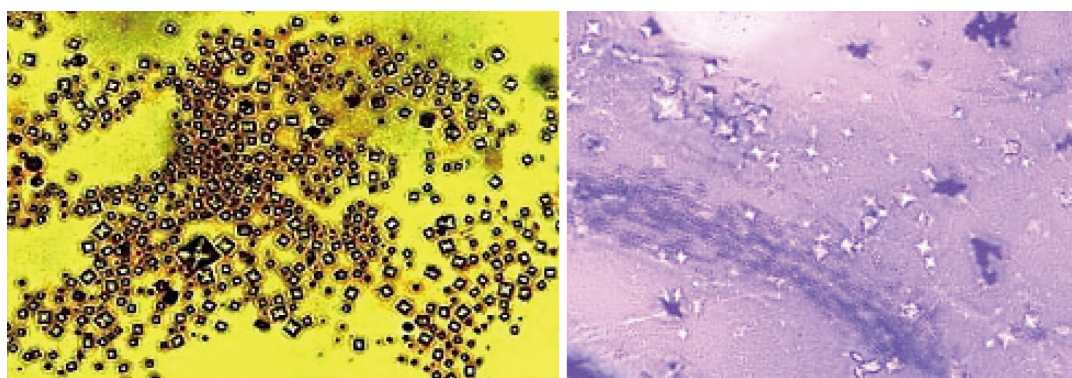
It is a non invasive process of extraction of MSCs from dental pulp tissue. DPSCs can be cryopreserved and revived whenever; they are needed for future regenerative therapies^[13]. Some of the diseases which are being cured by DPSCs include type 1 diabetes, neurological diseases, Immunodeficiency diseases and diseases of bone and cartilages^[14-16].

During the development of teeth, there is an interaction between epithelial cells of dental pulp which lead to the differentiation of ameloblasts and odontoblasts, resulting into deposition of specialized mineralized matrices, *i.e.*, enamel and dentin respectively^[17]. The inner area of dental pulp chamber contains a highly proliferative stem/progenitor cells possessing a self-renewal and differentiation properties^[17]. It has been shown that after teeth eruption, there is an induction of reparative dentin formation which protects dental pulp from further degradation^[17]. MSCs constitutes a heterogeneous population of cells which are found first in BM and later in multiple tissues like adipose tissue^[2,12], skin^[18], cartilage, umbilical cord^[19], placenta^[20,21], and now in dental pulp^[13,16]. As DPSCs have comparable therapeutic potential similar to BMMSCs, DPSCs is another alternative noninvasive source to be used for future regenerative therapies^[22].

DPSCs or stem cells from human exfoliated deciduous teeth (SHED) cells require a longer time for initial colony formation than other somatic cells^[23]. Third molar teeth derived cells differentiate into odontoblasts and secrete 3 D like crystal structure *in vitro*. Figure 2 shows calcium phosphate crystals secreted from DPSCs cells in culture experiment from my laboratory.

Table 1 Normal differentiation pathways of adult stem cells from various tissues and cells

Stem cell	Source	Types of cells produced
Hematopoietic	All types of blood cells	Red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, macrophages and platelets
Bone marrow Stromal cells (mesenchymal)	Connective tissues	Tendons, osteocytes (bone cells), adipocytes (fat cells), chondrocytes (cartilage cells)
Neural	Parts of the nervous system	Neurons, astrocytes and oligodendrocytes
Epithelial	Lining of the digestive tract	Absorptive cells, goblet cells, paneth cells and endocrine cells
Epidermal	Basal layer of epidermis	Keratinocytes and dermal cells
Follicular	Base of hair follicles	Hair follicles and epidermis
Hepatic	Liver	Hepatocyte cells

**Figure 1** Phase contrast and Giemsa stained picture of Dental pulp stem cells growing in monolayer culture (Dr. Potdar's, laboratory).**Figure 2** Silver and Giemsa stained Calcium Phosphate crystals secreted by Dental pulp stem cells in culture (Dr. Potdar's Laboratory).

It has been shown that DPSCs can be differentiated by modulation with growth factors, transcriptional factors, extracellular matrix proteins and receptor molecules into different cell types include odontoblast, osteoblast, chondrocyte, cardiomyocytes, neuron cells, adipocyte, corneal epithelial cell, melanoma cell and insulin secreting Beta cells^[24].

DPSCs usually remain quiescent when they are within the dental pulps, but respond quickly after injury. These DPSCs have a high proliferative capacity and immediately differentiate into odontoblasts, osteoblasts, and chondrocytes to produce dentin, bone, and cartilage tissues respectively for this repair process. It has been shown that the potentiality of

DPSCs differentiation into odontoblasts reduced after passage 9 and they can only differentiate into osteoblast cells^[24,25]. DPSCs are mostly derived from cranial neural crest cells because they expressed GFAP, HNK-1, Nestin, P75 and S-100 which are neural crest-stem cell markers^[26]. Our recent studies on DPSCs have shown the differentiation of DPSCs into neuron like cells, cardiomyocytes, adipocytes and insulin secreting beta cells as shown in Figures 3 and 4.

Almushayt *et al.*^[26] have confirmed the expression of odontoblast specific markers and presence of collagenous matrix and calcified deposits in DPSCs to show that these cells differentiate into odontoblast and form dentin material. Mokry *et al.*^[27] have

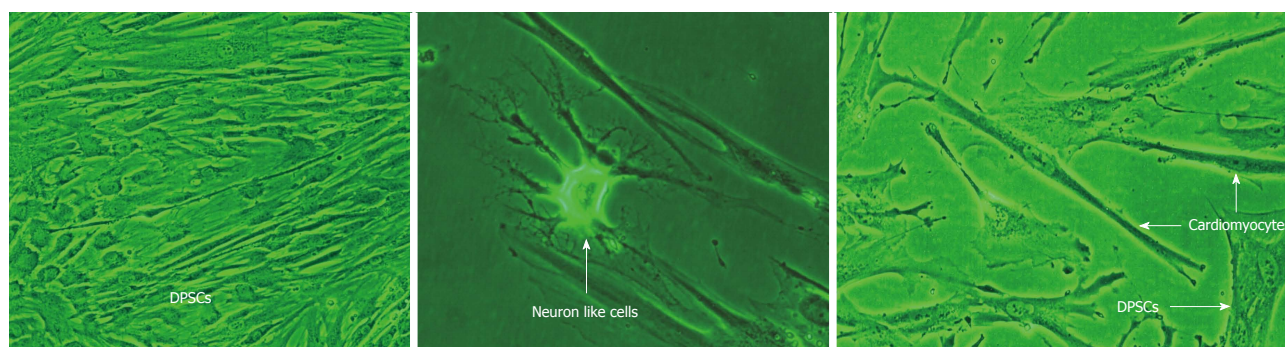


Figure 3 Differentiation of Dental pulp stem cells into neuron like cells and cardiomyocytes (Dr. Potdar's Laboratory). DPSCs: Dental pulp stem cells.

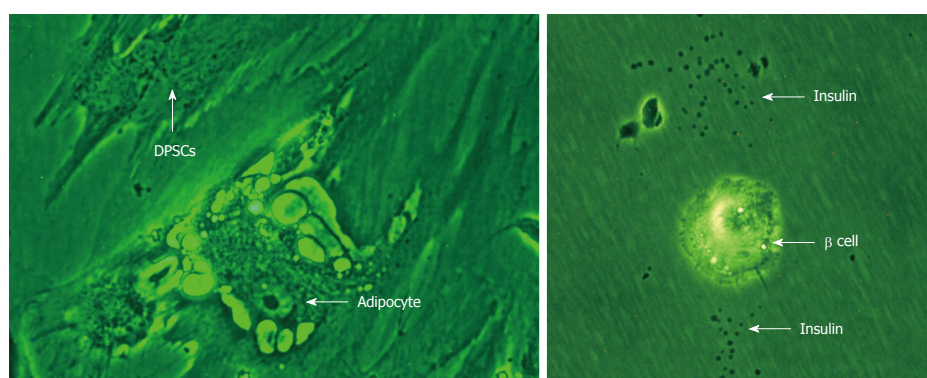


Figure 4 Differentiation of Dental pulp stem cells into adipocytes and insulin secreting beta cells. DPSCs: Dental pulp stem cells.

shown that due to telomere attrition, human DPSCs have shown extensive cell proliferation *in vitro* but progressive shortening of telomere length diminishes transplantation capacity of DPSCs and therefore these cells are less suitable for therapeutic applications. They have further proposed that the determination of telomere length and age of these stem cells are as important biomarkers to be assessed before utilization of these cells for regenerative therapies^[27]. Smith *et al.*^[28] have further shown that molecular signaling is responsible for the differentiation of DPSCs into odontoblast-like cells for reparative dentinogenesis. Therefore, determination of the molecules, responsible for signaling their DPSCs differentiation, may provide a powerful tool for the clinicians to evaluate a regenerative response of these cells appropriately for their application in regenerative therapies^[28]. d' Aquino *et al.*^[29] have shown that human DPSCs can co-differentiate into osteoblasts and endotheliocytes and form adult bone tissue after transplantation *in vivo*. This suggests an interesting area for development of three-dimensional tissue scaffold for reconstructing bone tissue engineering using DPSCs in dental disorder^[29]. Further it has shown that DPSCs and SHEDs are differentiated into neuron like cells and also shown expression of Nestin as a confirmation of this differentiation. These cells also have constitutive expression of Nestin^[30]. Thus, DPSCs have high potential to serve as a good resource for treatment in

neurodegenerative diseases^[31].

Human DPSCs which are grown by explant culture method have better proliferative capacity and also differentiate into various cell types *in vitro*^[32]. Recent study by Paino *et al.*^[33] have shown that DPSCs spontaneously differentiate *in vitro* toward the melanocytic lineage too. However, SHEDs differ from DPSCs with respect to their growth rate and their property to differentiate *in vivo*^[34]. Similarly, DPSCs have shown the greatest potential to produce a high volume of mineralized matrix suggesting that these cells also show promise for use in regenerative dental therapies^[35].

Stem cells from human exfoliated deciduous teeth

Stem cells can be isolated from the pulp of human exfoliated deciduous teeth. These cells induce bone formation and differentiate into other non-dental mesenchymal cells *in vitro*. SHED have higher proliferation rates, form a sphere-like clusters and differentiate into osteoblasts but they are not able to regenerate complete dentin and pulp-like complexes *in vivo*. These cells can repair calvarial defects in mice due to their ability to differentiate into osteoblasts. SHED secretes neurotrophic factor for repair of motor neurons following dental injury and therefore it has proposed that SHED can be useful for the treatment of neurodegenerative diseases^[23]. Jeon *et al.*^[36] have shown differences in *in vitro* and *in vivo* characteristics

of SHED isolated *via* enzymatic disaggregation (SHED) and outgrowth (o-SHED) by primary culture method. SHEDs have stemness characteristics, but o-SHEDs are mainly suitable for bone tissue regeneration therapy.

Stem cells from apical papilla

Stem cells from apical papilla (SCAP) are the cells which are found at the tooth root apex. They have higher proliferation rates as well as have a differentiation property *in vitro* similar to DPSCs. They are capable of differentiating into odontoblast cells and produce dentin *in vivo*^[23]. Due to their higher proliferative potential, SCAPs are also suitable for cell-based therapy for formation of apex roots. White Mineral Trioxide Aggregate (WMTA) is a capping material has a property to differentiate SCAPs into osteoblast cells and thus SCAPs are involved in this regenerative process. WMTA significantly increase the proliferation of SCAPs within 1-5 d whereas, calcium-enriched medium take 7 d to proliferate these cells. SCAPs migrate and proliferate steadily in the presence of 2% and 10% FBS. Thus, this data suggests that WMTA induces early migration and proliferation of SCAPs as compared with late induction by calcium chloride or fetal bovine serum (FBS)^[37].

Periodontal ligament stem cells

Human periodontal ligament stem cells (PDLSCs) can differentiate into cementoblast-like cells. They also have a capacity to form connective tissue which is riched in collagen I fiber. Human PDLSCs when seeded on 3D scaffolds such as fibrin sponge, generate bone *in vivo* and retain stem cell properties and tissue regeneration capacity^[23]. It has further been shown that Proteasome inhibitor, Bortezomib has a property of inducing differentiation of PDLSCs into osteoblasts. The main mechanism involved behind this is the mineralization of PDLSCs cells by accumulation of β catenin and significant expression of *BMP2* gene. Thus, it is clear that the Bortezomib plays important role in periodontal regenerative therapy^[38].

Dental follicle precursor cells

Dental follicle precursor cells (DFPCs) are derived from dental follicle tissue which is a loose connective tissue that surrounds the developing tooth. These cells have an ability to produce bone and cementum. Therefore, these stem cells have great potentiality to use in periodontal and bone regeneration therapies. DFPCs derived from human third molars teeth are rapidly attached to the culture plates and form a calcified nodules. They also exhibits a better plasticity than other dental stem cells^[39]. Dental pulp and dental follicle stem cells have similar mesenchymal stem cell characteristics, but DFPCs are easily accessible for cell culture and have a higher proliferation capacity

than DPSCs. Therefore it appears that DFPCs might have more advantages as a stem cell resource for regenerative therapies in dental abnormalities^[40].

ISOLATION OF DPSCs

Several investigators have described various methods for isolation of stem cells from human dental pulp. Raoof *et al*^[41] have used three different methods for isolation of DPSCs from dental pulp tissue: (1) Dental pulp tissue is digested with collagenase or dispase enzyme and isolated trypsinised cells are plated in culture dishes; (2) They have explanted undigested dental pulp small tissue pieces directly to petridishes; and (3) Dental pulp tissues are initially trypsinised and then small tissue pieces are explanted to petridishes for their outgrowth. They have grown these cultures in Minimum Essential Medium (MEM) supplemented with 20% FBS at 37 °C with 5% CO₂ and 90% humidity in CO₂ incubator. They have recommended the third method for isolation of DPSCs from dental pulp. This method gives better cell outgrowth with achieving confluency at 60% within 2 d of culture. They have further checked the pluripotency of these cells by studying the expression of Nanog, OCT-4, and Nucleotoxin markers by RT/PCR analysis. Thus, this study proposes the third method for obtaining better DPSCs with high efficacy in a short time^[41].

Lindemann *et al*^[42] have evaluated the effect of cryopreservation on DPSCs characteristics. They have isolated dental pulp cells from 7-d old non-cryopreserved and cryopreserved human deciduous teeth and culture them simultaneously. They found that there is no change in differentiating and immunophenotype properties of both these cells. There is a change in the morphology, proliferative capacity of cryopreserved cells than non- cryopreserved cells^[42].

Successful and efficient cryopreservation of living cells and organs is a key clinical application of regenerative medicine. Recently, Lin *et al*^[43] 2014 have reported magnetic cryopreservation for intact tooth banking and dental tissue. Human DPSCs isolated from extracted teeth are frozen and then stored at -196 °C for 24 h. During freezing, the cells are suspended in freezing media containing 10% DMSO. The results have shown that when the freezing medium is DMSO-free, the survival rates of revived DPSCs increase by 2 to 2.5-folds^[43]. Gioventù *et al*^[44] have developed a new method of cryopreservation of whole dental pulp by using laser beam. They have studied 4 human deciduous whole teeth, cryopreserved by making micro-channels into the tooth with the help of laser beam and then preserve these cells at -80 °C. This method saves a time in isolating DPSCs before cryopreservation and thus reduces the initial costs and workload of tooth banking. The DPSCs cells isolated by this method have shown normal morphology, cell

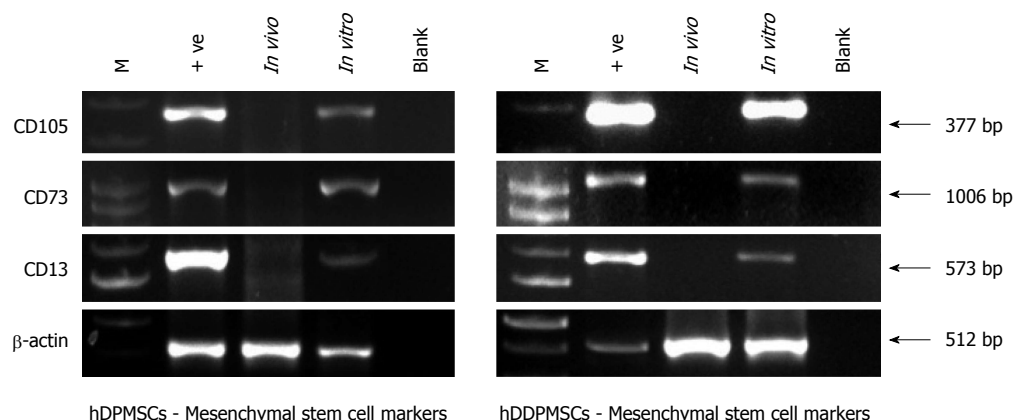


Figure 5 Mesenchymal stem cell markers in dental pulp stem cells.

viability and proliferation rate as well as maintain normal mesenchymal phenotype, similar to those of cells isolated from fresh non-cryopreserved teeth. They have further shown that DPSCs isolated without laser piercing have significant loss of cell viability and proliferation rate as compared to teeth cryopreserved by leaser piercing. Thus, this data has suggested the use of Gioventù *et al.*^[44] method for whole tooth banking.

MOLECULAR MARKERS FOR DPSCs

Stem cell markers

Since completion of human genome project in year 2003, several molecular markers are established to identify specific cell type. Several investigators have already established many markers but there are few markers which tell us their phenotypes, pluripotency status and differentiating characteristics. Dental pulp cells also should be characterized by such types of markers, some of which are explained in next paragraph of this review.

Mesenchymal stem cell markers

The mesenchymal phenotypes of stem cells can be confirmed by using 3 major genes, CD105 is termed as Endoglin (ENG). This protein is a component of the Transforming Growth Factor β Receptor Complex having high affinity in binding to TGF β 1 and TGF β 3. Figure 5 shows the expression of CD105 in DPSCs suggesting it mesenchymal phenotype.

It has been reported that there is a significant decrease in expression of *CD105* gene in differentiated osteoblasts, chondrocytes, adipocytes. Therefore there is a need to check for expression of *CD105* gene before use of these stem cells for stem-cell therapy^[45,46]. CD13 is another marker which is termed as alanyl (membrane) aminopeptidase (ANPEP). This gene plays very important role in a causation of various types of leukemia or lymphoma^[15]. CD73 (Cluster of Differentiation 73) also known as ecto-5'-nucleotidase

or 5'-nucleotidase (5'-NT). This is an enzyme, which encodes *NT5E* gene^[27,36]. This enzyme is used as a marker of lymphocyte differentiation. Barry *et al.*^[47] (2001) have reported expression of CD73 on MSCs. In our study, we have shown that all three markers such as CD105, CD13 and CD73 are expressed in human dental pulp cells as shown in Figure 5.

Hematopoietic stem cell markers

Blood cells mainly expressed two important hematopoietic markers, *i.e.*, CD45 and CD34^[48,49]. CD45 is also called as a Protein Tyrosine Phosphatase, Receptor Type C (*PTPRC*) gene. CD34 is other marker specifically expressed on human hematopoietic progenitor cells. It is also termed as a *RP11-328D5.2* gene. It is a cell surface glycoprotein and functions as a cell-cell adhesive factor. It has function in attaching stem cells to extracellular matrix of bone marrow as well as it can attach directly to stromal cells also^[49].

Pluripotency markers

Stem cells are pluripotent cells and express 3 major genes such as OCT4, NANOG and SOX2. The official symbol of *OCT4* gene is POU5F1B. OCT4 is a transcriptional factor involved in early embryogenesis and very much essential for maintenance of pluripotency of stem cells. It is well established marker for confirming undifferentiated status of stem cells and always involved in self-renewal capability of undifferentiated stem cells^[50]. Study has already shown that gene knockdown of OCT 4 brings about differentiation of undifferentiated stem cells^[51]. NANOG is termed as NANOG homoeobox. It is a transcription factor and well involved in self-renewal capacity of undifferentiated stem cells. It maintains pluripotency property of stem cells. The cells with NANOG protein have an ability to form any cell type of three germ layers of human body^[52,53]. The third pluripotency gene is Sex determining region Ybox 2 (*SOX2*). It is also a transcription factor and maintains self-renew capacity of undifferentiated stem cells. This is a intronless gene and is involved in the regulation of

embryonic development^[54].

Differentiation markers

LIF gene is called as Leukemia Inhibitory Factor and involved in the induction of hematopoietic differentiation in normal and myeloid leukemia cells. It plays a role in immune tolerance at the maternal-fetal interface. LIF derives its name from its ability to induce the terminal differentiation of myeloid leukemic cells^[55]. The other differentiating marker is a Keratin18. It is a KAP protein which forms a matrix of keratin intermediate filaments of cell cytoskeleton structure. Keratin 8, Keratin 18, and keratin 19 are used as a marker for epithelial cells and differentiate from hematopoietic cells^[56].

Specific markers for DPSCs

Specific markers for Dental pulp cells have been well described by researchers^[57,58] 2013.

Dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1) are the markers of odontoblast differentiation^[58]. DSPP is a major non-collagenous dentin specific protein expressed and secreted by odontoblasts. It is an essential protein for normal tooth development^[58]. DMP1 is an another extracellular matrix protein which is involved in differentiation of DPSCs into odontoblasts. It is a member of the small integrin-binding ligand N-linked glycoprotein family and has a critical role for proper mineralization of bone and dentin. It plays a role in regulating expression of osteoblast-specific genes during osteoblast cell differentiation^[58]. Telomerase is another dental pulp marker, which is known to play key roles in understanding the differentiating status of DPSCs. It has been reported that undifferentiated DPSCs express high telomerase activity, whereas, it is gradually decreased in differentiated cells^[59]. This is an important marker to be taken into consideration while therapy of patients to know undifferentiated status of DPSCs for transplantation^[59]. Alkaline Phosphatase (ALP) is also one of the markers of differentiation of DPSCs and plays important role in formation of calcified tissue and extracellular matrix^[60]. Similarly, Osteopontin (OPN) is an earlier marker of osteogenic differentiation^[61] and Bone Sialoprotein (BSP) is one of the late markers of mineralized tissue differentiation^[62]. Recent studies by Yu *et al*^[24] has shown that STRO-1⁺ DPSCs can differentiate into odontoblasts to form dentin, osteoblasts to form bone and chondrocytes to form a cartilage tissue respectively. This suggests that STRO 1 can be used as a marker for understanding the differentiating potentiality of isolated DPSCs^[24].

DIFFERENTIATION OF DPSCs

Biodentine is a bioactive dentine substitute which is used in direct contact with pulp tissue. Luo *et al*^[63] have investigated the response of DPSCs to

Biodentine and shown that this material acts *via* 3 major signaling pathways to induce odontoblast differentiation in DPSCs. They have also shown that Biodentine significantly increases alkaline phosphates activity, OCN, DSPP, DMP1, and *BSP* gene expression and mineralized nodule formation. Hence they have concluded that Biodentine is a bioactive and biocompatible material capable of inducing odontoblast differentiation of DPSCs^[63].

Studies have shown that optimal mechanical compression brings about the induction of cell differentiation^[64]. Miyashita *et al*^[64] have shown that the optimal mechanical compression significantly increases expression of the odontoblast-specific markers, *i.e.*, DSPP in dental pulp cells *via* MAPK signaling pathways. This also results into the expression of the *BMP7* and *Wnt10a* genes^[64]. These transcription factors have been implicated in regulating the differentiation of odontoblasts from DPSCs but their regulatory role is not completely understood. New transcriptional factors involved in odontoblast differentiation of DPSCs are analyzed by using a Microarray analysis^[65]. Choi *et al*^[65] have also shown that DPSCs strongly express Bobby sox homolog (*BBX*) gene during odontoblast differentiation. This gene is also expressed by adult molar odontoblasts cells and other tissues of our body. So overall, it is suggested that *BBX* plays an important role during the odontoblast differentiation of human DPSCs.

Kim *et al*^[66] have cultured dental pulp cells on a conventional surface and nano-patterned surface. It has been shown that cell plated on nano-patterned surface are in linear arrangement as compared to cells plated on a conventional surface. Gene expression analysis also has shown that there is a significantly higher expression of *LPL* in the nano-patterned group than in the conventional group. Whereas, there is a higher expression of *RUNX2* gene in the conventional group than in the nano-patterned group. This study overall suggests that nano-patterned surface enhances adipogenic differentiation of hDPSCs, whereas, normal surface may inhibits osteogenic differentiation of these cells^[66,67].

APPLICATIONS OF DPSCs IN REGENERATIVE MEDICINE

Presently therapies for dental pulp degradation are done by conventional methods such as dental pulp capping or by root canal therapy. However, advancement in dental research, dental scientists are focusing on using some of the medical devices in dental tissue engineering and also can use potential dental cells, extracted from dental pulp of patient. They can use biocompatible material as direct capping agents that can supply growth factors or molecules to stimulate reparative dentin formation. As DPSCs cells

have property to differentiate into odontoblasts, these cells can be used directly for dental therapy as well as very well used as *in vitro* model system to evaluate or optimize newly developed bioactive materials for future dental therapy^[17]. Regenerative property of the pulp-dentin complex is mainly depending on formation of tertiary dentin, reactionary dentin and reparative dentin. There are two different approaches implemented in regeneration of dentin by the use of tissue engineering techniques^[17]. The first approach includes a device which can use as a filling material into a deep cavity of tooth with partial layer of dentin on top of the pulp. In this process, they used some growth factors or molecules that can form reparative dentin. The second approach is to put scaffold on open pulp along with odontoblast-like cells to grow on it. These cells will synthesis reparative dentin. This is somewhat difficult and challenging approach and is being studied extensively for curing dental disorders. It is a mandatory that the scaffold used for clinical application should have capacity to adhere the cells to the surface of this scaffold and should proliferate and differentiate these dental pulp cells into dentin forming odontoblasts. It is also essential to have a good mobility of these cells on this scaffold^[17]. Similarly after implantation, the scaffold should be replaced by regenerated tissue without alteration of volume and size of this scaffold material^[17].

DPSCs and Deciduous teeth stem cells (DTSCs) are being used as stem cells for regenerative therapies for bone related diseases and orthopedics surgeries^[68] because DPSCs and DTSCs can be differentiated into multiple cell types including bone cells such as osteoblast and chondrocytes^[68] as well as we can make iPSCs cells for these treatment. Similarly DPSCs can express neural markers and differentiate into functionally active neurons suggesting, the use of DPSCs in cell based therapy for neuronal disorders and in many cases of accidental brain injury^[23].

MSCs show different pluripotency *in vitro* depending on their source of origin, which suggests that they could behave differently *in vivo*. DPSCs constitute an alternative to BMSCs in the armamentarium for cardiac repair because DPSCs are able to repair infarcted myocardium, due to their ability to secrete proangiogenic factor. It has also been reported that cardiac repair seen with DPSCs is similar to repair seen by using BMSCs. Therefore, DPSCs is considered as a good source of stem cells for regenerative therapies of ischemic heart diseases^[69].

The clinical studies by d'Aquino *et al.*^[70] have shown the use of DPSCs cells in oro-maxillo-facial (OMF) bone repair. They have used DPSCs on collagen sponge's scaffolds which produces an effective bio-complex which can give an optimal support for regeneration of DPSCs cells for OMF bone repair. Thus autologous transplantation of DPSCs can be used in a low-risk and effective therapeutic strategy for the

repair of bone defects^[70].

Vasculogenesis is a potential treatment for ischemic heart disease and it is an exciting area of research in regenerative medicine. Iohara *et al.*^[71] has shown that side population (SP) of dental pulp cells has a property of Vasculogenesis. They have isolated a highly vasculogenic subfraction of SP cells from dental pulp which are positive for *CD31* and *CD146* genes. Thus they have further suggested that these SP cells are a new source of stem cells which stimulate angiogenesis/vasculogenesis in tissue and can be used in cell based treatment of ischemic heart diseases^[71]. Under steady condition, EphB/ephrin-B molecule restricts DPSCs cells for their attachment, migration and to maintain within their stem cell niche and thus Eph/ephrin interactions may contribute to the localization and maintenance of DPSCs within adult human teeth. Following injury, the mobilization of DPSCs to the dentine surfaces may be mediated by EphB/ephrin-B interactions within in the adult dental pulp tissue. Therefore this result suggests a role of EphB/ephrin-B molecule in dental pulp development and regeneration^[72].

In dentistry, replacement of damaged tooth by functional and living tooth is one of the most promising areas of research in dental therapy. Recent advances in biotechnology have encouraged researchers to explore the possibility of regenerating living teeth with functional properties by use of scaffold and DPSCs cells. It has been seen that dental implants require high-quality bone structures for their support and reconstruction of teeth in these patients without adequate bone support would be a major problem. Thus, Stem cell-mediated root regeneration technology along with clinical crown technology may be a promising approach for functional tooth restoration in these patients^[73].

TGPCs cells from the human third molar have high proliferation activity than MSCs isolated from bone marrow. These cells can be frozen and use as per need for auto graft in the process of regenerative medicine. Studies have shown that TGPCs cells can help to stop malignant progression to HCC in hepatitis patients receiving antiviral treatment^[74]. Fibroblastic Growth Factor (FGF) helps in enrichment of DPSCs cells in culture and may be useful for regenerative medicine. It has been shown that bFGF signaling is unconditionally required for maintenance of self-renewal and pluripotency of hESCs^[75,76]. The DPSCs cultured in the presence of bFGF may obtain more undifferentiated characteristics than those cultured in its absence^[75,76].

CLINICAL STATUS OF USES OF DPSCs IN CLINICAL STUDIES

DPSCs have been extensively used in clinical studies by various investigators. Nakashima *et al.*^[77] have examined the effect of growth/differentiation factor 11

(GDF11) on differentiation of dental pulp cells in animal model and shown that GDF11 can differentiate DPSCs into odontoblasts. The odontoblasts differentiation is further confirmed by studying the expression of *DSPP* gene. *GDF11* gene is a morphogen and has property of enhancing process of wound healing in dental pulp tissue. They have further shown that *in vivo* transfer of GDF11 stimulates reparative dentin formation during dental pulp wound healing. Therefore they have suggested that GDF11 can be used for gene therapy for endodontic treatments in dental pulp disorder^[77]. Recently Yang *et al.*^[78] have studied TGF- β signaling pathways in mesenchymal and epithelial dental stem cells and shown that TGF- β signaling in homeostasis of mesenchymal types dental cells is *via* Wnt signaling^[78]. In regenerative endodontic, it is believed that EDTA induces odontoblast differentiation by releasing growth factors from the dentin matrix. After 3 d of culture, both the cell density and fibronectin expression level are shown to be significantly higher in the EDTA-treated Dental pulp cells. However, after three weeks, EDTA treated DPSCs have shown higher expression of DSPP and DMP1 indicating role of EDTA in inducing cell attachment and differentiation of DPSCs into odontoblasts or osteoblasts. Thus, it suggests an importance role of EDTA in achieving successful outcomes in regenerative endodontics^[79].

Estrela *et al.*^[8] have shown that DPSCs are differentiated into active neuron like cells in culture condition as well as these cells have expressed neural marker Nestin. This clearly indicates that there is a great use of DPSCs in regenerative therapies in neurological disorder especially, when there is a brain injury^[23]. Periodontitis is a chronic inflammatory disease leading to alveolar bone destruction resulting into tooth loss due to infection with periodontopathogenic bacteria as well as some genetic or environmental factors. DPSCs are known to be potent immunomodulators and they can be very well suitable for tissue regeneration^[80]. Thus, DPSCs can improve treatment outcome by promoting bone regeneration in these patients when used in combination with conventional treatment modalities for this disorder^[80].

Last few decades, the management of facial defects has rapidly changed. This defect is mainly caused due to Loss of vertical alveolar bone height which gives a stability of dental implants in adult patients. At present, there is no cure for the loss of vertical alveolar bone height and scientists are trying to achieve optimal pre-implantological bone regeneration before placement of dental implant in these patients. Recently, it has been shown that stem cells isolated from the dental pulp, dental follicle, and periodontal ligament can be used to treat alveolar bone defects in humans^[81].

Amir *et al.*^[82] have shown that the Chitosan added in the growth medium significantly increase DPSCs metabolism within 7 to 14 d in culture. Chitosan is responsible to increase in the release of ALP hydrolytic

enzyme activity into the medium during the first week of culture resulting into proliferation and early osteogenic differentiation of DPSCs. However, it has been shown that mineralization remains unaffected by Chitosan treatment. Chitosan also has its role as a 3D scaffold for estrogenic cells differentiation *in vivo* and acts similarly as in *in vitro* condition^[82].

Regenerative endodontic is a replacement of diseased or missing tooth and traumatized dental pulp by new dental tissue or cells. Recently new protocol for management of these cases has been introduced. Shieh-zadeh *et al.*^[83] 2013 have studied 3 cases of necrotic or immature teeth with periradicular periodontitis with dental MSCs and they are successful in bone healing within 3-4 wk after treatment. They have also discussed the mechanism of bone healing process and development of formation of root end in this paper. The regenerative endodontic techniques involve combination of disinfection or debridement of infected root canal systems along with use of stem cells, scaffolds, and growth factors. The new protocol is possibly involved in combination of disinfection or debridement of infected root canal systems along with the use of stem cells, scaffolds, and growth factors to permit the revascularization of this pulp. Therefore they have suggested that dental stem cells can be used successfully for this type of regenerative endodontic^[83].

Human PDLSCs can differentiate into cementoblast-like cells. They also have a capacity to form connective tissue which is richer in collagen I fiber. Human PDLSCs when seeded on 3D scaffolds such as fibrin sponge, generate bone *in vivo* and retain stem cells differentiating properties^[23]. Recent study by Bright *et al.*^[84] have shown the use of PDLSCs for periodontal regenerative therapy. This evidence is proved with their latest experimentation of injecting PDLSCs into animal model system. They are successful in periodontal regeneration therapy and shown the formation of bone, cementum and connective tissue fibers in the animals treated with PDLSCs^[84]. They have further postulated that this regeneration process is not depending on any type of periodontal ligament defect. Exiting results of this study encourages scientists to go further with transplantation of PDLSCs for periodontal regeneration therapy in human after examining its efficacy, safety, feasibility in clinical trials.

FUTURE DIRECTIONS

The stem-cell-based tissue-engineering approaches are widely applied in establishing functional organs and tissues. Rapid progress in advancement in technology such as development of iPSCs has provided great hopes in regenerative therapies for various diseases. Liu *et al.*^[85] have previously reported that iPS could be an appealing stem cells source contributing to tooth regeneration. The application of iPS technology in

dental bioengineering for whole tooth regeneration is an interesting area for future work^[85]. Advancement in stem cell and scaffold technology, damaged or lost teeth can be replaced by the use of regenerative therapies. Similarly, discovery of iPSCs technology has revolutionized complete treatment protocols in the field of dentistry by using a concept of autologous transplantation^[86].

Now day, dentist can very well manage periodontal diseases by using stem cell and scaffold technology^[87,88]. However, making whole artificial tooth and periodontal frame work by this technology is a challenge for scientists working in the field dental regenerative therapies. It is now well established that most of the dental related problems can be treated by using DPSCs alone or in combination with scaffold technology^[88]. Similar to periodontal disorder, advancement in use of DPSCs have added advantage in the field of Endodontic where, we can develop human dental pulp in the laboratory. These outcomes provide evidence suggesting that it might be feasible to restore viability in a necrotic young permanent tooth by engineering a new dental pulp. The potential impact of such therapies is immense and may allow for the completion and reinforcement of the tooth structure by biological regeneration in near future^[89].

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Histone deacetylases and cardiovascular cell lineage commitment

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Abstract

Cardiovascular diseases (CVDs), which include all

diseases of the heart and circulation system, are the leading cause of deaths on the globally. During the development of CVDs, choric inflammatory, lipid metabolism disorder and endothelial dysfunction are widely recognized risk factors. Recently, the new treatment for CVDs that designed to regenerate the damaged myocardium and injured vascular endothelium and improve recovery by the use of stem cells, attracts more and more public attention. Histone deacetylases (HDACs) are a family of enzymes that remove acetyl groups from lysine residues of histone proteins allowing the histones to wrap the DNA more tightly and commonly known as epigenetic regulators of gene transcription. HDACs play indispensable roles in nearly all biological processes, such as transcriptional regulation, cell cycle progression and developmental events, and have originally shown to be involved in cancer and neurological diseases. HDACs are also found to play crucial roles in cardiovascular diseases by modulating vascular cell homeostasis (*e.g.*, proliferation, migration, and apoptosis of both ECs and SMCs). This review focuses on the roles of different members of HDACs and HDAC inhibitor on stem cell/ progenitor cell differentiation toward vascular cell lineages (endothelial cells, smooth muscle cells and Cardiomyocytes) and its potential therapeutics.

Key words: Histone deacetylases; Stem cell; Endothelial cell; Smooth muscle cell; Cardiovascular diseases

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Core tip: Stem cell differentiation toward vascular cell lineages is an area of important active research at present. Histone deacetylases (HDACs) are found to play important roles in cardiovascular diseases. Through modulating the homeostasis of acetylation status in histone and non-histone proteins and regulating grow factor activities, HDACs participate in stem cell differentiation and vascular cell homeostasis. In this review we provide an update on the roles of HDACs

and HDAC inhibitors on stem cell differentiation toward vascular cell lineages.

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INTRODUCTION

Cardiovascular diseases (CVDs) are the number one killer of human beings globally, which is a class of disorders of the heart and blood vessels. CVDs include all diseases of the heart and circulation system, including coronary heart disease, angina, heart attack, congenital heart disease and stroke. Coronary heart disease (angina and heart attack) and stroke may be caused by the same problem - atherosclerosis^[1].

Atherosclerosis is a chronic inflammation process marked with thickened artery wall as a result of invasion and accumulation of fatty material (called atheroma) inside arteries. During this process, the endothelium, as the major regulator of vascular homeostasis, shows a number of vasoprotective effects, such as vasodilation, suppression of smooth muscle cell growth, and inhibition of inflammatory responses^[2]. Accumulating evidence suggests that endothelial dysfunction is an early marker for atherosclerosis and can be detected and repaired prior to structural changes within the vessel wall^[3].

Normally the repairing treatments for injured vascular endothelium include physical exercise and drug treatments aimed at reducing cardiovascular risk factors, such as lowering cholesterol, antihypertensive therapy, smoking cessation and some regulator replacement strategies such as angiotensin-converting enzyme 2 inhibitor therapy, estrogen replacement therapy in postmenopausal women, supplementation with folic acid^[4]. Recently, a new therapeutic strategy, that designed to regenerate the damaged myocardium and injured vascular endothelium and improve recovery by the use of stem cells, attracts more and more public attention^[5]. Till now, there has not established a specific cell type to serve best the treatment of CVDs. Many stem cell types such as embryonic stem cells (ESCs), adult stem cells and induced pluripotent stem cells have the ability to differentiate into vascular cell lineages *in vitro*.

Stem cell therapy is based on the stem cell characters, the potential to differentiate into specific cell types under different stimulation and proliferate to replace damaged cells with healthy functional ones^[6]. Like any biological process, the differentiation of stem cell involves gene expression reprogramming *via* histone acetylation homeostasis mediated chromatin

remodelling. Histone deacetylases (HDACs) are key components of the regulating system that controls histone acetylation homeostasis. HDACs play an essential role in multiple biology processes regulating cell survival, proliferation and apoptosis *via* histone and non-histone protein modification. Accumulating evidence showed that HDACs play an important role in vascular remodelling^[7].

HDACS

Histone acetylation is a reversible process characterized by histone and non-histone protein acetyl-transferase transferring the acetyl moiety from acetyl co-enzyme A to lysine residues. HDACs are a family of enzymes that remove acetyl groups from the N-acetylated lysine residues on histones^[8] and non-histone proteins. Histone acetylation/ deacetylation alters chromosome structure and affects transcription factors access to DNA^[9]. Accumulating evidence indicates that HDACs play a fundamental role in transcriptional regulation, cell cycle progression, and contribute to developmental events.

Mammalian genome encodes 18 HDACs, which can be grouped into four classes based on the homology with yeast histone deacetylases^[10]. All members share a highly conserved deacetylase domain but differ in structure, subcellular localization and expression pattern, which results in different cellular functions^[11]. Class I HDACs (homologous to yeast Rpd3) comprise of HDAC1, -2, -3 and -8, which are widely expressed in many human tissues and cell lines. Among them both HDAC3 and HDAC8 can shuttle between the cytosol and the nucleus, while HDAC1 and HDAC2 are exclusively located in nucleus^[12,13]. The deacetylation of histones to repress gene transcription is the main function of class I HDACs. Class I HDACs form large multi-subunit complex that associates with transcription factors and other chromatin modifiers, except HDAC8 whose function is not clearly clarified^[14]. In mammals, HDAC1 and -2 can bind to each other forming the catalytic core of three different complexes: Sin3, nucleosome remodelling deacetylase and corepressor of RE1-silencing transcription factor. HDAC3 can form two different multi-protein complexes, nuclear receptor corepressor and silencing mediator of retinoic acid and thyroid hormone receptors. Class I HDACs are mainly found in such nuclear complexes. Furthermore, the association with several different proteins show an essential role in regulating their activity, which is highly pronounced in developmental processes that requires a global modulation of transcriptional programmes. With the exception of HDAC8, knockout of class I HDACs in mice have indeed resulted in embryonic lethality^[15].

Class II HDACs can be divided into two sub-class groups. Class II a members include HDAC4, -5, -7, -9, while class II b comprises of HDAC6 and -10. Class II HDACs differ from Class I mainly for their tissue-

specific expression pattern and the considerable lower deacetylase activity. Besides, their length (almost the double of Class I HDACs) and cellular localization are all different. Class II HDACs can shuttle between nucleus and cytoplasm. Class IIa HDACs have conserved binding sites for the transcription factor myocyte enhancer factor 2 and the chaperone protein 14-3-3, which help HDACs shuttle from the nucleus to the cytoplasm. The nuclear-cytoplasm shuttling and occurrence of post-translational modification such as phosphorylation, indicates their primary role as signal transducers in numerous tissues during development and disease^[16].

Class IIb family includes HDAC6 and HDAC10. HDAC10 presents a leucine-rich domain at the C-terminal. Recent reports showed HDAC10 might be upregulated on human myeloma cell lines^[17]. HDAC6 is different from all other HDACs, as it harbours two deacetylase domains and a C-terminal zinc finger, being the important cytoplasmic deacetylase in mammalian cells^[18]. Cytoskeletal proteins such as α -tubulin and cortactin, transmembrane proteins such as the interferon- α receptor, and chaperones are all targets of HDAC6^[19,20].

Class III HDACs comprises a group of protein called Sirtuins (SIRT1-7). Although they possess a deacetylase domain, they diverge from classical HDACs as their enzymatic activity requires the cofactor NAD⁺. This feature suggests their involvement in metabolic functions^[21]. HDAC11 is the last discovered and the only member of Class IV HDACs^[22]. This review will focus on the classical HDACs, class I and II HDACs. The protein length, cellular location and potential cellular functions of these HDACs can be referred to references^[11,23].

HDACS AND ENDOTHELIAL CELL DIFFERENTIATION

In response to vascular injury, endothelial cell (EC) migration and proliferation contribute to the repairing of the damaged EC or denuded endothelium. Recent reports show that circulating or local resident stem/progenitor cell differentiation is also involved in this process^[24]. Through modulating chromatin structures and non-histone transcription factors, HDACs are involved in the gene expression reprogramming in multiple biological processes such as cell-cycle, cell differentiation and survival^[25]. Therefore, the involvement of HDACs in EC differentiation is expected.

The first evidence came from studies with HDAC inhibitors (HDACi). HDACi decreases endothelial lineage commitment of endothelial progenitor cells^[26,27]. Rössig *et al.*^[27] found that the suppressive effect of HDACi on EC differentiation was mediated by the down-regulation of homeobox transcription factor HoxA9, which directs the transcription of EC markers such as eNOS, VEGFR2 and VE-cadherin, suggesting that the

HDAC-dependent activation of Hox-A9 is essential for EC differentiation^[27].

There is no direct evidence that HDAC1, 2 and 8 are involved in EC differentiation, although indirect evidence indicates that HDAC1 may suppress EC differentiation. Rajasingh *et al.*^[28] showed that HDACs inhibitor, trichostatin A, improved AceH3K9 and reduced HDAC1 expression in bone marrow progenitor cells, leading to differentiation into myocytes and ECs, which suggests that HDAC1 plays a suppressive role in bone marrow progenitor cell differentiation toward EC lineage^[28]. Different from other members within class I HDACs, accumulating evidence suggests that HDAC3 possesses a pivotal function in stem cells differentiation into ECs, which is capable of repairing the damaged endothelium. VEGF is a well-known EC differentiation inducer. Xiao *et al.*^[29] reported that VEGF up-regulated HDAC3 in ESC-derived Sca1⁺ cells. Over-expression of HDAC3 *via* adenoviral gene transfer increased, while trichostatin A or HDAC3 siRNA abolished VEGF-induced EC marker expression in Sca1⁺ cells, suggesting HDAC3 may function downstream of VEGF signal pathway^[29]. Our study^[29] and reports from Illi B^[30] demonstrated that laminar flow enhanced ESC-derived progenitor cell differentiation into EC lineage in an HDAC dependent manner. We found that laminar flow stabilized and activated HDAC3 through the Flk-1-PI3K-Akt pathway in a ligand independent manner, which in turn deacetylated p53, leading to p21 activation, contributing to EC differentiation^[31]. Similar mechanism is involved in VEGF-induced EC differentiation, in which HDAC3 modulates differentiation process *via* regulating non-histone proteins. Our recent study found that unconventional splicing of HDAC3 might change HDAC3 function, inducing endothelial-to-mesenchymal transition^[32]. These reports suggest a critical role of HDAC3 in EC fate determination.

Class II HDACs seem not directly involved in EC differentiation. Several groups tried to link class II HDACs with EC differentiation, but no solid evidence has been obtained. In Spallotta *et al.*^[33,34]'s reports, nitric oxide induced a cross-talk between class I HDACs (HDAC3) and class II HDACs (HDAC4 and 7), which might contribute to the neovascularization in ischemic tissue and skin repairing^[33,34]. However, the effect of nitric oxide on EC differentiation may be largely derived from HDACs-mediated global hypoacetylation on pluripotency maintaining genes like Oct4, Nanog, KLF4, *etc.* Considering class II HDACs have only weak deacetylase activity, the histone hypoacetylation might be mainly caused by HDAC3 in the complex. Reports from other groups indicate that HDAC7 may participate in EC proliferation and cell-to-cell contact but is not involved in EC differentiation^[35,36]. A recent report from Song *et al.*^[37] showed that AMPK activation participated in endothelial colony forming cells differentiation. During this process, HDAC5 could be phosphorylated by AMPK. However, there is no direct evidence on the involvement of HDAC5.

HDACS AND SMOOTH MUSCLE CELL DIFFERENTIATION

Smooth muscle cells (SMCs) are the major cellular components within vessel wall, located in the tunica media controlling the calibre of the vessel. It is widely believed that in response to vascular injury SMCs in the tunica media undergo from contractile to synthetic phenotype change, followed by the migration into the intima and proliferation, contributing to the neointima formation. However, recent reports show that stem/progenitor cell-derived SMCs play an important role in neointima formation and atherosclerosis, but the origin of the progenitor cells is controversial. Reports from Shimizu *et al.*^[38], Han *et al.*^[39] and Li *et al.*^[40] support the notion that the circulating bone-marrow-derived progenitor cells contribute to SMC differentiation and neointima formation, but reports from Hu *et al.*^[41] and Tang *et al.*^[42] exclude the involvement of bone marrow-derived progenitor cells. They demonstrated that SMCs in the lesion area were mainly derived from local resident progenitor cells.

HDACs play diverse roles in SMC differentiation depending on the HDAC species, stem cell source and stimulus, exerting promoting or suppressive effects. Except HDAC1, all other three members of class I HDACs are reported to be involved in SMC differentiation. In response to oxidized lipids or PDGF, HDAC2 forms a complex with Class II HDACs (HDAC4/5) to deacetylate histone H4 in SMC marker gene promoter region, thereby suppressing SMC differentiation^[43,44]. Recently, Liu *et al.*^[45] reported that HDAC2 exerted suppressive effect on bone marrow mesenchymal stem cells to SMCs differentiation^[45]. In contrast, HDAC3 and 8 may play a positive role in SMC differentiation. Loss of HDAC3 in neural crest caused deficiency of aortic arch artery SMCs in midgestation during embryonic development *via* down-regulation of Notch signalling, suggesting a positive regulatory role of HDAC3 in the neural crest-derived smooth muscle lineage^[46]. Several reports demonstrate that HDAC8 is critical for SMC differentiation, therefore HDAC8 is even regarded as a novel SMC differentiation marker^[13,47,48]. The involvement of class II HDACs in SMC differentiation seems restricted to HDAC4, 5 and 7. Different from the suppressive role of HDAC4 and 5 from the reports by Yoshida *et al.*^[43,44], studies from Glenisson *et al.*^[49] revealed that HDAC4 is necessary for SMC marker expression in TGFβ1-induced myofibroblast differentiation^[49]. Our group found that different isoforms of HDAC7 might play opposite function in SMC differentiation. During ESC differentiation toward SMC lineage, HDAC7 mRNA underwent further splicing, which was enhanced by PDGF. The full spliced HDAC7 increased SMC marker expression through modulating the SRF-myocardin complex while the partially spliced HDAC7 decreased SMC marker expression by degrading myocyte enhancer factor 2C^[50].

HDACS AND CARDIOMYOCYTES DIFFERENTIATION

Cardiomyocytes (CMs) build the atria and ventricles of the heart, which are critical to the proper form during the beating of the heart. So the differentiation towards CMs is a prerequisite and an essential part of heart development^[51].

The effect of class I HDACs on CM differentiation seems a bit controversial, especially on HDAC1. Opposite effect of HDAC1 on CM differentiation has been reported by different groups. Dovey *et al.*^[52] reported that HDAC1 deficiency in ESCs increases CM marker expression in the embryoid bodies, suggesting a suppressive role of HDAC1 in CM differentiation. Lu *et al.*^[53,54] also reported that HDAC1 exerted negative effect on cardiac cell differentiation from mesenchymal stem cells under a myocardial microenvironment. The suppressive role of HDAC1 was further supported by Liu *et al.*^[55] as down-regulation of HDAC1 promoted cardiac cell differentiation. However, Hoxha *et al.*^[56,57] reported that HDAC1 knockdown decreased CMs marker expression in ES and induced pluripotent stem cells, suggesting a supportive role in CMs differentiation. HDAC1 favours stem cell differentiation *via* turning off pluripotency genes by modulating histone acetylation and DNA methylation. HDAC2 may play a positive role in CM differentiation, contributing to angiotensin II-induced cardiac hypertrophy^[58]. In contrast, HDAC3 suppresses CM differentiation through modulating transcription factor Tbx5, preventing the pre-maturation of cardiac progenitor cells^[59].

Current reports revealed that Class II HDACs mainly played a suppressive role in CMs differentiation. The inhibition of HDAC4 facilitates c-kit(+) cardiac stem cells to differentiate into cardiac lineage, leading to cardiogenesis and the restoration of cardiac function in myocardial infarction mouse model^[60]. Ha *et al.*^[61] reported that retention of HDAC5 in nucleus suppressed cardiac foetal gene expression and CMs hypertrophy. Gene disruption studies from Chang *et al.*^[62] revealed that HDAC5 or 9 deficiency induced cardiac hypertrophy in mice.

Depending on the stem cell source and stimulus, different HDACs may play different roles in stem cell differentiation toward different cell lineages. HDACs and their potential roles in cardiovascular lineage commitment are summarized in Table 1. Different HDACs (HDAC1, 2, 3, 4, 5, 7, 8) play different roles in stem cell differentiation toward different cell lineages, HDACs and their potential roles in cardiovascular lineage (ECs, SMCs, CSCs) commitment are summarized in Table 1.

HDACS INHIBITORS

The role of HDACs in injured vessel repairing and stem/progenitor cell differentiation derived originally from

Table 1 Summary of histone deacetylases' effect on endothelial cell, smooth muscle cell and cardiomyocyte differentiation

Commitment	HDACs	Characterization	Ref.
EC	HDAC1	EC differentiation markers ↓	[28]
	HDAC3	EC differentiation markers ↑	[29]
		<i>In vitro</i> and <i>in vivo</i> angiogenesis	[31]
		<i>In vivo</i> ability to repair injured vessels	
	HDAC4	EC differentiation	[33,34]
	HDAC5	Endothelial colony forming cells differentiation	[37]
	HDAC7	EC differentiation Maintenance of vascular integrity Promote angiogenesis	[33-35]
SMC	HDAC2	Involved in suppressing SMC differentiation	[43,44]
	HDAC3	Neural crest-derived smooth muscle cell differentiation ↓	[43]
	HDAC4	Involved in suppressing SMC differentiation	[44]
	HDAC5	Involved in suppressing SMC differentiation	[44]
	HDAC7	Full spliced HDAC7 SMC differentiation markers ↑	[50]
	HDAC7	Unspliced HDAC7 SMC differentiation markers ↓	[50]
	HDAC8	Marker of smooth muscle differentiation	[13]
CM	HDAC1	CM differentiation ↑	[56,57]
	HDAC1	CM differentiation ↓	[52-55]
	HDAC2	Induce CMs hypertrophy	[58]
	HDAC3	Regulate early cardiogenesis	[59]
	HDAC4	CSC-derived cardiac regeneration Restoration of cardiac function ↑	[60]

EC: Endothelial cell; SMC: Smooth muscle cell; CM: Cardiomyocyte; CSC: Cardiac stem cell; HDAC: Histone deacetylase.

investigations on HDACi, which are a large number of natural and synthetic compounds. Generally, HDACi can be divided into 4 classes according to their chemical structure: hydroxamic acids, short chain fatty acids, cyclic tetra-peptides, and benzamides^[63]. These HDACi share the presence of an active group targeting a three-part structure consisting of a zinc-binding group of HDACs and inhibits their enzymatic activity. Some HDACi are able to discriminate HDACs among different classes and a few of them can even inhibit a single member^[64,65]. In addition to suppress the deacetylase activity of HDACs, HDACi may exert functions in HDAC-independent way, like through SP1^[66]. HDACi has been shown to regulate multiple cellular processes, including cell apoptosis and survival, proliferation and growth arrest, senescence, differentiation, immunogenicity, *etc.* Some kinds of HDACi are used in clinical trials as therapeutic drug against cancers. Detailed information on this aspect won't be discussed in this review.

SUMMARY AND PERSPECTIVES

CVDs characterized by vascular injury are one of the greatest health challenges we have been facing for decades. During the development of CVDs,

HDACs play a critical role in various signal pathways by modulating vascular cell homeostasis (*e.g.*, proliferation, migration, and apoptosis of both ECs and SMCs) and stem cell differentiation towards vascular cells. HDAC3 and HDAC7 have been proved to be capable of directing stem cell differentiation towards ECs and SMCs respectively. Through modulating the homeostasis of acetylation status in histone and non-histone proteins and regulating grow factor activities, HDACs participate in stem cell differentiation and vascular cell homeostasis. Targeting HDACs activities and their inhibitors' effect on injured vessel and heart will undoubtedly provide new therapeutic strategies for the treatment of CVDs.

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Hepatic stem cells: A viable approach for the treatment of liver cirrhosis

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Abstract

Liver cirrhosis is characterized by distortion of liver architecture, necrosis of hepatocytes and regenerative nodules formation leading to cirrhosis. Various types of cell sources have been used for the management and treatment of decompensated liver cirrhosis.

Knowledge of stem cells has offered a new dimension for regenerative therapy and has been considered as one of the potential adjuvant treatment modality in patients with end stage liver diseases (ESLD). Human fetal hepatic progenitor cells are less immunogenic than adult ones. They are highly propagative and challenging to cryopreservation. In our earlier studies we have demonstrated that fetuses at 10-18 wk of gestation age contain a large number of actively dividing hepatic stem and progenitor cells which possess bi-potent nature having potential to differentiate into bile duct cells and mature hepatocytes. Hepatic stem cell therapy for the treatment of ESLD is in their early stage of the translation. The emerging technology of decellularization and recellularization might offer a significant platform for developing bioengineered personalized livers to come over the scarcity of desired number of donor organs for the treatment of ESLD. Despite these significant advancements long-term tracking of stem cells in human is the most important subject nowadays in order to answer several unsettles issues regarding the route of delivery, the choice of stem cell type(s), the cell number and the time-point of cell delivery for the treatment in a chronic setting. Answering to these questions will further contribute to the development of safer, noninvasive, and repeatable imaging modalities that could discover better cell therapeutic approaches from bench to bedside. Combinatorial approach of decellularization and nanotechnology could pave a way towards the better understanding in determination of cell fate post-transplantation.

Key words: Hepatic stem cells; Bioengineering; Liver cirrhosis; Labeling and tracking

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Core tip: Liver cirrhosis is characterized by distortion of liver architecture, necrosis of hepatocytes and regenerative nodules formation leading to cirrhosis. The

available treatment modalities are not very effective against liver cirrhosis. Stem cells are considered one of the potential adjuvant treatment modality in liver cirrhosis patients. Fetal hepatic stem cells transplantation in liver cirrhosis has emerged as an alternative to organ transplantation. However long-term stem cells labeling and tracking is needed for cell fate determination after transplantation. Decellularization technology provides a novel tool to develop bioengineered personalized livers to accomplish the shortage of donor livers.

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INTRODUCTION

The liver is a central metabolic and highly specialized detoxifying organ. Approximate 70%-75% of liver functions are being carried out by the hepatocytes which together with cholangiocytes (5%-10% of hepatic cells) constitute the liver parenchyma. Liver regeneration is a very rapid and well synchronized phenomenon. Liver responds to initial injury compensating the loss of parenchymal mass. If damage perseveres commencement of petite terminal peri-portal oval cells activates which engross mobilization of several vital factors.

EVOLUTION OF CELL THERAPY AND PRECLINICAL INVESTIGATION

Various strategies have been explored to provide functional support to the fading liver. Hepatic cell transplantation has emerged as one of the alternative modality. The experimental data of hepatocyte transplantation in different animal models has proved its efficacy. In early 1980s hepatocyte transplantation in D-galactosamine induced acute liver failure (ALF) model showed survival rate of more than 60%^[1-4]. Different routes were used for delivery of cells like intraportal, intrasplenic and intraperitoneal. Intra-peritoneal cell transplantation was found to be more appropriate site for transplantation as large number of cells can be delivered and also provides better engraftment of infused cells^[5]. In view of larger number of cells required, Habibullah *et al*^[5], infused 6×10^6 hepatocytes according to body weight (per kg) in ALF animal model and observed > 60% survival post-transplantation.

CLINICAL INVESTIGATION OF HEPATOCYTES TRANSPLANTATION

After preclinical investigations in animal models, first

attempt of hepatocytes transplantation in human with liver cirrhosis was carried out by Mito *et al*^[6] which provided proof for functional benefits post-transplantation. This was a landmark study proving a new paradigm for shifting hepatocytes transplantation towards the clinical perception and further followed by studies from our centre. In our preliminary clinical study, human fetal hepatocytes were infused intra-peritoneally into seven ALF patients^[7]. The study have demonstrated beneficial effects in ALF patients with grade III and grade IV hepatic encephalopathy because transplanted hepatocytes by virtue of their synthetic, metabolic and detoxifying functions causes clinical and functional improvements. Several other studies have also provided evidence for active bridging of orthotropic liver transplantation in patients though post-transplantation of human hepatocytes^[8]. Besides this various other studies have also demonstrated the functional benefits using different other routes of cell transplantation^[1,3,5]. We performed a pioneer study by intra-peritoneal hepatocytes transplantation in a 26 year old pregnant patient having acute fatty liver to investigate the functional benefit after transplantation^[9]. This study demonstrated significant improvements after 2 d of hepatocytes transplantation and provided an alternative option of liver transplantation and new hope for the patients suffering with end stage liver failure. After this several other studies have been reported from various centers for hepatocytes transplantation in different types of metabolic disorders^[10,11]. However, transplantation of large number of human hepatocytes for adequate liver function remains a challenge before its wide clinical applications.

CHRONIC LIVER DISEASE (LIVER CIRRHOSIS)

This category has produced a large burden on the society and has been considered as more appropriate type for cell therapeutic approaches. Liver cirrhosis is being characterized by distortion of liver architecture, necrosis of hepatocytes and regenerative nodules formation leading to cirrhosis. Various types of cell sources have been applied for the management and treatment of decompensated liver cirrhosis.

HEPATIC STEM CELLS AND LIVER CIRRHOSIS

Discovery of stem cells has provided a new dimension for regenerative therapy and are considered one of the potential adjuvant treatment modality in patients with ESLD. With the knowledge of embryonic stem cells, the area of cell biology has revolutionized for its potential in regenerative medicine. It gave a new hope and opened a new area of cellular therapy. However, a variety of stem cell types have been identified from various sources, hepatic stem cells (HSCs) has been

potential therapeutic option for the treatment of liver cirrhosis. HSCs are able to give rise mature progenies and have self-renewing capacity. Several studies have demonstrated isolation of HSCs from adult tissues followed by *in vitro* proliferation and differentiation into functional hepatocytes^[12-14]. However, regeneration of diseased liver by human HSCs is still awaited.

Hepatic progenitor cells (HPCs) isolated from human fetal liver is less immunogenic, highly propagative and more challenging for cryopreservation than the adult ones. In our previous study we have demonstrated that fetuses at 10-18 wk of gestation age contain a large amount of dynamic HPCs which possess bi-potent nature giving rise to both hepatocytes and bile duct cells^[15]. Fetal liver stem cells has been identified as transition between embryonic cells and adult ones which is mostly non-teratogenic^[16]. During third trimester of fetal development, plasticity to form liver parenchyma cells makes fetal HSCs an excellent resource for cellular therapeutic approaches.

SOURCES OF STEM CELLS IN LIVER DISEASES

There are two major sources of stem cells (1) embryonic; and (2) adult which have been considered to have potential in the treatment of various types of diseases. However, fetal liver derived stem cells are the transition between embryonic and adult sources and have several advantages on these two sources. Fetal liver derived cells are also termed as hepatoblasts which appears after the specialization of liver endoderm and growing liver bud. Various types of adult stem cell sources have been investigated for their application in liver regeneration such as mesenchymal stem cells (MSCs) derived from umbilical cord blood/tissue and bone marrow, induced pluripotent cells derived from somatic cells. These extra-hepatic sources have been reported to provide trophic support in injured liver. They also induce the endogenous cell proliferation and survival under diseased environment. But the control of their lineage specificity and plasticity needs more investigation in order to apply them in clinical settings. In this regard, MSCs from bone marrow has already been recognized as major player in clinical regeneration and resolution of liver cirrhosis due to its immunomodulation and regenerative potential.

ISOLATION AND DEPICTION OF HUMAN HEPATIC PROGENITORS FROM FETAL LIVER

After obtaining a human fetus in better clinical form, isolation of cells is performed by two step collagenase digestion method as earlier described by Habibullah *et al.*^[7]. Cells within the fetal liver are generally well connected with intercellular links rooted in extracellular matrix (ECM) which is required to be destroyed

for single cell isolation either by mechanical or enzymatic dissociation. The human fetal liver cells thus isolated can be enriched further for specific progenitor cells using magnetic activated cell sorting (MACS) or fluorescence activated cell sorting (FACS). Enrichment can be done either based on cell size and density or based on cell surface/functional markers. Enriched human HPCs further need to be distinguished from biliary and mature hepatocytes^[17]. In our earlier studies human HPCs were first enriched with epithelial cell adhesion molecule (EpCAM) and further identified for the expression of cytokeratin-18; a marker for liver epithelium, cytokeratin-19; a biliary marker and finally albumin and alfa-feto protein for mature hepatocytes using Real-Time quantitative polymerase chain reaction and immunocytochemical (ICC) staining. These explanations were further supported by FACS results indicating positivity for above markers including CD49f, CD29, CD90 and CD34 and negative for CD45 and HLA class II.

IN VIVO DELIVERY OF CELLS WITHIN LIVER

Portal vein

Portal vein is the most often used route for the stem cell transplantation due to multiple vascular accesses. It is admitted either by trans-jugular or trans-hepatic percutaneous approach. However accessing this route by above approaches is difficult in the presence of ascites and has major risk of bleeding, diathesis and coagulopathy.

Accessing through hepatic artery

In our knowledge this is a more suitable route for cellular transplantation as compared to the portal vein^[17]. It is accessed through trans-radial, trans-femoral and trans-branchial approaches.

Splenic infusion

Intrasplenic approach has been suggested as better approach compared to the splenic arterial infusion of hepatocytes transplantation^[18]. A recent study has proved this concept where the fetal hepatocytes were transplanted via intrasplenic infusion through the splenic artery in a patient with end stage liver cirrhosis leading to the improvement in clinical conditions^[19]. The translocation of infused cells through intrasplenic injection has also been demonstrated where cells after infusion first reaches to the splenic veins and then to hepatic sinusoids^[20].

CLINICAL STUDIES OF HUMAN HEPATIC STEM/PROGENITOR CELL THERAPY

HSCs-based therapies for the treatment of ESLD are in their early stages of translation. We have done the pioneering studies using HSCs transplantation

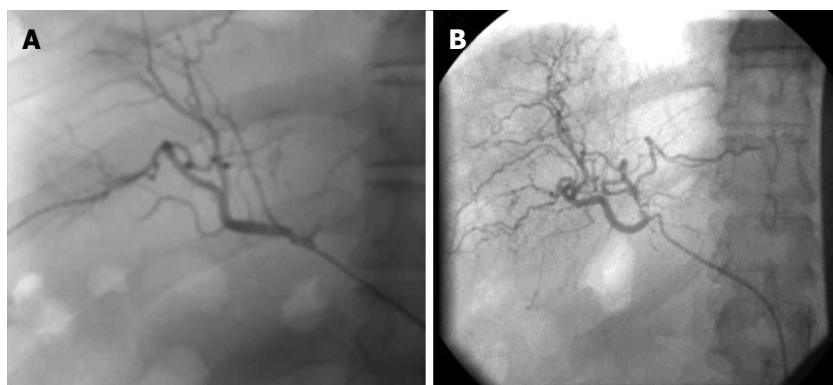


Figure 1 Hepatic artery characterization using angiograph of patients post transplantation after 6 mo (A) and after 12 mo (B).

in the treatment of patients with inborn metabolic errors, liver cirrhosis, hepatitis B/C, and other liver disorders^[17,20]. We used EpCAM+ve enriched human HSCs from human fetal livers following the approach described by Schmelzer *et al.*^[14]. Remarkably no immune suppressant was required and recipient was not matched for histocompatibility antigens. All the patients with decompensated liver cirrhosis receiving HSCs transplantation through hepatic artery showed significant decrease ($P < 0.01$) in the mean Mayo-End stage Liver Disease (MELD) score and improvement in multiple diagnostics and biochemical parameters after six-months follow-up^[17]. After 6 mo to one year of HSCs post-transplantation angiogram analysis of the patients showed significant improvement in branching of hepatic arteries and veins (Figure 1).

Recently we accessed cellular immune response in 5 patients with decompensated liver cirrhosis after transplantation of human HPCs. The study showed marked clinical recovery with decline in the MELD score and there was no significant variation found in T-cell subpopulation (CD3, CD4, CD8 and CD4/CD8 ratio), natural killer cells and in serum cytokine levels between pre and post transplantation demonstrating the safety of EpCAM+ve HPCs transplantation for liver cirrhosis (Unpublished).

Future efforts to use human HSCs/HPCs clinically will be facilitated by production of cells at large-scale. The sourcing of donor cells may be fetal tissue in those countries that permits their use. The cells may be utilized as directly isolated, or after *in vitro* expansion.

IN VIVO TRACKING AND MONITORING OF TRANSPLANTED HEPATIC STEM/PROGENITOR CELLS

Since last few decades' vast developments have been made for the management of liver disease. However more investigations are desired to direct the better tissue imaging approaches to identify the fate of grafted cells. It is essential for better understanding of cell infusion routes, migration and efficacy of

desired cells post-transplantation. Currently various magnetic, fluorescence or radio imaging methods have been used to evaluate efficacy of transplanted cells. However, few of them have proved their prospective in clinical settings but majority of them are still in their preclinical phases of investigations.

Tracking the fate of transplanted cells is vital to monitor the delivery and viability of the grafts over extended time. It could be achieved by merging optimal molecular imaging methods with stem cells. Through advancements in experimental and molecular imaging technologies, it would provide heavy motivation to improvise the molecular imaging approaches for better understanding of the regenerative mechanisms involved in stem cells transplantation.

Molecular imaging

Stem cells labeling and tracking in human tissues/organs is the most important subject nowadays in order to answer several unsettled issues regarding the cell delivery routes, stem cell choice, number of cells to be infused and the time-point of cell delivery for the treatment in a chronic setting. Answering to these questions will further contribute to the development of safer, non-invasive, and repeatable imaging modalities that could discover better cell therapeutic approaches from bench to bed-side. Long-term monitoring of transplanted stem cells continuously with high temporal resolution and good biocompatibility will allow us to better understand the precise regeneration mechanisms in different organs. Nanobiotechnology has emerged as the most immense area for labeling and tracking of cells both *in vitro* and *in vivo*.

HM-PAO based tracking of transplanted HSCs:

Our initial effort was made to monitor transplanted HSCs *in vivo* by using HM-PAO (lipid-binding dye). However, existence of short life-span of HM-PAO within the cells, we were able to identify transplanted cells only till 24 h of post-transplantation^[18]. However, other methods such as hepatic scintigraphy demonstrated that infused human HPCs homes evenly in both liver lobes with elevated engraftment, thus yet again

recalling the efficiency of cell delivery route through hepatic artery. During and after 6 mo of follow-up no other clinical complications were observed^[17].

Di-D labeling and tracking of transplanted cells:

Due to short life of HM-PAO, it was quite difficult to monitor transplanted cells in long-term. Hence, further we used live cell imaging dye (*i.e.*, Di-D) for labeling and long-term tracking of human HSCs post-transplantation. DiD-labeled EpCAM+ve human HPCs were infused in liver of nude mice to identify its engraftment and survival rate. Post-transplantation labeled cells were tracked and located at different time points by using an animal imaging system (KODAK Fx-PRO). Post-transplantation persistence DiD-labeled cells were observed up to 110 d. X-ray images of animals receiving cells were also captured and overlaid on fluorescence imaging to investigate better consignment (Unpublished). However, additional studies are required with regard to safety and efficacy of such dyes before applying into the medical conditions.

Magnetic resonance imaging of transplanted cells using high contrast nanoparticles: Magnetic resonance imaging (MRI) is widely used technique in preclinical and clinical studies. Particular type of stem cells are enriched with a contrast agent or directly labeled with super paramagnetic iron oxide (SPIO) to track them in damaged tissues post-transplantation. Non-invasive *in vivo* monitoring can be achieved by MRI using SPIO-labeled cells due to its significant temporal and spatial contrast^[21-24]. We have synthesized such high contrast nanoparticles by combining SPIO with Gadolinium which is also detectable in 1.5 T MRI machine. This approach represents one such tool that can provide insight into cell survival and proliferation following transplantation into the tissues.

LIVER BIOENGINEERING: A POTENTIAL APPROACH TO UNDERSTAND THE REGENERATIVE MECHANISMS OF HSCS POST-TRANSPLANTATION

Liver bioengineering is one of the most exciting areas in regenerative medicine. In last few decades various strategies have been attempted to support the liver tissue regeneration either by using artificial devices or two-dimensional culture system. However, three-dimensional architecture, vascular network and dense cellular masses are required to support the functional damage of liver. Hepatocytes are known to be attachment-dependent cells and loss very quickly in absence of suitable medium composition, ECM and cell-cell contacts. Cellular proliferation and differentiation is also influenced by three-dimensional organ architecture^[25-27]. Employment of naturally derived

matrices have proved their potential in hepatocyte culture^[26]. Several studies have demonstrated long-term heterotopic hepatocytes transplantation in a variety of matrices^[27,28]. Nonetheless, initial engraftment rates are suboptimal. Hence development of a whole tissue engineered liver construct is in urgent need for orthotopic transplantation.

Repopulation of human HSCs in whole decellularized liver scaffold to generate humanized organ

Decellularization is defined as the technology used to remove all parenchyma cells, endothelial cells, myofilaments and other cellular components from the organ while retaining its three dimension architecture and vascular tree. The use of naturally vasculature and rapid separation of debris and waste fluid from organ or tissue significantly improves the quality of organ scaffolds for reconstitution of new organ/tissue. Because of the above facts decellularization technology has been considered an ideal tool for regeneration of whole organ/tissues. Different types of stem cells from a variety of sources either alone or in combination with other cells have been tried to develop the whole functional organ architecture to provide a considerable approach for organ bioengineering.

Recellularization is a process of generating functional cells within the decellularized organ scaffold in order to get fully functional bio-artificial organ. Seeding of specific type of cells with high proliferation and controlled differentiation potential is necessary to repopulate the decellularized organ scaffold. Despite using various types of organs for decellularization and recellularization, none of them have proved their absolute potential to replace the damaged organ/tissue.

Since last two decades' most of the tissue engineering approaches have involved in reconstruction of thin masses of cells, for instance skin, arteries and bladder^[29-31]. Whereas construction of thick masses of cells/tissues such as muscles, liver and kidney, etc. has been proved difficult to mimic due to fractional distribution of oxygen and nutrients inside the engineered scaffold^[32]. Several recent studies have proved decellularization and recellularization technologies as a most significant tool for the creation of fully humanized functional organs^[33-37].

In our view, these decellularized liver bio-scaffolds have a great potential to ensure an advanced *in vitro* natural liver cell culture system for toxicology, pharmacology and drug discovery. This may also establish an excellent tool to cram the normal development of organs and diseased tissues. The technology of decellularization and recellularization passes much potential to reach the significant aim of engineering bio-artificial personalized livers for the treatment of acute and chronic liver diseases. Therefore lead has been taken by our centre to utilize the technology of decellularization and repopulation further to improvise the research quality and to reach the goal

for generating personalized whole functional liver for patients suffering with end stage liver failure.

CONCLUSION

Human HSCs-based therapies for the treatment of ESLD are in their early stage of the translation. Long-term stem cells labeling and tracking *in vivo* is the most important subject nowadays. High contrast and paramagnetic nanoparticles with high biocompatibility have emerged a better option in order to answer several unsettled issues regarding the cell delivery routes, better stem cell choice, number of cells to be infused and the time-point of cell delivery for the treatment in a chronic setting. The technology of decellularization and recellularization might offer a significant platform for developing bioengineered personalized livers to come over scarcity of desired number of donor organs for the treatment of ESLD. Combinatorial approach of decellularization and nanotechnology could pave a way towards the better understanding in determination of cell fate post-transplantation.

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Hair follicle stem cells: *In vitro* and *in vivo* neural differentiation

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Abstract

Hair follicle stem cells (HFSCs) normally give rise to keratinocytes, sebocytes, and transient amplifying progenitor cells. Along with the capacity to proliferate rapidly, HFSCs provide the basis for establishing a putative source of stem cells for cell therapy. HFSCs are multipotent stem cells originating from the bulge area. The importance of these cells arises from two important characteristics, distinguishing them from all other adult stem cells. First, they are accessible and proliferate for long periods. Second, they are multipotent, possessing the ability to differentiate into mesodermal and ectodermal cell types. In addition to a developmental capacity *in vitro*, HFSCs display an ability to form differentiated cells *in vivo*. During the last two decades, numerous studies have led to the development of an appropriate culture condition for producing various cell lineages from HFSCs. Therefore, these stem cells are considered as a novel source for cell therapy of a broad spectrum of neurodegenerative disorders. This review presents the current status of human, rat, and mouse HFSCs from both the cellular and molecular biology and cell therapy perspectives. The first section of this review highlights the importance of HFSCs and *in vitro* differentiation, while the final section emphasizes the significance of cell differentiation *in vivo*.

Key words: Hair follicle; Stem cells; Bulge area; Neuron; Differentiation

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Core tip: Hair follicle stem cells (HFSCs) can proliferate *in vitro* and retain the label for a long time. Various types of stem cells, including epidermal-neural crest stem cells, nestin-positive, keratin 15-negative cells, and CD34-positive cells have been demonstrated in

hair follicles. HFSCs normally give rise to keratinocytes, sebocytes, and transient amplifying cells *in vivo*. In addition, these cells differentiate into ectodermal lineages including oligodendrocytes, astrocytes, and neurons. Neural cells derived from HFSCs can replace lost cells in neurodegenerative diseases. Their easy accessibility along with their potential for neural differentiation makes HFSCs an ideal stem cell source for treatment of neurodegenerative disorders.

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INTRODUCTION

Skin stem cells reside in the stratum basale of the epidermis, sebaceous glands, and bulge area of the hair follicles. A promising start to derive hair follicle stem cells (HFSCs) was made in the early 1990s, when it was found that label-retaining cells were located in the upper portion of the hair follicle (bulge area). Subsequently, it was clarified that during the early anagen phase, the bulge cells grew downward in response to the stimulating factors of the dermal papilla cells and formed half of a hair follicle, which was degenerated during the catagen phase. Studies on hair follicles suggest that mouse HFSCs occupy a relatively fixed location in the hair follicle called the bulge area, which is important for hair follicle cycling^[1]. HFSCs continuously supply new cells to the bulb during the anagen phase^[2], and have the ability to differentiate into most of the ectodermal lineages^[3]. These characteristics make them a promising cell source for grafting in various skin and nervous system diseases^[4].

Kobayashi *et al.*^[5] has shown that the bulge area of rat whisker follicles contains colony-forming stem cells that can be expanded continuously on the feeder cell layer and form 95% of the total colonies. Moreover, the bulge cells form larger colonies than the non-bulge keratinocytes^[6]. HFSCs have been seen to be able to differentiate either *in vitro* or *in vivo* upon grafting into a spinal cord injury (SCI)^[7,8] and Alzheimer disease (AD)^[9] animal models. The isolation of human HFSCs has generated tremendous interest, because the information gained from the study of human HFSCs, particularly those regarding cell differentiation, is of particular importance^[10].

The neural potential of HFSCs was first reported by Sieber-Blum *et al.*^[11] and Amoh *et al.*^[12] who demonstrated that the population of HFSCs could be converted into Schwann cells after pretreatment with neuregulin-1 or upon transplantation into a severed

sciatic nerve and SCI. Since then, many studies used inducers for neural differentiation of HFSCs, including neurotrophin-3 (NT-3)^[13], a glial cell line-derived neurotrophic factor (GDNF), a brain-derived neurotrophic factor (BDNF)^[14], a serum-free medium, *all trans* retinoic acid (RA), and other chemical neural inducers^[15].

In this review, we have summarized the progress achieved in both the proliferation and neural lineage differentiation of HFSCs. Research on the application of stem cells for the treatment of neurodegenerative diseases such as peripheral nerve lesions, SCI, and AD has created a growing interest in the field of biology. Ultimately, a good knowledge of the wide variety of cell differentiation will provide the therapeutic application of HFSCs in degenerative nervous system diseases and skin pathologies.

MAINTAINING HFSCS

HFSCs were initially identified in the bulge area of rat hair follicles^[5]. In 1990, Cotsarelis *et al.*^[1] suggested that follicular stem cells retain the label and are slow cycling. Similarly, Morris *et al.*^[16] demonstrated that bulge cells are quiescent and maintain a label for a long time following induction of anagen. The label-retaining cells divide asymmetrically into both identical copies of themselves and transient amplifying cells^[1,17]. The initial HFSCs culture is established and maintained by a co-culture, with a feeder layer of 3T3-J2 fibroblasts^[5]. Subsequent studies have revealed that the epidermal growth factor (EGF) and a basic fibroblast growth factor (b-FGF) are the feeder molecules that play a significant role in the maintenance and proliferation of these cells^[18,19]. HFSCs can also be grown in a medium supplemented with EGF, cholera toxin, insulin, and hydrocortisone^[13]. Sieber-Blum *et al.*^[11] suggests that epidermal neural crest stem cells (Epi-NCSCs) are pluripotent stem cells residing in the bulge area and can also be expanded continuously in the presence of an alpha-modified MEM medium, 5% chick embryo extract, and 10% of fetal calf serum. With these new culture conditions, it is now possible to grow HFSCs with defined factors in the absence of a feeder cell layer.

CELL SURFACE MARKERS

HFSCs can be routinely isolated from hair follicles and expanded *in vitro* to cell populations that are similar to the adult stem cells in respect to morphology and cell surface markers. In fact, numerous subsets of stem cells with varying differentiation potentials have been demonstrated in hair follicles, such as, Epi-NCSCs^[9,11], nestin-positive, and keratin 15-negative cells^[20], CD34-positive cells^[15,21], and CD200 -positive cells^[22].

Mouse bulges cells express specific markers including CD34 and K19, as well as CD200 and

K15^[23]. Expression of CD200 protects the bulge area from inflammation and hair loss in alopecia areata^[24] and these cell surface protein is the best positive marker for the human bulge stem cells^[22]. A central problem is the lack of a specific and reliable marker for the identification of HFSCs. Among the different markers, CD34 is possibly the most promising marker for HFSCs^[25]. Previously, bulge cells were isolated from mouse, rat, and human cells by manual dissection, but the purity of the isolated cells was unclear. Several years ago, Ohyama *et al.*^[22] used laser capture microdissection for precise bulge cell isolation. Now different isolation methods for bulge stem cells are accessible using CD34 as a hair follicle stem cell surface marker, by fluorescence-activated cell sorting (FACS)^[26] and magnetic activated cell sorting (MACS)^[8,27]. Therefore, the precise isolation of HFSCs having the ability to differentiate into many different cell types has generated a promising field for therapeutic application.

Some bulge stem cells are K15-positive and nestin-negative and differentiate into keratinocytes^[28]. More recently, Snippert *et al.*^[29], reported Lgr6 expression in HFSCs, which differs from Lgr5/CD34⁺ HFSCs. Lgr6⁺ HFSCs particularly express Sca-1, $\alpha 6$ -integrin, $\beta 1$ -integrin, sox9, and Lhx2, and contribute to wound repair. Sieber-Blum *et al.*^[7], showed that the other type of HFSCs express neural crest genes as well as neural stem cell markers, indicating a neural crest origin and neural differentiation potential. The other types of HFSCs in mouse hair follicles are called nestin-positive stem cells. These cells express some stem cell markers such as CD34, and can differentiate into non-follicular cell types, such as, neural cells^[30], which are maintained in the presence of FGF2 and a low concentration of B27^[20]. Nestin-positive cells exhibit a colony forming potential *in vitro* and are small oval or round shaped, with a dendrite-like structure. At the anagen stage, nestin-expressing cells form the lower part of the hair follicle^[30].

MULTIPOTENCY OR PLURIPOTENCY

This is an open question of whether bulge stem cells isolated from the hair follicle are pluripotent or multipotent cells and whether truly pluripotent cells can be isolated from the hair follicle by expansion, *in vitro*. HFSCs are considered to be multipotent cells because they differentiate into ectodermal derivatives such as neural cells, astrocytes, oligodendrocytes, and Schwann cells^[14], as well as mesodermal lineages, including chondrocytes^[11], myocytes^[31], adipocytes, and osteocytes^[17]. However, the expression of differentiation markers is often insufficient to conclude whether a cell has converted to a new state of differentiation^[32]. Bulge cells can proliferate and differentiate into the bulb keratinocytes at the onset of the anagen phase^[2,33]. In addition it is shown that these

stem cells can also emigrate and continuously provide progenitors, regenerating the epidermis and sebaceous gland^[34-36].

EP-NCSCs derived from the mouse vibrissae hair follicle express neural stem cell markers, such as Nanog and Oct4^[37]. The neural crest transcription factors such as nestin, Slug, Snail, Twist, Sox9, Sox10, Wnt-1 and BMP4 play essential roles in maintaining the undifferentiated state of bulge cells^[11,31]. The role of these transcription factors in neural crest stem cell renewal has been recently reviewed^[38,39] and we are not going to discuss further. Together, HFSCs has an extensive developmental potential, however, it is necessary to evaluate the multipotency of HFSCs by differencing, *in vivo*, after cell grafting. For this purpose, HFSCs are transplanted into the skin^[40], SCI^[8], peripheral nerve injury and AD^[9] models. The results suggest that HFSCs have extensive developmental potential. Further studies are required to determine whether the population of HFSCs can give rise to endodermal derivatives. Finally, the pluripotency of all subpopulations of HFSCs remains an unproven concept, but it seems that only Epi-NCSCs express two pluripotency factors, such as, Oct-4 and nanog^[11].

IN VITRO NEURAL DIFFERENTIATION OF HFSCS

HFSCs attract attention not only in dermatology, but also in the treatment of nervous system diseases. It has been shown that HFSCs retain the ability to generate differentiated progeny *in vitro*. Many differentiation approaches have been optimized using growth factors and other inducing factors. These protocols can also successfully generate certain lineages. HFSCs can be induced to produce an enriched population of glial and neuronal lineages following incubation in exogenous growth factors^[14]. On account of the diversity of HFSCs, differentiation and the proliferative potential of HFSCs is strongly dependent on the exogenous growth factors. Neuroregulin-1 has been found to be required for differentiation of HFSCs to Schwann cell and β -III tubulin positive neural cells. The Epi-NCSCs can also differentiate into smooth muscle cells in response to bone morphogenetic protein-2 (BMP-2)^[11].

Of late, Ghoroghi *et al.*^[13], isolated rat HFSCs and cultured them on a poly-L-lactic acid (PLLA) scaffold to provide a suitable microenvironment for neural differentiation, and then used NT-3 to induce neural differentiation in the HFSCs.

New protocols have been developed to generate neural cells from HFSCs. Of late, we have isolated CD34⁺ cells from the mouse hair follicle, using MACS, and then we applied RA, serum-free medium, and chemical treatments such as β -mercaptoethanol (BME), butylated hydroxyanisole (BHA), and dimethyl sulfoxide

(DMSO), for the neural differentiation in these cells. We have found that the serum-free condition and 1 $\mu\text{mol/L}$ RA appropriately triggered neurogenesis in HFSCs^[15]. In addition, it seems that prolonged serum deprivation induces formation of the ectodermal derivatives^[41]. However, chemical treatments with DMSO, BME, BHA, and potassium chloride (KCL) induced rapid changes in cell morphology, which led to the CD34⁺ cells dying within seven days of treatment^[15]. It will be important to determine whether the neural cells derived from HFSCs possess the functional characteristics of neurons.

Therapeutic application of HFSCs

HFSCs have advantages and disadvantages regarding the potential use of cell-based regenerative therapies. The main potential advantage of the HFSCs is that they are easily accessible adult stem cells, which have ectodermal differentiation potential^[15]. Moreover, HFSCs are attractive tools for future burn and neurodegenerative disease treatments.

HFSCs are similar to embryonic stem cells and have a high proliferative potential *in vitro*. Similar to other types of adult stem cells, they do not form tumors. Without a doubt, autologous transplantation and avoiding immune rejection has been demonstrated in HFSC-treated animal studies^[7,12,42].

HFSCs have the properties to differentiate into various neural cell types including neurons, astrocytes, oligodendrocytes, and Schwann cells. Among them some specific cell types can be produced in high purity, such as, Schwann cells, motor neurons, and oligodendrocytes, which are the main cells that degenerate in SCI, peripheral nerve lesions, and AD. One of the goals of the researchers in this field is to generate neural cells from stem cells, with the aim of replacing lost tissue in degenerative disorders. Therefore, we have used HFSCs as an alternative cell source for cell therapy in nervous system diseases. AD is one of the most common neurodegenerative disorders affecting about four million people throughout the world each year. AD brains have been characterized by amyloid β peptide plaque and neurofibrillary tangle formation, which leads to axonal transport defects and synaptic loss responsible for cholinergic neuron degeneration^[43].

HFSCs have the potential to constitute an anatomical nervous system structure. Thus, in the case of an AD model, these cells are capable of replacing the degenerated neurons. In a recent study, researchers transplanted a subtype of HFSCs (Epi-NCSCs) to differentiate into cholinergic neurons in the AD rat model. These stem cell-derived cholinergic neurons improved learning and memory deficits in the AD model. In spite of rare studies on the therapeutic effects of Epi-NCSCs in an AD model^[9], it will be possible to modify HFSCs to deliver neurotropic factors that modify the course of the disease, in the future.

It is well known that SCI induces paralysis in

about 2.5 million people, affecting the patients and society. Unfortunately, no effective treatment has been discovered for SCI to date^[44]. However, some studies are under way to promote regeneration and neural repair in patients with SCI^[45]. Cell therapy plays a major role in axonal regeneration and neuronal replacement in AD^[9], SCI, and other nervous system diseases. Potential strategies to repair neurodegenerative diseases include neuronal and glial replacement, axonal regeneration and remyelination, and increased production of neurotrophins by grafted cells^[46,47]. Various cell types have been assessed to repair central and peripheral nervous system disorders, such as, fetal tissue, olfactory ensheathing glia, Schwann cells, skin derived precursors, mesenchymal stem cells, and HFSCs^[48]. To this end, in this part of the review, we have focused on the transplantation of HFSCs in SCI.

Preclinical studies have shown a novel type of HFSCs known as Epi-NCSCs, which survive and differentiate into β III-tubulin, glutamic acid decarboxylase (GAD67), RIP positive, and myelin basic protein-positive neural cells, but not Schwann cells, when transplanted into the contusion model of the SCI; the cells can be even integrated with host neurites in the contused spinal cord. However, the transplanted cells do not form tumors^[7]. In another study, extensive Schwann cell differentiation of nestin-positive HFSCs has been reported after transplantation into a severed spinal cord. They have found that the differentiated cells facilitated the repair of SCI and promoted hind limb function recovery^[49]. Likewise, when rat HFSCs are transplanted into a compression model of SCI, they generate oligodendrocytes and neuron-like cells expressing RIP and β -III tubulin, respectively. Walking scale, limb coordination, and plantar stepping improved following HFSC transplantation^[8]. Consistent with this study, Hu *et al.*^[50], also demonstrated that unilateral bulge-derived Epi-NCSC transplantation into a contused spinal cord promoted a 24% recovery in sensory connectivity and touch perception. Moreover, some grafted Epi-NCSCs differentiated into functional motor neurons. *In vivo* studies using Epi-NCSCs isolated from the mouse hair follicle showed similar results^[7]. Briefly, these studies showed the safety of HFSC transplantation and enhanced recovery in animal models of SCI.

Damage to the peripheral nerve interrupts the axonal pathways and causes partial or total loss of motor, sensory, and autonomic functions. An important aim for the ongoing research is the development of therapeutic strategies that enhance axonal regeneration and replace lost neural cells, to bridge the gap of the lesion^[51].

HFSCs possess a self-renewal ability and neural differentiation potential. Amoh *et al.*^[12,52] transplanted HFSCs in the severed sciatic nerves of mice. Eight weeks after grafting, many spindle cells grew in the severed sciatic nerve rather than in the control, differ-

entiated extensively into Schwann cells, and promoted motor function.

In another study, extensive neuronal differentiation of the nestin-expressing cells was reported after transplantation of RA-pretreated cells into the transected distal sciatic nerve. In this study, the nestin-positive bulge cells grew in the distal sciatic nerve stump and caused locomotor recovery owing to the presence of many Tuj1, Is1^{1/2}, and EN1-positive cells and nerve fibers. Muscle atrophy was also reduced after grafting, and re-innervation was promoted^[53]. HFSCs transplanted to the subcutis and severed sciatic nerve also differentiated into blood vessels^[40] and Schwann cells^[12], respectively. It seemed that the differentiation of HFSCs supported neural regeneration and motor function of the lower extremity muscles, such as, the gastrocnemius^[12].

There are still many problems that need to be resolved before HFSCs can be widely used clinically. The main problem is that approximately only a few neural cells derive from these stem cells *in vivo* and further studies are required to determine whether the differentiated neurons are functionally integrated into the AD brain, SCI, and peripheral nerve lesion tissues. Some other forms of cell differentiation, such as keratinocytes, myocytes, and melanocytes are the other disadvantages of HFSC transplantation in neurodegenerative disorders. The successful directed differentiation of HFSCs into specific neurons with the help of different neural induction methods may make neural transplantation widely available for neurodegenerative disorders at some point in the future.

Collectively, these results indicate that HFSCs can promote the recovery of peripheral nerve injury, SCI, and AD. Thus, these cells have the desirable properties of neural replacement and remyelination.

CONCLUSION

In summary, we have explained that the hair follicle contains various multipotent stem cells. The ability of HFSCs to proliferate *in vitro* and the directed differentiation toward ectodermal and mesodermal lineage cell types render them potential for clinical application. These include the modeling of SCI, sciatic nerve injury, and AD, studies of cell therapy on progression, and amelioration of symptoms.

At present, HFSC transplantation provides the best chance of cure for many diseases. Future studies seek to characterize the signaling genes that regulate stem cell behavior.

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Basic Study

Utility of tricalcium phosphate and osteogenic matrix cell sheet constructs for bone defect reconstruction

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Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Nara Medical University.

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Abstract

AIM: To determine the effects of transplanting osteogenic matrix cell sheets and beta-tricalcium phosphate (TCP) constructs on bone formation in bone defects.

METHODS: Osteogenic matrix cell sheets were prepared from bone marrow stromal cells (BMSCs), and a porous TCP ceramic was used as a scaffold. Three experimental groups were prepared, comprised of TCP scaffolds (1) seeded with BMSCs; (2) wrapped with osteogenic matrix cell sheets; or (3) both. Constructs were implanted into a femoral defect model in rats and bone growth was evaluated by radiography, histology, biochemistry, and mechanical testing after 8 wk.

RESULTS: In bone defects, constructs implanted with cell sheets showed callus formation with segmental

or continuous bone formation at 8 wk, in contrast to TCP seeded with BMSCs, which resulted in bone non-union. Wrapping TCP constructs with osteogenic matrix cell sheets increased their osteogenic potential and resulting bone formation, compared with conventional bone tissue engineering TCP scaffolds seeded with BMSCs. The compressive stiffness (mean $\pm$ SD) values were 225.0 ± 95.7 , 30.0 ± 11.5 , and 26.3 ± 10.6 MPa for BMSC/TCP/Sheet constructs with continuous bone formation, BMSC/TCP/Sheet constructs with segmental bone formation, and BMSC/TCP constructs, respectively. The compressive stiffness of BMSC/TCP/Sheet constructs with continuous bone formation was significantly higher than those with segmental bone formation and BMSC/TCP constructs.

CONCLUSION: This technique is an improvement over current methods, such as TCP substitution, and is useful for hard tissue reconstruction and inducing earlier bone union in defects.

Key words: Bone marrow stromal cells; Osteogenesis; Bone regeneration; Tissue engineering; Calcium phosphate

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Core tip: The treatment of bone defects is a common clinical problem for orthopedic surgeons. Artificial bone combined with cultured bone marrow stromal cells (BMSCs) has been examined and applied for the treatment of clinical cases such as small bone defects. In the present study, we developed constructs of BMSCs and beta-tricalcium phosphate (TCP) combined with osteogenic matrix cell sheets (BMSC/TCP/Sheet constructs), which showed a vigorous osteogenic potential compared with conventionally engineered bone tissue structures consisting of BMSC/TCP constructs. The BMSC/TCP/Sheet constructs will be useful for reconstruction of hard tissues, such as bone defects, as new tissue-engineered bone.

Ueha T, Akahane M, Shimizu T, Uchihara Y, Morita Y, Nitta N, Kido A, Inagaki Y, Kawate K, Tanaka Y. Utility of tricalcium phosphate and osteogenic matrix cell sheet constructs for bone defect reconstruction. *World J Stem Cells* 2015; 7(5): 873-882 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i5/873.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i5.873>

INTRODUCTION

The treatment of bone defects is a common clinical problem for orthopedic surgeons. Autologous bone grafts including vascularized bone grafts are performed for treatment of osteonecrosis and bone defects in clinical practice^[1,2]. However, it is necessary to sacrifice intact bone such as the pelvis or fibula. Moreover, there is limited bone available for autologous transplantation.

Artificial bone such as hydroxyapatite (HA) and beta-tricalcium phosphate (TCP) can be applied as a scaffold for the treatment of small bone defects. Although these biomaterials provide a physical matrix for deposition of new bone and may guide the extension of bone, they do not induce ectopic bone formation^[3]. To achieve osteogenesis in artificial bone, constructs of artificial bone combined with cultured bone marrow stromal cells (BMSCs), which are designated as engineered bone tissue structures^[4-6], have been examined and applied for the treatment of clinical cases such as avascular necrosis of the femoral head and benign tumors^[7-9]. However, some cases require a long period of time to replace autologous bone, which subsequently delays bone union.

Previously, we developed a mechanical retrieval method to fabricate cell sheets of cultured BMSCs with osteogenic potential, designated osteogenic matrix cell sheets, that could be transplanted without a scaffold, and resulted in bone formation at subcutaneous sites^[9-12]. The utility of transplantation of engineered bone tissue structures for hard tissue reconstruction will increase if such treatment can achieve sufficient bone formation for early bone union after transplantation. In our previous study, we reported that a construct of osteogenic matrix cell sheets and artificial bone results in extensive bone formation compared with a construct containing a BMSC suspension. A construct with osteogenic matrix cell sheets allows bone formation inside of the pores as well as outside of the artificial bone. Outside bone formation can unite two artificial bones by bridging bone formation^[11]. These results suggested that transplanting a combination of osteogenic matrix cell sheets and engineered bone tissue structures may advance bone formation for reconstruction of bone defects. Marcacci *et al*^[9] reported the clinical outcomes of long bone repair using macroporous bioceramics with BMSCs. However, they used a BMSC suspension to prepare BMSC/bioceramic composites. Therefore, in the present study, we prepared TCP constructs with or without osteogenic matrix cell sheets and assessed their osteogenic potential after transplantation subcutaneously and at bone defect sites. We compared the bone formation patterns between TCP constructs with or without osteogenic matrix cell sheets.

MATERIALS AND METHODS

Preparation of bone marrow cells

The experimental procedures were approved by the Animal Experimental Review Board of Nara Medical University. Male Fisher 344 (F344) rats were purchased from Japan SLC (Shizuoka, Japan) for use as donors and recipients. The method for bone marrow cell preparation has been reported previously^[12-14]. Briefly, bone marrow cells were obtained from the femoral shafts of 7-wk-old F344 rats by flushing with 10 mL of

culture medium. The cells were collected in two T-75 flasks (Costar, Cambridge, MA, United States) containing 15 mL of minimal essential medium (MEM; Nacalai Tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, United States) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Nacalai Tesque), and incubated at 37 °C in a humidified atmosphere with 5% CO₂. At confluency (approximately day 14), the primary cultured cells were harvested using a 0.25% trypsin/EDTA solution (Nacalai Tesque) to obtain in a cell suspension.

Cell sheet preparation

To prepare an osteogenic matrix cell sheet, a cell suspension was seeded at a density of 1×10^4 cells/cm² in a 100-mm cell culture dish (100 × 20 mm; BD Falcon, NJ, United States) and cultured with medium containing 10 nmol/L dexamethasone (Dex; Sigma, MO, United States) and 82 µg/mL ascorbic acid phosphate (AsCP; L-ascorbic acid phosphate magnesium salt n-hydrate; Wako Pure Chemical Industries, Kyoto, Japan) until confluency (approximately 2 wk)^[10,13]. The cells were then rinsed twice with phosphate-buffered saline (PBS; Gibco), and the sheet was lifted by a mechanical retrieval method using a scraper. Because the osteogenic matrix cell sheet was sufficiently robust to handle with a scraper and forceps, handling procedures for the cell sheet were performed easily, such as combination of the cell sheet with TCP. Although β-glycerophosphate (β-GP) was added to create osteogenic matrix cell sheets in our initial study^[10], recent investigations have revealed that osteogenic matrix cell sheets with high osteogenic potential can be prepared without β-GP^[15,16]. Therefore, the osteogenic matrix cell sheets in the present study were created without β-GP.

Construction of TCP containing BMSCs

Sterilized porous TCP ceramics (Osferion; disks: 5 mm diameter and 2 mm thick; cylinders: 5 mm outer diameter, 1 mm inner diameter, 5 mm in length, and 60% porosity) were provided by Olympus Terumo Biomaterials (Tokyo, Japan). After immersion in MEM, the air bubbles in the TCP were aspirated and each ceramic was soaked in a cell suspension (1×10^6 cells/mL) for 2 h in a CO₂ incubator at 37 °C^[13]. The constructs were then cultured with osteoinductive supplements including Dex, AsCP, and 10 mmol/L β-GP (Sigma) for 2 wk in a CO₂ incubator at 37 °C to obtain BMSC/TCP constructs.

Experimental groups

We prepared three experimental groups according to the type of implant received. The C group received BMSC/TCP constructs, the S group received TCP combined with an osteogenic matrix cell sheet (Sheet/TCP constructs), and the SC group received BMSC/

TCP constructs combined with an osteogenic matrix cell sheet (BMSC/TCP/Sheet constructs). Constructs with an osteogenic matrix cell sheet were prepared by simply wrapping the sheet around the constructs just prior to transplantation. The TCP ceramics used for subcutaneous transplantation and bone defect model experiments were the disk and cylinder types, respectively.

Subcutaneous implantation of constructs

Constructs with a TCP disk were implanted subcutaneously onto the back of syngeneic 7-wk-old male rats using a previously described procedure^[10,17]. Each group included six disks for one recipient rat as the subcutaneous implantation model. After 4 wk, the implanted constructs were harvested and evaluated radiographically, histologically, and biochemically. For radiographic and histological evaluations, the harvested disks were fixed in buffered formalin (Wako Pure Chemical Industries). After obtaining X-ray images, each disk was decalcified with a KCl solution (K-CX solution; Falma, Tokyo, Japan), embedded in paraffin, cut at the middle of the disk parallel to the base, and then stained with hematoxylin and eosin (HE) for histological evaluation.

Biochemical analysis

The alkaline phosphatase (ALP) activities and osteocalcin contents of harvested constructs were measured as described previously^[17,18]. Briefly, each disk was crushed and homogenized using a microhomogenizer in 1 mL of 0.2% Nonidet P-40 (NP-40), and then centrifuged at 12000 rpm for 10 min at 4 °C. A 10-µL aliquot of the supernatant was combined with 56 mmol/L 2-amino-2-methylpropanediol buffer containing 10 mmol/L p-nitrophenylphosphate and 1 mmol/L MgCl₂. After incubation for 30 min at 37 °C, 2 mL of 0.2 N NaOH was added to stop the reaction and the ALP activity was calculated by measuring the absorbance of p-nitrophenol released at 410 nm using a spectrophotometer.

After extraction with 0.2% NP-40 in 4 mL of 20% formic acid for 2 wk at 4 °C, osteocalcin was extracted from the sediment. A 0.5-mL aliquot of the formic acid extract was applied to a prepacked Sephadex G-25 column (NAP-5 column; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and eluted with 10% formic acid. The protein fractions were pooled and dried. After dissolution in 0.5 mL of ELISA sample buffer, the osteocalcin content was assayed using a Rat Osteocalcin ELISA Kit (DS Pharma Biomedical, Osaka, Japan)^[18].

Implantation of constructs into the bone defect model

Bone defects of the femur were created in F344 rats (300–350 g body weight) under anesthesia. Briefly, a lateral incision was made on the hind limb and the vastus muscle was divided longitudinally to expose the femur. The femoral shaft was removed from

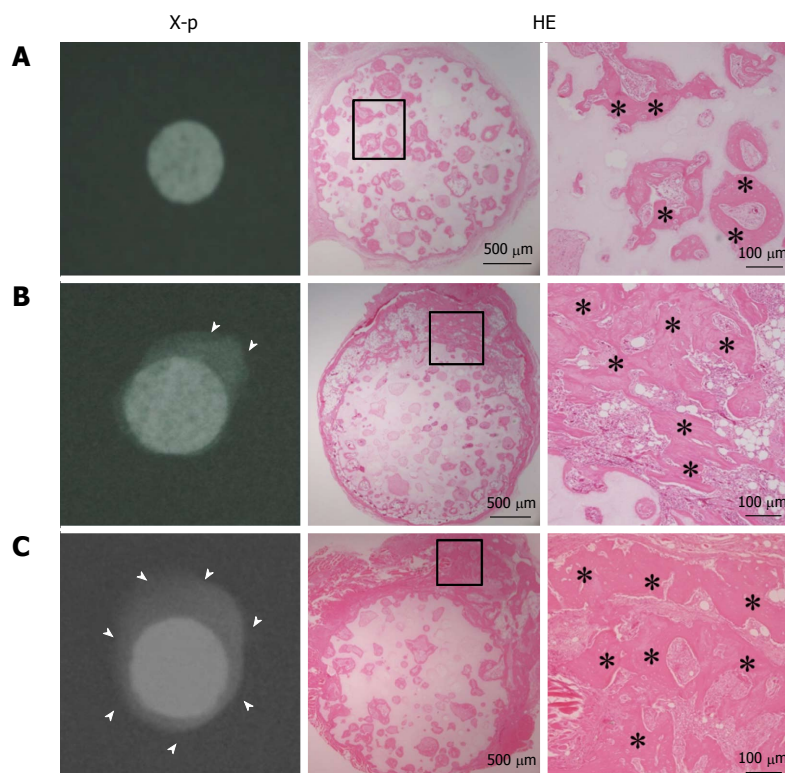


Figure 1 X-ray images and hematoxylin and eosin-stained sections at 4 wk post-implantation. A: BMSC/TCP constructs (C group); B: Sheet/TCP constructs (S group); C: BMSC/TCP/Sheet constructs (SC group). The S and SC groups showed abundant calcification around the TCP disk. Arrowheads indicate the area of calcification. Bone formation was observed histologically in the pores of the TCP disk in all groups. The S and SC groups showed bone formation on the surface of the disk. Higher magnification images of the boxed areas are shown at the right side of each panel. Asterisks indicate bone tissue. HE: Hematoxylin and eosin.

the distal lesser trochanter to a length of 10 mm to create a bone defect that was filled with a TCP-cylinder construct. The cylinder was fixed with an 18 G needle inserted into the intramedullary femoral shaft to achieve ridged fixation. A BMSC/TCP cylinder (BMSC/TCP construct) was transplanted into the bone defect of the left femur, while a BMSC/TCP cylinder with an osteogenic matrix cell sheet (BMSC/TCP/Sheet construct) was transplanted into the defect of the right femur. To evaluate bridging bone formation, X-ray images were taken under anesthesia at 2, 4, and 8 wk postoperatively.

Evaluation of bone formation in the bone defect model

At 8 wk post-transplantation, the rats were euthanized and both hind limbs were harvested. After removal of the intramedullary pins, micro-CT images were taken using a Microfocus X-ray CT system (SMX-160CTS; Shimadzu, Kyoto, Japan). Five of the harvested femurs were then dissected from the surrounding muscle, fixed in 10% formalin, decalcified with K-CX solution, and embedded in paraffin. For histology, the femurs were cut longitudinally and stained with HE. Eight of the harvested femurs were applied to compression testing using a universal testing machine (5566; Instron, Canton, MA, United States) equipped with a computer for data acquisition. The harvested femur was compressed longitudinally and the primary

yield point for fracture of the TCP was calculated. The crosshead speed was 10 mm/min. Compressive stiffness was calculated by dividing the compressive stress by the compressive strain in the linear portion to the primary yield point of the stress-strain curve.

Statistical analysis

The values for ALP activity, osteocalcin content, and compressive stiffness were calculated as the mean and SD. Statistical significance was determined by one-way ANOVA with post-hoc multiple comparisons using Tukey's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Subcutaneous implantation

Figure 1 shows the radiographic images and histology (HE staining) at 4 wk after subcutaneous implantation. Abundant calcification around the disks was observed in the radiographic images of the S and SC groups, whereas no calcification was observed in the C group. Histology showed bone formation in all harvested disks. Although the C group showed bone formation only in the pores of the ceramics, the S and SC groups showed bone formation in the pores and around the disks. Less bone formation appeared to be in the central area of the disks in the S group compared with

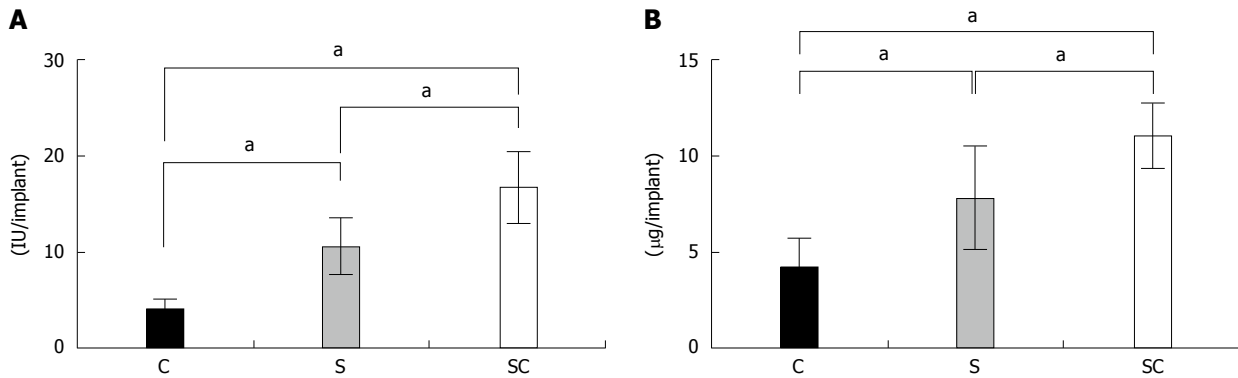


Figure 2 ALP activity (A) and osteocalcin content (B) of harvested constructs. The C, S, and SC groups indicate BMSC/TCP (black column), Sheet/TCP (gray column), and BMSC/TCP/Sheet (white column) constructs, respectively. Values are means $\pm$ SD ($n = 6$). ^a $P < 0.05$. BMSCs: Bone marrow stromal cells; TCP: Tricalcium phosphate.

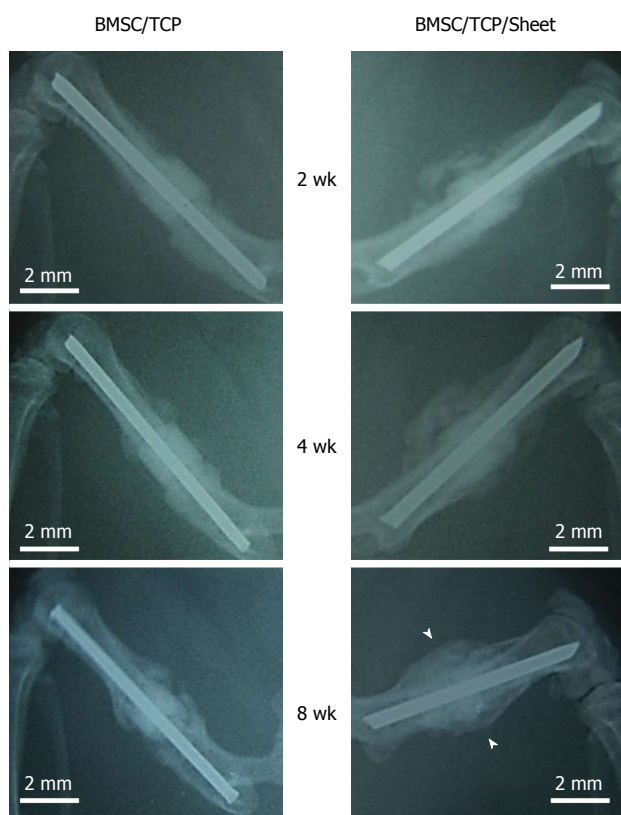


Figure 3 X-ray images of constructs implanted into femurs. The femoral shaft was removed and replaced with BMSC/TCP constructs with or without cell sheets. Callus formation appeared around the BMSC/TCP constructs with cell sheets (BMSC/TCP/Sheet) at 2 wk post-implantation, was extended at 4 wk, and continued to the host bone at 8 wk. In contrast, the BMSC/TCP constructs showed non-union at 8 wk. Arrowheads indicate continuous bone formation. Bar = 2 mm. BMSCs: Bone marrow stromal cells; TCP: Tricalcium phosphate.

the SC group.

The ALP activities (Figure 2A) and osteocalcin contents (Figure 2B) in the SC group were significantly higher than those in the other groups at 4 wk post-implantation. In the S group, these values were significantly higher than those in the C group.

Bone defect model

Radiographic images were taken at 2, 4, and 8 wk

post-implantation (Figure 3). At 2 wk, obvious callus formation was observed around the implanted constructs with osteogenic matrix cell sheets. At 8 wk post-implantation, callus formation had developed and bridging callus formation between host bones resulted in bone union. In contrast, no bridging callus formation was observed around constructs without osteogenic matrix cell sheets at 8 wk post-implantation.

Figure 4 shows the micro-CT images and histology of each construct implanted into femurs at 8 wk. Implanted BMSC/TCP/Sheet constructs showed bone formation around the TCP cylinder ($n = 8$). However, micro-CT revealed two patterns of bone formation, continuous bone formation to host bones (Figure 4A, $n = 4$) and segmental bone formation over the cylinder (Figure 4B, $n = 4$). The ratio of segmental and continuous bone formation among the samples was 1:1. We defined BMSC/TCP/Sheet constructs showing bridging bone formation between host bones across the TCP by micro-CT as continuous bone formation. Bone formation observed around the constructs, but not continuing to the host bones, was defined as segmental bone formation. Bone formation was not observed around implanted BMSC/TCP constructs ($n = 8$) in which soft tissue interposition between the cylinder and the host bone resulted in non-union (Figure 4C). Micro-CT also revealed that BMSC/TCP/Sheet constructs with either continuous or segmental bone formation were intact, whereas broken TCP cylinders were found in BMSC/TCP constructs (Figure 4C).

Figure 5 shows the results of biomechanical compression testing. The compressive stiffness (mean $\pm$ SD) values were 225.0 ± 95.7 , 30.0 ± 11.5 , and 26.3 ± 10.6 MPa for BMSC/TCP/Sheet constructs with continuous bone formation, BMSC/TCP/Sheet constructs with segmental bone formation, and BMSC/TCP constructs, respectively. The compressive stiffness of BMSC/TCP/Sheet constructs with continuous bone formation was significantly higher than those with segmental bone formation. The stiffness of constructs with segmental bone formation was similar to that of constructs without osteogenic matrix cell sheets (BMSC/TCP constructs).

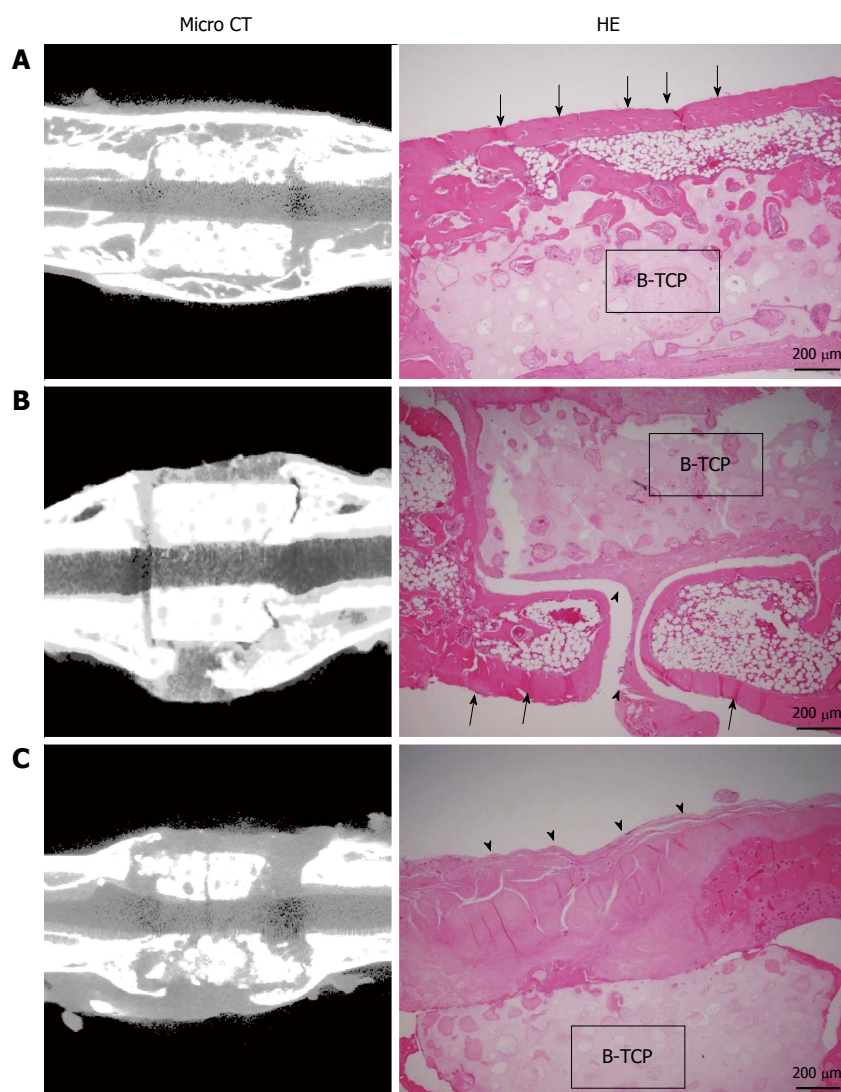


Figure 4 Micro-computed tomography images and hematoxylin and eosin-stained sections at 8 wk post-implantation. A: BMSC/TCP/Sheet constructs with continuous bone formation to host bone. Both micro-computed tomography (CT) and histology showed bridging bone formation over the TCP to host bone (arrows); B: BMSC/TCP/Sheet constructs with segmental bone formation over the TCP. Soft tissue interposition was observed between the TCP and the callus (arrowheads); C: BMSC/TCP constructs implanted into femurs. The TCP cylinders of the BMSC/TCP constructs were broken. Arrowheads indicate soft tissue interposition between the TCP and the autologous femur. BMSCs: Bone marrow stromal cells; TCP: Tricalcium phosphate.

DISCUSSION

The present study clearly demonstrated that the osteogenic potential of the BMSC/TCP/Sheet construct was significantly higher than that of the BMSC/TCP construct, namely a conventional engineered bone tissue structure. When the conventional engineered bone tissue structures were combined with an osteogenic matrix cell sheet, the newly formed bone area spread from the center to the surface of the TCP. We observed bridging bone formation over the TCP cylinder implanted between both ends of autologous bones. Moreover, biomechanical examination showed significantly higher strength against compressive stress in the BMSC/TCP/sheet construct with continuous bone formation to host bones, indicating that the BMSC/TCP/sheet construct may be useful for hard tissue reconstruction.

Some scaffolds such as TCP can be replaced with

autologous bone by bone remodeling after implantation into bone defects^[19]. Although osteogenesis does not occur in artificial bone itself, HA and TCP are common scaffolds for artificial bone in hard tissue reconstruction^[20]. Ohgushi *et al.*^[21] and Akahane *et al.*^[22] reported a transplantation technique using artificial bone combined with cells. The cells combined with artificial bone can be freshly prepared bone marrow cells or cultured mesenchymal stem cells (MSCs). In clinical cases, differentiated osteoblasts have been used in combination with scaffolds for the treatment of patients with osteoarthritis and osteonecrosis^[8,9,23]. When a BMSC suspension is combined with such scaffolds, bone formation is observed but only in the pores of the artificial bone. Therefore, when artificial bone is combined with BMSCs for transplantation into a bone defect, soft tissue interposition between the construct and the host bone might occur and result in non-union.

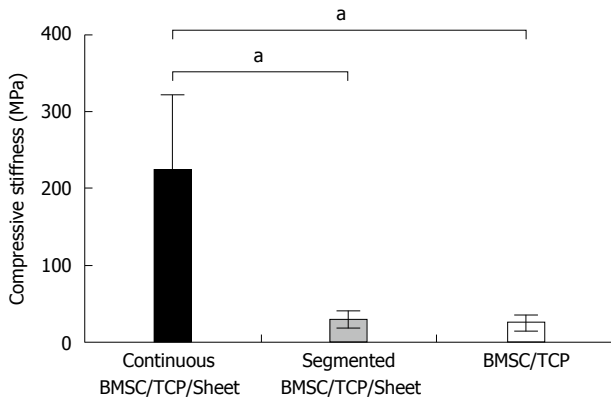


Figure 5 Results of biomechanical compression testing. The stiffness was highest in the BMSC/TCP/Sheet constructs with continuous bone formation (black column), while the stiffness of the BMSC/TCP/Sheet constructs with segmental bone formation (gray column) was similar to that of the BMSC/TCP constructs (white column). Values are means $\pm$ SD. ^a $P < 0.05$. BMSCs: Bone marrow stromal cells; TCP: Tricalcium phosphate.

Several studies have reported tissue-engineered bone combined with BMSCs for reconstruction of bone defects^[24-26]. Kadiyala *et al.*^[26] showed that a critical-size segmental defect of the femur in a canine model can be healed by transplantation of culture-expanded cell-loaded artificial bone. However, defects that received no implant, cell-free artificial bone, or artificial bone loaded with fresh marrow failed to heal at 8 wk. Dallari *et al.*^[25] reported critical-size defect reconstruction using BMSCs, platelet-rich plasma (PRP), and freeze-dried bone allografts alone and in combination. They concluded that the combination of freeze-dried bone allografts, BMSCs, and PRP accelerates bone healing and remodeling processes. However, the bone healing was evaluated by radiology, histology, and histomorphometry in these previous studies. In contrast, we evaluated bone reconstruction by mechanical testing (compression testing) as well as micro-CT, radiology, and histology because the mechanical strength of the reconstructed site is an important clinical outcome.

The starting cell numbers were different for the three groups in this study. It would be very difficult to control the cell numbers because of the different modalities used for construct preparation. Considering clinical cases of bone defect reconstruction, tissue-engineered bone with a vigorous osteogenic potential should be used for better clinical outcomes. Therefore, we first evaluated the osteogenesis of the three types of constructs, BMSC/TCP, Sheet/TCP, and BMSC/TCP/Sheet, using the subcutaneous implantation model to determine the highest osteogenic construct, and found that the BMSC/Sheet/TCP construct had the most vigorous osteogenic potential. The number of cells that can be combined with artificial bone materials like TCP is limited because suspended cells remain in the pores of the TCP. In contrast, cell sheets can be wrapped around TCP, resulting in a large amount of loaded cells on the TCP. Moreover, the Sheet/TCP

constructs showed bone formation on the surface of the construct, which is greatly advantageous for bridging bone formation.

The bone union of conventionally engineered bone tissue structures was improved by combining the construct with an osteogenic matrix cell sheet. The BMSC/TCP construct with an osteogenic matrix cell sheet (BMSC/TCP/Sheet construct) had an excellent ability to form bone, as indicated by bone formation on the TCP surface as well as in the pores of the central area. Bone formation around the TCP was continuous with the host bone when BMSC/TCP constructs with osteogenic matrix cell sheets were implanted into bone defects of the femur. This finding may have occurred because the BMSC suspension entered the pores of the central area and the osteogenic matrix cell sheet was located on the surface of the TCP ceramic. The subsequent combination of osteogenesis by the BMSC suspension and osteogenic matrix cell sheet resulted in vigorous bone formation in the subcutaneous and bone defect sites. Prevention of soft tissue invasion and achieving early bone formation at the bone defect site is clinically important for reconstruction of bone tissue. Accordingly, our transplantation technique using constructs with osteogenic matrix cell sheets is useful because the defect is filled with the osteogenic matrix cell sheet to prevent the invasion of soft tissue, resulting in early bone formation.

MSCs have the potential to replace or regenerate damaged and diseased tissue by multipotent differentiation into osteogenic, chondrogenic, and adipogenic cells among others^[4,7,27]. Recently, although the detailed molecular mechanisms remain unclear, it was reported that MSCs, including BMSCs, can interact with cells of the immune system and modulate immune responses *in vitro* and *in vivo*^[28,29]. MSCs can drive immune responses toward immunosuppression and anti-inflammation. In the present study, we used TCP constructs to repair bone defects in the femur. Scaffolds such as TCP might cause inflammatory responses when they are transplanted *in vivo*^[30]. However, the transplantation model in the present study used a combination of TCP ceramics and cell sheets created from BMSCs, and therefore inflammatory responses could be reduced by the anti-inflammatory function of BMSCs. Further studies are necessary to clarify this point.

We used an osteogenic matrix cell sheet created in a 100-mm cell culture dish to prepare BMSC/TCP/Sheet constructs. When BMSC/TCP/Sheet constructs were transplanted into bone defects of the femur, half of the constructs (four of eight constructs) formed a segmental callus. Similar to the TCP cylinders, the mechanical properties of constructs with segmental bone were still fragile. This result indicates that coverage of the entire TCP cylinder with an osteogenic matrix cell sheet is important for continuous bone formation around the TCP cylinder. Two or more sheets

or a larger sheet can be used to cover the TCP surface and increase the compressive stiffness. Further studies will establish the numbers of sheets needed to cover certain sizes of TCP cylinders.

A thermosensitive cell culture plate is commonly used to fabricate cell sheets^[31]. Memon *et al.*^[32] used culture dishes coated with thermosensitive polymers to fabricate cell sheets for implantation into a heart disease model and for clinical treatment of corneal surface dysfunction^[33]. However, other methods have been reported, such as those using magnetite nanoparticles and magnetic force^[34]. Anil Kumar *et al.*^[35] reported a method for preparation of cell sheet/HA constructs. In their study, they showed rapid and complete cellularization of HA by wrapping it with a cell sheet. Gao *et al.*^[36] reported the results of osteogenic cell sheet transplantation using rabbit MSCs. They used osteogenic cell sheets to generate bone grafts, but did not find any bone formation on the outer HA surface in their model. We^[10,13] and others^[24,37] reported a method to prepare osteogenic cell sheets cultured in osteogenic medium. Our mechanical retrieval method only requires the use of regular cell culture equipment, including a cell scraper, to fabricate the osteogenic matrix cell sheet, in which cells form the sheet during culture with Dex and AscP. On the other hand, the methods of Anil Kumar *et al.*^[35] and Gao *et al.*^[36] require specialized culture dishes coated with thermosensitive polymers. The osteogenic matrix cell sheets used in this study were sufficiently robust to handle with forceps. The cell sheet could be easily handled for processes such as combination with artificial bone, and has osteogenic potential for osteoblastic differentiation induced by Dex and AscP. Therefore, we propose that this technique can be applied to bone defects that occur because of tumor resection or wounding.

In our study, there are a few limitations to be acknowledged. First, we only used TCP cylinders of 5 mm in length. We need to conduct further studies with longer TCP cylinders to assess their bone formation ability for cell sheet transplantation. Second, the experimental period in the present study was relatively short. Therefore, a further follow-up study is needed to determine whether the implanted TCP is replaced by autologous bone. Third, the TCP was transplanted into a bone defect site that was created by bone resection. We need to assess whether the cell sheet can form bone tissue when it is applied to a bone defect in a region with a poor blood supply, such as a large bone defect with soft tissue injury after severe trauma. Finally, we need to show the osteogenic ability using human BMSCs for future clinical application.

In conclusion, the present study clearly demonstrates that constructs of BMSCs and TCP combined with osteogenic matrix cell sheets show a vigorous osteogenic potential compared with conventionally engineered bone tissue structures consisting of a BMSC/TCP construct. Because bridging bone formation over the

implanted TCP can unite the ends of the cylindrical TCP to autologous bone, the construct will be useful for hard tissue reconstruction as new tissue-engineered bone.

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COMMENTS

Background

Bone defects are difficult to treat. Effective treatment options for bone defects have been reported that include tissue engineering of bone tissue structures using bone marrow stromal cells (BMSCs). Recently, many researchers have reported methods for creating cell sheets, such as thermo-responsive polymer-grafted culture dishes and mechanical retrieval.

Research frontiers

Tissue engineering for regenerative medicine using cell sheets are becoming much more common. The transplantation of cell sheets created from BMSCs will most likely be incorporated into clinical cases in the near future. Osteogenic matrix cell sheets have the potential to greatly advance the treatment of critical size bone defects.

Innovations and breakthroughs

Constructs comprised of BMSCs, tricalcium phosphate (TCP) and osteogenic matrix cell sheets show an increased osteogenic potential compared with conventionally engineered bone tissue structures consisting of a BMSC/TCP construct. Bridging bone formation by placing osteogenic matrix cell sheets over the implanted TCP can unite the ends of the cylindrical TCP to autologous bone.

Applications

The construct will be useful for hard tissue reconstruction, such as critical bone defects after tumor resection or traumatic injuries and avascular necrosis of femoral head, by acting as new tissue-engineered bone.

Terminology

Osteogenic matrix cell sheets are cell sheet created from BMSCs. The cell sheet is lifted by a mechanical retrieval method using a scraper. Beta-TCP is one of the most commonly used artificial bone in clinical treatments.

Peer-review

The article describes about the application of tricalcium phosphate and osteogenic matrix cell sheet for bone defect reconstruction. The efficacy of bone formation with matrix cell sheets, BMSC, and beta-tricalcium phosphate has been examined. Although the result section should be described more in detail, this study provides helpful insights for understating overall regenerative medicine technique.

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Basic Study

New model for cardiomyocyte sheet transplantation using a virus-cell fusion technique

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Ethics approval: The study was reviewed and approved by the Animal Care Committee of Shinshu University, which belongs to an institution of the university as well as the Animal Center of Shinshu University and referees a research plan on the view of ethics, science and animal welfare.

Institutional animal care and use committee: All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Animal Center of Shinshu University. It is the same institution as The Animal Care Committee of Shinshu University (Approved protocol number: 210018; 240025).

Conflict-of-interest: All authors have declared that no conflicts of interest exist.

Data sharing: No additional data are available.

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Abstract

AIM: To facilitate close contacts between transplanted cardiomyocytes and host skeletal muscle using cell fusion mediated by hemagglutinating virus of Japan envelope (HVJ-E) and tissue maceration.

METHODS: Cardiomyocytes (1.5×10^6) from fetal rats were first cultured. After proliferation, some cells were used for fusion with adult muscle fibers using HVJ-E. Other cells were used to create cardiomyocyte sheets (area: about 3.5 cm^2 including 2.1×10^6 cells), which were then treated with Nile blue, separated, and transplanted between the latissimus dorsi and intercostal muscles of adult rats with four combinations of HVJ-E and/or NaOH maceration: G1: HVJ-E(+), NaOH(+), Cardiomyocytes(+); G2: HVJ-E(-), NaOH(+), Cardiomyocytes(+); G3: HVJ-E(+),

NaOH(-), Cardiomyocytes(+); G4: HVJ-E(-), NaOH(-), Cardiomyocytes(-). At 1 and 2 wk after transplantation, the four groups were compared by detection of beating domains, motion images using moving target analysis software, action potentials, gene expression of *MLC-2v* and *Mesp1* by reverse transcription-polymerase chain reaction, hematoxylin-eosin staining, and immunostaining for cardiac troponin and skeletal myosin.

RESULTS: *In vitro* cardiomyocytes were fused with skeletal muscle fibers using HVJ-E. Cardiomyocyte sheets remained in the primary transplanted sites for 2 wk. Although beating domains were detected in G1, G2, and G3 rats, G1 rats prevailed in the number, size, motion image amplitudes, and action potential compared with G2 and G3 rats. Close contacts were only found in G1 rats. At 1 wk after transplantation, the cardiomyocyte sheets showed adhesion at various points to the myoblast layer in the latissimus dorsi muscle. At 2 wk after transplantation, close contacts were seen over a broad area. Part of the skeletal muscle sarcoplasm seemed to project into the myocardiocyte plasma and some nuclei appeared to share both sarcoplasmas.

CONCLUSION: The present results show that close contacts were acquired and facilitated the beating function, thereby providing a new cellular transplantation method using HVJ-E and NaOH maceration.

Key words: Cardiomyocyte sheet; Latissimus dorsi; Hemagglutinating virus of Japan envelope; Cell fusion; NaOH; Maceration; Cellular transplantation method

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Core tip: It is very important to produce close contacts between transplanted cell sheets and host cells without fibrous invasion for tissue reconstruction and function. Unfortunately, cellular transplantation research has not focused on this problem and convenient methods have not yet been developed. In this study using hemagglutinating virus of Japan envelope, transplanted cardiomyocyte sheets were placed in direct contact with host skeletal muscles. By adding NaOH maceration of connective tissues, rich, largely-movable, beating domains were observed. Histological observations revealed successful formation of close contacts between the two cell types. This paper proposes a new idea of introducing artificial cell fusion into cellular transplantation methods.

Takahashi Y, Tomotsune D, Takizawa S, Yue F, Nagai M, Yokoyama T, Hirashima K, Sasaki K. New model for cardiomyocyte sheet transplantation using a virus-cell fusion technique. *World J Stem Cells* 2015; 7(5): 883-893 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i5/883.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i5.883>

INTRODUCTION

Advances in regenerative medicine have realized clinical applications of pluripotent stem cell-derived differentiated cells^[1], and have simultaneously allowed recognition of the importance and potential of cellular transplantation and the necessity for its further improvement.

One of the issues is how to establish and maintain morphological, functional junctions or dense contacts without fibrous invasion between the transplanted and host cells. Regarding cardiomyocyte transplantation into the heart in particular, if direct electrical coupling through gap junctions is not established between the transplanted and host cells, the transplanted area becomes a source of arrhythmia and/or disordered contractions^[2,3].

Simple cellular injection does not always lead to the formation of electrical coupling between the transplanted and host cells. Instead, it can produce scarring, granulation, or connective tissue formation through tissue reactions such as phagocytosis of necrotic host cells, ingrowth of fibroblasts and capillaries^[4], and interposition of a layer of non-cardiomyocyte cells between the graft and host cardiomyocytes^[5], which may interrupt direct cell-cell interactions, electrical coupling, and integration. To overcome these disadvantages, there are two requirements: removal of the matrix and connective tissues surrounding the host tissue cells before transplantation to facilitate direct contacts between the transplanted and host cells, and close cellular contacts or fusion at the interface between the transplanted and host cells.

Another issue is how to obtain sufficiently large numbers of purified differentiated cardiomyocytes. Many methods using pluripotent stem cells have proposed improvements for maintaining cellular function for transplantation^[6]. However, if a method can be established that allows the production of more effective systolic function with fewer pluripotent stem cell-derived cardiomyocytes, it will be preferable and more ideal.

The notion, trialing, and practice of using skeletal muscles or myoblasts instead cardiac muscles have repeated vicissitudes. The latissimus dorsi muscle has been used as a cardiac bioassist in clinical settings, but requires continuous external stimulation^[7]. Some difficulties may remain in harmonizing the contraction of the muscle with the host heart. Skeletal myoblast transplantation has also been focused upon^[8,9] and clinically applied^[10]. However, other papers reported that myoblast transplantation caused arrhythmogenicity compared with human stem cell-derived cardiomyocytes (SC-CMs) because of the lack of gap junctions and presence of islet-like localized cell-clusters resulting in disturbance of the electrical conduction^[11], leading to questions regarding its value. Recently, a new therapy involving epicardial placement of generated

myoblast sheets in temperature-responsive dishes was developed and prevented arrhythmogenic substrate production through paracrine effects without islet-like cell-cluster formation causing electrical conduction disturbances^[12,13]. However, it remains unknown how long its effectiveness continues or whether it continues to function until complete recovery.

If cardiomyocytes can fuse directly with skeletal muscles, integrated systolic forces may be retained and produce tough contractions without requiring large numbers of differentiated cells from pluripotent stem cells. A previous report *in vitro* showed the potential of spontaneous fusion between mouse neonatal cardiomyocytes and primary neonatal skeletal myoblasts^[14]. Furthermore, mouse fetal cardiomyocytes and mouse embryonic stem cells were fused using polyethylene glycol, and survived to become chimeric cells^[15].

On the basis of the above descriptions, we planned to establish a new transplantation model using a safe virus-cell fusion technique of cardiomyocytes with the latissimus dorsi muscle in addition to maceration of the cellular matrix and connective tissue of the host tissues.

MATERIALS AND METHODS

Animals

Pregnant ICR mice (SLC, Hamamatsu, Japan) at embryonic day 14 and 6-wk-old ICR mice were used for *in vitro* experiments. In addition, 6-wk-old BALB/cSLC-nu/nu (SLC) were used as recipients for *in vivo* experiments. This study was approved by the Animal Care Committee of Shinshu University.

In vitro experiments

Preparation of fetal cardiomyocytes: Pregnant female mice were euthanized by cervical dislocation. After opening the abdomen under sterile conditions, the uterus was removed and washed three times with PBS containing 50 U/mL penicillin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50 µg/mL streptomycin (Wako Pure Chemical Industries Ltd.). Fetuses were removed from the uterus and transferred to D-Hanks solution. After opening the thorax, the fetal heart was removed under a SZ-ST stereo microscope (Olympus, Tokyo, Japan), cut into small pieces, and centrifuged at 1200 rpm for 3 min. The supernatant was aspirated, and the pellet was resuspended with PBS and centrifuged again. This process was repeated three times. Subsequently, the pellet in a 15-mL tube was immersed in 5 mL of 0.05% trypsin/0.01% EDTA and shaken in a thermostatic bath at 37 °C for 10 min. The digested tissue was diluted with 5 mL of DMEM containing 10% FBS (Equitech-Bio Inc., Kerrville, TX, United States) and centrifuged as described above. After aspiration of the supernatant, the pellet was resuspended with the medium and filtered through a 70-µm Nylon Cell Strainer (BD, Franklin Lakes, NJ,

United States). The cells were adjusted to 1.5×10^6 cells/mL and transferred into 60-mm gelatin-coated dishes.

Preparation of skeletal muscle fibers: Six-week-old ICR mice were euthanized by cervical dislocation. After disinfection with 70% ethanol, the skin of the leg was cut and separated. The muscles surrounding the legs were removed *en bloc*, almost completely cleared of connective tissue and fat tissue in D-Hanks solution under a stereo-microscope, cut into small pieces, and centrifuged at 1200 rpm for 3 min. The supernatant was aspirated, and the pellet was resuspended with PBS and centrifuged again. This process was repeated three times. Subsequently, 10 g of the pellet was immersed in 0.6 g/L collagenase type 1 (Wako Pure Chemical Industries Ltd.) including 0.05% trypsin/0.01% EDTA in 30 mL, divided into 5-mL aliquots in 15-mL tubes, and shaken in a thermostatic bath for 10 min at 37 °C. The digested tissues were diluted with 5 mL of DMEM containing 10% FBS and centrifuged under the same conditions. After aspiration of the supernatant, the pellet was resuspended with 10 mL of the medium, filtered through a 70-µm Nylon Cell Strainer, transferred into a 100-mm gelatin-coated dish, and preincubated at 37 °C under 5% CO₂ for 3 h. After pipetting, the supernatant was transferred into another dish and preincubated for 2 h. This process was repeated twice. The final cell numbers were adjusted to 70 cells/mm², transferred into 60-mm dishes, and incubated. The medium was exchanged for fresh DMEM containing 10% FBS every day.

Fusion of the two types of muscle cells using an HVJ envelope cell fusion kit: Cultured skeletal muscle fibers were washed three times with PBS, resuspended in Vybrant DiI Cell-labeling Solution (5 µL/mL) (Molecular Probes, Eugene, OR, United States), and incubated for 15 min. After aspiration, the solution was exchanged for DMEM containing 10% FBS. Cultured cardiomyocytes were treated with Vybrant DiO Cell-labeling Solution (Molecular Probes) using the same process.

The treated cardiomyocytes were separated as a sheet using a TPP Cell Spatula (TPP-US, St. Louis, MO, United States) and transferred into 60-mm dishes containing cultured skeletal muscle fibers. Subsequently, 5 µL/mL of the fusogen (HVJ Envelope Cell Fusion Kit GenomONE-CF: Ishihara Sangyo Kaisha Ltd., Osaka, Japan) was added to them. After then, the dishes were incubated for 15 min. The medium was then changed and the dishes were observed at 6, 12, 24, 36, and 48 h using an Axio Observer Z1 (Carl Zeiss, Oberkochen, Germany). For negative controls, the fusogen was omitted.

In vivo experiments

Preparation of transplanted cardiomyocytes: At 2 d before transplantation, cardiomyocytes were

Table 1 Four experimental groups

G/T	HJV-E	NaOH	CMT
G1	+	+	+
G2	–	+	+
G3	+	–	+
G4	–	–	–

HJV-E: Hemagglutinating virus of Japan envelope; CMT: Cardiomyocytes.

cultured in medium containing $2.5 \times 10^{-5}\%$ Nile blue (Wako Pure Chemical Industries Ltd.) for staining. Alternatively, they were stained with DiO at 1 d before transplantation as described in *in vitro* experiments.

Transplantation: Depending on the treatments, the animals were divided into four groups as shown in Table 1. After peritoneal anesthesia (0.25 mL of 1% Nembutal Solution; Dainihon Sumitomo Pharma Co. Ltd., Osaka, Japan), the animals were fixed in a propped position. The dorsal skin was cut along the spine and separated to expose the latissimus dorsi muscle. After confirming the internal edge, the muscle was separated from the deeper intercostal muscle and its abdominal aspect was placed in contact with cotton wool immersed in 5 mol/L NaOH for a few seconds until the color of the muscle changed slightly. To inhibit any NaOH-associated damage to the intercostal muscle, a nylon film was inserted between the two muscles before maceration. The area was washed immediately with saline after the maceration. In control animals, no NaOH maceration was performed.

Nile blue-stained cardiomyocytes were separated from dishes with a scraper. A cell sheet was inserted between the latissimus dorsi muscle and intercostal muscle and expanded as extensively as possible with or without addition of HVJ-E (Figure 1). The incised skin was closed. At 1 and 2 wk after transplantation, the rats were deeply anesthetized, and the transplanted domains were analyzed by the following cell biological methods. As controls, the domains on the opposite side were evaluated.

Cell biological analyses

Observation with a fluorescence stereo-microscope:

To confirm that the cardiomyocytes survived and remained in the transplanted domain, cardiomyocytes were treated with DiO, transplanted under the latissimus dorsi muscle, and observed under a fluorescence stereo-microscope SZX12 after 2 wk.

Motion image analysis: To compare the beating strengths among groups, the variation distances followed by contraction seen on the latissimus dorsi muscle were estimated and computationally analyzed. After anesthesia, the dorsal skin was longitudinally cut and separated toward both sides to expose the latissimus dorsi muscle. A motion image of the transplanted domain was photographed

with an AE-AF Digital Camera including a P-TTL stroboscope (Ricoh Imaging Company Ltd., Tokyo, Japan). Motion image analyses were performed using the moving target analysis software VW-H1MA (Keyence, Osaka, Japan).

Electrophysiological analysis: To assess whether each motion was caused by an action potential, electrophysiological analyses were performed. Two probes were separately placed at each end of the motional domain of the latissimus dorsi muscle, but not directly over the transplanted cardiomyocytes. The action potentials were recorded with a PowerLab SP (AD Instruments Japan Inc., Nagoya, Japan).

Reverse transcription-polymerase chain reaction:

To confirm that the transplanted cardiomyocytes survived and did not lose their features, the gene expression in samples removed from the transplanted areas was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) for cardiomyocyte markers (*MLC-2v*, marker of the ventricle; *Mesp1*, early marker of cardiac development). The primer sets, annealing temperatures, and predicted product sizes were as follows: *MLC-2v*: 5'-cctcgaactctccagaggtg-3' and 5'-cttcctgtttatttgccgac-3', 53 °C, 612 bp; *Mesp1*: 5'-cctgggatgctgtttctgacg-3' and 5'-acctgaccaagatcgagacg-3', 60 °C, 291 bp; *β-actin*: 5'-ttccttcttggtatggaat-3' and 5'-gagcaatgatcttgatcttc-3', 55 °C, 200 bp. Total RNA was extracted using TRIzol reagent (Invitrogen Japan, Tokyo, Japan). DNase-treated total RNA was used to prepare the first-strand cDNA with SuperScript II (Invitrogen Japan), following the manufacturer's protocol. The cDNA samples were subjected to PCR amplification with the specific primer sets under linear conditions to approximate the original amounts of the specific transcripts. The amplification conditions included denaturation at 94 °C for 1 min, annealing at the temperature specified for each primer for 30 s, and elongation at 72 °C for 30 s. A total of 30 cycles were performed. The PCR products were size-fractionated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Hematoxylin-eosin staining: *En bloc* removed samples from the beating domains were fixed with 4% PFA in 0.1 mol/L phosphate buffer (pH 7.4) overnight. After dehydration through a graded ethanol series, the samples were prepared for hematoxylin-eosin (HE) staining using standard methods.

Immunohistochemical double-staining for skeletal myosin and cardiac troponin

PFA-fixed tissue sections on slides were deparaffinized, rehydrated, washed three times with 0.02 mol/L PBS (pH 7.4), and treated with 20 µg/mL Proteinase K solution (Wako Pure Chemical Industries Ltd.) for 25 min at 37 °C. After three washes with PBS, the sections were

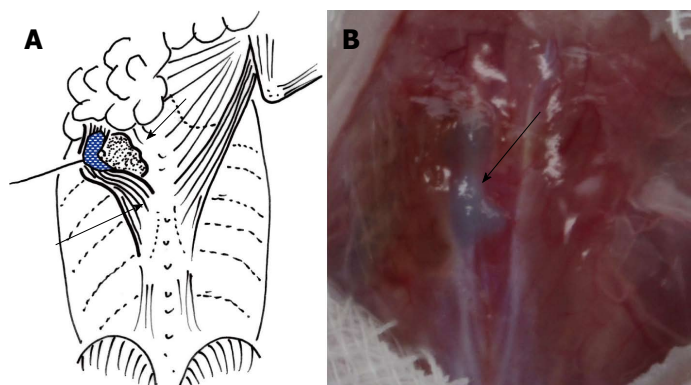


Figure 1 Method for cardiomyocyte transplantation. A: The cardiomyocyte sheet (short arrow) is inserted under the latissimus dorsi muscle (long arrow); B: The sheet stained with Nile blue (arrow) can be seen through the muscle.

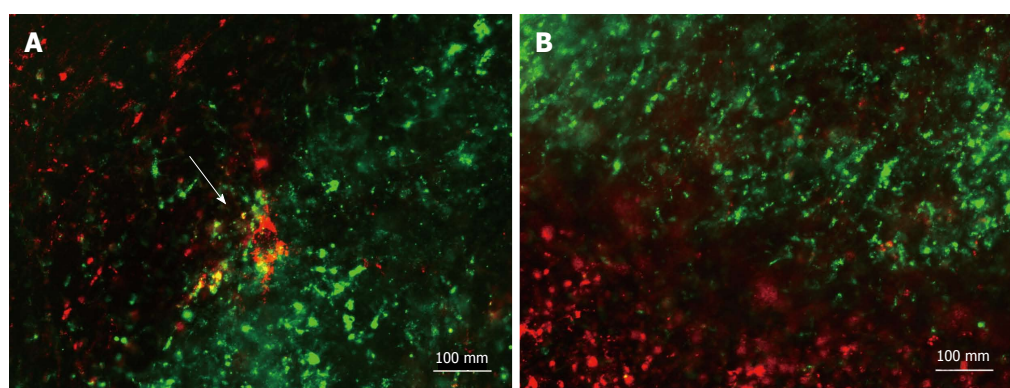


Figure 2 Cell fusion *in vitro*. DiO-stained cardiomyocytes (green) and DiI-stained muscle fibers (red) were examined. A: In G1 HVJ-E(+), the red and green staining shows partial merging (arrow); B: In G3 HVJ-E(-), the red and green staining is separated and no merging is observed. HVJ-E: Hemagglutinating virus of Japan envelope.

immersed in 0.1% Triton X-100 (Nakalai Chemical Ltd., Kyoto, Japan) at room temperature for 1 h. After another three washes with PBS, the sections were immersed in blocking solution I (5% goat normal serum in 0.02 mol/L PBS) at room temperature for 1 h. The treated cells were stained with a mixture of primary antibodies comprising mouse anti-cardiac troponin (1:100; Chemicon, Billerica, MA, United States) and rabbit anti-skeletal myosin (1:500; Sigma-Aldrich Japan, Tokyo, Japan) at 4 °C for 12 h. After washing, the sections were reacted with one secondary antibody (1:1000; Alexa Fluor 568-conjugated goat anti-rabbit IgG; Molecular Probes) in 0.02 mol/L PBS containing 5% goat normal serum at room temperature for 1 h. After washing, the sections were immersed in blocking solution II (5% rabbit normal serum in 0.02 mol/L PBS) at room temperature for 1 h and then reacted with another secondary antibody (1:1000; Alexa Fluor 488-conjugated rabbit anti-mouse IgG; Molecular Probes) at room temperature for 1 h. After washing with PBS and reaction with Prolong Gold antifade reagent containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen Japan), the sections were observed under an Axio Observer Z1 fluorescence microscope (Carl Zeiss).

RESULTS

In vitro experiments

Cell fusion: To confirm the effectiveness of HVJ-E, DiO-stained cardiomyocytes were transferred onto DiI-stained muscle fibers in dishes, and HVJ-E was added. After 24 h, merged areas of DiO and DiI appeared (Figure 2A). In control dishes, no merged areas were observed (Figure 2B).

In vivo analyses

Before transplantation, the prepared cardiomyocyte sheets were peeled directly from the dishes using a scraper. The sheets were 50-100 μm in thickness and included rich dotted nuclei and eosinophilic cytoplasm. The nucleus had a spindle-type shape and showed a strong basophilic appearance with heterochromatin (Figure 3A). Immunostaining revealed cardiac troponin-positive cells (Figure 3B).

DiO-treated cardiomyocytes occupied the same position under the latissimus dorsi muscle as the first transplanted domain at 2 wk after transplantation, and did not extend or move into other areas around the muscle (Figure 3C and D).

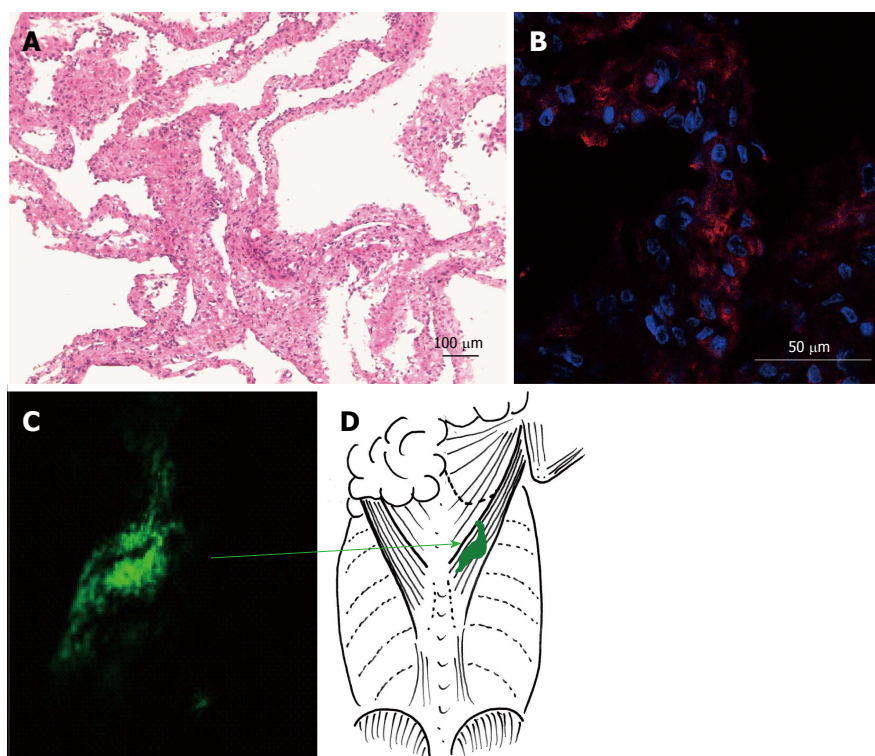


Figure 3 Representative cardiomyocyte sheets. A: HE staining of a cardiomyocyte sheet separated from the dish; B: The sheets contain cardiac troponin-positive cells (red); C: At 2 wk after transplantation, the DiO-stained sheet (green) occupies the same position as the primary transplanted site (green arrow); D: Schematic diagram showing the transplanted area under the latissimus dorsi muscle.

Under four different experimental conditions (Table 1), cardiomyocyte sheets were inserted between the latissimus dorsi and intercostal muscles, and examined by motion image, action potential, RT-PCR, fluorescent dye, HE staining, and immunostaining analyses at 1 and 2 wk after transplantation.

Motion image and action potential analyses

After confirming arrest of the host heart beats, the motion variation seen in the transplanted domains was analyzed computationally. The effectiveness of NaOH maceration and HVJ-E addition was compared in different conditions (Table 1). The G1 [HVJ-E(+), NaOH(+), Cardiomyocytes(+)], G2 [HVJ-E(-), NaOH(+), Cardiomyocytes(+)], and G3 [HVJ-E(+), NaOH(-), Cardiomyocytes(+)] rats all showed a beating-like motion (Figure 4A-C). However, the G1 rats showed the most numerous motion areas (Figure 4A), with a larger amplitude ($2.5 \pm 1.80 \mu\text{m}$) compared with the other groups of rats. In G3 rats, the small amplitudes were seen more frequently (Figure 4B). The motion had a particular feature, in that some amplitudes did not return the basal line. The mean amplitude was $0.73 \pm 0.55 \mu\text{m}$. In G2 rats, no motion was found in the transplanted area at 1 wk after transplantation, while a few small motion areas were found at 2 wk after transplantation (Figure 4C). The amplitude was small (0.62 ± 0.36).

The action potentials were compared between G1 and G3 rats at 2 wk after transplantation. Consistent

with the low fluttering motion amplitudes in G3 rats, their action potentials were also disordered (Figure 5A). On the other hand, the action potentials in G1 rats were relatively ordered and larger, but contained varied patterns (Figure 5B).

RT-PCR

After transplantation, the motion samples under the latissimus dorsi muscle clearly expressed *MLC-2v* in G1 and G3 rats (Figure 6). *Mesp1* showed weak expression at 2 wk after transplantation compared with 1 wk after transplantation.

Histology of the interrelationship between the transplanted and host skeletal cells in vivo

The interfaces between the NaOH-macerated skeletal muscle fibers and transplanted cardiomyocyte sheets showed marked differences. In the case of HVJ-E addition in particular, the inserted sheet showed close contacts with the myoblast areas. NaOH maceration destroyed the skeletal muscle fibers and brought about marked myoblast differentiation (Figure 7A). Myoblasts were observed as small round cells with a round clear nucleus, typical nucleolus, and paler cytoplasm than skeletal muscle fibers. The transplanted cells closely adhered to this active zone at several points. At 2 wk after transplantation, myoblasts were rarely found, but the sheet extended among the skeletal muscle fibers. Without NaOH maceration, the inserted cardiomyocyte sheet still survived, but the interface area was quiet

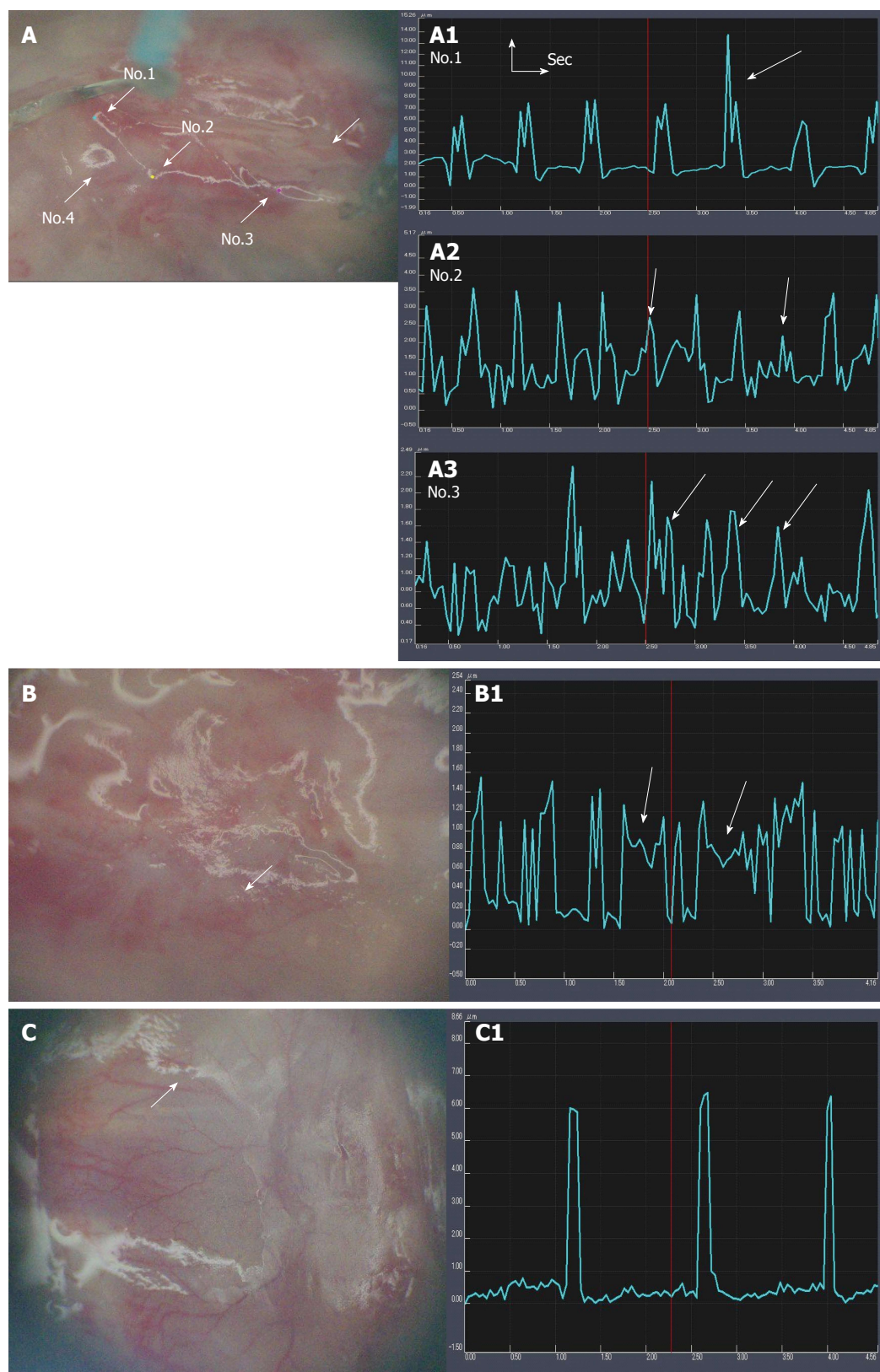


Figure 4 Motion image analysis of transplanted domains. A: Rich motion areas (arrows) in G1 NaOH(+), HVJ-E(+), Cardiomyocytes(+). Length: μm ; width: time (s); A1: The amplitudes are ordered. Large amplitudes rarely appear (arrow); A2: The amplitudes are more frequent, and their width is mostly ordered, but their amplitudes are both high and low. Low amplitudes (arrows) are fewer than high amplitudes; A3: The amplitude width is disordered. The amplitudes consist of three kinds: high, middle (arrows), and low. The frequency of each size is as follows: low > middle > high; B: Poor beating areas in G3 NaOH(-), HVJ-E(+), Cardiomyocytes(+); B1: The width between the amplitudes is irregular. High and low amplitudes are mixed. In particular, some amplitudes do not return to the baseline, and stop on the way for a short time (arrows); C: Poor motion areas in G2 NaOH(+), HVJ-E(-), Cardiomyocytes(+); C1: The amplitude width is long. The amplitudes maintain the same height. HVJ-E: Hemagglutinating virus of Japan envelope.

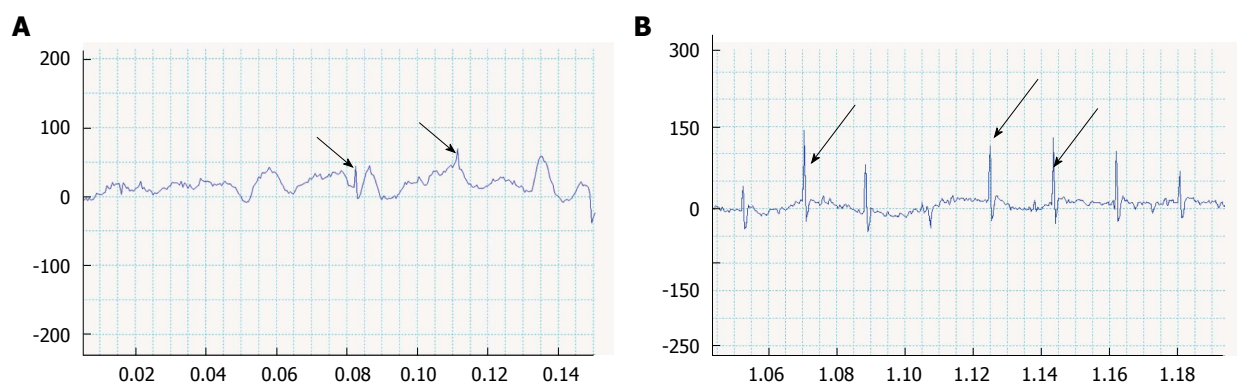


Figure 5 Action potentials of transplanted domains. A: Definite action potentials are scarcely detected in G3 NaOH(-), HVJ-E(+), Cardiomyocytes(+) (arrows), but the few action potentials observed are varied in waveforms and sizes; B: In G1 NaOH(+), HVJ-E(+), Cardiomyocytes(+), the waveforms are definite and mostly the same (arrows), although there are a few different waveforms. HVJ-E: Hemagglutinating virus of Japan envelope.

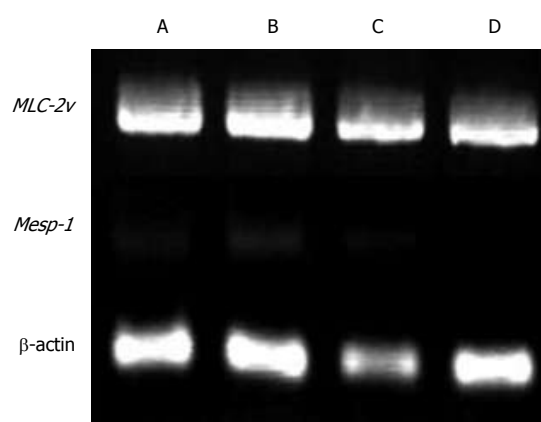


Figure 6 Gene expression in transplanted sheets. *MLC-2v* expression is maintained at 2 wk after transplantation, but *Mesp1* expression decreases at 2 wk compared with 1 wk after transplantation. A: G3 at 1 wk; B: G1 at 1 wk; C: G3 at 2 wk; D: G1 at 2 wk.

without myoblast differentiation and showed definite separation (Figure 7B).

At 2 wk after transplantation, HE staining showed close contacts between the inserted cardiomyocyte sheet and the skeletal muscle fibers, in which collagenous fibers and fibroblasts were not found (Figure 8A). Immunostaining of the same area revealed that, although the interface was extremely close, cardiac troponin and skeletal muscle actinin did not merge (Figure 8B).

DISCUSSION

Cell transplantation is different from organ transplantation in that the transplanted cells interact with the surrounding host tissues and need to restructure themselves. The environments encircling transplanted cells are not always preferable for this restructuring. Transplanted cells are always close to cell death, because they cannot rapidly achieve a blood supply. If such cells are not in a sheet-like form, they need to conjugate with one another through a junctional apparatus, while fibroblasts or other free cells can

invade the cells and inhibit their interactions. Meanwhile, it is even more difficult for transplanted cells to create new structures with host tissue cells, because basement membranes demarcate both the host and transplanted cells or tissues as the first barrier, and fibroblasts, collagenous fibers, elastic fibers, fat cells, and other free immunopotent cells can invade the interface easily as a second barrier. Although injected neonatal or pluripotent stem cell-derived cardiomyocytes conjugate with host heart cardiomyocytes^[11], it remains uncertain whether these findings hold true, because extremely small domains were demonstrated by immunostaining.

In this study, cardiomyocytes were transplanted as sheets to avoid the cardiomyocyte restructuring process. Tough cardiomyocyte sheets can easily be acquired, because cardiomyocytes conjugate strongly with one another through intercalated disks and the sheets are easily peeled off using a scraper without requiring enzymatic digestion or other new techniques such as sheet engineering^[13]. The peeled sheets do not become broken and the cells remain alive.

In addition, NaOH was used to remove the connective tissues and basement membranes. This procedure was derived from a method based on the morphology, which involved maceration of basement membranes, collagenous fibers, and elastic fibers applied to fixed specimens^[16,17]. The myoblasts in the NaOH-macerated skeletal muscle fibers showed marked differentiation. The scrub of muscles alone did not produce as much reaction (data not shown). Myoblasts originate from satellite cells, which are self-renewing stem cells that become activated by products such as growth factors produced by crushed muscles to migrate to the injured areas, differentiate into myoblasts, and fuse with each other and the damaged cells to restore skeletal muscle cells^[18]. Although it is well known clinically that NaOH devastates the tissue, penetrates easily followed by an acute necrotic phase, coagulation of intracellular proteins, and intense inflammatory reactions, and causes serious complications^[19], no definite invasion of immunopotent cells and no fibroblasts, at least, were observed in this

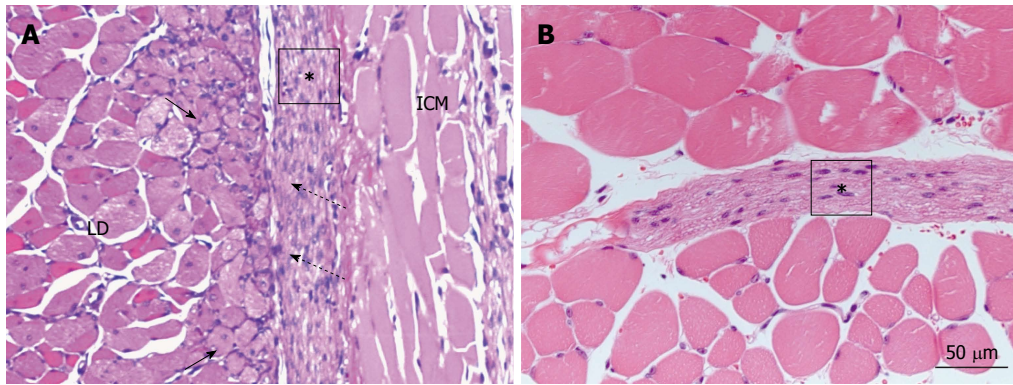


Figure 7 Histology of transplanted domains at 1 wk after transplantation. A: In G1 NaOH(+), HVJ-E(+), Cardiomyocytes(+), myoblasts (dotted long arrows) are marked in the latissimus dorsi muscle (LD). The cardiomyocyte sheet (asterisk) adheres to the myoblast layer at various points (short arrows); B: There are no myoblasts, and no adhesion of the cardiomyocyte sheet (asterisk) to the skeletal muscles in G3 NaOH(-), HVJ-E(+), Cardiomyocytes(+). The interspace between the cardiomyocyte sheet and the skeletal muscle fibers is wider. ICM: Intercostal muscle.

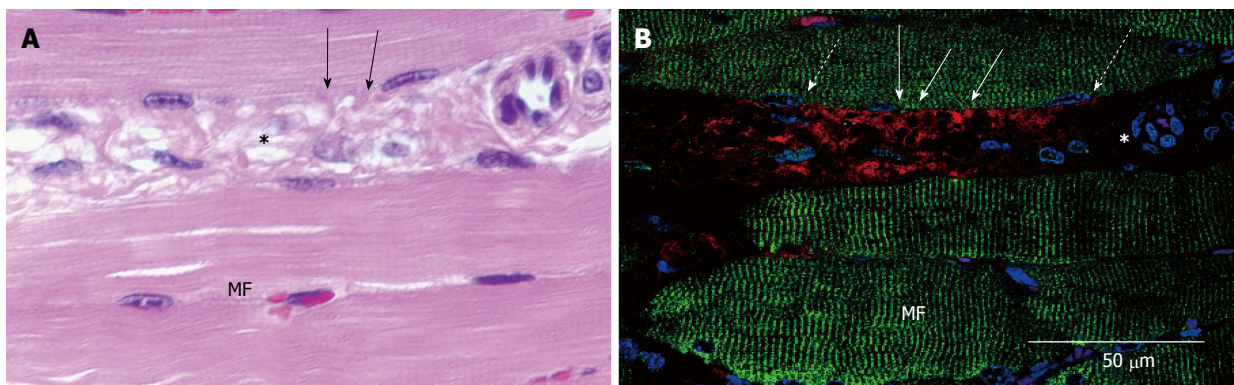


Figure 8 HE staining and immunostaining of cellular close contacts in G1. A: The myofibrils of cardiomyocytes (asterisk) are looser than those of skeletal muscle fibers (MF). The boundary is not differentiated between the cardiomyocytes and skeletal muscle fibers. A part of the myoblast sarcoplasm seems to project into the cardiomyocyte plasma (arrows); B: Immunostaining for cardiac troponin (red), skeletal myosin (green), and nuclei (blue). Small projections of skeletal myosin produce invaginations of cardiac troponin (short arrows). Cardiac troponin and skeletal myosin sandwich some nuclei (dotted long arrows) from each side.

study for 2 wk after transplantation. While the marked myoblast recruitment and few invading cells created a better environment for cardiomyocyte transplantation, they were not sufficient because, although the cardiomyocyte sheets showed beating, their domains were smaller and they did not adhere closely to the latissimus dorsi muscle.

The procedure for cardiomyocyte sheet transplantation also required some improvements in this study. First, when the sheet was overlaid on the latissimus dorsi muscle under the skin, *i.e.*, between the skin and the muscle, the procedure failed, in that no beating was observed with or without NaOH maceration (data not shown). This site under the skin, as opposed to under the muscle, may not be as good probably because of the poor vessel supply. Therefore, for subsequent survival, use of cardiomyocyte sheets necessitates some suitable conditions. Although the sheets constantly showed beating in NaOH maceration, when they were inserted between the latissimus dorsi and intercostal muscles, the beating sites were smaller and less frequent, the motion amplitude was smaller, and the action potentials were disordered. Moreover,

close contacts between the transplanted sheets and the host muscular tissues were not formed despite the fact that the sheets were packed like a sandwich between the two muscular tissues. Thus, the space between the two muscles is good for transplantation, but not sufficient for close contact formation.

To obtain close contacts between a sheet and the latissimus dorsi muscle, artificial fusion of the membranes of cardiomyocytes and skeletal muscle fibers by HVJ-E was investigated. HVJ-E is composed of the envelope of Sendai virus and causes high fusion potency between cellular membranes^[20]. It is easier and safer to handle compared with other viruses. After its effectiveness was confirmed *in vitro*, the fusogen was applied to transplantation *in vivo*. Its addition alone was not effective, as the motion amplitude was smaller, and the interspaces were histologically wider. This result was within our expectation, because the lack of removal of fibroblasts, collagenous fibers, and other connective tissues would have interrupted any close contact formation between the transplanted and host cells.

The combination of HVJ-E and NaOH maceration

brought about dramatic changes, *i.e.*, increased beating sites, larger amplitudes, albeit varied, and ordered action potentials. Although there were areas where the interfaces were separated, close contacts between cardiomyocytes and skeletal muscle cells were often found at various points, in which collagenous fibers and fibroblasts were not observed. This morphological feature, *i.e.*, close contacts between skeletal muscle fibers and cardiomyocytes, may bring about complicated motion variance.

Cell fusion produces chimeric cells that include features of both cell types, as seen in hybridomas^[15,21]. Intracellular organelles, including the nucleus, pass back and forth between both cell types after the formation of weak cytoplasmic bridges^[22]. Although the close contact areas observed in this study seemed to be maintained as fused membranes, because both the cytoplasm shared one nucleus and seemed to be partially mingled with each other, while both cytoskeletal systems maintained independent forms from one another and did not undergo reconstruction. However, to prove the interrelationship between the membrane fusion and the cytoskeleton, other cellular morphological methods, such as transmission electron microscopy and deep-etching, may be required. This complicated cell biological theme exceeds the present study and needs to be further examined in future studies.

In conclusion, the combined method involving NaOH and HVJ-E treatments is effective for cellular transplantation as a new technique.

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COMMENTS

Background

Advances in regenerative medicine requires further improvements of a cellular transplanted technique to realized clinical applications, because it is important to establish and maintain functional junctions or dense contacts without fibrous invasion between the transplanted and host cells.

Research frontiers

In particular regarding cardiomyocyte transplantation into the heart, if direct electrical coupling is not established between the transplanted and host cells, the transplanted area becomes a source of arrhythmia and/or disordered contractions. It is challenging to overcome this issue, which has been performed in this research.

Innovations and breakthroughs

The most important innovations of the research propose a new invention of myocardiocyte sheet transplantation, which is to fuse face-to-face areas between two tissues, after NaOH maceration, using virus envelopes and not to intervene connective tissues between them. This method will protect a host heart from conduction disturbance.

Applications

This transplantation method using cell fusion will be applied clinically as a new one to establish cell biological function between host and donor tissues without intervention of connective tissues.

Terminology

HVJ-E is a simplified form of hemagglutinating virus of Japan envelope. This virus is usually murine parainfluenza virus type 1 and is known as Sendai virus. The most novel feature is that it has an ability to fuse cells, which is a useful tool in the scientific world to produce hybridoma. In this experiment, its envelope is used, which guarantees safety as well as effectiveness.

Peer-review

The current study provided the evidence that a new method using HVJ-E and NaOH may facilitate the cell transplantation into the tissues.

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**EDITORIAL**

- 894 Enhancing endothelial progenitor cell for clinical use
Ye L, Poh KK

REVIEW

- 899 Importance of being Nernst: Synaptic activity and functional relevance in stem cell-derived neurons
Bradford AB, McNutt PM
- 922 Stem cell autotomy and niche interaction in different systems
Dorn DC, Dorn A
- 945 New insights into the epigenetic control of satellite cells
Moresi V, Marroncelli N, Adamo S

ORIGINAL ARTICLE**Basic Study**

- 956 Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells
Ribeiro J, Pereira T, Caseiro AR, Armada-da-Silva P, Pires I, Prada J, Amorim I, Amado S, França M, Gonçalves C, Lopes MA, Santos JD, Silva DM, Geuna S, Luís AL, Maurício AC

SYSTEMATIC REVIEWS

- 976 Mesenchymal stem cells in maxillary sinus augmentation: A systematic review with meta-analysis
Mangano FG, Colombo M, Veronesi G, Caprioglio A, Mangano C

Contents

World Journal of Stem Cells
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Enhancing endothelial progenitor cell for clinical use

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endothelial dysfunction and cardiovascular risk factors. However, translation of basic research into the clinical practice has been limited by the lack of unambiguous and consistent definitions of EPCs and reduced EPC cell number and function in subjects requiring them for clinical use. This article critically reviews the definition of EPCs based on commonly used protocols, their value as a biomarker of cardiovascular risk factor in subjects with cardiovascular disease, and strategies to enhance EPCs for treatment of ischemic diseases.

Key words: Endothelial progenitor cells; Cell therapy; Enhancing function and number; CD34; Clinical trials

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Core tip: Circulating endothelial progenitor cells (EPCs) have important potential for use in the treatment of ischemic diseases. However, their clinical application is limited by the lack of unambiguous and consistent definitions. This article reviews the definition of EPCs, their status in subjects with cardiovascular disease and discusses strategies to enhance EPCs for treatment of ischemic diseases. In patients with cardiovascular conditions who may require EPC administration, EPC numbers are low and EPCs are dysfunctional. Augmenting these cells may eventually improve their clinical efficacy.

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Abstract

Circulating endothelial progenitor cells (EPCs) have been demonstrated to correlate negatively with vascular

INTRODUCTION

Circulating endothelial progenitor cells (EPCs) are bone marrow - derived mononuclear cells that have the capacity to migrate, proliferate, and differentiate into mature endothelial cells (ECs)^[1]. Asahara *et al*^[2],

reported that CD34⁺ hematopoietic progenitor cells from adults can differentiate *ex vivo* to an endothelial phenotype, expressing EC markers and incorporating into neovessels. Since then, many publications have demonstrated the phenotype and function of these cells and use them in animal models and clinical studies^[3-7].

HETEROGENEITY OF EPC

The translation of basic research into the clinical practice of EPCs has been limited, in part, by the lack of unambiguous and consistent definitions of EPCs. In general, two methods have been used to isolate EPCs: (1) cell selection based on surface markers; and (2) cell culture and then selection. Besides CD34, a variety of cell surface markers have been shown to be associated with EPCs.

Both CD14⁺ monocytic and CD14⁻ non-monocytic mononuclear cells have been used as the starting population for cultivation of EPCs^[6]. Fernandez Pujol *et al.*^[8], demonstrated that CD14⁺ monocytic cells underwent a morphological transformation to oval cells and showed a clear expression of EC markers after 1 wk. These markers include von Willebrand factor (vWF), VE-cadherin, CD105, acetylated low-density lipoprotein (ac-LDL)-receptor, CD36, vascular endothelial cell growth factor receptor-1 (VEGFR-1, Flt-1), and VEGF receptor-2 (VEGFR-2 or KDR). However, Urbich *et al.*^[6], showed that CD14⁻ non-monocytic mononuclear cells also expressed endothelial marker proteins and were able to form colonies. CD14⁻ cells were found to incorporate into vascular structures of nude mice after hind-limb ischemia and significantly improved neovascularization.

Kalka *et al.*^[9], cultured human peripheral blood mononuclear cells and characterized adherent EPCs. EPCs were able to up-take ac-LDL and expressed endothelial surface markers such as vWF, VEGFR-2, VE-cadherin, CD146, and CD31. In a similar approach, Hill *et al.*^[10], found that colonial formation EPCs expressed KDR, CD31, and Tie2.

Interestingly, Hur *et al.*^[3], identified two types of EPCs from the same source and labelled them as early EPC and late EPC. They expressed different level of VE-cadherin, Flt-1, KDR, and CD45 markers. Late EPC produced more nitric oxide, incorporated more into human umbilical vein ECs monolayer, and are able to better form capillary tube than early EPC^[3]. However, early EPC secreted more angiogenic cytokines (VEGF and interleukin-8) than late EPC at culture^[3]. These suggest that two types of EPC might have different roles in neovascularogenesis.

More recently, besides CD34, CD133, KDR and Ac-LDL up-take have been used more frequently as EPC markers for selection^[11-14]. CD34 is a stem cell marker, CD133 an early EPC marker, KDR a marker for ECs, and Ac-LDL up-take, an endothelial function. Controversy remains on what are the most suitable makers for selecting EPC and what type of EPCs are most optimal for clinical application of cell therapy. Regardless of their

cell surface markers, EPCs shall have the capacity to form tubular structure on Matrigel^[3,4], take up Ac-LDL^[3,4], form colonies^[4-6] and have the capacity to form vessels *in vitro*^[2,9,15,16].

EPC NUMBER AND FUNCTION IN CARDIOVASCULAR DISEASES

Clinical studies documented a decreased number of circulating EPCs in coronary artery disease (CAD) patients, suggesting that levels of circulating EPCs might be associated with vascular endothelial function and cardiovascular risk factors^[17-21]. Hill *et al.*^[10] measured colony-forming units of EPCs from patients with different degrees of cardiovascular risk but without history of cardiovascular disease. A strong correlation was found between the number of circulating EPCs and the subjects' Framingham risk factor score and between the endothelial function and the number of EPCs. It was also found that EPCs from subjects at high risk for cardiovascular events had higher rates of *in vitro* senescence than cells from subjects at low risk.

Subsequently, it was demonstrated that reduced level of circulating EPCs independently predicts atherosclerotic disease progression and development of cardiovascular events^[17-19]. Sen *et al.*^[17], suggested that EPC determines the quality of the coronary collateral circulation. Georgescu *et al.*^[18] found that hypertension with hypercholesterolemia is accompanied by the alteration of vascular tone, the expression of pro-inflammatory molecules by the vessel wall, and reduced circulating EPC number.

However, contrasting results were reported, by Güven *et al.*^[22] and Werner *et al.*^[23], on the association between the level of circulating EPCs and severity of CAD. Similarly, contrasting reports were also published in patients with peripheral arterial disease (PAD)^[13,24-26]. This difference might be explained by different sampling time during the studies at different part of the disease process^[27]. Higher circulating EPC may indicate early stage of CAD and PAD, while lower circulating EPC indicates more severe and late stage of disease, and is associated with poorer outcome of these patients. It become clear that EPC cell number and function are reduced in subjects with cardiovascular risk factors and more severe cardiovascular disease^[28-32]. These are also subjects whom may benefit from EPC harvest for autologous transplantation. Thus it is important to enhance EPC cell number and function for clinical use.

STRATEGIES TO ENHANCE EPC POTENCY

Though animal studies of autologous EPC transplantation is feasible in both coronary and peripheral artery diseases, clinical application of these therapies are limited. Asai *et al.*^[33] demonstrated that topical Sonic hedgehog (*Shh*) gene therapy enhanced wound healing

by promoting recruitment of bone marrow derived EPCs. Besides migration, Shh also enhances EPC proliferation, adhesion, and tubule formation. In contrast, Jujo *et al.*^[34], showed that AMD3100, a CXCR4 antagonist, enhanced neovascularization and functional recovery after myocardial infarction through enhanced mobilization of EPC. Interestingly, the combinational therapy of Shh gene transfer with AMD3100 improved cardiac functional recovery by enhanced progenitor cell mobilization^[35].

Yao *et al.*^[12], modified EPCs with tissue kallikrein (TK) and found that TK protected EPCs from oxidative stress-induced apoptosis *via* inhibition of activation of caspase-3 and -9, induction of Akt phosphorylation, and secretion of vascular endothelial growth factor^[12]. Moreover, Fu *et al.*^[36], demonstrated that TK can enhance EPC migration and adhesion by up-regulating the expression of integrin- α 3. Thus, TK-modified EPCs may be another strategy to enhance therapeutic potency of EPC for tissue repair.

Other agents such as statin, adiponectin, thymosin β 4 (T β 4), losartan, aliskiren, hydrogen sulfide, GTP cyclohydrolase I, and ephrin-B2/Fc have been used to enhance EPC^[37-42]. Among these, statin, losartan, aliskiren, and T β 4 have good potential to improve quality and quantity of EPCs in patients with diabetes. Statins, a commonly used class of drugs to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, have pleiotropic beneficial effect on EPC^[37,43], atorvastatin increased circulating EPC after just 4 wk of administration in patients with ischemic heart failure^[37]. Rosuvastatin mobilized EPC which is shown to be Akt/endothelial nitric oxide synthase dependent^[43].

Besides statins, losartan, an angiotensin II receptor antagonist drug, commonly used in hypertension, significantly improved impaired EPC function in hypertensive patients^[42]. T β 4, an actin sequestering protein, is found to possess angiogenic activity^[44,45] and anti-diabetic property^[46]. T β 4 is angiogenic and can promote EC migration and adhesion, tubule formation, aortic ring sprouting, and angiogenesis^[44,45]. T β 4 ameliorates hyperglycemia and improves insulin resistance in mouse model of type 2 diabetes^[46]. We showed that EPCs derived from Zucker diabetic fatty rat have reduced angiogenic potential. Treatment with T β 4 *in vitro* improved EPC function and survival. It also appears to be beneficial *in vivo* (unpublished results). T β 4 is a potential drug that can improve function of diabetic EPCs for cell therapy.

Clinically, EPC has been used in clinical trials^[7,21,47]. In early phase clinical trials using EPCs for intramyocardial transplantation in subjects with refractory angina, Losordo *et al.*^[7,48] used G-CSF to mobilize bone marrow cells and selected CD34⁺ cells using magnetic beads post-apheresis. Subsequently these are injected into the ischemic myocardium without enhancing the cells^[7,48]. Feasibility and safety appears to be established. However, efficacy remains to be demonstrated in sizeable clinical trials, preferably in a randomized and blinded manner. As subjects who need EPC therapy has lower EPC numbers and more dysfunctional EPC, these cells may need to be

augmented prior to autologous transplantation. There are already several agents discussed above, which can enhance mobilization (*e.g.*, AMD3100) and function of these cells. For the latter, post-processing after harvesting, may be employed. However, it will be important to make sure the cells improve and no adverse effect on these cells as a result. *In vitro* assays and animal experiments appear to show enhancement. Whether these strategies can be translated into clinical use remain to be demonstrated. It is also important to define relevant clinical end-points and surrogate parameters. Beyond statistical significance in improving markers, these need to be clinical significance. Thus, EPC migration may increase by a few millimeter *in vitro* in a dish or the left ventricular longitudinal strain may increase by a couple of percent, it is important to show clinical relevance in translation. Besides autologous strategies, allogeneic transplantation, which has been used in animal models, may also be considered for clinical use^[45,49].

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Importance of being Nernst: Synaptic activity and functional relevance in stem cell-derived neurons

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Abstract

Functional synaptogenesis and network emergence are signature endpoints of neurogenesis. These behaviors provide higher-order confirmation that biochemical and cellular processes necessary for neurotransmitter release, post-synaptic detection and network propagation

of neuronal activity have been properly expressed and coordinated among cells. The development of synaptic neurotransmission can therefore be considered a defining property of neurons. Although dissociated primary neuron cultures readily form functioning synapses and network behaviors *in vitro*, continuously cultured neurogenic cell lines have historically failed to meet these criteria. Therefore, *in vitro*-derived neuron models that develop synaptic transmission are critically needed for a wide array of studies, including molecular neuroscience, developmental neurogenesis, disease research and neurotoxicology. Over the last decade, neurons derived from various stem cell lines have shown varying ability to develop into functionally mature neurons. In this review, we will discuss the neurogenic potential of various stem cells populations, addressing strengths and weaknesses of each, with particular attention to the emergence of functional behaviors. We will propose methods to functionally characterize new stem cell-derived neuron (SCN) platforms to improve their reliability as physiological relevant models. Finally, we will review how synaptically active SCNs can be applied to accelerate research in a variety of areas. Ultimately, emphasizing the critical importance of synaptic activity and network responses as a marker of neuronal maturation is anticipated to result in *in vitro* findings that better translate to efficacious clinical treatments.

Key words: Synapses; Neurotransmission; *In vitro* techniques; Induced pluripotent stem cells; Neuronal networks; Neurogenesis; Neural stem cells; Embryonic stem cells; Induced neurons

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Core tip: During stem cell neuronal differentiation, functional synaptogenesis and the emergence of coordinated, networked activity are critical behaviors in confirming that cells have developed into a relevant neuronal population. As the number of stem cell-derived neuron (SCN) models continues to proliferate, the use

of specific functional readouts to evaluate SCN maturity will become increasingly important compared to morphological or proteomic characterization of neuronal maturation. The review provides diverse options for reliably assaying the development of synaptic neurotransmission in derived neurons and describes the strengths, weaknesses and potential applications of several stem cell-based neuron models.

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INTRODUCTION

Over the last few decades a large variety of *in vitro* models have been developed for use in basic and applied neuroscience. These neurogenic models originate from diverse sources, including dissociated primary neurons, immortalized cell lines derived from neuronal and non-neuronal tissues and, most recently, stem cells. The predictive value of these models is critically dependent on their ability to recapitulate fundamental neuronal behaviors exhibited by primary neurons. This is particularly important given the profound effects that subtle changes in neuron development or maturation can have on emergent network properties.

In vivo, the differentiation of neural precursors into synaptically active, post-mitotic neurons involves a complex developmental cascade of gene expression and morphological changes^[1-3]. These changes ultimately orchestrate synaptic, neuronal and network behaviors to produce the emergent properties responsible for sustained central nervous system (CNS) function. Given the complex cellular behaviors involved in producing synaptically active neurons, it is not surprising that synaptogenesis and maintenance of synaptic activity are highly sensitive to genetic and environmental perturbation^[4,5]. While many dissociated primary neuron cultures reliably form functioning networks that exhibit physiological behaviors, their use is limited by several factors, including the difficulty of dissection, variability among cultures, poor viability for longer-term studies and the regulatory, administrative and ethical burdens imposed by animal studies. In contrast, while immortalized cells have been extensively used as a replacement for primary neurons, they uniformly fail to recapitulate many neurotypic properties^[6].

The advent of neurons derived from stem cells offers the potential for a unique experimental platform that combines the relevance of primary neurons with the flexibility and scalability of immortalized cells^[6]. Stem cell-derived neuron (SCN) models that produce functionally mature neurons have multiple characteristics that render them exceedingly well-suited to the study

of neural development, neuron function and human disease. For example, SCNs can recapitulate functional behaviors that are characteristic of primary neurons, such as synaptic neurotransmission and network emergence. Many stem cell lines can be maintained in culture for prolonged periods prior to differentiation, enabling scalable expansion to accommodate the demands of high-throughput approaches and endowing differentiated neurons with reduced inter-experimental phenotypic and genetic variability. The ability to convert primary cells to patient-specific induced pluripotent stem cells (iPSCs) has kindled the extraordinary potential of personalized medicine, in which iPSC-derived neurons (i-neurons) expressing cellular correlates of particular neurological pathologies can be studied *in vitro* in the context of the patient's genome^[7]. Finally, SCNs have also been proposed to have a direct application in cell-based therapies, whereby partially differentiated neural progenitor cells or post-mitotic immature neurons can be directly injected into the CNS to integrate into existing architecture, supplement endogenous neurogenic processes and promote the repair of damaged neural tissues^[8,9]. However, SCN models must be shown to be competent to form context-appropriate, functioning neurons before these approaches can be used as intended.

The signature characteristic of CNS neurons is action potential (AP)-induced synaptic neurotransmission that synchronizes neuron firing to give rise to emergent circuit behaviors. Since synaptic activity is a principal endpoint of neurogenesis, detection of synaptic events and/or synaptically driven network behaviors serves as a higher-order readout that confirms the proper elaboration of the full range of biochemical, proteomic and morphological properties that are required for neuron function. However, in many cases the rigor and specificity of techniques used to characterize the physiological relevance of SCNs have been highly variable^[10,11]. Frequently, characterizations have been limited to expression of small sets of neurotypic genes or electrophysiological assessment of intrinsic electrical excitability, without evaluation of functional synaptogenesis or network formation^[12,13]. SCNs are frequently described as physiologically relevant based on insufficient or incomplete characterizations, therefore producing data of uncertain value. These inconsistencies illuminate a critical need for the identification of appropriate assays to evaluate the functional maturity and physiological relevance of derived neuron models.

In this review we will discuss methods to characterize the progression of *in vitro* neurogenesis and propose specific functional assays to confirm the physiological relevance of SCNs. We will focus on SCNs derived from four sources (summarized in Figure 1): embryonic stem cells (ESCs); restricted-potency neural stem cells (NSCs); iPSCs; and direct conversion of post-mitotic cells into induced neurons (iNs). Note that although iNs do not explicitly incorporate a pluripotent phase, the derivation of iNs uses principles and techniques involved

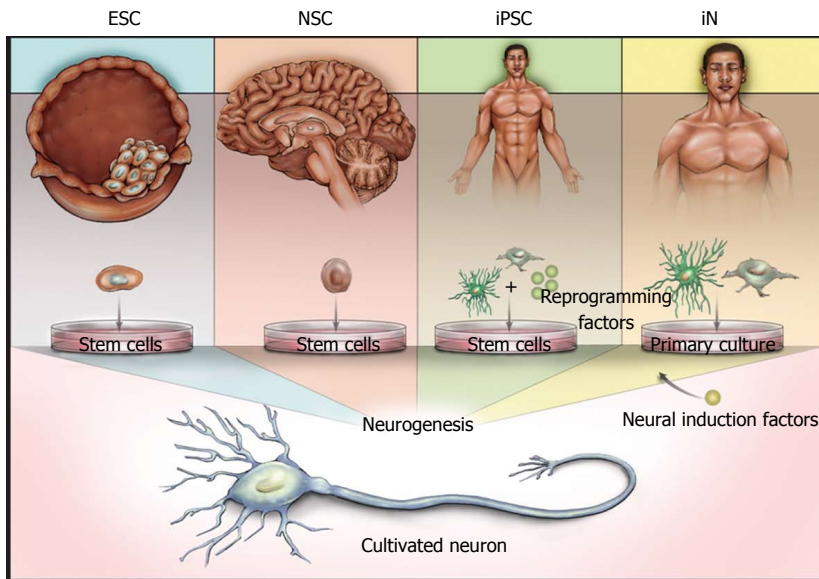


Figure 1 Illustration of the sources of derived neurons. Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts, whereas neural stem cells (NSCs) are derived from several defined niches in the developing or adult brain. Both ESCs and NSCs are capable of neurogenesis without the forced expression of induction factors. Induced pluripotent stem cells (iPSCs) and induced neurons (iNs) can be derived from various tissues, and proceed to neuronal states via either reprogramming to a stem cell phenotype (iPSCs) or direct conversion using neuronal induction factors (iNs).

in production of other SCN models and therefore will be addressed in this review. We will also describe the current status of existing SCN models, and elaborate on reasons why synapse and network formations are critically important to SCN applications, even in cases where applications may not directly rely on neuronal function.

METHODS TO CHARACTERIZE NEUROGENESIS AND NEURONAL MATURATION

Measuring the maturation and relevance of neurogenic models

Developmentally regulated changes in proteomic, transcriptomic, biochemical and functional properties during embryonic neurogenesis can be repurposed to evaluate developmental progression *in vitro*^[14]. The specific markers used may vary depending on the ultimate application, but at a minimum should include morphological characterization of neurotypic compartments; the development of electrical excitability; the detection of post-synaptic responses to pre-synaptic release of neurotransmitter; and, when appropriate, network-level behaviors (Figure 2). While functional confirmation of synaptic neurotransmission represents indisputable evidence of synaptogenesis, supplemental studies such as transcriptomic analysis, planar multi-electrode array (MEA) analysis or protein expression studies can also provide valuable contributions to the overall understanding of the maturity and nature of derived neuronal populations, as described below.

Although methods to validate the progression

of neuronal maturation have been well-described in dissociated cultures of primary neurons, it is important to recognize that the source tissues for these neurons are often comprised of functionally mature neurons that are dissociated and plated^[15]. In these cases, re-establishment of synaptic activity is primarily contingent on the temporal re-elaboration of appropriate morphological structures. In contrast, the differentiation of pluripotent stem cells into mature SCNs requires recapitulation of the full range of transcriptional, morphological and proteomic changes involved in functional neurogenesis. Neurogenic progression is an intricate process that is susceptible to misdirection *in vitro*, and unlike in dissociated primary neurons, there is a lower correlation between expression of intermediate developmental markers and the probability of functional maturation. Inappropriate differentiation conditions can cause neurogenic models to become developmentally arrested, expressing immature neurotypic properties but lacking the functional correlates of active synapses and network emergence^[16]. Dissociated primary neuron cultures can also undergo developmental arrest if not properly maintained, despite having been functionally mature prior to dissociation^[1]. Once neurons become functionally mature, their survival continues to depend on synaptic activity-induced neurotrophic signaling^[17-20], as demonstrated by neurotoxicity following impaired synaptic function in neurodegenerative diseases^[21-23]. Therefore, researchers characterizing SCN differentiation should be careful to avoid phenomenologically conflating the expression of intermediate developmental markers with the eventual likelihood of producing functionally mature neurons. For example, the ability to fire repeated APs with hyperpolarizing after shoots in response to

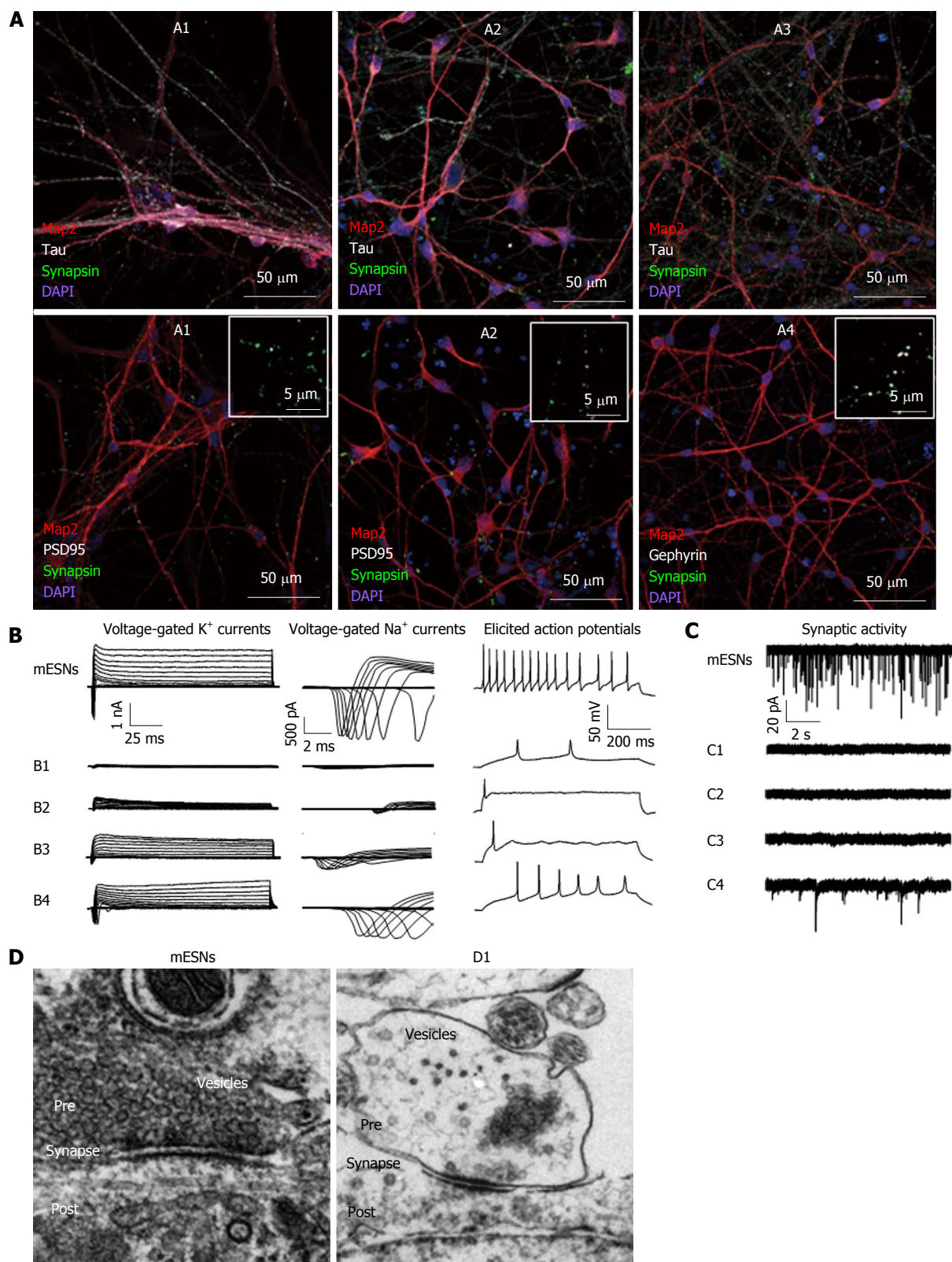


Figure 2 Neurotypic markers and passive membrane responses are not predictive of functional synaptogenesis. A: Representative fluorescent immunocytochemistry of i-neurons from several protocols (A1-A4) imaged at 43 d after plating (DAP). SCNs display neurotypic morphologies, including distinct dendrites (Map2) and axons (Tau) around cell nuclei [4',6-diamidino-2-phenylindole (DAPI)], with distributed and punctate synapsin (top panel) and PSD-95 or gephyrin (bottom panel) often expressed in close proximity to synapsin (insets); B: Although all four i-neuron models displayed intrinsic excitability, including voltage-gated currents, and produced action potentials following current injection, responses were smaller in amplitude and depolarization led to fewer repeated action potentials compared to responses displayed by mouse embryonic stem cell-derived neurons (mESNs, top row); C: Only one of four human i-neuron models displayed miniature synaptic activity on DAP35 or later as indicated by miniature post-synaptic currents in the presence of tetrodotoxin, and those currents were smaller in amplitude, less frequent and slower to return to baseline when compared to mESNs; D: Transmission electron microscopy of single synapses in mESN and i-neuron models (D1) indicates that pre-synaptic compartments of mESNs are densely loaded with vesicles, while i1 is not. This indicates that while the model is capable of producing the morphology, synaptic markers and intrinsic characteristics of synaptogenesis, it lacks the capacity to maintain spontaneous activity and therefore is unable to model physiological synaptic transmission or network activity.

current injection is often associated with neuronal maturity. This may be valid in many well-characterized primary neuron cultures. However, there is no causal requirement that SCNs with this intrinsic behavior will exhibit functional synapses with synaptically driven network responses.

As the number of SCN models continues to proliferate, the deliberate use of specific functional readouts to evaluate SCN maturity will become increasingly important. By precisely and accurately associating neurotypic behaviors with stage-specific markers, researchers will be able to better ascertain the degree of relevance and applicability of SCN populations. Efforts to develop techniques to reliably determine the neurogenic potential of SCN differentiation models are currently underway^[11,24]. However, in the absence of such functional validation, claims of neuronal maturation and therefore relevance should be reviewed with care.

Approaches for morphological and structural characterization of neurogenesis

Much of the current understanding of neuronal maturation comes from longitudinal studies of the formation of specialized neuronal structures in dissociated primary neurons^[25]. These studies established that the progressive establishment of functionally specialized compartments (e.g., axons, hillocks, dendrites, and pre- and post-synaptic compartments) provided the cellular substrates for polarized neurotransmission *in vitro*. Many of these morphological features can be distinguished by the localization expression of specific proteins and therefore immunocytochemistry (ICC) is often used to characterize developmental progression^[26,27]. Morphological characterization may start with general neuronal markers, such as NeuN (post-mitotic neuronal nuclei) or β III-tubulin (immature neuronal marker), and progress to more specialized markers of cellular compartments, such as synapsin (pre-synaptic), tau (axon), MAP2 (somatodendritic) and gephyrin or PSD-95 (inhibitory or excitatory post-synaptic markers, respectively). It can then extend to the use of well-described antibodies to distinguish among neuronal subtypes, such as markers of glutamatergic (vesicular glutamate transporter-1 or -2), cholinergic (choline acetyltransferase), GABAergic (glutamate decarboxylase 1), serotonergic (serotonin transporter), aminergic (vesicular monoamine transporters 1 or 2), or dopaminergic and/or adrenergic (tyrosine hydroxylase) neurons. Longitudinal studies using such markers are a regular feature of SCN differentiation studies and are an important aspect of verifying neuronal maturation, even though expression of specific markers are not indicative of functional behaviors^[28,29].

In addition to providing information on the establishment of synaptic compartments, the morphological apposition of pre- and post-synaptic proteins can provide clear evidence of synaptic assembly^[30]. Synaptic assembly begins with the contact of complementary cell adhesion molecules between potential pre- and post-synaptic compartments, which in turn requires

the previous elaboration of axons and dendrites (with the exception of axosomatic synapses^[31]). From this point, proteins necessary for synaptic architectures and function are recruited to the site of contact in both compartments^[32,33]. In mature neuron cultures and *in vivo*, the establishment of new synapses can occur very quickly, with synaptic activity apparent almost immediately^[34,35]. However, as demonstrated in Figure 2 and discussed above, SCNs can form synaptic structures but fail to develop synaptic function. Based on this positional apposition, ICC for synapsin and PSD-95 (for example) have been used to provide a low-resolution confirmation that pre- and post-synaptic compartments are in close proximity. More recently, the mammalian GFP reconstitution across synaptic partners (mGRASP) method has been used as a fluorescent indicator of the co-localization of synaptic adhesion proteins. mGRASP is based on functional complementation of split GFP separately expressed from pre- and post-synaptic membranes, and therefore is a highly specific marker of synaptic proximity^[36]. Ultrastructural imaging using transmission electron microscopy (TEM) can also be used for advanced morphological analysis of synaptogenesis. TEM enables a definitive visualization of pre- and post-synaptic compartments, allowing researchers to search for characteristic intra-synaptic structures, such as pre- and post-synaptic densities, the presence of synaptic vesicles in the pre-synaptic compartment and possibly even vesicles docked or fused with the pre-synaptic membrane, suggesting neurotransmitter release. It is important to emphasize that positive confirmation of synaptic architectures by any of these methods is not *prima facie* evidence of synaptic function. Synapse assembly precedes onset of synaptic activity and is not indicative of the current or future development of synaptic function^[37-39]. However, pairing these types of morphological studies with electrophysiology may allow the identification of benchmarks that can be used to correlate synaptic function with gene expression (e.g., the onset of synaptic activity cannot precede the localization of protein X, etc.). While structural characterizations have rarely been used in the study of SCN maturation, they have the potential to offer highly specific and detailed correlations between structural and functional synaptogenesis when combined with synaptic activity assays.

While morphological characterization provides researchers with a visual confirmation of protein expression and compartmentalization, some neuronal markers (e.g., β III-tubulin^[40], synapsin^[41] or NeuN^[42]) may also be expressed in non-neuronal or neoplastic cells. Morphological characterization of neurogenesis should therefore be used as a secondary method to confirm neurogenesis and be corroborated by more direct methods to measure the production of neurons. For example, we have found transcriptomic analysis to be invaluable in reconciling neuronal fate with expression of neurotypic markers and evaluating neurotypic responses, including: (1) longitudinal characterization

of neurogenic progression; (2) identification of neuronal subtypes; (3) determination of which neurotransmitter receptors subunits are expressed; and (4) measurement of activity-dependent responses to treatment with pharmacological regulators of network activity^[43].

Measurements of intrinsic excitability

Functional evaluation of neuron maturation begins with electrophysiological characterization of intrinsic excitability, which is the ability to undergo the rapid, defined changes in ion flux that are necessary to repeatedly fire APs. These include measurements such as quantitation of Na⁺ and K⁺ voltage-gated responses, the ability to fire evoked APs, membrane resistance, membrane capacitance, sag (or I_h) currents, hyperpolarization-activated currents and establishment of a stable resting membrane potential. Another intrinsic neuronal characteristic that can be used as a developmental marker of maturation is the γ -aminobutyric acid (GABA) reversal potential (E_{GABA}). Immature neurons have high intracellular Cl⁻ levels, such that GABA_A receptor activation triggers Cl⁻ efflux^[44]. This excitatory GABA current has been proposed to contribute to the developmental activation of nascent excitatory synapses^[45]. Neuron maturation is associated with increased expression of the potassium chloride co-transporter KCC2, which is an electroneutral Cl⁻ exporter that decreases intracellular Cl⁻ concentration, thereby shifting E_{GABA} to a value that is close to the mature resting membrane potential^[46]. The developmental shift in E_{GABA} potentiates a transition from excitatory post-synaptic GABA currents to inhibitory GABA currents^[47]. While the development of E_{GABA} is rarely used for neuronal characterization because of the relative difficulty in measuring Cl⁻ reversal potentials, the shift in E_{GABA} is an elegant marker of the developmental changes that are required for the establishment of network-level activities in many neuron subtypes.

Once neurons have been determined to exhibit mature characteristics of intrinsic excitability, analysis then expands to measuring cell behaviors under non-evoked conditions, such as spontaneous production of APs and miniature post-synaptic currents. It is important to note that the mere presence of spontaneous APs does not necessitate or signify synaptic function^[48]. For example, dissociated primary neurons exhibit mature-appearing intrinsic characteristics and spontaneously fire APs prior to formation of active synapses^[27]. In fact, spontaneous, non-evoked AP firing appears to be a developmental phenomenon involved in the maturation of ionic gradients^[49,50]. Furthermore, the detection of APs can reflect a neuron with a membrane potential that is near to the threshold potential and therefore fires APs in response to subtle changes in the conductance of membrane ion channels, independent of synaptic activity^[51,52]. Since APs may occur in the absence of synaptic function, pharmacological antagonists of neurotransmitter receptors must be used to experimentally confirm that APs originate from synaptically driven events as described below.

Synaptic activity is a functional signature of successful synaptogenesis

In some cases, SCNs produce morphological characteristics of synapse formation, exhibit evidence of intrinsic excitability and fire spontaneous APs, yet fail to develop synaptic activity^[29,53]. As discussed below, this is currently a particularly vexing problem with human iPSC-derived neurons, which exhibit a wide array of morphological markers of neurogenesis, but often appear to be developmentally delayed or even arrested prior to the onset of synaptic activity. Consequently, measurements of synaptic activity are critical to providing unimpeachable confirmation of functional synaptogenesis.

The production of excitatory and/or inhibitory post-synaptic currents is a higher-order representation of the spatiotemporally precise elaboration of a large number of developmental processes required for synaptic activity, such as juxtaposition of pre-synaptic and post-synaptic compartments; loading and docking of neurotransmitter vesicles; and the appropriate localization and function of voltage-dependent ion channels. The two dominant modes of synaptic neurotransmission are spontaneous release and AP-evoked release. Under physiological conditions stochastic Ca²⁺ currents trigger synapses to spontaneously release neurotransmitter at approximate rates of 10⁻³ quanta per second (Hz) per CNS synapse^[54,55] and 0.79 Hz per motor neuron synapse^[56]. In a typical central neuron, which has 10²-10⁴ synapses, monosynaptic release probabilities result in a post-synaptic current detection rate of 0.1-10 Hz^[57,58]. Similar rates have been reported in mouse SCNs^[59]. In contrast, AP-induced pre-synaptic depolarization increases instantaneous quantal release rates increase to 10³ Hz^[54,55]. While either spontaneous or AP-evoked neurotransmission can be used to identify the presence of synaptic neurotransmission, the large currents generated by AP propagation can complicate rigorous characterization of post-synaptic behaviors, as described below. Consequently, detection and characterization of spontaneous post-synaptic events often provides a more reliable and quantifiable indicator of synaptic function than detection of synchronized release from many synapses following an AP.

Detection of post-synaptic events using whole-cell patch-clamp electrophysiology can involve characterization of excitatory post-synaptic potentials (EPSPs) in current-clamp mode or spontaneous miniature post-synaptic currents in voltage-clamp mode. For a number of technical reasons related to quantitation and characterization that will not be addressed here, we recommend the latter approach. Spontaneous release is a salient feature of all synapses *in vivo* and *in vitro* and direct measurement of spontaneous monosynaptic activity *via* detection of miniature excitatory or inhibitory post-synaptic currents (mEPSC or mIPSC, respectively) in the presence of tetrodotoxin (TTX) is an unambiguous indicator of synaptic function in neuron subtypes that utilize ionotropic neurotransmitter receptors (Figure

2)^[54,60-62]. TTX blocks voltage-gated Na⁺ channels, eliminating the large whole-cell currents caused by AP firing and enabling the detection of small post-synaptic currents resulting from the spontaneous activation of individual synapses. The addition of pharmacological agonists or antagonists for specific neurotransmitter receptors allows the contributions of different neuron subtypes to post-synaptic responses to be precisely determined.

Characterization of miniature post-synaptic currents (aka, minis) should include analysis of event rates and kinetics; functional responses to the addition of pharmacological modulators of post-synaptic function; and current: voltage relationships for well-characterized receptors^[59]. Much is known about the functional mechanisms of synaptic neurotransmission in primary CNS and peripheral neurons and an extensive pharmacopeia is available to manipulate and characterize post-synaptic responses for most synapse subtypes. Thus, there is a wide array of resources to identify and develop electrophysiological approaches to evaluate synaptic function in SCNs.

While the kinetic analysis of minis can be used to unambiguously distinguish between synaptic and non-synaptic neurotransmission, in some instances (e.g., synaptic potentiation studies) it would be valuable to simultaneously evaluate both pre- and post-synaptic behaviors. Paired recordings offer one means to do so. In paired recordings, two synaptically connected neurons are simultaneously patched, such that stimulation of one neuron elicits post-synaptic responses in the other. While this is a highly rigorous and reliable indicator of connectivity, it is a technically difficult method. Paired recordings are relatively feasible under conditions in which neuronal connectivity is well defined and the polarization of synaptic connections are known, such as in hippocampal slices^[63]. In contrast, trans-synaptic connectivity and polarization are stochastic in dissociated neuron cultures, making it difficult to identify and record from synaptically coupled neurons, even when plated at very low densities^[64]. Thus, while paired recordings are an effective method to characterize evoked synaptic neurotransmission, it is technically challenging to accomplish in neuron cultures and should be anticipated to have a high failure rate. Alternatively, the use of bipolar stimulating electrodes allows for the repeated stimulation of specific local synapses *in vitro* without requiring multiple patches^[65]. However, this approach relies on the stimulation of multiple processes in proximity to a patched neuron and therefore may result in field stimulus-like artifacts and difficult to interpret post-synaptic responses.

The use of post-synaptic miniature currents to measure synaptic neurotransmission is not feasible in neuron subtypes that rely on metabotropic neurotransmitter receptors, such as dopaminergic or serotonergic neurons. While methods such as ultrafast voltammetry or the use of fluorescent false neurotransmitters can directly measure synaptic release of neurotransmitters^[65,66], they are not a confirmation of synaptic neurotransmission,

but rather are limited to providing information about the pre-synaptic release of neurotransmitter. Alternatively, indirect measurements of the activation of metabotropic synapses in cultures containing multiple neuron subtypes can be conducted based on changes in network behavior, as described in the next section.

Table 1 lists exemplar SCN models that have been evaluated for the existence of spontaneous post-synaptic currents in the absence of co-cultured primary neurons. The latter were excluded due to the difficulty in disambiguating neurotransmission and neuro-excitation between SCNs and primary neurons^[67]. Table 1 does not include references for putative SCNs that have only been characterized for morphological markers or for intrinsic excitability, since as described above these are poor surrogates for assaying functional synapses.

Network behaviors as higher-order signatures of neuron function

Similar to synaptic activity, emergence of network activity confirms successful elaboration of the developmental processes required for functional synaptogenesis. We define "network potential" as the ability of derived neurons to integrate post-synaptic events into APs (excitability), to transmit outgoing synaptic signals to other neurons (neurotransmission), and to elicit activity-dependent post-synaptic behaviors (adaptability). While the emergence of network activity is a function of the neuronal subtypes present, and therefore is not a required feature of mature neuron cultures, it does provide unambiguous confirmation of synaptic activity. Ideally, neuron cultures will incorporate both excitatory and inhibitory neuron subtypes whose activity is modulated by feedback mechanisms to produce an excitatory/inhibitory (E/I) balance. This condition is maintained by adaptation of pre- and post-synaptic properties and can be considered functional evidence of synaptic plasticity^[68]. Alternatively, monotypic cultures comprised of excitatory or inhibitory neurons will result in unbalanced signaling and network inactivity^[69] or seizurogenic bursting^[70], conditions in which long-term neuronal viability may be compromised^[71,72].

Mature network activity is typically observed in SCNs as synchronous AP propagation among multiple neurons, a phenomenon often referred to as network bursting. Since AP production is a stochastic process caused by the near-simultaneous occurrence of a sufficient number of excitatory EPSPs to exceed the firing threshold, *in vitro* quantitation of APs is influenced by a number of factors which determine neuron connectivity, such as the number, weight and type of functioning synapses. Several methods are available to quantify network activity. The most specific is the use of electrophysiological methods to directly measure AP production^[59,73,74]. This can occur *via* simultaneous, multiple patch-clamp experiments, where the synchronicity of spontaneous AP production is compared among patched neurons. Alternatively, the effects of network modulators (e.g., bicuculline, aminopyridine or CNQX, as described below) on the

Table 1 Stem cell-derived neurons characterized for synaptic activity

Cell type	Source	Technical approach	Post-synaptic measurement (days after differentiation)	Additional notes	Ref.
ESC	Human (BG01, BG03)	Stromal cell factors applied	mEPSCs (21)	Also dopaminergic Continued expression of NPC marker <i>Msx1</i>	[254]
	Human (WiCell H9, H1)	SHH application on ESCs or NPCs	mEPSCs, mIPSCs (30)	Also dopaminergic	[110]
	Human (H9 and SA121)	Dual SMAD inhibition, variable SHH, several additional neurotrophic factors	sEPSCs, sIPSCs (35)	SHH concentration-dependent GABAergic and glutamatergic, or dopaminergic fates	[240]
	Mouse (R1)	RA treatment, suspension culture	mEPSCs, mIPSCs (21)		[6,59,226]
	Mouse (J1)	FACS sorting for dopaminergic NPCs, SHH treatment	sEPSCs (22)	Also dopaminergic	[255]
NSC	Human (NSI-566RSC from fetal spinal cord)	9 component differentiation media	mEPSCs (12-48)		[256]
iPSC	Mouse (Adult C57/Bl6 SEZ)	BDNF treatment	mEPSCs, mIPSCs (11-28)	Perforated patch recording	[171]
	Human (normal and ALS fibroblasts)	Dual SMAD inhibition, RA and purmorphamine treatment	sEPSCs (21-42)	sEPSCs only detected with picrotoxin and 4-aminopyridine	[86]
iN	Human (normal and AD fibroblasts)	FACS sorting of NPCs, BDNF, GDNF and cAMP treatment	mIPSCs, mEPSCs (26)	Currents detected in approximately 40% of cells	[148]
	Human (postnatal and adult fibroblasts)	Two-stage Dox, bFGF, Noggin, GDNF, BDNF, forskolin treatments	mEPSCs (30)	Currents detected in 25%-43% of converted cells	[158]
	Mouse (perinatal tail tip fibroblasts)	Glial co-culture, FACS sorting	sEPSCs (15-27)		[156,157]
ESC + iPSC	Human (HES3, BioTime ESI, and WiCell H9 ESCs + melanocyte hiPSCs)	5 morphogenic factors, FACS sorting, glial co-culture	sIPSCs (42-84)	% of cells with currents increases over time	[28]
	Human (HUES9 ESCs + fibroblast hiPSCs)	Noggin and dorsomorphin treatment, recombinant human <i>Dkk1</i> treatment of hiPSC EBs, glial co-culture	mEPSCs (35)	Implanted neurons also received EPSCs Currents detected in 33% of differentiated ESCs and 46% of differentiated iPSCs tested	[111]
	Human (WiCell H9 and SHEF4 ESCs + fibroblast hiPSCs)	3% O ₂	mEPSCs (35)	Mature AMPA and GABA profiles	[47,116]

ESC: Embryonic stem cell; NSC: Neural stem cell; iPSC: Induced pluripotent stem cell; ALS: Amyotrophic lateral sclerosis; AD: Alzheimer's disease; SHH: Sonic hedgehog; NPC: Neural precursor cell; SMAD: Sma and Mad related proteins; RA: Retinoic acid; FACS: Fluorescence-activated cell sorting; BDNF: Brain-derived neurotrophic factor; GDNF: Glia-derived neurotrophic factor; iN: Induced neuron; cAMP: Cyclic adenosine monophosphate; bFGF: Basic fibroblast growth factor; mEPSC/mIPSC: Miniature excitatory/inhibitory post-synaptic current; sEPSC/sIPSC: Spontaneous excitatory/inhibitory post-synaptic current; GABA: Gamma aminobutyric acid; AMPA: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

frequency or nature of synaptically driven APs can be evaluated in a single neuron in current-clamp mode, providing indirect evidence of the contribution of synaptic activity to AP production. A relatively new approach is the use of MEAs to quantify synchronized bursting behavior in two- and three-dimensional cultures, which provides a unique method for characterizing the effects of neuromodulatory compounds on longitudinal network responses^[75,76]. A more limited alternative to MEAs is the use of live-imaging assays to quantify bursting behavior using fluorescence-based detection of neuronal activity reporters, such as fluorescent genetically encoded calcium indicators (GECIs) or voltage-gated dyes^[77-79]. Calcium imaging is particularly useful for studying network activity, as it is capable of simultaneously monitoring up to thousands of neurons and their associated processes^[80]. While the temporal resolution and sensitivity

of calcium imaging can be limited by fluorophore kinetics, level of gene expression or equipment, it is an excellent complement to electrophysiology^[81]. Recently described GECIs have been found to be able to detect individual APs^[82]; however, improved detection of the small potential differences driven by sub-threshold events is still needed before calcium imaging can approach the resolution offered by whole-cell patch-clamp electrophysiology. The development of optical electrophysiology techniques that offer the throughput of GECIs with the sensitive of whole-cell recordings would represent a transformative approach in large population analyses that would be immediately applicable to synaptically active SCN models.

In all cases the presence of network activity must be confirmed by treatment with pharmacological modulators that alter synaptic activity without directly activating

post-synaptic receptors. This is particularly important for MEAs and calcium imaging, in which it is currently impractical to directly measure synaptic function. Such treatments could include bicuculline, which disinhibits the network by blocking GABAergic inhibitory activity; aminopyridines, which prolong APs by blocking K⁺ efflux; or CNQX, which blocks AMPAR activation and therefore eliminates excitatory signaling in glutamatergic networks^[83-85]. Depending on neuron maturity and the relative proportions of neuron subtypes in SCN cultures, the use of network modulators may be the only possible means to reveal excitatory synaptic activity exceeding the threshold of AP detection^[86]. Thus, the inability to detect APs by methods such as fluorescent calcium imaging or MEAs should not be considered evidence of the absence of synaptic activity.

Activity-dependent responses as an indirect measure of synaptic function

A signature *in vivo* characteristic of network function is the elaboration of activity-induced changes in post-synaptic neurons. In healthy networks a variety of mechanisms are used to balance excitatory and inhibitory inputs, producing circuits that can respond to increases or decreases in input^[68,87]. The best-described of these is Hebbian plasticity, which allows for the constant formation and strengthening of synchronously active synapses and weakening of asynchronous synapses and is mediated by altered phosphoproteomic signaling, changes in receptor function and expression and differential gene expression^[88,89]. Functional Hebbian plasticity can be indirectly evaluated in cultured neurons by measuring changes in the biochemical correlates of plasticity under conditions that up- or down-regulate network activity. Molecular correlates of plasticity include the surface localization of glutamate-responsive AMPA receptors, which can be measured by surface biotinylation or antibody labeling of extracellular residues^[47,90], or the phosphorylation state of post-synaptic kinases such as CaMKII, Akt or CREB, which can be quantified using routine immunoblot methods^[91-93]. Activity-induced changes in the expression of plasticity-related immediate early genes (IEGs) can be monitored by transcriptomic and/or proteomic methods, including Arc, the EGR family, c-Fos, Jun and Homer-1a^[69,70,94-96]. However, it is important to note that many of these genes have multiple functions and are regulated during development and other neuromodulatory treatments^[97,98]. Thus, researchers should be careful to ensure they are evaluating IEG expression under conditions that precisely modulate network activity without inducing acute cytotoxicity or other forms of neuronal stress. Synaptic plasticity can be directly measured by whole-cell patch-clamp electrophysiology in neuron cultures using trans-synaptic patch clamping or bipolar stimulating electrode techniques^[65]. Plasticity- and activity-related assays have rarely been described in SCN models or in primary neuron cultures, but do represent relevant physiological

correlates of network function and they should be considered to be a valuable confirmatory tool when characterizing and validating activity in SCNs.

SCNS: TYPES AND CHARACTERISTICS

Approaches to SCN differentiation

The yield, neuronal subtype(s) and purity of SCNs are heavily influenced by an array of biochemical factors that affect the differentiation process by modulating progression through neurodevelopmental stages. In some cases these factors can be controlled very precisely, such as by the addition of small molecules to promote exit from the pluripotency state, whereas others are less amenable to rational control, such as longitudinal changes in neurogenic potential caused by the spontaneous emergence of mutated subpopulations during routine culturing of stem cell lines. Thus identifying confirmatory markers of neurogenic progression is critically important to validate that differentiation of specific stem cell lines successfully results in the desired neuronal products. Derivation of neurons from human and mouse stem cells lines has been shown to recapitulate many ontological markers described during *in vivo* neurogenesis^[43]. These include markers of the different stages of neurodevelopment, starting from a stem cell state and expressing characteristics of a NSCs stage, neural progenitor cell (NPC) stage and an immature neuron stage before ultimately developing into functional mature neurons.

Neuronal differentiation typically starts with the induction of a neuroepithelial NSC phenotype by withdrawal of pluripotency factors, such as LIF (mouse stem cells) or FGF-2 (human stem cells). The resulting NSCs may be expanded in culture for a limited number of passages to increase neuronal yield^[99-101]. Identifying compounds that sustain the neurogenic competency of NSCs in culture will be valuable for manufacturing purposes, since this will both streamline the differentiation process and increase the total yield. Methods of neural patterning vary wildly. Many protocols involve the use of retinoic acid (RA), which is an early developmental signal for rostral-caudal patterning of the embryonic brain^[102-104]. Differentiation can be further enhanced or directed by a variety of techniques, including supplementation with growth factors, such as brain-derived neurotrophic factor or glia-derived growth factor^[100,105-107]; forced expression of transcription factors that control neuronal fates^[108,109]; or addition of small molecules such as Noggin, Sonic Hedgehog or dorsomorphin^[74,110,111]. Neural patterning can also be influenced by secreted factors, such as the increased production of motor neurons by co-culture of NSCs with muscle cells^[112]. While neuronal maturation can proceed without the deliberate addition of conditioned media or the presence of support cells, some studies suggest that synaptogenesis is significantly enhanced by co-culture with astrocytes^[113,114].

ESCs

ESCs are derived from totipotent cells collected from the inner cell mass of embryos at the blastocyst stage^[115]. Mouse ESC are frequently differentiated to neurons *via* the 4/4 method, which involves neural induction by the withdrawal of LIF and neural patterning by the addition of RA^[116,117]. The resulting ESC-derived neurons (e-neurons) have been shown to produce highly active glutamatergic and GABAergic synapses with emergent network responses^[59] and activity-dependent gene expression^[74]. ESC differentiation protocols can be modified by the addition of exogenous growth factors to generate other neuronal phenotypes, ranging from a mixture of dopaminergic, glutamatergic, cholinergic and adrenergic neurons^[118] to a homogenous GABAergic neuron population with immature, excitatory GABA signaling^[119]. Mouse e-neurons have been found to reliably form functioning synapses and robust emergent networks within weeks, whereas methods to produce comparable levels of network activity in human e-neurons are still under investigation^[28]. It has recently been suggested that many extant human ESC lines exhibit epigenetic markers of partially differentiated epiblasts rather than naïve ESCs, which could provide a plausible mechanism for their relatively poor and inconsistent neurogenic potential^[120]. In contrast, neurons derived from monkey, canine, pig, chicken, worm or fly ESCs^[121-127] have been found to develop neurotypic morphologies, although characterization of the resulting neurons has not included functional measure of synaptic activity and network emergence to date.

NSCs

NSCs are proliferating stem cells that are restricted to neural lineages. NSCs are isolated from fetal and adult CNS, and therefore their availability and use is limited and subject to ethical concerns^[128-131]. One advantage of NSCs is that they appear to replicate *in vivo* mechanisms of adult neurogenesis and therefore may represent a more physiologically appealing model than neurons derived from pluripotent cell lines^[132]. In the brain, slow cycling NSCs in the sub-ventricular zones and in the subgranular zone of the dentate gyrus produce neurogenic progeny that pass through sequential developmental stages with structurally and functionally distinct cellular properties^[133]. For example, in the well-studied rodent hippocampal subgranular zone, GFAP⁺ NSCs progressively differentiate into Trb2⁺ transiently amplifying NPCs, DCX⁺ migratory neuroblasts and finally into post-mitotic NeuN⁺-neurons which functionally integrate into existing hippocampal circuits within 3 wk and adopt mature dentate gyrus characteristics within 6-8 wk^[134]. A similar developmental progression has been shown to occur in cultured NSCs based on morphological analysis^[132]. Some NSCs have been observed to be restricted to specific neural lineages *in vitro*, such as lines that only generate astrocytes and oligodendrocytes^[135,136]. The mechanisms underlying this limited potency are unknown, and whether this is a consequence of culture conditions or representative of

in situ behaviors is unclear. As with iPSC- or ESC-derived NSCs, primary NSCs have a limited ability to proliferate in culture before losing their neurogenic competence^[137,138]. Despite the potential value of NSC models, few functional assays have been performed on neurons differentiated from these sources, making it unclear whether they reliably produce active synapses. Furthermore, the collection of human NSCs is only possible under limited conditions, such as from aborted fetuses, during brain surgery or immediately post-mortem, and consequently their use is likely to have limited clinical utility^[139].

iPSCs

iPSCs are reprogrammed from differentiated tissues through the exogenous expression of pluripotency genes, such as the original Yamanaka reprogramming factors *Oct4*, *Sox2*, *Klf4* and *cMyc* or subsequently described variants^[140,141]. While neurogenic iPSCs have been generated from a wide variety of animals and tissues, this review will specifically focus on human iPSCs, predominantly derived from fibroblasts. For reasons that appear to be attributable to stochastic variability in epigenetic changes that occurs during reprogramming, even iPSC clones derived from the same tissue typically exhibit a range of neurogenic potentials, requiring neurogenesis to be characterized from each individual line^[142,143]. As with human ESCs, there has been a recent focus on converting human iPSCs to a naïve epigenetic state (aka, ground-state), with the objectives of establishing a truly pluripotent phenotype that exhibits greater reliability and reduced variability during differentiation^[144-146]. Despite their current technical limitations, two characteristics of iPSCs render iPSC-derived neurons (i-neurons) highly valuable for clinical research. First is the ability to generate patient-specific i-neurons that express the specific genotypic and phenotypic characteristics for a given disease in the context of the patient's own genome. Second, the decreased risk of immunoreactivity following engraftment of autologous cells back to the patient renders iPSCs a viable model for cell replacement therapies. The clinical implications of these characteristics are described further in next section.

The production of i-neurons is relatively new and protocols to reliably generate synaptically active i-neurons are not yet currently available^[147]. For example, neurons differentiated from both normal and Alzheimer's disease patient iPSCs display neurotypic intrinsic electrophysiological properties within a week after differentiation from NPC cultures, but derived neurons were unable to fire repetitive APs following current injection, so they were considered immature^[148]. Similarly, iPSCs differentiated for 10 wk with standard stem cell derivation and differentiation directed towards cortical neurons were found to express abundant synaptic markers, but electron micrographs indicated sparse vesicles in pre-synaptic compartments, and no activity was reported^[16]. In another example, the ability to fire repeated APs was not observed in differentiated iPSC neurons until

about 12 wk after plating^[28]. These findings suggest that i-neurons produced using current methods tend to be developmentally arrested or undergo functional synaptogenesis much less quickly than rodent SCNs. In contrast, there are reports suggesting the robust production of synaptically active i-neurons; however, reproducible protocols have not been published nor have other labs validated these findings, making it unclear exactly what element(s) of the differentiation protocols promoted functional synaptogenesis^[111,116].

Rationally designed improvements to the speed and efficiency of i-neuron differentiation would be exceedingly valuable for a wide variety of uses, including pharmaceutical screening and cell replacement strategies. The ideal approach would enable the reproducible yield of large quantities of synaptically active neurons of defined lineages in a relatively short period of time. Recent experiments have focused on the ability of transgenic transcription factors and/or small molecules to increase the speed and efficiency of differentiation. For example, overexpression of neurogenin (Ngn1 or 2) transcription factors accelerates maturation of intrinsic neuronal characteristics in i-neurons, but has mixed results on the appearance of post-synaptic currents, with one study finding such currents at 2-3 wk^[149] while another did not find any until 7 wk^[150]. The addition of small molecules to iPSCs during neuronal differentiation increases the efficiency of differentiation into intermediate neuronal precursors^[151]. Small molecules may also be used to influence neuronal patterning, but to date this approach has only been successful in directing cells towards dopaminergic populations, and the resulting neurons have not been assessed for network activity^[152,153]. While these approaches are still immature, the identification of methods that improve the speed and efficiency/specificity of neuronal differentiation has the potential to render i-neurons a transformative tool in basic and clinical sciences.

iNs

Recent work suggests that a neuronal phenotype can be differentiated directly from non-neuronal tissue by the expression of specific genes. Direct conversion, or trans-differentiation, works on many of the same principles as iPSCs, involving the forced expression of transcription factors associated with the target neurons or neuronal intermediates. As with iPSCs, a variety of transcriptional and biochemical factors have been used as neural induction factors for iNs. Initially, the transcription factors Ngn2, Ascl1 (Mash1), and Dlx2 were found to directly convert non-proliferative mouse astrocytes into populations of glutamatergic and GABAergic neurons^[154,155]. Ascl1, along with the factors Brn2 and Myt1l (collectively BAM), can convert mouse fibroblasts into active neuronal populations^[156,157]. Human postnatal and adult fibroblasts appear to require expression of Brn2, Myt1l and microRNA-124 to produce active glutamatergic synapses^[158]. Alternatively, Nurr1, Lmx1a, Ngn2, Sox2 and Pitx3 have been found to induce

fibroblasts to express dopaminergic markers^[159-161], while BAM plus Lhx3, Hb9, Isl1, and Ngn2 promotes expression of motor neuron markers^[162]. Like iPSC models, iNs can be used to replicate functional pathologies. For example, neuroligin-3 mutations associated with autism cause post-synaptic dysfunction in iNs when co-cultured with primary neurons^[163].

The iN field is still new, and many of the challenges facing iPSC-based neuronal differentiation are likely to affect iN approaches as well. Only a small percentage of cells are successfully converted to neurons and most iNs retain epigenetic features of their non-neuronal origins^[161]. Unlike differentiation of iPSCs which can be expanded during the stem cell stage iN conversion has frequently proven to have low efficiency, with only 1%-20% of starting cells successfully reprogrammed to become neurons, although some more recent work indicates conversion rates that exceed 200% (indicating a period of proliferation during the conversion process)^[162,164]. Low success rates may complicate functional neurogenesis, leading to a sparser neuron population, variable intrinsic properties and little to no network activity, as demonstrated in electrophysiological profiling of iN populations with as few as five neuron-like conversions per coverslip^[165]. Conversely, the limited scalability may also render iNs less likely to become tumorigenic following implantation or conversion *in vivo*. One potential way around limited scalability might be to induce differentiated cells into neuronal precursor cells rather than directly into neurons. While this approach has been used to produce presumptive precursors (based on gene expression studies and morphology), it remains to be seen if induced progenitors cells can differentiate into functionally mature neurons^[166,167].

APPLICATIONS OF SCNS FOR NEUROSCIENCE AND THERAPEUTICS

General properties of SCN models that facilitate research applications

In addition to the clinical potential, SCNs have extensive potential for basic and applied neuroscience studies. In many cases, stem cells are genetically tractable, typically clonal and amenable to culturing in large numbers, facilitating the production of scalable quantities of synchronized SCNs that are genetically homogenous and amenable to forward and reverse genetics approaches as well as functional engineering for biotechnological studies^[101,168,169]. While the stem cell stage of SCN differentiation is the most obvious target for genetic manipulation, allowing for isolation and expansion of genetically identical clones, post-mitotic neurons can also be modified using a variety of techniques that are well-described in primary neuron cultures^[170]. Genetic manipulations have been used for a variety of studies. For example, exogenous expression of developmentally regulated transcription factors has been used to explicate the genetic regulators that influence neuronal patterning

and neuron subtype specification^[171,172]. In addition to influencing cell type or development, functionality can be added to SCNs by the expression of transgenic proteins. These functional modifications enable a particular readout, such as fluorescent reporters of gene expression, synaptic assembly, synaptic activity, or metabolic function^[77,173-175]. Recent advances that provide control over neuron activity *via* biologically orthogonal inducers, such as biophotonic or chemogenetic approaches, make it possible to measure and control synaptic activity in networked neuron cultures^[176,177]. For example, expression of channelrhodopsin under the control of a dopaminergic promoter allows for the light-activated stimulation of grafted dopaminergic neurons^[67]. The effects of these controlled stimuli on *in vitro* network behavior could be measured by whole-cell patch clamp or in MEAs, providing an indirect electrophysiological measure of the function of metabotropic synapses. Genetic manipulation may also be used to improve the therapeutic potential of SCNs. Targets include repairing the mutated huntingtin protein in models of Huntington's disease^[178,179] or expressing factors enhancing neuron growth and repair in implanted SCNs^[180,181]. Despite the utility of genetic modulation of stem cells, care must be taken in characterizing the resultant lines. For example, genetic modifications may alter differentiation outcomes, potentially producing cells that are morphologically and structurally neuronal, but lack intrinsic or synaptic electrophysiological characteristics^[182]. Although below we will focus on applications that are not dependent on genetic modification, it is important to remember that in most applications the incorporation of genetically modified SCNs would complement basic and clinical studies.

Study of disease mechanisms in personalized SCN models

The *in vitro* analysis of i-neurons produced from patient-derived iPSCs is expected to be one of the early successes of personalized medicine. Autologous SCN models are already in use for applications such as population-wide phenotypic comparisons and drug screening studies^[183]. Shortly after protocols became widespread to generate iPSCs, laboratories rushed to establish cell banks of iPSCs derived from patients with a wide variety of neurodevelopmental and neurodegenerative disorders, with the goal of developing diagnostics and assessing treatment efficacies *in vitro*^[184-186]. This approach has the distinct advantage of studying the phenotypic disease in the native genetic environment and avoiding confounds that may influence disorder penetrance and prognosis. One example of a disease-specific approach is i-neurons derived from a patient with Dravet syndrome epilepsy, which is caused by a single protein mutation in the Nav1.1 sodium channel. Dravet SCNs exhibited altered excitability, with reductions in evoked AP amplitude and probability of firing^[187]. Autologous iPSCs also facilitate the study of neuropsychiatric diseases with complex polygenic or unknown genetic origins, something

difficult or impossible by the rational introduction of mutations^[188]. For example, i-neurons derived from schizophrenia (SZ) patients have fewer post-synaptic markers and less neurite growth than controls, despite similar intrinsic electrophysiological profiles^[189]. When maintained in an immature NPC-like state, SZ cells were also found to have abnormal cytoskeletal development, increased oxidative stress, and aberrant migration, indicative of developmental abnormalities^[190]. These discoveries would have been considerably more difficult without access to a developmental SCN model.

Many neurodegenerative disorders originate from multiple genetic loci, including Alzheimer's disease, Parkinson's disease and other dementias^[191]. They often exhibit variable penetrance, which is further compounded by a lifetime of unknown environmental interactions. Patient-derived iPSCs offer the potential to study the effects of complex and subtle genetic contributions to these variable disease phenotypes by replicating the genomic context from which the disease is expressed. In some cases, disorders have displayed relatively consistent pathophysiologies in SCN models, enabling high resolution characterization of the disease pathophysiology. For example, i-neurons derived from Parkinson's disease patients from diverse genetic backgrounds exhibit initial morphological characteristics of synaptogenesis, but subsequently degenerate *in vitro*, accumulating α -synuclein and other toxic intermediaries^[192,193]. Other disorders have shown inconsistent pathophysiology *in vitro*, including iPSCs derived from Huntington's disease patients^[194,195] and multiple sclerosis patients^[196]. However, while sometimes confounding, this inconsistent pathology may also contribute to an improved understanding of disease mechanisms. For example, iPSC-derived motor neurons from a subset of amyotrophic lateral sclerosis (ALS) patients exhibit pathophysiological characteristics, while others ALS patient-derived motor neurons appear normal^[186,197-199]. Derived ALS motor neurons cocultured with astrocytes have suggested that some ALS neuropathology is due to aberrant glial function^[200-202]. Together, these studies suggest that ALS may originate from the interactions of multiple cell types and genetic loci, with potentially tissue-specific diagnoses and treatment requirements.

Two potential caveats to the general appeal of iPSCs for disease modeling are the possibility of selecting a non-representative line during reprogramming and increased risk of genomic changes during maintenance of reprogrammed iPSCs. For example, cultured iPSCs have been found to exhibit increased copy number variation, which is associated with a wide range of neuropsychiatric diseases^[203]. Unfortunately, while a variety of assays have been used to characterize the phenotype of neuropsychiatric i-neuron models, few have emphasized differences in synaptic and network activity, in large part because reproducible protocols to generate synaptically active i-neurons are not widely available. In fact, recent reviews have noted that functional assays, such as calcium imaging or electrophysiology are infrequently

performed compared to gene expression studies^[192]. This lack of rigor in characterizing SCN models may further contribute to discrepancies in translational interpretation. While i-neurons are not likely to be viable models for all neuropsychiatric diseases, particularly those that rely on multi-tissue interactions, the pathophysiological relevance of patient-specific SCN models has extraordinary potential to explicate complex disease mechanisms.

Neuro-reparative studies

Unlike the comparatively robust reparative mechanisms of the peripheral nervous system, intrinsic regenerative processes in the CNS do not appear to be capable of repairing damaged nervous tissue and restoring normal brain function in cases of severe or large-scale neural disease or injury^[204]. Consequently, clinical management of traumatic brain injury or degenerative neurological diseases is predominantly limited to supportive or palliative care. One of the most promising treatment strategies for regeneration of neural function is targeted cell replacement (TCR), in which exogenously derived cells are administered to the CNS to ameliorate the disease or injury process, protect from further decline of function and possibly facilitate repair of lost capacity. In one aspect of TCR, *in vitro*-derived neural cells of defined maturity and fate are delivered to specific CNS locations, where in theory they will appropriately integrate into existing circuits. This approach is complicated by several factors, including the intricate and poorly understood physiology of the nervous system; the fact that ontogenic processes such as axonal pathfinding and circuit formation may not be appropriately recapitulated in adult brains; and the difficulty in specifying the character of the grafted cells. Nonetheless, in preliminary studies centrally administered SCNs have exhibited potential as a regenerative CNS therapy. Administration of human NSCs, such as the NSI-566RSC line, have proven safe and effective in animal models of ALS, spinal cord injury, and ischemia^[205], and are currently undergoing clinical trials^[206]. These NSCs appear to form functioning neurons that integrate into existing neuronal networks *in vivo*^[207]. Studies using more mature dopaminergic neurons derived from fetal NSCs have also found that long-term integration and transplanted neurons conferred resistance to PD in mouse models^[208].

TCR has been made more technically feasible by the recent development of autologous iPSCs, which are less likely to evoke undesired immune responses. The development of autologous SCN-based TCR therapies would be immensely valuable for traumatic CNS injury as well as for neurodegenerative diseases such as Parkinson's disease and ALS. However, despite the promise of SCN therapies, early studies suggest that implantation of stem cells and stem cell-derived tissue suffers from several complications, most notably cell survival and functional integration into target circuits^[209,210]. For example, inappropriately integrated cells may disrupt existing circuits while cell death may trigger counterproductive inflammatory responses that further degrade central

function^[211,212]. More severe complications may arise if mitotically active donor cells proliferate to form non-neuronal, neoplastic tissues^[213] or otherwise damage the engraftment site, provoking responses that impair the function of implants over time^[214,215]. Despite these complications, SCN therapies have transformative potential now, and as protocols are established for improved graft survival and integration with reduced adverse host responses, such therapeutic modalities will be realized.

In vitro neurotoxicology

One of the most attractive uses of SCNs is as a scalable, relevant cell model of neuronal responses in pharmacological and toxicological testing^[216]. For example, the physiological relevance imparted by SCNs for *in vitro* toxicology is anticipated to significantly reduce dependence on the use of animals to detect, diagnose or study lethal toxins, such as with the mouse lethality assay, or to conduct initial efficacy testing of candidate therapeutics^[217,218]. Neuron-like immortalized cell lines, such as neuroblastomas and pheochromocytomas, have historically been used in *in vitro* toxicity studies and countermeasure screening protocols for the Tier 1 select agents collectively known as botulinum toxins (BoNTs)^[219-222]. However, as described above, these cell lines have poor neurogenic competence and thus are not suitable to study neurotoxic substances whose modes of action are mediated by neurotypic behaviors, act in specific neuronal compartments or are evidenced through altered neurotransmission^[223,224]. The various BoNT serotypes are good examples of neuron specific agents, as they are internalized at the pre-synaptic compartment and then specifically target and cleave SNARE proteins responsible for neurotransmitter release^[225]. In the absence of a functioning pre-synaptic compartment, it is doubtful that non-neuronal models faithfully reflect the biochemistry and host interactions involved in intoxication. In contrast, mouse e-neurons differentiated into a synaptically active population of glutamatergic and GABAergic neurons with emergent network behavior undergo synaptic blockade within hours after intoxication with femtomolar concentrations of botulinum neurotoxins serotype A^[6,226,227]. This represents the first *in vitro*-derived, cell-based model to manifest the functional pathophysiology responsible for the clinical symptoms of botulism, enabling novel *in vitro* studies on host: toxin interactions. This e-neuron model has also been shown to be highly sensitive to other neurotoxins, such as glutamate and latrotoxin, with mechanisms of injury, toxin sensitivities and cellular responses that are identical to those of primary neurons^[59]. Similar glutamate excitotoxicity has been demonstrated in human e-neurons and i-neurons^[228]. As these models are shown to be increasingly relevant neurotoxicological models, they are anticipated to rapidly become the primary approach for target discovery, drug screening and detection/diagnosis of neurotoxins and neurotoxic effects.

Another valuable application of SCNs in neurotoxin

studies is the modeling of developmental neurotoxicity. While primary neuron cultures may not recapitulate developmental vulnerabilities that occur during early stages of differentiation, SCNs are well-suited for studying specific mechanisms of early developmental toxicity, such as the effects of reactive oxygen production and alterations to specific cell signaling pathways during early stages of neurogenesis^[229-232]. SCNs can also be used to evaluate toxicant-induced changes to relative neurogenic and gliogenic outcomes during neural differentiation, as well as to test for persistent changes in synaptic function, such as the chronic functional deficits resulting from developmental exposure to ethanol^[233-235]. Taking advantage of the scalability of SCN models and high throughput detection platforms such as MEAs or calcium imaging, multiple neurotoxic compounds can be screened and compared in SCN systems over multiple stages of development to assess vulnerable stages and mechanisms^[236-238]. In addition, the use of MEAs facilitates high-throughput monitoring of the development and interruption of network activity^[75,76,239]. Finally by combining scalability with transgene expression, moderate-to-high throughput assays can be developed to rapidly assess the efficacy of candidate therapeutics on injury or disease at various stages of neurogenesis.

Histogenic models: A step beyond networks

Relevance is a key goal of neuronal models. In addition to functional relevance of neuronal monolayers, a relevant histotypic model can be generated by simultaneously producing or combining multiple nervous tissue types in two- or three-dimensional cultures. Depending on the differentiation protocol, multiple neural types, including glia and oligodendrocytes, can be produced simultaneously with neurons^[137,138]. Multiple neuronal subtypes are generated in many SCN differentiation protocols^[43,110,171,240]. The introduction of physcobiological techniques to differentiation methods may further enable the defined production of diverse neural cell types, such as the three-dimensional culture of neocortical-like organoids^[241-245] and use of chemical or physical micro-patterning of plates, including microfluidics^[246,247].

Histogenic neuronal models are anticipated to produce more complex neuronal circuits by facilitating synaptic interactions among multiple neuron types. For instance, complex retinal tissue has been generated from iPSCs^[248-250], including the formation of three-dimensional superstructures that facilitate the development of photoreceptor cells, multiple neuron types and other cells in the retina. The cells themselves respond to incident light with electrochemical and biochemical responses that are similar to those produced *in vivo*. Further elaboration of this retinal model would require optic nerve tissue as well as visual cortex tissue to more comprehensively model the circuits involved in sight. It may be that in producing SCN models with multiple active neuronal and glial types, researchers have incidentally begun to form specific regional patterning and developmental forms of neuroectodermal tissue that essentially produce many

of the outcomes of a histogenic model. For example, the rosettes formed during neurogenesis *in vitro* may be patterned similar to neural tubes or cortical plates *in vivo*^[251]. Similarly, three-dimensional cultures of some ESCs allow for the formation of stratified structures similar to fetal cortex^[252], and even recapitulates the folding of neuroepithelium^[253]. The functional generation of complex tissues such as retina and multi-layered cortex and neuroepithelium solely from stem cells suggests that generation of other complex CNS tissues may soon be feasible.

CONCLUSION

With available stem cell derivation and SCN differentiation techniques growing rapidly, researchers must be able to critically analyze and compare these models to *in vivo* neurons and circuits. Many SCN models, particularly those derived from human origins, still require optimized protocols to reproducibly generate synaptically active neurons. It may be a challenge for some researchers to functionally validate SCN models, but validation is essential for physiological relevance and to increase the translatability of findings regarding neurological development, function and dysfunction. It is encouraging to see newer research in the literature recognizing that functional endpoints, such as the establishment of a neuronal network, are highly sensitive and powerful tools. This review presents some variations in the approaches used to establish synaptic and network activity, but for now it should be apparent to those pursuing electrophysiological approaches that post-synaptic activity must be measured alongside intrinsic characteristics, followed by assays for networked activity. It is hoped that as techniques improve, the means of proving functional synaptogenesis and networked activity in SCNs will also expand. With expanding collections of validated models available, researchers will have excellent options for both basic neuroscience and therapeutic applications.

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Stem cell autotomy and niche interaction in different systems

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Abstract

The best known cases of cell autotomy are the formation of erythrocytes and thrombocytes (platelets) from progenitor cells that reside in special niches. Recently, autotomy of stem cells and its enigmatic interaction with the niche has been reported from male germline stem cells (GSCs) in several insect species. First described in lepidopterans, the silkworm, followed by the gypsy moth and consecutively in hemipterans, foremost the milkweed bug. In both, moths and the milkweed bug, GSCs form finger-like projections toward the niche, the apical cells (homologs of the hub cells in *Drosophila*). Whereas in the milkweed bug the projection terminals remain at the surface of the niche cells, in the gypsy moth they protrude deeply into the singular niche cell. In both cases, the projections undergo serial retrograde fragmentation with progressing signs of autophagy. In the gypsy moth, the autotomized vesicles are phagocytized and digested by the niche cell. In the milkweed bug the autotomized vesicles accumulate at the niche surface and disintegrate. Autotomy and sprouting of new projections appears to occur continuously. The significance of the GSC-niche interactions, however, remains enigmatic. Our concept on the signaling relationship between stem cell-niche in general and GSC and niche (hub cells and cyst stem cells) in particular has been greatly shaped by *Drosophila melanogaster*. In comparing the interactions of GSCs with their niche in *Drosophila* with those in species exhibiting GSC autotomy it is obvious that additional or alternative modes of stem cell-niche communication exist. Thus, essential signaling pathways, including niche-stem cell adhesion (E-cadherin) and the direction of asymmetrical GSC division - as they were found in *Drosophila* - can hardly be translated into the systems where GSC autotomy

was reported. It is shown here that the serial autotomy of GSC projections shows remarkable similarities with Wallerian axonal destruction, developmental axon pruning and dying-back degeneration in neurodegenerative diseases. Especially the hypothesis of an existing evolutionary conserved "autodestruction program" in axons that might also be active in GSC projections appears attractive. Investigations on the underlying signaling pathways have to be carried out. There are two other well known cases of programmed cell autotomy: the enucleation of erythroblasts in the process of erythrocyte maturation and the segregation of thousands of thrombocytes (platelets) from one megakaryocyte. Both progenitor cell types - erythroblasts and megakaryocytes - are associated with a niche in the bone marrow, erythroblasts with a macrophage, which they surround, and the megakaryocytes with the endothelial cells of sinusoids and their extracellular matrix. Although the regulatory mechanisms may be specific in each case, there is one aspect that connects all described processes of programmed cell autotomy and neuronal autodestruction: apoptotic pathways play always a prominent role. Studies on the role of male GSC autotomy in stem cell-niche interaction have just started but are expected to reveal hitherto unknown ways of signal exchange. Spermatogenesis in mammals advance our understanding of insect spermatogenesis. Mammal and insect spermatogenesis share some broad principles, but a comparison of the signaling pathways is difficult. We have intimate knowledge from *Drosophila*, but of almost no other insect, and we have only limited knowledge from mammals. The discovery of stem cell autotomy as part of the interaction with the niche promises new general insights into the complicated stem cell-niche interdependence.

Key words: Stem cell-niche interaction; Male germline stem cells; Spermatogenesis; Erythropoiesis; Stem cell autotomy; Thrombopoiesis

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Core tip: A new mode of stem cell-niche interaction has been observed in insects. Male germline stem cells (GSCs) undergo autotomy by serial segregation of vesicles from finger-like projections. These vesicles either accumulate at the niche surface or are phagocytized by the niche cells. Autotomized projections are apparently replaced by newly sprouting ones. It is suggested that the unprecedented dynamics of GSC autotomy are involved in a not yet known form of information exchange between GSCs and niche. Apoptotic pathways and autodestruction programs could be involved in GSC autotomy.

Dorn DC, Dorn A. Stem cell autotomy and niche interaction in different systems. *World J Stem Cells* 2015; 7(6): 922-944 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i6/922.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i6.922>

EARLY OBSERVATIONS ON STEM CELL-NICHE RELATIONSHIPS PRIOR TO THE ESTABLISHMENT OF THE STEM CELL-NICHE HYPOTHESIS

When, in 1978, Schofield^[1] put forward the hematopoietic stem cell (HSC)-"niche" hypothesis it was solely based on the assumed requirements the niche must fulfill, but he had no knowledge concerning the physical identity of the niche: "The location of the stem cell niche can, of course, only be a matter of speculation, although there are several items of data which suggest that they may well be in intimate association with the bone". It is astonishing how accurate some of his predictions turned out to be. However, up to date the HSC niche is not fully understood and is apparently composed of a variety of different cell types: osteogenic cells, endothelial cells, perivascular mesenchymal cells and adipocytes^[2,3]. Recently, Chasis *et al.*^[4] pointed out that the first description of a hematopoietic niche actually took place 20 years earlier when, in 1958, Bessis^[5] described erythroblastic islands. These represent microenvironmental niches for erythropoiesis. Erythroblasts, which represent oligopotent progenitors derived from a small population of HSCs, are arranged rosette-like around a reticular cell (macrophage) where they proliferate and differentiate (Figure 1A). Erythroblastic islands offer striking structural similarities with another significant model system for research on stem cell niche-interactions, the male germline stem cells (GSCs) and its niche in insects (Figure 1B).

Due to its anatomical simplicity and the advantageous genetic access, the male GSC-niche system of *Drosophila* proved as an invaluable tool to study stem cell-niche interactions on the molecular level^[6]. In insects the GSC niche is located in the apex of testicular follicles and consists of the somatic apical cells (ACs) (called hub cells in *Drosophila*) and the cyst stem cells (CySCs). As in the case of the erythroblastic islands, apical complexes were described long before *Drosophila* became the model system. Figure 2 demonstrates the testicular GSC-niche complex of butterflies published in 1889 by Verson^[7] and in 1911 by Zick^[8], respectively (The first record of apical complexes stems from Spichardt^[9] published in 1886 from studies on butterflies). The drawings in Figure 2, based on light microscopical observations, indicate intricate physical relationships between AC and GSCs which could not be clearly resolved with the techniques available at that time. It is remarkable that several of the early investigators already suggested that the ACs might regulate the fate of the GSCs: Zick^[8] in 1911 believed that the spermatogonial pathway could only be entered after detachment of the germ cells from the AC, and Buder^[10] and Schneider^[11] in 1915 postulated that ACs release an inhibitory factor which prevents the differentiation of GSCs into spermatogonia; Nelsen^[12] in 1931 considered the ACs as an activation center which controls the mitotic activity

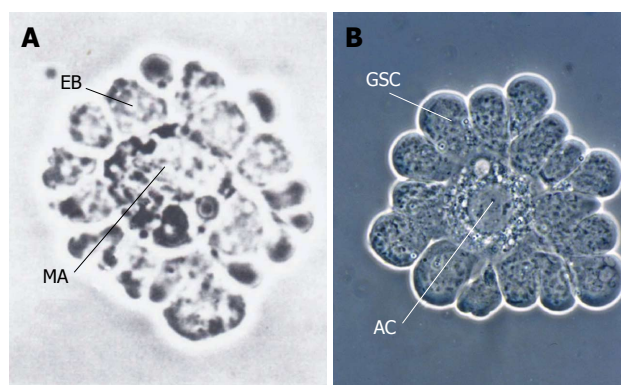


Figure 1 Simple systems of the stem cell/progenitor-niche interaction: isolated erythroblastic island and isolated apical complex from an insect exhibit strong resemblances. A: Erythroblastic island from dissociated rat bone marrow. A corona of erythroblasts (EB) has extensive cell-cell contact with their niche, the central macrophage (MA) (from Bessis *et al.*^[93] with kind permission of Springer Verlag, Berlin, Heidelberg); B: Isolated testicular apical complex from *Locusta migratoria*. Germline stem cells (GSC) surround the apical cell (AC), which represents the niche (besides the peripheral cyst stem cells, which were removed) (From Dorn *et al.*^[64]).

of the GSCs. Thus, main characteristics of a niche were already hypothesized at the beginning of cell research. Since the early investigations on butterfly apical complexes developmental studies have ascertained the identity of GSCs and the role of ACs as their niche (together with CySCs). Electron microscopic studies revealed complex physical interactions between ACs and stem cells which points to a hitherto not elucidated communication between these cells. Figure 3D demonstrates the complicated structure of lepidopteran apical complexes. In fact, enigmatic physical relationships between male GSCs and their niche have been observed in a number of insect species. One of the most astonishing phenomena is the controlled autotomy of GSC projections which are directed toward the ACs. Examples of these cases are examined for their functional significance in this review. They are compared with other stem cell-niche systems where cell autotomy takes place, such as enucleation of erythroblasts and megakaryocyte fragmentation are discussed in respect to the suggested “autodestruction program” of neurons.

GSC-NICHE INTERACTION IN THE TESTIS OF THE MODEL INSECT *DROSOPHILA MELANOGASTER*

The testis of *Drosophila* has become one of the most successful models for the exploration of molecular stem cell-niche interaction. Comprehensive reviews on this matter have been published recently^[13,14]. A summary of the structural and molecular relationships within the apical complex of *Drosophila* is presented here and compared with observations in other insects.

Figure 3A shows the apex of a testicular follicle of *Drosophila*. A longitudinal section of the testicular follicle shows the three cell types that constitute the germinal

proliferation center. A small cluster of somatic cells, the hub cells (*i.e.*, ACs), is located in the follicular apex (the hub). Hub cells, together with CySCs, represent the niche for the row of bordering GSCs. Except for the region where hub cells and GSCs contact one another; each GSC is embraced by a pair of CySCs whose tips also contact the hub cells. CySCs are of somatic origin and, besides their niche function for GSCs, represent stem cells that generate the cyst cells by asymmetrical division. Hub cells only function as the CySC niche. Prior to an asymmetric division of a GSC (which produces a gonialblast that is directed toward the periphery of the apical complex and a daughter GSC that remains in contact with the niche) its two associated CySCs undergo a synchronized division resulting in a pair of daughter cells which encloses the forming gonialblast and becomes the cyst wall. The two cyst cells forming the wall no longer divide during subsequent spermatogenesis although the cysts enlarge considerably.

The fate of GSCs - maintenance, self-renewal (asymmetric and symmetric division), frequency of mitotic activity - is orchestrated by a multitude of factors and processes: (1) Short-range signaling between niche and GSCs. This was the first factor to be elucidated and proved to be exemplary for other stem cell-niche systems; (2) Niche-stem cell adhesion. Adherens junctions were found to play a crucial role in the regulation of signaling and asymmetric GSC division in addition to its physical adhesion function; (3) Cell intrinsic regulation. This has more recently come into focus; and (4) Systemic regulation. This may affect all aspects of niche-stem cell interaction, but is to date the least understood.

Short-range signaling

GSC maintenance and self-renewal are supported by a wide range of signals from the hub. The cytokine ligand unpaired (Upd), secreted by the hub cells activates Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling in GSCs and CySCs^[15,16]. Gonialblast differentiation is caused by lower levels of Upd. In aging flies the number of GSCs and their proliferation rate declines in correlation with declining Upd levels in the hub cells. Upd secreted by hub cells also activates the JAK/STAT signaling in CySCs. Whereas JAK/STAT activation is sufficient for CySC maintenance and self-renewal, GSC self-renewal requires additional signals. Hub cells and CySCs both secrete glass bottom boat (Gbb) and decapentaplegic (Dpp). Both ligands activate the bone morphogenetic protein (BMP) signaling pathway in GSCs. BMP represses the transcription of the differentiation factor bag of marbles (Bam). Thus inhibition of differentiation to gonialblasts contributes to GSC self-renewal^[17,18]. Hub cells also produce the ligand Hedgehog (Hh) that supports the self-renewal of CySCs in addition to JAK/STAT activation. GSC maintenance does not require Hh signaling^[19,20]. Gbb and Dpp produced from CySCs contribute to the activation of BMP

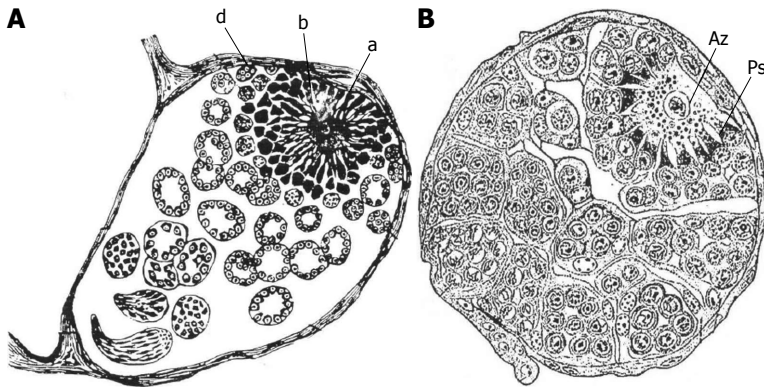


Figure 2 Early histological studies on testicular follicles in butterflies (Lepidoptera) that depict the complex structures of the apical complexes. A: Testicular follicle of the silkworm *Bombyx mori* which includes the apical complex (a, b, d). The limited light microscopical resolution caused some misinterpretation concerning the identity of cell types: the central apical cell (a) was considered to be a "germ cell" ("Keimzelle") with radial extensions. The germline stem cells were described as clumps of protoplasm with nuclei (b, d) (from Vernon^[7]); B: Testicular follicle of the cabbage white butterfly *Pieris brassicae*. The relationship between apical cell (Az) (also called Vernon cell) and germline stem cells (Ps) is correctly described: The germline stem cells send projections toward the apical cell and their tips penetrate the apical cell. A layer of cells surround the germline stem cells, the cyst stem cell which, however, were not identified as such (from Zick^[8]).

in GSCs (besides Gbb and Dpp signaling from the hub cells)^[18]. They are conceivably activated by STAT and/or zinc-finger homeodomain protein 1 which is targeted by STAT as is chinmo (chronologically inappropriate morphogenesis)^[21,22] (Figure 4).

Niche-stem cell adhesion, adherens junctions, E-cadherin

GSCs and CySCs are both connected with hub cells via adherens junctions^[23]. Hub cell-GSC connection plays a crucial role in GSC behavior. Tight contact of the GSCs with hub cells is correlated with high levels of E-cadherin and β -catenin at the interface (adherens junctions). Accumulation of both proteins at the interface is dependent upon guanine nucleotide exchange factor 26 (Gef26) for the Rap GTPase (Rap0-GEF)^[24]. The intracellular domains of the cadherin molecules interact with cytoskeleton-associated proteins. JAK/STAT signaling is required in GSCs to maintain E-cadherin expression, niche anchorage and self-renewal and in CySCs to control BMP expression^[25]. In addition, the leukocyte-antigen-related-like receptor tyrosine phosphatase has been proposed to regulate the attachment of GSCs to the hub cells^[26]. It is responsible for the proper localization of tumor suppressor Adenomatous polyposis coli 2 (Apc2) and E-cadherin and the proper orientation of centrosomes in GSCs^[23]. The BMP receptor complexes are localized to E-cadherin rich adherens junctions at the stem cell-niche junction, which might help restrict BMP signaling activity to the GSC niche interface^[27]. Localized BMP signaling might be also affected by BMP signaling modulators that accumulate in the extracellular matrix such as the protein Magu (known to be involved in life span extension and late age female fecundity) which is transcribed in hub cells^[28] and the heparin sulfate proteoglycans Dally (division abnormally delayed) and Dally-like^[29]. Recently it was demonstrated that the actin-binding protein profilin is required cell autonomously to maintain GSCs, possibly facilitating

localization or maintenance of E-cadherin to the GSC-hub cell interface^[30].

The age dependent loss of GSC is accompanied by a decline in E-cadherin expression. Increased E-cadherin expression slows down GSC loss^[31].

E-cadherin is also required in CySCs to maintain their adhesion to the hub. In addition, integrin-mediated adhesion exists between the hub and CySCs, and is limited by a negative regulator of STAT signaling^[32].

Integrins: In the *Drosophila* testis, competition exists between GSCs and CySCs and among CySCs themselves for occupancy of the hub^[33]. Interestingly, the CySCs with higher JAK/STAT signaling activity, which can be achieved experimentally by removing the function of the JAK/STAT negative regulator SOCS36E, can outcompete normal CySCs and can also push GSCs out of their niche. This JAK/STAT-regulated stem cell competition is dependent on the cell adhesion protein β PS integrin, but not E-cadherin. Integrin-mediated cell competition is thus thought to play a crucial role in balancing two stem cell populations in the same niche^[33]. Integrins are also required for positioning the hub in the apical testis tip, but are dispensable for GSC or CySC anchorage to the niche^[34]. The extracellular domains of integrins can bind directly to extracellular matrix (ECM) proteins, such as laminin, but there is no ECM between hub cells, GSCs and CySCs.

Gap junctions: The gap junction protein zero population growth is required for GSC maintenance and differentiation in *Drosophila* testes^[35]. But it remains to be seen whether the function of gap junctions in the regulation of stem cell maintenance derives from their adhesion role, intercellular molecular transfer or electrical communication.

Cell intrinsic factors, hub cells

Upd levels in hub cells are regulated by IGF-II mRNA

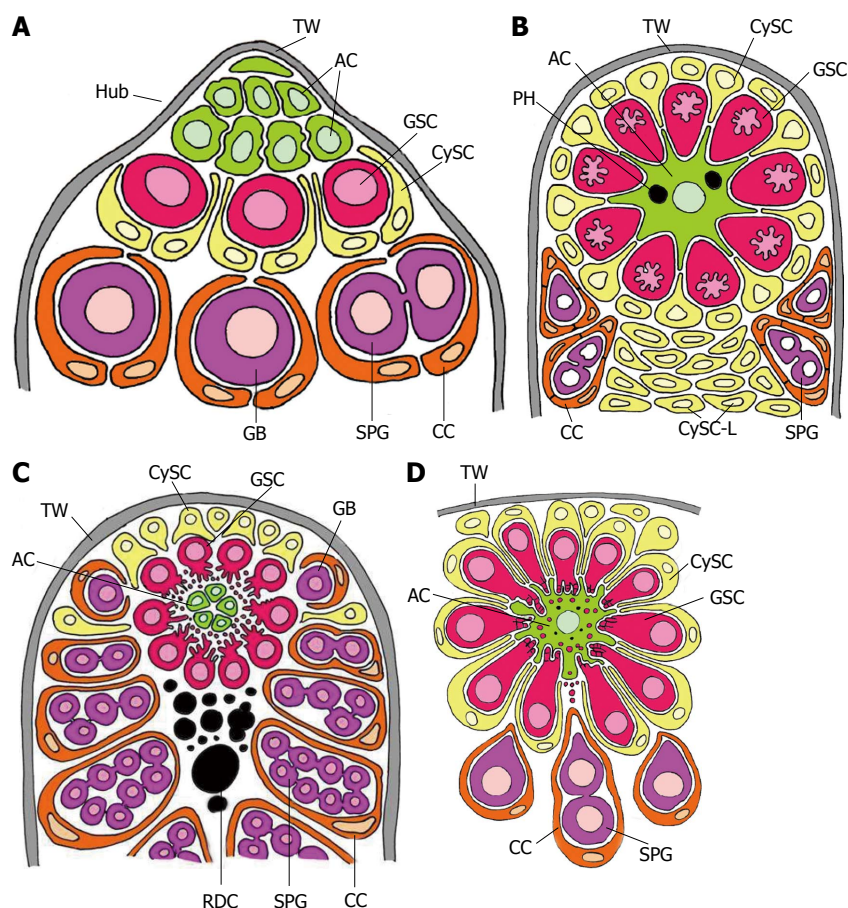


Figure 3 Schematized longitudinal sections through the apices of testicular follicles of four insect species. The order of the images from (A) to (D) is arranged according to increasing complexity of the structural relationships between germline stem cells (GSCs) and their niche. It should be noted that the order is not in congruence with the positions of the species in the natural insect system based on evolutionary progress. **A:** *Drosophila melanogaster*. The ACs (hub cells) are located in a small terminal appendix of the follicle, the hub (HUB), where many of them border the testicular wall (TW). (The testicular wall consists of an outer pigment layer resting on a basal lamina and a muscle cell layer sitting on an inner basal lamina.) The testicular wall does not provide a hemolymph-testis barrier. A spermatogonial is constituted by two CCs which protect the spermatogonia against hemolymph. Both GSCs and CySCs contact ACs (Adapted from Hardy *et al.*^[61]); **B:** *Locusta migratoria*. Depicted is the rosette-like arrangement of GSCs and CySCs with the single AC in the center. The AC of this example includes two phagocytized GSCs (see above). Besides GSCs also CySCs contact extensions of the star-like AC. A plug of CySC-like cells (CySC-L) is located below the apical rosette. The wall of the spermatogonial cysts is composed of numerous CCs (Adapted from Szöllösi *et al.*^[154] and Dorn *et al.*^[64]); **C:** *Oncopeltus fasciatus*. The cellular extensions of GSCs and segregated vesicles surround the surface of ACs. CySCs cover only the distal part of the apical rosette; they do not make contacts with the ACs. Proximal to the rosette, remnants of degraded GSCs and probably young cysts amass (RDC). Cysts form at the lateral parts of the rosette (Adapted from Schmidt *et al.*^[65]); **D:** *Lymantria dispar*. Note that the cellular extensions of GSCs protrude into the large singular AC. The extensions autotomize and the segregated vesicles are phagocytized by the AC. Each GSC is affiliated with one CySC. During early larval development the AC is attached to the TW [comparable to the situation in *Drosophila*, where ACs (i.e., hub cells) are lifelong attached to the TW], but separates with progressing development. The apical complex then adopts a spherical organization. (Adapted from Klein^[66]). AC: Apical cell (often called hub cell in *Drosophila*) (green); CC: Cyst cell (orange); CySC: Cyst stem cell (yellow); GB: Gonialblast (purple); GSC: Germline stem cell (red); SPG: Spermatogonia (purple); TW: Testicular wall (grey).

binding protein (Imp) that binds to upd mRNA and protects it from degradation caused by short interfering RNAs. Imp itself is repressed by let-7 microRNA (miRNA) that is expressed at higher levels in aging male GSCs^[36]. epidermal growth factor (EGF) signaling negatively regulates GSC division frequency in adults, but not in larvae, and promotes gonialblast differentiation and enclosure of germ cells by somatic cyst cells. Stg (string, a Cdc 25 homolog-phosphatase) is essential for activating cyclin-dependent kinases and promoting the cell cycle and is therefore required for proliferation and maintenance of GSCs and CySCs. The transcriptional regulator lola (longitudinals lacking) is cell autonomously required for GSC (and CySC) maintenance^[37]. MiRNAs control the stem cell differentiation pathway by re-

gulating Bam^[38]. Recently, the impact of epigenetic factors on male GSCs has been analyzed. Nucleosome remodeling factor promotes STAT expression while repressing Bam thus contributing to the maintenance of GSCs^[39]. Additionally chromatin-associated proteins, such as no child left behind and PHD finger protein 7 are necessary for GSC maintenance^[40,41]. CySCs. JAK/STAT signaling in CySCs is suppressed by suppressor of cytokine signaling at 36E (Socs36E) which may harmonize self-renewal of CySCs with that of GSCs^[33,42]. Ken (ken and barbie, a transcriptional repressor) also promotes CySC identity^[43]. Restriction of proliferation and maintenance of CySC identity are affected by polycomb repressive complex 1 genes posterior sex combs and suppressor of zeste two [Su(z)2]^[44].

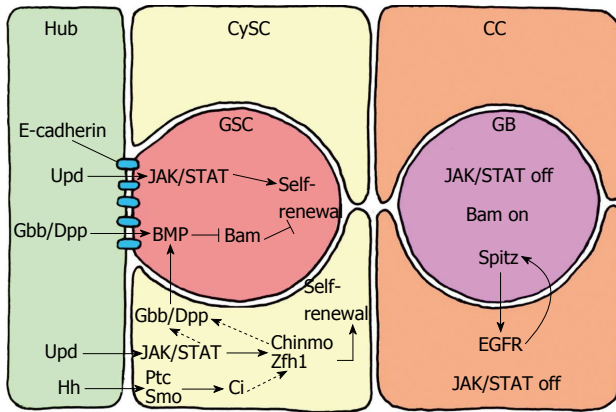


Figure 4 Short range signalling between the germline stem cells and their niche, which consists of the hub cells and the cyst stem cells. The hub cells secrete the ligand Upd, which activates JAK/STAT signalling in GSCs and CySCs. Whereas JAK/STAT activation is sufficient for CySC maintenance and self-renewal, GSC self-renewal requires additional signals. Hub cells and CySCs secrete the ligands Gbb and Dpp, which activate BMP in GSCs. BMP suppresses the transcription of Bam that inhibits the differentiation of GSCs to gonialblasts. BMP activation in GSCs is also supported by Gbb and Dpp produced by the CySCs. Gbb and Dpp in CySCs are conceivably activated by STAT and/or Zfh1 and Chinmo. Hub cells also secrete the ligand Hh (hedgehog), which supports CySC self-renewal in addition to, but independently of, JAK/STAT activity. Hh binds to the transmembrane receptor PTC (patched) of CySC, which releases Smo (smoothened) from repression. This leads to the activation of the transcription factor Ci (cubitus interruptus), which activates the transcription of target genes that support maintenance and self-renewal of CySCs besides JAK/STAT activity. The Hh signalling pathway in CySCs may also affect Zfh1 and, via Gbb/Dpp, influence BMP signalling in GSCs. Thus, Hh signalling in the testis niche apparently has a dual role. CC: Cyst cell; GSCs: Germline stem cells; CySCs: Cyst stem cells.

The histone variant His2Av and the ATP-dependent chromatin-remodeling factor Domino are also required for GSC and CySC maintenance. Furthermore, in a recent review Zoller *et al.*^[45] listed about 50 genes that were found (or expected) to be involved in the direction of CySC specification, CySC self-renewing divisions, cyst cell differentiation, and soma-germline interactions.

Systemic factors

Numerous environmental factors, such as changing seasons or periods of drought and rain, nutritional conditions (irregular food availability or starvation), injury or illness all affect tissue homeostasis. As a consequence niche and stem cell activity must be adapted to these changing demands. This is primarily accomplished *via* systemic factors that may influence any of the regulatory entities of the stem cell-niche complex.

Best known is the support of GSCs maintenance by insulin signaling^[46,47]. The source of some insulin-like peptides is the brain others are synthesized in the fat body and other tissues probably including the GSCs themselves. Effects of the nutritional status on GSC maintenance are apparently exerted by insulin signaling pathway^[48]. In the *Drosophila* female it has been shown that the nutrient-sensing insulin/FOXO signaling directly controls Notch activation in the GSC niche which maintains the niche and GSC identity^[49]. In maintaining embryonic stem cell pluripotency and the modulation of

adult stem cell quiescence nutrient-sensing pathways play an important role. They maintain energy production by inhibition and stimulation of crucial processes like oxidative phosphorylation and glycolysis. "This interplay is key to the maintenance of stem-ness"^[50].

Recently it has been found that day-night cycles and alterations in sleep can influence the daily dynamics of GSC divisions in male *Drosophila*^[51]. The GSC division rate increases, when the sleep-promoting factor, Sleepless, is lacking. This is mediated, in part, by the GABAergic signaling pathway.

A systemic signal that presumably plays a decisive role in testis development and spermatogenesis is the steroid hormone ecdysone^[52]. It is synthesized in testicular tissue of many insects: *Heliothis virescens*^[53], *Lymantria dispar*^[54], *Ostrinia nubilalis*^[55], *Spodoptera littoralis*^[56], *Melanoplus sanguinipes*^[57]. However, its significance in the regulation of male GSC proliferation and self-renewal in *Drosophila* is not known.

Ecdysteroids play a role in female GSC regulation in *Drosophila*. Ecdysteroids are synthesized by developing follicles of ovarioles and regulate directly GSC maintenance, proliferation and self-renewal. Ecdysteroids interact with the intrinsic epigenetic factor ISWI, a chromatin remodeling factor^[58]. The survival of ecdysone-producing follicles of ovarioles depends on the availability of food which points to an interaction of the hormonal with the nutrient-sensing signaling pathways^[59].

INCREASING COMPLEXITY OF STRUCTURAL RELATIONSHIPS BETWEEN MALE GSCS AND THEIR NICHE IN DIFFERENT INSECT SPECIES: DROSOPHILA MELANOGASTER, LOCUSTA MIGRATORIA, ONCOPELTUS FASCIATUS, LYMANTRIA DISPAR, AND THE CASE OF LAMPYRIS NOCTILUCA

Insects with their long evolutionary history may be expected to present great variations on a theme, in this case the organization of GSCs and their niche. And in deed, the Figure 3 exhibits an increasing complication of the physical interactions of the cells of the apical complex. However, the complexity of the structures is not correlated with the systematic position of a given species. In fact, whereas the hemipteran *Oncopeltus fasciatus* (Figure 3C) shows an astonishingly irregular and dynamic anatomy of GSCs^[60], another hemipteran, *Corizus hyoscyami*, has a relatively "simple" apical complex which harbors only one small AC, sparsely equipped with cell organelles, surrounded by a corona of pear-shaped GSCs (Klein personal communication). Nonetheless, it appears self-evident that the enigmatic structures expressed especially in the GSCs of *Oncopeltus fasciatus* and the lepidopteran *Lymantria dispar* (Figure 2D) - as well as in many other lepidopterans (Figure 2) - have a

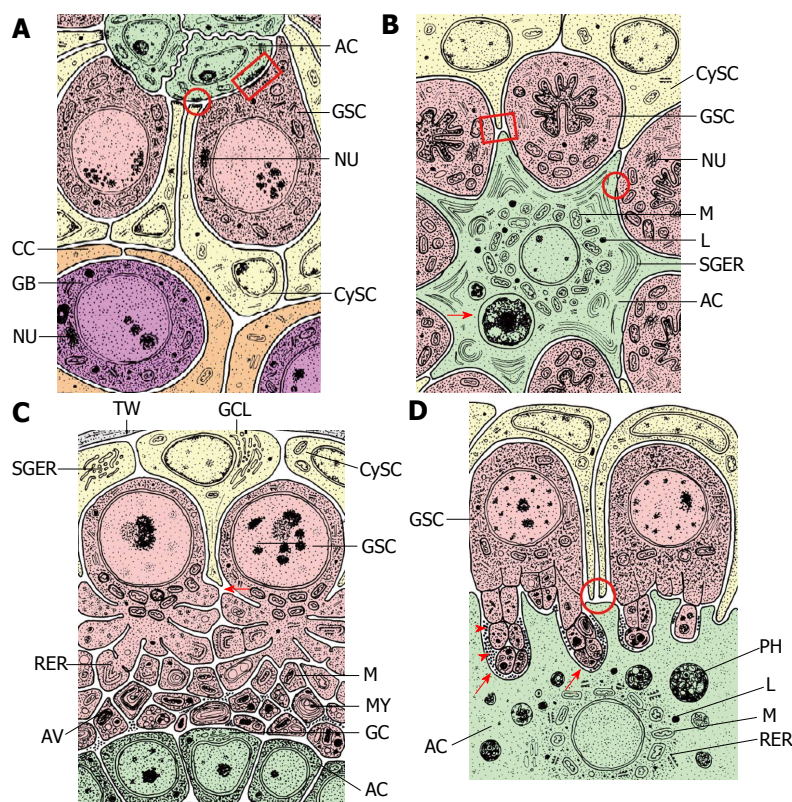


Figure 5 Fine structural organization (schematized) of the apical complexes (longitudinal sections) of four insect species. The order (A) to (D) is arranged according to the order in Figure 3. A: *Drosophila melanogaster*. ACs are "light" and interdigitate richly between themselves. Their organelle equipment is scarce. GSCs are "dark" due to many free ribosomes and characterized by "spongy bodies" which may represent nuage. Adherens junctions are expressed between ACs and GSCs (marked by a rectangle) and between ACs and CySCs (encircled). CySCs are "light" and include few cell organelles (Adapted from Hardy *et al.*^[61]); B: *Locusta migratoria*. The single apical cell of an apical complex is "light" and harbors a complex organelle equipment: in particular a ring of mitochondria (M) and lysosomes (L) around the nucleus, and stacks of sparsely granulated endoplasmic reticulum (SGER) at the cell periphery. Arrow marks a phagocytized and partly lysed GSC. ACs and GSCs are connected by gap-like junctions (encircled). The "dark" GSCs include extremely irregularly shaped nuclei and nuage-like material (NU). Extensions of the "light" CySCs reach to extensions of the star-shaped AC (marked by square) (Adapted from Dorn *et al.*^[64]); C: *Oncopeltus fasciatus*. The ACs are rather small and very "dark". Cell organelles are inconspicuous besides Golgi complexes (GC) that face bordering GSC vesicles. The "dark" GSCs form cytoplasmic projections toward the ACs that undergo progressing autotomy. In the course of vesicle segregation rough endoplasmic reticulum (RER) and mitochondria (M) increase. Later autophagic vacuoles (AV) and myelin-like bodies (MY) are formed. Arrow points to a presumably newly sprouting cell projection. The "light" CySCs are characterized by extensive Golgi complex-like structures (GCL) that are often associated with sparsely granulated endoplasmic reticulum (SGER) (Adapted from Dorn *et al.*^[60]); D: *Lymantria dispar*. The single AC of an apical complex is "light" and exhibits a spherical organization: mitochondria (M), lysosomes (L) and rough endoplasmic reticulum (RER) surround the nucleus. The cell periphery shows deep indentations caused by invading, autotomizing GSC projections (arrows). Segregated GSC vesicles are taken up by the AC, and phagosomes (PH) accumulate in the cytoplasm. The "dense" GSCs exhibit projection formation and projection autotomy that closely resembles that of *Oncopeltus* (see Figure 4C). GSC projections that indent the AC are often surrounded by extracellular granules (arrow heads). CySCs are "light", and thin extensions reach the surface of the GSCs (encircled) (Adapted from Klein^[66]). AC: Apical cell (green); CC: Cyst cell (orange); CySC: Cyst stem cell (yellow); GB: Gonialblast (purple); GSC: Germline stem cell (red); TW: Testicular wall (grey); AV: Autophagic vacuole; GC: Golgi complex; GCL: Golgi complex-like structure; L: Lysosomes; M: Mitochondrion; MY: Myelin-like body; NU: Nuage; PH: Phagosomes; RER: Rough endoplasmic reticulum; SGER: Sparsely granulated endoplasmic reticulum.

specific function (see below), in reverse to the principle: no function without structure. We will briefly describe the portrayed apical complexes and characterize the cell types.

Drosophila melanogaster

Figure 3A demonstrates the rather "ordinary" organization of the apical complex and Figure 5A the fine structural characteristics of its different components. The niche consists of 8-16 small hub cells, which richly interdigitate and are anchored to the testicular wall. They are characterized by a "light" appearance and scarcity of cell organelles. No mitoses were observed. The GSCs are, in contrast, rather "dark" due to many free ribosomes. They have a spheroidal shape but are flattened where they contact the hub cells. Their

cytoplasm includes "spongy bodies"^[61] which may represent nuage material that is typical for germ cells^[62]. Mitoses of GSCs were rarely observed by the above authors, *i.e.*, one mitotic GSC in 50 GSCs. E-cadherin-mediated adherens junctions (Figure 4A) attach GSCs to hub cells^[26]. The CySCs are rather "light" and have inconspicuous organelle equipment. They also form adherens junctions with GSCs (Figure 5A). Among the 76 CySCs observed by Hardy *et al.*^[61] only two were in mitosis.

Locusta migratoria

The longitudinal section through the follicular apex (Figure 3B) shows that the apical complex has the shape of a rosette with a singular AC in the center. The pear-shaped GSCs reach deeply into the large star-like AC.

The CySCs are irregularly arranged around the GSCs and it is not clear how many CySCs are affiliated with a GSC. The ACs touch the protrusions of the CySCs between the GSCs. Remarkably, a plug of CySC-like cells is located beneath the rosette formed by AC, GSCs and CySCs. The CySC-like cells presumably participate in the formation of the cyst wall which consists of a higher number of cyst cells, up to 7 were counted on serial sections^[63].

In contrast to the hub cells in *Drosophila* the large AC of *Locusta* (also "light") shows a complex organelle equipment (Figure 5B). Around the large centrally located nucleus there is a broad ring of mitochondria that also includes lysosomal bodies. The periphery of the AC shows an abundance of sparsely granulated endoplasmic reticulum, often arranged in stacks and whorls that are also found in the cellular extensions that reach deeply between the basal parts of GSCs. The AC includes regularly one or two engulfed and more or less lysed GSCs (Figures 3B and 5B). The GSCs, "darker" than the ACs, show a polar organization. The nuclei are located in broader peripheral parts of the cells and present extremely irregular outlines. In the direction to the AC, mitochondria aggregate and fibrous nuage-like material is discernible. Free ribosomes are abundant. AC and GSCs form gap-like junctions (Figure 5B) but adherens junctions are not apparent^[63]. No specific junctions seem to exist between CySCs and GSCs. CySCs and CySC-like cells have the same fine structural characteristics but only the CySCs contact the AC. Both cell types are rather "light". Their cytoplasm is scant and shows no specifications. In mature males, many dividing GSCs, CySCs and CySC-like cells can be observed^[64]. Both, asymmetrical and symmetrical divisions occur at the same time. Sometimes whole clusters of these cells are seen in mitosis.

Oncopeltus fasciatus

The longitudinal section through the follicular apex of *Oncopeltus* (Figure 3C) reveals an organization of the apical complex similar to that of *Locusta* (Figure 3B), however, with important cytological differences of the different cell types. The niche is represented by a small cluster of "dark" cells with a relatively thin cytoplasmic lining. Small areas of the cytoplasm include a Golgi complex in continuation with some strands of smooth endoplasmic reticulum which is directed toward the periphery where GSCs are bordering (Figure 5C). Specialized cell-cell contacts were not seen^[60]. The GSCs reveal an extraordinary structure and astonishing dynamics. The GSCs have a polar structure, with a lobular perikaryon oriented toward the CySCs and prominent cytoplasmic projections toward the ACs (Figure 5C), which are reminiscent of neurons. The projections exhibit trabecular or septum-like ingrowths that are most advanced at their tips, next to the ACs. The process results in the segregation of free vesicles that amass around the niche. During the autotomy of the projection terminals, the number of mitochondria

increases in these segments, lysosomal bodies and autophagic vacuole-like vesicles become abundant and rough endoplasmic reticulum is often arranged in whorls (Figure 5C). Degradation processes proceed, indicated by the presence of acid phosphatase and TPPase, resulting in myelin- and autophagosome-like bodies^[60]. Autotomized vesicles aggregate at the surface of the ACs (Figure 6A). They finally rupture and release their content in the vicinity of the ACs. None of the debris is taken up by the ACs. Remarkably, intact-looking cytoplasm contains free ribosomes grouped in clusters. These clusters break up in slightly advanced stages of autotomy and the ribosomes are then evenly distributed. It is suggested that they give rise to electron dense granules of about 25 nm in diameter. These grana are sometimes enclosed in projection terminals; grana of lesser diameter occur free in the extracellular space between projection terminals and ACs (Figure 6A). This is of special interest, since morphologically similar grana were reported to be present at the same location in a number of apical complexes of different insect species (see *Lymantria dispar*, below). It is not clear whether or not these grana are taken up by ACs. After advanced autotomy of GSC projections new projections sprout at the "neck" of GSCs, where the projections arise from the perikaryon (Figure 5C). In this "neck" area of the cell there is an accumulation of mitochondria. CySCs surround only the apical part of the apical complex (Figure 3C). The ratio CySCs:GSCs is roughly 1:1, and only one cyst cell grows around a gonialblast. The cyst cell does not divide any more after its generation by division of a CySC but becomes highly polyploid as it enlarges during spermatogenesis^[60]. Divisions of GSCs and CySCs are rarely observed. Asymmetrical divisions of GSCs, where the spindle axis is oriented perpendicularly to the niche, were never observed. During symmetrical GSC divisions, the cell projections are persistent. In the process of gonialblast formation one of the GSCs, that shows no structural difference to the remaining GSCs, moves away from the niche toward the periphery. It loses its projections as the cyst cell encloses it. The spermatogonial cysts now move proximally. Cyst cells, which are rather "light", like the CySCs, develop striking organelles, composed of multiple complexes consisting of a meshwork of branching and anastomosing tubules and budding off vesicles which partly enclose electron-dense material. The Golgi complex-like structures (about 2.8 µm long and 1.1 µm in diameter) are often associated with sparsely granulated endoplasmic reticulum. The mitochondria are exceptionally long and branched. Cyst cells take up apoptotic spermatogonia^[65]. Follicular apices include regularly extensive clusters of degenerating GSCs and spermatogonia (Figure 3C).

Lymantria dispar

The niche of the apical complex consists of only one large AC (Figure 3D). The shape of the apical complex changes during development. During the first three larval stages the AC is attached to the envelope of the follicular apex,

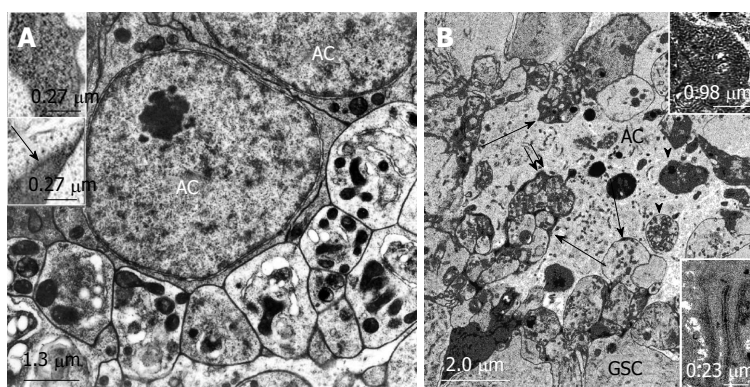


Figure 6 Structural relationships between apical cells and projections and autotomized vesicles of germline stem cells in *Oncopeltus fasciatus* and *Lymantria dispar*. A: Electron micrographs. *Oncopeltus fasciatus*. Vesicles that are segregated from GSC projections accumulate at the surface of ACs. The vesicles show signs of degeneration. Mitochondria are abundant and appear electron dense. (From Schmidt *et al.*^[65]). Upper inset: Intracellular granules in autotomizing GSC projections. Lower inset: Extracellular granules (arrow) between autotomizing GSC projections; B: Electron micrograph. *Lymantria dispar*. Numerous autotomized GSC projections protrude deeply into the AC (long arrows). Some of the segregated vesicles were apparently taken up by the AC and are being digested (arrow heads). Double arrow points to extracellular granules between GSC vesicles and the AC. Upper inset: Extracellular granules at higher magnification. Lower inset: Tubular indentations into the AC include electron dense material. (From Klein^[66]). GSCs: Germline stem cells; AC: Apical cell.

and only the distal part of it is contacted by GSCs^[66]. From third to fourth larval stage the AC detaches from the follicle envelope and moves somewhat distally. Then it adopts a concentric organization, and the GSCs attach from all sides (Figure 6A). Each GSC is accompanied by one CySC whose perikaryon covers the peripheral part of the GSC and sends delicate projections to the AC, separating neighboring GSCs. Whereas the symmetry of the apical complex changes during larval development (from bipolar to rotationally symmetrical), the intricate relationships between AC and GSCs are largely similar until the pupal stage when signs of senescence become apparent and cyst formation seizes^[66].

The large, "light" AC includes a centrally located nucleus around which the cell organelles are concentrically arranged (at progressed developmental stages) (Figure 5D). Mitochondria, rough endoplasmic reticulum and lysosomes are especially abundant. As a rule the cell contains phagosomes, exceedingly numerous during early larval stages, with cell fragments of variable degree of degradation (Figure 5D and 6B). The periphery of the AC shows many Golgi complexes. The pear-shaped GSCs are "darker" than the AC apparently due to the presence of many free ribosomes. Striking are the cell projections of GSCs that deeply invade the AC. Similar to the GSC projections in *Oncopeltus*, GSC projections of *Lymantria* autotomize. But, unlike to *Oncopeltus*, the separated vesicles are phagocytized by the AC (Figure 6B). The GSC projections contain whorls of rough endoplasmic reticulum, many free ribosomes, mitochondria and multivesicular bodies. Phagosomes of the AC include similar cell organelles before they are digested indicating their origin from GSC projections. The electron micrograph Figure 6B demonstrates the extremely complex interactions between GSCs and AC. Extracellular space between GSC projections and AC regularly contains electron dense granules of 25-38 nm in diameter (Figure 6B inset 1). Tubular invaginations of the AC also contain electron dense material which often

exhibits a fibrillar consistency (Figure 6B). A relationship with the dense granules is unclear. The large nucleus of the GSCs is located in the peripheral part of the cells. The organelles in the perinuclear cytoplasm are inconspicuous. Symmetrical as well as asymmetrical divisions have been observed (see below). CySCs are "light". They divide apparently prior to the associated GSC^[66].

Lampyrus noctiluca

The glowworm represents a special case in as far as no niche cells (ACs) for the GSCs could be identified^[67]. During early larval stages the gonadal follicles only include ("dark") GSCs but no ACs and no CySCs; male and female gonads can not be differentiated. The onset of testis differentiation is marked by the appearance of "light" cyst progenitor cells (CPCs) segregated from the apical part of the follicle wall^[67,68]. Whereas a cluster of these cells is located and multiplies in the apex of the follicle, a cluster of dividing GSCs is located at the basal part of the follicle. The ratio of GSCs/CPCs is about 1/1. At that stage of development there are no associations between individual GSCs and individual CPCs. ACs are never observed. In later larval development, CPCs form cell projections, move toward and between the germ cells, which now may represent gonialblasts, contact and ensheath them, thus forming spermatogonial cysts^[67]. During transformation from CPCs to cyst cells, the cells develop conspicuous stacks and whorls of smooth endoplasmic reticulum. It was speculated that the cyst cells may produce hormones, *i.e.*, juvenile hormone or ecdysone. Since all GSCs/gonialblasts and CPCs engage in cyst formation at approximately the same time, a precise temporal regulation of GSC division and gonialblast differentiation seems obsolete and the function of a niche therefore not necessary. The absence of ACs has also been reported from several other insect species^[69].

In summary primarily electron microscopic studies

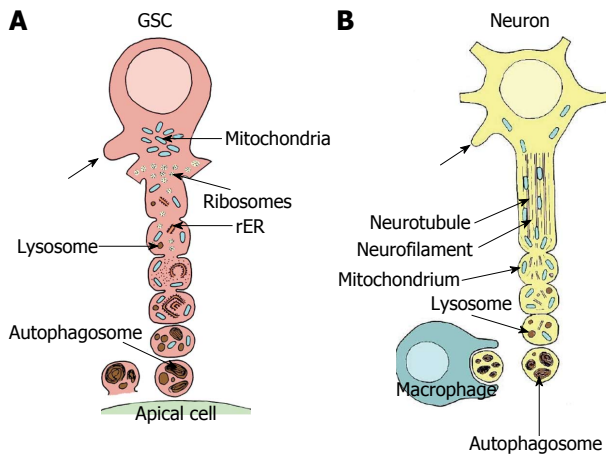


Figure 7 Comparison of autotomizing germline stem cell projection with autodestruction of an injured axon. A: Sequence of vesicle formation of a GSC projection in *Oncopeltus fasciatus*. The area of projection formation is characterized by an accumulation of mitochondria (blue). At the base of the projection ribosomes form small clusters (yellow), mitochondria are infrequent. Rough endoplasmic reticulum (rER, orange) and lysosomes (brown) are scarce. With progressing vesicle segregation mitochondria become more frequent and swollen, ribosomes form no longer clusters, rER becomes more prominent and lysosomal bodies increase. Segregated vesicles show many autophagosomes and myelin bodies. They accumulate at the surface of the apical cells (green) and disintegrate. Arrows point to newly sprouting projections (adapted from Dorn *et al.*^[60]); B: Sequence of progressive axonal fragmentation after injury. First neurotubules and neurofilaments break down. Then mitochondria accumulate and lysosomes (brown) become more abundant. Finally vesicles which mainly include autophagosomes and myelin bodies are segregated and taken up by macrophages. Arrows points to newly sprouting axon (adapted from Lingor *et al.*^[155], Beirowski *et al.*^[156], Kerschensteiner *et al.*^[157]). GSC: Germline stem cell.

revealed the enigmatic relationship between GSCs and their niche in numerous insects. Identification of the different cell types and their interactions were facilitated by the profound differences in electron density between the components of the apical complex: GSCs, ACs and CySCs. In almost all species studied, the ACs and the CySCs were "light" and the GSCs "dark". *Oncopeltus* is an exception: here, ACs are "very dark" whereas GSCs are "dark" and the CySCs "light". But, whereas the "light" and "dark" marking facilitates identification of the cell types, the functional significance of these characteristics remains unclear. In contrast, the intriguing processes of GSC autotomy and the interaction of autotomized GSC vesicles with niche cells (ACs) point to a to date unknown form of communication between GSCs and their niche. Next we will examine the process of GSC autotomy and compare it with morphologically similar processes in other systems: axon autodestruction, erythropoiesis and thrombopoiesis.

THE AUTOTOMY OF GSC PROJECTIONS: ARE THERE COMPARABLE PROCESSES IN OTHER CELL TYPES?

Autotomy of GSC projections in *Oncopeltus fasciatus* and *Lymantria dispar*

In both species the autotomy process follows an apparently standardized pattern (see above). Figure 7A

gives a schematized view of the process in *Oncopeltus*. At the base of the cell projections the cytoplasm exhibits similar organelle equipment as the GSC perikaryon: scattered mitochondria, few strands of rough endoplasmic reticulum, many free ribosomes, often forming typical clusters and few lysosomes. The fractionation of the projections that leads to vesicle formation starts with the sequential ingrowth of the plasma membrane from the periphery (described by Schmidt *et al.*^[65] in *Oncopeltus*). With progressing vesiculation of a projection, mitochondria and rough endoplasmic reticulum that often form concentric whorls, become more abundant, clusters of ribosomes dissolve and the ribosomes are scattered evenly. Mitochondria and vacuoles accumulate. With progressing projection segregation mitochondria become swollen and show signs of degradation and the number of lysosomal bodies increase. Finally, autophagosomal activity becomes evident^[60] and secondary lysosomes and myelin-like become abundant. The terminal vesicles then completely segregate from the GSC projections.

In the case of *Oncopeltus* the separated vesicles amass at the surface of the ACs, where they eventually rupture and release their contents. In the case of *Lymantria* the GSC projections are embedded in the AC and the segregated vesicles are phagocytized and digested by the AC (Figure 6B).

The process of projection segregation of GSCs exhibits remarkable similarities with degenerative processes of axons that take place either after injury, in neurodegenerative diseases or during developmental axon pruning. In the following it will be discussed whether an autodestruction program that may underlie the axonal destruction processes could possibly also be active in the process of GSC autotomy.

INJURY-INDUCED AXON DESTRUCTION (WALLERIAN DESTRUCTION) AND THE HYPOTHESIS OF AN AUTODESTRUCTION PROGRAM

Programmed cell autotomy seems to be a rather rare process. It is best known in axon degeneration that occurs after nerve injury in vertebrates and invertebrates (Wallerian degeneration), developmental neuron pruning and as pathological symptoms of neurodegenerative diseases (multiple sclerosis, Parkinson's disease and others)^[70]. Other examples of programmed cell autotomy are the formation of blood platelets and erythrocytes in mammals which are discussed later. Wallerian degeneration (Figure 7B) shares morphological similarities with axon degeneration during normal brain development (axon pruning) and with the "dying back" degeneration in neurodegenerative diseases.

Recent studies on the degeneration of injured axons strongly suggest that an active autodestruction program exists akin to apoptosis, and that the autodestruction pathway may be conserved between fly and human^[71,72]. The sequence of progressing autodestruction of the

stumps of transected giant nerve fibers in the cockroach *Periplaneta americana*, for instance, follows closely the segregation of GSC projection terminals in *Oncopeltus* (Figure 7): swelling of mitochondria, accumulation of myelin bodies, accumulation of lysosomal vacuoles and sequential segregation of vesicles^[73]. Vesicle formation at the axon stump starts like in GSC projections with cell membrane ingrowths. Whereas the distal nerve stump degenerates, the proximal stump produces sprouts that have been interpreted as axonal regeneration. This resembles the expected outgrowth of new GSC projections after exhaustive vesicle segregation (Figures 5C and 7A).

Although still little is known concerning the signaling pathway directing the autodestruction program, some progress has been reported recently. The *Wlds* gene protects severed axons from degeneration. It encodes dNmnat (nicotinamide mononucleotide adenylyltransferase 1). Nmnat is a critical enzyme in the NAD⁺ biosynthesis pathway and is essential for many cellular processes^[74]. Nmnat is likely essential for normal axon maintenance. Down regulation of Nmnat in the wing nerve of *Drosophila* leads to robust dying back fragmentation that markedly resembles Wallerian degeneration, whereas upregulation of Nmnat protects axon degeneration. The function of WldS/Nmnat may involve its essential role in NAD⁺ biosynthesis. The question arises if the mitochondria are the site of Nmnat-mediated action and mechanism^[71]. Also the ubiquitin proteasome system (UPS) plays a crucial role in axon and dendrite maintenance and neuropathology, but the precise effects of WldS/Nmnat and the effect of the UPS vary depending upon the situation, an acute injury, developmental pruning or disease context (for review see Fang *et al.*^[71]).

The UPS is a potent regulatory mechanism used to control protein stability in numerous cellular processes, including neural development^[75]. Many neurodegenerative diseases are featured by the accumulation of UPS-associated proteins, suggesting the UPS dysfunction may be crucial for pathogenesis. Recent experiments have highlighted the UPS as a key player during synaptic development. Recent discoveries center on the role of UPS in synapse remodeling and draw attention to the potential link between synaptic UPS dysfunction and the pathology of neurodegenerative diseases: Parkinson, Alzheimer, Huntington's disease^[75]. In *Drosophila*, the E3 ubiquitin ligase RPM-1 (disease resistance protein) targets DLK1 (delta homolog 1) which acts in the mitogen activated protein kinase (MAPK) cascade consisting of the MAPK MKK4 and the p38 kinase pMK3 or the MAPK c-Jun N-terminal kinase. Thereby RPM-1 regulates the organization and stabilization of presynaptic terminals and axon termination in mechano-sensory and motor neurons^[76].

Regeneration of injured neurons can restore function, but most neurons regenerate poorly or not at all. The failure to regenerate in some cases is due to a lack of activation of cell-intrinsic regeneration pathways. These

pathways might be targeted for the development of therapies that can restore neuron function after injury or disease. Hammarlund *et al.*^[77] showed that the DLK-1 MAPK pathway is essential for regeneration in *Caenorhabditis elegans* motor neurons. Loss of this pathway eliminates regeneration, whereas activating it improves regeneration. Further, these proteins also regulate the later steps of growth.

Osterloh *et al.*^[78] demonstrated that the ortholog genes sterile alpha and TIR motif-containing protein 1 (Sarm 1) in mouse and dSarm (sterile alpha/Armadillo/Toll-Interleukin receptor homology domain protein) in *Drosophila* promote cell autonomous axon destruction. The genes otherwise involved in innate immune response, are also players in a highly conserved axon destruction pathway. dSarm and Sarm 1 exhibit a punctate localization in neuronal cell bodies and a broad localization in neuritis of *Drosophila* and mouse respectively. An early event in the axon self-destruction pathway is the increase of intra-axonal calcium levels followed by a calcium-dependent cytoskeletal breakdown^[72].

There is evidence that WldS enhances physiological functions of the mitochondria and that axonal mitochondria are required for WldS-dependent axon protection. WldS/Nmnat activity enhances mitochondrial motility and Ca²⁺ buffering and that the mitochondrion is an organelle necessary for WldS/Nmnat-mediated axonal protection^[79,80].

DEVELOPMENTAL AXON PRUNING, DYING-BACK DEGENERATION AND NEURODEGENERATIVE DISEASES

Developmental axon pruning occurs at a large scale during metamorphosis of holometabolous insects, including *Drosophila*, where the process was studied in detail. Metamorphosis and axon pruning are controlled by ecdysone. Interestingly, in the mushroom body of the fly brain glia cells participate actively in axon pruning. Ecdysone stimulated axons extrinsically activate glial cells to infiltrate the axon branches and eliminate varicosities actively. They induce the fragmentation of axons, and engulf the fragments^[81,82]. The process resembles the interaction of GSCs with the AC in *Lymantria* (Figure 6B). In both cases, the perikarya survive whereas the autotomy of cell projections/axons proceeds, and fragments are taken up by the AC and glia cells, respectively. In *Oncopeltus*, the GSC projections also autotomize but the severed and degrading vesicles are almost never phagocytized. It has been suggested that new projections sprout from the perikarya of *Oncopeltus* comparable to the sprouting of new neurites from pruned neurons. The neuron-glia interaction has an indispensable role in the pruning process of neurons in the mushroom body. The pruning proceeds in a neuron-autonomous manner. It resembles the interaction between phagocytes and apoptotic cells^[81,82]. It was shown that dendrite-

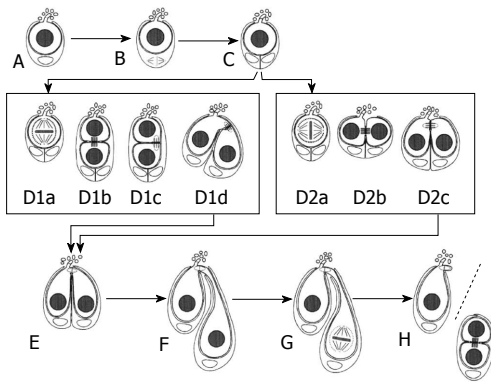


Figure 8 Symmetric and asymmetric germline stem cell division in *Lymantria dispar*. Both symmetric and asymmetric GSC divisions occur in this insect species. Both are preceded by the division of the single cyst stem cell that is associated with a GSC (A to C). Asymmetric division is depicted in D1a to D1d. One of the daughter cells is oriented toward the AC and still forms projections, whereas the other daughter cell has no contact with the AC and is devoid of projections (D1b). Then this daughter cell “swivels” round toward the AC (D1d) and adopts a similar position as daughter cells have after a symmetric GSC division (D2a to D2c). In each case, the daughter cell that doesn’t form projections becomes the gonialblast (E to H) (from Klein^[66]). GSCs: Germline stem cells; AC: Apical cell.

specific remodeling of *Drosophila* sensory neurons is controlled by two intracellular mechanisms: the ecdysone pathway and ubiquitin-proteasome system^[83]. It should be noted that components of the ubiquitin-proteasome pathway has been linked to apoptosis^[84].

A dying-back degeneration of axons can be induced in culture of mice neurons by removing nerve growth factor from the chamber. In the process, several distal ends of axons atrophy and undergo fragmentation while the neuronal somata survive. The controlling events are obviously confined to neurites and occur autonomously from the soma^[85]. Many neurological diseases are accompanied by neurodegeneration. Although different factors might contribute to axon pathology in each case, what is clear is that the end result is always the same, the axon degenerates in a process that resembles Wallerian degeneration^[86].

Wallerian degeneration is an evolutionary highly conserved process that is central to neurite autotomy occurring either regularly in developmental remodeling of neurons or pathologically in neurological diseases and experimental manipulations. The mechanism of serial autotomy of GSC projections in *Oncopeltus* and *Lymantria* (and probably butterflies in general) resembles closely axon autotomy described above. Molecular studies are necessary to uncover whether similar signaling pathways are involved.

CONSEQUENCES OF GSC AUTOTOMY ON SIGNAL EXCHANGE WITH THE NICHE AND GSC DIVISION (SYMMETRIC VS ASYMMETRIC DIVISION)

Until recently it was accepted that adult male GSCs in

Drosophila only undergo asymmetric divisions^[87]. GSCs attached to hub cells by adherens junctions provide a polarity cue that orients stem cells. The centrosome is oriented toward the hub cell-GSC interface throughout the cell cycle. The mother centrosome is always located close to the hub cells whereas the daughter centrosome moves to the opposite side. Consequently, GSC spindle orientation is predetermined during interphase^[88]. The correct centrosome orientation toward the hub cells requires the adherens junction which is composed of E-cadherin and β -catenin, centrosomin, and Apc2. Apc2 is believed to connect astral microtubules to the adherens junction/actin cytoskeleton network formed between hub cells and GSCs thereby anchoring the centrosome^[89]. Centrosome orientation prior to mitosis is accomplished by Par-1 (a serine/threonine kinase that regulates polarity in many systems) that regulates cyclin A localization^[90]. Recently “symmetric renewal” of male GSCs in *Drosophila* has been observed^[91]. In this process, GSC division starts like a typical asymmetric mitosis (the mitotic spindle is perpendicularly oriented toward the hub surface) but then the still interconnected pair of cells “swivel” such that both cells contact the hub. Studies on other insect testes revealed still different modes of GSC divisions and gonialblast differentiation.

Unique processes have been described in male GSCs of *Lymantria* (Figure 8). GSCs undergo either symmetrical divisions (the spindle is oriented parallel to the AC surface) or asymmetrical divisions (the spindle is oriented perpendicularly to the AC surface). Both types of mitosis can result in gonialblast formation but, surprisingly, after asymmetrical division GSC and daughter cell - interconnected by a fusome - “swivel”, comparable to the process in *Drosophila* described by Sheng *et al.*^[91] in 2011, and both contact the AC. Whereas the mother GSC maintains its intense interaction with the AC, the daughter and presumptive gonialblast does not form cell projections. Finally, the daughter cell moves further to the periphery and differentiates to a gonialblast. Alternatively, after symmetrical division the daughter cell is again characterized by the lack of cell projections, develops to a gonialblast in a similar fashion as in the case of asymmetric GSC division^[66]. Each GSC is associated with one CySC. CySC division precedes GSC division and the mitotic spindle is always oriented parallel to the AC surface. The mechanism destining the centrosome location is not known and adherens junctions between GSCs and AC have not been described - and are not likely to exist, given the complex relationship between GSCs and AC. Consequently, the regulation of spindle orientation must differ in *Lymantria* from *Drosophila*.

Also in *Oncopeltus* adherens junctions could not be identified in electron microscopic studies^[92]. In this species, only symmetrical divisions of GSCs have been observed. GSC projections persist during division. Gonialblasts are formed when GSCs migrate toward the periphery of the apical complex. Its projections elongate, become thin and gradually degenerate before one cyst

cell encloses the gonialblast^[92]. *Locusta* shows both symmetrical and asymmetrical divisions^[64]. But again, besides tight junction-like connections, no adherens junctions have been detected between GSCs and AC. It should be mentioned, however, that GSCs and AC are extremely difficult to separate, either enzymatically or mechanically, in *Locusta*^[64].

In summary, the mechanism for spindle orientation appears variable in insect male GSCs. But up to date, *Drosophila* is the main species studied in this respect in the evolutionary highly diversified group of insects.

Interactions between GSCs and ACs in the testes of various insects are diverse and it is evident that the mode of short range signaling between stem cell and niche differ in distinct species from that in *Drosophila*. In *Oncopeltus*, ACs are surrounded by vesicles segregated from GSC projections (Figure 6A). This poses the questions: how is the communication and information exchange organized between GSCs and AC? and how is the sequential projection autotomy, progressing degradation, and sprouting of new projections programmed? There are no studies on the molecular level concerning these questions. However, the ancient conserved axon destructing Wallerian pathway bears many similarities with GSC projection autotomy. Further, the outgrowth of new projections near the perikaryon of *Oncopeltus* GSCs parallels the outgrowth of new neuritis after Wallerian axon destruction. As described above, developmental axon pruning and neurite dying-back in neuronal diseases all appear to be governed by a related axon-autonomous program that carries characteristics of apoptotic processes. Extensive fragmentation of megakaryocyte projections takes place during platelet formation as described below. Also in this case apoptotic processes take place, *i.e.*, fragmentation of the nucleus. The segregation of platelets, however, appears to follow a different pattern than Wallerian axon fragmentation and GSC projection autotomy. In the latter cases, concentrically transverse ingrowing plasma membrane that finally fuses, cuts off vesicular fragments. Pinching off platelets apparently involves transverse microtubule arrangement and vesicle widening at the constriction zone (see below).

In *Lymantria*, segregation of GSC projection terminals resembles that in *Oncopeltus*. Thus, the underlying cell autotomy program is expected to be similar as in *Oncopeltus*. But in *Lymantria* and several other butterflies (see above) the AC embraces the GSC projections and engulfs and digests separated vesicles. In *Locusta*, ACs almost constantly include one or two phagocytized GSCs. The interactions of GSCs and ACs (their niche) are puzzling in *Oncopeltus* as well as in butterflies. We speculate that the AC sends signals that promote GSC autotomy and that in return the AC receives information concerning the surrounding GSC population. In response to that information the AC may regulate GSC self-renewal and maintenance. Unfortunately, none of these aspects have yet been tested. In about all insect apical complexes studied

by electron microscopy, "dark" granules have been described in the interface between AC and GSCs (Figure 6). In *Lymantria*, the AC shows tubular invaginations filled with "dark" material of unknown fate. Due to the phagocytic processes and material exchange early investigators suggested a trophic role of ACs^[92]. We believe that these processes are part of the information exchange and signaling pathways. In erythroblastic islands, after release of the reticulocyte, the pyrenocyte is phagocytized by the central macrophage. This is mandatory for continued erythropoiesis (see below). This indicates that the involvement of phagocytic processes in stem cell-niche interaction is existent but needs further investigation.

STEM CELL/PROGENITOR AUTOTOMY IN ERYTHROCYTE AND PLATELET FORMATION

Erythroblastic islands and enucleation of erythroblasts

In vivo, erythropoiesis occurs in specific units, the erythroblastic islands in the bone marrow of mammals^[93]. Erythroblastic islands were also described in the spleen, yolk sac and fetal liver. They harbor a central macrophage that arises from a resident monocyte precursor with a unique immunophenotypic signature^[94]. The central macrophage, representing the niche, is surrounded by one or more synchronously maturing cohorts of erythroid cells that undergo four or five divisions between proerythroblast and orthochromatic erythroblast stage. In their fine structural study Allen *et al*^[95] describe gap junction-like contacts between the macrophage and erythroblasts and possible reciprocal vesicular activity. Several molecules indicate adhesive interactions within the erythroblastic islands^[4]: (1) Erythroblast macrophage protein (Emp) forms macrophage/erythroblast attachments *via* hemophilic binding; (2) $\alpha 4\beta 1$ integrin in erythroblasts and vascular cell adhesion molecule-1 in the central macrophage mediate receptor/counter receptor cell-cell interactions; (3) Macrophage α integrin and erythroid intercellular adhesion molecule-4 are expected to contribute to the island integrity; and (4) Other macrophage adhesion glycoproteins, *i.e.*, CD69 and CD 163, have been detected^[96] although their erythroid binding partners are unknown. It is expected that adhesive connections between erythroblasts and macrophages play a crucial role in signaling pathways as they do in *Drosophila* testes. The central macrophage secretes soluble factors, cytokines, that promote proliferation and maturation of erythroblasts (insulin-like growth factor-1 and others) and also negative regulatory factors [transforming growth factor- $\beta 1$ (TGF- β), TNF- α , ILG and others]^[4] (Figure 9).

The most striking event in mammalian erythrocyte maturation is the enucleation of orthochromatic erythroblasts at the last stage of erythroblast differentiation. It results in multilobulated non-nuclear reticulocytes and

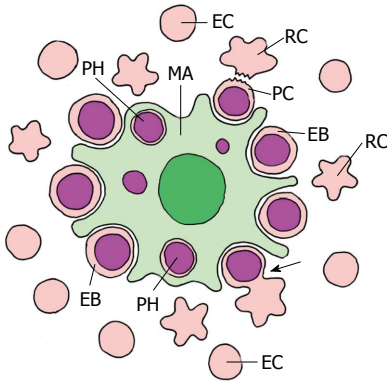


Figure 9 Erythropoiesis in mammal bone marrow (schematized). The erythroblastic island consists of a central macrophage (MA) that functions as the niche, and the peripheral erythroblasts (EB), that represent stem/progenitor cells. Erythroblasts undergo an enucleation process (arrow) that results in the pyrenocyte (PC) that mainly consists of the erythroblast nucleus, and the nucleus-free reticulocyte (RC). The pyrenocyte is phagocytized by the macrophage (PH, phagosome), whereas the reticulocyte develops to the erythrocyte (EC) (adapted from Chasis *et al.*^[4]; Keerthivasan *et al.*^[96]).

pyrenocytes which mainly consist of the nucleus and the enwrapping plasma membrane. Enucleation is regulated by retinoblastoma protein (Rb) but other signaling molecules, *e.g.*, p38 MAPK (p38) and Rac-1 GTPase, have been found to be involved in the enucleation^[97]. Interestingly, sorting of protein and vesicle trafficking in the orthochromatic erythroblast in concert with nuclear positioning are essential for the enucleation process^[98]. The pyrenocytes express phosphatidylserine, a recognition signal similar to apoptotic cells, on their surface that signals macrophages to engulf and digest them. The taken up DNA is digested by DNase II from the macrophage^[99]. The lack of DNase II in DNase II knockout mice is lethal *in utero* due to embryonic anemia. Digestion of engulfed pyrenocyte nuclei is vital for continued erythropoiesis^[100].

Already in the 1990ties Hanspal *et al.*^[101,102] found that the interaction between erythroblasts and macrophages is needed for normal erythroblast proliferation and for enucleation. The authors showed that this interaction is mediated by Emp that prevents apoptosis of developing erythrocytes. Nonetheless, erythroblasts cultured *in vitro* in the absence of macrophages undergo complete differentiation including nuclear extrusion^[103,104]. Although erythropoietin was used to start erythroblast amplification (and other factors were eventually added) erythropoiesis proceeded apparently normal without macrophages, however, at a much lower pace as it does in erythroblastic islands^[93]. Thus the question arises: have macrophages merely a trophic function? As mentioned above, genetic manipulations of macrophage activity resulted in lethality due to anemia. It may be speculated that in these cases erythropoiesis was (only) insufficient (but not completely) suppressed. The "true" function of island macrophages is to optimize and accelerate erythrocyte production allowing effective erythrocyte supply and rapid adjustment to the actual need. Erythrocyte homeostasis might be largely

regulated by systemic factors that convey the need or surplus of erythrocytes and affect short-range signaling within the erythroblast island which determines the pace of erythrocyte production.

We suggest that spermatocyte production in insect testes is regulated in a similar way. The example of *Lampyrus* shows that no ACs are needed for GSC differentiation *per se*. The apparent reason: all GSCs differentiate at the same time and the process does not need any temporal regulation. Onset of GSC differentiation and production of CC progenitors occurs during larval development of *Lampyrus* and is expectedly put in motion by the release of morphogenetic hormones, *e.g.*, ecdysone and juvenile hormone, representing systemic signals^[67].

Another conspicuous interaction shared by the niche of erythroblastic islands and the niche of butterfly testes are spectacular phagocytic processes. In the case of erythroblastic islands, macrophages engulf the pyrenocytes; in the case of butterflies, ACs phagocytise large vesicles segregated from GSC projections. DNA taken up by macrophages plays a role in signaling, as described above. And there are also vesicle interactions with expected receptor exchange. In butterflies, the vivid autotomic activity of GSCs and the phagocytotic uptake of the autotomized vesicles are not understood. We propose that it represents an interaction/communication between niche and stem cell hitherto unknown. Noteworthy, whereas autotomy of GSC projections also takes place in *Oncopeltus* the segregated vesicles are not taken up by ACs, but degenerate in a distinct pattern as described above. We suggest that the degenerating vesicles provide specific signals that are recognized by the AC. Interestingly, the ACs of *Locusta* include, as a rule, one or two phagocytized GSCs (see above). Phagocytosis here may play a similar role as it does in *Lymantria*. The variations in stem cell-niche relation seem highly variable in insect testes, and the analysis of species beyond *Drosophila* might provide new insights.

Platelet formation by megakaryocyte fragmentation

The probably most spectacular case of programmed cell autotomy is the shedding of 5000-10000 platelets from one megakaryocyte. Platelets are characterized by the absence of a nucleus and by the accumulation of three types of granules: (1) Dense (or delta) granules, with a diameter of 150 nm, contain ADP or ATP, Ca and Serotonin. They are secreted to recruit other platelets; (2) Alpha-granules, with a diameter of 200-400 nm, contain P-selectin, platelet factor 4, TGF- β 1, platelet-derived growth factor, fibronectin, B-thromboglobulin, von Willebrand factor (VWF), fibrinogen, coagulation factors V and XIII. They are responsible for adhesion and healing processes; and (3) Lambda granules, with a diameter of 175-250 nm, resemble lysosomes. They contain several hydrolytic enzymes that are able to eliminate circulating platelet aggregates (for review see Rendu *et al.*^[105]) (Figure 10).

Megakaryocytes mature from megakaryoblasts

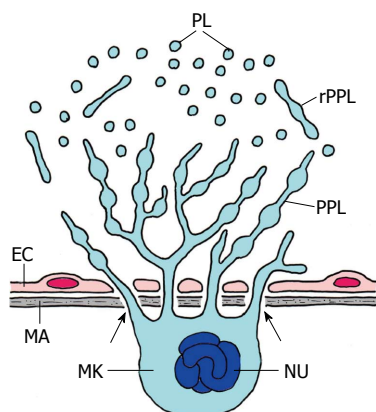


Figure 10 Thrombopoiesis (platelet formation) in mammal bone marrow (schematized). Platelets are produced by a most spectacular form of cell autotomy performed by the megakaryocyte (MK). Maturation and fragmentation of the megakaryocyte is orchestrated by the vascular niche which consists of the endothelial cells (EC) of sinusoids and the extracellular matrix (MA) of the endothelium. The megakaryocyte is located at the outer surface of the sinusoid epithelium and sends pseudopods through the pores of the endothelium into the lumen of the sinusoids (arrows). The pseudopods branch and form proplatelets (PPL). These are released into the sinusoids (rPPL) followed by the release of platelets (PL). Nu, polyploidy nucleus of the megakaryocyte (Adapted from Patel *et al*^[115]).

via promegakaryocytes in the bone marrow. Megakaryoblasts reside in the osteoblastic niche where osteoblasts secrete the cytokine thrombopoietin (TPO) that regulates megakaryopoiesis and thrombopoiesis, *i.e.*, platelet formation, as well^[106]. Stromal cell derived factor-1 from stromal fibroblasts and fibroblast growth factor-4 direct megakaryocyte interaction with the bone marrow stroma and regulate cytokine-independent megakaryocyte maturation^[107]. A number of cytokines [interleukin-3 (IL-3), IL-6, IL-11, IL-13, leukemia inhibiting factor (LIF), stem cell factor, and others] affect megakaryopoiesis which was mostly tested in *in vitro* systems^[108,109]. In the process of maturation, the precursors of the megakaryocytes migrate from the osteoblastic to the vascular niche along an oxygen gradient to the higher oxygen of the vascularized compartment of the bone marrow^[110]. During maturation megakaryocyte progenitors undergo endomitosis, up to 128 times. Reaching the endothelium of the marrow sinusoids the strongly enlarged megakaryocytes form cytoplasmic projections that protrude through endothelial pores into the lumina of the sinusoids. There, the projections presumably (as inferred from *in vitro* observations) branch repeatedly forming formidable trees of proplatelets which segregate and release the platelets into the lumina of the sinusoids. The vascular niche that promotes platelet formation and shedding consists of the endothelial cells and the extracellular matrix of the endothelium^[111].

The dynamic interactions of megakaryocytes with different extracellular matrix proteins seem to orchestrate their maturation in specific sites^[111]. In the vascular niche such proteins include collagen type IV, fibronectin, laminin, fibrinogen and VWF. (VWF is secreted by endo-

thelial cells and megakaryocytes into the blood and has a function in adhesion and aggregation of platelets.) In mice (*in vitro*) fibrinogen binding to the fibrinogen receptor $\alpha_{IIb}\beta_3$, which is expressed in megakaryocytes, is essential for proplatelet formation. However, the role of the interaction in humans is not fully understood^[112]. Astonishingly, *in vitro* studies have shown that the cytokine TPO alone is required for thrombopoiesis and that extracellular matrix and other cytokines are not essential, although they may have regulatory functions *in vivo* that accommodates platelet homeostasis^[113]. This is reminiscent of the niche function in erythroblastic islands and testes of insects where stem cell differentiation can proceed without niche but is adjusted to the actual need by niche interaction.

The process of proplatelet formation and platelet shedding is highly complicated and still not fully understood^[114,115]. Electron microscopic studies on cultured megakaryocytes demonstrated that prior to proplatelet formation mature megakaryocytes have already a well-developed demarcation membrane system^[116]. This elaborate membrane system is - as commonly believed - formed by invaginations of the plasma membrane, shows open cisternae and is at first randomly distributed throughout the cytoplasm. Randomly scattered α -granules and some dense granules are also present. Preparing for active platelet shedding, the peripheral demarcation membranes dilate and align at the cell periphery. Cytoplasmic sheets unfold and cell projections extend. The extensions display a beaded appearance with constriction points separating discrete platelet-like territories. A bundle of longitudinal microtubules runs through the center of the extensions. At the constriction zone transverse microtubules are observed near the longitudinal microtubules. Also, a vacuole of increasing size is formed at the constriction zone which may lead to the detachment of the platelet fragment. These observations of Cramer *et al*^[116] suggest that vesiculation and microtubule force attribute to autotomy. Other studies emphasize even more the role of microtubules in platelet segregation^[115,117]. Before proplatelet formation, microtubules align into bundles beneath the surface of megakaryocytes and, at projection formation, fill the cortex of outgrowing cones. Cell organelles are in direct contact with microtubules and are transported along these elements. Microtubules coil at the end of the projections, but their exact role in platelet segregation remains unresolved.

It should be mentioned that in an opposing view the membrane boundaries of platelets are not provided by involutions of the megakaryocyte plasma membrane but by vesicles from Golgi complexes. So-called proplatelets constitute within the megakaryocyte whose plasma membrane finally ruptures and releases the platelets. Extensions of the megakaryocyte with proplatelets and segregation of terminal platelets are considered as artifacts by some authors^[114,118,119].

It is, however, the prevailing view that platelets segregate from ends of the megakaryocytes extensions that

offer a beaded structure, in as much platelet-sized and platelet-structured proplatelets are connected by string-like connections with longitudinally running microtubules (Figure 10). Interestingly, the process of platelet assembly is accompanied by some characteristics associated with apoptosis: cytoskeletal reorganization, membrane condensation and chromatin condensation. Microtubules and F-actin play supposedly a major role in proplatelet formation and fragmentation. The kinetics of platelet release *in vitro* corresponds to the onset of apoptosis in the megakaryocyte. Maximal platelet production and megakaryocyte apoptosis are closely related events^[115,120,121]. Molecular evidence of apoptotic processes in megakaryocytes provided the detection of caspase 3. Before the platelet formation caspase 3 shows a punctuate cytoplasmic distribution (in a presumably inactive state) and a diffuse staining pattern (in a presumably active state) in senescent megakaryocytes. It was concluded, that proplatelet formation is regulated by caspase activation limited to only cellular compartments^[122]. Further evidence for an involvement of apoptotic processes in platelet formation comes from the presence of the antiapoptotic protein BclxL which is upregulated during megakaryocyte differentiation but absent during late megakaryopoiesis. BclxL overexpression causes a strong decrease in proplatelet formation^[123]. Other apoptotic-related genes such as TGF- β 1 and SMAD proteins are expressed during thrombopoiesis which supports the significance of apoptotic signaling in the process^[124]. Besides, NO in conjunction with TPO facilitates platelet production^[125]. Nagata *et al.*^[126] report that estradiol synthesized in megakaryocytes triggers proplatelet formation by autocrine action. Few transcription factors were reported to play major roles in thrombopoiesis. GATA-1, which interacts with friend of GATA-1 controls proliferation during megakaryopoiesis, and NF-E2 regulates platelet biosynthesis^[127-129].

Cell autotomy in the process of erythrocyte formation (*i.e.*, enucleation) and platelet generation (by megakaryocyte fragmentation) appear to follow quite different strategies. However, in both cases apoptotic processes play a prominent role. This offers parallels to the autodestructive processes in neurons (see above). Concerning GSC autotomy, no attempts have been made to demonstrate apoptotic processes in GSC projections. Schmidt *et al.*^[92] described apoptosis of GSCs and spermatogonia in *Oncopeltus* but its significance - apart from removal of surplus spermatogonia - remains obscure. Dying germ cells in *Drosophila* revealed mixed morphologies of apoptosis and necrosis that may indicate an alternative developmental cell death pathway^[130]. The role of the cytoskeleton in cell autotomy is little understood although it may play a major role in all cases. Concerning megakaryocyte fragmentation, the cytoskeleton is obviously decisively involved in platelet segregation. Neurofilaments and microtubules are the first cell organelles that break down in axonal autodestruction following intracellular calcium

increase after nerve injury. The role of the cytoskeleton in GSC autotomy is not known but is conceivably important and should be analyzed.

COMPARISON OF THE INSECT AND MAMMAL SPERMATOGENESIS

Testes of insects offer a rather simple architecture. One testis is composed of one to many testicular follicles, blind ending tubules that join into a common seminal duct. GSCs and niche exhibit a globular arrangement, mostly in the form of a rosette at the apex of a testicular follicle, representing the apical complex. Spermatogonial cysts move distally during spermatogenesis. The follicular epithelium that envelopes the apical complex and cysts is mostly thin and has, so far, not been considered as part of the niche for GSCs. However, as pointed out above, in a number of species, the envelope synthesizes and releases the steroid ecdysone at some point of development^[53]. Although effects of ecdysone on spermatogenesis have been reported, a specific function or signaling pathway has not been elucidated. Nonetheless, it parallels the production of steroids, androgens, in Leydig cells of the testes of mammals.

Mammalian testes exhibit a complex epithelial organization (for review see Yoshida^[131]). The long seminiferous tubules are convoluted and both ends open into the rete testis. Figure 11 shows the organization of a seminiferous tubule. The high epithelium of the tubules consists of the larger Sertoli cells, and the smaller spermatogonia and spermatocytes. The epithelium rests on a basement membrane, and below it stretches peritubular myoid cells. Located in the interstitial between the seminiferous tubules are Leydig cells, macrophages, lymphoid epithelial cells and connective tissue. Blood vessels form a network around the tubules and run in the interstitial spaces. Besides the germ cells all mentioned cell types and structures may be part of the GSC niche. The complexity of the niche, which doesn't offer spatial specifications, is reflected by the difficulty to define GSCs. They represent obviously a small population of spermatogonia. According to de Rooij *et al.*^[132] and Russell *et al.*^[132,133], spermatogenesis progresses uniformly all over the inner surface of the seminiferous epithelium, and stem cells are scattered all over. But how are GSCs identified? First, they are located in the basal compartment of the epithelium. All neighboring Sertoli cells form tight junctions at a distinct height separating a basal compartment which has contact with blood vessels and an adluminal compartment without blood contact. Thus, a blood-testis-barrier is installed at the level of tight junctions. Located in the basal compartment are "undifferentiated spermatogonia" (A_{undiff}), comprising singly located spermatogonia (A_s) and such that have undergone up to four mitotic divisions. The mitotic spermatogonia form syncytia due to incomplete cytokinesis. A_s have contact with the basement membrane and a subpopulation of them might represent yet-to-

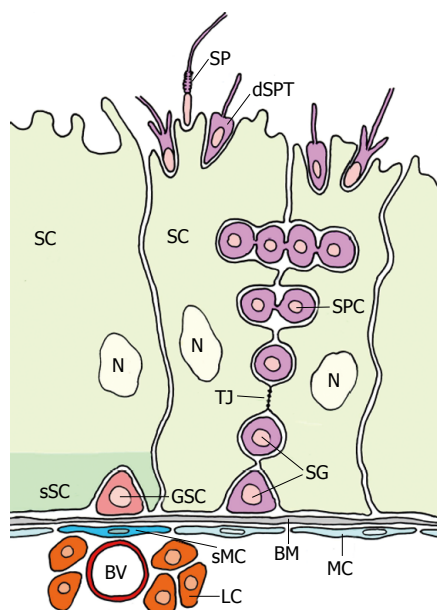


Figure 11 Organization of the mammalian testicular germline stem cell niche (adapted from Yoshida^[131]). The seminiferous tubules exist of large sertoli cells (SC) and spermatocytes (SPC). The putative germline stem cell (GSC) is located at the basement membrane (BM) at an interstitium. It is in close vicinity of a blood vessel (BV) and leydig cells (LC). Myoid cells (MC) that line the BM are thought to be "specialized" MC (sMC) below the GSC. The basal part of the SC that surrounds the GSC presumably performs a specialized "domain" acting as (the main) part of the complex niche. The niche allegedly consists of several additional entities besides the specialized SC domain: the BM, the sMC, the BV, the LC and probably others (see text). Tight junctions (arrow) between SCs establish a blood-testis barrier: GSCs and spermatogonia are exposed to the vascular system, whereas primary spermatocytes (SPC) and the following stages of Spermatogenesis proceed behind the barrier. dSPT: Differentiating spermatid; SP: Spermatozoon; N: Nucleus of SC; Red: GSC; Light green: SC; Dark green: sSC domain surrounding the GSC; Purple: Spermatogonia and different stages of spermatogenesis; Dark blue: sMC; Light blue: MC; Light red: BV; Orange: LC; Grey: BM.

be-identified GSCs. At the transition from mitotic to meiotic divisions, spermatogonia move to the adluminal compartment and are now called spermatocytes. From there on spermatogenesis takes place behind the blood-testis-barrier. Round spermatids move to the luminal surface where they elongate. The accumulation of stags of spermatogonia and spermatocytes among the Sertoli cell epithelium results in a multilayered organization of the seminiferous tubules.

Since A_s are spread all over the tubules the question arises: which mechanisms provide uneven features within the basal compartment to specify the niche microenvironment for stem cells? Yoshida *et al.*^[100] demonstrated that A_{undiff} (and possibly GSCs) are preferentially localized to the area adjacent to the interstitium at branching points of blood vessels of medium thickness. Yoshida *et al.*^[131] suggests that Sertoli cells and myoid cells in this region might be "specialized". Theoretically, all parts of the niche could control stem cells directly by short-range signaling. Systemic signals could arrive *via* blood directly at the GSCs or indirectly by modulating the short-range signaling of other parts of the niche. The niche region might not be fixed.

Several signaling factors are known to have effects on spermatogenic cells (Figure 12). Most important is glial cell line-derived neurotrophic factor (GDNF), a member of the TGF- β superfamily^[134]. The ligand GDNF is expressed in Sertoli cells and its receptor c-Ret and co-receptor GFR α 1 is expressed in the least mature subsets of A_{undiff} , the putative GSC^[135,136]. Thus, *via* GDNF signaling Sertoli cells control self-renewal, survival, and maintenance of GSCs. Another receptor highly expressed in GSCs is colony stimulating factor 1 receptor. Its ligand is expressed in Leydig cells of the interstitium and a subset of myoid cells^[137]. It is suggested that CSF1 may cooperate with GDNF in supporting the self maintenance of GSCs. The tyrosine kinase receptor c-kit and its ligand KitL, which is expressed in Sertoli cells, have been recently shown to be involved in proliferation, survival and migration of spermatogonia. However, only KIT(-) spermatogonia have stem cell activity. Several factors have effects on GSCs *in vitro*: FGF, EGF and LIF, in the presence of GDNF, support the proliferation of GSCs^[138]. LIF is probably also involved in the maturation of gonocytes into spermatogonia^[139]. But a possible function *in vivo* is uncertain. The transcriptional regulator Ets related molecule has been detected in nuclei of Sertoli cells of adult testes. It is assumed that it regulates Sertoli cell function that mediates germ cell self-renewal^[140].

Stem cells and spermatogonial populations express α 6 and β 1-integrin^[136] which mediates the attachment to the basement membrane *via* binding of laminins, probably as a heterocomplex with α 6-integrin^[141]. Its significance in signaling *in vivo* is not known; GSCs lacking β 1-integrins fail to develop spermatogenic colonies after transplantation^[142]. E-cadherin is expressed in A_{undiff} but is dispensable for the normal functioning of stem cells^[143].

Despite the profound differences between the organization of the insect *Drosophila* and the mammal mouse testis, several important common principles can be observed. In both cases, the stem cell-niche complex is exposed to blood: in insects hemolymph freely surrounds the testicular follicles whose envelope allows the passage of larger molecules^[144]. In mammals blood vessels run through the niche of GSCs and release molecules in the vicinity of GSCs. In several adult stem cell-niche systems blood vessels and endothelial cells are an integral part of the niche: the HSC niche and the vascular niche of platelet producing megakaryocytes (see above), the neural stem cell (NSC) niche the B1 NSCs within the ventricular-subventricular zone send out a basal process ending in a specialized end-foot that contacts blood vessels; blood-borne factors and endothelial-derived factors may act on B1 cells in this domain^[145], intestinal stem cell and probably other niches^[146,147]. Recently, it has been reported that an important function of endothelial cells in glioblastoma multiforme is to create a niche that helps to promote self-renewal in cancer stem-like cells^[148]. In liver regeneration, endothelial cells establish an instructive vascular niche, which through elaboration of paracrine trophogenesis stimulates organ regeneration,

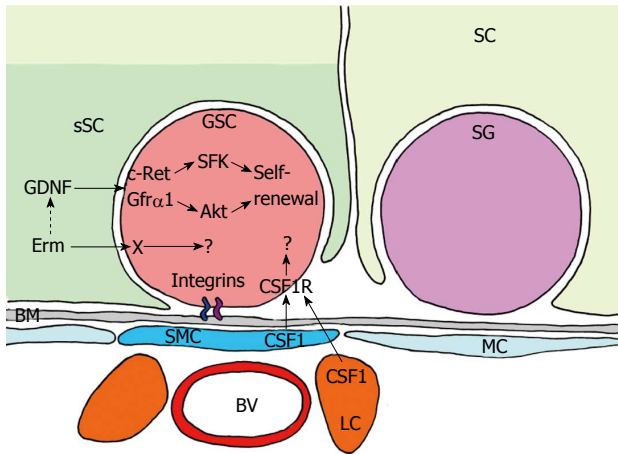


Figure 12 Short range signalling between germline stem cell and its niche. GDNF is the most important signal produced by the “specialized” basal compartment of SC. Its receptor - c-RET (transmembrane tyrosine kinase receptor) and co-receptor GFR α 1, which functions as a glycosylphosphatidylinositol-anchored docking receptor that is needed for GDNF to bind with c-RET - is expressed in GSC, and SFK and Akt pathway are initiated. They control self-renewal and maintenance of GSCs. GSCs also express CSF1R (colony stimulating factor 1 receptor). Its ligand CSF1 is synthesized in “specialized” MCs (sMC) and in leydig cells (LC). Thus, CSF1 may cooperate with GDNF in supporting self-maintenance. Integrins attach the GSCs to the BM and may also contribute to signalling. Red: GSC; Light green: SC; Dark green: sSC domain surrounding the GSC; Purple: Spermatozoon (SG); Dark blue: SMC; Light blue: MC; Light red: BV; Orange: LC; Grey: BM.

in a manner similar to endothelial-cell-derived angiocrine factors that support hematopoiesis^[149].

Meiotic divisions of spermatogonia in mammals and all mitotic and meiotic divisions after gonialblast formation in insects take place behind the blood-testis-barrier. Whereas tight junctions between neighboring Sertoli cells establish the barrier in mammals, it is the cyst cells that isolate the developing germ cells from the hemolymph in insects. There is little information on the necessity for a compartment in which spermatogenesis is protected from blood-borne factors.

Mouse and *Drosophila* differentiating germ cells share another astonishing potency: they can generate GSCs, *in vivo*. In mice “spermatogonial progenitors committed to differentiation” can generate functional GSCs that can repopulate germ cell-depleted testes when transplanted into adult males. GDNF and FGF2 are able to reprogram *in vitro* spermatogonial progenitors for reverse differentiation^[150,151]. Amazingly, posttransplantation homing GSCs have to perform multiple steps: attachment to the Sertoli cell surface, retrograde translocation to the basal compartment across tight junctions, migration to the presumptive stem cell niche, survival, proliferation, and self-renewal within the niche, expansion of the transient amplifying spermatogonia and differentiation into sperm^[131]. β 1-integrin, which is expressed in spermatogonial cells, plays an essential role in GSC homing^[142]. In *Drosophila* testes spermatogonia undergoing transit-amplifying divisions can be reverted to stem cell identity by conditionally manipulating Jak-STAT signaling^[152]. In the process, the spermatogonia, which

are enclosed in cysts and interconnected by fusomes, have to break up their connections, and the cyst wall has to disintegrate in order to release the dedifferentiating germ cells. These germ cells populate the orphaned niche and reestablish normal spermatogenesis. Spermatogonial dedifferentiation can be genetically induced by conditional loss of STAT or misexpression of the differentiation factor Bam within the testes. This causes the differentiation of all GSCs and free niche space. If normal signaling is restored differentiating spermatogonia revert to stem cells, as described above, adhere to hub cells and function normally^[14,152]. Remarkably, dedifferentiation of oögonia has also been shown in the *Drosophila* ovary^[153].

CONCLUSION

This review summarizes the current knowledge of a novel mode of interaction between GSCs and their niche, the ACs, in insects. In several insect species (*Oncopeltus*, *Lymantria* and other moths) male GSCs undergo autotomy of cell projections, which are directed toward the ACs. The segregated GSC vesicles degrade at the surface of the niche cells or are phagocytized by them. This unique stem cell-niche relationship has been compared with known examples of stem cell/progenitor autotomy (*i.e.*, erythrocyte and thrombocyte formation) and autotomy of neurons in developmental axon pruning or neurodegenerative processes. In all the cases described, apoptotic signalling is involved. Studies on injury-induced axon destruction (Wallerian degeneration) suggest that an active autodestruction program exists akin to apoptosis and that the autodestructive pathway maybe conserved between fly and human^[71,72]. We propose that this pathway also exists and is active in male GSCs of *Oncopeltus*, *Lymantria* and other species. The analysis of signal exchange between autotomized GSC vesicles and niche cells is expected to reveal a new mechanism of stem cell-niche interaction.

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New insights into the epigenetic control of satellite cells

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Abstract

Epigenetics finely tunes gene expression at a functional

level without modifying the DNA sequence, thereby contributing to the complexity of genomic regulation. Satellite cells (SCs) are adult muscle stem cells that are important for skeletal post-natal muscle growth, homeostasis and repair. The understanding of the epigenome of SCs at different stages and of the multiple layers of the post-transcriptional regulation of gene expression is constantly expanding. Dynamic interactions between different epigenetic mechanisms regulate the appropriate timing of muscle-specific gene expression and influence the lineage fate of SCs. In this review, we report and discuss the recent literature about the epigenetic control of SCs during the myogenic process from activation to proliferation and from their commitment to a muscle cell fate to their differentiation and fusion to myotubes. We describe how the coordinated activities of the histone methyltransferase families Polycomb group (PcG), which represses the expression of developmentally regulated genes, and Trithorax group, which antagonizes the repressive activity of the PcG, regulate myogenesis by restricting gene expression in a time-dependent manner during each step of the process. We discuss how histone acetylation and deacetylation occurs in specific loci throughout SC differentiation to enable the time-dependent transcription of specific genes. Moreover, we describe the multiple roles of microRNA, an additional epigenetic mechanism, in regulating gene expression in SCs, by repressing or enhancing gene transcription or translation during each step of myogenesis. The importance of these epigenetic pathways in modulating SC activation and differentiation renders them as promising targets for disease interventions. Understanding the most recent findings regarding the epigenetic mechanisms that regulate SC behavior is useful from the perspective of pharmacological manipulation for improving muscle regeneration and for promoting muscle homeostasis under pathological conditions.

Key words: Histone methylation; Histone acetylation; Muscle stem cells; Adult stem cells; Noncoding RNAs; Satellite cell activation; Satellite cell differentiation; Satellite cell quiescence

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Core tip: Skeletal muscle needs to efficiently respond to internal and external stimuli, and satellite cells (SCs), the stem cells of muscle, play key roles in the preservation of muscle mass under both physiological and pathological conditions. Epigenetic pathways participate in coordinating the precise time-dependent expression of different subsets of myogenic genes in SCs. Thus, these pathways represent promising targets for therapeutic interventions. In this review, we focus on the epigenetic changes mediated by histone modifications - methylation or acetylation - and by noncoding mRNAs throughout SC differentiation.

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SATELLITE CELLS

The first report and ultrastructural description of satellite cells (SCs) was in 1961 when Mauro reported the presence of cells containing a scant cytoplasm that were "intimately associated with the muscle fiber wedged between the plasma membrane of the muscle fiber and the basement membrane"; Mauro termed them SCs^[1]. In the same report, despite the absence of direct functional evidence, Mauro proposed that SCs "might be pertinent to the vexing problem of skeletal muscle regeneration".

In subsequent decades, Mauro's hypothesis proved true, and a number of studies demonstrated that SCs are the key mediators of post-natal skeletal muscle growth, homeostasis and repair^[2-4]. The core functions of SCs are the repair of damaged muscle fibers and the maintenance of an adequate pool of stem cells. In fact, dividing SCs were shown by autoradiographic studies to supply both new nuclei within growing or regenerating muscle fibers and new SCs adjacent to the muscle fibers^[5,6].

Quiescent SCs express the transcription factor Pax7^[7]. However, quiescent SCs constitute a heterogeneous population: most SCs are committed to the myogenic lineage (Pax7⁺, Myf5⁺), whereas a small subpopulation of SCs (Pax7⁺, Myf5⁻) are interpreted as representing satellite stem cells, whose asymmetric division produces both Pax7⁺, Myf5⁻ stem cells and Pax7⁺, Myf5⁺ committed SCs. SCs are also capable of maintaining or expanding their number *via* symmetric division^[8,9]. The observation that SC proliferation contributes to both the growth or repair of the muscle fiber and the maintenance of the SC pool provided the basis for considering SCs as muscle stem cells^[10]. The equilibrium between asymmetric

and symmetric division is therefore relevant to the maintenance of a homeostatic population of stem cells. In SCs, this equilibrium is influenced by signaling that includes WNT7A and its receptor Frizzled 7 (Fzd7) *via* the -catenin-independent, non-canonical planar cell polarity pathway^[11]. Such signals dictate the polarity (parallel or perpendicular) of the orientation of mitotic division with respect to the fiber sarcolemma (and the basal lamina). WNT7A activity induces stem cells to divide in a planar orientation, parallel to the fiber sarcolemma, thereby favoring symmetric division, which produces two Pax7⁺, Myf5⁻ stem cells. Conversely, in the absence of WNT7A activity, the mitotic spindle is oriented perpendicular to the fiber sarcolemma, thereby favoring asymmetric division into two daughter cells; the daughter cell that contacts the basal lamina retains stem cell characteristics (Pax7⁺, Myf5⁻), whereas the other daughter cells, which contacts the fiber sarcolemma, becomes a Pax7⁺, Myf5⁺ committed SC.

SCs in muscle regeneration

Many stimuli, such as those present in injured or diseased muscle, induce SCs to activate, expand and contribute to new fiber formation. Importantly, SC activation is not restricted to the damaged area; rather, SC proliferation and migration to the regeneration site have been observed along entire fibers of injured muscles^[12]. Muscle regeneration is absolutely dependent upon muscle-resident Pax7⁺ cells^[13], which predominantly, although not exclusively, consist of SCs^[14]. The absolute requirement of Pax7⁺ SCs for muscle regeneration was demonstrated in different studies^[13,15,16]. In the absence of Pax7⁺ SCs, regeneration does not take place; instead, fibro-adipogenic cells invade the tissue. In keeping with the notion that SC-dependent muscle regeneration processes are similar to those of embryonic myogenesis, SC activation involves the upregulation of myogenic basic helix-loop-helix transcription factors and SC differentiation^[17]. In particular, at the molecular level, the activation of SCs is characterized by the rapid expression of *MyoD* and *Myf5*, which is triggered by Pax7 (and its paralog Pax3)^[18] and is modulated by epigenetic mechanisms^[19].

After the proliferation phase, the expression of the myogenesis regulatory factor (MRF) members myogenin and MRF4 is upregulated, leading to terminal SC differentiation^[20]. This event is concomitant with the activation of the cell cycle arrest protein p21 and permanent exit from the cell cycle. The completion of the SC differentiation program includes the activation of muscle-specific proteins, such as myosin heavy chains, and the fusion of SCs to each other or the repair of damaged muscle. SC fusion, which is a complex and tightly controlled process^[21], is regulated by numerous proteins involved in cell-cell adhesion and actin dynamics^[22-25], as well as muscle-specific membrane proteins^[26].

The depletion of SCs is a common occurrence in

chronic muscle degenerative diseases, and these cells cannot be replaced. Thus, the regulation of SC renewal is central to the promotion of muscle regeneration in muscle diseases, such as muscular dystrophies.

EPIGENETIC MECHANISMS OF GENE REGULATION

Epigenetics is responsible for the identity of distinct cell types despite the same genetic information by modulating gene expression without altering the genetic code. In other words, the ensemble of epigenetic characteristics, referred to as the epigenome, determines the gene expression pattern that defines the distinct characteristics and functions of each cell type^[27]. In fact, although their genomes are essentially identical, the cell types in a multicellular organism perform strikingly different behaviors over extended periods. Lineage commitment during development is the most extreme example of epigenetics. During embryogenesis, cells progress from totipotency to terminal differentiation, and each step of this progression involves the establishment of a stable state in which specific developmental commitments that can be transmitted to daughter cells are encoded. The understanding of the epigenome of different cell types and the complexity of the post-transcriptional control of gene expression is constantly expanding due to the development of new technologies and the continuous discovery of noncoding RNAs that participate in epigenetic regulation.

Histone methylation

The eukaryotic genome is packaged into chromatin, a chain of nucleosomes composed of four core histones - H2A, H2B, H3, and H4 - whose amino-terminal tails are exposed on the surface of nucleosomes and are subjected to a wide range of post-translational modifications^[28,29]. Gene activation and repression, as well as transcriptional initiation and elongation, are regulated by many such histone modifications. In the last decade, studies of human and mouse embryonic stem cells have delineated the role of the histone methyltransferase (HMT) families Polycomb group (PcG) and Trithorax group (TrxG) in modulating the pluripotency and lineage restriction of several cell types^[30]. For instance, numerous trimethylations of histone 3 lysine 4 (H3K4me3) mediated by the TrxG family surrounding the transcription start sites indicate transcriptional gene activation, and trimethylations of histone 3 lysine 36 (H3K36me3) in the gene body are generally associated with active gene transcription; alternatively, the trimethylation of histone 3 lysine 27 (H3K27me3) mediated by the PcG complex is associated with transcriptional repression^[28]. Although the repressive H3K27me3 mark is transmitted to daughter cells^[31] and is dominant over the permissive H3K4me3 mark^[32], transcriptional gene activation requires the demethylation of H3K27me3, which is mediated by the demethylase

families lysine-specific demethylase 6A (Kdm6a) and KDM1 lysine-specific demethylase 6B (Kdm6b)^[33-35]. Thus, whereas polycomb repressive complex 2 (PRC2) establishes gene silencing at developmentally regulated loci, the TrxG and Kdm6a/Kdm6b families work together to antagonize the repressive activity of PRC2 and to promote gene expression in specific cell types.

Histone acetylation

Histone acetylation modulates transcription *via* multiple mechanisms. The acetylation of lysine residues within histone tails neutralizes their positive charge, thereby facilitating chromatin relaxation and increasing the accessibility of transcription factors to their target genes^[36]. Acetylated histones are also recognized as binding sites for transcriptional activators. Conversely, histone deacetylation induces transcriptional repression by compacting the chromatin structure^[37]. The combined activities of two enzyme families, histone acetyltransferases (HATs) and deacetylases (HDACs), determine the overall levels of histone acetylation in the genome. Both HATs and HDACs act on chromatin by associating with a variety of DNA-binding transactivator proteins. In some cases, DNA targeting involves other chromatin-modifying activities, such as histone methylation. Thus, the effects of HATs and HDACs on gene regulation depend on the cell type and the spectrum of available partners^[38].

Noncoding RNAs

Advances in the field of gene regulation mediated by single-stranded noncoding RNA molecules have demonstrated their importance in gene regulatory networks. Until recently, small noncoding RNAs (miRNAs) were believed to solely negatively regulate target mRNAs^[39]. However, published studies are increasingly indicating that miRNAs can also stimulate gene expression in response to specific cellular conditions or cofactors^[40]. miRNAs are able to reduce gene expression *via* multiple mechanisms. At the transcriptional level, miRNAs repress gene expression by pairing nucleotides 2 to 8, termed the seed region, to the seed match site in the target mRNA, typically positioned at the 3' UTR or, less frequently, at the 5' UTR or the coding region^[41]. In addition to transcriptional effects, miRNAs can repress translation initiation *via* multiple mechanisms, such as promoting mRNA degradation or interfering with the formation of closed-loop mRNA or other translation initiation factors^[41]. Moreover, increasing evidence indicates that some miRNAs can upregulate gene expression in specific cell types and under certain conditions *via* the direct action of miRNAs or *via* the indirect inhibition of repressive miRNA activity^[42]. Another class of noncoding RNAs, long noncoding RNAs, has been shown to take part in many transcription regulatory processes^[43] and post-transcriptional events, such as mRNA stability and translational control^[44-46], and to function as competing endogenous RNA^[47-49] by acting as an miRNA sponge to participate with coding RNA in a regulatory circuit that controls the binding of RNA to

miRNA.

Reciprocal and dynamic interactions between different epigenetic mechanisms and transcription factors modulate gene expression. For instance, changes in histone modifications are caused by the recruitment of chromatin-modifying enzymes, such as HATs or HMTs, by transcription factors and the RNA polymerase II complex^[28]; at the same time, combinations of histone modifications in the proximity of consensus sequences may anticipate and direct the binding of the transcription factor, facilitating the transcriptional activation of a given gene^[50].

Numerous epigenetic mechanisms regulate the different phases of myogenesis, including chromatin remodeling and post-transcriptional gene regulation mediated by noncoding RNAs^[51-53]. Such orchestrated regulation permits the correct timing of muscle-specific gene expression and influences the fate of muscle progenitors into muscle or non-muscle cell lineages^[54,55]. In this review, we report and discuss the recent literature about the epigenetic regulation of the myogenic process from activation to proliferation and SC commitment. We focus on the epigenetic changes, specifically those mediated by chromatin methylation or acetylation and noncoding RNA function to regulate gene expression, that occur during the different steps of SC differentiation.

EPIGENETICS IN SCs

Epigenetics mediates most of the signaling integrating the regeneration cues released by interstitial cells and by the external environment within the SC niche. Our understanding of the highly coordinated layers of epigenetic regulation of SC maintenance, activation and differentiation and cross-talk of epigenetic regulatory mechanisms with muscle-specific transcriptional machinery has tremendously increased due to the recent results obtained *via* next-generation genome-wide sequencing^[56]. Using ChIP-Seq experiments, chipped chromatin can currently be entirely mapped across the genome to identify the regions that are over-represented among these sequences, revealing the interactions between chromatin-remodeling enzymes, transcription factors and DNA, thereby facilitating the production of chromatin-state maps.

Epigenetic control of SC quiescence

In quiescent SCs, while the *Pax7* gene must be expressed, modulators of cell cycle progression and transcription factors of the myogenic lineage need to remain silenced. Increasing studies have suggested that quiescent SCs are not in a dormant state but rather are primed for activation and differentiation in response to external stimuli^[57,58]. At the chromatin level, this primed state is maintained by the general lack of the repressive mark H3K27me3 across the genome and the concomitant presence of H3K4me3 at the transcription start sites of a large number of genes (nearly 50% of all annotated genes)^[59], including myogenic regulatory

factors such as MyoD, SRF and Myf5^[60], whose encoded proteins are the primary activators of the myogenic program. Consistent with the notion that H3K4me3 alone does not predict the transcriptional state of a gene but rather marks the gene for transcriptional activation^[61], neither the number nor the identity of genes marked by H3K4me3 is significantly different between activated and quiescent SCs. Indeed, SC activation is accompanied by the retention of H3K4me3 and the acquisition of H3K27me3 *via* PcG members^[62], often in association with the transcriptional repressors YY1 and HDAC1. Interestingly, low levels of H3K27me3 are associated with the pluripotency of embryonic stem cells^[56,63]. The current understanding is that the general lack of repressive H3K27me3 marks and the concomitant presence of H3K4me3 at the transcription start sites of a large number of genes may establish a permissive chromatin state that underlies and permits the pluripotency of stem cells. In addition, numerous transcription start sites across the genome of quiescent SCs contain bivalent chromatin domains^[59], which are characterized by the concomitant presence of both H3K4me3 and H3K27me3 marks. Consistent with the presence of H3K27me3 marks, these genes are either not transcribed or transcribed at very low levels. Interestingly, bivalent domains correspond to genes that are associated with the development of other organs and tissues aside from muscle, suggesting that SCs retain the potential to adopt a non-myogenic fate because of the presence of bivalent domains that contribute to the determination of cell lineage^[59].

miRNAs also contribute to the generation of an epigenetic state that enables the maintenance of the myogenic lineage in quiescent SCs and that facilitates the activation of muscle gene expression and the formation of differentiated myotubes in response to SC activation. An important role in the maintenance of muscle stem-cell quiescence has been demonstrated for microRNA-489, which is highly expressed in quiescent SCs, in which it suppresses the expression of the oncogene *DEK*, and which is rapidly downregulated upon SC differentiation^[57]. Additionally, miR-31 has been demonstrated to play an important role in quiescent SCs^[58]. In quiescent SCs, the *Myf5* gene has already been transcribed but cannot be expressed because miR-31 functionally inactivates *Myf5* mRNA by retaining it inside cytoplasmic mRNP granules, thereby preventing its translation and blocking myogenic differentiation^[58].

Epigenetic control of SC activation

In response to different stimuli, *e.g.*, muscle damage, SCs become activated, begin to express cell cycle markers, which are readily marked by the permissive H3K4me3^[64], and re-enter the cell cycle. SCs that divide in a parallel orientation to the myofibers, undergo a symmetrical cell division and give rise to two SCs that can return to the quiescent state^[8]. In contrast, cells that divide in the sagittal orientation undergo asymmetric cell division to produce one cell that returns to the

quiescent state and one proliferating myoblast^[8]. The latter cell expresses the *Myf5* and *MyoD* genes, as well as genes that regulate cell cycle progression, all of which are characterized by the enrichment of the transcriptionally permissive H3K4me3 mark within their associated chromatin^[19]; Pax7 is progressively silenced while transitioning from a transcriptionally permissive state of H3K4me3 to a repressive state of H3K27me3 throughout cell differentiation^[65]. Additional mechanisms that regulate SC proliferation include p38-gamma MAPK, which phosphorylates the MyoD protein to reinforce the interaction between MyoD and the HMT KMT1A, and the consequent inhibition of the premature expression of the myogenin gene. Consistently, in p38-gamma MAPK-null SCs, KMT1A cannot associate with the myogenin promoter; therefore, myogenin is expressed earlier, resulting in decreased SC proliferation and defective differentiation^[66].

During myoblast proliferation, distinct classes of HDACs are also involved in the repression of muscle gene transcription by countering the activities of HATs. Whereas local hyper-acetylation at consensus MyoD-binding sites in myoblasts likely predetermines the regions of chromatin accessibility, class I and II HDACs contribute to the hypo-acetylation of the *MyoD* gene and the inhibition of *MEF2* transcription and activation, respectively. In undifferentiated SCs, *MyoD* interacts with HDAC1, and this interaction is responsible for silencing the MyoD-dependent transcription of p21 and muscle-specific genes^[67,68]. Moreover, class II HDACs are localized to the nucleus during SC proliferation and are responsible for blocking the activity of the myogenic co-factor MEF2^[69]. A recent study of HDAC4 function and SC proliferation reported that the HDAC4 levels positively correlate with the expression of *Pax7* and *Lix1*, both of which are important for appropriate SC proliferation^[70]; however, the molecular mechanism underlying this phenomenon remains unclear.

SC proliferation is also promoted and maintained by miR-27a/b, which targets and downregulates myostatin mRNA^[71]. Consistently, SCs treated with antagomirs specific to miR-27a/b displayed increased myostatin expression and reduced proliferation. In activated SCs, miR-27b plays an important role in determining the appropriate timing of myogenic gene expression and regulates the Pax3 protein levels to control the entry of these cells into the myogenic differentiation program^[72]. SC proliferation is also promoted by miR-133a, which represses the expression of serum response factor^[73], and by miR-682, which is highly upregulated during myoblast proliferation both *in vitro* and *in vivo*; the inhibition of miR-682 results in reduced myoblast proliferation^[74]. Moreover, in activated SCs, tissue inhibitor of metalloproteinase 3 (TIMP3), an inhibitor of tumor necrosis factor (TNF)-alpha-converting enzyme, regulates TNF-alpha release and acts as a switch for myogenic differentiation. miR-206 promotes TIMP3 downregulation^[75] and suppresses Pax3 expression^[74], thereby promoting SC differentiation. Paradoxically,

quiescent SCs express high levels of both Pax3 and miR-206. An additional layer of gene regulation explains these contradictory data. In fact, it has been shown that in quiescent SCs, Pax3 transcripts are alternatively polyadenylated and are expressed as shorter 3' UTR transcripts, thereby resulting in the resistance of Pax3 expression to miR-206-mediated regulation^[76].

Epigenetic control of SC differentiation

As a general rule, genes no longer required for lineage progression are targeted for stable repression^[59,62]. Accordingly, during differentiation, SC chromatin converts to a more repressed state by accumulating H3K27me3 across the genome at both transcription start sites and intergenic regions. In fact, in contrast to the level of H3K4me3, the level of the repressive mark H3K27me3 is low in quiescent SCs and is dramatically increased in differentiating SCs. In particular, when SCs differentiate, PRC2 is released from muscle differentiation genes (*MyoD* and *SRF*) to translocate to loci that are typically repressed in differentiated myotubes, e.g., Pax7. By inducing a transition from the transcriptionally permissive mark H3K4me3 to the repressive mark H3K27me3 on the Pax7 gene, PRC2 contributes to the switching off of SC proliferation^[65]. Similarly, a switch from the permissive mark H3K4me3 to the repressive mark H3K27me3 on genes involved in the cell cycle is mediated by the E2F family of transcription factors and by the retinoblastoma protein as the SC exits the cell cycle to terminally differentiate^[64,77,78]. Moreover, Pax7 associates with the Wdr5-Ash2L-MLL2 HMT complex, which mediates H3K4me3^[19]. The binding of the Pax7-HMT complex to Myf5 results in the formation of H3K4me3 on the surrounding chromatin. Thus, Pax7 also participates in the induction of chromatin modifications that stimulate transcriptional activation of target genes to regulate the entry into the myogenic developmental program. Concomitantly, lysine-specific demethylase 4A, together with heterochromatin protein 1 alpha, promotes the demethylation of H3K9me3 at myogenic promoters, facilitating myoblast commitment^[79].

The Ezh2 subunit of PRC2 complex has been demonstrated to play a critical role in mediating SC differentiation into the skeletal muscle lineage by suppressing a subset of regulators of non-muscle cell fate. Indeed, Ezh2-mediated H3K27me3 marks are specifically present on genes associated with alternative lineage selection, although Ezh2 does not suppress terminal differentiation into skeletal muscle^[80]. In contrast to PRC2-Ezh2, PRC2-Ezh1 is required for the myogenic differentiation of SCs; specifically, PRC2-Ezh1 replaces PRC2-Ezh2 on the myogenin promoter to regulate the appropriate timing of the transcriptional activation of myogenin^[81].

When SCs differentiate, HDAC1 downregulation and pRb hypo-phosphorylation occurs, enabling the formation of the pRb-HDAC1 complex in differentiated myotubes. The pRb-HDAC1 interaction coincides with the disruption of the MyoD-HDAC1 complex, the

transcriptional activation of muscle-specific genes, and the differentiation of myoblasts^[68]. Muscle differentiation also induces the nuclear-to-cytoplasmic translocation of class II HDACs, thereby releasing the inhibitory constraints on MEF2 and consequently activating the expression of muscle-specific genes^[69].

Over the course of differentiation, p38 alpha/beta MAPK activity increases and is required for complete myoblast differentiation and fusion, which is partially due to its regulation of the epigenetic mechanisms controlling gene expression^[82]. In particular, p38alpha/beta MAPK, by selectively enabling the recruitment of SWI-SNF to the gene promoters of myogenin and creatine kinase, facilitates chromatin remodeling and the consequent expression of muscle genes. Indeed, p38-alpha-null SCs display increased Pax7 expression, persistent proliferation, and impaired differentiation and fusion^[83], highlighting the distinct role of different members of the p38 MAPK family in SC proliferation and differentiation. It appears likely that the relative abundance of p38-alpha and p38-gamma MAPK activity in activated SCs serves as a balance between SC proliferation and differentiation.

As SCs differentiate, the downregulation of the enzymatic subunit of PRC2-Ezh2 and its partner YY1 is mediated by the combined action of miR-214^[84], miR-26a^[85] and miR-29^[86], thereby relieving PRC2-mediated repression of muscle genes. Once SCs differentiate, miR-128a, miR-1 and miR-206 cooperate to block cell proliferation by inhibiting the expression of several targets in the insulin signaling pathway and Pax7 expression^[87,88]. Indeed, the loss of miR-1 and miR-206 increases Pax7 expression, enhances SC proliferation and significantly inhibits myoblast differentiation^[87,89]. The inhibition of cell proliferation is also achieved *via* the downregulation of the *Ccnd1* gene by both miR-26a and miR-1^[90]. In addition, miR-133a and miR-133b inhibit cell proliferation and promote myoblast differentiation by negatively regulating the FGFR1 and PP2AC proteins, which participate in ERK1/2-mediated signal transduction^[91]. In addition to inhibiting Pax7, miR-206 promotes SC differentiation and fusion to muscle fibers *via* the suppression of a collection of negative regulators of myogenesis, such as notch3, igfbp5, Meox2, RARB, Fzd7, MAP4K3, CLCN3, and NFAT5^[89,92]. An important role in determining SC lineage commitment has been attributed to miR-133^[93]. By targeting the mRNA of PRDM16, a master gene for brown fat determination, miR-133 modulates the choice between the myogenic and brown adipose lineages during SC differentiation. In SCs, HDAC4 expression correlates with that of miR-133, and HDAC4 inhibition induces SCs to partially differentiate into adipocytes^[70]; however, the link between HDAC4 and miR-133 has not been yet characterized. Moreover, by modulating the insulin-like growth factor (IGF)-1 pathway *via* the downregulation of the IGF-1 receptor, miR-133a promotes SC differentiation^[94] and sarcomeric actin organization^[95]. In differentiating myoblasts, the repression of tumor

growth factor-beta signaling, which is known to negatively affect SC differentiation, is ensured by the combined activity of miR-26a, which represses Smad1 and Smad4 expression^[96], miR-206 and miR-29, which are capable of inhibiting Smad3 expression^[97]. Together with myomiRs whose expression is upregulated during SC differentiation, the expression of other microRNAs needs to be down-regulated in order to achieve SC terminal differentiation. One such example is miR-23a, which directly regulates the expression of myosin heavy chain genes^[98].

CONCLUSION

The maintenance of skeletal muscle homeostasis is a key survival factor, considering that skeletal muscle accounts for approximately 40% of body mass. Skeletal muscle needs to efficiently respond to internal and external stimuli - such as changes in levels of blood sugar, circulating hormones, or growth factors or mechanical insults - or to pathological conditions to maintain homeostasis, particularly in muscle. Unequivocally, SCs play central roles in adult regenerative myogenesis and in the preservation of muscle mass. Different epigenetic marks contribute to the coordination of the precise time-dependent expression of different subsets of myogenic genes in SCs (Table 1 and Figure 1). For instance, the PcG catalytic subunit Ezh2 is specifically distributed on the regulatory regions of late but not early muscle genes^[60], whereas the methyltransferases PRMT5 and CARM1 are detected on promoters of early and late muscle genes, respectively^[99].

Despite the observation that most muscular diseases do not have an epigenetic cause *per se*, the importance of epigenetic pathways in modulating muscle-specific gene expression renders them as excellent candidate targets for disease interventions. For instance, an elegant study demonstrated that epigenetic commitment mediated by SMARCD3, a member of the SWI/SNF family of proteins, and MyoD is required for the efficient generation of skeletal muscle cells from human embryonic stem cells^[100]. Several drugs that target epigenetic mechanisms are currently undergoing clinical trials for many diseases. These drugs include HDAC inhibitors^[101] and HMT inhibitors alone^[102] or in combination^[103]. In amyotrophic lateral sclerosis, HDAC inhibitors have been proposed as potential drugs to ameliorate patient symptoms^[104,105]. In the case of muscular dystrophy, HDAC inhibitors have been extensively studied using the mdx mouse model^[106]; currently, these drugs are under review in a clinical trial for muscular dystrophy^[107]. Their effects are believed to be primarily due to the inhibition of class I HDACs^[108]. However, the prolonged treatment of patients with drugs that inhibit these ubiquitously required chromatin-modifying enzymes is a potential concern. An alternative approach ideally relies on the identification of small molecules that interfere with the epigenetic enzymes to specific loci within the genome. This approach may provide similar benefits

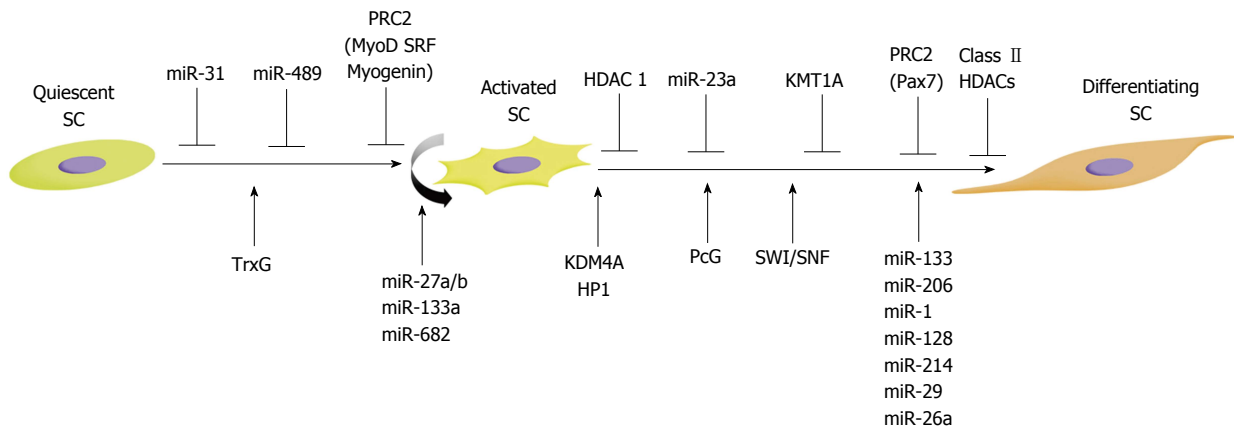


Figure 1 Schematic diagram of the epigenetic control of satellite cells during their activation and differentiation. SC: Satellite cells; PRC2: Polycomb repressive complex 2; TrxG: Trithorax group; PcG: Polycomb group; HDAC: Histone deacetylase.

Table 1 Summary of the main epigenetic events regulating the satellite cells status during myogenesis

SC status	Epigenetic regulation	Ref.
Quiescent	<p>↓ H3K27me3¹ and ↑ H3K4me3²</p> <p>Bivalent domains (H3K27me3 + H3K4me3)</p> <p>↑ miR-489 ↓ DEK³</p> <p>↑ miR-31 ↓ Myf5⁴</p>	<p>[59,60]</p> <p>[59]</p> <p>[57]</p> <p>[58]</p>
Activated	<p>↑ H3K4me3 on cell cycle genes</p> <p>↑ H3K27me3 on the Pax7⁵ gene</p> <p>↑ P38 γ-MAPK⁶ → p-MyoD⁷ + KMT1A⁸ ↓ myogenin</p> <p>MyoD + HDAC1⁹ ↓ p21¹⁰ and muscle genes</p> <p>HDAC4/5¹¹ ↓ MEF2¹²</p> <p>HDAC4 → Pax7, Lix1¹³</p> <p>↑ miR-27a/b ↓ myostatin and Pax3¹³</p> <p>↑ miR-133a ↓ serum response factor</p> <p>↑ miR-206 ↓ TIMP3¹⁴ and Pax3</p>	<p>[19]</p> <p>[65]</p> <p>[66]</p> <p>[67,68]</p> <p>[69]</p> <p>[70]</p> <p>[71,72]</p> <p>[73]</p> <p>[75,76]</p>
Differentiating	<p>↑ H3K27me3 on cell cycle and alternative fate genes</p> <p>↑ H3K43me on Myf5</p> <p>↓ HDAC1 → MyoD-dependent genes</p> <p>HDAC4/5 in the cytoplasm → MEF2</p> <p>↑ P38 α/β-MAPK¹⁵ → SWI-SNF¹⁶ → myogenin and creatine kinase genes</p> <p>↑ miR-214, miR-26a, and miR-29 ↓ Ezh2¹⁷ and YY1¹⁸</p> <p>↑ miR-128a, miR-26a, miR-1, miR-206, miR-133a, and miR-133b ↓ cell cycle</p> <p>↑ miR-206 ↓ notch3¹⁹, igfbp5²⁰, Meox2²¹, RARB²², Fzd7²³, MAP4K3²⁴, CLCN3²⁵, and NFAT5²⁶</p> <p>↑ miR-133a ↓ PRDM16²⁷ and the IGF-1²⁸ receptor</p> <p>↑ miR-26a ↓ Smad1²⁹ and Smad4³⁰</p> <p>↑ miR-206 and miR-29 ↓ Smad3³¹</p> <p>↓ miR-23a ↓ myosin heavy chain</p>	<p>[59,64,65,76-78]</p> <p>[19]</p> <p>[68]</p> <p>[69]</p> <p>[82,83]</p> <p>[84-86]</p> <p>[87,88,90,91]</p> <p>[59,89]</p> <p>[78,80]</p> <p>[96]</p> <p>[97]</p> <p>[98]</p>

¹Trimethylated histone H3 lysine 27 (H3K27me3); ²Trimethylated histone H3 lysine 4 (H3K4me3); ³DEK oncogene; ⁴Myogenic factor 5; ⁵Paired box 7; ⁶p38 gamma mitogen-activated protein kinase (P38 MAPK); ⁷Phospho-myogenic differentiation; ⁸Also referred to as suppressor of variegation 3-9 homolog 1 (Suv39h1); ⁹Histone deacetylase 1; ¹⁰Cyclin-dependent kinase inhibitor 1A; ¹¹Histone deacetylase 4/5; ¹²Limb expression 1 homolog (chicken); ¹³Paired box 3; ¹⁴TIMP metalloproteinase inhibitor 3; ¹⁵p38 alpha/gamma mitogen-activated protein kinase; ¹⁶SWI/SNF complex; ¹⁷Enhancer of zeste homolog 2; ¹⁸YY1 transcription factor; ¹⁹Neurogenic locus notch homolog protein 3 gene; ²⁰Insulin-like growth factor binding protein 5; ²¹Mesenchyme homeobox 2 (Meox2); ²²Retinoic acid receptor, beta (RARβ); ²³Frizzled class receptor 7 (Fzd7); ²⁴Mitogen-activated protein kinase kinase kinase 3 (MAP4K3); ²⁵Chloride channel, voltage-sensitive 3 (CLCN3); ²⁶Nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5); ²⁷PR domain containing 16 (PRDM16); ²⁸Insulin-like growth factor 1 (IGF-1); ²⁹SMAD family member 1; ³⁰SMAD family member 4; ³¹SMAD family member 3. HDAC: Histone deacetylase.

without exerting side effects caused by the modification of gene expression in other cell types. In the last few years, numerous studies involving RNA-seq or ChIP-seq have contributed to provide a picture of the epigenetic characteristics of muscle-specific gene promoters during the different stages of myogenesis. However, most of these studies were performed using muscle cell lines

and remain to be validated using *in vivo* models, for instance by analyzing mice in which individual epigenetic regulators are inactivated in a muscle-specific manner. The elucidation of the epigenetic mechanisms regulating SC function might reveal new targets for pharmacological manipulation to improve muscle regeneration and to promote muscle homeostasis under pathological

conditions.

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Basic Study

Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells

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Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress. Animals were housed for two weeks before entering the experiment.

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Abstract

AIM: To study the therapeutic effect of three tube-guides with electrical conductivity associated to mesenchymal stem cells (MSCs) on neuro-muscular regeneration after neurotmesis.

METHODS: Rats with 10-mm gap nerve injury were tested using polyvinyl alcohol (PVA), PVA-carbon nanotubes (CNTs) and MSCs, and PVA-polypyrrole (PPy). The regenerated nerves and tibialis anterior muscles were processed for stereological studies after 20 wk. The functional recovery was assessed serially for gait biomechanical analysis, by extensor postural thrust, sciatic functional index and static sciatic functional

index (SSI), and by withdrawal reflex latency (WRL). *In vitro* studies included cytocompatibility, flow cytometry, reverse transcriptase polymerase chain reaction and karyotype analysis of the MSCs. Histopathology of lung, liver, kidneys, and regional lymph nodes ensured the biomaterials biocompatibility.

RESULTS: SSI remained negative throughout and independently from treatment. Differences between treated groups in the severity of changes in WRL existed, showing a faster regeneration for PVA-CNTs-MSCs ($P < 0.05$). At toe-off, less acute ankle joint angles were seen for PVA-CNTs-MSCs group ($P = 0.051$) suggesting improved ankle muscles function during the push off phase of the gait cycle. In PVA-PPy and PVA-CNTs groups, there was a 25% and 42% increase of average fiber area and a 13% and 21% increase of the "minimal Feret's diameter" respectively. Stereological analysis disclosed a significantly ($P < 0.05$) increased myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fiber diameter (d/D; g-ratio) in PVA-CNT-MSCs group ($P < 0.05$).

CONCLUSION: Results revealed that treatment with MSCs and PVA-CNTs tube-guides induced better nerve fiber regeneration. Functional and kinematics analysis revealed positive synergistic effects brought by MSCs and PVA-CNTs. The PVA-CNTs and PVA-PPy are promising scaffolds with electric conductive properties, bio- and cytocompatible that might prevent the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction.

Key words: Nerve regeneration; Neurotmesis; Tube-guides; Mesenchymal stem cells; Carbon nanotubes; Functional assessment; Gait biomechanical analysis; Histomorfometry; Polyvinyl alcohol

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Core tip: The rat sciatic injury neurotmesis injury model is an appropriate model to evaluate the nerve regeneration when electric conductive tube-guides of polyvinyl alcohol (PVA)-carbon nanotubes (CNTs) and PVA-polypyrrole (PPy) associated to mesenchymal stem cells (MSCs) are used as scaffolds. The results obtained revealed that treatment with MSCs associated to PVA-CNTs tube-guides induced an increased number of regenerated fibers and thickening of the myelin sheet. Functional and kinematics analysis revealed positive synergistic effects brought by MSCs and PVA-CNTs. The PVA-CNTs and PVA-PPy are promising scaffolds with electric conductive properties, bio- and cytocompatible that might prevent the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction.

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INTRODUCTION

Functional recovery is rarely satisfactory in patients where peripheral nerve repair is needed, remaining a challenging task in neurosurgery^[1,2]. Direct repair is the procedure of choice but only when a tension-free end-to-end suture is possible. However, for patients with loss of nerve tissue, resulting in a gap it is necessary to reconstruct the injured nerve using an autograft or a graft from a compatible donor^[1,3]. One of the disadvantages of grafting is the necessity of a second surgery for collecting the nerve sample and respective donor site morbidity. In addition, non-matching donor and recipient nerve diameters often occur, which might be the reason for an incomplete functional recovery^[4]. Entubulation offers advantages compared to graft implantation, including the potential to manipulate and to improve the regeneration environment within the tube-guide by adding to the lumen, growth factors and/or cellular systems^[5]. Consequently, guidance of regenerating axons is improved by a mechanical effect but also by cellular growth factors, a chemical^[6] and electrical cues^[7]. Natural and synthetic biomaterials have been used as tube-guides, being the later sub-divided into two major groups: biodegradable and non-biodegradable^[8,9]. The development of tube-guides is a consequence of the limitations inherent in the use of grafts, in terms of length, diameter and type of fiber, preventing damage to the sampling local, as it was previously referred^[10]. For a correct nerve regeneration some important physical properties of tube-guides have been listed and referred by several authors like: the tube-guides should be made of biodegradable biomaterials with appropriate porous dimensions; the ability to deliver growth factors and drugs and allow the incorporation of cellular systems; an internal geometry to support an organized cell migration or intraluminal structures similar to nerve fascicles; and with some electrical activity in order to promote axon regeneration^[11]. The use of electric conductive biomaterials to achieve nerve regeneration is a promising research area^[12]. The goal of this work was to evaluate the therapeutic effect (by morphological and functional analysis) of three previously developed tube-guides with electrical conductivity for nerve regeneration associated to mesenchymal stem cells (MSCs) isolated from the Wharton's jelly of umbilical cord (UC) on neuro-muscular regeneration after neurotmesis injury using the rat sciatic nerve model. The three tube-guides used *in vivo* where those made of polyvinyl alcohol (PVA), PVA loaded with MWCNTs [functionalized carbon nanotubes (CNTs), PVA-CNTs], and PVA loaded with polypyrrole (PVA-PPy). Such composites with electric conductivity can be used to

host cell therapies, and beneficial regenerative electrical stimulation can be applied directly to the cells through the composite^[13]. PVA is a polymer used as a biomaterial due to its biocompatibility, non-toxic, non-carcinogenic, swelling properties, and bio-adhesive characteristics. PVA can also be used as host material in order to increase the solubility as well as the mechanical strength of conductive materials, and it is approved by Food and Drug Administration^[14,15]. PPy and CNTs are two of the most studied conductive polymers for tissue engineering, especially for nerve tissue engineering^[16]. The authors previously to *in vivo* application, focused on tube-guides design and *in vitro* characterization. The PVA-CNTs and PVA-PPy tube-guides presented conductivity advantages, important for nerve regeneration. The developed electrical conductive nerve tube-guides was achieved by loading PVA with 0.05% of PPy or COOH-functionalized CNTs. The inclusion of CNTs and PPy brought a significant increase of electrical conductivity of the simple PVA tube-guide. The PVA-CNTs tube-guides showed the rougher topography. The DSC and X-ray diffraction (XRD) studies revealed that all materials have similar low crystallinity. The wettability studies indicated a hydrophilic behaviour of all nerve tube-guides, being the PVA-PPy slightly more hydrophobic. In terms of surface charge, the zeta potential measurements revealed that both COOH-functionalized CNTs and PPy loads turned the surface charge slightly more positive. Regarding the elastic behaviour, the COOH-functionalized CNTs load caused a slight decrease of the rigidity of PVA. PVA-CNTs and PVA-PPy might present a regenerative potential and were tested in the rat sciatic nerve neurotmesis injury model^[17]. Also, since the PVA-CNTs presented the rougher topography, which is important for the association and viability of the associated cellular systems^[9], where also tested associated to the MSCs-based therapy^[3,18].

Considering the peripheral nerve system (PNS), MSCs are promising cell-based therapies to be applied alone or associated to scaffolds. MSCs have a high plasticity, proliferative and differentiation capacity, and also have the advantage of presenting immunosuppressive properties^[3,19,20]. For these reasons, MSCs have been used in experimental trials as cell-based therapies, including pathologies of PNS and central nervous system (CNS). Furthermore, nowadays the characterization of MSCs is well defined by recommendations and standards stated by the international society for cellular therapy (ISCT)^[19]. The MSCs therapeutic effect resides on their capacity to replace original cells of damaged tissues, and also by the physiologic secretion of growth factors and cytokines that modify the microenvironment inducing the activity of endogenous progenitor cells within the injured tissue, and by modulating the inflammatory and immune responses. The inflammatory modulation includes the Wallerian degeneration, a crucial step for nerve regeneration after neurotmesis injury^[3]. Therefore, the use of cellular systems is a rational approach for promoting nerve



Figure 1 Synthetic biodegradable tubes of polyvinyl alcohol, polyvinyl alcohol loaded with COOH-functionalized multiwall carbon nanotubes MWCNTs (polyvinyl alcohol-carbon nanotubes, polyvinyl alcohol-carbon nanotubes tube-guides), and polyvinyl alcohol loaded with polypyrrole (polyvinyl alcohol-polypyrrole, polyvinyl alcohol-polypyrrole tube-guides).

regeneration by delivering growth-promoting factors and cytokines at the nerve lesion site^[9]. MSCs can be isolated from several tissues, including bone marrow (BM), umbilical cord blood (UCB) and umbilical cord tissue (UCT), dental pulp, and adipose tissue^[9]. BM collection has several disadvantages, since the BM include a high percentage of adipocytes and a heterogeneous cell population, there is the possibility of morbidity associated to donor collection, as well as it is observed a decreasing number of BM-MSCs along the adult life and available for therapeutic applications. The research concerning other sources of MSCs has been intensively performed for the past years for identifying tissues that will allow MSCs isolation, and safe and controlled *ex vivo* expansion of these cells for potential allogeneic and autologous application^[9]. MSCs isolated from the Wharton's jelly UCT have been tested for the clinical application in CNS and PNS, including neurodegenerative diseases^[21]. It has been reported that MSCs from the UCT are still viable 4 mo after transplantation and there is no need for immunological suppression of the patients and experimental animals^[22]. The reason for this advantage is the fact that these cells are negative for major histocompatibility complex (MHC), and have low expression of MHC class I^[19] with potential application for MSC-based therapies in allogeneic treatments. *In vivo* studies involving these MSCs are limited but encouraging, especially what concerns PNS. Also, there are an increasing number of high quality cryopreserved cord tissue units in Cord Blood Banks worldwide. In addition, these cells represent a non-controversial source of MSCs, without neither ethical nor religious issues that are routinely harvested after birth, cryogenically stored, thawed, and can be expanded for therapeutic uses^[9]. On the other hand, it was demonstrated in some studies, that grafted MSCs have a signalling role which initiates the recruitment and direction of endogenous cells by growth factors production, promoting the local regeneration and modulating the inflammatory process^[23]. Nowadays it is believed that factors secreted

by MSCs are primarily responsible for their therapeutic action, so it is particularly important to understand and fully characterize the MSCs secretome^[18,24,25]. Recent studies demonstrated that MSCs produce and secrete multiple paracrine factors like interleukine-2 (IL-2), IL-6, IL-8, IL-12, IL-15, monocyte chemoattractant protein-1, macrophage inflammatory protein-1 β , regulated on activation, normal T cell expressed and secreted, and platelet-derived growth factor-AA. The several secreted factors previously listed that are present in MSCs conditioned medium (CM) was already described by others^[26] and more recently, by our research group^[18]. These paracrine factors are important to promote the MSCs therapeutic effects identified by the scientific community, like immunomodulatory and chemoattractive, anti-apoptotic, anti-fibrotic, angiogenic, and anti-oxidants activities. The hypothesis that MSCs and the growth factors produced during expansion in culture (CM) is an appropriate therapeutic product for local application in severe peripheral lesions seemed to be a rational approach and was studied in the present experimental work.

MATERIALS AND METHODS

Tube-guide preparation

Synthetic biodegradable tubes of PVA (Aldrich, Mowiol 10-98), PVA loaded with COOH-functionalized multiwall carbon nanotubes MWCNTs (Nanothinx, NTX5, MWCNTs 97% - COOH) (PVA-CNTs tube-guides), and PVA loaded with PPy (Aldrich, 10-40 S/cm of conductivity) (PVA-PPy tube-guides) were prepared using a casting technique to a silicone mould. A 15% (%w/v) aqueous solution of PVA was prepared. Then the solution of PVA was mixed with 0.05% of COOH-functionalized MWCNTs and 0.05% of PPy. The tube-guides were produced by freezing/thawing process. The treatment consisted in three cycles of freezer (-30 °C)/ incubator (25 °C), and an annealing treatment started with a stage of 14 h on an incubator (25 °C) followed by a ramp rate of 0.1 °C /min until 80 °C, and then a stage of 20 h at 80 °C. The tube-guides were sterilized by gama-radiation and hydrated in a sterile saline solution during 2 h before microsurgical application in the rat neurotmesis injuries (Figure 1).

MSCs cell culture and in vitro characterization

MSCs culture and expansion: Human MSCs isolated from the Wharton jelly (WJ) of UCT from PromoCell GmbH (C-12971, lot-number: 8082606.7) were cultured and expanded in a humidified atmosphere with 5% CO₂ at 37 °C by replacing the mesenchymal stem cell medium, PromoCell (C-28010) every 48 h. At 80% confluence, normally obtained after 4 d in culture (Figure 2), MSCs were harvested with 0.25% trypsin with EDTA (GIBCO) for further expansion at an initial concentration of 1 × 10⁴ cells/cm². Immediately previously to *in vivo* application, a MSCs suspension was prepared in 1 mL syringes. Each syringe contained MSCs at a concentration

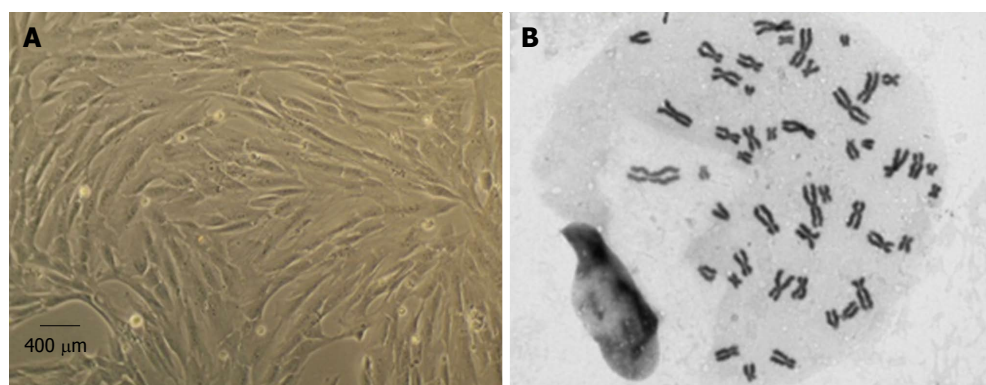


Figure 2 Mesenchymal stem cells culture and expansion. A: Isolated mesenchymal stem cells (MSCs) from the Wharton's jelly of umbilical cord presented a fusiform, fibroblast-like morphology in culture (magnification: 100 ×); B: Selected metaphases from MSCs in culture, showing normal number of chromosomes (46, XY) (magnification: 1000 ×).

of $1 \times 10^6/\mu\text{L}$ for posterior intra-operatively nerve injection.

The MSCs lot used in the present experimental study was characterized by flow cytometry analysis for a comprehensive panel of markers by PromoCell as previously reported^[18,25]. The MSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology during cell culture expansion. Their phenotype was evaluated by flow cytometry for PE anti-human cluster of differentiation-105 (CD105); APC anti-human CD73; PE anti-human CD90; PerCP/Cy5.5 anti-human CD45; FITC anti-human CD34; PerCP/Cy5.5 anti-human CD14; Pacific Blue anti-human CD19 and pacific-blue anti-human Human Leukocyte Antigen-DR (HLA-DR) (antibodies and their respective isotypes from BioLegend).

MSCs karyotype by cytogenetic analysis: Cytogenetic analysis was carried out before *in vivo* application between passages 4 and 5 as previously reported^[18,25]. Chromosome analysis was performed by one scorer on 20 Giemsa-stained metaphases and each MSC cell was scored for chromosome number. For determination of the karyotype, a routine chromosome G-banding analysis was also carried out and no structural alterations were observed which demonstrates the chromosomal stability to the cell culture procedures and also that the *in vivo* applied MSCs are not neoplastic (Figure 2).

Cytocompatibility evaluation of biomaterials: Intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by using dual wavelength spectrofluorometry as previously described^[27] was measured in Fura-2-loaded MSCs cells. $[\text{Ca}^{2+}]_i$ from MSCs cultured without the presence of any biomaterial, sub-cultured over PVA discs, over PVA loaded with CNTs (PVA-CNTs) and PVA loaded with PPy (PVA-PPy) discs of 10 mm diameter are results obtained from epifluorescence technique confirming that the MSCs did not begin the apoptosis process.

Reverse transcriptase polymerase chain reaction to confirm that MSCs are undifferentiated: Reverse

transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) targeting specific genes expressed by the MSCs that were *in vivo* applied was performed to certify that the MSCs used *in vivo* were not differentiated. For that, primers were designed targeting seven human genes based on the literature^[18]. DNA sequences from growth associated protein-43 (GAP-43), neurofilament-H (NF-H), Nestin, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), β -actin, neuronal nuclear (*NeuN*) and glial fibrillary acidic protein (*GFAP*) genes from mice (*Mus musculus*), rat (*Rattus norvegicus*) and human (*Homo sapiens*) were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank) and aligned using the Clustal Omega bioinformatic tool from EMBL-EBI (<http://www.ebi.ac.uk/Tools/msa/clustalo>). MSCs culture was harvested with 0.25% trypsin EDTA solution (Gibco) and centrifuged at 2000 rpm 4 °C during 5 min. Cell pellets were used for total RNA extraction using an adequate extraction kit, high pure RNA Isolation kit (Roche). Briefly, cell pellets were lysed with a lysis buffer, loaded into a High Pure Filter Tube, DNA was removed with DNase I enzyme, washed twice on column, and eluted with 100 μL of Elution Buffer. RNA was quantified and its quality assessed by using a Nanodrop ND-1000 Spectrophotometer and reads from 220 nm to 350 nm, and then stored at -80 °C until further use. In the following step, cDNA was synthesized from the purified RNA. To fulfill that issue, the kit Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) was used following the manufacturer instructions. Briefly, 1.5 μg of total RNA was used and diluted in DEPC-treated water to a 30 μL final volume in a RNase-free microcentrifuge tube; then heated at 65 °C for 10 min and then chilled in ice; transfer the RNA solution to the kit tube containing the first-strand reaction mix beads; add 0.2 μg of Oligo(dT) primer and DEPC-treated water to a 33 μL final volume; mix the content and incubate at 37 °C for 60 min. cDNA was synthesized and stored at -20 °C until further use. Of referring that, due to the use of the Oligo(dT) primer, the synthesized cDNA corresponds to the mRNA present in the sample at the time of collection. cDNA synthesized from undifferentiated MSCs was used to

check the expression of seven genes, two housekeeping genes (β -actin and *GAPDH*) and five specific of neuronal cells (GFAP, NeuN, Nestin, NF-H and GAP-43). Primers were designed in house and then synthesized in an external laboratory (MWG Operon, Germany). The primers were rehydrated in DNase/RNase free water in a concentration of 100 pmol/ μ L. qPCR was performed in a iCycler[®] iQ5TM (BioRad) apparatus using the iQTM SYBR[®] Green Supermix (BioRad). Each pair of primers targeting a gene was used to analyze its expression in the MSCs cDNA, in triplicate, along with a negative control. The plates containing the mix targeting the seven genes for both types of cells were submitted to the following cycles of temperatures: 95 °C during 4 min, 35 cycles comprising 95 °C during 20 s, 55 °C during 20 s and 72 °C during 20 s ending with Real-Time acquisition, and final extension of 75 °C for 7 min. After cycling temperatures, the number of cycle threshold for each well was recorded. The plate containing the amplified genes or qPCR products was kept in ice and observed in a 2% agarose gel to check and reinforce the identity of the amplicons. Briefly, 2 g of NuSieve[®] 3:1 Agarose (Lonza) were mixed with 100 mL Tris-Acetate-EDTA buffer, melted, mixed with ethidium bromide in a final concentration of 0.2 μ g/mL, and loaded in a horizontal electrophoresis apparatus. After solidification, 15 μ L of the qPCR products were loaded in the agarose wells, and submitted to a 120 V potential difference during 40 min to separate the amplicons. Gel was then observed under UV light and pictures recorded using the GelDoc[®] 2000 (BioRad) and Quantity One[®] software (BioRad). In the MSCs, the molecular analysis showed a very small amplification of *GFAP* gene, absence of amplification of the *NF-H* and *GAP-43* genes, and reasonable amplification of *NeuN*, β -actin, *GAPDH* and *Nestin* genes. Amplification of a given gene is correlated with its expression seeing that the template DNA is the one generated from mRNA. The RT-PCR results confirmed that the MSCs used *in vivo* were not differentiated into neuro-glial cells.

Surgical procedure for the rat sciatic nerve model for neurotmesis

The article describes a basic research study involving animal subjects and was approved by the Veterinary Authorities of Portugal [Direcção Geral de Alimentação e Veterinária (DGAV)] in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals have been observed. Also all the authors involved in the *in vivo* tasks have a degree in Veterinary Medicine and are accredited by the Veterinary Authorities of Portugal (DGAV) and by Felasa - Category C for working with laboratory animals.

The OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000) were always followed by the authors

by taking adequate measures to minimize pain and discomfort.

Polycarbonate cages type 3 were used for housing under standard laboratory conditions adult male Sasco Sprague Dawley rats with 300 g (Charles River Laboratories, Barcelona, Spain) always in a temperature and humidity controlled room with 12-12 h light/dark cycles. The rats were fed with standard chow and water *ad libitum* until the day of surgery. For surgery, the rats were anesthetized with ketamine 9 mg/100 g and xylazine 1.25 mg/100 g (body weight), by intraperitoneal administration. In lateral recumbence, the right sciatic nerve was exposed unilaterally and a transection injury was performed above the terminal nerve ramification using a straight microsurgical scissors, for creating a neurotmesis injury with 10 mm gap. Six experimental groups were studied: in groups 1, 2 and 3 after neurotmesis the proximal and distal nerve stumps were inserted 3 mm into PVA (group 1: PVA), PVA loaded with 0.05% (%w/v) of COOH-functionalized MWCNTs (group 2: PVA-CNTs), and PVA loaded with PPy 0.05% (%w/v) tube-guides (group 3: PVA-PPY), respectively, and tube-guides were sutured with two epineural sutures using 7/0 polypropylene monofilament, maintaining a nerve gap of 10 mm; in group 4 after neurotmesis, an autologous 180° inverted graft was sutured between both nerve stumps (group 4: Graft); in group 5 after neurotmesis, immediate coaptation with 7/0 monofilament polypropylene suture (group 5: End-to-End); in group 6 after neurotmesis the proximal and distal nerve stumps were inserted 3 mm in a PVA loaded with 0.05% (%w/v) of COOH-functionalized MWCNTs tube-guide kept in place with 2 epineural sutures using 7/0 monofilament polypropylene suture and the interior of the tube-guide was filled with 100 μ m of MSCs at a concentration of 1×10^6 cells/ μ L in culture medium, also 100 μ m of MSCs at a concentration of 1×10^6 cells/ μ L in culture medium were infiltrated in both nerve stumps inserted in the tube-guide (group 6: PVA-CNTs-MSCs) (Figure 3).

Functional assessment

After injury and sciatic nerve microsurgery reconstruction using the developed scaffolds in standardized neurotmesis injuries, a follow-up consisting in functional parameters measurements are important to evaluate the regeneration process and recovery. The animals were tested in an appropriate environment to minimize stress and handled by a single operator already trained for that purpose. Animals have been tested before the surgery at week 0, and after the surgery, at week 1, at week 2 and then every two weeks until the end of follow-up time of 20 wk.

Evaluation of motor performance and nociceptive function:

Motor performance was evaluated by measuring extensor postural thrust (EPT) and nociceptive function using the withdrawal reflex latency (WRL). The EPT included in the neurological recovery evaluation

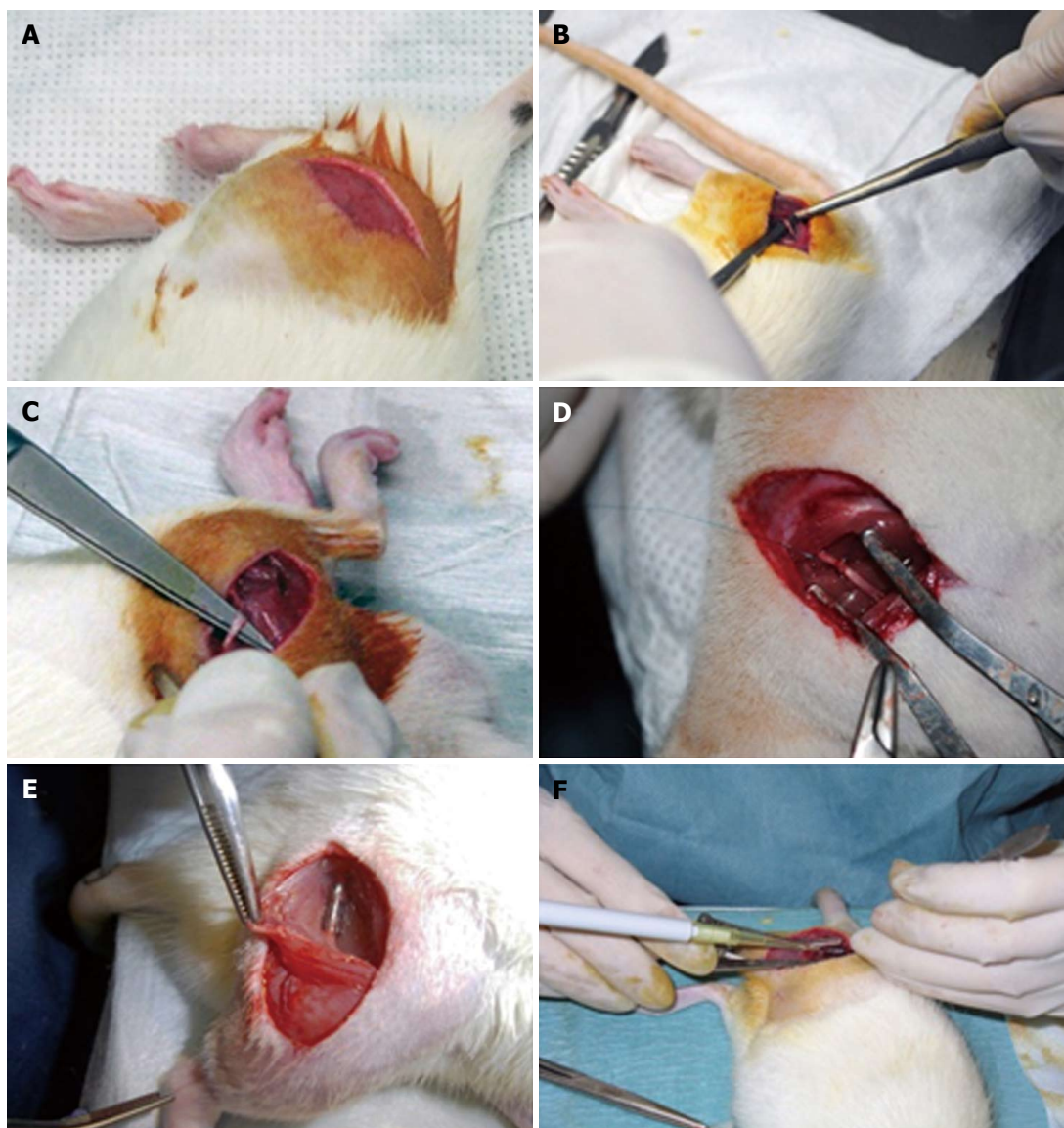


Figure 3 Surgery of the rat sciatic nerve neurotmesis injury model. Under deep anesthesia the right sciatic nerve was exposed unilaterally through a skin incision extending from the greater trochanter to the mid-half distally followed by a muscle splitting incision (A). After nerve mobilization (B), a transection injury was performed (neurotmesis) using a straight microsurgical scissors (C). In group 5 (End-to-End) after neurotmesis, immediate coaptation with 7/0 monofilament polypropylene suture was performed (D). Implantation of the tube-guide in the 10 mm gap (E). Local application of the mesenchymal stem cells (MSCs), the MSCs suspension filled the polyvinyl alcohol (PVA)-carbon nanotubes (CNTs) tube-guide lumen (group 6: PVA-CNTs-MSCs) (F).

of the rat after sciatic nerve injury was first described by^[28]. EPT is induced by lowering the affected hind-limb towards the platform of a digital balance supporting the animal by the thorax. During the test, the rat extends the hind-limb and the distal metatarsus and digits contact with digital platform balance. The force in grams (g) applied is recorded individually and the results for both hind-limbs (affected and un-affected hind-limb) are taken for equation 1 calculation. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values considered for equation (1) and the percentage of functional deficit is calculated^[9,28-31].

$$\% \text{ Motor deficit} = [(NEPT - EEPT)/NEPT] \times 100 \quad (1)$$

To assess the nociceptive WRL, the hotplate test

described by Masters *et al.*^[32] was used with some modifications and previously described by the authors^[3,9,17,25,31,33]. Also, it is considered by the scientific bibliography that rats without sciatic nerve injury withdraw their paws from the hotplate within 4.3 s or less^[9,17,31,33]. If there was no paw withdrawal after 12 s, the animal was assigned the maximal WRL of 12 s and the heat stimulus was removed to prevent tissue damage^[34-36].

For sciatic functional index (SFI), a confined walkway measuring 42 cm long and 8.2 cm wide with a dark shelter at the end (own fabrication) that the rats cross was used. The footprints from the experimental (E) and normal (N) sides are measured: (1) distance from the heel to the third toe, the print length; (2) distance from the first to the fifth toe, the toe spread (TS); and (3) distance from the second to the fourth toe,

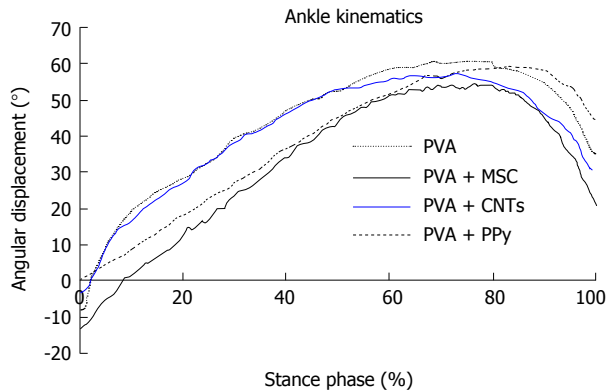


Figure 4 Kinematic plots in the sagittal plane for the ankle angle (°) as it moves through the stance phase, during the transection injury study. The mean of each group is plotted. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells.

the intermediary toe spread (ITS). In the static sciatic functional index (SSI) is a simpler test where only the parameters TS and ITS are the measurements included in equation (6). Prints for measurements are chosen at the time of walking based on precise, clear and completeness of footprints^[9,36,37]. The mean distances of 3 measurements are used to calculate the following factors (dynamic and static):

$$\text{TSF} = (\text{ETS} - \text{NTS})/\text{NTS} \quad (2)$$

$$\text{ITSF} = (\text{EITS} - \text{NITS})/\text{NITS} \quad (3)$$

$$\text{PLF} = (\text{EPL} - \text{NPL})/\text{NPL} \quad (4)$$

TSF: Toe spread factor; ITSF: Intermediate toe spread factor; PLF: Print length factor; ETS: Experimental toe spread; NTS: Normal toe spread; NITS: Normal intermediary toe spread; EITS: Experimental Intermediary toe spread; NPL: Normal print length; EPL: Experimental print length.

SFI is calculated as described by Bain *et al.*^[38] according to the following equation:

$$\text{SFI} = -38.3 \times (\text{EPL} - \text{NPL})/\text{NPL} + 109.5 \times (\text{ETS} - \text{NTS})/\text{NTS} + 13.3 \times (\text{EITS} - \text{NITS})/\text{NITS} - 8.8 = (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8 \quad (5)$$

Considering the SFI measurements, it was not possible to obtain values during the 20 wk follow-up after the surgery procedure, due to autotomy of the fingers. As a matter of fact, the rat sciatic nerve model for neurotmesis has this disadvantage; the autotomy observed in several experimental animals even when a repellent substance is applied routinely^[9]. SSI was calculated as described by^[39] according to the following equation (equation 6):

$$\text{SSI} = 108.4 \times \text{TSF} + 31.85 \times \text{ITF} - 5.49 \quad (6)$$

For SFI and SSI, when no footprints are measurable,

the index score of -100 is given considering total impairment, on the other hand, a score of 0 means that there is no alteration and the footprint is normal.

Kinematic analysis: Ankle kinematics was carried out at the end of the 20-wk follow-up time in the following experimental groups: PVA, PVA-CNTs, PVA-PPy, Graft, End-to-End, and PVA-CNTs-MSCs. A Perspex track with length, width (adjustable) and height of 120, 12, and 15 cm, respectively was used to record the rat locomotion in a straight line. The animals' gait was video recorded at a rate of 300 Hz images per second (Casio Exilim PRO EX-F1, Japan) by a camera with a visualization field of 14 cm wide, that was positioned at the track half-length, and 1 m distant from the track, where gait velocity was steady. The software APAS® (Ariel Performance Analysis System, Ariel Dynamics, San Diego, United States) was used for data analysis. The authors used a two-segment model of the ankle joint, adopted from the model firstly developed by^[40] (2D biomechanical analyses in sagittal plan), and the rat ankle angle was determined using the scalar product between a vector representing the foot and a vector representing the lower leg. Dorsiflexion and plantarflexion is considered for positive and negative values of position of the ankle joint (θ°). Initial contact (IC), opposite toe off (OT), and heel rise (HR) and toe-off (TO)^[33,40] were the time points analyzed for each step cycle, the values were time normalized for 100% of step cycle. The normalized temporal parameters were averaged over all recorded trials (Figure 4). Since the animal's normal walking velocity is 20-60 cm/sa^[33,40], stance phases lasting between 150 and 400 ms were considered for analysis.

Morphological analysis and histopathology: For histomorphometric analysis, nerve samples obtained from the 10-mm-long sciatic nerve segments distal to the neurotmesis site and from un-operated controls^[41-44]. The histological nerve preparation followed the previously reported protocol^[3,9,17,25,31,33,35] and a systematic random sampling and D-disector were adopted^[41-44].

At the end of the healing period tested (week 20), tibialis anterior (TA) muscles of all experimental (PVA, PVA-CNTs, PVA-PPy, Graft, End-to-End, and PVA-CNTs-MSCs) and Control (no lesion) groups were collected, and the tissue samples were fixed in 10% buffered formalin, routinely processed, dehydrated and embedded in paraffin wax. Consecutive 3 μm transverse sections from the mid-belly of each muscle were cut, stained with haematoxylin and eosin (HE) and used for morphometry evaluation and determination of the degree of atrophy. For the morphometric analysis, an unbiased sampling procedure was applied and the following measures were calculated: area, perimeter, "Feret's angle" and "minimal Feret's diameter" (which is the minimum distance of parallel tangents at opposing borders of the muscle fiber). These parameters were evaluated from the cross sections using the ImageJ® software (NIH) which allowed to apply this set of individual fiber measurements. A

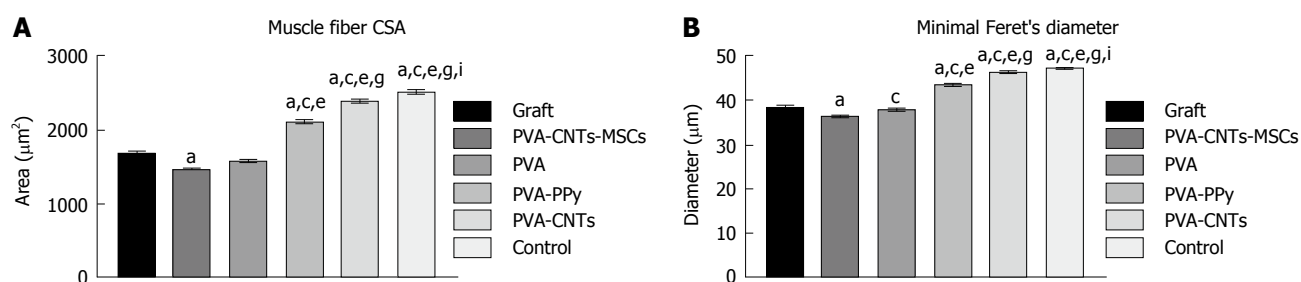


Figure 5 Graphical representation of the mean of area (A) and "minimal Feret's diameter" (B) of control, regenerated tibialis anterior muscle fibers at week-20 after neurotmesis (Graft) and neurotmesis with the proximal and distal nerve stumps sutured to a polyvinyl alcohol (polyvinyl alcohol) tube and polyvinyl alcohol coated tubes (polyvinyl alcohol, polyvinyl alcohol polypyrrole and polyvinyl alcohol carbon nanotubes). Values are presented as mean $\pm$ SEM. ^a $P < 0.05$ vs Graft; ^c $P < 0.05$ vs Graft PVA CNTs MSCs; ^e $P < 0.05$ vs Graft PVA; ^g $P < 0.05$ vs Graft PVA PPy; ⁱ $P < 0.05$ vs Graft PVA CNTs. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells.

minimum of 1000 skeletal muscle fibers was measured from each group. This assessment was performed by 2 independent operators. Each one of the operators measured, blindly and randomly, an average of 50 fibers in each section. Images were acquired using a Nikon® microscope connected to a Nikon® digital camera DXM1200, at low magnification (100 $\times$) under the same conditions that were used to acquire a reference ruler.

At the end of the study, all animals were subjected to a complete necropsy examination in order to evaluate the presence of possible internal anomalies and/or injuries. Lung, heart, kidneys, liver and spleen were collected and weighed and then submitted to histological analysis to check whether there were related microscopic changes such as inflammation, degeneration, or accumulation of biological material. The organs were fixed in 10% buffered formalin and processed for routine histology with HE stain. Microscopically, massive carbon deposits generally appear as well-recognized anthracotic black pigment, especially in the lung. A recent report described that some types of carbon nanotubes may appear as small punctuated accumulations inside Kupfer cells (liver) and in the intermediate zone of the spleen, when intravenously administered^[45]. Special stains, such as Von Kossa (method that demonstrates phosphates and carbonates) and Masson-Fontana (method used for distinguish carbon deposits from melanin) were also performed in consecutive sections.

Statistical analysis

The statistical review of the study was performed by a biomedical statistician [PAS Armada-da-Silva, CIPER-Faculdade de Motricidade Humana (FMH): Centro Interdisciplinar de Estudo de Performance Humana, FMH, Universidade de Lisboa, Estrada da Costa, 1499-002, Cruz Quebrada - Dafundo, Portugal].

A mixed model repeated measures ANOVA was used to test for differences across time and sciatic nerve treatment. Sphericity was assessed by Mauchly's test and Greenhouse-Geisser degrees of freedom correction was used in cases sphericity could not be assumed or when corrected P -values were below the accepted level of significance ($P < 0.05$). Tukey's HSD test was used for pairwise comparisons. All data is presented as mean

and standard deviation, unless otherwise stated. These statistical tests were carried out with IBM SPSS Statistics version 19. For stereology, statistical comparisons of quantitative data were subjected to one-way ANOVA test, followed by pairwise comparisons using Tukey's HSD test. Statistical significance was established as $P < 0.05$. Stereological data was analyzed using the software using the SPSS version 19.0 (SPSS, Chicago, IL). For muscle morphometry, statistical analysis was performed using the SPSS version 19.0 (SPSS, Chicago, IL). Results are presented as mean $\pm$ scanning electron microscope (SEM) in Figures 5 and 6. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey's HSD post hoc test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Development and characterization of MSCs isolated from UC WJ

MSCs are defined by the ISCT which includes the following characteristics: (1) their capacity to adhere to plastic surfaces during cell culture; (2) expression of the specific surface markers (positive for CD73, CD90, and CD105, and no expression of CD14, CD19, CD34, CD45 and HLA-DR); and (3) are able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts^[19]. MSCs isolated from the UCT were expanded to P5-P6 where the culture appeared homogeneous and cells presented their typical fusiform, fibroblast-like, morphology (Figure 1). Flow cytometry analysis performed previously to *in vivo* application to expanded MSCs showed that over 95% of the cells in the population were consistently positive for the cell surface markers CD44, CD73, CD90 and CD105 and less than 2% positive for CD14, CD19, CD31, CD34, CD45 and HLA-DR. Also, the phenotype of MSCs was assessed by PromoCell by flow cytometry analysis for an additional comprehensive panel of markers: PECAM (CD31), HECAM (CD44), CD45, and Endoglin (CD105). Overall, these results are the ones expected for MSC-type stem cells according to ISCT^[19].

Giemsa-stained cells of MSCs at P5 were analyzed

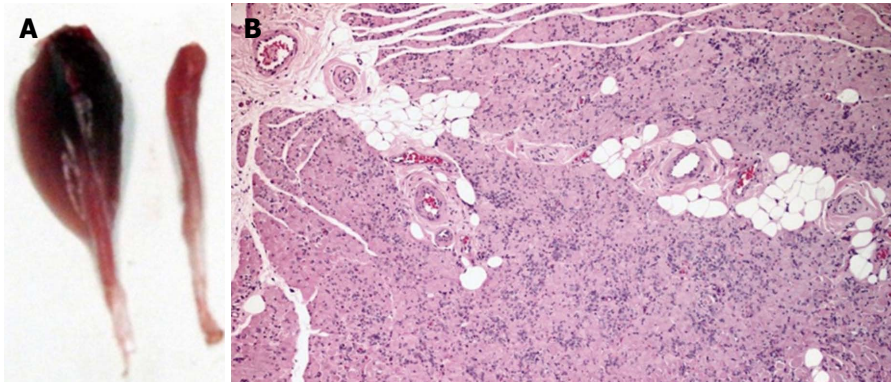


Figure 6 Macroscopic appearance of control tibialis anterior muscle and histological haematoxylin and eosin staining of a transverse section of tibialis anterior muscle. A: Macroscopic appearance of control tibialis anterior (TA) muscle (left) and 20 wk following neurotmesis and surgical treatment with PVA CNTs MSCs (right); B: Histological haematoxylin and eosin staining of a transverse section of TA muscle from PVA CNTs MSCs group. Magnification 40 ×. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; MSCs: Mesenchymal stem cells.

for cytogenetic characterization. The karyotype of MSCs was determined previously to *in vivo* application to ensure that no neoplastic cells were used proving the cell therapy safety. The karyotype analysis demonstrated that no structural alterations were found, as well as chromosomal stability in terms of number and structure of the somatic and sexual chromosomes, to the cell culture procedures. The transplanted MSCs also presented normal morphology and flow cytometry markers for MSCs according to ISCT^[19].

Results obtained from epifluorescence technique confirmed that MSCs did not begin the apoptosis process, showing that the *in vivo* applied MSCs were viable even in the presence of the tube-guides biomaterials. The $[Ca^{2+}]$ measured was 47.9 ± 4.5 ($n = 25$), 46.2 ± 3.5 ($n = 25$) and 48.1 ± 3.9 ($n = 25$) for MSCs cultured in the presence of PVA, PVA-CNTs, and PVA-PPy discs after 7 d of culture, respectively. The MSCs cultured and expanded in the presence of the three tested biomaterials reached confluence and exhibited a normal star-like shape with a flat morphology in culture. According to these results, it is reasonable to conclude that the three biomaterials (PVA, PVA-CNTs, and PVA-PPy) are viable substrate for MSCs culture and survival and may be used in the pre-clinical trials.

The MSCs were harvested and its RNA purified and converted to cDNA using adequate procedures. Primers targeting markers, two housekeeping genes (β -actin and *GAPDH*) and five specific of neuronal cells (GFAP, NeuN, Nestin, NF-H and GAP-43) were used to support the fact that the MSCs used *in vivo* were not differentiated into neuro-glial cells. The molecular analysis showed a very small amplification of GFAP gene, absence of amplification of the NF-H and GAP-43 genes, and reasonable amplification of *NeuN*, β -actin, *GAPDH* and Nestin genes. Amplification of a given gene is correlated with its expression seeing that the template DNA is the one generated from mRNA. According to the results of RT-PCR, the molecular analysis showed a small expression of *GFAP* gene and absence of expression of the NF-H and *GAP-43* genes in the expanded MSCs.

Biomaterials characterization

The electrical conductivity achieved for the different tube-guides (PVA, PVA-CNTs and PVA-PPy) was the following: $1.5 \pm 0.5 \times 10^{-6}$ S/m, $579 \pm 0.6 \times 10^{-6}$ S/m, and $1837.5 \pm 0.7 \times 10^{-6}$ S/m, respectively. Therefore these three tube-guide [simple PVA, PVA loaded with 0.05% (%w/v) of COOH-functionalized CNTs and PPy] compositions were chosen for further characterization and for *in vivo* application in the rat sciatic nerve neurotmesis injury model. The thermal characteristics of simple PVA and loaded PVA materials was examined by differential scanning calorimetry and enthalpy of fusion (ΔH) was calculated, and the percentage of crystallinity was near 7.4% for all analyzed nerve guide tubes. Fourier transform infrared spectroscopy analysis the bands identified for PVA loaded with COOH-functionalized CNTs were similar of the bands detected for simple PVA. For PVA loaded with PPy new bands appeared at 1313/cm (C - N stretching vibration in the ring) and 1170/cm (C - H in-plane deformation). Compared with simple PVA the other nerve guide tubes showed a less intensity of the peaks, especially between 2237 and 2380/cm (unpublished data). Considering the XRD analysis previously performed, the broad peak observed at 20° corresponded to a typical diffraction peak of PVA, and it could be also observed in all tube-guides. Near 26° a broad scattering peak appeared for the tube loaded with PPy, and it was an indication of the presence of PPy as supported in literature^[46]; (unpublished data).

Both PVA and PVA loaded with PPy when analyzed by SEM exhibited similar surface appearance. On the other hand, the PVA loaded with COOH-functionalized CNTs showed a rougher surface as expected due to the presence of CNTs on PVA matrix, with oriented features. This characteristic was determinant to choose this biomaterial to be associated to the MSCs (PVA-CNTs-MSCs group). The Wettability analysis showed a hydrophilic behavior for the three biomaterials used for tube-guide tested, also the three materials showed negative zeta potential being the PVA the most negative

Table 1 Static sciatic functional index

SSI		Time											
		T0	T1	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20
Group 1: PVA (<i>n</i> = 7)	Mean	-3.13	-40.52	-52.76	-69.60	-60.30	-56.59	-63.51	-54.89	-40.25	-44.03	-57.34	-48.23
	SD	24.17	19.25	17.78	9.56	19.69	13.19	8.05	11.96	13.33	9.07	21.81	7.25
Group 2: PVA-CNTs (<i>n</i> = 7)	Mean	12.10	-41.85	-45.81	-68.07	-51.18	-52.22	-49.31	-44.52	-48.73	-37.65	-38.47	-38.99
	SD	34.10	20.82	23.21	17.00	11.41	41.96	21.11	17.62	26.31	20.78	17.97	31.11
Group 3: PVA-PPy (<i>n</i> = 7)	Mean	5.31	-41.40	-50.95	-45.76	-47.40	-34.56	-39.51	-31.52	-32.47	-43.17	-50.00	-36.08 ^b
	SD	17.51	17.52	23.99	29.92	22.53	14.00	25.05	15.10	20.78	25.52	11.77	16.09
Group 4: Graft (<i>n</i> = 4)	Mean	-6.29	-56.46	-52.18	-55.41	-63.81	-59.79	-75.56	-53.15	-64.53	-67.22	-57.95	-52.07
	SD	1.59	28.10	27.54	27.99	19.06	11.00	10.45	15.44	12.14	12.17	8.31	9.76
Group 5: End-to-End (<i>n</i> = 5)	Mean	-5.78	-44.21	-33.72	-58.28	-73.92	-70.22	-52.47	-68.04	-41.91	-46.56	-43.04	-59.97
	SD	12.91	15.34	15.72	22.25	6.82	6.44	27.43	11.02	24.55	16.79	12.31	2.64
Group 6: PVA-CNTs-MSCs (<i>n</i> = 7)	Mean	3.46	-31.48	-23.75	-28.57	-39.97	-40.30	-62.32	-34.71	-26.28	-42.87	-37.54	-36.16 ^b
	SD	12.35	21.89	19.70	9.96	9.20	21.72	18.07	16.44	15.31	25.75	10.06	14.87

Static sciatic functional index (SSI) measured pre-operatively (week-0), and every week after the surgical procedure until week-20. An index score of 0 is considered normal and an index of 100 indicates total impairment. The measurements of the toe spread (TS), and the intermediary TS, were taken from the experimental (E) and normal (N) sides. Results are presented as mean and SD. *n* corresponds to the number of rats within the experimental group. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells. ^b*P* < 0.01 *vs* Graft.

surface (-4.97 mV) (Figure 2).

Functional analysis during the healing period of 20 wk

SFI and SSI analysis: A few SSI data were missing due to inability to collect visible fingerprints as it was previously referred. As a result of the limited number of animals and the limitations of repeated measures analysis, missing SSI values were replaced using interpolation. Nine of a total of 275 SSI values were interpolated with only one animal having two missing values replace using the linear interpolation procedure. Also because of missing values, the 2-wk time point was not considered for statistical analysis. Sciatic nerve neurotmesis caused a steep decrease in SSI scores in every experimental group and therefore a significant effect of time could be found [$F(10, 200) = 20.445$; $P < 0.001$]. SSI scores remained negative and with little changes throughout the time following sciatic nerve neurotmesis and independently from sciatic nerve treatment, leading to a non-significant time *vs* treatment interaction effect [$F(40, 190) = 279.581$; $P < 0.651$]. However, there was significant differences in the extent of SSI scores' decrease between the experimental groups [$F(4, 20) = 5.848$; $P < 0.01$], with PVA-PPy tube-guides ($P < 0.01$) and PVA-CNTs-MSCs ($P < 0.01$) groups showing less severe SSI scores compared with Graft group (Table 1).

The motor performance by SFI was not possible to perform due to autotomy observed in all treated animals.

Evaluation of motor performance (EPT) and nociceptive function (WRL): In the weeks following sciatic nerve neurotmesis there was a large increase in the time latency to withdraw the paw in response to the thermal stimulus, with most animals reaching the cut-off time of 12 s and leading to a significant effect of time [$F(11, 341) = 17.944$; $P < 0.001$]. A mild improvement in latency times occurred over the weeks of recovery

at a rate that was similar between the experimental groups so that no significant interaction effect existed [$F(55, 341) = 5.727$; $P < 0.503$]. According to ANOVA results, differences between treated groups (PVA, PVA-CNTs, PVA-PPy, PVA-CNTs-MSCs, Graft and End-to-End) in the severity of changes in WRL times existed [$F(5, 31) = 2.942$; $P < 0.05$], but these differences were not large enough to be detected by the pairwise comparisons (Table 2).

The motor performance by EPT was not possible to perform due to autotomy observed in all treated animals.

Ankle joint kinematics: Measures of ankle joint angle at the four selected instants of the stance phase (IC, OT, HR and TO) were collected at the end of 20-wk recovery period. This analysis was carried out only for biomaterial (PVA, PVA-CNTs and PVA-PPy groups, *n* = 7) and biomaterial plus MSCs-treated animals (PVA-CNTs-MSCs group, *n* = 7). Ankle joint angle values were similar across the experimental groups at the times of IC, OT and HR (P = non-significant). However, at TO less acute ankle joint angles were seen for PVA-CNTs-MSCs group, compared with PVA-PPy group ($P < 0.01$) and with PVA-CNTs group showing similar trend also compared with PVA-PPy group ($P = 0.051$). Such less acute ankle joint angles might suggest improved ankle muscles function during the push off phase of the rat's gait cycle (Table 3 and Figure 4).

Morphological analysis

Muscle results: After the healing period of 20 wk, TA muscle of all treated rats from the experimental groups (PVA, PVA-CNTs, PVA-PPy, PVA-CNTs-MSCs, Graft and End-to-End) was collect for histological analysis and morphometry to evaluate the secondary neurogenic muscle atrophy associated to neurotmesis injury. It was also evaluated the muscle healing process that followed the sciatic nerve regeneration where the scaffold

Table 2 Withdrawal reflex latency

WRL		Time											
		T0	T1	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20
Group 1: PVA (n = 7)	Mean	3.71	10.95	10.81	10.68	9.74	10.23	9.85	8.15	7.78	9.99	7.88	6.98 ^a
	SD	0.93	1.45	2.04	2.26	2.89	2.50	2.81	2.70	3.50	2.49	3.72	3.72
Group 2: PVA-CNTs (n = 7)	Mean	5.36	9.91	8.98	7.50	8.40	9.48	9.51	6.92	6.60	8.43	5.88	5.83 ^a
	SD	1.50	3.57	3.65	3.36	3.22	3.41	3.01	3.49	3.41	3.40	3.09	2.80
Group 3: PVA-PPy (n = 7)	Mean	4.86	11.42	9.37	7.10	7.85	8.82	9.72	6.87	7.02	7.09	6.06	7.02 ^a
	SD	1.69	1.54	3.31	2.51	3.40	2.53	2.35	3.50	3.92	3.23	4.43	3.89
Group 4: Graft (n = 4)	Mean	4.53	12.00	10.14	11.17	11.50	10.67	10.83	11.16	9.81	10.46	9.92	9.68 ^a
	SD	1.14	0.00	3.72	1.66	1.01	2.65	2.35	1.27	2.84	1.78	1.82	1.88
Group 5: End-to-End (n = 5)	Mean	4.28	12.00	11.85	12.00	10.22	10.45	8.78	10.48	9.26	8.57	9.82	7.50
	SD	0.86	0.00	0.34	0.00	2.50	2.12	2.22	2.86	3.07	0.98	2.02	0.87
Group 6: PVA-CNTs-MSCs (n = 7)	Mean	3.93	12.00	8.89	9.23	9.30	9.90	7.65	7.41	10.06	8.33	8.28	8.02 ^a
	SD	0.86	0.00	2.36	1.63	2.72	2.66	2.51	2.33	1.85	2.86	2.34	1.20

Values in seconds were obtained performing withdrawal reflex latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week-0), at week 1, week 2 and every 2 week after the surgical procedure until week-20, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SD). *n* corresponds to the number of animals within each experimental group. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells. ^a*P* < 0.05 *vs* End-to-End.

Table 3 Ankle kinematics

Temporal events	Group	Week 20
IC	Group 1: PVA	-3.23 ± 20.65
	Group 2: PVA-CNTs	-8.32 ± 17.36
	Group 3: PVA + PPy	0.82 ± 26.33
	Group 6: PVA-MSCs	-13.68 ± 8.57
OT	Group 1: PVA	28.30 ± 19.52
	Group 2: PVA-CNTs	27.67 ± 12.91
	Group 3: PVA + PPy	17.66 ± 13.20
	Group 6: PVA-MSCs	11.43 ± 9.69
HR	Group 1: PVA	46.67 ± 14.81
	Group 2: PVA-CNTs	47.69 ± 11.19
	Group 3: PVA + PPy	36.46 ± 10.14
	Group 6: PVA-MSCs	34.07 ± 6.85
TO	Group 1: PVA	33.99 ± 9.57
	Group 2: PVA-CNTs	27.01 ± 10.99
	Group 3: PVA + PPy	43.90 ± 8.76
	Group 6: PVA-MSCs	20.56 ± 11.31

Values were obtained performing video analysis at 20-wk of the healing period. Results are presented as mean and standard deviation (SD) (*n* = 7). For each step cycle the following time points were identified: Initial contact (IC), opposite toe off (OT), heel rise (HR) and toe-off (TO). PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole.

was applied. It was possible to observe by muscle morphometry that there was a significant difference (*P* < 0.05) in terms of the increase in mean fiber size between some of the treatment (PVA-PPy and PVA-CNTs) groups and the Graft group. In fact, in the PVA-PPy group there was a 25% increase in terms of average fiber area and a 13% increase in terms of the "minimal Feret's diameter", when compared to the Graft group. Whereas in the PVA-CNTs treatment group there was a 42% increase in terms of average fiber area and a 21% increase in term of the "minimal Feret's diameter", when compared to the Graft group. Although both these two treatment groups (PVA-PPy and PVA-CNTs) exhibited significant improvement in fiber size, approaching the values for normal muscles (collected from rats with no sciatic nerve neurotmesis lesion), both of them were

still significantly different (*P* < 0.05) from the control group (without lesion) at 20 wk after lesion. PVA treatment without any coating (PVA group) revealed no regeneration benefit since the mean fiber size was lower (with significant difference in terms of "minimal Feret's diameter"; *P* < 0.05) when compared to the Graft group (Figure 5). However, unexpected results were observed with PVA-CNTs-MSCs, which showed the worst results in terms of muscular atrophy after denervation, results not correlated with functional analysis (considering mostly kinematics gait analysis and SSI) (Figure 5). The decreased fiber size observed in these animals' TA muscles was coincident with the smaller sized muscles that were collected at 20 wk after surgery macroscopically evaluated during the collection for histological evaluation. Histologically it was also possible to detect a considerable amount of necrosis with delayed muscle regeneration in this group's muscles (Figure 6).

Histomorphometry of the regenerated sciatic nerve:

Histological analysis showed that nerve fiber regeneration occurred in all reconstructed nerves. In comparison to controls (sciatic nerve without neurotmesis injuries, data not shown)^[3], in all repaired nerves regenerated fibers showed small axons with thin myelin sheaths and microfasciculation (Figure 7). Microfasciculation was more evident in the PVA-CNTs-MSCs repaired group (Figure 7 and Table 4). Also, stereological analysis disclosed a significantly (*P* < 0.05) larger axon diameter, but not fiber diameter, in the regenerated nerves from PVA-PPy group compared with those from PVA-CNT-MSC group (Table 4). On the other hand, as it was previously reported^[3,25], the application of MSCs-based cell therapy to neurotmesis nerve injuries resulted in a significantly increased myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fiber diameter (d/D; g-ratio) when compared to PVA, PVA-CNTs, PVA-PPy groups (*P* < 0.05). The results clearly demonstrate a

Table 4 Stereological quantitative assessment

Group		Density	Total number	Axon diameter (d)	Fiber diameter (D)	Myelin thickness (M)	M/d	D/d	d/D (g-ratio)	Area (mm ²)
Group 1: PVA (n = 7)	Mean	42872	16596	2.95	3.56	0.31 ^a	0.11 ^a	1.23	0.82 ^a	0.3862
	SD	4007	1741	0.25	0.28	0.03	0.01	0.02	0.01	0.0772
Group 2: PVA-CNTs (n = 7)	Mean	36261	16049	2.98	3.71	0.37 ^a	0.14 ^a	1.28	0.79 ^a	0.4455
	SD	4267	1172	0.25	0.27	0.01	0.01	0.03	0.01	0.1856
Group 3: PVA-PPy (n = 7)	Mean	40289	22588	3.03 ^a	3.70	0.33 ^a	0.12 ^a	1.24	0.81 ^a	0.5720
	SD	2686	3157	0.21	0.23	0.02	0.01	0.01	0.00	0.2565
Group 4: Graft (n = 4)	Mean	37041	21939	2.58	3.56	0.49	0.21	1.41	0.71	0.5936
	SD	1214	1302	0.06	0.08	0.02	0.01	0.02	0.01	0.0694
Group 5: End-to-end (n = 5)	Mean	38762	22729	2.65	3.58	0.47	0.19	1.38	0.73	0.5990
	SD	1524	2308	0.14	0.16	0.01	0.01	0.01	0.01	0.2082
Group 6: PVA-CNTs-MSCs (n = 7)	Mean	43373	25731	2.44	3.34	0.45 ^{c,e}	0.21 ^{c,e}	1.41	0.72 ^{c,e}	0.5942
	SD	3881	3386	0.20	0.21	0.06	0.04	0.08	0.01	0.1940

Density, total number, axon diameter (d), fiber diameter (D), myelin thickness (M), ratio myelin thickness and axon diameter (M/d), ratio fiber diameter and axon diameter (D/d), ratio axon diameter and fiber diameter (d/D, g-ratio) of regenerated sciatic nerve fibers at week-20 after neurotmesis. Values are presented as mean ± SD. *n* corresponds to the number of animals within each experimental group. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole. ^a*P* < 0.05 vs PVA-CNT-MSC; ^c*P* > 0.05 vs End-to-End; ^e*P* > 0.05 vs Graft.

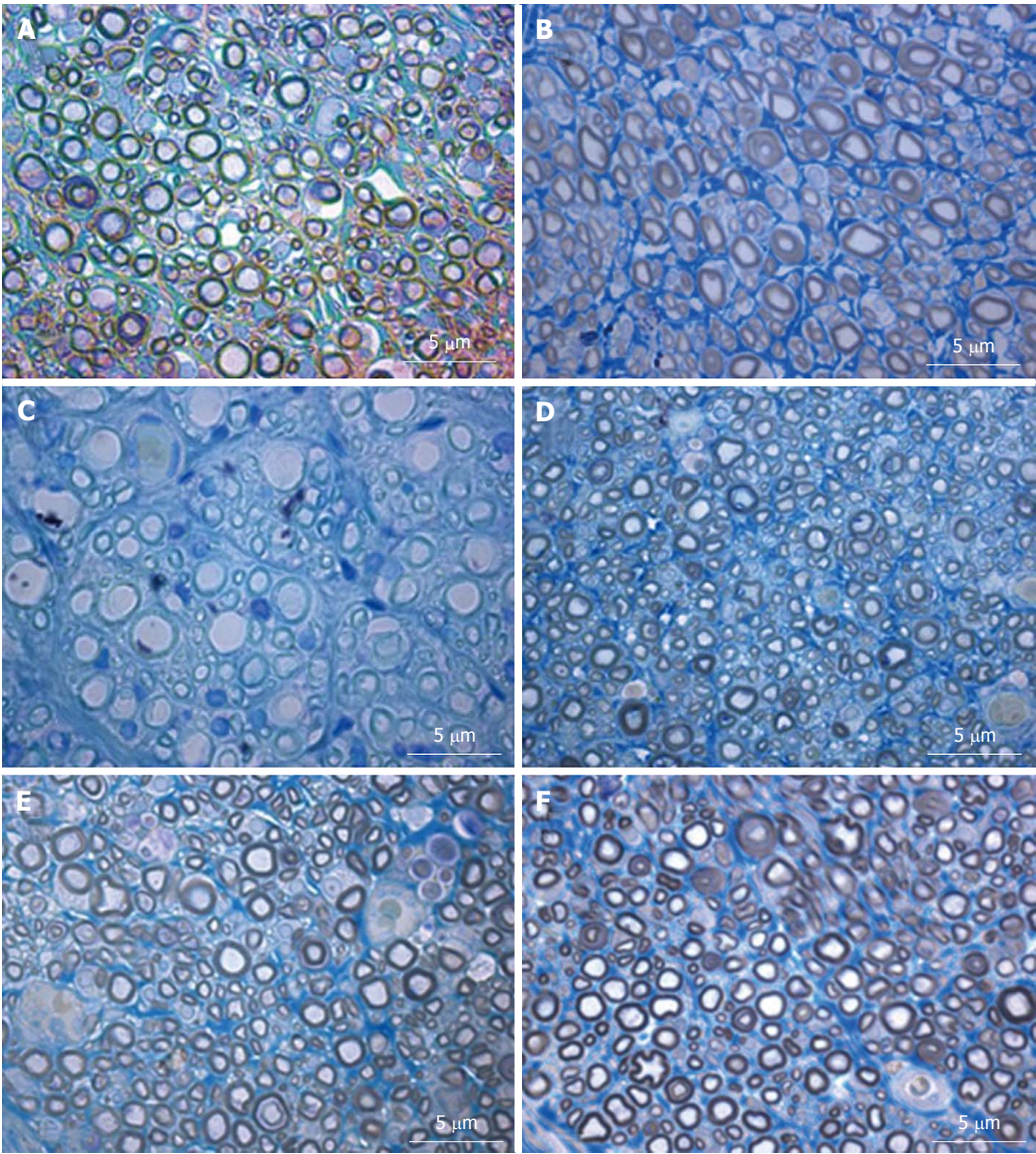


Figure 7 Histological appearance of regenerated nerve fiber in the different groups of neurotmesis. A: PVA; B: PVA-CNTs; C: PVA-PPy; D: PVA-CNTs-MSCs; E: Graft; F: End-to-end suture (magnification: 1000 ×). PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells.

synergistic positive effect on regenerated nerve fibers resulting from combined use of MSCs with the PVA-CNTs tube-guides. Also, in the PVA-CNTs-MSCs group these three stereological parameters (M, M/d, and g-ratio) were not significantly different from the values obtained from End-to-End and Graft groups (Table 4). The total number of nerve fibers and nerve fibers density were similar in all groups.

Histology of internal organs: At the microscopic exam, no alterations were observed. The histology sections of all organs confirmed the absence of inflammation, cell degeneration, necrosis and fibrosis. No signs of nanotubes, neither carbon deposits were detected in all organs.

DISCUSSION

An organ re-innervation and functional recovery after nerve injury has been intensively studied worldwide and regenerative medicine methods including cellular systems and new biomaterials concerning the peripheral nerve have been developed. The involved mechanisms in the regeneration process and the ideal scaffold have not been discovered yet. Peripheral nerve might regenerate after some types of injury like axonotmesis but it is difficult to achieve functional recovery after neurotmesis, especially when there is loss of nerve tissue and a gap is created. In the peripheral nervous system, nerves after axonotmesis injuries can regenerate, without any treatment but in most clinical cases a muscle regional neurogenic atrophy occurs. When neurotmesis injuries occur, the nerves must be surgically treated by direct end-to-end suture^[33,47,48], using appropriate microsurgery techniques and suturing material. Nowadays most tissue engineered nerve grafts are composed of a neural scaffold prepared with a variety of biomaterials, and surgically applied in neurotmesis injuries with loss of nervous tissue where the direct end-to-end suture is not possible creating tension in the suture line and compromising the nerve regeneration^[9]. The introduction of cell therapies should be delivered by appropriate vehicles promoting an important biochemical and physical cue. MSCs represent an appealing source of adult stem cells for cell therapy and tissue engineering including peripheral nerve. Because MSCs are present in low percentage in the adult BM and also because of some of the disadvantages previously listed, alternative sources have been studied, like the UCT (also called Wharton's jelly). MSCs were defined by the ISCT in 2006, and since that time it is considered that MSCs must adhere to plastic; have a specific profile for the specific surface markers (positive for CD73, CD90, and CD105, and negative for CD14, CD19, CD34, CD45 and HLA-DR). Other important characteristic is that MSCs are also able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts^[19]. *In vivo* and *in vitro* studies referred that MSCs have the capacity to induce the regeneration of cartilage, bone,

tendon, and meniscus^[18]. The MSCs are also able to differentiate into neuro-glial cells, which have been studied *in vitro* and *in vivo* using animal models, by our research group^[25]. Children and adult clinical trials have been performed where MSCs were used to treat a wide range of pathologies, including neurological disorders, PNS and CNS injuries (access www.clinicaltrials.gov). In fact, nowadays, the cryopreservation of UCB, and UCT is performed worldwide in private and public cord blood banks. Furthermore, the MSCs from the UCT and hematopoietic stem cells from the UCB have positive clinical outcomes not only in hematologic malignancy patients, where the MSCs work as a co-adjuvant for the hematopoietic transplant success, but also in PNS injuries and neurologic disorders. For that reason, the crucial role of MSCs in tissue renewal and regeneration has been well established for several limiting pathologies, not treated by the traditional therapeutic approaches^[49]. The *in vivo* application of MSCs performed by the authors was intended to improve the regeneration process in the rat sciatic nerve after a neurotmesis injury which was surgically reconstructed using electric conductive tube-guides of PVA-CNTs composition. At that moment there are several nerve tube-guides in the market, however they still have limitations and the functional outcome of the patients is still not complete. In this work the previously developed tube-guides with high electrical conductivity for nerve regeneration (unpublished data) were used *in vivo* using the rat sciatic nerve neurotmesis injury model. A matrix of PVA was used loaded with the following electrical conductive materials: COOH-functionalized multiwall carbon nanotubes (MWCNTs) and PPy PVA-CNTs and PVA-PPy tube, guides, respectively. The tubes production was carried out by a freezing/thawing process (physical cross-linking) and a final annealing treatment. After producing the tube-guides, the physicochemical characterization was performed. The most interesting results were achieved by loading PVA with 0.05% of PPy or COOH-functionalized CNTs, by combining the electrical conductivity of CNTs and PPy with the biocompatibility of PVA matrix, which seems to have potential to be used in peripheral nerve regeneration. For that reason, these two biomaterials were chosen to proceed for *in vivo* pre-clinical trials using the rat sciatic model. As a matter of fact, the microscopic exam performed to lung, heart, kidneys, liver and spleen collected after animal euthanasia, confirmed the absence of inflammation, cell degeneration, necrosis and fibrosis, and no signs of nanotubes, neither carbon deposits were detected in all organs, confirming the biocompatibility of these biomaterials loaded with CNTs. The evaluation of cytocompatibility was performed by measuring the $[Ca^{2+}]_i$ in Fura-2-loaded MSCs cells by using dual wavelength spectrofluorometry, sub-cultured over PVA discs, over PVA loaded with carbon nanotubes (PVA-CNTs) and PVA loaded with PPy (PVA-PPy) discs of 10 mm diameter, as previously described^[3,25,27]. Results correspond to $[Ca^{2+}]_i$ from viable MSCs that did not begin the apoptosis

process, confirming the MSCs ability to expand and viability when associated to PVA, PVA-PPy and PVA-CNTs tube-guides.

The MSCs isolated from the WJ used as cell-based therapy for promoting nerve regeneration when associated to electric conductive tube-guides was previously *in vitro* validated concerning flow cytometry profile, RT-PCR, and cytogenetic analysis. MSCs were expanded to P5-P6 where the culture appeared homogeneous and cells presented their typical fusiform, fibroblast-like, morphology (Figure 1). Also, the flow cytometry analysis confirmed their profile according to ISCT^[19] and the karyotype demonstrated that no structural alterations were present demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures. RT-PCR and qPCR targeting specific genes expressed by the MSCs *in vivo* applied was performed to certify that the MSCs used *in vivo* were not differentiated into neuro-glial cells. For the MSCs, the molecular analysis showed a very small amplification of *GFAP* gene, absence of amplification of the *NF-H* and *GAP-43* genes, and reasonable amplification of *NeuN*, β -actin, *GAPDH* and *Nestin* genes, confirming that MSCs used *in vivo* were undifferentiated and not neuro-glial differentiated. In fact, the small detection of the *GFAP* gene expression may be due to the high sensitivity of the molecular tests in comparison with immunocytochemistry tests previously performed using undifferentiated and neuro-glial type differentiated MSCs^[3,18]. Moreover, the expression of the remaining genes, *NeuN*, β -actin, *GAPDH* and *Nestin* was also observed in undifferentiated MSCs. The expression of the housekeeping genes, β -actin and *GAPDH*, is expected to occur. As per the *NeuN* and *Nestin* gene, the observation of its expression in undifferentiated MSCs is not new; Bertani *et al.*^[50] showed that naïve MSCs express at a constitutive level *NeuN* gene, which increases when these cells are chemically induced to differentiate to pre-neuronal cells. Furthermore, compared gene expression profiles before and after MSCs induction for a number of germ layers, and observed that even before neuronal induction, MSCs population and clonal lines expressed a mixture of mesodermal, germinal, endodermal and ectodermal genes, including several whose expression was thought to be restricted to neuronal cells^[51]. Molecular analysis previously performed^[18] on these same genetic markers over the differentiated MSCs showed an increase in the expression of *GFAP*, *NF-H* and *GAP-43* genes. These genes were not expressed, or expressed at very low levels, in the undifferentiated MSCs transcriptome. Overall, these results support the fact that the MSCs used *in vivo* were not differentiated but presented a neuro-glial potential for differentiation confirming previous results where the same MSCs undifferentiated and *in vitro* differentiated into neuro-glial cells were tested in axonotmesis and neurotmesis injuries associated to other biomaterials namely poly(DL-lactide- ϵ -caprolactone), hybrid chitosan and collagen^[3,25,31].

The rat sciatic nerve model has been widely used in experiments on peripheral nerve regeneration and it has been demonstrated that it is reliable and reproducible model, but research on peripheral nerve injury needs to include both functional and morphological analysis^[52]. Since it is not generally agreed which type of evaluation tool is the most useful for evaluation of functional recovery; our research group like others has been using different methods for an overall assessment of nerve function, which has been widely recommended^[53]. The sciatic nerve regeneration after neurotmesis studies described by the authors were followed during 20 wk after the surgical procedure based on the previous experimental work^[17,29,54,55]. The morphological evaluation together with functional data has been used to assess neural regeneration after induced neurotmesis injuries, but some subjective evaluation, depending on the operator/research analysis is observed^[9]. Some methods for evaluation of nerve recovery, like peroxidase and retrograde fluorescent labeling, histomorphometry, and retrograde transport of horseradish^[47,56] fail in assessing the functional recovery, which is essential to evaluate the success of a scaffold application^[57,58]. The present experimental work includes a variety of independent evaluation tools considering the morphologic and functional recovery, in order to understand and estimate the potential therapeutic benefit of a nerve repair strategy^[9]. EPT, WRL, SSI and SFI, have been proven to be valid methods and to give some information to determine functional recovery following sciatic nerve injury, including motor and nociceptive evaluation^[59]. The use of biomechanical techniques and rat's gait kinematic evaluation was a progress in documenting functional recovery, largely published by our research group^[3,9,17,31,33,35]. Indeed, the use of biomechanical parameters allows an accurate analysis of the sciatic denervation/reinnervation process, permitting to understand the integration of the neural control acting on the ankle and foot muscles, and thus allowing to evaluate the nerve and muscle regeneration after neurogenic muscle atrophy associated to neurotmesis injuries^[60,61]. At the end of the 20-wk healing period, it was performed kinematic gait analysis of rats from the groups where it was applied the biomaterial alone or associated to MSCs-based therapies (PVA, PVA-CNTs, PVA-PPy, and PVA-CNTs-MSCs groups) and measures of ankle joint angle at the four selected instants of the stance phase (IC, OT, HR and TO) were obtained (Table 3 and Figure 4). Considering the histological analysis, it was performed stereology of the regenerated sciatic nerves and morphometry analysis of the TA muscles, trying in this way, not only correlate the neurotmesis injury and the secondary neurogenic regional atrophy, but also consider and validate the morphometry of regional muscles (for instance, by biopsy of the injured muscle) as a method to assess functional recovery after peripheral nerve injuries^[18,25]. The restoration of locomotor activity following damage of the PNS has emerged as one of the most important and critical clinical problem in

neuroscience and neurosurgery fields. Regional muscle weakness and impairment of joint control and mobility occurs in many patients with peripheral nerve or spinal cord injuries which results in gait disorders only evaluated with accuracy by kinematics analysis. During the past 10-15 years, experimental work has been carried out on rat gait analysis which may significantly contribute in the future for a more precise peripheral nerve research. Indeed, the use of biomechanical parameters permits an integration of the neural control acting on the ankle and foot muscles, relevant for the analysis of the sciatic denervation/reinnervation effects. The biomechanical analysis is very useful and accurate to evaluate different therapeutic approaches, describing high number of kinematic variables including positions, velocities, and accelerations, often using high speed digital cameras^[35,40,62,63]. There is high variability between individual joint kinematics and between different animals from the same the same experimental group^[64]. The high level of variability observed in normal quadruped walking, affects significantly the precision of joint kinematic measures of functional recovery after nerve injury. It is important to reduce this variability in kinematics analysis to assess functional recovery after neurogenic muscle atrophy and neurotmesis/axonotmesis injuries. One evident advantage is that using a treadmill the operator reduces step-by-step variability in joint kinematics^[59] which has the disadvantage of being expensive equipment. Also the possibility of combining kinematic analysis with other data, such as ground reaction forces, should be consider for a more accurate evaluation of nerve regeneration^[59,64,65]. Our research group has recently analyzed hip, knee and ankle joint kinematics during recovery of rat sciatic nerve axonotmesis injury, using reflective markers attached to the rat hind-limb to track the motion with infra-red capture cameras, to better assess the rat sciatic nerve model hind-limb joint kinematics during walking. Due to physiological constraints and muscle actions it was observed that different joints have different motion patterns within motion planes, more evident when a 3D segmental kinematic analysis using a 3D reconstruction of the rat hind-limb was performed^[9]. This method allowed a more complete segmental kinematic analysis using both planar angles computation (2D) and a 3D reconstruction of the rat hind-limb but unfortunately, in the present experimental work it was only possible to perform a 2D gait analysis^[9]. In the present work, 2D biomechanical analysis (sagittal plan) was carried out applying a two-segment model of the ankle joint, adopted from the model firstly developed by Varejão *et al.*^[40]. For that reason, ankle joint angle values were similar across the experimental groups at the times of IC, OT and HR. However, less acute ankle joint angles observed in PVA-CNTs-MSCs group, compared with PVA-PPy and PVA-CNTs groups might suggest improved ankle muscles function during the push off phase of the rat's gait cycle (Figure 4 and Table 3). It is well known and demonstrated by the scientific bibliography that neuromuscular pathologies

are related to important clinical signs or motor deficits that should be observed, qualified, and quantified, only possible with a precise kinematic analysis^[65,66]. In the field of peripheral nerve research using the rat sciatic nerve model, an improved walking analysis might include several methods combination like joint kinematics, ground reaction forces and electromyographical data of muscle activity. These methods refinements might be important to differentiate the regenerative potential of different scaffolds used, that are not evident when using the traditional standardized methods previously referred^[9,66].

The recent published paper by di Summa *et al.*^[67] where collagen tube-guides (Neurogen®) were used *in vivo* to promote peripheral nerve regeneration, combined with schwann cells (SCs), it was possible to demonstrate an improved distal stump sprouting^[68]. This sprouting was more pronounced in the experimental group where the SCs were derived from BM-MSCs when compared to SCs derived from adipose tissue MSCs (AT-MSCs). On the other hand, no significant differences were observed in proximal regeneration among all the experimental groups. BM-MSCs and AT-MSCs -loaded conduits induced a diffuse sprouting pattern of the axons. On the other hand, the tube-guides loaded with SCs induced an enhanced cone pattern and a typical sprouting along the collagen type I conduits walls, showing improved regenerating results. This observation is important and should be related to results obtained from the innervated muscle morphometry analysis. It should be bear in mind that the sprouting is also evaluated and a constant observation in the histomorphometry analysis of the regenerated peripheral nerve after axonotmesis and neurotmesis lesions, where different reconstruction strategies were tested *in vivo* in the rat model by our research group for the past years^[3,25,31,68].

The morphometry and histological analysis of TA muscles collected from rats where the neurotmesis injury was reconstructed with PVA-CNTs-MSCs tube-guides is in fact somehow surprising since in the treatment groups presented in a previous published study (Gärtner *et al.*^[3], 2014), no adverse effect was noticed when MSCs isolated from UC WJ were used. The TA muscles from PVA-CNTs-MSCs group presented the worst results in terms of muscular atrophy after denervation, results not correlated with functional analysis (considering mostly kinematics gait analysis and SSI) (Figure 5). The decreased fiber size observed in these TA muscles was coincident with the smaller sized muscles that were collected at 20 wk, macroscopically evaluated during the collection for histological evaluation. Histologically it was also possible to detect a considerable amount of necrosis with delayed muscle regeneration in this group's TA muscles (Figure 6). Some toxic metabolite resulting from the interaction between the vehicle where the MSCs are suspended (PBS) which has calcium and magnesium, and PVA-CNTs tube-guide, could explain the histological results. These results may occur, not because there is a deleterious effect of the cellular

system, but because the vehicle is not appropriate to the cells microenvironment. As a matter of fact these MSCs were previously tested associated to Floseal® and some local nerve necrosis could be observed by histological analysis. On the other hand, the myelin sheath of the MSCs-treated regenerated nerves was thicker, suggesting that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and consequent axonal regeneration^[3]. From the data obtained from the stereology analysis of PVA, PVA-CNTs, PVA-PPy, Graft, End-to-End and PVA-CNTs-MSCs groups, it was also demonstrated a synergistic positive effect on regenerated nerve fibers resulting from combined use of MSCs with the PVA-CNTs tube-guides, where PVA-CNTs-MSCs presented higher myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fiber diameter (d/D; g-ratio) when compared to PVA, PVA-CNTs, PVA-PPy groups (Table 4). Also, these three stereologic parameters (M, M7d, and g-ratio) measured in PVA-CNTs-MSCs regenerated nerves, were not significantly different from the values obtained from End-to-End and Graft groups. In conclusion, as it was previously reported^[3,25], in the MSCs-based cell therapy applied to neurotmesis nerve injuries, it is observed a significantly higher myelin thickness, and similar to Graft and End-to-End regenerated nerves, which are considered the Gold Standards in nerve neurosurgery. These results clearly are consisted with functional data, from SSI and kinematic gait analysis. The PVA-CNTs and PVA-PPy groups presented significantly higher axon diameters (d) and fiber diameters (D), suggesting also the positive effect of these electric conductive materials on axon regeneration and reestablishment of the neuro-muscular junction in agreement with the TA muscle morphometry analysis.

In conclusion, the results revealed that treatment with MSCs associated to PVA-CNTs tube-guides induced an increased number of regenerated fibers and thickening of the myelin sheet. Functional and kinematics analysis has revealed positive synergistic effects brought by MSCs and PVA-CNTs. The PVA-CNTs and PVA-PPy are promising scaffolds with electric conductive properties, bio and cytocompatible, that might prevent in some degree the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction. Since the present studies were carried out in rats with human MSCs, it is important to continue these studies to evaluate the therapeutic potential of these MSCs in human nerve injuries. However, the results discussed herein show a promising effect of MSCs and PVA-CNTs tube-guides in neurodegenerative diseases that are typified by demyelination since this scaffold induced myelin production in surgically reconstructed nerves after a neurotmesis injury.

COMMENTS

Background

The sought for effective new therapeutic strategies for improving peripheral

nerve regeneration represents one of the hot topics in biomedicine because of the high number of lesions affecting peripheral nerves (much higher than lesions to the spinal cord).

Research frontiers

In this study, the authors have tested *in vitro* and *in vivo* mesenchymal stem cells (MSCs) derived from the umbilical cord matrix [Wharton's jelly (WJ)] associated to electric conductive tube-guides developed by their research group, focusing on its effect in promoting nerve regeneration in the rat neurotmesis model. In particular, *in vivo* assessment of nerve regeneration and functional recovery was carried out using a comprehensive battery of complementary methods of analysis that the authors have developed along their previous experience in the study of nerve repair and regeneration and which include behavioral analysis of motor and nociceptive function, kinematic analysis using high speed cameras and histological analysis of nerve fiber regeneration.

Innovations and breakthroughs

The results of this study open interesting perspectives for the clinical application of biodegradable membranes in peripheral nerve reconstruction associated to MSCs isolated from the umbilical cord matrix. Interestingly, these cells, which are major histocompatibility complex class II negative, not only express both an immune-privileged and immune-modulatory phenotype, but their major histocompatibility complex class I expression levels can also be manipulated, making them a potential cell source for MSC-based therapies. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses.

Applications

The results revealed that polyvinyl alcohol (PVA)-carbon nanotubes (CNTs) and PVA-polypyrrole (PPy) are promising scaffolds with electric conductive properties, bio and cytocompatible, that might prevent in some degree the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction. Also, a promising effect of MSCs and PVA-CNTs tube-guides in promoting myelin production in surgically reconstructed nerves after a neurotmesis injury was demonstrated. A new gateway it therefore opened for using these cells in neurodegenerative diseases that are typified by demyelination.

Terminology

Neurotmesis (in Greek tmesis signifies "to cut") is part of Seddon's classification used to classify nerve damage. It is the most serious nerve injury. In this type of injury, both the nerve and the nerve sheath are disrupted. Nowadays, when a nerve is damaged and there is a neurotmesis injury with a gap, it can be repaired using a nerve tube-guide, whenever it is not possible to perform an end-to-end suture without tension or there isn't a graft available. This work focused on the development of electrical conductive nerve tube-guides, which was achieved by loading PVA with PPy or COOH-functionalized CNTs and their *in vivo* application in the rat sciatic nerve neurotmesis injury model. The inclusion of CNTs and PPy brought a significant increase of electrical conductivity of the simple PVA tube-guide. MSCs as defined by the international society for cellular therapy in 2006, are cells characterized by: (1) their capacity to adhere to plastic; and (2) expression of specific surface markers, namely, CD73, CD90, and CD105, and no expression of CD14, CD19, CD34, CD45 and human leukocyte antigen-DR. Additionally, MSCs are able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts.

Peer-review

The article is very interesting. The experimental part is very well designed.

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Mesenchymal stem cells in maxillary sinus augmentation: A systematic review with meta-analysis

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Abstract

AIM: To investigate the effectiveness of mesenchymal stem cells (MSCs) in maxillary sinus augmentation (MSA), with various scaffold materials.

METHODS: MEDLINE, EMBASE and SCOPUS were searched using keywords such as sinus graft, MSA, maxillary sinus lift, sinus floor elevation, MSC and cell-based, in different combinations. The searches included full text articles written in English, published over a 10-year period (2004-2014). Inclusion criteria were clinical/radiographic and histologic/ histomorphometric studies in humans and animals, on the use of MSCs in MSA. Meta-analysis was performed only for experimental studies (randomized controlled trials and controlled trials) involving MSA, with an outcome measurement of histologic evaluation with histomorphometric analysis reported. Mean and standard deviation values of newly formed bone from each study were used, and weighted mean values were assessed to account for the difference in the number of subjects among the different studies. To compare the results between the test and the control groups, the differences of regenerated bone in mean and 95% confidence intervals were calculated.

RESULTS: Thirty-nine studies (18 animal studies and 21 human studies) published over a 10-year period (between 2004 and 2014) were considered to be eligible for inclusion in the present literature review. These studies demonstrated considerable variation with respect to study type, study design, follow-up, and results. Meta-analysis was performed on 9 studies (7 animal studies and 2 human studies). The weighted mean difference estimate from a random-effect model was 9.5% (95%CI: 3.6%-15.4%), suggesting a positive effect of stem cells on bone regeneration. Heterogeneity was measured by the I^2 index. The formal test confirmed the presence of substantial heterogeneity ($I^2 = 83\%$, $P < 0.0001$). In attempt to explain the substantial heterogeneity observed, we considered a meta-regression model with publication year, support type (animal vs humans) and

follow-up length (8 or 12 wk) as covariates. After adding publication year, support type and follow-up length to the meta-regression model, heterogeneity was no longer significant ($I^2 = 33\%$, $P = 0.25$).

CONCLUSION: Several studies have demonstrated the potential for cell-based approaches in MSA; further clinical trials are needed to confirm these results.

Key words: Mesenchymal stem cells; Maxillary sinus; Sinus floor augmentation; Scaffolds; Bone regeneration

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Core tip: Cell-based approaches, utilizing adult mesenchymal stem cells, may overcome the limitations of conventional bone augmentation procedures. The present review of the current literature aims to systematically review the available evidence on the characteristics and clinical effectiveness of cell-based maxillary sinus augmentation, compared to current evidence-based methods.

Mangano FG, Colombo M, Veronesi G, Caprioglio A, Mangano C. Mesenchymal stem cells in maxillary sinus augmentation: A systematic review with meta-analysis. *World J Stem Cells* 2015; 7(6): 976-991 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i6/976.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i6.976>

INTRODUCTION

Implant dentistry is a successful treatment procedure, as demonstrated by more than 20 years of clinical evidence^[1-3].

However, the edentulous posterior maxilla is often characterized by a lack of bone because of severe post-extraction alveolar crest resorption coupled with age-linked sinus pneumatization^[4,5]. This anatomic limitation often dictates the need for reconstructive osseous surgery to re-establish adequate bone volume for implant positioning^[4,5].

Accordingly, different augmentation approaches have been introduced to obtain more maxillary bone volume, placing various grafting materials in the maxillary sinus^[6-9]. Nowadays maxillary sinus augmentation (MSA) has become a reliable, commonly used procedure to increase bone volume in the posterior maxilla^[4,5,9,10].

Autogenous bone (AB) is still the best grafting material in bone reconstructive surgery, and MSA has been originally carried out with it^[10,11]. In fact, AB exhibits osteogenic and osteoconductive potential, since it contains living cells and growth factors^[10-12]. However, additional surgical procedures are needed to harvest bone chips from other skeletal sites (intraorally or from the iliac crest); the available AB supply is limited, and morbidity at donor site is often a problem^[11,12]. In

addition, AB shows resorption patterns proportional to the quantity of harvested material, and this may result in a significant loss of grafted bone in large defect fillings over time^[11,12].

To overcome these limitations, several osteoconductive materials have been used in MSA, such as autogenous bone [allografts (AL)]^[13,14], xenografts [bovine bone mineral (BBM)]^[9,15,16], synthetic bone grafts [calcium phosphate ceramics (CPC)]^[4,17] or composite materials^[18]. However, these bone grafting substitutes don't contain living cells, so their healing times are longer than AB^[12,19].

Bone tissue engineering (BTE) procedures may help to overcome these limits^[20]. According to BTE, a bone substitute should have biological and morphological features as similar as possible to AB, and the fabrication of the ideal bone graft requires the manipulation of three essential components: osteogenic cells, growth factors and osteoconductive scaffolds^[20,21]. In particular, BTE aims to achieve bone augmentation without surgical AB harvesting from other donor sites, through the use of specific scaffolds seeded with osteogenic cells^[12,19,21].

The aim of the present review was to evaluate the effectiveness of cell-based approaches in MSA, associated with various scaffold materials, in animals and humans.

MATERIALS AND METHODS

Study design

The protocol of this review is in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses^[22], the Cochrane Collaboration^[23] and CheckReview^[24] checklists. It was developed a priori, covering rationale, design of the study, focused question, inclusion/exclusion criteria, search strategy, data synthesis. The protocol was examined and refereed by researchers with experience in systematic reviews. The focused questions was: "What is the effectiveness of cell-based approaches in MSA, with different materials?"

Inclusion and exclusion criteria

Most important study designs to address the focused question were randomized controlled trials (RCTs) and controlled trials. Although this, both experimental and observational studies (RCTs, controlled trials, case series, case reports and prospective cohort trials) were included in this review. Inclusion criteria were studies of BTE methods in MSA using different scaffolds, in animals and humans, with analysis using either radiographic or histologic/histomorphometric approaches. Exclusion criteria were studies where there was no information about the surgical team or the location (private practice/hospital/university) where MSA was performed.

Search strategy

Systematic searches were performed in MEDLINE, EMBASE and SCOPUS databases including full text articles published in English between October 2004 and October 2014, presenting either radiographic or

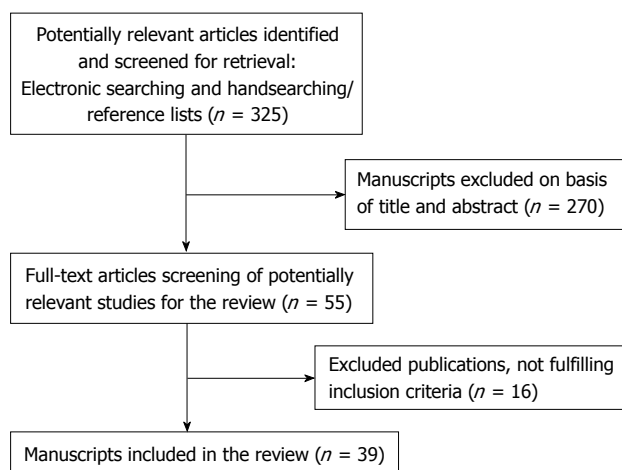


Figure 1 Flow chart of manuscripts screened through the review process.

histologic/histomorphometric evaluations.

Keywords such as sinus graft, MSA, maxillary sinus lift, sinus floor elevation, mesenchymal stem cell (MSC) and cell-based, in different combinations, were used. Titles and abstracts were examined and then expert reviewers (Mangano FG and Colombo M) evaluated full text of publications extrapolating relevant information. Data extraction procedures affected title, authors, year of publication, type of study, cells and scaffold used, design, randomization/blinding if present, number of procedures performed, treatment phase, follow-up, radiographic and/ or histologic/histomorphometric outcomes, statistical findings, conclusions. In addition, the reference lists of included studies were hand searched.

Statistical analysis

Experimental studies (RCTs/CTs) on MSA with an outcome measurement of histologic/histomorphometric evaluation were subjected to meta-analysis. Mean and standard deviation values of new bone formation were used; to overcome the differences of sample size between studies weighted mean was calculated. Test and the control groups values were compared by calculating the differences of bone gain in mean, and 95% CIs. Software package R version 2.14 (Package Metafor; Wolfgang Viechtbauer, Maastricht, The Netherlands) was used to perform all statistical analyses.

RESULTS

Result of search and included studies

Of the 325 eligible articles initially identified, 270 were excluded following assessment of the title and/or abstract. In total, 39 studies (18 animal studies and 21 human studies) were eligible for inclusion in the present literature review (Figure 1). All these publications were issued between 2004 and 2014, and were variable with respect to study type, design, follow-up, and results.

MSCs

A stem cell is an undifferentiated cell with the capability

to renew itself and to get a specific cell-phenotype if exposed to proper stimuli^[25-28]. The more relevant stem cells types in clinical researches are embryonic stem cells (ESCs), typical of embryonic blastocyst, and adult stem cells, also defined as pluripotent MSCs^[28-31].

ESCs have unlimited proliferation potential and are able, under appropriate inductive conditions, to produce all three germ layers cellular phenotypes^[28-31]. ESCs can be cultured indefinitely *in vitro* for more than two years, with approximately 400 doubling cycles, without the loss of differentiation potential. They can be re-implanted into a host embryo giving rise to progenies that differentiate into all kind of tissues^[29-31]. Although their clinical potentials several issues remain to be addressed with ESCs^[30,31]. The use of these cells, in fact, presents the potential risks of immunorejection or teratogenesis^[30]. Moreover, despite the pluripotency of ESCs, moral and legal controversies concerning their use for therapeutic and clinical application have encouraged to find the reservoirs of progenitor cells in adult tissues^[28,32,33].

Adult stem cells or pluripotent MSCs, derived from different adult tissues, have a wide self-renewal and proliferation capability, whereas if correctly stimulated have the ability to differentiate into specific cell-lines^[25-31]. Although MSCs display a finite life span and get into senescence faster than ESCs, current *in vitro* techniques allow to expand them in sufficient number for clinical uses maintaining the undifferentiated phenotype^[25-31]. MSCs lack immunogenic or tumorigenic features^[26-28]; moreover, there is no ethical or legal concern for the clinical use of MSCs^[32]. For all these reasons, these cells can be used in cell-based approaches in bone regeneration^[25-31,33].

MSCs can be extracted from different tissues such as bone marrow [bone marrow stem cells (BMSCs)]^[25-31,34], periosteum (periosteal derived stem cells)^[35], trabecular bone^[36], adipose tissue [adipose stem cells (ADSCs)]^[37] or skeletal muscle^[38], umbilical cord^[39], amniotic fluid [amniotic fluid stem cells (AFSCs), and amniotic epithelial stem cells (AESC)]^[40,41], skin [skin-derived stem cells (SDSCs)]^[42], dental pulp (dental pulp stem cells)^[43,44], deciduous teeth [deciduous tooth stem cells (DTSCs)]^[45] and periodontal ligament [periodontal ligament stem cells (PDLSCs)]^[46].

The bone marrow aspirates (BMA), from the iliac crest of the pelvis, has always been considered the first source for MSCs^[25-34]. The tibial and femoral marrow compartments are also available as alternative sources. Due to the morbidity and the operative difficulties of this procedure the possibility to harvest MSCs from other tissues, such as periosteum or maxillary tuberosity has become of interest^[35,36]. At present, MSCs can be also achieved from adipose tissue by liposuction^[37] or from the dental pulp^[43,44]. The latter represents a very interesting option in the field of oral surgery^[43,44].

As the number and the concentration of transplanted MSCs are critical to induce a significant clinical outcome, an adequate number of cells for cell culture/replication is

needed. BMA represent a heterogeneous cell population; the amount of MSC is very small compared to that of hematopoietic cells and averaging 0.001%-0.01% of the total nucleated cells^[47], thus requiring extensive *in vitro* separation steps and expansion. Moreover, the number of MSCs that can be collected is inversely correlated to patient age and to his/her systemic health state. Younger donors tend to provide higher yield of stem cells in the aspirate, and the age of the donor seems to be directly associated with detrimental effects in term of proliferation and differentiation, such as senescence^[47]. Cell density varies with different skeletal sites of the donor: on average, human BMA yield 400 to 500 cells/mL with an estimated total volume of 600 cc in the iliac crest^[48].

At present, using standard cell culture techniques, MSCs can be isolated and expanded with great efficiency, inducing to grow into multiple lineages if exposed to the appropriate culture conditions^[25]. For *in vitro* separation and expansion, cultured MSCs undergo successive passages. Cells are cultured in a medium supplemented with autogenous serum or fetal bovine serum and growth factors^[49]. MSCs have fibroblastic morphological features in a monolayer culture and tend to adhere to the tissue culture substrate^[49]; changing the medium, non-adherent hematopoietic cells are discharged^[28,50]. MSCs are identified by their adhesion to plastic and their expression of peculiar membrane epitopes (CD73, CD90, CD105), with a lack of expression of human leucocyte antigen-DR and the hematopoietic markers (CD14, CD34, CD38, CD45)^[49,50]. Unfortunately, MSCs display finite life spans. Recent studies have shown that extensive *in vitro* proliferation can affect MSCs replicative potential and differentiation capability: long-term culture and sequential passages affect the quality of MSCs in that their proliferation rate decreases and gradually lose their progenitor properties due to senescence and telomere shortening^[51]. Since MSCs undergo limited mitotic divisions *in vitro*, the number of passages in *in vitro* expansion should not exceed five^[51]. The addition of fibroblast growth factor-2 to the culture medium can stimulate the proliferation of MSCs maintaining their osteogenic potential, keeping the cells in a more immature state^[52].

Usually, the osteogenic differentiation is initiated by supplementing the medium with dexamethasone, ascorbic acid and beta-glycerolphosphate^[53]. In addition, soluble signals like bone morphogenetic proteins (BMPs) such as BMP-2, BMP-6 and BMP-9 can be used^[53,54].

Scaffold

Cells are usually seeded onto a three dimensional (3D) scaffold that guides their growth and proliferation^[55]. At present, there are at least 4 different strategies for the delivery of MSCs into the recipient site: MSCs can be replicated and differentiated in culture, then seeded on a 3D solid scaffold that can be implanted, after a stabilization time to obtain cell adhesion; MSCs can be replicated in culture and seeded into the 3D solid

scaffold, then the cell-scaffold composite is placed in differentiation medium to stimulate the shift into the osteoblastic phenotype, ready to be implanted into the site; MSCs can be replicated and differentiated directly in the 3D scaffold *in vitro*, and the cell-scaffold composite can be implanted after a maturation time; finally MSCs can be replicated and differentiated *in vitro*, and the cell-scaffold composite can be incubated for a short time to prepare an injectable bone preparation.

In all these strategies, the scaffold has a key role providing a substrate into which bone cells migrate, proliferate, differentiate and make new bone^[55,56]. The scaffold should be characterized by specific physical structure and chemical composition in order to mimic the hierarchical architecture and biological functions of native extracellular bone matrix^[55,56]. Native extracellular matrix offers a physical substrate for cells, but also a biological environment for cell adhesion and chemotaxis through specific ligands. Providing specific growth factor, bone matrix regulates cellular proliferation and function^[55,56]. First generation biomaterials were designed promoting mechanical resistance and stability over time, bioinertness or biocompatibility; nowadays new generation scaffold materials are developed with biologically-inspired approach; these new materials should incorporate signals into the scaffold, to modulate proliferation and differentiation^[55-57]. Architectural characteristics define the ultimate shape of the new tissue. Highly porous structures with many interconnection and with large surface areas related to volumes, can aid cell ingrowth and their diffusion throughout the scaffold as well as the passage of nutrients and waste products^[55,57].

Angiogenesis is a pre-requisite for osteogenesis^[57]. Accordingly, pore sizes in the range of 200-800 µm have good results in these situations: in fact, they stimulate osteoprogenitor and endothelial cells to migrate into the matrix. Endothelial cells produce the vascular vessels for new bone nourishment^[55-58]. Surface roughness, surface energy and the presence of cell attachment sites all influence specific proteins expression, quantity and structural conformation which adsorb onto materials surfaces, modulating cells behaviours^[55,57]. Finally, substrate degradation by matrix enzymes^[57] is critical. The ideal material should incorporate controlled resorption, and the regenerated tissue should assume function while the scaffold is slowly degraded^[55,57].

Scaffolds and stem cells: Animal studies

In total, 18 animal studies evaluating the histologic/histomorphometric results obtained with MSCs combined with different scaffold materials were found in the literature^[40-42,59-73]. Among these, one single study was on MSCs with allogeneic bone matrix (AL)^[42], 6 studies were on MSCs with xenografts (BBM)^[59-64], 10 studies were on MSCs with synthetic bone grafts (CPC)^[40,41,65-71], and 2 studies were on MSCs with platelet-rich-plasma (PRP)^[72,73].

AL (bone tissue from human cadavers), provides an osteoconductive scaffold which offers structural support

for vascular and perivascular tissues growth, and for osteoprogenitor cells migration from the adjacent environment^[13,14,42]. In a study on minipigs, Kang *et al.*^[42] evaluated *in vivo* osteogenesis of SDSCs with scaffolds composed by allogeneic demineralized bone (AL) and fibrin glue. The animals were allocated in two groups: in one group, MSA was performed with SDSCs + AL/fibrin glue (test), while in the other group the scaffold without cells was used^[42]. They observed better trabecular bone formation and osteocalcin expression with scaffolds seeded with SDSCs compared with controls^[42]. The authors found that autogenous SDSC grafting with a AL and fibrin glue scaffold can provide an adequate alternative to bone grafting in MSA procedures^[42].

BBM associated with bone marrow aspirate concentrate (BMAC) could provide a substitute for AB to stimulate new bone formation^[9,15,16,59-64]. Sununliganon *et al.*^[59] investigated the bone regeneration capacity of autologous BMAC mixed with BBM in MSA. Twenty-four white New Zealand rabbits were randomly subdivided into groups and when subjected to maxillary sinus floor elevation and augmentation with four different materials: saline solution, AB, BBM and BMAC + BBM^[59]. Four MSA procedures were performed per each material. The animals were sacrificed at 2, 4 and 8 wk, and rates of new bone apposition in augmented surgical sites were evaluated; bone histomorphometry was also examined. Significant increase in the quantity of nucleated cells and colony forming unit-fibroblasts were confirmed in BMAC. MSCs in BMAC retained their *in vitro* multi-differentiation capability. BMAC + BBM showed a similar benefit to AB in term of acceleration, since higher (though not significantly different) rates of mineral appositions in the early period were detected in BMAC + BBM and AB than BBM alone^[59]. Furthermore, graft volume/tissue volumes in BBM and BMAC + BBM resulted to be higher than in AB and saline solution. The results of this study suggest benefit in early bone formation *in vivo* using immediate autologous BMAC transplantation^[59]. In a similar study, Yu *et al.*^[60] compared the potential of tissue-engineered bone derived from different stem cell sources for canine MSA. Bilateral MSA were performed in six beagle dogs and were randomly repaired with three graft types: BBM granules alone ($n = 4$), a complex of osteoblasts derived from BMSCs + BBM ($n = 4$), and a complex of osteoblasts derived from PDLSCs and BBM ($n = 4$). After 12 wk, the animals were sacrificed and fluorescent labeling, maxillofacial computed tomography (CT), scanning electron microscopy, and histologic/histomorphometric analyses were used to evaluate new bone deposition, mineralization, and healing processes in the augmented area^[60]. At the end of the study, the osteogenic capacity was greater with BMSCs + BBM and PDLSCs + BBM than with BBM alone^[60]. The level tended to be higher with PDLSCs than with BMSCs; however, the difference was not statistically significant^[60]. Oshima *et al.*^[61] investigated the *in vivo* osteogenic potential of a novel gabapentin-lactam (GBP-L) in MSA. Bilateral MSA in 10 adult sheep were conducted. BBM and MSCs

combined with novel GBP-L were placed into the test sinus of each sheep; MSCs + BBM alone served as the control on the contralateral side^[61]. The animals were sacrificed after 8 and 16 wk, and the amount of newly formed bone was analysed using histology/histomorphometry. The histologic evaluation showed newly formed bone connected with the original bone in the control and test groups; however, the amount of newly formed bone was not significantly different between the test and control sites^[61]. The authors concluded that the application of GBP-L did not induce faster new bone formation. However, GBP-L did not alter the multipotency of the MSCs or impair bone formation^[61]. Jhin *et al.*^[62] evaluated the potential of BMP-2 gene-transduced BMSCs to facilitate osseous healing after MSA in rabbit. BMSCs derived from New Zealand white rabbits were cultured and some of these cells were transduced with BMP-2 (BMP-2/BMSCs) using an adenovirus vector. Then, BMSCs and BMP-2/BMSCs were seeded on a BBM scaffold. Twenty-seven animals were randomly allocated into three groups: MSA with BMSCs + BBM, MSA with BMP-2/BMSCs + BBM and MSA with BBM alone^[62]. During all these procedures, a mini-implant was placed in the floor of each sinus^[62]. Animals were sacrificed at 2, 4, and 8 wk after surgery, and new bone area and bone-to-implant contact (BIC) were evaluated histomorphometrically. The histomorphometric evaluation revealed that at 2 and 4 wk, the BMP-2/BMSC group showed more new bone and higher BIC than the other two groups; however, at 8 wk, there was no difference in new bone area or BIC among the three groups^[62]. The authors concluded that BMP-2 delivery using BMSCs may result in earlier and increased bone formation in MSA; nevertheless, limitations in the stimulatory effect of BMP-2/BMSCs were evidenced in later healing stages^[62]. Gutwald *et al.*^[63] compared the efficacy of mononuclear cells (MNCs, including MSCs) plus BBM with AB in MSA in sheep. Bilateral MSA were performed in 6 adult sheep. MNCs + BBM were mixed together and used into one sinus, AB in the other sinus^[63]. After 8 and 16 wk, animals were sacrificed. Sites of augmentation were evaluated through radiographic and histological methods. After 8 wk, no difference in new bone formation was noticed between the two groups, but after 16 wk, sites grafted with MNCs + BBM showed 29% of newly formed bone vs 16% in sites grafted with AB^[63]. The authors concluded that MNCs combined with BBM have the potential to stimulate new bone formation in MSA^[63]. Finally, in a similar study, bilateral MSA were performed in 6 adult sheep by the same group of authors^[64]. BBM and MSCs were used in test side and only BBM in the contra-lateral control side of each animal^[64]. Animals were sacrificed after 8 and 16 wk. The regenerated areas were evaluated by CT, histology and histomorphometry. They observed that the newly formed bone was closely connected to BBM particles; furthermore its apposition was significantly faster in the test sides^[64]. The authors concluded that mixture of BBM and MSCs could stimulate new bone

deposition in MSA^[64].

Synthetic CPC such as porous hydroxyapatite (HA), beta-tricalcium phosphate (beta-TCP) and biphasic combinations of these two are excellent bone alternatives^[40,41,65-71]. CPC are considered biocompatible, non-immunological, osteoconductive (they act as scaffolds which allow internal growth of vessels from neighbouring bone tissue)^[40,41,65-71]. Zhao *et al.*^[65] investigated the effect of MSA with engineered bone constructs from DTSCs and CPC in goats. Eighteen bilateral maxillary sinuses of nine goats were randomly assigned into three groups (6 sinuses per group). In the first group, MSA was performed with DTSCs + CPC, while in the second and in the third group MSA was performed with CPC alone and AB, respectively^[65]. All augmentation sites were analysed using CT, histology and histomorphometry. After 12 wk of healing, CT analysis evidenced that the volume of new bone with DTSCs + CPC was greater than that in the other two groups^[65]. In addition, the histological/histomorphometrical evaluation indicated that the DTSCs + CPC compound significantly stimulated new bone formation and mineralization, when compared with CPC and AB alone^[65]. The authors concluded that DTSCs can stimulate new bone formation and maturation in the maxillary sinus of goats, and that the engineered mixture of DTSCs and CPC should represent a possible substitute for MSA procedures^[65]. Zhang *et al.*^[66] compared the bone formation capacity of ADSCs and BMSCs in a canine MSA model. Bilateral MSA were performed in nine beagle dogs using randomly three graft material combinations: BMSCs + CPC ($n = 9$), ADSCs + CPC ($n = 9$) and CPC alone ($n = 9$)^[66]. After 6 wk, the animals were sacrificed and the histological/histomorphometric evaluation suggested that BMSCs might be more advantageous than ADSCs for fast bone regeneration in MSA^[66]. Barboni *et al.*^[41] evaluated the bone regenerative property of AESCs seeded on a CPC synthetic bone substitute (fabricated using rapid prototyping techniques) in MSA in sheep. Two blocks of CPC, engineered with ovine AESCs or alone, were grafted bilaterally into maxillary sinuses of six adult sheep^[41]. The sheep were randomly divided into two groups and sacrificed at 45 and 90 d after surgery. Micro-CT, morphological, biochemical and morphometric analyses were performed to evaluate tissue regeneration in the sinus explants. AESCs seem to influence positively scaffold integration and new bone deposition^[41]. Engineered scaffolds, derived from explanted sinuses grafted with AESCs, displayed an accelerated process of angiogenesis; moreover, AESCs significantly promoted osteogenesis^[41]. These results confirmed those of a previous study by the same group of authors^[40] in which bilateral MSA was performed on eight adult sheep, in order to evaluate the bone regeneration process at 6 and 12 wk after implantation of AFSCs combined with a magnesium-enriched HA/collagen-based scaffold (test) with the scaffold alone (control). In fact, the use of AFSCs increased bone apposition and promoted a faster angiogenesis^[40]. The authors concluded that AFSCs may be a new, easily accessible source of MSCs to develop

cell-based therapy for oral augmentation procedures: in fact, the osteoinduction of a biomimetic commercial scaffold may be significantly enhanced by these cells^[40].

Zeng *et al.*^[67] evaluated the efficacy of BMSCs seeded on CPC, magnesium phosphate cement (MPC), and a calcium-MPC (CMPC), in MSA in rabbit. In test groups augmentation procedures were performed using BMSCs in addition to CPC, MPC, and CMPC; the same materials (CPC, MPC and CMPC), without cells, were used for surgical procedures as control^[67]. In each group new bone formation was investigated histologically and by fluorochrome labeling at weeks 2 and 8 after MSA^[67]. The authors found that CMPC cement could better help new bone formation and mineralization than CPC or MPC cements, and that the addition of BMSCs could further promote its osteogenic capacity significantly^[67].

Zou *et al.*^[68] assessed the potential of BMSCs when combined with CPC in MSA in goats. They randomly allocated nine goats in three different groups: the first group received BMSCs + CPC, the second CPC alone and the third AB. Each animal underwent a bilateral MSA procedure. Implants were also placed in order to evaluate BIC^[68]. After 12 wk, the histological/histomorphometric evaluations showed that the BMSCs + CPC composite could foster earlier bone formation and mineralization, and could preserve more volume and height after MSA^[68]. In addition, BIC was significantly higher in the BMSCs + CPC group than in the other two groups. The authors concluded that BMSCs + CPC seems to be a good graft material for MSA, allowing faster healing in augmented sites and a better stability of implants^[68]. In a study by Xia *et al.*^[69] 36 rabbits were randomly allocated in 4 groups, to test CPC scaffolds associated with recombinant BMP-2 and BMSCs, in different combinations. Although the authors found no significant difference among groups for augmented height, histomorphometric analysis showed significantly less residual graft material and more new bone formation and mineralization in BMP-2/BMSCs + CPC than in other groups^[69]. Based on these outcomes, they suggested that combining BMP-2/BMSCs with CPC could enhance new bone formation and maturation as compared with BMP-2 + CPC or BMSCs + CPC^[69]. In two different studies, Sun *et al.*^[70,71] evaluated the outcome of MSA with CPC and BMSCs in rabbits. In the first study^[70] 16 MSA were performed bilaterally in 8 animals and randomly grafted by BMSCs + CPC, CPC alone, AB and blood clot (4 sites per group). The animals were sacrificed 2, 4 and 8 wk after the surgery and studied histologically and histomorphometrically^[70]. After 8 wk, the authors observed a significantly higher amount of new bone in the test groups (BMSCs + scaffold and scaffold alone) than in control groups (AB and blood clot). An increase in bone height along time was found for the test groups, while control groups showed a constant decrease of augmented height^[70]. Surgical sites augmented with BMSCs + CPC showed more bone than areas grafted with CPC alone, but this difference was not statistically significant. These results suggested that CPC could be used as a bone graft substitute in

bone augmentation procedures and that adding BMSCs to this material could successfully promote new bone formation in maxillary sinus elevation^[70]. In a second, similar study^[71] the same authors evaluated the effects of MSA by a tissue engineered bone composite with *BMP-2* and *EGFP* gene modified BMSCs and CPC. In this study, eight rabbits were allocated in two groups (four rabbits per group), and subjected to bilateral MSA with two different materials: *BMP-2*/BMSCs + CPC (test) and *EGFP*/BMSCs + CPC (control)^[71]. Histological/histomorphometric evaluation was performed 2 and 4 wk after surgery. The vertical bone gain was maintained, over all the experimental period, for both groups, while new bone volume increased over time for test group^[71]. Four weeks after surgery, bone area in test group was significantly more than that in control group^[71]. The authors concluded that BMSCs modified with *BMP-2* gene can stimulate new bone formation in MSA in rabbit animal model, and that CPC scaffold can be a valid vector for gene improved BTE^[71].

Finally, in a split-mouth controlled study on eight minipigs, Pieri *et al.*^[72] investigated whether BMSCs and PRP loaded on a HA substrate can influence bone formation and BIC in MSA, when compared to HA scaffold alone. Bilateral MSA procedures were performed in eight minipigs: each animal received BMSCs + PRP + HA in one sinus (test) and HA alone in the other (control)^[72]. In addition, distal to the augmented site, one endosseous implant was placed per sinus, to evaluate BIC. The animals were sacrificed 12 wk after surgical procedures. Block sections of the implant sites were extracted and prepared for histologic/histomorphometric analysis. The histomorphometric observations revealed statistically significant increase both in bone quantity and in the BIC for the test sites^[72]. This study showed that the use of BMSCs and PRP with a HA scaffold can significantly promote bone growth in MSA techniques, and can enhance the osseointegration of endosseous dental implants positioned in the augmented sites, in comparison with HA alone^[72]. In another, similar study, Ohya *et al.*^[73] evaluated the outcomes of BMSCs associated with PRP vs particulate cancellous bone and marrow (PCBM) with PRP. Bilateral MSA procedures were performed in 18 adult Japanese white rabbits. Each rabbit received BMSCs + PRP in one sinus (test) and PCBM + PRP in the other (control)^[73]. The animals were sacrificed at 2, 4, and 8 wk after surgery, and histological/histomorphometric evaluation was executed. Both test and control sites displayed newly formed bone and neovascularization at 2 and 4 wk in histological preparations; at 8 wk, authors observed large areas of fatty marrow inside lamellar bone in both sites. The analysis of bone volume and augmented height did not highlight any significant differences between BMSCs + PRP and PCBM + PRP groups. On the other hand, significant differences in bone volume and augmented height between 2 and 8 wk in PCBM + PRP or BMSCs + PRP groups were found, as well as in bone volume between 4 and 8 wk in the PCBM + PRP group^[73]. The

authors concluded that the use of a BMSCs + PRP compose could give good outcomes in osteogenesis and bone volume gain comparable to that achieved by particulate cancellous bone in MSA^[73].

The histologic/histomorphometric results of all these animal studies are reported in Table 1.

Scaffolds and stem cells: Human studies

In total, 21 human studies evaluating the histologic/histomorphometric results obtained in MSA with MSCs combined with different scaffold materials were found in the literature^[74-95]. Among these, 2 studies were on MSCs with allogeneic bone matrix (AL)^[74,75], 9 studies were on MSCs with xenografts (BBM)^[77-85], 2 were on MSCs with synthetic grafts (CPC)^[86,87], 6 were on MSCs with synthetic polymers [polylactid-co-glycolic acid, (PLGA)]^[88-93], and 2 were on MSCs with PRP^[94,95].

A commercially available AL containing native MSCs has been clinically used for MSA in 2 different studies^[74,75]. McAllister *et al.*^[74] evaluated the bone formation following MSA using an AL containing native stem cells. After a healing period of 4 mo, biopsy and histologic evaluation were performed. The histologic and histomorphometric evaluation for the five cases reported revealed a high percentage of vital bone content, after a relatively short healing period^[74]. A recent clinical study by Gonshor *et al.*^[75] evaluated bone formation in MSA sites using either an AL cellular bone matrix containing MSCs or a conventional AL. Statistically significant difference were found between the two groups, in terms of vital and residual bone content, using histomorphometric comparison^[75]. In AL, cryopreservation is used to maintain cell viability and multipotential characteristics^[75]. This might help the healing process stimulating bone formation directly from within the graft material, allowing earlier and larger quantity of available vital bone^[74,75]. However, there is a high level of uncertainty around the possibility of iatrogenic transmission of prion or viral infections with AL, due to a lack of evidence-based research on this problem^[76].

BBM has excellent osteoconductive properties^[9,15,16] and the encouraging results emerged from pre-clinical studies^[59-64] have led to the clinical use of this material in MSA, with the cell-based approach^[77-85]. Unfortunately, however, human studies^[77-85] on MSCs + BBM have not confirmed the excellent outcomes originally emerged from animal studies. In a recent clinical study on implant survival after MSA, Duttenhoefer *et al.*^[77] concentrated MSCs with either Ficoll (control group, $n = 6$ sinus) or BMAC (test group, $n = 12$ sinus) and transplanted in combination with BBM. 50 dental endosseous implants were positioned with other surgical procedures (17 Ficoll/33 BMAC) and loaded after 4 mo. At the end of the study, implant survival of the Ficoll group was 100% compared with 93.4% survival of the BMAC group; however, both cell isolation methods were found to be efficient because the difference between the groups was not statistically significant^[77]. In a randomized, controlled, split-mouth design study by Rickert *et al.*^[78], a

Table 1 Histomorphometric results of the animal studies included in the review

Ref.	Animal model	Study design	Histomorphometry (% of newly formed bone)
Kang <i>et al</i> ^[42]	Minipig	SDSCs + AL	NR
		AL alone	NR
Sununliganon <i>et al</i> ^[59]	Rabbit	BMSCs + BBM	59.2 ± 2.1 (4 wk)
			55.9 ± 3.6 (8 wk)
		BBM alone	54.3 ± 2.8 (4 wk)
			51.5 ± 2.6 (8 wk)
		AB	NR
Yu <i>et al</i> ^[60]	Dog	Saline solution	NR
		BMSCs + BBM	NR
		PDLSCs + BBM	NR
		BBM alone	NR
Oshima <i>et al</i> ^[61]	Sheep	BMSCs + GBP-L + BBM	17.0 ± NR (8 wk)
			23.0 ± NR (16 wk)
		BMSCs + BBM	18.0 ± NR (8 wk)
			23.0 ± NR (16 wk)
Jhin <i>et al</i> ^[62]	Rabbit	BMP-2/BMSCs + BBM	12.6 ± 2.8 (2 wk)
			29.3 ± 4.6 (4 wk)
			25.7 ± 3.8 (8 wk)
		BMSCs + BBM	6.2 ± 2.6 (2 wk)
			25.7 ± 2.8 (4 wk)
			27.7 ± 8.2 (8 wk)
		BBM alone	4.2 ± 2.0 (2 wk)
			15.1 ± 2.9 (4 wk)
			22.7 ± 3.3 (8 wk)
Gutwald <i>et al</i> ^[63]	Sheep	MNCs + BBM	19.0 ± 11 (8 wk)
			29.0 ± 12 (16 wk)
		AB	20.0 ± 13 (8 wk)
			16.0 ± 6 (16 wk)
Sauerbier <i>et al</i> ^[64]	Sheep	MSCs + BBM	NR
		BBM alone	NR
Zhao <i>et al</i> ^[65]	Goat	DTSCs + CPC	41.8 ± 6.2 (12 wk)
		CPC alone	30.1 ± 8.0 (12 wk)
		AB	23.0 ± 10.2 (12 wk)
Zhang <i>et al</i> ^[66]	Dog	BMSCs + CPC	NR
		ADSCs + CPC	NR
		CPC alone	NR
Barboni <i>et al</i> ^[41]	Sheep	AESC + CPC	NR
		CPC alone	NR
Berardinelli <i>et al</i> ^[40]	Sheep	AFSCs + MgHA/collagen	NR
		MgHA/collagen alone	NR
Zeng <i>et al</i> ^[67]	Rabbit	BMSCs + CPC	11.7 ± 1.8 (2 wk)
			25.4 ± 3.4 (8 wk)
		CPC alone	5.9 ± 1.4 (2 wk)
			20.5 ± 3.6 (8 wk)
		BMSCs + MPC	5.1 ± 1.7 (2 wk)
			13.5 ± 3.5 (8 wk)
		MPC alone	4.0 ± 1.2 (2 wk)
			6.5 ± 2.0 (8 wk)
		BMSCs + CMPC	12.7 ± 1.9 (2 wk)
			30.9 ± 3.1 (8 wk)
		CMPC alone	6.8 ± 1.3 (2 wk)
			25.5 ± 4.1 (8 wk)
Zou <i>et al</i> ^[68]	Goat	BMSCs + CPC	35.6 ± 9.4 (12 wk)
		CPC alone	22.4 ± 4.2 (12 wk)
		AB	28.2 ± 8.0 (12 wk)
Xia <i>et al</i> ^[69]	Rabbit	BMSCs + rhBMP-2/CPC	17.9 ± 4.3 (2 wk)
			30.5 ± 5.7 (4 wk)
			42.2 ± 4.0 (8 wk)
		BMSCs + CPC	13.9 ± 2.5 (2 wk)
			21.8 ± 4.4 (4 wk)
			28.7 ± 3.7 (8 wk)
		rhBMP-2/CPC	12.8 ± 3.0 (2 wk)
			18.9 ± 2.6 (4 wk)
			31.1 ± 4.5 (8 wk)
		CPC alone	8.0 ± 2.0 (2 wk)
			12.2 ± 3.1 (4 wk)
			22.7 ± 5.7 (8 wk)
Sun <i>et al</i> ^[70]	Rabbit	BMSCs + CPC	21.0 ± 2.6 (2 wk)

Sun <i>et al</i> ^[71]	Rabbit	CPC alone	23.4 ± 3.0 (4 wk)
			35.3 ± 10.5 (8 wk)
			19.2 ± 2.2 (2 wk)
			22.9 ± 2.1 (4 wk)
			19.5 ± 2.4 (8 wk)
		AB	34.7 ± 7.1 (2 wk)
			28.7 ± 5.8 (4 wk)
			NR (8 wk)
		Blood clot	NR (2 wk)
			17.5 ± 3.3 (4 wk)
Pieri <i>et al</i> ^[72]	Minipig	BMP-2/BMSCs + CPC	13.8 ± 4.0 (8 wk)
			21.2 ± 2.1 (2 wk)
		EGFP/BMSCs + CPC	31.9 ± 2.2 (4 wk)
			18.9 ± 1.9 (2 wk)
Ohya <i>et al</i> ^[73]	Rabbit	BMSCs + PRP + HA	23.19 ± 1.9 (4 wk)
			42.5 ± 7.0 (12 wk)
		HA alone	18.9 ± 0.9 (12 wk)
			29.1 ± 4.4 (2 wk)
		BMSCs + PRP	24.1 ± 3.6 (4 wk)
			20.9 ± 4.1 (8 wk)
			35.0 ± 5.2 (2 wk)
		PCBM + PRP	28.6 ± 3.4 (4 wk)
			20.6 ± 4.0 (8 wk)

SDSCs: Skin-derived stem cells; AL: Allograft; NR: Not reported; BMSCs: Bone marrow stem cells; BBM: Bovine bone mineral; AB: Autogenous bone; PDLSCs: Periodontal ligament stem cells; GBP-L: Gabapentin-lactam; MNCs: Mononuclear cells; DTSCs: Deciduous tooth stem cells; CPC: Calcium phosphate ceramics; AECs: Amniotic epithelial stem cells; AFSCs: Amniotic fluid stem cells; Mg/HA: Magnesium/hydroxyapatite; MPC: Magnesium phosphate cement; CMPC: Calcium-magnesium phosphate cement; Rh-BMP-2: Recombinant human bone morphogenetic protein 2; PRP: Platelet rich in plasma; HA: Hydroxyapatite; PCBM: Particulate cancellous bone and marrow; ADSC: Adipose stem cell.

bilateral MSA procedure was executed in 12 edentulous patients. At random, one side was treated with BMSCs + BBM (test side) and the other with BBM mixed with AB (control side)^[78]. Three to four months after MSA, 66 implants were placed. Implant survival, plaque, gingival, and bleeding indices, probing depth, and peri-implant radiographic bone levels were assessed at baseline and 1 year after functional loading. During osseointegration, 3 implants failed on the test side and no implants failed on the control side, resulting in 3-mo survival rates of 91% and 100%, respectively. No other implants were lost after 1 year of functional loading^[78]. Even if the two reconstructive techniques were reliable in providing new bone for implant placement in the posterior maxilla, a higher implant failure rate was reported in MSA procedures with BMSCs + BBM^[78]. In a radiographic study, Kühl *et al*^[79] investigated BMA and BMAC influence on graft materials stability when added to BBM within the first 6 mo after MSA. Using a 3D reconstruction software, CT data of 13 patients undergoing bilateral MSA in a split-mouth study, were processed to evaluate graft volumes 2 wk after the sinus lift procedure and 6 mo later^[79]. The comparison between volumes at 2 wk and 6 mo showed a statistically significant decrease in all single groups between 15% and 21%. However, changes in volumes between the different groups were not statistically significant^[79]. Since an evident decrease in graft volume over the first 6 mo of healing has to be expected, over-augmentation of the sinus is recommended with this cell-based approach^[79]. In an interesting split-mouth design study, Wildburger *et al*^[80] evaluated early bone formation in BBM sinus

grafts using also BMSCs in test group, after 3 and 6 mo. Seven patients, with a posterior maxilla characterized by atrophic bone, were included in this study^[80]. In test side, augmentation procedures were performed with BMSCs mixed to BBM; control sides were grafted using pure BBM. At 3 and 6 mo, biopsies of augmented sites were taken^[80]. The histologic/histomorphometric evaluation found no significant difference in new bone formation between the test and control group^[80]. These results confirmed those of a previous multicentric, controlled study by Sauerbier *et al*^[81]; they found that BMAC + BBM or a mixture of AB + BBM, used in sinus augmentation, give similar new bone formation values, after 3-4 mo of healing. However, these outcomes are partially in contrast with those of a previous histologic/histomorphometric study^[82], where adding MSCs to BBM led to more new bone formation compared with BBM combined with AB. In this study, Rickert *et al*^[82], in fact, described how BMSCs seeded on BBM particles could bring sufficient volume of new bone to allow clinicians to place endosseous implants with a comparable timing regarding to the use of AB or a mixture of AB + BBM. The slow resorption rate of BBM permits an adequate bone integration before scaffold resorption^[81-83]; however, the rate of non-mineralized material is generally high, even 6 mo after augmentation procedure^[81-83]. Finally, Fuerst *et al*^[84] examined the 12-mo histologic/histomorphometric and radiologic outcomes after MSA with autogenous culture-expanded bone cells and BBM. In total, 22 sinuses of 12 patients were grafted with AB cells seeded on BBM. Six months after MSA, during endosseous implants ($n = 82$) placement procedures, a

biopsy was taken from each sinus^[84]. The percent newly formed bone was determined on undecalcified histologic preparations. Graft stability was estimated using dental CT scans after MSA (CT 1), after implant insertion (CT 2) and after implant uncover (CT 3)^[84]. Despite a considerable reduction of the graft volume along time, AB cells and BBM provided an adequate bone volume, which permitted implant placement and tolerated functional loading^[84].

Synthetic porous CPC ceramics can support new bone apposition by MSCs *in vivo*^[86,87]. Osteoconductive scaffolds such as HA and beta-TCP have the ability to attract fibronectin and vitronectin, which are ligands for the integrin family of cell adhesion receptors; these proteins mediate adhesion of MSCs and osteoblast precursors^[20,21,86,87]. In addition, HA/beta-TCP degradation products may favour an alkaline microenvironment and provide calcium and phosphate ions requested for the mineralization of extracellular matrix phases during ossification process^[20,21,86,87]. Finally, microporosity of synthetic CPC (given by pores with a controlled size, communicating through interconnections) supports angiogenesis^[20,21,86,87]. Blood vessels carry cells and soluble signals that promote new bone apposition^[86,87]. In a recent study by Shayesteh *et al.*^[86], CPC has been used in combination with BMSCs in MSA. Six patients underwent MSA with BMSCs + HA/beta-TCP. Three and twelve months after MSA, a radiographic evaluation was performed^[86]. In total, 30 fixtures were inserted and a biopsy was taken from each implant site. Prosthetic rehabilitation were delivered after 4 mo. Clinical successful implant rate was 93% (28 implants over 30)^[86]. Histologic evaluation showed several areas of osteoid and bone formation without any inflammatory cell infiltration. Mean bone regenerate was 41.34%. No complications were clinically observed. Mean bone height was 12.0 mm, 10.0 mm, 3 and 12 mo after MSA, respectively^[86]. The authors concluded that sinus grafting with HA/beta-TCP seeded with BMSCs can offer reliable results^[86]. A previous study by Smiler *et al.*^[87] evaluated the effect of bone marrow aspirate added to xenografts or alloplast graft matrix scaffold (beta-TCP) to enhance bone formation in MSA. Clinical procedures involved harvesting four cc of bone marrow aspirate from the anterior iliac crest; these materials were seeded on matrix scaffold before sinus augmentation operations. Seven graft sites were evaluated in five patients; various MSA techniques were performed such as particulate onlay graft of the maxilla *via* a tunneling procedure, and particulate onlay graft of the maxilla stabilized with titanium mesh. Biopsies at 4 mo showed, in beta-TCP scaffolds, 40% a newly formed bone completely vital^[87]; there was 57% of interstitial material and 3% of residual graft scaffold^[87]. With these histological preparations, the authors presented evidence that stem cells aspirated from bone marrow and seeded onto beta-TCP scaffolds can be a useful method to obtain new bone in augmentation procedures^[87].

Synthetic polymeric materials are an interesting

category of materials. PLGA copolymer and its homopolymer derivatives have been considered for potential use in bone reconstruction procedures. MSCs have been associated to PLGA in MSA^[88-93]. MSCs were isolated from periosteum, re-suspended and cultured; the suspension was soaked in polymer fleeces and the cell-polymer association were used in MSA^[88-93]. Under specific conditions, periosteum-derived, tissue engineered bone grafts showed typical osteogenic differentiation characteristics such as: expression of alkaline phosphatase activity, bone gene expression and mineralization^[93]. Trautvetter *et al.*^[88] performed MSA with simultaneous dental implant placement; they used an autologous tissue-engineered periosteal bone grafts based on bioresorbable PLGA scaffolds. Ten patients were radiologically assessed 5 years after MSA; in addition, histologic evaluation was performed^[88]. The authors reported excellent outcomes after only 4 mo from surgical procedure; they observed significantly greater bone height over the 5-years follow-up observation period. Furthermore histological preparation from bone biopsies of two patients six months after surgery showed trabecular bone with osteocytes and active osteoblasts^[88]. Accordingly, the authors concluded that the use of autologous periosteal bone grafts with simultaneous dental endosseous implants placement is a valid procedure, with excellent clinical, radiographic and histologic outcomes^[88]. Although this clinical study^[88] and those of previous researches^[89,93] have reported that the newly formed bone provided by augmentation procedures, using tissue engineered bone grafts, allowed proper initial stability for dental implant placement^[89,90], the degradation rate of the PLGA scaffold may be too fast to maintain an optimal substrate to support bone formation^[90-92]. This was evidenced by a recent MSA study, where AB transplants from the iliac crest were compared with tissue engineered grafts (BMSCs loaded on PLGA scaffolds): this research showed considerable graft resorption (approximately 90%, in a 3 mo observation period) and less mineralization density in the sites augmented with tissue engineered bone^[92]. Two other comparative studies revealed that coral-derived HA^[90] and AB^[91] show greater volume maintenance than PLGA scaffolds cultured with MSCs. These studies showed that the significant resorption of the PLGA grafts may be an important problem in the clinical scenario, with potential failure of augmentation particularly in large areas^[90,91]. The fast resorption rate of the PLGA, in fact, represents an unfavourable factor for bone regeneration, making it impossible to provide mechanical stability to MSCs transplanted in the augmentation site. Osteoblasts must adhere to a stable structure to produce a new bone matrix that will be interested by consequent mineralization and maturation processes. In this way, a too fast and extended degradation of the supporting scaffold determines an instability of augmented area and then the probably failure of bone regeneration because of the collapse of newly formed, immature bone matrix^[90,91]. Supply of oxygen and nutrients is essential

Table 2 Histomorphometric results of the human studies included in the review

Ref.	Patients	Study design	Histomorphometry (% of newly formed bone)
McAllister <i>et al</i> ^[74]	5	5 MSA: MSCs + AL	33.0 ± NR (16 wk)
Gonshor <i>et al</i> ^[75]	18	18 MSA: MSCs + AL	32.5 ± 6.8 (12 wk)
		8 MSA: AL	18.3 ± 10.6 (12 wk)
Duttenhoefer <i>et al</i> ^[77]	11	12 MSA: BMAC + BBM	NR
		6 MSA: Ficol1 + BBM	
Rickert <i>et al</i> ^[78]	12	12 MSA: BMSCs + BBM	NR
		12 MSA: AB + BBM	
Kühl <i>et al</i> ^[79]	13	13 MSA: BMA + BBM	NR
		13 MSA: BMAC + BBM	
Wildburger <i>et al</i> ^[80]	7	7 MSA: BMSCs + BBM	7.4 ± 4.1 (12 wk)
			13.5 ± 5.4 (24 wk)
		7 MSA: BBM	11.8 ± 6.2 (12 wk)
			13.9 ± 8.5 (24 wk)
Sauerbier <i>et al</i> ^[81]	26	34 MSA: BMAC + BBM	12.6 ± 1.7 (12 wk)
		11 MSA: AB + BBM	14.3 ± 1.8 (12 wk)
Rickert <i>et al</i> ^[82]	12	12 MSA: BMSCs + BBM	17.7 ± 7.3 (14 wk)
		12 MSA: AB + BBM	12.0 ± 6.6 (14 wk)
Schmelzeisen <i>et al</i> ^[83]	1	2 MSA: BMAC + BBM	26.9 ± NR (12 wk)
Fuerst <i>et al</i> ^[84]	12	22 MSA: BMSCs + BBM	17.9 ± 4.6 (24 wk)
Beaumont <i>et al</i> ^[85]	3	6 MSA: PDSCs + BBM	NR
Shayesteh <i>et al</i> ^[86]	6	6 MSA: BMSCs + HA/β-TCP	41.3 ± NR (24 wk)
Smiler <i>et al</i> ^[87]	4	2 MSA: BMSCs + β-TCP	40.0 ± NR (16 wk)
		1 MSA: BMSCs + BBM	13.0 ± NR (16 wk)
		1 MSA: BMSCs + HA	31.0 ± NR (16 wk)
Trautvetter <i>et al</i> ^[88]	10	10 MSA: PDSCs + PLGA	NR
Mangano <i>et al</i> ^[89]	1	1 MSA: PDSCs + PLGA	28.8 ± NR
Voss <i>et al</i> ^[91]	35	50 MSA: PDSCs + PLGA	NR
		63 MSA: AB	
Mangano <i>et al</i> ^[90]	5	5 MSA: PDSCs + PLGA	37.3 ± 19.5 (24 wk)
		5 MSA: HA	54.6 ± 21.1 (24 wk)
Zizelmann <i>et al</i> ^[92]	20	14 MSA: PDSCs + PLGA	NR
		17 MSA: AB	
Schimming <i>et al</i> ^[93]	27	27 MSA: PDSCs + PLGA	NR
Yamada <i>et al</i> ^[94]	23	23 MSA: BMSCs + PRP	NR
Ueda <i>et al</i> ^[95]	6	6 MSA: BMSCs + PRP	NR

MSA: Maxillary sinus augmentation; MSCs: Mesenchymal stem cells; AL: Allograft; NR: Not reported; BMAC: Autologous bone marrow aspirate concentrate; BBM: Bovine bone mineral; BMSCs: Bone marrow stem cells; AB: Autogenous bone; BMA: Bone marrow aspirate; PDSCs: Periosteal derived stem cells; HA/β-TCP: Hydroxyapatite/beta-tricalcium phosphate; PLGA: Polylactid-co-glycolic acid; PRP: Platelet rich in plasma.

to cells embedded within large cell-polymer constructs, in order to sustain their survival and proliferation^[89-92]. In addition, PLGA resorption generates a low pH that is detrimental to osteoblasts^[89-92].

Finally, two different clinical studies used an injectable tissue engineered bone, composed by BMSCs and PRP, to conduct MSA^[94,95]. In a recent study, Yamada *et al*^[94] evaluated the effects of an injectable tissue engineered bone on osteotome technique with simultaneous implant placement. Injectable bone, composed of BMSCs and PRP, was used as bone graft in 23 cases of MSA^[94]. The osteotome technique was used^[7]: after dental implant sites were pre-prepared with pilot drills and/or using the osteotomes, the injectable bone was inserted and then endosseous implants were placed. The bone regeneration technique was effective, as the lift-up bone height by injectable bone using BMSCs showed an increase of 6.1 ± 1.5 mm; the authors concluded that the application of injectable bone using osteotome technique can stably predict the success of bone formation and dental implants, providing also minimally

invasive cell therapy^[94]. These results confirmed those of a previous study by Ueda *et al*^[95] in which the height of mineralized tissue after 2 years showed a mean gain of 8.8 mm compared to pre-operative values. However, more studies are needed to understand the efficacy of injectable bone as a graft for MSA: this material has poor compressive and tensile strength^[94].

The histologic/histomorphometric results of all these human studies are reported in Table 2.

In vivo experiment meta-analysis

A meta-analysis of 9 studies (7 animal and 2 human studies) was performed to give a quantitative estimate of the mean difference of the newly formed bone between the two groups (stem cells + scaffold vs scaffold alone). We considered the mean difference in % newly formed bone at 12 wk as it was the most frequently reported time period; the latest examination period was at 8 wk in 4 studies, all of them on animals. All the analyses were conducted using Package Metafor (R version 2.14). The weighted mean difference estimate from a

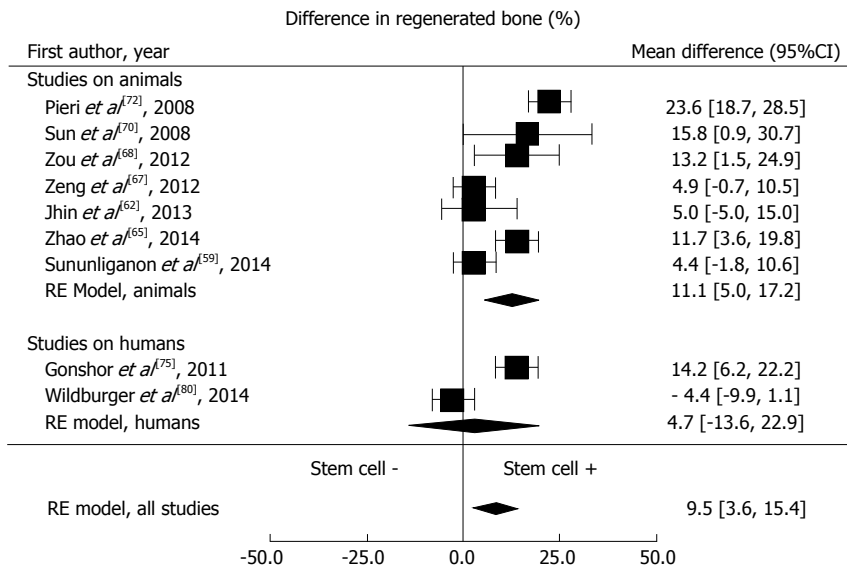


Figure 2 Differences in regenerated bone between test and control groups from meta-analysis. RE: Reference.

random-effect model was 9.5% (95%CI: 3.6%-15.4%), suggesting a positive effect of stem cells on the bone re-growth (Figure 2). Heterogeneity was measured by the I^2 index. The formal test confirmed the presence of substantial heterogeneity ($I^2 = 83\%$, $P < 0.0001$). In an attempt to explain the substantial heterogeneity observed, we considered a meta-regression model with publication year, support type (animals vs humans) and follow-up length (8 or 12 wk) as covariates. After adding publication year, support type and follow-up length to the meta-regression model, heterogeneity was no longer significant ($I^2 = 33\%$, $P = 0.25$).

DISCUSSION

The finding that adult MSCs can be manipulated *in vitro* and subsequently form bone *in vivo* provides new therapeutic strategies for bone regeneration in dentistry.

Several researches have demonstrated that MSCs can be used in MSA: controlled experimental and clinical studies showed higher bone regeneration applying MSCs compared with controls. However, further clinical trials, which clearly demonstrate benefits of cell-based approach compared to conventional treatments are still needed: these studies should evaluate patient-based outcomes, including the time and cost-effectiveness of cell-based approaches.

In the future, the use of stem cells seeded on appropriated scaffold materials will dictates advances in bone regeneration: as a consequence, improvements upon current therapeutic strategies will depend on innovations in material science. It will be mandatory to search for appropriate scaffolds for MSCs, with adequate resorption rate and osteoconductive surface, over which new bone formation can occur.

The merger between these two disciplines - stem cell research and scaffold engineering - will draw the future of regenerative medicine.

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COMMENTS

Background

Adult mesenchymal stem cells (MSCs) that can be obtained from several tissues represents the new frontier for bone regeneration, due to their proven ability to differentiate into functional osteoblast, capable to produce new bone. Maxillary sinus augmentation (MSA) enables rehabilitation with oral implants in the posterior maxilla. Fresh autogenous bone (AB) has been always considered the gold-standard for MSA, but limited availability and donor-site morbidity reduce its application. Several osteoconductive scaffolds, such as allografts, bovine bone mineral and synthetic bone grafts (calcium phosphate ceramics) have been used in MSA with clinically successful results, but these materials do not have cells and require more time for healing. The science of bone tissue engineering (BTE) aims to overcome this problem, as it promises to obtain bone regeneration through the use of scaffolds seeded with osteogenic cells, without harvesting AB from other anatomical sites.

Research frontiers

According to BTE, a bone substitute should possess the same biological and structural properties as native bone, and the fabrication of the ideal bone graft requires the manipulation of three essential elements: scaffold, growth factors and osteogenic cells. BTE is a multidisciplinary science, based on harvesting of living cells that are expanded and differentiated in laboratory, then seeded on an appropriate scaffold, capable to mimic the structures and physiological behaviour of natural tissues. Ultimately, these "engineered scaffold" are implanted in patients. In this context, cells are the basic unit for the regeneration strategy. Several cell types have been investigated for their application in bone regeneration: MSCs can be a suitable for this aim. The authors' present review aimed to investigate the effectiveness of MSCs in MSA, with different scaffold materials, in animals and humans.

Innovations and breakthroughs

In total, 39 studies (18 animal studies, 21 human studies) published over a 10-year period (between 2004 and 2014) were included in our present review; these studies were variables with respect to type, study design, follow-up, and results. Meta-analysis was performed on 9 studies (7 animal studies and 2

human studies): the weighted mean difference estimate from a random-effect model was 9.5% (95%CI: 3.6%-15.4%), suggesting a positive effect of MSCs on bone regeneration. These results are similar to those of previous reviews of the literature on the same topic, where a positive influence of MSCs on bone regeneration was evidenced.

Applications

The use of scaffolds seeded with MSCs seems to represent a safe and successful treatment procedure to achieve bone regeneration in MSA. In the coming years there will be a further, huge flood of new studies on MSA with MSCs. Accordingly, dental professionals/surgeons need to change the way they think and work, to adapt to a new challenging scenario that is increasingly driven by the fascinating concepts of BTE. BTE will change the world of dentistry, changing patients' expectations towards dental treatments: waiting to adopt or integrate these new techniques would leave oral surgeons decades behind.

Terminology

MSA is a surgical procedure to increase the amount of bone in the posterior maxilla by sacrificing some of the volume of the maxillary sinus. In case of tooth/teeth loss (due to caries, periodontal disease or traumatic injury) the alveolar process undergoes remodelling, usually losing both height and width; in addition, the floor of the sinus gradually becomes lower. This represents a problem for the correct placement of dental implants, which rely on osseointegration. The aim of MSA is to graft extra bone into the maxillary sinus, in order to support dental implant. BTE is a new science that combines cells, materials and growth factors, with the aim to obtain bone regeneration in the clinical field. While most definitions of tissue engineering cover a broad range of applications, in practice the term is closely associated with applications that repair or replace portions of or whole tissues (*i.e.*, bone, BTE: science that aims to achieve bone regeneration, without harvesting AB from other anatomical sites, through the use in combination of scaffolds, osteogenic cells and soluble signals).

Peer-review

Several studies have reported the potential for cell-based approaches in MSA; however, most of these are animal studies. Accordingly, further clinical studies, which clearly demonstrate benefits of cell-based approach compared to conventional treatments are still needed.

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**EDITORIAL**

- 992 Signaling involved in stem cell reprogramming and differentiation
Tanabe S

REVIEW

- 999 Stem cell guidance through the mechanistic target of rapamycin
Maiese K
- 1010 Allogenic banking of dental pulp stem cells for innovative therapeutics
Collart-Dutilleul PY, Chaubron F, De Vos J, Cuisinier FJ
- 1022 Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors
Heiblig M, Elhamri M, Mauricette M, Thomas X

MINIREVIEWS

- 1039 Advancement in high dose therapy and autologous stem cell rescue in lymphoma
Isidori A, Clissa C, Loscocco F, Guiducci B, Barulli S, Malerba L, Gabucci E, Visani G
- 1047 Stem cells: Sources, and regenerative therapies in dental research and practice
Aly LAA
- 1054 Rat embryonic stem cells create new era in development of genetically manipulated rat models
Kawaharada K, Kawamata M, Ochiya T

ORIGINAL ARTICLE**Basic Study**

- 1064 Simplified three-dimensional culture system for long-term expansion of embryonic stem cells
McKee C, Perez-Cruet M, Chavez F, Chaudhry GR

Contents

World Journal of Stem Cells
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Signaling involved in stem cell reprogramming and differentiation

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Abstract

Stem cell differentiation is regulated by multiple signaling events. Recent technical advances have revealed that differentiated cells can be reprogrammed into stem cells. The signals involved in stem cell programming are of major interest in stem cell research. The signaling mechanisms involved in regulating stem cell reprogramming and differentiation are the subject of intense study in the field of life sciences. In this review,

the molecular interactions and signaling pathways related to stem cell differentiation are discussed.

Key words: Stem cell; Signaling; Differentiation; Gene; Genome; Reprogramming

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Core tip: Signals in stem cell are regulated both genetically and epigenetically by many molecules. The programming of stem cell signaling is an important aspect of understanding stem cell phenotype transitions and functions. The differentiation process as well as intra- and inter-cellular signaling of stem cells is described in this article. The epigenetic regulation of these cells is also discussed.

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INTRODUCTION

Stem cells are the source of all other cells, and they have the potential to differentiate into many types of cells that contribute to the various organs, such as the heart, lungs, liver, and blood. There are many kinds of stem cells: totipotent embryonic stem cells (ESCs) found in the embryo; mesenchymal stem cells (MSCs) that give rise primarily to bone marrow stroma, adipocytes, and cord blood; epithelial stem cells that reside in the intestine; induced pluripotent stem cells that are artificially reprogrammed from differentiated cells; and cancer stem cells (CSCs). In this article, signaling in stem cells and the role of signaling molecules in the differentiation and reprogramming of stem cells are discussed.

STEM CELL DIFFERENTIATION

Stem cell differentiation is tightly regulated. Among the various pathways related to cellular differentiation, epigenetic regulation is the key mechanism that controls midbrain dopaminergic neuron differentiation^[1]. Urocortin, which is expressed in the developing ventral midbrain, mediates dopaminergic neuron differentiation *via* the up-regulation of acetylated histone H3 and the dopaminergic regulators *Nurr1*, *Foxa2* and *Pitx3*^[1]. Dimethyl sulfoxide down-regulates the pluripotency genes *OCT4* (also known as *POU5F1*) and *NANOG* in human embryonic stem (ES) cells during definitive endoderm differentiation and controls hepatic differentiation^[2].

A previous report indicated that *N*⁶-methyladenosine (*m*⁶A) transferase (*Mettl3*; methyl transferase-like 3) regulates murine naïve pluripotency^[3]. mRNA methylation is a key RNA modification and an essential factor in epigenomic regulation and cell fate determination^[3]. Pluripotent cells are affected by environmental factors such as culture conditions, and such factors can affect the spatial regulations of transplanted stem cells^[4]. Several signaling pathways are involved in the transformation of stem cells, such as fibroblast growth factor 2 (FGF2)/Activin-A signaling and histone 3 lysine 4 trimethylation alterations^[4]. Matrix-bound heparan sulfate (HS) proteoglycans are needed for the differentiation of stem cell-derived endodermal cells into airway epithelial cells^[5]. The three-dimensional extracellular matrix scaffold is essential for effective functional expression of lung epithelial cells^[5], and HS proteoglycans also appear to play an important role in this process. Human ES cells differentiate into osteogenic cells, which can be identified by *RUNX2* expression^[6]. Monitoring the osteogenic differentiation of human ES cells is useful for the therapeutic strategies used in bone regeneration^[6]. The three-dimensional poly(L-lactic acid) scaffold culture of human ES cells on a nano-fibrous matrix results in enhanced osteogenic differentiation^[7]. *RUNX2* expression in nano-fibrous matrix culture is significantly higher than in control cultures^[7]. Additionally, an efficient method for the osteogenic differentiation of human ES cells using primary bone-derived cells has been reported^[8]. Some cellular factors from bone-derived cells may be involved in human ES cell differentiation^[8]. Cell surface proteins play roles in stem cell culture differentiation^[9]. The use of functionalized hyaluronic acid surfaces with either fibronectin or collagen enhances the attachment of human ES cells^[9]. During the osteogenic differentiation of human ES cells, hyaluronic acid surfaces with collagen I and collagen IV have inductive effects on differentiation compared to fibronectin alone^[9]. Transcription factors are regulated dynamically during human ES cell differentiation^[10]. Transcription factor binding dynamics are classified into static, dynamic, enhanced, and suppressed states^[10]. Transforming growth factor (TGF)- β and WNT signals are involved in human ES

cell differentiation into mesendoderm and endoderm, whereas bone morphogenetic protein (BMP), vascular endothelial growth factor, and FGF2 are key regulators of the differentiation of ES cells into mesoderm. The inhibition of TGF- β , WNT or BMP signals result in ES cell differentiation into ectoderm^[10]. *RUNX2* is down-regulated in late-stage cultures of MSCs^[11,12]. The osteogenic differentiation capacity is decreased in late-stage cultures of MSCs compared to early-stage cultures; thus, gene expression levels can be used as an indicator of stem cell differentiation capacity^[11]. Neural differentiation is regulated by epigenetic mechanisms^[13]. Deep cortical layer neuronal markers such as *BCL11B* are expressed in differentiated neurons derived from neuroepithelial and early radial glia, and superficial layer neuronal markers such as *POU3F2/POU3F3* and *MEF2C* are expressed in differentiated progeny derived from mid radial glia^[13]. Transcription factor activity is modulated dynamically during each stage of the neural progenitor population^[13]. In addition to intracellular molecular network signaling, intercellular communication is also an important factor in stem cell signaling. Human neural stem/progenitor cells (hNSCs) and peripheral blood mononuclear cells (PBMCs) interact with each other when co-cultured under allogenic conditions. hNSCs increase the production of regulatory T cells, while PBMCs promote the differentiation of hNSCs^[14]. The effect of intercellular communication on stem cell differentiation is an interesting topic for investigation. Cartilaginous tissue can be generated from human induced pluripotent stem (iPS) cells^[15]. In severe combined immunodeficiency (SCID) mice transplanted with human iPS cell-derived differentiated cartilaginous cells, cartilage formation without tumor formation is observed^[15].

STEM CELL SIGNALING

Tools for bioinformatics

A wealth of "big data" in gene and genome information, which has been collected *via* integrative profiling and is publically available on the internet, combined with recent advances in bioinformatics, have enabled us to analyze gene and signaling information. These advances include a useful web-accessible tool called the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (<http://david.abcc.ncifcrf.gov/home.jsp>) and the cBioPortal for Cancer Genomics (<http://www.cbioportal.org/index.do>)^[16,17]. The open-source tool Cytoscape is a molecular network analysis tool for integrating the networks with annotation and gene expression profiles (http://cytoscape.org/what_is_cytoscape.html).

Hedgehog pathway

Hedgehog (Hh) signaling is an important player in epithelial and mesenchymal regulation during embryonic limb and bone development and cell fate determination^[18-20]. Sonic Hh signaling is involved in

vascular differentiation and regulates human CD34-positive cell function^[20]. Hh signaling also has an important role in hematological CSCs^[21]. Targets of the Hh signaling pathway include the Notch pathway, the epithelial-mesenchymal transition (EMT) pathway, the WNT signaling pathway, the TGF- β signaling pathway, and the regulation of cell cycle, adhesion, fate determination, and stem cell signaling^[21].

Ephrin pathway

Ephrin signaling is involved in inflammatory bowel diseases^[22]. Bone marrow stem cells and stem cell factors play important roles in mucosal regeneration. In the intestine of a rat model of inflammatory bowel disease, ephrin-B3 is up-regulated in bone marrow MSCs during mucosal regeneration^[22]. Ephrin type-B receptor 3 (*EPHB3*), a tumor suppressor gene, is important in cancer cell metastasis, which is regulated by Notch signaling in colorectal cancers^[23]. *EPHB3* down-regulation in tumorigenesis correlates with Wnt/ β -catenin, Notch and mitogen-activated protein kinase (MAPK) signaling^[23]. EphrinA1 expression decreases as β -catenin expression is inhibited in liver tumor cells^[24]. The treatment of hepatocellular cancer with cell-permeable, gamma guanidine-based peptide nucleic acid antisense oligonucleotides resulted in the inhibition of β -catenin and Wnt target gene expression^[23]. The EphrinA1 receptor has been reported to be an independent prognostic marker for different survival endpoints in clear cell renal cell carcinoma^[25]. The use of Ephrin and ephrin receptor signaling as either a prognostic marker or for revealing cancer progression mechanisms is an interesting topic for future study.

WNT pathway

The WNT pathways regulate stem cell differentiation and proliferation. WNT3-WNT9B signaling is involved in the regulation of neural differentiation^[26]. An increase in WNT9B is reported to control the switch between pluripotent and differentiated states *via* noncanonical Rho/JNK signaling, whereas canonical WNT3/ β -catenin signaling promotes proliferation^[26]. WNT5A is known to promote cancer cell invasion and proliferation^[27]. WNT5A activation of the Wnt/ β -catenin-independent pathway, including Src family kinases, induces the proliferation of certain cancer cell types, such as HeLa S3 cervical cancer cells and A549 lung cancer cells^[27]. Interestingly, WNT5A is not involved in the proliferation of KKLS gastric cancer cells, although cancer invasion is dependent on the WNT5A pathway^[27]. Wnt5A up-regulation is reported to induce EMT and metastasis by pancreatic cancer cells^[28]. In this case, β -catenin-dependent canonical Wnt signaling is involved in promotion of EMT^[28]. Graphene oxide is reported to target CSCs by inhibiting a number of signaling pathways including the WNT, Notch and STAT pathways^[29]. Graphene oxide selectively inhibits CSC proliferation but does not affect normal fibroblast viability^[29]. STAT3 signaling alters the tumor microenvironment and angiogenesis and

promotes KRAS-induced lung tumorigenesis^[30]. The Wnt signaling pathway is reported to be down-regulated in irradiated tumor cells^[31]. Wnt signaling may be involved in tumor repopulation after radiotherapy^[31]. Notch1 and Delta-like 4 signals are important factors for cell fate decisions^[32]. The Notch pathway is a therapeutic target in cancer treatment^[32]. Interleukin-27 (IL-27) down-regulates EMT- and stemness-related genes such as *SONIC HEDGEHOG* in adenocarcinoma and *OCT4A*, *SOX2*, *NOTCH1*, *KLF4*, *Nestin*, *SNAI1/SNAI1*, *SNAI2/SLUG* and *ZEB1* in squamous cell carcinoma^[33]. *SNAI2*, an EMT-related gene, and stemness genes are known to be down-regulated by IL-27^[33]. WNT signaling modulation also mediates the long-term expansion and chondrogenic differentiation of adult human MSCs^[34]. WNT3A and FGF2 maintain the phenotype and multipotency of MSCs^[34]. In ES cells, cyclin-dependent kinase 1 (CDK1) inhibition is reported to prevent teratoma formation^[35]. CDK1 signaling and its association with other cancer signaling pathways such as the WNT pathway would be interesting topics for future study of the mechanisms of tumor formation by stem cells.

MicroRNA signaling

MicroRNAs (miRNAs) are involved in cancer cell signaling^[36]. The tumor microenvironment that maintains cancer cells is regulated by microRNA-mediated gene expression^[36]. Anti-miRNAs, antisense oligomers that inhibit onco-miRNAs, can be used as anti-cancer drugs^[36]. The conserved, well-established promoter of the terminal differentiation *let-7a* miRNA induces mitochondrial reactive oxygen species production and up-regulates oxidative stress-responsive genes^[37]. The regulation of energy metabolism in cancer cells *via let-7a* is a potential target of anti-cancer therapy because *let-7a* miRNA plays a tumor-suppressive role^[37]. Human MSCs that support the tumor microenvironment deliver tumor regulatory miRNAs *via* extracellular vesicular trafficking^[38]. The extracellular vesicles of hMSCs contain proteins and lipids on the vesicle membrane, and miRNAs and metabolites inside the vesicles^[38]. The internalization of these vesicles by cancer cells may be one of the key mechanisms for cancer cell survival^[38]. During the hypoxia-induced myogenic differentiation of ES cells, miRNA-26a is up-regulated and inhibits the mRNA expression of histone deacetylase 6 (*HDAC6*) and of stemness genes such as *Oct4* (also known as *Pou5f1*) and *Nanog*^[39]. miRNA-26a-mediated signaling may be important for stem cell differentiation^[39]. Hypoxia contributes to the development and phenotype of CSCs *via* miRNA- and cytokine-mediated microenvironmental regulation^[40]. The vascular CSC niche is regulated by miRNAs, which may induce CSC generation and EMT *via* the NFkB, PI3K/Akt/mTOR, NOTCH, Wnt/ β -catenin and Hedgehog signaling pathways^[40].

In the murine cortex and hippocampus, subclasses of neurons and glial cells have been characterized by the expression of regulatory genes using large-scale

single-cell RNA sequencing^[41]. These cells exhibit differential gene expression and epigenetic regulation and miRNAs have recently become a focus of these processes as selective cell markers^[42]. Glioma cells expressing miR-302 are enriched by serum deprivation, and miR-302 may be a marker of CSCs^[42]. miRNA-30c plays a role in sphere formation, self-renewal and neural differentiation in C6 glioma cells^[43]. In these cells, the astrocyte marker glial fibrillary acidic protein (GFAP) is up-regulated during differentiation. However, GFAP expression decreases in miRNA-30c-overexpressing cells^[43]. The fate of neural stem cells is also regulated by miRNAs^[44]. The differentiation of neural stem cells into neurons is promoted by kuwanon V, a phytochemical isolated from mulberry tree (*Morus bombycis*) roots that may up-regulate the expression of miR-9, miR-29a and miR-181a^[44]. Cancer stem cell self-renewal, differentiation and tumorigenesis are regulated by the Notch pathway, in which miRNAs such as the miR-34 family, miR-200 family, and miR-199-5p, miR-146a, miR-1 and miR-143 play important roles^[45]. Notch signaling cross-talk with miRNAs may be involved in cancer cell proliferation, which suggests possible therapeutic approaches *via* miRNA re-expression in cancer^[45]. Moreover, genome-wide analysis has revealed that long noncoding RNAs (lncRNAs) are regulated by Notch signals in acute leukemia^[46]. lncRNAs are defined as transcripts of greater than 200 nucleotides that function by means other than coding for proteins, and regulate active and silent chromatin states^[47]. Pluripotent states are epigenetically regulated by lncRNAs^[47]. Epigenetic modifications in pluripotency are conducted *via* several steps such as histone modifications, DNA methylation and chromatin remodeling^[48]. miRNA expression is also regulated in atherosclerosis^[49]. Endothelial miRNA expression profiling *in silico* is one area for future study of miRNA signaling^[49].

CELLULAR REPROGRAMMING

Stem cells can be reprogrammed from differentiated cells^[50]. The abundant genes including *CDH1*, *RGS1*, and *NOTCH1* are related to the cell characteristics such as MSCs and gastric cancer cells^[51]. A recent study revealed that neural stem cells could be reprogrammed from adult fibroblasts, senescent somatic cells, and blood cells by HMGA2/let-7^[52]. These induced neural stem cells could be successfully differentiated into neurons, astrocytes, and oligodendrocytes *in vitro* and *in vivo*^[52]. The clinical application of reprogrammed iPS cells has also been attempted^[53]. The transplantation of reprogrammed cells efficiently overcame spinal cord injury in mice; however, the possibility of tumorigenesis is a concern during long-term therapy^[53]. One of the mechanisms of tumor development in transplanted iPS cells seems to be the acquisition of mesenchymal features such as either snail up-regulation or epithelial-mesenchymal transition^[53]. Careful investigation and gene expression analysis are needed before iPS cells can

be utilized in clinical settings^[53]. The selective targeting of human iPS cells to avoid tumorigenesis has been described using a stem cell-specific lectin probe and *Pseudomonas aeruginosa* exotoxin A fusion proteins^[54]. iPS cells are reprogrammed from cancer cells that are used as a carcinogenesis model^[55]. The expression of pluripotency associated genes such as either *Oct4* (also known as *Pou5f1*) or *Nanog* is regulated by the bromodomain and extraterminal domain family member BRD4^[56]. BRD4 regulates transcription by binding to acetylated histones^[56]. The induced differentiation of human iPS cells by interleukin-3/granulocyte colony-stimulating factor results in CD45⁺CD11b⁺CD14⁺CD163⁺CD68⁺ monocyte/macrophage-type cell formation^[57]. Mesenchymal-amoeboid transition is a suggested mechanism by which cancer cells might adopt a migration mode^[58]. The cell migration mode is characterized by three qualities: adhesion, confinement, and contractility^[58]. The change in cell phenotype that occurs during reprogramming may be related to migration parameters. *WNT5A* is reported to be up-regulated in MSCs compared to diffuse-type gastric cancer cells^[59]. Genes related to EMT such as *WNT5A* or *NOTCH2* may have roles in maintaining mesenchymal features^[59]. A genome-wide analysis of chromatin interactions in human ES cells, ES cell-derived mesendoderm, MSCs, neural progenitor cells and trophoblast-like cells revealed that 36% of active and inactive chromosomal compartment alterations occur during differentiation^[60]. An integrative analysis of 111 reference human epigenomes has been performed by the NIH Roadmap Epigenomics Consortium; this analysis profiled histone modification patterns and gene regulation in several cell types and found that each identified mechanism of genetic regulation targets different tissues and cell types in human biology and disease^[61].

The analysis of adipose-derived stem cell differentiation has revealed that keratinocyte progenitor cells are present in adipose tissue^[62]. The reprogramming of fibroblasts into iPS cells requires WNT signaling^[63]. The efficiency of cell reprogramming is regulated by WNT signaling in fibroblasts^[63]. Single-cell mass cytometry has revealed that iPS cell reprogramming is related to mesenchymal-epithelial transition (MET)^[64]. A time-resolved progression analysis of iPS cell reprogramming has revealed that the expression patterns are altered at each time point and that some similarities exist between Oct4-GFP, Nanog-Neo, and Nanog-GFP mouse embryonic fibroblast reprogramming systems^[64]. NANOG, which regulates the pluripotency and reprogramming of iPS cells, binds to the OCT4 promoter, and its mutation enhances ES cell self-renewal^[65].

In early-stage diabetic endothelial progenitor cells, the expression of anti-oxidative enzymes compensates for oxidative stress levels^[66]. The relationship between oxidative stress and progenitor cell function may be an interesting future topic for future study of the reprogramming process. The expansion of human

ES cells can be successfully maintained using MSC feeder layers^[67]. Considering that xenogeneic-free culture conditions are necessary for safe advances in regenerative medicine, the mechanisms that maintain pluripotent status and induce differentiation in the cellular microenvironment should be investigated.

CONCLUSION

In conclusion, stem cells have diverse internal signaling and communicate with surrounding cells. This communication among cells, such as immune cells and cancer cells, through signaling proteins and molecules leads to dramatic alterations in cell responses and pathological physiology. The intra- and inter-cellular signaling pathways in the cellular microenvironment must be elucidated to safely manipulate stem cells and program signaling cascades for therapeutic applications. Abundant data about cellular gene expression, genomics and epigenomics, which are “big-data”, and database analyses are publically available *via* the internet and will contribute to our understanding of cell phenotype transition and the mechanisms that regulate various cell populations in the context of disease and therapy.

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Stem cell guidance through the mechanistic target of rapamycin

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Abstract

Stem cells offer great promise for the treatment of multiple disorders throughout the body. Critical to this premise is the ability to govern stem cell pluripotency, proliferation, and differentiation. The mechanistic target of rapamycin (mTOR), 289-kDa serine/threonine protein kinase, that is a vital component of mTOR Complex 1 and mTOR Complex 2 represents a critical pathway for the oversight of stem cell maintenance. mTOR can control the programmed cell death pathways of autophagy and

apoptosis that can yield variable outcomes in stem cell survival and be reliant upon proliferative pathways that include Wnt signaling, Wnt1 inducible signaling pathway protein 1 (WISP1), silent mating type information regulation 2 homolog 1 (*Saccharomyces cerevisiae*) (SIRT1), and trophic factors. mTOR also is a necessary component for the early development and establishment of stem cells as well as having a significant impact in the regulation of the maturation of specific cell phenotypes. Yet, as a proliferative agent, mTOR can not only foster cancer stem cell development and tumorigenesis, but also mediate cell senescence under certain conditions to limit invasive cancer growth. mTOR offers an exciting target for the oversight of stem cell therapies but requires careful consideration of the diverse clinical outcomes that can be fueled by mTOR signaling pathways.

Key words: Apoptosis; Autophagy; Cancer; Cardiovascular; Erythropoietin; Mechanistic target of rapamycin; Neurodegeneration; Progenitor stem cells; Silent mating type information regulation 2 homolog; Wnt1 inducible signaling pathway; Wnt

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Core tip: Mechanistic target of rapamycin, the mechanistic target of rapamycin, can directly impact stem cell maintenance, proliferation, and differentiation to offer new therapeutic strategies for multiple disease entities.

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MECHANISTIC TARGET OF RAPAMYCIN SIGNALING

The mechanistic target of rapamycin (mTOR) is a

289-kDa serine/threonine protein kinase that is encoded by a single gene *FRAP1*^[1,2]. mTOR, also known as the mammalian target of rapamycin and the FK506-binding protein 12-rapamycin complex-associated protein 1, oversees a complex array of cellular functions that involve gene transcription, cellular proliferation, senescence, metabolism, survival, and cellular death. The target of rapamycin (TOR) was initially identified in *Saccharomyces cerevisiae* with the genes *TOR1* and *TOR2* that encode two isoforms in yeast Tor1 and Tor2 through the use of rapamycin-resistant TOR mutants^[3]. Rapamycin is a macrolide antibiotic derived from *Streptomyces hygroscopicus* that can inhibit TOR as well as mTOR activity.

mTOR is a vital component for the function of the protein complexes mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) (Figure 1)^[4-7]. Rapamycin primarily inhibits mTORC1 by blocking mTOR phosphorylation^[8]. However, mTORC2 activity can be limited during chronic administration of rapamycin. mTORC1 is composed of Raptor (Regulatory-Associated Protein of mTOR), the proline rich Akt substrate 40 kDa (PRAS40), Deptor (DEP domain-containing mTOR interacting protein), and mLST8/GβL (mammalian lethal with Sec13 protein 8, termed mLST8). Phosphorylation of Raptor through the protein Ras homologue enriched in brain (Rheb) leads to mTORC1 activation. PRAS40 is inhibitory to mTOR activity and can prevent the binding of mTORC1 to Raptor^[9]. Phosphorylation of PRAS40 by protein kinase B (Akt) frees PRAS40 from Raptor and allows PRAS40 to be sequestered by the cytoplasmic docking protein 14-3-3 to activate mTORC1^[4-7]. Similar to PRAS40, Deptor inhibits mTORC1 activity through the binding of the FAT domain of mTOR (for FKBP associated protein, Ataxia-telangiectasia, and Transactivation/transformation domain-associated protein). In contrast to PRAS40 and Deptor, mLST8 fosters mTOR kinase activity through p70 ribosomal S6 kinase (p70S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) that bind to Raptor^[10]. PRAS40 can block mTORC1 activity by preventing p70S6K and 4EBP1 to associate with Raptor^[9,11].

mTOR activity also is controlled by Akt and AMP activated protein kinase (AMPK) through the hamartin (tuberous sclerosis 1)/tuberin (tuberous sclerosis 2) (TSC1/TSC2) complex (Figure 1)^[12,13]. TSC2 is considered to be a principal site to govern the activity of the TSC1/TSC2 complex that is an inhibitor of mTORC1. As a GTPase-activating protein (GAP) that can convert Ras homologue enriched in brain (Rheb-GTP) to the inactive GDP-bound form (Rheb-GDP), TSC2 prevents the activity of Rheb-GTP and blocks mTORC1 activity by limiting binding of 4EBP1 to mTORC1. Akt can phosphorylate TSC2 to disrupt the TSC1/TSC2 complex, force TSC2 to be sequestered by the cytoplasmic protein 14-3-3, and activate mTORC1^[14]. It should be noted that under some cellular protection scenarios, a limited activity of TSC2 as well as AMPK appears necessary since complete knockdown of TSC2 can prevent cellular

protection^[15].

AMPK also provides a mechanism to control the activity of the TSC1/TSC2 complex, but in contrast to Akt serves to promote TSC2 activity and block mTORC1 function. AMPK phosphorylates TSC2 to enhance GAP activity to process Rheb-GTP into Rheb-GDP that can then block mTORC1 activity. Interestingly, AMPK can influence sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*) (SIRT1) activity that can be critical for stem cell survival and proliferation^[16]. AMPK increases the cellular NAD⁺/NADH ratio that results in the deacetylation of the SIRT1 targets peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1α) and forkhead transcription factors FoxO1^[17] and FoxO3a^[18]. AMPK also can increase nicotinamide phosphoribosyltransferase (NAMPT) activity that catalyzes the conversion of nicotinamide to nicotinamide mononucleotide^[19], increases nicotinamide adenine dinucleotide (NAD⁺) levels, decreases levels of the SIRT1 inhibitor nicotinamide, and promotes SIRT1 transcription^[20-22]. SIRT1 up-regulation in combination with AMPK activation promotes the induction of autophagy that can protect endothelial cells exposed to oxidized low-density lipoproteins^[23]. Similar to AMPK that is an inhibitor of mTOR, SIRT1 appears to exert its effects over cellular proliferation through blockade of mTOR^[24]. SIRT1 inhibits mTOR activity to preserve the integrity of embryonic stem cells during oxidant stress^[25]. SIRT1 also inhibits mTOR signaling to foster neuronal growth^[26] and assist with mesangial cell proliferation during high glucose exposure^[27].

In relation to mTORC2, this complex consists of Rictor (Rapamycin-Insensitive Companion of mTOR), mLST8, Deptor, the mammalian stress-activated protein kinase interacting protein (mSIN1), and the protein observed with Rictor-1 (Protor-1)^[28-33]. Rictor and mSIN1 through mTORC2 can activate Akt to promote cell survival^[11,29,34]. Protor-1 is a Rictor-binding subunit of mTORC2 and is believed to activate serum and glucocorticoid induced protein kinase 1 (SGK1), since loss of Protor-1 reduces the hydrophobic motif phosphorylation of SGK1 and the substrate N-Myc down-regulated gene 1 in the kidney (NRDG1)^[35]. mTORC2 is a member of the protein kinase A/protein kinase G/protein kinase C (AGC) family of protein kinases and is activated by growth factors to control ion transport. mTORC2 also controls cytoskeleton remodeling through protein kinase C-α (PKCα) and oversees cell migration through the Rac guanine nucleotide exchange factors P-Rex1 and P-Rex2 and through Rho signaling. In contrast to mTORC1, mTORC2 is activated by the TSC1/TSC2 complex through the N-terminal region of TSC2 and the C-terminal region of Rictor^[36].

MECHANISTIC TARGET OF RAPAMYCIN AND STEM CELL PROGRAMMED CELL DEATH

mTOR is an important component in the control of

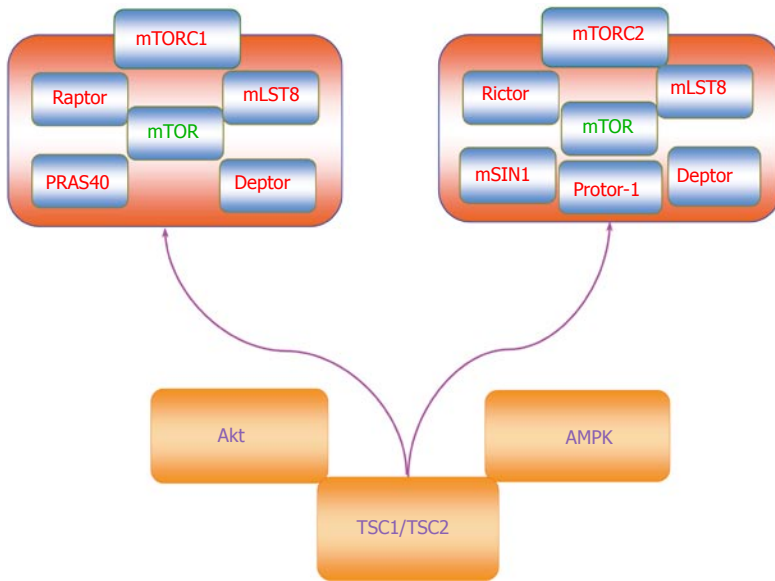


Figure 1 The components of the mechanistic target of rapamycin regulatory pathways. The mechanistic target of rapamycin (mTOR) is an important component of mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). The function and activity of mTOR is controlled by multiple pathways that include protein kinase B (Akt), AMP activated protein kinase (AMPK), and the hamartin (tuberous sclerosis 1)/tuberin (tuberous sclerosis 2) (TSC1/TSC2) complex. mTORC1 is consists of Raptor (Regulatory-Associated Protein of mTOR), the proline rich Akt substrate 40 kDa (PRAS40), Deptor (DEP domain-containing mTOR interacting protein), and mLST8/GβL (mammalian lethal with Sec13 protein 8, termed mLST8). mTORC2 is composed of Rictor (Rapamycin-Insensitive Companion of mTOR), mLST8, Deptor, the mammalian stress-activated protein kinase interacting protein (mSIN1), and the protein observed with Rictor-1 (Protor-1).

programmed cell death that involves autophagy and apoptosis for stem cell proliferation and survival (Figure 2). The process of autophagy recycles cytoplasmic components to remove defective organelles that can no longer be used by the cell^[37]. Autophagy has three categories of chaperone-mediated autophagy, microautophagy, and macroautophagy^[38]. Chaperone-mediated autophagy uses cytosolic chaperones that transport cytoplasmic components across lysosomal membranes^[39]. Microautophagy sequesters components of the cytoplasm through invagination of the lysosomal membrane for digestion^[40]. The most prevalent of the three categories is macroautophagy that sequesters cytoplasmic proteins and organelles into autophagosomes. These autophagosomes then fuse with lysosomes for degradation and are recycled for future use^[24,41].

In yeast and mammals, TOR and mTOR are associated with genes that control autophagy^[4,6,42]. At least 33 autophagic related genes (*Atg*) have been identified in yeast that can affect multiple disorders including cancer, diabetes, vascular disease, and neurodegenerative disorders^[37,38,40,43-48]. In this group, Atg1 and Atg13 (also known as Apg13) are associated with phosphoinositide 3-kinase (PI 3-K), Akt, and the TOR pathways. When Atg13 is dephosphorylated such as during starvation, Atg1 is active to promote autophagy. Phosphorylation of Atg13 through a TOR dependent pathway releases it from Atg1 and lessens Atg1 activity. In mammals, the homologues of Atg1 are UNC-51 like kinase 1 (ULK1) and ULK2^[4]. Mammalian Atg13 binds to ULK1, ULK2, and FIP200 (focal adhesion kinase family interacting protein of 200 kDa) to activate

ULKs, promote phosphorylation of FIP200 by ULKs, and lead to the induction of autophagy^[49]. Activation of mTOR prevents the induction of autophagy by phosphorylating Atg13 and ULKs to inhibit the ULK-Atg13-FIP200 complex.

Autophagy can become a vital determinant for stem cell survival and proliferation. In some stem cell populations, activation of autophagy can lead to stem cell demise. Breast cancer stem cells have been shown to succumb to apoptosis during the activation of autophagy and the inhibition of Wnt signaling^[46]. Wnt proteins are cysteine-rich glycosylated proteins that oversee stem cell proliferation and tumor cell growth^[50-56]. Reduction in autophagy also may prevent the development of cellular senescence^[57]. Endothelial progenitor cells that lead to the regeneration of vascular endothelium become dysfunctional during exposure to elevated glucose as a result of autophagy activity^[58].

However, under other conditions, autophagy appears critical for stem cell survival. In endothelial progenitor cells, SIRT1 activity prevents apoptotic cell death during oxidative stress through the induction of autophagy^[59]. In human embryonic stem cells challenged with oxidative stress, autophagy was found to be protective and required SIRT1 activity with the down-regulation of mTOR^[25]. Furthermore, activation of SIRT1 is necessary to promote autophagy to maintain proteostasis, produce energy during nutrient deprivation, and maintain muscle stem cell activation^[60]. In such cases, SIRT1 may have an inverse relationship with mTOR to foster stem cell survival^[16,20].

The programmed cell death pathway of apoptosis also has an important role with mTOR signaling and

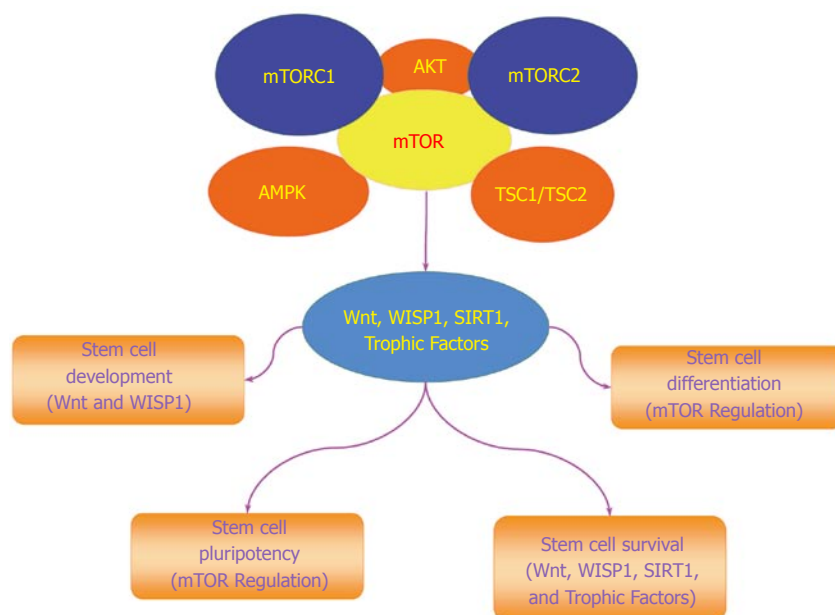


Figure 2 Mechanistic target of rapamycin governs stem cell development, pluripotency, survival, and differentiation. The mechanistic target of rapamycin (mTOR), mTOR Complex 1 (mTORC1), mTOR Complex 2 (mTORC2), protein kinase B (Akt), AMP activated protein kinase (AMPK), and the hamartin (tuberous sclerosis 1)/tuberin (tuberous sclerosis 2) (TSC1/TSC2) complex each play a role in stem cell development and proliferation with downstream pathways. Stem cell maintenance and survival is reliant upon mTOR signaling pathways that work in concert with Wnt signaling, Wnt1 inducible signaling pathway protein 1 (WISP1), silent mating type information regulation 2 homolog 1 (*S. cerevisiae*) (SIRT1), and trophic factors such as erythropoietin (EPO). Stem cell development is under the regulation of Wnt and WISP1. Regulation of mTOR can control stem cell differentiation and stem cell pluripotency. As a result, mTOR signaling pathways have oversight of stem cell development, pluripotency, survival, and differentiation.

stem cell survival^[61]. During the early stages of apoptotic cell injury, the loss of plasma membrane lipid phosphatidylserine (PS) asymmetry occurs^[62-64]. If membrane PS externalization is not reversed and allowed to remain, inflammatory cells are activated that seek out membrane PS positive cells to engulf and remove these cells. Under such circumstances, these membrane PS positive cells may remain functional but their ultimate loss leads to tissue injury^[65-71]. During the later phase of apoptotic cell injury, cellular DNA is destroyed which is usually not considered a reversible process^[72-75]. During most conditions, activation of mTOR and its related pathways of PI 3-K and Akt can block apoptotic cell death in stem cells. Inhibition of mTOR, such as with rapamycin, leads to endothelial progenitor cell apoptotic death that may be related to inhibition of growth factor signaling^[76]. Growth factors that include erythropoietin (EPO)^[77,78] are cytoprotective against apoptosis through mTOR activity against sepsis-associated encephalopathy^[79], oxidative stress^[80], cerebral microglial injury^[81], and beta-amyloid (A β) toxicity^[82]. EPO has been shown to protect retinal progenitor cells from apoptotic cell death during oxidative stress through activation of the PI 3-K, Akt, and mTOR pathways (Figure 2)^[83]. Interestingly, protection with EPO and mTOR may be lost with high exogenous EPO concentrations, since elevated concentrations of EPO result in decreased phosphorylation and activity of mTOR with subsequent increased apoptotic cell death^[84]. Similar to EPO, other growth factors rely upon mTOR to maintain stem cell

integrity (Figure 2). In murine experimental models, mTOR is used by the growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF) that are protective of stem cells and neurons^[85-89] to maintain the proliferation of neural stem and progenitor cells^[90]. EGF also uses the PI 3-K, Akt, and mTOR pathways to block cell injury such as during metabolic stress^[91] and prevent memory impairment^[92]. The growth factor brain derived neurotrophic factor (BDNF) relies upon mTOR activation for memory consolidation^[93]. However, in some experimental models with growth factors, mTOR blockade with the induction of autophagy may take precedent over the inhibition of apoptosis to prevent cellular injury. During oxygen deprivation, cortical neurons are protected by BDNF through the induction of autophagy and the inhibition of mTOR^[94].

MECHANISTIC TARGET OF RAPAMYCIN CONTROL OF STEM CELL PROLIFERATION AND MAINTENANCE

mTOR governs the proliferation and maintenance of stem cell in multiple systems of the body (Figure 2)^[4]. The loss of the *mTOR* gene leads to limited trophoblast growth, faulty implantation, and inability to establish embryonic stem cells^[95]. A decrease in proliferation of embryonic stem cells occurs during the deletion of the C-terminal six amino acids of mTOR that blocks the kinase activity of mTOR^[96]. mTOR can maintain long-term undifferentiated growth of human embryonic

stem cells. Inhibition of mTOR promotes pluripotency, cell proliferation, and blocks mesoderm and endoderm activities in embryonic stem cells^[97]. mTOR activity also leads to mesenchymal stem cell senescence^[98]. Yet, under some conditions, activation of mTOR signaling components can lead to cell differentiation. In embryonic stem cells, mTOR signaling with p70S6K is limited, but once this signaling is increased, differentiation ensues^[99].

In the nervous system, loss of mTORC1 activity in neural stem cells leads to reduced lineage expansion, prevention of differentiation, and blocked neuronal production^[100]. Loss of mTOR activity during aging may influence decreased neurogenesis. In the aged brain, mTOR signaling is reduced which leads to a reduction in the proliferation of active neural stem cells^[101]. mTOR activity appears important for the timing and control of neurogenesis. Inhibition of mTOR through the RTP801/REDD1 pathway delays neuronal differentiation. However, in newborn and mature neurons, levels of RTP801/REDD1 are diminished with increased mTOR activity to allow for the maturation of neurons^[102]. Expression of mTOR is necessary for the neuronal phenotype of post mortem neuronal precursors^[103]. Yet, the degree of mTOR activity may independently affect different populations of stem cells since in this model inhibition of mTOR activity leads to cell differentiation into astrocytic cells^[90]. Akt and mTORC1 inhibition also has been shown to result in reduced neuronal stem cell self-renewal and earlier neuronal and astroglial differentiation^[104]. Neighboring cells also may influence the growth of neuronal stem cells. Endothelial cells can promote mTOR activity and lead to the expansion of long-term glioblastoma stem-like cells^[105].

In the cardiovascular system, mTOR is one of several components necessary for the proliferation of human embryonic stem cell-derived cardiomyocytes^[106]. The activity of mTOR also controls the proliferation of hematopoietic stem and progenitor cells^[107]. Maintenance of hematopoietic stem cells and inhibiting differentiation is tied to mTOR signaling and the reduction in phosphorylation of p70S6K^[108]. Failure of endothelial progenitor cell development may be the result of decreased growth factor signaling and loss of mTOR activity^[76]. Growth factors such as EPO have been shown to require mTOR activation to regulate bone homeostasis with osteoblastogenesis and osteoclastogenesis^[109]. Differentiation of neural precursor cells that may be used for neurodegenerative disorders also is dependent upon EPO and mTOR^[103]. mTOR may be necessary to increase angiogenesis from endothelial progenitor cells that may provide neuroprotection during cerebral ischemia^[110]. The ability of human amniotic fluid stem cells to influence the differentiation of embryonic kidney cells is dependent upon mTOR activity^[111].

During tumorigenesis, mTOR activation may affect neural precursor and oligodendroglial precursor cells to promote high-grade glioma proliferation^[112]. Blockade of

mTOR can prevent the conversion of astrocytoma cells to oligodendroglioma cells that can lead to glioblastoma multiforme^[113]. Inhibition of mTOR signaling may reduce the population of cancer stem cells that can lead to disease recurrence and therapeutic resistance^[114].

Under some conditions, mTOR may be protective against tumor cell growth by inhibiting proliferative pathways of Wnt. Wnt signaling can lead to rapid cell proliferation and cancerous growth, but in epithelial stem cells this process is blocked by mTOR that maintains cell senescence and prevents tumor growth^[115]. Wnt may result in malignant melanoma^[116], metastatic disease^[117-120], and glioma proliferation^[121,122]. It should be recognized that Wnt signaling also leads to beneficial and cytoprotective effects^[52,54,123-125]. Loss of Wnt can result in human monocyte injury^[126], impairment in bone repair^[127], spinal cord injury^[125], loss of neurogenesis^[128], inhibition of wound healing^[129], loss of stem cell differentiation^[130], programmed cell death^[38,66,131], and defects in placental development^[132]. Wnt signaling activation can block inflammatory cell loss during neurodegenerative disorders^[66,70,82,133], prevent cerebral ischemia^[134,135], and protect cells during experimental diabetes^[136-138]. Furthermore, trophic factors employ cytoprotective pathways of Wnt to prevent cerebral endothelial cell injury^[137], preserve mesenchymal stem cells^[139], block apoptosis during forkhead transcription factor activation^[136,140], promote the maintenance of immune cells in the nervous system^[81], and prevent A β toxicity in cerebral microglia^[82]. However, prolonged exposure of growth factors such as EPO that rely upon Wnt signaling can have ill effects with the proliferation of cancer^[141-143], inflammation, and blood-brain barrier injury^[144].

In the Wnt signaling pathway, Wnt1 inducible signaling pathway protein 1 (WISP1), also known as CCN4, is a member of the six secreted extracellular matrix associated CCN family of proteins that are involved in cellular survival and stem cell proliferation^[145]. WISP1 can activate the components of the mTOR pathway that determine stem cell survival (Figure 2)^[21]. WISP1 increases mTOR activity by blocking the inhibitory actions of the mTOR component PRAS40^[146]. WISP1 controls the post-translational phosphorylation of AMP activated protein kinase (AMPK), a pathway involved in stem cell proliferation and cellular metabolism^[12,147]. WISP1 differentially decreases phosphorylation of TSC2 at Ser¹³⁸⁷, a target of AMPK, and increases phosphorylation of TSC2 at Thr¹⁴⁶², a target of Akt^[15].

As a tightly linked pathway to mTOR, WISP1 can significantly influence stem cell survival and proliferation. During stem cell migration, WISP1 expression is increased^[148]. WISP1 is differentially regulated during human embryonic stem cell and adipose-derived stem cell differentiation. WISP1 expression is increased during human adipocyte differentiation^[149] and is repressed in adipose-derived stem cells during hepatic differentiation^[51]. WISP1 can modulate induced pluripotent stem cell reprogramming^[150,151]. WISP1 is

one of several genes that are over-expressed during pancreatic regeneration^[152]. WISP1 also may support vascular regeneration during saphenous vein crush injury^[153]. WISP1 oversees cellular senescence^[154] and does not appear to foster excessive cellular proliferation under circumstances involving aging vascular cells^[155]. However, as a proliferative agent, WISP1 can lead to excessive cell growth. WISP1 may promote distant metastatic disease^[156] and WISP1 expression is increased in neurofibromatosis type 1 tumorigenesis^[157]. Variants of WISP1 can be extremely aggressive in promoting cell growth^[158] in comparison to non-variant WISP1 expression that may be protective under specific scenarios and block tumor cell invasion, motility, and metastases^[159].

FUTURE CONSIDERATIONS

Stem cells represent an important strategy as well as a vital experimental tool for the treatment of multiple disorders that can affect diverse systems of the body that include the brain, cardiovascular system, metabolism, and tumor cell growth. mTOR, a 289-kDa serine/threonine protein kinase and a critical component for the protein complexes of mTORC1 and mTORC2, oversees cellular development, proliferation, and senescence that can directly impact stem cell maintenance, proliferation, and differentiation.

Although mTOR is a highly attractive target to control stem cell maintenance and differentiation, the complexity of this system raises a number of considerations. How does mTOR interface with programmed cell death pathways that can directly affect stem cell populations? mTOR regulates the programmed cell death pathways of autophagy and apoptosis that have a complex outcome in stem cell survival. Through the modulation of Wnt signaling, activation of autophagy that necessitates inhibition of mTOR can block breast cancer stem cell growth. Yet, activation of autophagy that may work in concert with SIRT1 has been shown to play a vital role to maintain muscle stem cell activation and the protection of endothelial progenitor cells. Apoptosis that consists of both an early stage with membrane PS externalization and a late stage involving the destruction of genomic DNA usually relies upon activation of mTOR and its related pathways of PI 3-K, Akt, and growth factors such as EPO, EGF, FGF, and BDNF to block apoptotic cell death in stem cells. However, during some toxic environments, stem cells that become differentiated may require the induction of autophagy with mTOR inhibition to prevent apoptotic cell death.

Another consideration for mTOR is its variable role in the maintenance of stem cell populations and the eventual differentiation of cells into specific phenotypes. mTOR is necessary for trophoblast growth, implantation, the establishment of embryonic stem cells, and the maintenance of pluripotency. Loss of mTOR, such as in the aged brain, may be a factor that results in the

reduction of the proliferation of active neural stem cells. Yet, mTOR signaling that can involve p70S6K also affects the modulation of stem cell genesis and cellular differentiation. The activation of mTOR rather than its inhibition can be necessary for stem cell differentiation as well as the ability to selectively promote the maturation of specific cell phenotypes. The control of stem cell development, migration, and proliferation by mTOR can be dependent upon both Wnt and WISP1 signaling.

Ultimately, consideration also must be given for the role mTOR plays to block tumorigenesis and the ability of mTOR signaling to at times accelerate tumor cell growth. Given its proliferative role, mTOR can foster cancer stem cell development and the conversion of differentiated cells into cells that have invasive growth. The degree of mTOR activity may be critical during tumorigenesis, since mTOR in some cell populations can either maintain cell senescence and prevent tumor growth or conversely promote cancer stem cell development that can lead to disease recurrence and therapeutic resistance. By clearly addressing the challenges that lie ahead, targeting mTOR and its signaling pathways offer an exciting approach to translate the development and utilization of stem cells into new therapeutic strategies for multiple disease entities.

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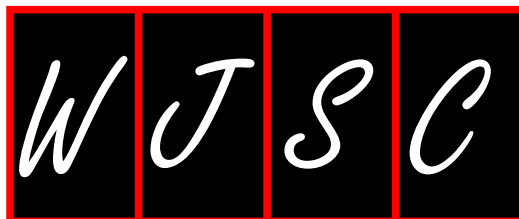
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Allogenic banking of dental pulp stem cells for innovative therapeutics

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Abstract

Medical research in regenerative medicine and cell-based therapy has brought encouraging perspectives for the use of stem cells in clinical trials. Multiple types of stem cells, from progenitors to pluripotent stem cells, have been investigated. Among these, dental pulp stem cells (DPSCs) are mesenchymal multipotent cells coming from the dental pulp, which is the soft tissue within teeth. They represent an interesting adult stem cell source because they are recovered in large amount in dental pulps with non-invasive techniques compared to other adult stem cell sources. DPSCs can be obtained from discarded teeth, especially wisdom teeth extracted for orthodontic reasons. To shift from promising pre-clinical results to therapeutic applications to human, DPSCs must be prepared in clinical grade lots and transformed into advanced therapy medicinal products (ATMP). As the production of patient-specific stem cells is costly and time-consuming, allogenic biobanking of clinical grade human leukocyte antigen (HLA)-typed DPSC lines provides efficient innovative therapeutic products. DPSC biobanks represent industrial and therapeutic innovations by using discarded biological tissues (dental pulps) as a source of mesenchymal stem cells to produce and store, in good manufacturing practice (GMP) conditions, DPSC therapeutic batches. In this review, we discuss about the challenges to transfer biological samples from a donor to HLA-typed DPSC therapeutic lots, following regulations, GMP guidelines and ethical principles. We also present some clinical applications, for which there is no efficient therapeutics so far, but that DPSCs-based ATMP could potentially treat.

Key words: Adult stem cells; Multipotent stem cells; Cell-based therapy; Cell tissue bank

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Core tip: To achieve clinical applications, stem cell-based therapy must shift from lab experimentation to clinical grade stem cells. We present here the development of advanced therapy medicinal products (ATMP) by the banking of dental pulp stem cells (DPSCs) for allogenic use. The dental pulp represents an efficient tool for industrial applications due to its accessibility after wisdom teeth extraction for orthodontic purpose. DPSC therapeutic batches can be produced in good manufacturing practice condition after human leukocyte antigen typing and stored in allogenic biobanks. We propose some clinical applications, for which there is no efficient therapeutics so far, but that DPSCs-based ATMP could potentially treat.

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INTRODUCTION

Adult organisms contain postnatal somatic stem cells that are involved in symmetrical and asymmetrical cell divisions, allowing stem cell compartment maintenance and cell differentiation^[1]. Thus, adult stem cells provide replacement and repair cells for normal turnover or injured tissues^[2]. As these stem cells are able to renew particular tissues, they have motivated research on how to apply them in the clinic. Because of their self-renewal and ability to regenerate tissue, stem cells could provide long-lasting clinical benefits to recipients. Among these potentially beneficial cells, mesenchymal stromal cells are spindle-shaped, plastic-adherent cells isolated from bone marrow, adipose tissue, dental pulp and many other tissue sources^[3,4]. They are also called mesenchymal stem cells (MSCs) in reference to their significant self-renewing properties and ability to form skeletal and connective tissue, and are suggested to be responsible for the normal turnover and maintenance of adult mesenchymal tissues^[2,5]. MSCs are now the focus of intensive efforts in order to elucidate their nature and properties, and to develop cell-based therapies with real clinical applications^[6]. Moreover, MSCs provide promising therapeutic benefits as they primarily mediate positive effects through paracrine mechanisms independent of cell differentiation^[7]. Many preclinical and clinical trials have been completed and the major hurdles are now cell engraftment and survival, stem cell fate control, and donor-patient compatibility for allogenic applications. Several current efforts are directed at promoting the registration and banking of stem cell lines and providing associated data^[8,9]. Banking MSCs, with shared materials and data, is an important step for the efficient progress of stem cell research and clinical translation. Emphasis on clinical applications is increasing, with

an aim of establishing clinical grade, human leukocyte antigen (HLA)-matched banks for clinical translation^[10].

DENTAL PULP STEM CELLS

Teeth are formed of two main parts, the crown and the root, that can be defined by anatomic criteria. They are linked by the periodontal ligament to the supporting alveolar bone, which is composed of both compact and trabecular bone. The dental crown consists of enamel, dentin, and dental pulp tissue. During tooth growth and development, ameloblasts form enamel and odontoblasts generate primary dentin. After tooth eruption, ameloblasts disappear from the surface of the enamel; consequently, enamel formation ceases to occur naturally *in vivo*. In contrast, odontoblasts, along the inner surface of the dentin inside the pulp chamber, continue to deposit dentin matrix to form secondary dentin throughout life. In addition to secondary dentin, odontoblasts can form tertiary (reparative) dentin in response to several stimuli, such as mechanical, chemical, and/or bacterial stimulation. Even when odontoblasts have been damaged, the reparative dentin can be formed in the dental pulp to protect against further disruption of the pulp tissue. This reparative dentinogenesis has been thought to be mediated by newly generated odontoblasts arising from dental pulp tissue. These findings led to the speculation that odontogenic progenitor cells or stem cells may exist in dental pulp tissue^[11]. The first type of dental stem cell was subsequently isolated from the human pulp tissue and given the name dental pulp stem cells (DPSCs)^[12]. Dental pulp is a soft connective tissue entrapped within the dental crown, and divided into four layers. The external layer is made up of odontoblasts producing dentin; the second layer is poor in cells and rich in collagen fibers; and the third layer contains progenitor cells and undifferentiated cells, some of which are considered stem cells. From this layer, undifferentiated cells migrate to various districts where they can differentiate under different stimuli and make new differentiated cells and tissues. The innermost layer is the core of the pulp and comprises the vascular area and nerves^[13]. Dental pulp is an interesting source of adult stem cells because of the large amount of cells present and the non-invasiveness of the isolation methods compared to other adult tissue sources^[13-15]. MSCs defined as dental stem cells can be obtained from human permanent and primary teeth, human wisdom teeth^[12], human exfoliated deciduous teeth^[16], apical papilla^[17], the periodontal ligament^[18,19] and the dental follicle^[20,21].

Dental pulp tissue from human third molar, exfoliated deciduous or supernumerary teeth represent an easily accessible source for harvesting MSCs as these teeth are often discarded.

Stem cells that reside in dental pulp (DPSCs) have been described as a population of MSCs, as they match the definition given by the Mesenchymal and Tissue

Stem Cell Committee of the International Society for Cellular Therapy^[3]: DPSC are plastic-adherent when maintained in standard culture conditions; they express some specific surface molecules such as CD105, CD73, CD90 and lack expression of CD45, CD34, CD14, CD19 and HLA-DR surface molecules; and they have the ability to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*^[11,12,18,22-24]. Moreover, DPSCs can differentiate into a large array of cells and tissues^[25-28] and a comparison of their multipotency with Bone Marrow Stem Cells has demonstrated that proliferation, availability, and cell number of DPSCs were greater than for bone marrow MSCs^[24,29].

In addition, DPSCs were also found to undergo myogenic and neurogenic differentiation capacities *in vitro*, expressing respective gene markers and exhibiting neuron-like cell morphologies. The plasticity and multipotential capability of DPSCs can be explained by the fact that dental pulp is made of both ectodermic and mesenchymal components, and contains neural crest-derived cells^[13]. Concerning cell surface molecules, the persistence of negative results for CD45 demonstrates that these cells are not derived from a hematopoietic source, although they are of mesenchymal origin^[25]. Like all MSCs, DPSCs are also heterogeneous and the various markers may be expressed differently by subpopulations of these stem cells^[24]. A selected subpopulation of CD34⁺/CD45⁻ DPSCs, which represented roughly 10% of dental pulp cells, has also been described. These cells displayed an increased capacity of self-expanding and differentiating in pre-osteoblasts, and were able to self-maintain and renew for long time^[17]. Although MSCs were originally described as CD34 negative, it seems that this subpopulation of DPSCs expresses the CD34 cell surface antigen in the manner reserved for the most primitive stromal stem cells (other than hematopoietic) that was gradually lost after the differentiation of lineage committed progenitors^[30].

DPSCS BIOBANKING

The term "biobank" describes various facilities that store biological samples, from small tissue collections to wide repositories featuring a variety of tissues and biological sample types^[31,32].

Storage and collection of biological material might be accompanied by various medical and epidemiological data that are used for current research and for potential future works^[33]. Biobanking has been defined as a structured resource for genetic and medical research and their therapeutic applications. It includes human biological material and extensive associated information^[34,35].

Several types of biobanks can be distinguished, according to the purpose and target: case control, clinical trial, tissue, biomolecular resource center, stem cells^[36]. Stem cell biobanks have received much attention as a new biologic resource for both research

and clinical applications, leading to the development of stem cell banks around the world^[37]. Stem cells of dental origin represent a promising source of new stem cells as, in western countries, 80% of teenagers and/or young adults have their wisdom teeth extracted. Furthermore, dental pulp is naturally protected within the pulp chamber, the inner cavity of the tooth, in a sterile environment. Pulp from one wisdom tooth generally contains between 200000 and 300000 DPSCs^[38]. Studies have indicated that DPSC isolation was feasible for 5 d after tooth extraction^[39]. Efficient results were obtained by cryopreserving second-passage DPSC cultures, but could also be achieved by isolating and cryopreserving entire pulp tissues, with digestion and culture performed post-thaw^[40]. Such minimal processing may be of interest for the banking of samples for which there are no immediate plans for expansion and use. Furthermore, cell recovery could be achieved by mechanical disruption with a single-use device, in accordance with the GMP (European Good Manufacturing Practices) standard. Dental pulp and DPSC recovery is represented in Figure 1.

Immunologic considerations: Allogenic use and immunomodulation

Immune mechanisms confer immediate protection against foreign organisms (innate immunity) and specific immune responses to neutralize pathogens (adaptive immunity). The immune system recognizes tissue compatibility and can raise an effective immune response against pathogens or incompatible allogenic tissues. Tissue compatibility or incompatibility is determined from allelic similarities or disparities at genetic loci that encode the major histocompatibility complex (MHC) antigens, also called the HLA system. The HLA system encodes two major classes of highly polymorphic cell surface glycoproteins: HLA class I molecules are expressed on all nucleated cells and HLA class II molecules are expressed on antigen-presenting cells, thymic epithelial cells, and B lymphocytes. These immunological principles, which apply to organ or tissue transplantation, can be extended to transplantation of DPSCs or DPSC-derived tissue, especially for HLA class I molecules (HLA-A, HLA-B and HLA-C)^[41]. Thus, a major clinical challenge to DPSC banking will be to overcome the immunological barriers to the transplantation of DPSC-derived tissues in order to prevent rejection^[42,43]. Ensuring HLA compatibility is certainly the most interesting method of minimizing the risk of rejection. Embryonic stem cells have been shown to express very low levels of HLA class I proteins, with a moderate increase during differentiation^[44]. Regarding MSCs, HLA expression remains unclear, but they have been extensively studied for their immunomodulatory properties^[45-49]. Indeed, MSCs exert a profound inhibitory effect on T cell proliferation *in vitro* and *in vivo*, with similar effects on B cells, dendritic cells and natural killer cells^[50]. Moreover, T-cell inhibition is not restricted by HLA type, and immunosuppressive effects are

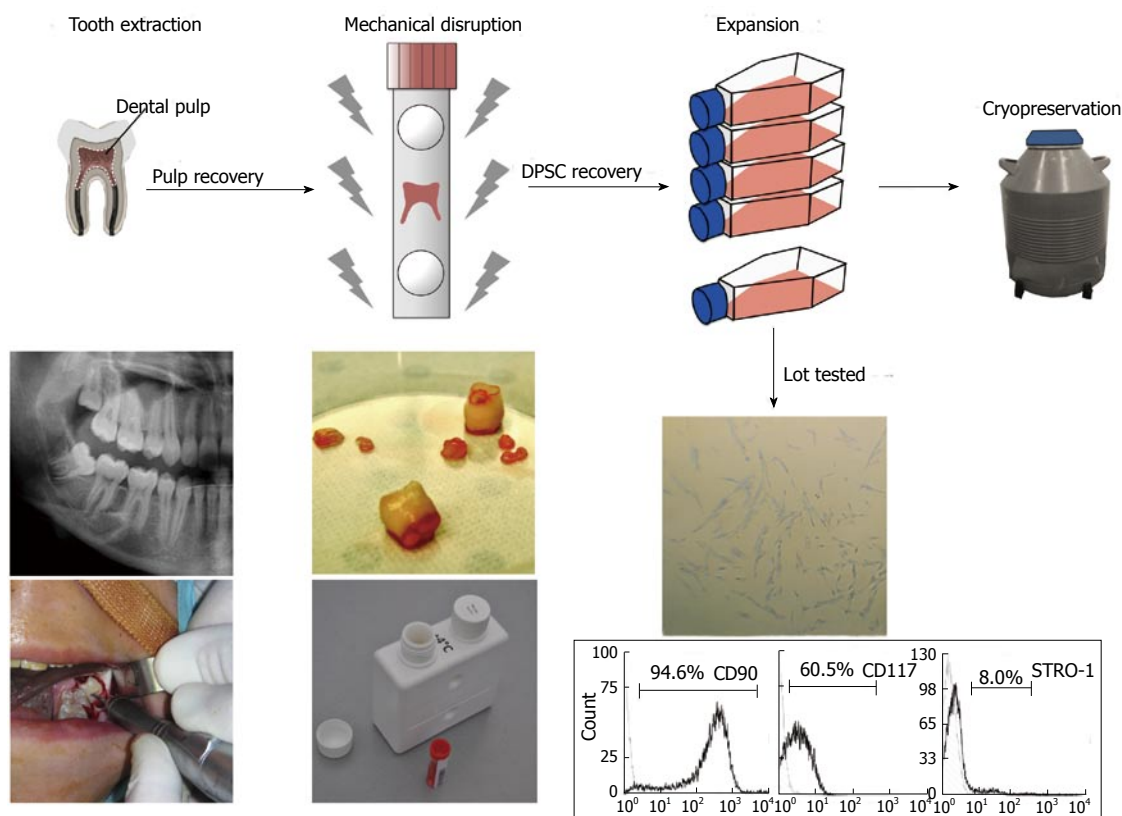


Figure 1 Dental Pulp tissue and dental pulp stem cell recovery. Wisdom teeth are extracted in aseptic conditions and transferred to the cell bank in a sterile transport tube. The teeth are then cracked opened and the pulps are mechanically disrupted in a tissue grinder/homogenizer. The cell suspensions obtained are screened for expression of stemness markers by flow cytometry, before storage in liquid nitrogen. DPSC: Dental pulp stem cell.

mediated through soluble factors and the generation of regulatory cells^[47,51]. These findings suggest that MSCs can induce peripheral tolerance, enhancing their potential for therapeutic applications^[45,48]. A higher immunosuppression of T-cell alloreactivity has also been demonstrated in DPSCs in comparison with bone marrow stem cells^[52]. These properties distinguish DPSCs as one of the most accessible cell sources for cell-based therapy in regenerative medicine and inflammation-related diseases^[46].

To overcome rejection and use DPSCs in transplantation medicine, the formation of a histocompatibility bank is an attractive option, where DPSCs are stored after HLA isotyping. Ideally, considering the large numbers of wisdom teeth extracted from genetically diverse populations, adequate levels of isotype matching with patients may be achieved. The establishment of an HLA-organized DPSC allogenic bank could be sufficient to provide stem cells for a large number of patients. The concept of haplobanking with HLA homozygous cell lines would also limit the number of HLA mismatches^[53]. Several studies have been conducted to determine the number of donors needed to cover the population of a country. These findings are dependent on ethnic disparities of the population and the type of stem cells considered. The creation of a bank containing highly selected homozygous lines is an attractive approach to HLA matching. Selected homozygous lines can provide

HLA matches for a wide percentage of populations. Estimates of the number of homozygous cell lines needed have mainly been established considering embryonic stem cells, for the main proteins HLA-A, HLA-B and HLA-DR. Data from cadaveric organ donors, cord blood bank or *in vitro* fertilization-derived embryos led to the estimate that approximately 150-190 human embryonic stem cell lines with various HLA genotypes, or a collection of 10-30 homozygous lines for the common HLA types, would be sufficient to provide HLA-matches for a wide part of the population in the United Kingdom^[54], Japan^[55,56], the United States^[57] or China^[58]. Because of the low incidence (1.5%) of HLA-homozygous individuals in the normal population^[54], a systematic collection of discarded wisdom teeth would be of prime interest. The determination of the HLA types of 100 DPSC lines from teeth collected in Japan revealed 2 homozygous lines for all the 3 considered HLA loci. These 2 homozygous lines therefore have the potential to cover approximately 20% of the Japanese population with a perfect match^[59].

Methods and good manufacturing practices

The production and marketing of stem cell-based therapy faces imperative steps, including product characterization, safety testing and clinical trials design. At both national and international levels, numerous standards and regulations must be followed in order

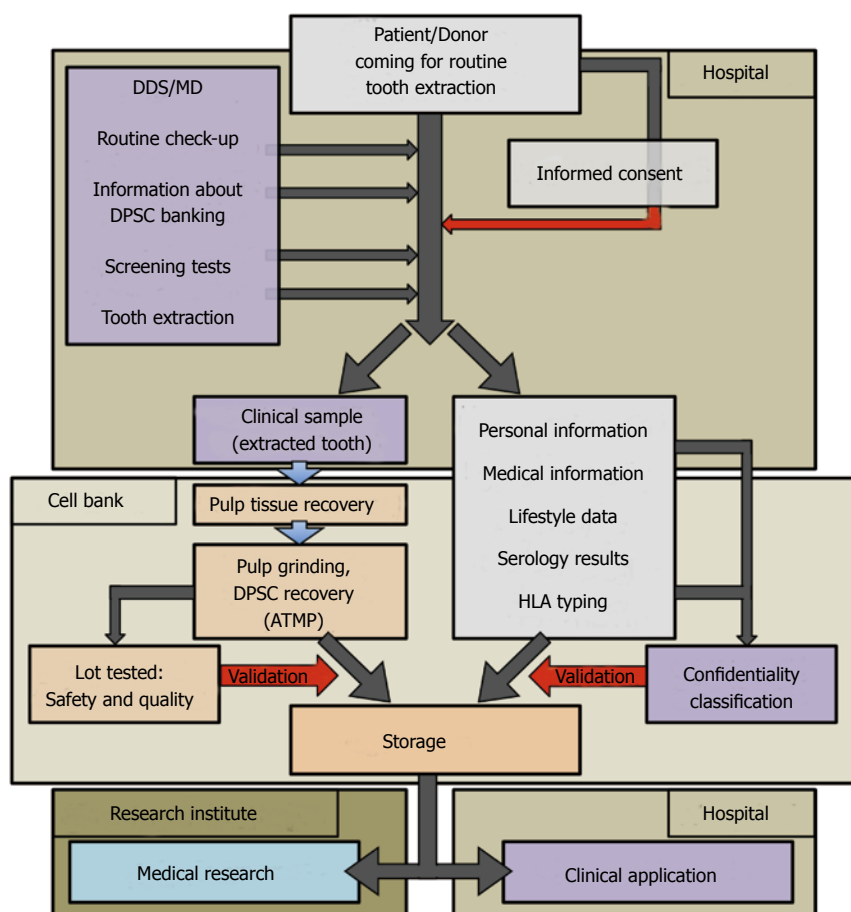


Figure 2 Process flowchart for current Good Manufacturing Practices manufacturing of dental pulp stem cell lots/therapeutic products. The chart is divided into 4 areas: Hospital for tooth recovery, Cell Bank, Research Institute and Hospital for clinical applications: (1) Hospital: direct contact between patients and authorized medical staff, such as Medical Doctor (MD) or Doctor of Dental Surgery (DDS). Clinical sample and donor's personal data are recovered; (2) Cell Bank: current Good Manufacturing Practices manufacturing of Advanced Therapy Medicinal Products (ATMP) from the biological samples (dental pulps). All data concerning the donors are anonymous; (3) Research Institute: dental pulp stem cell (DPSC) lots are used for animal experiments to develop new therapeutics; and (4) Hospital: clinical grade DPSC lots are used for therapeutics. Red arrows represent critical parameters related to each step of processing (informed consent, quality, safety, confidentiality). HLA: Human leukocyte antigen.

to translate DPSCs into clinical products. There are variations in these international and national guidelines, and in the regulations that are applied to the collection and storage of human tissue, personal data and medical records^[32]. The Food and Drug Administration, in the United States, and the European Medicines Agency (EMA), in Europe, are responsible for creating and enforcing these regulations. In Europe, stem cells for clinical therapies are classified under advanced therapy medicinal products (ATMP) unless they are minimally manipulated and intended for homologous use^[60]. A Committee for advanced therapies (CAT) has even been created to evaluate cell production marketing by assessing the quality, safety and efficacy of ATMPs, in accordance with the regulatory framework. EMA regulation defines the current Good Manufacturing Practices (cGMP) guidelines to manufacture ATMPs^[61]. Even though clinical grade production of DPSCs needs to be implemented, DPSCs can be isolated, stored, and eventually expanded by applying rational modifications to the commonly used methods^[15,62], in order to continue complying with good manufacturing prac-

tices^[63] from the donor (patient having his/her tooth extracted, in aseptic condition) to the storage tank. The critical step of enzymatic pulp tissue digestion can be replaced by mechanical disruption in single use devices, such as a tissue grinder/homogenizer. Fetal bovine serum usually required for *in vitro* expansion can be replaced by human serum supplements derived from peripheral blood serum, peripheral blood plasma, or platelet lysate^[64]. Moreover, genetic stability has been demonstrated for DPSCs for up to 9 cell passages^[65,66].

Legal and practical issues (consent, confidentiality, commercialization)

Translation of DPSC research into clinical applications relies on abundant *in vitro* and *in vivo* preclinical data. However, when it comes to potential therapeutic applications, some barriers can appear, due to restrictions specified in the consent document used for the collection of biological materials, questions about ownership of the collected DPSCs, and the confidentiality of the information associated with the cell lines^[10]. The constitution of an allogenic DPSC bank contains

procedures to ensure anonymity, although authorized parties can access some clinically relevant information.

The rights of donors and the interests of researchers are protected by incorporating relevant government legislation (ethical committee review) and procedures (e.g., anonymity and consent). It is crucial to explain the use and transfer of cells and data at the time of informed consent, especially highlighting features that distinguish collection for research from collection for a biobank^[67]. The whole process, from the patient coming for tooth extraction to storage of DPSC lots, is presented in Figure 2.

Allogenic DPSC biobanking brings together a multitude of data on individuals, including health and lifestyle. Thus, the way the informed consent is obtained should reflect the personal information used for medical research, taking into account that the patient was originally coming for a routine tooth extraction. As informed consent is derived from the standard that every donor has the right to self-determination, the patient must be informed about the nature of biobanking, the procedures in which the tooth he has donated might be involved, and the expected outcome of the research^[36,68]. The physical and intellectual property of biological samples collected must be clearly established and explained^[69].

INNOVATIVE THERAPEUTICS

Upon discovery of stem cells in the dental pulp, DPSCs demonstrated their ability to regenerate a complex consisting of a mineralized matrix of odontoblasts and connective tissue containing blood vessels similar to that observed in normal human tooth^[12]. Since then, the use range of potential medical applications based on DPSCs include the repair and regeneration of bone^[13,70], the central nervous system^[27,71,72], liver tissue^[73], heart tissue^[74], eyes^[75], muscles^[76,77], and salivary gland cells^[78,79]. Overall, it holds great potential in the field of regenerative medicine and tissue engineering^[13,23] alone or combined with various biomaterials^[80-84]. Some have proposed that DPSCs may have greater potential than the current MSC gold standard, the bone marrow-derived MSC^[29].

Allogenic banking of DPSCs could boost industrial and therapeutic innovations by providing tools for unsolved medical problems, including the production of advanced therapy medicinal products (ATMP). We present here some clinical applications, for which there is no efficient therapeutics so far, but that DPSCs-based ATMP could potentially treat.

Spinal cord injuries

Chronic medullary lesion, a result of spinal cord trauma, is characterized by neurologic deficiency without evolution. Six months after trauma, these lesions are considered chronic, with no chance of improvement. According to the Christopher Reeves foundation, these spinal cord lesions affect 1.2 million persons

in the United States and 300000 persons in Europe. Numerous preclinical studies have been conducted to graft stem cells of various origins into injured spinal cords, such as neural stem cells^[85], embryonic stem cells^[86-88], with encouraging results^[89]. DPSCs, due to their embryologic origin, express some markers of both mesenchymal and neuroectodermic origin^[12,16]. Indeed, DPSCs originate from migrating cranial neural crest cells. During embryonic development, these neural crest cells differentiate into a wide variety of cell types, including neurons of the peripheral nervous system^[27]. MSCs have been thought to be usable in the treatment of spinal cord injuries, and adult human DPSCs could provide an ideal source of stem cells for therapeutic applications in such neurological pathologies^[90]. The efficiency of DPSCs in improving neural regeneration has been shown *in vitro*^[72,73,91] and *in vivo* after spinal cord injury^[92-94]. These preclinical data enhance the therapeutic potential of intrathecally administrated HLA-typed DPSC lots in treatment of nerve tissue injuries.

Sjögren's syndrome

Sjögren's syndrome is an autoimmune pathology affecting 0.2% to 3% of the general population^[95]. It is a chronic inflammation of the salivary and lacrimal glands, characterized by lymphocytic infiltration of the exocrine glands with a polyclonal B cell activation^[96]. Although the pathogenesis of primary Sjögren's syndrome remains unclear, T cells and B cells have been shown to be involved. Pharmacological treatments have limited efficiency, with only the capacity to temporarily ameliorate symptoms, and with no modification of the overall course of the disease^[97]. Given the lack of disease-modifying drugs, treatment options are now focused on biotherapies^[98]. As detailed above, immunomodulatory properties of MSCs have been demonstrated *in vitro* and *in vivo*, suggesting a therapeutic potential for autoimmune disease treatments^[47], especially through anti-inflammatory cytokines production and T regulatory cells promotion^[99]. These immunomodulatory properties have also been demonstrated for DPSCs, identifying them as a cell source for cell-based therapy of immune and inflammation-related diseases^[47]. Intravenous injection and local injection of DPSC lots into salivary glands represents a potential novel immunotherapeutic tool for autoimmune Sjögren's syndrome.

Irradiated salivary glands

Cancers that originate from the aerodigestive epithelium, including carcinomas of the head and neck, are the leading causes of cancer-related mortality worldwide, accounting for about 2 million deaths and 500000 new cancers diagnosed annually^[100,101]. Treatment involves chemotherapy, radiotherapy, and surgery. Radiation-induced salivary hypofunction is one of the major developments that affect survivors. Even though radiotherapy is focused on the cancerous area, radiations often affect salivary glands, causing severe hyposialia and oral dryness after orofacial cancer

treatment; hyposalivation underlying xerostomia after radiotherapy is still a major problem in the treatment of head and neck cancer. To date, the only treatment for this oral dryness is the use of artificial saliva to supply salivary glands, a treatment with limited efficiency. Salivary stem cell (salisphere) transplantation has been shown to functionally restore salivary gland efficiency after radiation-induced impairment of salivary gland function and consequential xerostomia^[79]. Furthermore, it was demonstrated that DPSCs used as a cell source for the treatment of salivary gland hypofunction could partially revert this hypofunction^[80]. Thus, stem cell-based therapy has great potential in prevention or treatment of radiation-induced hyposalivation^[102]. New therapeutic strategies are now being considered using stem cells injected intravenously or directly into salivary glands to allow salivary gland cell reactivation^[103-106].

Acute periodontitis

Periodontal diseases are highly prevalent diseases that can affect up to 90% of the population worldwide. They have various forms, from gingivitis, the mildest form caused by dental plaque, to periodontitis, which induces the loss of connective tissue and bone support, and causes tooth loss in adults^[107]. Acute periodontitis is an inflammatory disease of the periodontium triggered by the host's immune response and resulting in the progressive loss of gingival tissue, periodontal ligament and supporting alveolar bone^[108]. Actual therapeutics consist of the control of bacterial infection and the stabilization of tissue loss. Regenerative treatment using bone grafts, gingiva grafts, and growth factors offer interesting possibilities, but only in specific indications^[109-111], and with unpredictable results^[112]. In this context, topical application of stem cells in periodontal lesions appeared to be a promising strategy to regenerate periodontium^[113]. *In vitro* studies demonstrated the ability of DPSCs to differentiate into osteoblast and cementoblast lineage, and to participate in periodontal ligament and cementum regeneration^[114,115]. *In vivo* experiments enhanced the therapeutic potential of dental stem cell grafting to regenerate periodontal tissues^[116-118]. Allogenic transplantation could enhance periodontal tissue repair and limit local inflammation through MSC immunomodulation^[108,119].

Endodontic regeneration

Dental pulp, the soft connective tissue described above, is the tissue entrapped within the teeth, in which are recovered DPSCs. In case of dental decay, this tissue can be infected and become necrotic, because it is encased in a thick dentin wall, and consists of a microcirculatory system originating from a very small opening at the apex of the root. This anatomical configuration limits the development vascular supply during pulp regeneration. Endodontic treatment, when needed, includes the removal of vital and necrotic tissues from the root canal system, along with infected

root dentine. It aims to prepare the canal space to facilitate disinfection by irrigants and medicaments. Prevention of reinfection is then achieved through the provision of a fluid-tight root canal filling and a coronal restoration^[120]. The potential possibility of regeneration of pulp tissue by cell therapy is a promising approach for the future treatment of pulpitis or peri-apical disease assuring longevity of teeth and improved quality of life. It has been demonstrated that transplantation of DPSC was capable of inducing complete pulp regeneration in a root canal after pulpectomy^[121]. Thus, the use of DPSC, combined with a supporting scaffold, could be used to treat and heal infected root canals, providing an interesting alternative to the actual inert fillings used in endodontics. Root canals anatomy limits the use of rigid scaffold systems in pulp regeneration: scaffolds for pulp regeneration should be injectable, with fibrous structures that ideally mimic the extracellular matrix of the pulp tissue and support stem cells growth.

Induced pluripotent stem generation from DPSC

Pluripotent stem cells can be induced from fibroblasts by retroviral introduction of Oct3/4, Sox2, c-Myc and Klf4^[122]. These induced pluripotent stem (iPS) cells are similar to embryonic stem cells in morphology, proliferation and differentiation capacities^[123]. They proliferate extensively and differentiate into virtually any desired cell type, providing an unlimited source of replacement cells for human therapy^[124]. It has been shown that DPSCs could be also reprogrammed into iPS cells, with a higher efficiency rate than dermal fibroblasts. DPSCs-derived iPS cells were indistinguishable from human embryonic stem cells, highlighting the potential of DPSCs as an alternative source for generating iPS cells^[125,126]. Many reprogrammed cell lines could easily be established from DPSCs obtained from young patients with a low risk of bacterial contamination and genetic modification, as extracted wisdom teeth are generally aseptically obtained from the mandible and are protected from ultraviolet and other damage by surrounding hard tissues. It was shown that iPS cells could be efficiently generated from DPSCs using the conventional 4 reprogramming factors (Oct3/4, Sox2, c-Myc and Klf4)^[59,125,126], as well as using only 3 factors (Oct3/4, Sox2 and Klf4)^[59], or even using only 2 non-oncogenic factors (Oct4 and Sox2)^[30]. Interestingly, the efficiency rate of reprogramming was related to the donor's age, with higher rate for younger patients with wisdom teeth still under maturation^[59].

With respect to safety, it would be ideal not to use retrovirus vectors for transient expression of the reprogramming genes. DPSCs are assumed to offer high efficiency of iPS cell generation even with the use of non-integrating vectors such as Sendai viruses or modified mRNA. Clinical use of iPS in regenerative medicine is very promising. However, time-efficiency and financial considerations argue in favor of the use of allogenic rather than autologous iPS lines. Similarly to DPSCs, biobanking of iPS lines would be a reasonable

strategy. In this setting, DPSC banking could be of great help to establish iPS cell banks with a sufficient repertoire of HLA types, since the establishment of clinical-grade iPS cell lines from individual patients would require much time and incur a high cost^[59].

From DPSC to successful therapy: Limitations and issues

The notion of stem cells as postnatal units of organ or tissue regeneration allows imagining therapies such as tissue engineering. A stem cell that could be expanded and modified *ex vivo*, and transplanted *in vivo* encourages attempts to treat severe or lethal diseases. However, even when the use of stem cells could replace the lost cells, it does not guarantee that the regenerated cells could circumvent the cell death caused by the disease. And replacing lost cells, even with cells expressing several specific cell markers, is far from a successful therapy with fully functional cells. Indeed, clinically successful translation of stem cell science into medicine has been conducted following a simple framework in which organ-specific stem cells were used for organ-specific diseases^[6]. The roles of DPSC, and MSCs in general, as niche cells, tissue organizers and skeletal or neural progenitors open opportunities and pose challenges. The molecular mechanisms by which stem cells become functional are still largely unknown. Its comprehension and identification may involve new methods that go far beyond the empirical injection of poorly characterized cultured cell strains^[6]. Such methods may involve modeling of disease mechanisms, identification of cell-derived bioactive factors and their use as drugs (including factors mediating the interactions with endothelial and hematopoietic cells), definition of targeted specific disease mechanisms and organ-specific strategies to deliver DPSC to a site of interest^[127].

CONCLUSION

DPSC-based therapy is now entering into a new stage of development, shifting from initial *in vitro* and *in vivo* studies to optimization of therapeutic products for clinical applications. As for other MSC, there are still many challenges concerning stem cell potency, age-related and disease-related tissue impairment, and production of clinical grade stem cells lots. The strategies presented in this review emphasize the potential of DPSC to be used for innovative clinical trials based on rational DPSC therapy, following GMP conditions.

Indeed, dental pulp is a remarkable site of stem cells, and the collection of stem cells from dental pulp is a non-invasive practice that can be performed after routine wisdom teeth extraction. DPSC can be recovered in GMP conditions and cryopreserved for long periods, after HLA typing. However, in the perspective of therapeutic use, optimization and better methods are still necessary for DPSC isolation, expansion and

banking.

Although producing and storing patient-specific stem cells could resolve immunological problems, this procedure would be costly, laborious, and time-consuming. Allogenic DPSC banking containing clinical grade stem cell lines offers an alternative and provides stem cell lines from which it will be possible to choose a HLA match for the patient to be treated. There are variations in national and international regulations for the collection and storage of human tissue, but ethical principles related to biobanks always include safety, informed consent and confidentiality. The recovery of DPSC doesn't involve any invasive procedure as they come from already extracted teeth. Thus, the main concerns are: (1) for the donor, clear explanations about the banking project and confidentiality of all personal and medical data; and (2) for the patient, safety of DPSC lots produced in accordance to guidelines. To date, despite promising preclinical data, clinical trials using DPSCs have not been widely reported. Allogenic biobanks represent a new strategy that aims to develop the clinical applications of the DPSC potential, involving both researchers and clinicians.

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Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors

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Abstract

Relapses remain a major concern in acute leukemia. It is well known that leukemia stem cells (LSCs) hide in hematopoietic niches and escape to the immune system surveillance through the outgrowth of poorly immunogenic tumor-cell variants and the suppression of the active immune response. Despite

the introduction of new reagents and new therapeutic approaches, no treatment strategies have been able to definitively eradicate LSCs. However, recent adoptive immunotherapy in cancer is expected to revolutionize our way to fight against this disease, by redirecting the immune system in order to eliminate relapse issues. Initially described at the onset of the 90's, chimeric antigen receptors (CARs) are recombinant receptors transferred in various T cell subsets, providing specific antigens binding in a non-major histocompatibility complex restricted manner, and effective on a large variety of human leukocyte antigen-divers cell populations. Once transferred, engineered T cells act like an expanding "living drug" specifically targeting the tumor-associated antigen, and ensure long-term anti-tumor memory. Over the last decades, substantial improvements have been made in CARs design. CAR T cells have finally reached the clinical practice and first clinical trials have shown promising results. In acute lymphoblastic leukemia, high rate of complete and prolonged clinical responses have been observed after anti-CD19 CAR T cell therapy, with specific but manageable adverse events. In this review, our goal was to describe CAR structures and functions, and to summarize recent data regarding pre-clinical studies and clinical trials in acute leukemia.

Key words: Chimeric antigen receptors; Adoptive immunotherapy; Acute leukemia; T cells; Immune surveillance

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Core tip: Leukemia cells ultimately escape to the immune system, due to various mechanisms such as limited availability of tumor specific T cells or down-regulation in major histocompatibility complex expression. Chimeric antigen receptor (CAR) T cell technology redirects immune reactivity towards a broad variety of chosen antigens in a human leukocyte antigen-independent manner. Recent introduction of co-stimulatory domains

in the CAR construct enhances significantly *in vitro* and *in vivo* expansion and persistence of these genetically modified T cells. First clinical trials, especially with anti-CD19 CAR T cells, report promising results in acute lymphoblastic leukemia.

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INTRODUCTION

Despite recent advances in therapeutics over the last decades, relapses remain a major concern in acute leukemia (AL). Despite complete remission (CR) achievement, leukemia stem cells (LSCs) resist to therapeutic strategies, hiding into bone marrow hematopoietic niches or other unknown sanctuaries^[1]. More than evading apoptosis and self-sufficiency of growth signals, these leukemia cells are also characterized by their ability to evade the immune system. Malignant cells escape such immune surveillance through the outgrowth of poorly immunogenic tumor-cell variants, known as immune selection, and/or through disruption of the immune system^[2,3]. A robust innate immune system is mandatory to avoid relapses by targeting chemoresistant malignant cells, underlining that bone marrow should be preserved as many as possible from aggressive chemotherapy agents. Allogeneic stem cell transplantation (ASCT) is a potential way to restore the tumor cell immunogenicity by transferring a brand new immune system. However, ASCT is largely unspecific and the benefit of graft versus leukemia is offset by the potential complications related to graft versus host disease (GVHD)^[4].

In order to achieve long-term survival and good quality of life, other types of immunotherapy have been developed, such as treatments using tumor-associated antigen (TAA)-monoclonal antibodies (mAbs) and more recently adoptive cellular therapies. Adoptive transferred tumor reactive T cells compared favorably with mAbs. They display direct tumor lysis, enhanced bio-distribution and synergism with the immune system through release of cytokines, and long-term memory properties. Cytokine induced killer (CIK) cells are *in vitro* manufactured T lymphocytes with natural killer (NK) cell properties. They can be extracted from human peripheral blood, bone marrow or cord blood mononuclear cells^[5]. They showed a non-major histocompatibility complex (MHC)-restricted lysis function on a broad spectrum of tumor targets *in vitro*, which was confirmed *in vivo*^[6,7]. However, first clinical results were not convincing, probably due to a lack of specificity, with a limited basal anti-leukemia activity and a rapid exhaustion of these cells^[8,9]. Adoptive transfer of

autologous or allogeneic manipulated T cells has proven to be safe and extendable in clinical practice. In patients presenting prolonged lymphopenia, adoptive multi-virus specific T cell transfers have showed promising data in reconstituting anti-viral immunity after SCT or in patients infected by human immunodeficiency virus^[10,11]. Another approach is to genetically engineer lymphocyte subsets to redirect their natural immune response, correct impaired immunity, and improve T cell anti-tumor effector response.

First described in 1989, the concept of the genetic redirection of T cells is based on chimeric antigen receptors (CARs), which are recombinant receptor molecules genetically transferred, redirecting T cells against a specific TAA^[12]. CARs are composed of 3 distinct domains, each displaying their own functions. The extra-cellular domain is generally constituted by a single chain variable fragment (scFv). Its targeting moiety derived from the fused variable heavy and light chains of an antibody. The trans-membrane domain is connected to the scFv through a "spacer" to provide flexibility and stable expression of the extracellular moiety. The intra-cellular signaling domain, usually derived from the CD3 ζ -chain of the T cell receptor (TCR)-CD3 complex, mediates activation of CAR T cells. CAR T cells overcome some primordial limitations of TCR by targeting antigens in a non-MHC manner, and can recognize tumor independently of human leukocyte antigen (HLA) molecules^[13]. After years of investigations to implement gene transfer tools and codify good manufacturing practices, CARs have been considered for human application. The first clinical trials have shown promising results in hematological diseases^[14-17].

In this review, we focused on the CAR backbone technology and its application in the setting of human AL therapy.

CHIMERIC ANTIGEN RECEPTOR T CELLS: STRUCTURE AND FUNCTIONS

CAR is an artificial T cell surface receptor that simulates the physiological response of a T cell receptor and targets native cell surface antigens. However, CARs have the ability to target molecules that can be recognized without requiring peptide processing or HLA presentation. Unlike regular T cells, CAR T cells can restore immunogenicity of tumor cells. They recognize antigens in any HLA background, independently of the patient haplotype, and without any cross-reactive action toward endogenous antigens^[18,19]. With a capacity of binding to any cell surface antigens, including proteins, carbohydrates and glycolipids, CARs can respond to a broader range of targets than native TCR. However, antibody-mediated recognition by CARs is not restricted to peptide antigens and does not exclude targeting MHC presented peptides. Engineered NK cells harboring a scFv specific for HLA*A2 (MHC class I) carrying a peptide derived from the Epstein-Barr virus latent

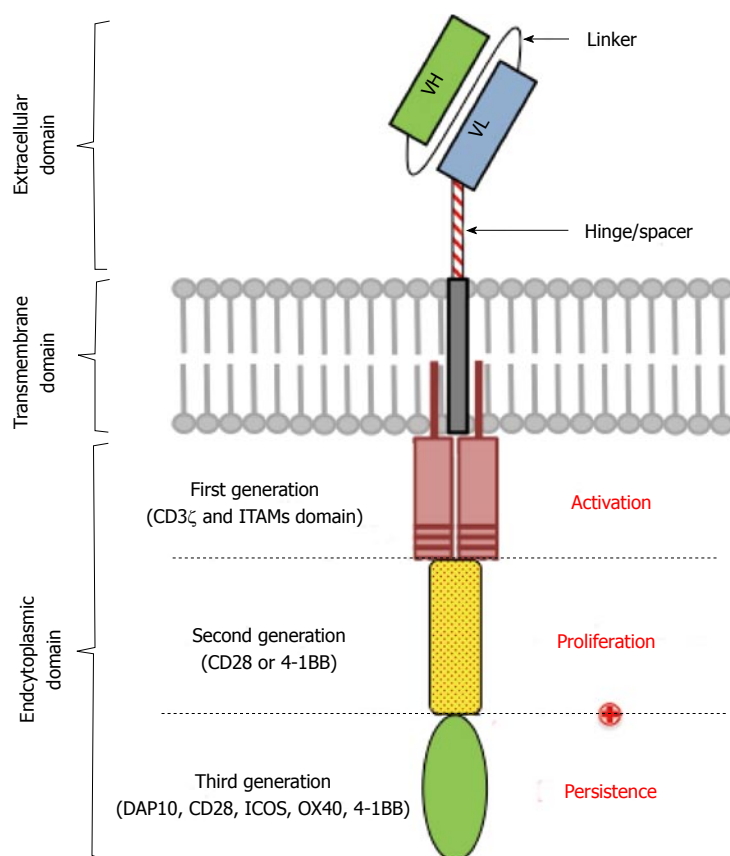


Figure 1 Schema of the general structure of a chimeric antigen receptor. The antigen recognition moiety is composed of the variable domain of heavy (VH) and light (VL) chains of the antibody, specific for a native tumor-associated antigen expressed on the surface of malignant cells. This structure is connected to the cytoplasm by a spacer, generally derived from CD8 or CD28 subunits, and a transmembrane domain. First generation chimeric antigen receptor T cells were solely composed of a single intracellular domain generally derived from CD3 ζ subunit and its immunoreceptor tyrosine-based activation motifs (ITAMs), essential for Lck recruitment and full downstream T cell receptor-like signaling transduction. In order to improve engineered T cells proliferation and persistence, investigators introduced successively one (second generation) or ≥ 2 additional intracellular signaling domains (third generation). ICOS: Inducible costimulator.

protein EBNA3C have been recently developed^[20]. These CAR NK cells showed substantial cytolytic activity against peptide-pulsed HLA-A2⁺ antigen-presenting lymphoblastic B-cell line in a peptide-specific, HLA-restricted manner without cross reactivity with native HLA*A2, paving the way toward highly sensitive and specific target cell killing^[21].

CARs engage the target *via* their extra-cellular recognition subunits, usually a scFv, but other strategies are actually explored, such as antigen-binding domains derived from natural ligand receptors (*i.e.*, NKG2D)^[22,23]. These TAA extra-cellular moieties are either derived from murine or humanized Fab's (variable fragment of an antibody) or synthesized *via* phage display libraries (Figure 1). Because of their accessibility, murine scFvs are the most frequently used, but they are considered more immunogenic than those derived from human libraries. The major risk is to induce humoral and/or cell mediated immune responses as previously reported^[24]. There is currently broad evidence that distinct epitopes of a same antigen, as well as their distance to the cell membrane, have not the same potential upon T cell activation. Based on the kinetic segregation model (KSM) relating TCR activation and ligands size-sensitivity, several reports support that this also occurs in CAR T cells^[25]. The KSM implied that TCR engagement through distal epitopes binding creates larger TCR-MHC-peptide complexes and that close-contact zone displays the synapse to phosphatase action such as CD45 or CD148 repressing TCR signaling. Conversely, targeting more proximal epitopes favored more potent TCR-MHC

interaction and more efficient downstream signaling^[26]. In a study assessing the anti-leukemia effect of anti-CD22 CAR T cells, it was showed that proximal targets have superior anti-leukemia effects^[27]. This was confirmed by further published data^[28]. An increased affinity for the target was not necessarily associated with an increased cytotoxicity. CARs with high affinity for an antigen had a higher proliferation rate than CARs with lower affinity, although this increased affinity was not correlated with higher T cell effector functions^[29,30]. Similarly, the effects of antigen density are not well understood but it seems that CARs exert various cytolytic activities according to antigen expression. In a CD123 CAR T cells model, it was demonstrated that engineered T cells eliminated CD123^{high} cells, while CD123^{low} targets survived in co-culture^[31]. A high affinity and density regarding a specific antigen is not an absolute condition for an optimal anti-tumor activity, but this should be considered as an advantage in order to reduce the severity of the "on target/off tumor" effect. Despite technical considerations, identifying the perfect target remains a matter of debate, specifically in AL. The ideal antigen target should be homogeneously expressed on malignant cells without ubiquitous expression on healthy tissues, and should be critical for tumor cell survival. CD19 is currently the best candidate in B cell precursor acute lymphoblastic leukemia (ALL). The situation is more complex in acute myeloid leukemia (AML), for which the best target still remains to be defined.

A short spacer region, which is the least aspect

of CAR design under discuss, generally follows the scFv's moieties. With the trans-membrane domain, these structures contribute to flexibility, accessibility and synapse formation of the extra-cellular complex. They also seem to influence CARs specificity^[32,33]. The simplest form of spacer region is that derived from human immunoglobulin (Ig) Fc (constant fragment) (*i.e.*, IgG4) potentially linked with CH₂CH₃ molecule(s). However, constant regions from human CD8 α and CD28 have been until now the most frequently used in pre-clinical assays (Figure 1). Following scFv binding moiety interaction with its target, CAR T cells need an active signaling transfer through their intra-cellular domain for proliferation, cytokine production, and acquisition of effector functions. In order to enhance T cell persistence and anti-tumor functions *in vivo*, research in CAR T cells has been focused on co-stimulation over the last decade.

CO-STIMULATORY DOMAINS IN CAR T CELLS: FIRST TO THIRD GENERATION

First generation CARs were composed only of one signaling domain such as the CD3 ζ -chain of the TCR-CD3 complex or the γ -chain of the high affinity IgE Fc receptor (Fc ϵ RI) initially associated with antigen-specific scFv derived from a murine antibody (Figure 1). They were associated with the activation of the phosphatidylinositol and tyrosine kinase pathways. However, they failed to mediate persistent and robust anti-tumor activity^[15,34,35]. Clinical trials with first generation CAR T cells have been conducted in different settings. Some of them are still ongoing, but first results were rather disappointing (Table 1). In a study exploring CD20 CAR T cells in 7 patients with relapsed or refractory indolent lymphoma, only a clinical partial response was obtained in one patient. Moreover, after three infusions of autologous CD20 specific T cells, T cell persistence was less than 10 wk (15-65 d), even after interleukin (IL)-2 administrations^[36]. In a cohort of 4 patients with non-Hodgkin lymphoma (NHL) treated by CD19-CD20 CAR T cells, no objective response and a very limited persistence of T cells was observed. In other patients, cellular anti-transgene immune rejection responses (specific immune response against transfected T cells) have been observed, showing that the immunogenicity of the transgene used is of major importance^[37]. In ALL, there is no clinical data available with first generation CAR T cells. However, two clinical trials based on CD19 scFv-CD3 ζ (19z1) CARs with or without IL-2 supplementation are currently recruiting (Table 1). Nevertheless, CD3 ζ alone is able to induce a TCR-like signal through immunoreceptor tyrosine-kinase based activation motif (ITAM) phosphorylation and lymphocyte-specific protein tyrosine kinase (Lck) recruitment, which are indistinguishable from those generated by an intact TCR currently used to provide

signal one.

It is well known that TCR engagement (signal one) without co-stimulatory signal (signal 2) lead to T cell anergy and rapid activated induced cell death (AICD). Signal 2 is provided by engagement of co-stimulatory domains, mediated essentially by the CD28 superfamily, involving receptors for the agonistic CD80 (B7.1) and CD86 (B7.2) ligands. However, these ligands are generally missing in most cancer cells. This plays a major role in the immune surveillance escape. In this setting, second generation CAR T cells have been designed by adding an additional intra-cellular co-stimulatory domain such as CD28 (forming at the end one polypeptide single chain) to the CAR backbone (Figure 1)^[38,39]. CD28, a disulfide-linked homodimer of the immunoglobulin superfamily is activated by binding either B7.1 or B7.2, expressed on the surface of antigen presenting cells. This represents one of the most potent co-stimulatory pathways. It facilitates Lck recruitment to ITAMs and the formation of the linker activation (LAT) complex. This structure is necessary for signal transduction through phospholipase C gamma (PLC γ) leading to IL-2 promoter activation and full T cell activation. Other co-stimulatory domains have been tested, such as members of the tumor necrosis factor (TNF)-receptor family [4-1BB (CD137) or OX40 (CD134)], ICOS or DAP10^[28,40-42]. Second generation CAR T cells generally show sustained polyclonal proliferation without B7-CD28 engagement, enhanced IL-2, interferon-gamma (IFN γ), TNF-alpha (α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine production and enabled resting primary T cells to survive^[43]. In two recent clinical studies, it was reported that CAR (scFv-4-1BB-CD3 ζ) T cells undergo 1000-fold amplifications *in vivo* compared to only 3.75-fold reported in first generation CAR clinical trials^[44,45]. This may be related directly on the co-stimulatory signal that seems to counteract the inhibition effect of tumor growth factor β 1 (TGF- β 1) secreted by regulator T cells (T_{reg}) on proliferating CAR T cells^[46]. Generally, TGF- β 1 represses IL-2-dependent T cell amplification, CD122 up-regulation (IL-2R- α), and IFN γ production. Dual signaling provided by second-generation CAR T cells enhanced engineered T cell persistence comparatively to their first generation counterparts. This was demonstrated in patients treated with either CD3 ζ or CD3 ζ -CD28 CARs^[47]. Besides overcoming PD1-mediated inhibition (an activation-induced inhibitory receptor expressed on T cells), co-stimulation through CD28 induces intrinsic survival signals independently of the exogenous survival signals mediated by IL-2. *In vitro*, CD28-B7 interaction leads to an up-regulation of Bcl_{xL}, an anti-apoptotic protein of the Bcl2 family, and to resistance to apoptosis by Fas (CD95, APO-1) cross-linking^[48]. This interaction enable protection from intrinsic and extrinsic cell death signals and promotes survival of the expanding lymphocyte population^[49]. Surprisingly, it seems that the adjunction

Table 1 Ongoing clinical trials recruiting patients with acute leukemia

Clinical trial.gov ID	Center	Disease	Antigen (scFv)	CAR signaling domain	Vector	Transplantation history	Origin	Lymphocyte depletion	Patients age
Acute lymphoid malignancies									
NCT01044069	MSKCC	B-ALL	CD19	CD3 ζ -CD28	γ -retrovirus	Autologous	Autologous	Chemo	≥ 18 yr
NCT01860937	MSKCC	B-ALL	CD19	CD3 ζ -CD28	γ -retrovirus	Autologous	Autologous	Cy	up to 26 yr
NCT01430390	MSKCC	B-ALL	CD19 VST (EBV)	CD3 ζ	Lentivirus	Relapse post-ASCT	Allogenic	Chemo	up to 19 yr
NCT01626495	CHOP	B-ALL	CD19	CD3 ζ -4-1BB <i>vs</i> CD3 ζ	Lentivirus	Relapse after ASCT	Autologous	Chemo	1-24 yr
NCT02030847	University of Pennsylvania	B-ALL	CD19	CD3 ζ -4-1BB	Lentivirus	Relapse $\pm$ after ASCT	Autologous	NS	≥ 18 yr
NCT00840853	BCM	B-ALL/NHL/CLL	CD19 VST (EBV)	CD3 ζ	γ -retrovirus	Post-ASCT	Allogenic	No	No limit
NCT00586391	BCM	B-ALL/NHL/CLL	CD19	CD3 ζ -CD28	γ -retrovirus	No ASCT	Autologous	NS	≥ 18 yr
NCT02132624 (not open)	Uppsala University	B-ALL/NHL	CD19	CD3 ζ -4-1BB-CD28	γ -retrovirus	Relapse $\pm$ after ASCT	Autologous	NS	≥ 18 yr
NCT01593696	NCI	B-ALL/NHL/CLL	CD19	CD3 ζ	γ -retrovirus	Relapse $\pm$ after ASCT	Autologous	Flu/Cy	1-30 yr
NCT01087294	NCI	B-ALL/NHL/CLL	CD19 VST (EBV)	CD3 ζ	γ -retrovirus	Relapse post-ASCT	Allogenic	Cy	18-75 yr
NCT01683279	Seattle Children Hospital	B-ALL	CD19 EGFRt ⁺	CD3 ζ -CD28	Lentivirus	No ASCT	Autologous	Cy	1-26 yr
NCT02028455	Seattle Children Hospital	B-ALL	CD19 EGFRt ⁺	CD3 ζ -4-1BB	Lentivirus	Relapse after ASCT <i>vs</i> no ASCT	Autologous	Cy/Flu/TBI	1-26 yr
NCT01865617	FHCRC	B-ALL/NHL/CLL	CD19	CD3 ζ	Lentivirus	> 1 st relapse	Autologous	NS	≥ 18 yr
NCT01475058	FHCRC	B-ALL/NHL/CLL	CD19 VST (CMV, EBV)	CD3 ζ	Lentivirus	Post-ASCT	Allogenic	No	18-75 yr
NCT02051257	City of Hope Medical Center	B-ALL/NHL/CLL	CD19 EGFRt ⁺	CD3 ζ -CD28	Lentivirus	Post-ASCT	Autologous	Chemo	≥ 18 yr
NCT02146924 (not open)	City of Hope Medical Center	B-ALL	CD19 EGFRt ⁺	CD3 ζ -CD28	Lentivirus	Relapse/progression $\pm$ after ASCT	Autologous	Cy	≥ 18 yr
NCT01195480	London University College	B-ALL	CD19 (EBV-CTL)	CD3 ζ	γ -retrovirus	Relapse after 1 st ASCT	Allogenic	NS	Up to 18 yr
NCT01864889	Chinese PLA General Hospital	B-ALL/NHL/CLL	CD19	CD3 ζ -4-1BB <i>vs</i> CD3 ζ	γ -retrovirus	Relapse $\pm$ after ASCT	Autologous	NS	5-90 yr
NCT01735604	Chinese PLA General Hospital	B-ALL/NHL/CLL	CD20	CD3 ζ -4-1BB <i>vs</i> CD3 ζ	γ -retrovirus	Relapse $\pm$ after ASCT	Autologous	NS	18-90 yr
NCT01362452	MDACC	B-ALL/NHL/CLL	CD19 (cord blood derived)	CD3 ζ	Transposon	Relapse post-ASCT	Allogenic	No	1-75 yr
NCT01497184	MDACC	B-ALL/NHL/CLL	CD19	CD3 ζ	Transposon	Post-ASCT	Allogenic	No	1-65 yr
Acute myeloid malignancies									
NCT01864902	Chinese PLA General Hospital	AML	CD33	CD3 ζ -4-1BB <i>vs</i> CD3 ζ	γ -retrovirus	Relapse $\pm$ after ASCT	Autologous/Allogenic	NS	5-90 yr
NCT02159495	City of Hope Medical Center	AML	CD123 EGFRt ⁺	CD3 ζ -CD28	γ -retrovirus	Relapse/refractory AML	Autologous	Cy	≥ 18 yr

CAR: Chimeric antigen receptor; AML: Acute myeloid leukemia; ASCT: Allogeneic stem cell transplantation; B-ALL: B-lineage acute lymphoblastic leukemia; BCM: Baylor College of Medicine; CHOP: Children Hospital of Philadelphia; Chemo: Chemotherapy prior CAR T cells infusion; CLL: Chronic lymphoid leukemia; Cy: Cyclophosphamide; FHCRC: Fred Hutchinson Cancer Research Center; Flu: Fludarabine; MDACC: MD Anderson Cancer Center; MSKCC: Memorial Sloan-Kettering Clinical Center; NCI: National Cancer Institute; NS: Not specified; NHL: Non Hodgkin lymphoma; VST: Virus specific T lymphocytes; EBV: Epstein-barr virus; CTL: Cytotoxic T-cell lymphoma.

of a co-stimulatory domain (*i.e.*, CD28) do not modify the threshold of antigen-dependent MHC-independent T

cell activation^[50]. It seems therefore that incorporation of at least one co-stimulatory domain in a CAR cons-

tract is mandatory for complete T cell activation. However, a major remaining issue concerns the potential superiority of one endodomain comparatively to another. Because of their heterogeneity, some CD28 or 4-1BB-based second generation CARs have been compared, essentially in mice models, and showed contradictory results. Regarding cell proliferation and cytokine production, second generation CD28 transfected CAR T cells were superior to CD19z1 (first generation CAR T cells), but also to constructs with 4-1BB, OX40 or DAP10 endodomains^[51]. Conversely, it was showed that CD28⁺ and 4-1BB-based CAR T cells, directed against the same epitope (SS1 scFv-based chimeric receptor), have the same anti-tumor activity^[52]. Other pre-clinical studies, containing 4-1BB signal transduction endodomain, exhibited a greatest anti-leukemia activity and prolonged *in vivo* survival^[53]. In AL, most of the current clinical trials use indistinctly CD28 or 4-1BB endodomains. Comparisons between intra-cellular signaling domains should take into account all representatives of distinct antigen epitopes, their location, their density, and their affinity for the target in order to clarify their exact contribution in the anti-tumor activity.

More recently, third generation CAR T cells have been developed, including more complex structures with three or more signaling domains, enabling wider T cell effector function in a specific fashion (Figure 1). In mice models, the inclusion of multiple endodomains showed enhanced functionality and greater potency of tumor targeted T cells *in vivo* and *in vitro*. However, this was not confirmed in other models^[54-56]. In a study comparing the consequences of CD28, OX40 and 4-1BB co-stimulation, it was showed that third generation CD3 ζ -CD28-OX40 CARs prevent AICD in memory T cells, and substantially improve effector functions in both naive and memory CD4 and CD8 T cells. They also increase cytokine production comparatively to second generation CAR T cells combination^[57]. Considering these results and the substantial improvement in terms of efficacy, the addition of a third endomain should be associated with increased long-term persistence and should delay anergy. However, a pilot study, using CD28-4-1BB-based CARs in 4 NHL patients, reported one partial response, one disease progression, and 2 cases free of disease at 12 and 24 mo, respectively. In this study, engineered T cells were detectable up to 12 mo after infusions by quantitative polymerase chain reaction (PCR) at low levels (< 1%)^[58]. Because of discrepancies among pre-clinical and clinical results, further investigations are warranted to optimize our understanding regarding CAR T cell signaling. Because massive activation and uncontrolled proliferation of genetically modified T cells has been associated with serious adverse events, investigators should take into account these features and find the right balance between efficiency of these engineered T cells and an acceptable tolerance.

CARS MANUFACTURING: A PERSONALIZED ASSEMBLY-LINE WORK

Investigators developed distinct approaches to introduce efficiently CAR constructs into T cells. Actually, most of them utilized viral transduction systems (mostly γ -retroviral) leading to permanent sequence integration into T-cell DNA. However, this kind of vehicle presents disadvantages: (1) high costs related to the production process; (2) risks of CAR expression silencing due to terminal repeat alterations; (3) risks of insertional mutagenesis; and (4) production of replication competent virus. Alternatively, lentiviral vectors, which are theoretically less genotoxic, can permanently transduce T cells, but display inferior yields of transgene integration^[59,60]. However, no genotoxic-related events related to viral vectors have been reported until now in human clinical trials using manipulated T cells^[61]. Moreover, residual vector sequences present in the genome lead to immunogenic epitope expression and can increase anti-CAR mediated response. First clinical trials in B-cell lineage ALL using viral vectors reported very limited gene transfection efficiency (5%-15%), without impairing anti-leukemia effect^[62,63].

Non-viral based DNA transfection represents an interesting alternative because of its low cost and its theoretically limited insertional mutagenesis [insertion at thymidine-adenosine (TA) dinucleotide base pairs and non-preference of integration into transcriptional units]. DNA plasmids were the first non-viral vehicles that have been tested. They seemed less immunogenic and independent of the sequence size comparatively to viral vectors. However, because of low chromosomal intake and sustained transgene expression, long-term cultures are required to obtain a sufficient number of CAR-modified T cells, despite a potential negative effect on T cell activity and *in vivo* expansion. One major issue with plasmid vectors is represented by the genomic integration of multiple copies and transfected DNA hypermethylation, leading to transgene silencing^[64]. Originally described in 1996, "Sleeping Beauty (SB)" transposase/transposon, based on the concept of "jumping genes" discovered by McClintock, enabled to overcome these issues and to restart non-viral DNA vectors^[65]. Generally, one plasmid is loaded with a transgene, named transposon, surrounded by inverted repeats that contain short direct repeats (Figure 2). These sequences are recognized by an enzyme (transposase) transported in a second plasmid, which cut the transposon out of the plasmid. The genetic cargo is then delivered into the targeted cell cytoplasm by any of the established non-viral delivery techniques, and inserted randomly into TA dinucleotide base pairs of the recipient (Figure 2). Costly but safer, the SB platform displays attractive features for human gene therapy, to such an extent that many protocols for manufacturing clinical grade T cells recently came to light^[66]. At the last European Bone Marrow Transplantation (EBMT)

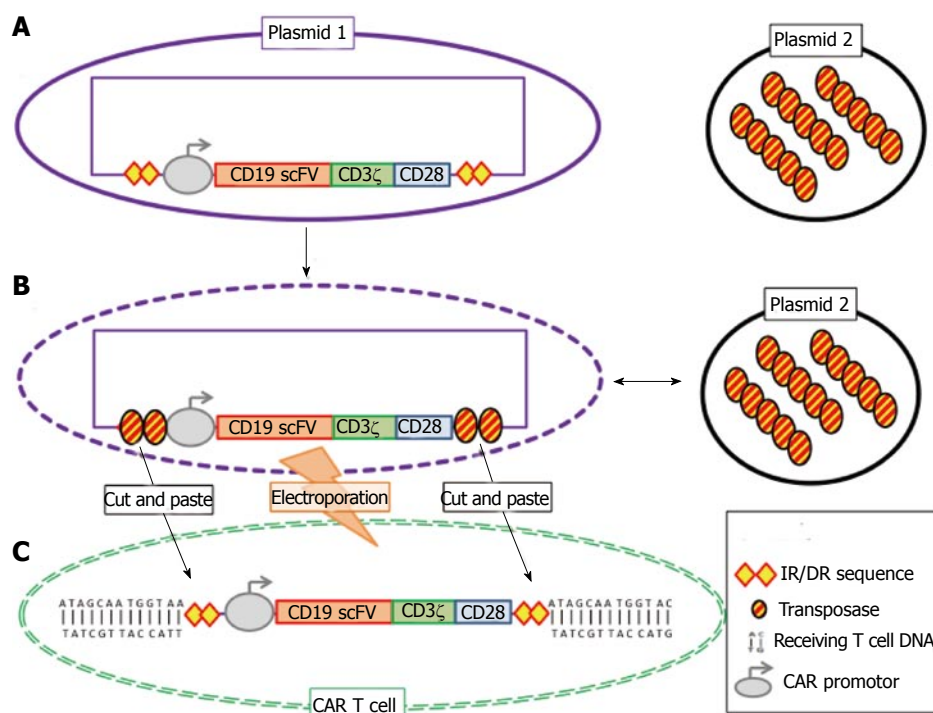


Figure 2 Schema of the transposon/transposase “sleeping beauty” system. A: The genetic cargo of a first plasmid (plasmid 1) is the chimeric antigen receptor (CAR) (anti-CD19 CD3 ζ -CD28) flanked by inverted/short direct repeats (IR/DR). The whole set composed the transposon. The enzyme transposase is loaded in a second plasmid, which is specific of the IR/DR sequence; B: Materials of the two plasmids are fused together by electroporation, an electric current opening pores or channels at the cell surface. Transposase binds to the IR/DR sequences; C: The enzyme cut out of the plasmid the flanked sequences (CAR transgene with its promoter) and transfect the genetic cargo into a random DNA sequence of the recipient (T cell).

meeting, promising results were reported about the production of third generation CD19/CD123 CIK CAR cells. With about 50% CAR transfection yields, investigators are now able to greatly expand CAR T cells and produce clinical grade within 3 wk (vs 90 d with retroviral vectors). To our knowledge, two human clinical trials using the SB system are currently ongoing at the MD Anderson Cancer Center (Table 1)^[67].

Clinical trials have reported serious adverse events during CAR T cells therapy in early clinical phase trials, which were related to uncontrolled T cells proliferation, cytokine storm, or “off tumor” effect^[68,69]. In this context, strategies to incorporate abortive mechanisms inside the genomic load have emerged. Based on *ex vivo* genetic modification of donor T lymphocytes, suicide gene therapy with the Herpes simplex thymidine kinase (Hsv-tk)/ganciclovir (GCV) suicide system in the context of ASCT has demonstrated its safety and efficacy^[70]. In order to facilitate its clinical development, this technology has been applied to CAR T cell development. Despite successes in the treatment of severe GVHD, Hsv-tk/GCV system showed some limitations: (1) the HSV-TK protein can be immunogenic in an immunocompetent situation; (2) the system can lead to unexpected elimination of modified T-cells in case of treatment with GCV; and (3) the system can show efficacy only on dividing cells^[71]. Other non-immunogenic suicide systems have been developed, especially through a modified caspase 9 (iCasp9) member of the intrinsic apoptosis

pathway^[72]. A modified FK506-binding protein (humanized FKBP12), belonging to a family of protein which display a propyl isomerase activity, has been combined with caspase 9. Infusion of a synthetic drug, AP1903, allows the dimerization of iCasp9 and activates the apoptosis cascade. Beside optimal bio-distribution of the dimerization inducer, iCasp9-based cell safety switch offers superior pharmacodynamical properties than the HSC-tk system, and leaves GCV available for antiviral therapy^[73]. The major issue of this suicide gene strategy is that anti-tumor T cells could be definitely eliminated, impairing the efficacy of cellular therapy. However, to date, no suicide gene therapy has been already used in human clinical trials with engineered CAR T cells. Although not as effective as CAR using integrating vectors technology, transient CAR expression is an interesting alternative approach currently under investigation. According to a recent study published by the University of Pennsylvania, CAR mRNA can be efficiently transduced into T cells by *in vitro* repeated electroporation procedure and administrated repeatedly safely, avoiding the risk of CAR T cells accumulation. This approach precludes transgene persistence for one week or two, and a rapid decrease of toxicity in case of adverse events after T cell discontinuation^[73]. The current stocks and production rates of adequate serum for good manufacturing procedure (GMP) are insufficient regarding the outgrowing demand, suggesting a further development of serum-free conditions for cell therapy cultures^[74].

CAR'S CULTURE: EXPANSION, PERSISTENCE AND TRAFFICKING

The technique and the duration of CAR T cell cultures prior CAR transfection and infusion of the product may be also critical in the setting of clinical activity. Autologous/allogeneic non modified-T cells are harvested from peripheral blood by leukapheresis, with heterogeneous threshold according to the underlying pathology. Then, naive T cells are expanded *ex vivo* generally with an artificial-antigen presenting cell system or anti-CD3/CD28 coated beads in combination with various cytokines (IL-2, IL-15, IL-17, IFN γ). As T cells are grown under GMP, expansion protocols and manufacturing input may vary among centers, depending on transduction efficiency yields, CD4/CD8 ratio, and final T cell phenotype. Most of culture protocols support the acquisition of a central memory phenotype (CD45RA⁺/CD122^{low}/CD62L^{low}/CCR7⁺), which is associated with self-renewal, high proliferation potential, and increased longevity. However, it was highlighted that proliferation, cytolytic activity, and persistence are markedly hampered by long-term *ex vivo* cultures, likely due to T cell exhaustion and telomere length shortening^[36,58]. In addition, the T cell subtype transduced with CARs seems to play a key role in the clinical efficacy of adoptive immunotherapy. Due to their well-known cytolytic activity through granzyme/perforine and Fas/FasL complexes, $\alpha\beta$ CD8⁺ CAR T cells have been considered as the effective component of CAR T cell-based therapy and have been the major T cell subset used in pre-clinical trials. However, recent data revealed that CD4⁺ T cell subset transduced with CARs showed cytolytic activity similar to that of CD8⁺ T cells toward their targeted antigen. They are also useful on CAR mediated activation^[75,76]. All T cell subsets play a key role in the anti-tumor immune response. The most powerful subset depends likely on tumor phenotype, its accessibility, and the tumor-cytokine microenvironment. In this setting, $\gamma\delta$, Th17, central memory, stem cell like memory, virus specific T cells and hematopoietic stem cells are also a part of CAR-based therapy^[77-80].

For a long-term disease control by adoptive immune surveillance, engineered T cell persistence is highly warranted. Preclinical studies indicate that tumor burden and the degree of lymphocyte depletion prior T cell infusion are likely to be critical requirements for proper T cell expansion and persistence. First clinical trials indicate that persistence and *in vivo* expansion of adoptively transferred T cells is strongly correlated with treatment outcome, as previously documented with tumor-infiltrating lymphocytes (TILs)^[45,69]. Conditioning chemotherapy is a way to enhance persistence and expansion of transferred CAR T cells. However, the optimal regimen is still a matter of debate. Although underlying mechanisms are not fully understood, this model probably involves homeostatic proliferation. Actually, T cell populations are tightly regulated by homeostatic mechanisms that maintain the T cell pool

at a near-constant level. These mechanisms mimic chronic T cell activation, depend upon TCR/MHC self-peptide interaction, and involve cytokines, such as IL-7 and IL-15. During the first clinical trials in chronic lymphoid leukemia (CLL) patients, anti-CD19 CAR T cell infusions without conditioning chemotherapy showed limited T cells persistence and disappointing results^[45]. Conversely, in a cohort of 5 relapsed ALL patients treated with 19-28z CAR T cells after lymphocyte depletion by cyclophosphamide, it was reported long-term CAR modified T cells persistence for 3 to 8 wk. Four of the five patients underwent ASCT and remained in minimal residual disease (MRD)-negative status. This study was the first to suggest an inverse relationship between CAR T cells clinical efficiency and initial tumor burden at the time of infusion. It was also shown that lymphocyte depletion prior CAR T cells infusion enhanced CAR T cells persistence^[62]. Lymphocyte depletion following chemotherapy (cyclophosphamide/fludarabine) or total body irradiation (TBI) suppresses repressive cell populations, such as T_{reg}, and cells competing for stimulatory cytokines (IL-2, IL-7, IL-15, IL-21), preventing rapid anergy of adoptively transferred T cells and transient "cytokine sink". "Cytokine sink" corresponds to the competition between transferred and host T cell subsets regarding homeostatic cytokines, and can decrease *in vivo* T cell expansion^[81]. IL-2, which has been widely used for adoptive T cell expansion *in vivo*, is essential for the maintenance of peripheral self-tolerance and is able to promote T cell effector functions. However, the administration of exogenous cytokines remains highly controversial. It has been shown that homeostasis and efficient suppressor functions of T_{reg} were essentially mediated by IL-2 receptor signaling. The infusion of anti-IL-2 plus IL-15 in tumor bearing mice increases effector functions of adoptively transferred T cells^[82]. Although IL-2 represents a key factor in the induction of terminal differentiation of effector T cells, its use for *in vivo* expansion may impair anti-tumor immunity. More recently, cyclophosphamide lymphocyte depletion has been shown to significantly reduce FoxP3 T_{reg} and to induce IL-12 and IFN γ secretion^[83]. IL-12 is a heterodimeric cytokine secreted by APCs, known to enhance T cell clonal expansion and T cell effector function in concert with TCR complex signaling (signal 1 and 2), serving as signal 3. This cytokine favors NK recruitment, but also avoid T cell anergy and T_{reg} action on effector T cells. This was illustrated by CAR targeted T cells (first generation 19z1) modified to produce autocrine IL-12 and to manage B lymphocyte depletion associated with tumor eradication^[84]. The exact schedule for infusing CAR T cells after lymphocyte depletion is still a matter of debate. Nevertheless, recent evidences support the superiority of a rapid transfer in term of T cell engraftment and immune reconstitution^[85,86].

AL is defined by an erratic clonal proliferation of immature hematopoietic stem cells, essentially localized in the bone marrow. Despite 60%-80% of CR

achievement after a first induction course of chemotherapy, most of AL patients will ultimately relapse, due to LSCs or persistence of resistant clones in unreachable reservoirs to standard chemotherapy. CAR T cells have to traffic in the entire body, after *ex-vivo/in vivo* activation, to clear central (bone marrow) and peripheral reservoirs. In solid tumors and lymphomas, preclinical studies and clinical trials have established that CAR T cells, specifically when using second generation CARs, can accumulate and persist over time at the site of disease^[47,62]. In a recent phase I study using anti-LeY CAR T cell therapy in AML, it was demonstrated by detection of a transgene derived PCR signal and by radio-labeled cells that CAR T cells have a systemic distribution and migrate to the bone marrow^[87]. Genetically engineered T cells seem to naturally traffic to the bone marrow, but also to the central nervous system (CNS), with however 5 to 10-folds less than what is observed in blood^[17,44]. The reasons why CAR T cells naturally cross the meningeal barrier remain intriguing. However, this is a major concern regarding B cell lineage-ALL, for which CNS involvement could have dramatic repercussions on treatment outcome. Lymphocyte depletion by cyclophosphamide or TBI stimulate the production of chemo-attractants by the microenvironment, and thus favor CAR T cells migration and engraftment in the bone marrow^[88]. Because chronic activation is associated with down-regulation of homing receptors such as CD62L, investigators are currently exploring genetic expression of chemokine/cytokine receptors (*i.e.*, CXCR2) and *ex vivo* cell surface glycan engineering (*i.e.*, modifications of fucosyltransferase, an enzyme involved in hematopoietic stem cell homing) in order to enhance bone marrow trafficking, which is critical for successful leukemia cell eradication^[89,90].

CARS IN ACUTE LEUKEMIA: A MATTER OF TARGET

Acute lymphoblastic leukemia

Historically, the first hematological malignancies treated with CAR T cells were CLL and lymphomas from the B cell lineage. As mentioned above, several issues have to be considered to select a target for CAR-mediated tumor cell recognition and unfortunately most of the TAA are self-antigens expressed on healthy tissues. In this setting, CD19 arise as a perfect target antigen, since it is expressed in almost all B cell malignancies (except 5% of undifferentiated immature B cell lineage-ALL) and long-term B cell depletion is generally well tolerated (*i.e.*, chimeric monoclonal antibody rituximab). CD19 is expressed on normal B lineage cells from pro-B-cells to mature B cells and plasma cells, but hematopoietic stem cells and other tissues lack this antigen expression. CD19 is thought to play a role in the balance between self-tolerance and antigen activation of the B cell receptor (BCR) complex in a specific and sensitive manner. Another hypothetical advantage of CD19 is

that CD19-positive cells are constantly produced in the bone marrow, thus providing an inexhaustible source of antigen stimulation. It was shown that CD19 was involved in Myc driven B cell oncogenesis in a BCR-independent manner, through paired box protein 5^[91]. Although they are sharing the same target, B cell lineage malignancies may respond differentially to adoptively transferred T cells. Based on promising pre-clinical data with first and second-generation CARs, first trials with anti-CD19 CARs were designed in patients with recurrent indolent NHL or CLL. They showed promising results with prolonged CR^[37,47,69]. However, the initial enthusiasm was hampered by further trials showing no CR achievement and very limited CAR T cells persistence^[47,62]. Discrepancies among studies could be attributed to the suppressor role of the tumor microenvironment, differences in treatment history, pretreatment tumor burden, potential tumor resistance to the lymphocyte depleting agent, and/or impaired T cell immunological function in lymphomas^[92,93]. CD-19-CAR T cells appeared as the best choice for the treatment of B-cell lineage-ALL with an overall CR rate of 80% (Table 2). The first case of treatment by CD19-CAR T cells in B cell lineage-ALL has been published by the Memorial Sloan Kettering Cancer Center. The patient was in early relapse and has been treated with second generation 1928z CAR T cells (second generation CAR T cells anti-CD19 scFv CD28-CD3 ζ). After CR achievement, the patient received cyclophosphamide (3.0 g/m²) followed after 2 d by a split dose of autologous 1928z T cells (1.8×10^8 cells/kg). He underwent ASCT 8 wk after T cell infusion. Prolonged B lymphocyte depletion was observed and related to 1928z CAR T cells persistence confirmed by immunohistochemistry on bone marrow aspirates through 6 to 8 wk post-infusion^[62]. This trial has been recently updated reporting about 13 patients (including the first one) with relapsed/refractory B cell lineage-ALL (of whom 3 patients with Philadelphia-positive B cell lineage-ALL). CR rate was 85% (10/13 patients) with complete molecular responses obtained 7 to 14 d after T cell infusion. Cytokine release syndrome (CRS) was observed in 6/13 patients and was controlled by corticosteroids or anti-IL-6R antibody therapy. Nine of the 13 patients underwent ASCT^[94]. Another phase 1 trial was reported in 5 adults with relapsed/refractory B cell lineage-ALL. They received one single dose of autologous 1928z-CAR T cells (1.5 to 3×10^6 cells/kg) after administration of high-dose cyclophosphamide. All patients became MRD-negative after CAR T cell infusion. Four of them underwent ASCT and were still MRD-negative at the time of the last report. The last patient, ineligible for ASCT and further CAR T cell infusion, relapsed 13 wk after T cell administration. Relapse was due to abrogation of CAR T cells persistence^[63]. Another phase 1 study, using virus-specific 1928z-CAR T cells (CD19 CAR-VSTs, cytotoxic T cells with a native receptor specificity directed to persistent human viruses) in patients relapsing after ASCT or with high-risk B cell malignancies, included 3 patients with B cell lineage-

Table 2 Completed chimeric antigen receptor T cells trials including acute leukemia patients

Indications	CAR construct	Vector	Cell dose	Pre-treatment	Patients	Responses	Relapses	CAR persistence (days median)	Toxicities	Ref.
Acute lymphoid malignancies										
Relapsed B-ALL ± post-ASCT	Anti-CD19 scFv 4-1BB-CD3ζ	Lentiviral	10 ⁶ -10 ⁷ cells/kg	Cy	30	90% CR (27/30)	6 relapses (one CD19)	145	CRS for all responding patients (fever, ARDS, MODS)	[17,97,98]
Relapsed B-ALL post-ASCT	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	10 ⁶ cells/kg	Cy + flu	2	100% CR (5/5)	1 transient CR	ND	Mild CRS, no GVHD	[117]
B-ALL relapsed	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	3 × 10 ⁶ cells/kg	Chemo	13 (3 Ph ⁺ B-ALL)	85% CR (10/13)	1 NR, 1 relapse	ND	6/13 CRS	[62,94]
B-ALL relapsed without prior ASCT	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	1.5-3 × 10 ⁶ cells/kg	Cy	5	100% CR (5/5)	1 relapse (no ASCT)	ND	3/5 mild CRS (MRD ⁺ or bulk)	[63]
Relapsed B-ALL/CLL post-ASCT	Anti-CD19 scFv CD28-CD3ζ VST (CMV, EBV, ADV)	γ-retroviral	1.5 × 10 ⁷ -1.2 × 10 ⁸ cells/m ²	No	4 (4/8)	75% CR (3/4), 25% PD (1/4)	1 relapse (no ASCT)	80	No CRS, No GVHD	[95]
Acute myeloid malignancies										
Relapsed AML	Anti-LeY scFv CD28-CD3ζ	γ-retroviral	1.3 × 10 ⁹ cells	Chemo	4	25% CR, 50% SD	4 relapses	14-120	Neutropenia, skin "flare" reaction, fever, rigors	[87]

CAR: Chimeric antigen receptor; ADV: Adenovirus; AML: Acute myeloid leukemia; ARDS: Acute respiratory distress syndrome; ASCT: Allogeneic stem cell transplantation; B-ALL: B-lineage acute lymphoblastic leukemia; Chemo: Chemotherapy prior CAR T cells infusion; CLL: Chronic lymphoid leukemia; CMV: Cytomegalovirus; CR: Complete remission; CRS: Cytokine release syndrome; Cy: Cyclophosphamide; EBV: Epstein Barr virus; GVHD: Graft versus host disease; MODS: Multi-organ dysfunction syndrome; ND: Not determined; NR: No response; PD: Progressive disease; SD: Stable disease; VST: Virus specific T lymphocytes.

ALL. No pre-conditioning regimen was administered. CD19 CAR-VSTs required several rounds of expansion (5-6 wk of cell culture) before infusion in order to reach clinical CAR T cell threshold. No GVHD was observed and all B ALL patients achieved CR, but relapsed between 2 to 8 mo after T cell infusion (Table 2)^[95]. The largest cohort was initially reported by the Children Hospital of Philadelphia in 2013 and updated at the 2014 EBMT meeting. The study included 30 patients (25 children and 5 adults) with chemo-refractory B cell lineage-ALL or ALL relapsing after ASCT. Patients received CTL019 CARs (second generation anti-CD19-scFv CAR T cells) coupled with 4-1BB endodomain and lentivirus transfected. The median dose of CTL019 was 3.7×10^6 cells/kg administered in 3 doses with 5×10^9 total cells as a target dose. Lymphocyte depletion regimens varied among patients, but were mostly based on cyclophosphamide administered during the week prior T cell infusion. Patients were at least in second relapse or were refractory to several lines of treatment. Overall, 90% of them achieved CR, and half of them underwent subsequently ASCT. With a median follow-up of 3.4 mo (range: 2-18 mo), only 6 patients relapsed. In one case, relapse occurred with emergence of CD19 negative blast cells. As previously reported with blinatumomab (a

bi-specific T-cell engager antibody designed to redirect CD3⁺ cytotoxic T cells to CD19⁺ malignant B cells), this relapse from CD19 negative leukemia cells illustrates the impact of such a targeted therapy on leukemia sub-clones^[96]. No such cases have been reported in CLL^[93]. CRs were observed independently of the level of tumor burden before T cell infusion. Among the 16 patients treated after ASCT, T cells were efficiently collected from recipient. No GVHD recurrence was observed after CAR T cell infusions. CTL019 cells expanded to levels that were more than 1000 to 10000 times as high as the initial levels. CAR T cell persistence was observed until 6 to 18 mo. This was concomitant of B lymphocyte depletion in responding patients, as previously reported in CLL patients treated with CTL019. Long-term persistence (≥ 145 d) was significantly associated with CR achievement. All responding patients developed some degree of delayed CRS, which was concomitant to T cell expansion and increased levels of IL-6. This seemed in relationship with the tumor burden prior CAR T cell infusion (Table 2)^[17,97,98]. Overall, these first results suggest that CD19 gene-modified T cell therapy is likely not enough efficient by itself, but it should be considered as a potentially life-saving bridge to ASCT. Recently, a multi-center clinical consortium was proposed in order

to harmonize practices and further export this new technology to academic institutions.

Alternative targets are likely to be developed. CD22, a type I trans-membrane sialo-glycoprotein expressed specifically on B lineage cells, is closely related to the BCR pathway. Moxetumomab pasudotox, an anti-CD22 covalently fused to a pseudomonas exotoxin, has shown promising results^[99], and could be considered as a suitable possible choice. On the other hand, in order to prevent antigen loss through selective clonal pressure and to improve tumor specificity, investigators are developing combinatorial antigen recognition strategy, and oncogene targeted CARs^[100,101].

Acute myeloid leukemia

Treatment of AML remains a great challenge. Whereas combined efforts in the field of intensified chemotherapy, SCT, and supportive care have yielded to improve survival, more than 50% of patients will relapse. Because 70% of AML patients are over 60 years, many of them are not eligible for ASCT^[102]. Recent preclinical reports demonstrated that CAR T cells have the potential to effectively and durably eradicate primitive myeloid blast cells^[103,104]. Although two trials are currently recruiting in China and in Australia, only one phase 1 trial, targeting Lewis Y (LeY) antigen coupled with cytoplasmic CD28 and CD3 ζ chain, has been reported so far in the literature regarding AML (Table 1). LeY antigen is a difucosylated carbohydrate antigen, part of the human blood system. It is widely expressed on AML cells, but has a limited expression in healthy tissues. However, its exact role and its functional significance for survival of the leukemia cells have to be elucidated. Four patients received 1.5 to 4.7 $\times 10^6$ cells/kg CAR T cells 6 wk after fludarabine-based chemotherapy regimen. A modest T cell expansion and persistence (1 to 10 mo) was observed and resulted in limited advantages. Only 2 out of four treated patients showed a reduction of leukemia cells. Beside these disappointing results, this study conveys major endpoints. Infusions were well tolerated with no CRS even in cases presenting with a high tumor burden. Second, it was demonstrated by SPECT imagery that anti-LeY CAR T cells can target bone marrow leukemia cells, but also peripheral lesions (leukemia cutis). The absence of down-regulation of LeY antigen after CAR T cell infusions suggests that this target is suitable for long-term immune control of the disease^[87]. However, the weak anti-leukemia activity of this antigen-based therapy should be balanced with combinatorial recognition strategy or loaded in more potent effectors such as CIK cells. Other antigens are also on the bench, such as CD33, CD123 or CD44v6, but only pre-clinical data are currently available, demonstrating a potential efficacy against AML blasts. CD33 is a member of the sialic acid-binding receptor family and is highly expressed on myeloid progenitor cells, and on the surface of 90% of AML blasts. Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 humanized mAb conjugated

to calicheamicin, was the first agent of its class and one of the first targeted therapies in AML. However, initial enthusiasm was tempered by inadequate efficacy, severe hepatotoxicity (*i.e.*, veino-occlusive disease) related to accumulation of the drug acting like an intercalating agent, and prolonged neutropenia^[105]. Moreover, drug resistance rapidly may occur by active efflux from leukemia cells through the P-glycoprotein pump. Because of the very strong non HLA-restricted NK-like cytotoxicity and the lack of allogeneic activity in the ASCT setting, CIK have been largely used in pre-clinical studies of CAR redirected T cells in AML. Anti-CD33 CAR-T cells might have several potential advantages over gemtuzumab ozogamicin. First pre-clinical data using CIK and EBV-specific T cells endowed promising features^[104,106]. Beside the anti-leukemia activity and its expected myelotoxicity, *in vitro* colony forming unit (CFU) assays showed remnant clonogenic activity of hematopoietic progenitors suggesting that toxicity is reversible. Furthermore, it was shown that anti-CD33 CAR T cells with CD28-OX40 endodomains exert cytolytic activity on KG-1 cell line, known to be resistant to gemtuzumab ozogamicin^[106]. In order to avoid off-target toxic effect on hematopoietic stem cells (HSC), CD123, also known as IL-3 receptor α -subunit, appears as an attractive target. Its functional role in AML is still unknown. CD123 is widely over-expressed among AML blasts and LSCs, while its expression is lower on HSCs, monocytes, and endothelial cells. The expression of CD123 at the time of diagnosis has been associated with a poor prognosis and resistance to apoptosis. Preliminary *in vivo* data showed that one single administration of anti-CD123 CAR T cells led to an immunologic memory associated with a specific anti-tumor response, yielding to long-term survival in mice engrafted with AML cell lines or fresh AML cells^[107]. Results of CFUs, regarding distinct anti-CD123 CAR constructs, were heterogeneous. However, one study revealed a limited activity against normal CD123^{low} expression on endothelial cells and monocytes^[31,103,104]. It is therefore hypothesized that suboptimal affinity of the scFv for CD123 allows recognition of targeted cells by CAR T cells, according to their antigen density and their expression of co-stimulatory molecules^[108]. The hyaluronase receptor CD44, a ligand for E-selectin, is broadly expressed on malignant cells in hematological malignancies. It plays a crucial role in the bone marrow homing of initiating leukemia cells and in interactions with the microenvironment. It was recently showed that CD44 inhibition drives leukemia cells into differentiation and apoptosis by dislodging them from the osteogenic niche^[109]. The isoform variant 6 (CD44v6) recently emerged as one of the most promising TAA for AML. It is absent on normal HSCs, but is over-expressed in 80% of AML cells^[110,111]. It has been demonstrated that anti-CD44v6 redirect T cells were able to efficiently kill leukemia cells, while sparing HSCs and keratinocytes that expressed low levels of CD44v6^[72]. These findings suggest that "off tumor" target expression levels do

not accurately predict susceptibility to CAR T cells^[72]. All together, these results suggest that anti-CD123 and anti-CD44v6 CARs are safer than anti-CD33, but *in vitro* data have to be confirmed in clinical practice (Table 1).

TOXICITY: NEW INSIGHTS IN CYTOKINE RELEASE SYNDROME MANAGEMENT

With the development of new therapeutic reagents, physicians have to face previously unknown toxicities. Immunotherapy adverse events are mainly related to autoimmune direct toxicity, known as “on tumor/off target effect”, and to an indirect cytokine-associated toxicity, called CRS.

Indirect toxicity: Cytokine release syndrome

CRS has been initially described following mAb infusions (*i.e.*, anti-CD52 or anti-CD20) and more recently with bi-specific antibodies and CARs^[112,113]. This syndrome is characterized by a massive non-antigen specific inflammatory response. CRS shares features with macrophage activation syndrome, such as cytopenia, fever, hyperferritinemia and hypofibrinogenemia. Regardless the TAA target, it seems that T cell expansion activates other hematological effectors (B cells, neutrophils, macrophages), and favors release of inflammatory cytokines, such as TNF- α , IL-2, IL-6, IL-13 and IFN γ ^[44,98]. Symptom onset has been reported with a widely variable timing among clinical trials, ranging from 1 d to 3 wk for anti-CD19 CARs. All CRS reported with anti-CD19 in ALL trials were related to the tumor burden prior the first infusion and corresponded with maximal *in vivo* T cell expansion. Symptoms of CRS were drastically mild or even absent with anti-LeY in AML and with anti-CD19 CARs in CLL^[45,87]. The magnitude of immune activation after engineered T cell infusion is therefore dependent upon the underlying condition, the remaining lymphocyte pool, and CAR T cell features. Symptoms are not specific and confusion can be made with those of infections. Fever appears as the hallmark of CRS, and arises generally concomitantly to rigors, myalgia, and gut disorders. However, other life threatening complications can occur. Recently, IL-6, a pleiotropic cytokine with ambivalent functions, emerged as the gatekeeper in the pathophysiology of CRS. In this setting, tocilizumab, an anti-IL-6R mAb initially developed for rheumatologic autoimmune diseases, has proven its efficacy in severe CRS. This agent showed a safe profile with few side effects at the recommended dose (one injection at 8 mg/kg for children and 4 mg/kg for adults). It did not seem to alter anti-leukemia functions of the transferred T cells. Alternative therapy in non-responders could involve anti-TNF- α mAb (infliximab), soluble TNF- α receptor (etanercept), or corticosteroids especially in patients presenting neurological symptoms^[114]. Cardiac complications (similar to stress cardiomyopathies) are non frequent and generally reversible events, although

potentially fatal. Neurological symptoms include mainly headache, dysphasia, confusion, seizure. Magnetic resonance imaging has shown abnormalities consistent with a mild encephalopathy with reversible splenial lesion syndrome, as observed in severe viral infections. The exact pathophysiology of neurological features during CRS is not fully understood, but seems to be related to a direct neurotoxicity of IL-6^[115]. IL-6 levels in cerebrospinal fluid have been monitored during CRS and have shown high levels. It can be hypothesized that systemic inflammatory response after CAR T cells infusion could lead to permeable blood-brain barrier, yielding trafficking of IL-6 and activated immune cells to CNS. At least for CTL019, CAR T cells revealed to cross the blood-brain barrier and to be evolve in the neurological symptoms, which can be overcome by tocilizumab therapy. However, the occurrence of neurological symptoms can be enhanced by the IL-6R inhibitor. Tocilizumab may inhibit the IL-6 receptor mediated clearance and may allow transient increase of IL-6 levels. In patients with severe neurological symptoms, but without other life-threatening organ failure, it has been recommended to treat with corticosteroids (especially dexamethasone)^[116]. Acute respiratory distress syndrome, hepatic/renal failure, disseminated coagulopathy have also been reported.

Direct toxicity: “On tumor/off target effect”

Prolonged B lymphocyte depletion illustrates perfectly the antigen driven direct toxicity, provided by anti-CD19 CAR T cells and hence a theoretical immunodeficiency. Although B lymphocyte depletion has been profound in reported clinical studies, its durability appears as a sign of prolonged anti-tumor response. This condition is very close to that of patients with X-linked agammaglobulinemia^[97]. While B lymphocyte depletion increases the risk of opportunistic infections, this may be improved by intravenous immunoglobulin therapy. Although patient follow-up is relatively short, patients treated with anti-CD19 CAR T cells did not show until now any increase of bacterial infections. However, the development of this new technique will certainly be accompanied by the description of further new adverse events.

CONCLUSION

Immunotherapy represents certainly a great step forward in the treatment of AL. Because of the complexity of T cell repertory and its interaction with the immune system, there is, however, a long way to go before achievement of a complete and optimal understanding of this technology, as illustrated by the “on tumor/off target” paradigm. Furthermore, production of CAR T cells is time consuming, and still requires tremendous financial resources. The next challenges will be to improve cell isolation and culture of CAR-modified T cell production, to reduce the cost of the technique,

and therefore facilitate CAR T cells production and delivery in most institutions, beyond the academic research environment.

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Advancement in high dose therapy and autologous stem cell rescue in lymphoma

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Abstract

Although advanced stage aggressive non-Hodgkin's

lymphomas and Hodgkin's disease are thought to be chemotherapy-responsive cancers, a considerable number of patients either relapse or never attain a remission. High-dose therapy (HDT) followed by autologous stem cell transplantation (ASCT) is often the only possibility of cure for most of these patients. However, many controversial issues still remain with respect to HDT/ASCT for lymphomas, including its role for, the optimal timing of transplantation, the best conditioning regimen and the potential use of localized radiotherapy or immunologic methods to decrease post-transplant recurrence. Recently, mainly due to the unavailability of carmustine, several novel conditioning protocols have been clinically developed, with the aim of improving the overall outcome by enhancing the anti-lymphoma effect and, at the same time, by reducing short and long-term toxicity. Furthermore, the better safety profiles of novel approaches would definitively allow patients aged more than 65-70 years to benefit from this therapeutic option. In this review, we will briefly discuss the most relevant and recent data available regarding HDT/ASCT in lymphomas.

Key words: Hodgkin lymphoma; Non-Hodgkin lymphoma; High dose therapy; Autologous stem cell transplantation; New drugs

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Core tip: High-dose therapy (HDT) followed by autologous stem cell transplantation (ASCT) is considered the golden standard for the vast majority of patients with both Hodgkin and non-Hodgkin lymphoma, who either relapse or never attain a remission. However, several questions about HDT/ASCT still remain unanswered, also comprising, but not limited to, its role in newly diagnosed patients with advanced stage disease. The incorporation of novel drugs in both salvage and conditioning regimens has recently

revitalized the HDT/ASCT area, with several phase I-II trials performed during the last 5 years. This review will focus on the most recent data regarding HDT/ASCT in lymphomas.

Isidori A, Clissa C, Loscocco F, Guiducci B, Barulli S, Malerba L, Gabucci E, Visani G. Advancement in high dose therapy and autologous stem cell rescue in lymphoma. *World J Stem Cells* 2015; 7(7): 1039-1046 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i7/1039.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i7.1039>

INTRODUCTION

High-dose therapy (HDT) followed by autologous stem cell transplant (ASCT) is the therapy of choice for patients with chemosensitive, aggressive, relapsed non-Hodgkin lymphoma, basing on the results of the PARMA and CORAL study (NHL)^[1,2]. Moreover, HDT/ASCT is considered the standard of care for Hodgkin lymphoma (HL) patients in chemosensitive relapse^[3]. Different HDT regimens followed by ASCT are able to produce rates of disease-free survival (DFS) and overall survival (OS) of approximately 30% to 70%. Despite HDT/ASCT prolonged DFS, few major drawbacks still limit the utility of this approach for a wide patient population. As an example, the introduction of Rituximab in every-day clinical practice has dramatically reduced the number of patients addressed to HDT/ASCT, specially in front-line therapy. Up to now, no regimen was demonstrated to be superior to another in randomized trials^[4]. Therefore, novel strategies are urgently required. As a consequence, and due to the sudden unavailability of carmustine, the International Investigators have developed novel HDT/ASCT protocol in resistant/relapsed aggressive NHL or HD within the last 5 years, aiming to improve the outcome while reducing toxicity. In this paper, we will discuss the data emerging from few recent clinical trials testing HDT/ASCT in aggressive lymphomas.

CONDITIONING REGIMENS PRE-ASCT IN LYMPHOMAS

High dose chemotherapy and ASCT is the standard of care for patients with recurrent HL and NHL who fail immunochemotherapy upfront, improving long-term survival in 30% to 50%. Several factors impact on survival of lymphoma patients after HDT/ASCT. In this regard, the most important predictive factor is disease status at transplant (chemosensitive vs chemoresistant). Nevertheless, transplant related morbidity and mortality still remain relevant in influencing the outcome of the transplant procedure. Therefore, when planning an autologous transplant, the efficacy of HDT to eradicate residual disease after salvage therapy may be well balanced with toxicity to normal tissues, in order to

maximize the probabilities of the procedure of being successful. Despite the efforts made to further increase the therapeutic window of new high-dose regimens, at present we do not have an evidence that clearly demonstrates the superiority of a specific HDT regimen to the others.

BEAM (carmustine, etoposide, cytarabine, melphalan) regimen is considered the regimen of choice for patients with HL and NHL submitted to ASCT, due to its acceptable safety profile and a quite high antitumor efficacy. The transplant related mortality (TRM) of the BEAM regimen is quite low, depending mainly on disease status at transplant, infectious history and prior lines of therapy. Early, non-hematological toxicities of carmustine, such as nausea, vomiting, mucositis, hepatotoxicity, diarrhea and nephrotoxicity are well known, and worldwide Clinicians know how to manage them. However, late toxicities are still a matter of concern, in particular second tumor and interstitial non-infectious pneumonitis, which have been reported in 16%-64% of patients receiving carmustine-based conditioning regimens, being fatal in approximately 9% of the patients.

Accordingly, various scientists and cooperative groups still continue in this search for the holy Grail, focusing mainly on the incorporation of novel drugs, radioimmunoconjugates, monoclonal antibodies and/or other stimuli for the immunologic system during the early pre- and post- transplantation phases.

Hodgkin lymphoma

The results of the most important trials with HDT/ASCT in HD are listed in Table 1.

Ramzi *et al*^[5] reported the results of non-cryopreserved ASCT of 45 HL patients receiving an alternative CEAM regimen in which iv carmustine was substituted by oral lomustine (CCNU: 2 chloroethyl cyclohexyl nitrosourea). Forty-five relapsed/refractory HL patients underwent conditioning regimen with: lomustine 200 mg/m² on day-3, etoposide 1000 mg/m² on day-3 and day-2, cytarabine 1000 mg/m² on day-3 and day-2, melphalan 140 mg/m² on day-1. All 45 patients showed engraftment of infused stem cell. Grade 2 and grade 3 mucositis was seen in 64.5% of patients. TRM at 100 d was 2.2%. Median DFS was 20 mo (range: 4-60 mo). After a follow up of more than 2-year, the 2-year DFS in 30 evaluable patients was 77% and the 2-year OS was 84% (25/30 patients).

Czyz *et al*^[6] have retrospectively evaluated the efficacy of a modified BEAM regimen followed by ASCT in 132 patients relapsed/refractory HL patients. The 10-year OS and progression free survival (PFS) were 76% and 66%, respectively. Age > 45 years, more than one salvage regimens and chemoresistant disease at transplant were all predictive for poor OS in multivariate analysis.

In 2011 Shafey *et al*^[7] have retrospectively evaluated 73 patients with refractory/relapsed HL treated in a 15 year period with a double high-dose therapy consisting

Table 1 High dose therapy followed by autologous stem cell transplantation in Hodgkin lymphoma

Ref.	Year	Patients (n)	Status of HL	Regimen	TRM (%)	PFS (%)	OS (%)	Follow-up (mo)
Ramzi <i>et al</i> ^[5]	2012	45	R/R	CEAM	2.2	77	84	24
Czyz <i>et al</i> ^[6]	2013	132	R/R	Modified BEAM	-	66	76	68
Shafey <i>et al</i> ^[7]	2012	73	R/R	DICEP	1	61	80	56
Sinha <i>et al</i> ^[9]	2013	30	R/R	VTEPA	0	67	81	32
Di Ianni <i>et al</i> ^[10]	2012	58	R/R	TECA	0	72	82	60

HL: Hodgkin lymphoma; DICEP: Dose-intensive cyclophosphamide, etoposide, cisplatin; VTEPA: Vinorelbine, paclitaxel, etoposide and cisplatin; TECA: Thiotepa, etoposide and carboplatin; R/R: Relapsed/refractory; OS: Overall survival; PFS: Progression free survival.

of dose intensified cyclophosphamide, etoposide and cisplatin reinduction, followed by high-dose melphalan and ASCT. TRM was 1%. The 5-year PFS and OS rates were 61% and 80%, respectively. In multivariate analyses, response to reinduction therapy and DICEP and International Prognostic System score at relapse were the only factors independently predicting PFS and OS.

As already stated, the standard of care for refractory or relapsed HL patients is a salvage therapy followed by HDT/ASCT. However, patients with chemoresistant disease after salvage therapy have a small probability of achieving a long lasting response and a long overall survival. In 2006, the Emory University group tested in a phase I study, the combination of cytarabine with fixed doses of vinorelbine, paclitaxel, etoposide and cisplatin (VTEPA) as second salvage therapy in patients with resistant/relapsed lymphoma, showing an overall response rate (ORR) of 33%^[8]. In 2013 the same group further examined the effectiveness of VTEPA in 30 patients with relapsed/refractory HL^[9]. Among 27 evaluable patients, ORR was 70% (7 CR, 12 PR). All but 1 responding patients (66%) subsequently underwent ASCT. This therapeutic strategy (VTEPA + ASCT) produced a median PFS and OS of 28 and 38 mo from transplant, respectively.

In 2012 Di Ianni *et al*^[10], reported their experience with a novel HDT regimen including thiotepa, etoposide and carboplatin (TECA) in HL patients. From March 1999 to December 2005, 58 patients with primary refractory or relapsed were enrolled in a phase II study. The conditioning regimen consisted of etoposide (250 mg/m² days 1-4), thiotepa (166 mg/m² days 2-4) and carboplatin (266 mg/m² days 2-4). After salvage therapy, 46 patients had chemosensitive disease (30 CR + 16 PR), whereas 12 were chemoresistant. At transplantation, 30 patients were in CR, 16 in PR and 12 showed a chemoresistance to salvage chemotherapy. TRM was 0%. The global ORR was 79.3% (37 CR, 7 PR), but 12 patients still did not respond to therapy neither after HDT/ASCT. The 5-year DFS and OS were superior for relapsed patients with respect to primary refractory ones. After 5-year of follow-up, approximately 75% of patients were alive. Even if the idea of combining thiotepa with more conventional drugs was interesting, the results of this study are in line with other trials, given the high percentage (80%)

of chemosensitive patients at transplant. Hard-to-respond, chemoresistant patients (12/58, 20%) did not show any benefit from the thiotepa-containing regimen.

Non-Hodgkin lymphoma

The results of the most relevant studies with HDT/ASCT in NHL are listed in Table 2.

As for HL, the standard salvage therapy of relapsed/refractory aggressive NHL mainly relies on HDT/ASCT, hopefully in patients with chemosensitive disease. Nevertheless, reaching of long lasting DFS remains not easy.

Kim *et al*^[11] reported the results of a novel NEAM regimen, administered prior to ASCT, to 69 patients with resistant/relapsed NHL. The NEAM regimen, another, novel variant of the standard BEAM, consisted of mitoxantrone (12 mg/m² iv on day-6 to day-4), etoposide (100 mg/m²) and cytarabine (100 mg/m² iv every 12 h from day-6 to day-3), melphalan (single 140 mg/m² dose at day-2). TRM at day-100 was 2.9%. Median event free survival (EFS) was 17.9 mo, whereas estimated 2-year OS was 64.2%.

In 2012 Falzetti *et al*^[12] reported the results of the TECA (thiotepa, etoposide and carboplatin) regimen administered to 45 patients with NHL at various disease stage. TRM was 4.4%. The ORR was 77.8% (30 CR, 5 PR). Ten patients (22.2%) did not respond. The mean 5-year OS was 71.1%. Patients with low (1) International prognostic index (IPI) at diagnosis had a better ORR and 5-year OS were than for those with intermediate IPI (2 and 3).

Another strategy to increase efficacy of the conventional BEAM regimen is to add novel drugs in a new reinforced BEAM combo. In this regard, the University of Nebraska Medical Center group designed a phase I/II trial testing the safety and the efficacy of the addition of a proteasome inhibitor to standard BEAM prior to ASCT in resistant/relapsed indolent or transformed NHL (including T cell lymphomas) or mantle-cell lymphoma (MCL, only in first CR)^[13]. Patients received 4 doses of escalating bortezomib (0.8, 1, 1.3, 1.5 mg/m²) on day-11, day-8, day-5 and day-2 prior to ASCT. After the maximum tolerated dose (MTD, 1 mg/m²) was defined, other 20 patients entered the phase II to determine a preliminary ORR, PFS and OS with this regimen. As a whole, 42 (13 + 29) patients were enrolled. Non-hematologic side effects were

Table 2 High dose therapy followed by autologous stem cell transplantation in non-Hodgkin lymphoma

Ref.	Year	Patients (n)	Disease	Regimen	TRM (%)	ORR (%)	OS (%)	Follow-up (mo)
Kim <i>et al</i> ^[11]	2012	44	Chemosensitive-NHL	NEAM	2.9	79	64	24
Falzett <i>et al</i> ^[12]	2012	45	HR NHL	TT-Vp-Car	4.4	77	71	60
William <i>et al</i> ^[13]	2014	42	R/R NHL	V-BEAM	0	87	91	12
Visani <i>et al</i> ^[14]	2011	43	R/R HL and NHL	BeEAM	0	82	81	18
Visani <i>et al</i> ^[15]	2014	43	R/R HL and NHL	BeEAM	0	72	88	41
Isidori <i>et al</i> ^[16]	2014	37	R/R NHL	BeEAM	2.7	88	94	9
Musso <i>et al</i> ^[17]	2010	84	R/R HL and NHL	FEAM	2.4	73	88	13
Kruger <i>et al</i> ^[19]	2012	16	R/R NHL	RIT + BEAM	6	94	75	44
Winter <i>et al</i> ^[20]	2009	44	R/R NHL	Z-BEAM	2.2	77	60	33
Shimoni <i>et al</i> ^[21]	2012	43	R/R NHL	Z-BEAM	0	97	91	24
Briones <i>et al</i> ^[22]	2014	30	R/R NHL	RIT + BEAM	3.5	70	63	31

HL: Hodgkin lymphoma; RIT: Radioimmunotherapy; BeEAM: Bendamustine, etoposide, ara-C, melphalan; FEAM: Fotemustine plus etoposide, cytarabine and melphalan; NEAM: Mitoxantrone, etoposide, cytarabine, and melphalan; NHL: Non-Hodgkin lymphoma; R/R: Relapsed/refractory; OS: Overall survival; TT-Vp-Car: Thiotepa, Vepeside, carmustine; V-BEAM: Velcade plus standard BEAM.

comparable to those observed with other regimen, with the relevant exception of an increase in grade III peripheral neuropathy, related to the use of bortezomib, and in grade III gastrointestinal toxicity. TRM at day 100 was 0%. At 1 year after ASCT, 38 patients were evaluable for response; 32 (84%) were in CR and 1 (3%) was in PR, resulting in an impressive ORR of 87%. these results were better among patients treated in the phase II, for whom ORR was 89% (84% CR, 5% PR). PFS was 83% at 1 year and 32% at 5 years. OS was 91% at 1 year and 67% at 5 years. The authors performed also an exploratory analysis to determine whether this regimen was more effective in a given histological pattern, finding no statistical difference. Conversely, by comparing the results of bortezomib-BEAM with standard BEAM in an historic cohort of patients matched for histology at their Institution, the authors showed an advantage in both PFS and OS at 5 years for MCL patients (57% and 72% vs 43% and 50%, respectively), even if not statistically significant. Even if promising, in particular in MCL patients, the bortezomib-BEAM regimen discourages, due to the lack of an evident benefit and higher than expected toxicity, its further exploration in a randomized, phase III study.

In 2011 Visani *et al*^[14] reported the efficacy of increasing doses of bendamustine (160 mg/m², 180 mg/m² and 200 mg/m² given on day-7 and day-6) in addition to fixed doses of etoposide, cytarabine and melphalan (BeEAM regimen) administered as preparative regimen to ASCT. Forty-three patients with resistant/relapsed HL (*n* = 15) and NHL (*n* = 28) were enrolled, 9 in the phase I and 34 in the phase II study. No patients experienced dose limiting toxicity. TRM at day 100 was 0%. The follow-up period at the time of publication was 18 mo, with 81% being alive and disease-free at that time. Disease type (HL vs NHL) and disease status at transplant (chemosensitive vs chemoresistant) significantly influenced DFS. Interestingly, the authors updated their experience in 2014^[15], reporting a 72% PFS at 3 years, that allowed them to met the primary end-point of the study. Median PFS and OS were still not reached. Disease status at

transplant (chemosensitive vs chemoresistant) was still a stron predictor of outcome. Conversely, disease type (HL vs NHL) was no longer affecting PFS nor OS^[15].

By riding the same wave, Isidori *et al*^[16] recently reported the preliminary data of a phase II study to confirm the effectiveness of BeEAM as a preparative regimen for autologous stem cell transplantation in resistant/relapsed aggressive B-cell non-Hodgkin lymphoma patients. Thirty-seven patients (median age 56 years, range 19-69) with resistant/relapsed aggressive B-cell NHL were enrolled, up to now, in the study. Briefly, 27 patients had advanced stage disease (III-IV), 12 were primary refractory and 25 had relapsed. Thirty-three patients had good performance status (WHO 0-1), and 11 patients presented with 1 or more relevant comorbidities (range: 1-5). Nineteen patients were in II or subsequent CR after salvage therapy, whereas 16 were in PR and 2 had progressive disease. All patients engrafted, with a median time to ANC > 0.5 × 10⁹/L of 10 d. TRM at day-100 was 2.7%. Eight out of 37 patients presented a fever of unknown origin (21.6%), whereas 19 patients (51%) presented a clinically documented infection. One patient died due to an incomplete hematological recovery after transplant, producing an overall transplant related mortality of 2.7%. Twenty-seven out of 37 patients are evaluable up to now for response: 22/27 (81.5%) obtained a CR, 2/27 a PR, resultin in an ORR of approximately 90%. After a median follow-up of 9 mo from transplant (range 2-24), 5/24 patients relapsed, whereas 19/24 (79.1%) are still alive, in continuous CR. The Authors concluded that the BeEAM regimen preliminary confirmed its safety and its promising efficacy in resistant-relapsed aggressive B-cell lymphomas.

In 2010 Musso *et al*^[17] substituted carmustine with the chloroethylnitrosurea fotemustine (150 mg/m² on day-7 and day-6) in the standard BEAM (FEAM regimen). 84 resistant/relapsed HL and NHL patients were enrolled in this study. Non-hematological side effects were superimposable to those of the BEAM regimen, with 7 patients experiencing grade 4 mucositis, without any other relevant grade 4 toxicity. TRM at day-100

was 2.4%. Even if the FEAM HDT regimen showed a favorable safety profile, it is not possible to draw any conclusion regarding survival or long-term efficacy due to the short follow-up period of 17-mo only.

Another question that still remains unanswered is related to the relative efficacy of HDT/ASCT in NHL patient population treated with chemoimmunotherapy comprising rituximab front-line. As a fact, this is really a burning question, as all patients with B cell malignancies are treated upfront, at present, with anti-CD20 monoclonal antibody plus chemotherapy. Nevertheless, the utility of HDT/ASCT for NHL patients in first CR is still a matter of great debate.

Recently, the Southwest Oncology Group tried to answer, at least partly, this question by conducting a large, randomized trial testing HDT/ASCT as consolidation therapy, in comparison to standard chemoimmunotherapy^[18]. 397 patients were enrolled, and 370/397 received five cycles of CHOP with (47%) or without (53%) rituximab. Responding patients (CR + PR) were subsequently randomized to receive 3 other cycles CHOP ± Rituximab (control group) or one additional cycle of CHOP ± Rituximab followed by ASCT (transplantation group), conditioned with standard BEAM regimen or total body irradiation (12 Gy). The primary efficacy end points were 2-year PFS and OS. Of 370 induction-eligible patients, 253 were randomly assigned to the transplantation group (125) or the control group (128). Like many of the randomized trials and several meta-analysis, this study showed an improvement in PFS for the combined high-risk and high-intermediate risk who are chemosensitive to induction therapy. However, again and again, this study was not able to demonstrate, in randomized fashion, an advantage in OS for HDT/ASCT, neither for high risk patients^[18]. On the other hand, we have to keep in mind that 29% of patients who had a relapse or progression after standard therapy, were rescued with HDT/ASCT, resulting in a relevant bias for the analysis of a statistical OS benefit for HDT/ASCT over the control group. Finally, the study was not designed and powered to address subgroup-related question, and therefore any point in favor of HDT/ASCT for high risk patients is merely speculative.

The incorporation of new drugs into HDT regimen prior to ASCT in B-cell lymphomas has recently been helped by the development of radioimmunotherapy (RIT). The potential advantage of using radioimmun-conjugates, with or without chemotherapy, prior to ASCT, relies on the opportunity of delivering localized radiation therapy to the site of tumor. This allows to minimize the toxicity of total body irradiation, with the goal of decreasing relapse rate without adding toxicity to the conditioning regimen. At present, no study comparing RIT and standard radiation therapy has been done. However, few preliminary phase II studies with RIT as a part of a preparative regimen have produced encouraging results by showing a high safety profile, a low TRM and a preliminary efficacy.

Kruger *et al.*^[19] enrolled 16 patients with resistant/relapsed NHL in a phase II study testing ¹³¹I-rituximab-BEAM and ASCT. A single dose of ¹³¹I-rituximab was given on day-15, whereas standard BEAM started on day-6 prior to ASCT. Non hematological side effects were mild in grade, without any grade IV toxicity. All patients engrafted, with a 0% TRM at day-100. Results were encouraging, with 75% of patients being alive and disease free after a median follow-up of 44 mo from ASCT (range 4-108). Interestingly, each patient received only a limited whole body radiation of only 0.75 Gy.

Winter *et al.*^[20] conducted a phase I-II study in 44 patients with resistant/relapsed NHL, by adding yttrium-90 (⁹⁰Y) ibritumomab tiuxetan to standard BEAM and ASCT (Z-BEAM regimen). A significant proportion of patients (30%) entering the study had chemoresistant disease after salvage therapy. Non hematological toxicities were similar to those reported with standard BEAM. Two dose limiting toxicities occurred at 17 Gy dose level, which made 15 Gy the recommended dose for the phase II of the study. After a median observation time of 33 mo, the estimated 3-year PFS and OS were 43% and 60%, respectively. When looking at these results, it has to keep in mind the significant proportion of chemoresistant patients, who perform extremely poor with conventional HDT regimen.

Shimoni *et al.*^[21] randomized 43 patients with CD20 positive aggressive B-cell lymphoma to receive either Z-BEAM (*n* = 22) or standard BEAM (*n* = 21). Ibritumomab tiuxetan was administered at 0.4 mCi/kg on day-14 prior to ASCT. Non hematological toxicities were mild and comparable within the 2 groups. TRM at day-10 was 0% in both groups. As a whole, Z-BEAM did not show a significant advantage in OS with respect to standard BEAM. However, there was a trend in 2-year PFS and OS in favor of Z-BEAM (59% and 91% vs 37% and 62%, respectively). The Authors speculated that Z-BEAM could be superior to standard BEAM for patients receiving frontline chemoimmunotherapy containing Rituximab. However, the sample size of the study was very small, and the statistical analysis did not allow to draw any conclusion regarding the superiority of a regimen to another.

Another study with yttrium-90 Ibritumomab tiuxetan was conducted and published by Spanish Group in 2013^[22]. It was a prospective, multicenter, phase II clinical trial which enrolled 30 patients with induction failure or refractory B-cell NHL. Patients received ⁹⁰Y-ibritumomab tiuxetan at a fixed dose of 0.4 mCi/kg, 14 d prior to the BEAM chemotherapy. Non hematological toxicities were similar to those reported with standard BEAM, and TRM at day-100 was 0%. Intriguingly, the vast majority of patients (25/30) underwent to HDT/ASCT with chemoresistant disease. Therefore, this regimen produced an outstanding ORR of 70%, with 60% of patients obtaining a CR; estimated 3-year PFS and OS were 61% and 63%, respectively, and the median time of observation for surviving

patients was 31 mo. The Authors enthusiastically concluded that ^{90}Y -ibritumomab tiuxetan based HDT regimen prior to ASCT results in a terrific response rate, with promising survival in a group of refractory lymphoma patients with extremely poor prognosis.

Taken together, these results are in favor of RIT-based HDT regimen prior to ASCT, given the good toxicity profile and the promising efficacy. However, none of these studies was able to demonstrate a statistical benefit for the RIT-based HDT, probably also due to the small sample size of the trials. In conclusion, a large, randomized trial comparing RIT-BEAM and standard BEAM is warranted before drawing any conclusion and before recommending the use of RIT-HDT outside from controlled clinical trials.

With regards to T-cell lymphomas, the use of HDT/ASCT is still considered an "experimental" practice. In fact, only a small amount of prospective trials evaluating the impact of HDT/ASCT as consolidation of first-line therapy have been reported up to now^[23-25]. Even if the results coming from these studies suggest an improve in both DFS and OS when compared with chemo alone^[23-25], an important amount of patients is not able to perform frontline transplant, principally due to disease progression during induction chemotherapy, thus underlying the need for a better induction regimen. In relapsed T-cell lymphomas, HDT/ASCT produced results similar to those obtained in relapsed, aggressive B-cell lymphomas. Reported long-term DFS approaches 30% to 50%, making HDT/ASCT a valuable option in the therapeutic armamentarium for this indication. Conversely, the use of HDT/ASCT in refractory T-cell lymphoma produce extremely poor outcome, and other strategies could be preferred for this setting of patients. In conclusion, we think that there is room for HDT/ASCT as a part of first-line treatment, especially in responding patients. On the other hand, resistant or relapsed patients may be better addressed to allogeneic transplantation or to clinical trials.

Late complications of HDT followed by ASCT in lymphomas

Late complications among HL survivors are still a matter of concern. Several papers have reported high rates of second cancers, heart disease, pulmonary fibrosis, and infections^[26]. The recognition of these risks has resulted in modification of chemotherapy regimens and of radiation fields and doses^[26,27]. HDT/ASCT by itself is also affected by late *sequelae* of treatment, but specific data for the HL population are limited^[26,27]. Few studies identified predictors of post-transplant long-term quality of life specifically for patients with HL, who have higher rates of treatment-related late morbidity and mortality than patients with other cancer diagnoses^[26,27].

The most relevant study that evaluated the risk of late morbidity and mortality, among patients with relapsed/refractory HL after HDT and ASCT was performed by the MSKCC group^[28]. this study, conducted on 153 HL

patients treated with HDT/ASCT between 1985 and 1998 who survived ≥ 2 years after ASCT, demonstrated a risk ratio of second malignancy equal to 6.5 (95%CI: 3.6-10.7) when compared with the general population, but limited to 2.4 (95%CI: 1.4-4.05) when compared with patients with HL^[28]. In other words, the risk of developing a second tumor after HDT/ASCT was elevated if compared with the cancer risk in the general population, but was less pronounced when compared with patients with HL in SEER registry^[29].

Data on NHL are quite similar, demonstrating a higher risk of developing a second malignancy mostly for patients receiving TBI as a part of the preparative regimen to ASCT. However, to the best of our knowledge, large studies have not been conducted in this patient population.

Our guess is that, in the era of the TBI-free conditioning regimens, what really counts for the development of a second malignancies or a late effect (*e.g.*, cardiomyopathy) in lymphoma patients, is mainly the type and the dose of chemo- and radiation therapy performed before transplant, and only in a minimal extent HDT/ASCT.

CONCLUSION

High dose therapy followed by autologous stem cell transplantation has still a major role in the treatment of resistant/relapsed HL and NHL. The relevant advancements made with the incorporation of novel drugs and/or radioimmunoconjugates into preparative regimens translated in a higher PFS rate with respect to the historical standards. Furthermore, TRM and overall toxicities seem to be lower. However, data on the possible overall survival advantage given by the novel agents are still controversial, and probably only large, randomized phase III trials could pick a winner between the plethora of new drugs recently incorporated in novel conditioning regimens. Our personal experience with Bendamustine, used both in Phase II trials and everyday clinical practice, indicate the favorable safety profile of the BeEAM regimen, coupled with a relevant efficacy in a hard-to-treat population of resistant/relapsed lymphoma patients.

A different scenario could be represented by maintenance therapy with new drugs, such as Brentuximab Vedotin in HL or Ibrutinib or Lenalidomide in aggressive NHL, in patients chemoresistant who obtain at least a PR after HDT/ASCT. In this setting of patients, the use of a drug with a different mechanism of action and a manageable safety profile could help the physician in the path to cure of a highly resistant subpopulation of lymphoma patients.

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Stem cells: Sources, and regenerative therapies in dental research and practice

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Abstract

Stem cells are considered to be among the principle scientific breakthroughs of the twentieth century for the future of medicine, and considered to be an important weapon to fight against diseases, particularly those that have resisted the efforts of science for a long time. Human dental tissues have limited potentials to regenerate but the discovery of dental stem cells have developed new and surprising scenario in regenerative dentistry. Stem cell treatments are one example of the

possibility using adult cells sourced from patients' own bodies' means that it can be expected that in the near future such treatments may become routine at dental practices. The hope is that it will become possible to regenerate bone and dental tissues including the periodontal ligament, dental pulp and enamel, and that the creation of new teeth may also become feasible. In view of this possibility of achieving restoration with regenerative medicine, it can be considered that a new era of dentistry is beginning. Thus the aim of this review is to give dental professionals a brief overview of different stem cells sources and the latest findings and their implications for improving oral health and treating certain conditions of the human mouth and face.

Key words: Stem cells; Regeneration; Dental research

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Core tip: Stem cells are considered to be among the principle scientific breakthroughs of the 20th century. Human dental tissue has limited potentials to regenerate but the discovery of dental stem cells have developed new and surprising scenario in regenerative dentistry. The hope is that it will become possible to regenerate bone and dental tissues and that the creation of new teeth may become feasible. Thus our review gives dental professionals a brief overview of different stem cells sources and the latest findings and their implications for improving oral health and treating certain conditions of the human mouth and face.

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INTRODUCTION

Stem cell therapies could lead to novel cures and palliative treatments. Stem cell research has clinical implications considering cell repair, replacement or regeneration to improve organ function. Thus future studies will require the collaboration of researchers from different specialties, including stem cell biology, material science, and of course, basic and clinical dentistry.

The loss of hard or soft dental tissue not only harms the patient psychologically, but also causes atrophy of the tooth supporting tissues. Regeneration may define as restoring a deficient part by complete regeneration of its architecture and function. Dramatic tooth extraction, sever periodontitis, implant dehiscence defects, tumor, or congenital anomalies may lead to alveolar bone deficiencies^[1]. Alveolar bone defect regeneration techniques have many advances using minimally morbid techniques enhanced the success and patient acceptance^[2,3]. Successful regeneration techniques of the craniofacial bone deficiencies, are used to optimize therapeutic approaches to bone regeneration^[4].

Autogenous bone graft remains the preferred reconstructive method but have some limitations such as increased operative time for bone graft harvesting, donor site morbidity, graft resorption, and limited availability. Various types of bone graft have become available to overcome these limitations^[5,6]. There have been recent interest in the development of new grafting materials using allogeneic, xenogeneic and synthetic bioimplants for reconstructive bony procedures, and have been used as alternative for autogenous bone grafts^[7-9]. A new combination of protein therapy, gene therapy and cell therapy and tissue engineering had been successfully developed and considered to be a more efficient and safer therapeutic system for bone and soft tissue regeneration^[10].

Stem cells are nonspecific cells with powerful self-regeneration properties and they are capable of organizing other cell types in the body. They are also play an important role as those undifferentiated cells have precursor properties, capable of forming many different cell types and have the property of unlimited self-renewal. Within dentistry, the hope is to regenerate both bone and dental tissues, including the periodontal ligament, dental pulp and enamel, so that new teeth could even be created, even in the presence of caries, pulpitis and periapical diseases, which are the primary causes of tooth loss^[11].

"Stem cells" are precursor cells that can give rise to multiple tissue types. Totipotent stem cells are cells that can give rise to a fully functional organism as well as to every cell type of the body. *Pluripotent* stem cells are capable of giving rise to virtually any tissue type, but not to a functioning organism. Multipotent stem cells are more differentiated cells that can give rise only to a limited number of tissues. For example, a specific type of multipotent stem cell called a mesenchymal stem cell

has been shown to produce bone, muscle, cartilage, fat, and other connective tissues^[12,13].

The effectiveness of stem cells can be attributed to many factors such as: Stem cells can be expanded *ex vivo*, thus a small number of them can be sufficient to heal large defects or to treat diseases. Stem cells in the presence of vasculature may elaborate and organize tissues *in vivo*. Also they may play an important role in regulating local and systemic immune reactions of the host favoring tissue regeneration.

STEM CELL TYPES AND SOURCES

Stem cells have important criteria of self-renewable and the ability to differentiate into at least two different types of cell. Types of stem cells are determined based on their source and differ in regard to the types of cells into which they differentiate^[14,15].

Embryonic stem cells, they are pluripotent cells that can differentiate into any other type of cell^[14].

Adult stem cells, also called somatic stem cells, lack the potency of their embryonic counterparts, but have been used successfully to treat diseases. They can be harvested from an individual and, be used to regenerate tissue by autologous or allogeneic transplant^[14].

Induced pluripotent stem cells - adult stem cells having the potential to serve as the source of a large number of autologous stem cells, but the main drawbacks are their capacity for proliferation and concomitant potential for carcinogenicity^[16].

Bone marrow-derived mesenchymal stem cells can self-replicate and have been differentiated into osteoblasts, chondrocytes, myoblasts, adipocytes and other cell types. MSCs are often viewed as a yardstick of adult stem cells (Figure 1)^[17].

Adipose-derived stem cells (AS) are typically isolated from lipectomy or liposuction aspirates. AS have been differentiated into adipocytes, chondrocytes, myocytes, neuronal and osteoblast lineages. They have many advantages over other adult stem cell populations, as adipose tissue is available in large number and become easily accessible^[18].

Dental stem cells - recently these cells have been found in various dental tissues, such as Stem Cells from Human Exfoliated Deciduous teeth^[19], dental Pulp Stem Cells^[20], Periodontal Ligament Stem Cells (PDLSCs)^[21,22], while in dental papilla of wisdom teeth Stem Cells from Apical Papilla is present^[23] and researchers termed stem cell found in dental follicles of developing wisdom teeth as Dental Follicle Precursor Cells^[24-26].

There are some advantages of dental stem cells: (1) they are readily accessible and provide an easy and least invasive way to obtain them; (2) stem cell banking is a reasonable and simple alternative to harvesting stem cells^[27]; (3) they show good interaction with scaffolds^[28]; and (4) they have a high proliferative and multidifferentiation potential^[29-31].

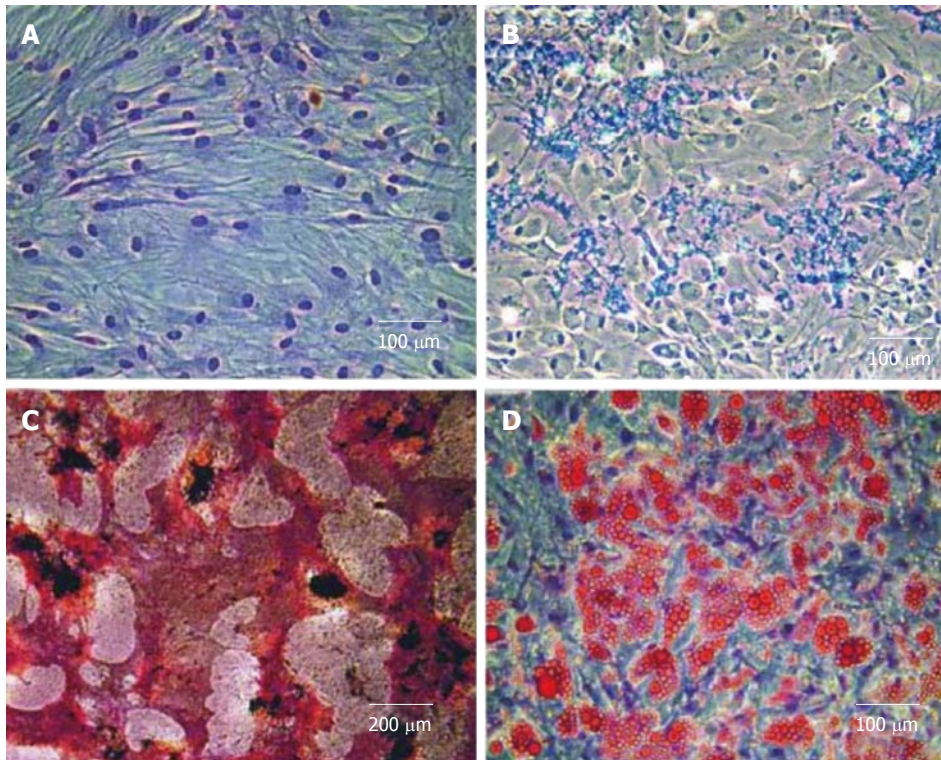


Figure 1 Mesenchymal stem cells are viewed as a yardstick of adult stem cells. A: Human mesenchymal stem cells (MSCs) isolated from anonymous adult human bone marrow donor after culture expansion (H and E staining); B: Chondrocytes derived from human mesenchymal stem cells showing positive staining to Alcian blue. Additional molecular and genetic markers can be used to further characterize MSC-derived chondrocytes; C: Osteoblasts derived from human mesenchymal stem cells showing positive von Kossa staining for calcium deposition (black) and active alkaline phosphatase enzyme (red). Additional molecular and genetic markers can be used to further characterize MSC-derived chondrocytes; D: Adipocytes derived from human mesenchymal stem cells showing positive Oil Red-O staining of intracellular lipids. Additional molecular and genetic markers can be used to further characterize MSC-derived chondrocytes^[16].

ROLE OF MESENCHYMAL STEM CELLS IN TISSUE REGENERATION

By chemotaxis Mesenchymal Stem Cells can migrate to tissues showing inflammation and injury in the organism^[32]. They can differentiate into different cell types, able to secrete a variety of cytokines, showing anti-inflammatory activity and create an anabolic microenvironment. Moreover, they play a role in regeneration of injured tissues by various means, as they directly differentiate into tissue-specific cells and thus substitute damaged or lost cells. On the other hand, they indirectly influence tissue regeneration by secretion of soluble factors. Thirdly, they are able to modulate the inflammatory response. So, they can effectively promote vascularization, cell proliferation, differentiation and modulate an inflammatory process^[33,34].

CHALLENGES IN STEM CELL BIOLOGY

Notable increase in the applications of craniofacial tissue engineering, among them is: (1) isolation of stem cells from several craniofacial tissues and their clinical therapeutic applications in the craniofacial structures; (2) several prototypes of the human-shaped temporomandibular joint condyle have been engineered with integrated cartilage and bone layers from a single

population of mesenchymal stem cells; (3) cell-based or non-cell-based tissue engineering of periodontium elements, including the periodontal ligament and cementum; (4) the application of stem cells, growth factors, and/or biomaterials in craniofacial bone engineering; and (5) adipose stem cells, with its efficient applications in facial soft and hard tissue reconstructive surgeries^[35].

Tissue engineering of the temporomandibular joint

Current approaches for replacing degenerated mandibular condyles suffer from deficiencies such as donor site morbidity, immune-rejection, implant wear and tear, and pathogen transmission. So, advances in the management of temporomandibular joint degenerative diseases have been established: (1) to promote matrix synthesis and tissue maturation of stem cell-derived chondrogenic and osteogenic cells (Figure 2)^[36]; (2) to enhance the mechanical properties of a tissue engineered mandibular condyle for ultimate in situ implantation into the human temporomandibular joint (Figure 3)^[37]; and (3) to facilitate the remodeling potential of a tissue-engineered mandibular condyle.

Periodontal tissue engineering^[35]

The modulation of the exuberant host response to microbial contamination that plagues the periodontal wound is considered as the main challenge. It clarified

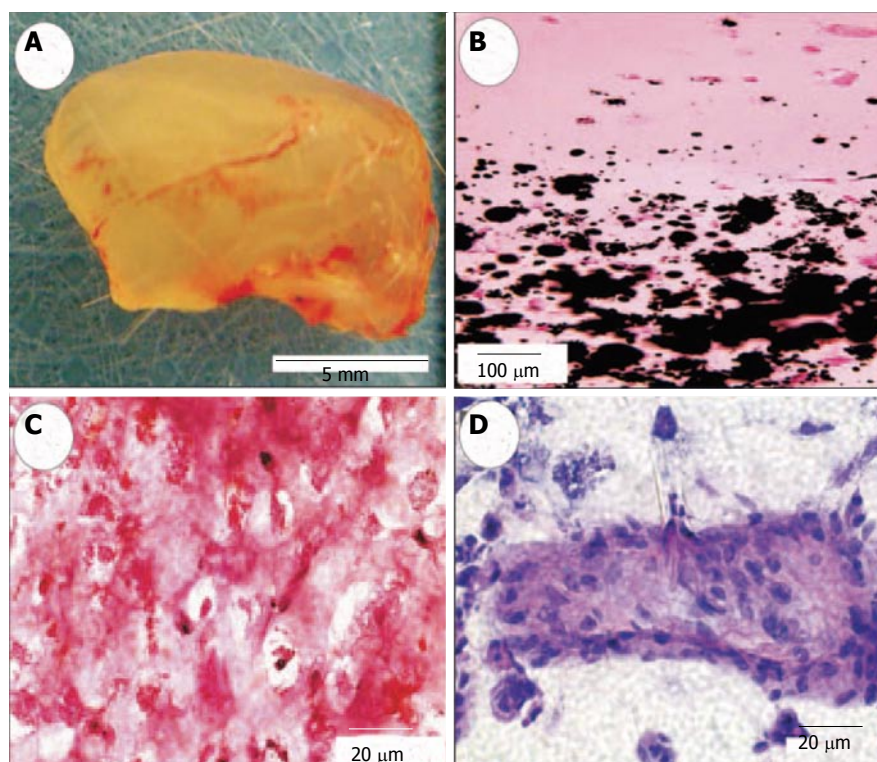


Figure 2 Engineered neogenesis of human-shaped mandibular condyle from mesenchymal stem cells. A: Harvested osteochondral construct retained the shape and dimension of the cadaver human mandibular condyle after *in vivo* implantation; B: Von Kossa-stained section showing the interface between stratified chondral and osseous layers. Multiple mineralization nodules are present in the osseous layer (lower half of the photomicrograph), but absent in the chondral layer; C: Positive safranin O staining of the chondrogenic layer indicates the synthesis of abundant glycosaminoglycans; D: H and E-stained section of the osteogenic layer showing a representative osseous island-like structure consisting of MSC-differentiated osteoblast-like cells on the surface and in the center. Reproduced with permission from Biomedical Engineering Society^[36].

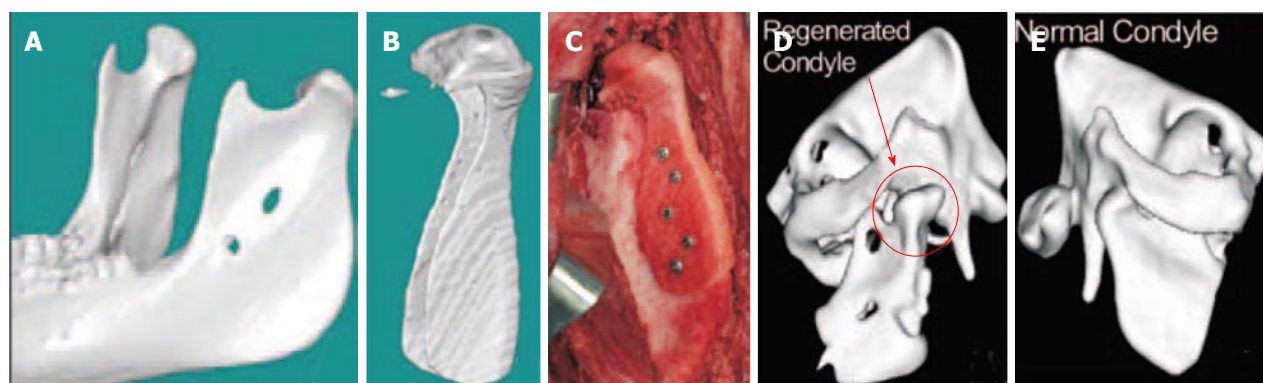


Figure 3 Design and engineering of minipig mandibular condyle. A: Original computed tomography scan of minipig mandible; B: Image-based design of condyle scaffold; C: Ppolycaprolactone (PCL) degradable polymer scaffold fabricated with selective laser sintering attached to the ramus; D: Regrowth of condyle following 3 months' implantation (new condyle shown in red circle); E: Comparison with normal condyle from contralateral side in Yucatan minipig^[37].

the interactions of multiple cell lineages including cementogenic cells, fibroblasts, and osteogenic cells. Regeneration of severe periodontal defects necessitates the attraction of endogenous periodontal tissue forming cells by growth factors.

As regard the periodontal ligament (PDL) function, a recent report identified stem cells in human PDL (PDLSCs) and found that PDLSCs implanted into nude mice generated cementum/PDL-like structures that resemble the native PDL as a thin layer of cementum that interfaced with dense collagen fibers, similar to

Sharpey's fibers (Figure 4)^[38,39]. Thus, the PDLSCs have the potential for forming periodontal structures, including the cementum and PDL.

Challenges in improving stem cell-based researches

Tissue engineered bone with customized shape and dimensions have the potential for the biological replacement of craniofacial bones, and segmental defects in the appendicular bones.

Stem cell therapy encompasses new technologies and therapies and poses many challenges in stem cell

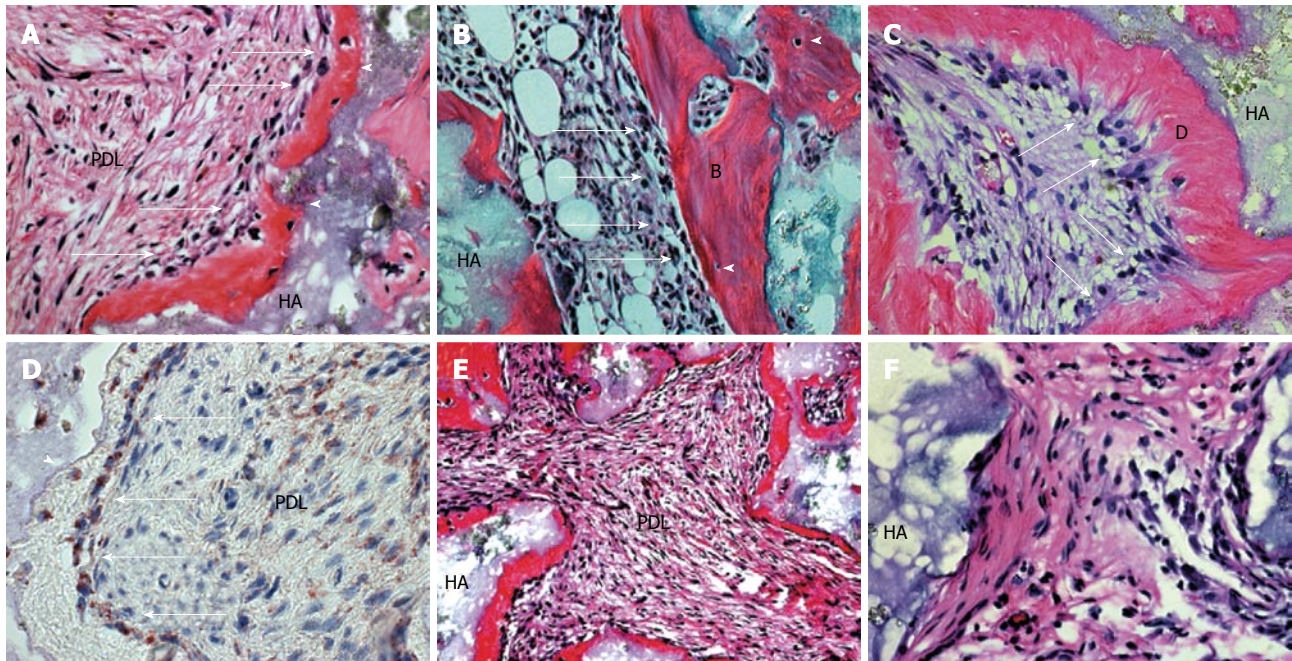


Figure 4 Generation of cementum-like and PDL-like structures *in vivo* by PDSCs. A: After 8 wk of transplantation, PDSCs differentiated into cementoblast-like cells (arrows) that formed a cementum-like structure (C) on the surface of the hydroxyapatite tricalcium phosphate (HA) carrier; cementocyte-like cells (arrowhead) and PDL-like tissue (PDL) were also generated; B: BMSSC transplant was used as control to show the formation of a bone/marrow structure containing osteoblasts (arrows), osteocytes (arrowhead), and elements of bone (B) and haemopoietic marrow (HP); C: DPSC transplant was also used as a control to show a dentin/pulp-like structure containing odontoblasts (arrows) and dentin like (D) and pulp-like (Pulp) tissue; D: Immunohistochemical staining showed that PDSCs generated cementum-like structure (C) and differentiated into cementoblast-like cells (arrows) and cementocyte-like cells (arrowhead) that stained positive for human-specific mitochondria antibody. Part of the PDL-like tissue (PDL) also stained positive for human specific mitochondria antibody (within dashed line); E: Of 13 selected strains of single-colony derived PDSC, only eight (61%) generated cementum/PDL-like structures *in vivo* as shown at lower magnification (approximately 20). New cementum-like structure (C) formed adjacent to the surfaces of the carrier (HA) and associated with PDL-like tissue (PDL); F: The other five strains did not generate mineralised or PDL-like tissues *in vivo*^[38].

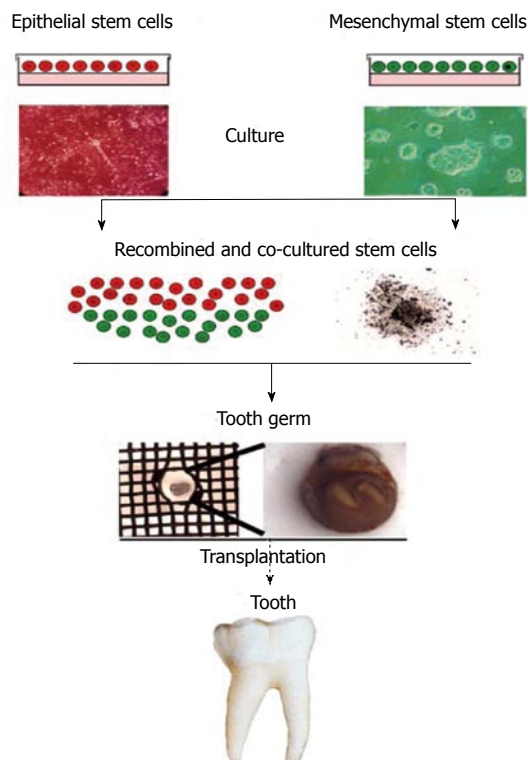


Figure 5 Use of stem cells for tooth formation *in vitro* and *ex vivo*. A tooth germ can be created *in vitro* after co-culture of isolated epithelial and mesenchymal stem cells. This germ could be implanted into the alveolar bone and finally develop into a fully functional tooth^[40].

research, such as: (1) biological challenges: Despite biological evidence showing that regeneration can occur in humans, complete and predictable regeneration still remains an elusive clinical goal (especially in advanced periodontal defects); (2) technical challenges: The technical challenges in stem cell therapy are associated with cell manipulations, scaffold materials and delivery systems; and (3) clinical challenges: Clinical challenges in stem cell-based periodontal therapy relate to immune rejection after administration, oncogenic properties of stem cells and functional integration of transplanted tissues into the host.

Stem cells for tooth engineering

Building a tooth logically requires the association/cooperation of odontogenic mesenchymal and epithelial cells. The recombination of dissociated dental epithelial and mesenchymal tissues leads to tooth formation both *in vitro* and *in vivo* (Figure 5). The bioengineered teeth have been produced in ectopic sites and with missing some essential elements such as the complete root and periodontal tissues that allow correct anchoring into the alveolar bone. Recently, a new approach has been proposed for growing teeth in the mouse mandible^[40].

CONCLUSION

For dental applications, stem cell therapy and tissue

engineering are gold solution for bone and soft tissue regeneration and provide hope of future application in humans within the next few years. Tissue engineering modalities will provide numerous clinical dental applications, including improved treatments for intraosseous periodontal defects, enhanced maxillary and mandibular grafting procedures, perhaps more biological methods to repair teeth after carious damage and possibly even re-growing lost teeth. This review highlights the sources and the regenerative therapies in the clinical practice of dentistry in the future.

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Rat embryonic stem cells create new era in development of genetically manipulated rat models

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Abstract

Embryonic stem (ES) cells are isolated from the

inner cell mass of a blastocyst, and are used for the generation of gene-modified animals. In mice, the transplantation of gene-modified ES cells into recipient blastocysts leads to the creation of gene-targeted mice such as knock-in and knock-out mice; these gene-targeted mice contribute greatly to scientific development. Although the rat is considered a useful laboratory animal alongside the mouse, fewer gene-modified rats have been produced due to the lack of robust establishment methods for rat ES cells. A new method for establishing rat ES cells using signaling inhibitors was reported in 2008. By considering the characteristics of rat ES cells, recent research has made progress in improving conditions for the stable culture of rat ES cells in order to generate gene-modified rats efficiently. In this review, we summarize several advanced methods to maintain rat ES cells and generate gene-targeted rats.

Key words: Embryonic stem cells; Transgenic rat

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Core tip: Rat embryonic stem (ES) cells are thought to be an essential tool for creating transgenic rats. Since the method for establishing rat ES cells using signaling inhibitors was reported, numerous approaches have been made to propagate rat ES cells efficiently. Additionally, recent investigations have demonstrated the usefulness of the signal inhibitors for microinjection. In this review, we summarize the several advanced methods to maintain rat ES cells and generate gene-targeted rats.

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INTRODUCTION

Rattus norvegicus, is widely used as a laboratory animal in many kinds of scientific field, such as biochemical, biomedical, and pharmacological studies. In 1828, albino mutants were brought into laboratories for physiological studies such as fasting studies. Since the nineteenth century, over 700 rat strains including inbred and outbred strains have been developed, and have been used for a multitude of studies^[1,2]. Rats offer some advantages over mice in some transplantation, behavior, and pharmacokinetic studies, because the larger size and greater intelligence of rats compared to mice enables ease of surgical operation, a large-volume of blood sampling, and assessment of high-level learning^[3,4].

In addition, it has been suggested that rats are a useful model for physiological studies owing to the heart rate of rats being closer than that of mice to the human heart rate^[2]. For these reasons, the publications involving rats outnumbered those involving mice for many years. However, far fewer scientific procedures have used transgenic rats than have used mice^[5]. One of the reasons for this disparity has been a lack of reliable methods to establish rat embryonic stem (ES) cells. Genetically modified mice have been routinely created using mouse ES cells with gene-manipulation technologies. These genetically modified mice have contributed greatly to scientific development since the first establishment of mouse ES cell technology in 1981^[6]. Meanwhile in rats, advancement of one of the gene-targeting technology using zinc-finger nucleases (ZFNs) allowed to generate the first knock-out rats in 2009^[7,8]. Microinjection of ZFNs into the pronuclei of rat embryos leads to the creation of knock-out rats. ZFNs are engineered proteins with DNA-binding and nuclease activity, which facilitates the targeted editing of genomes by creating double-strand breaks in the DNA at specified locations and promoting non-homologous end-joining. Moreover, a robust method to establish rat ES cells was reported in 2008^[9,10], and the generation of knock-out rats was achieved using rat ES cell-based technology in 2010^[11].

ESTABLISHMENT, CHARACTERIZATION, AND MAINTENANCE OF RAT EMBRYONIC STEM CELLS

Attempts for establishment of rat embryonic stem cells

Rat ES cells are isolated from the inner cell mass (ICM) of a blastocyst. Figure 1 shows a procedure for the establishment of rat ES cells (our unpublished data). The defining properties of ES cells are following; they are derived from an ICM with pluripotency; they have a stable, normal karyotype *in vitro*; they can be propagated indefinitely in theory without differentiation; they can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers,

both in teratomas after grafting and *in vitro* under appropriate conditions; and they can give rise to any cell type in the body, including germ cells, when injected into host blastocysts^[12]. Many researchers attempted to establish rat ES cells by using the same conditions as those of mouse ES cells, namely leukemia inhibitory factor (LIF) in combination with bone morphogenetic protein (BMP) or fetal bovine serum (FBS). The rat blastocyst-derived cells cultured under these conditions expressed various embryonic stem cell specific markers such as stage-specific embryonic antigen-1 (SSEA-1), Oct4, and alkaline phosphatase, whereas the chimeric rats or teratocarcinoma derived from the rat blastocyst-derived cells were not confirmed^[13-15].

Establishment of rat embryonic stem cells using small molecules

In 2008, a robust and efficient method to establish mouse ES cells was reported by Ying *et al.*^[16], which are also applicable to establish rat ES cells. To maintain the pluripotent state of mouse ES cells, LIF activates the Janus kinase/signal transducer and activator of transcription signaling pathway, while BMP and serum activate the Smad and Mad Related Family signaling pathway^[17]. Differentiation of mouse ES cells is induced *via* activation of mitogen-activated protein kinase/extracellular signal-related kinase (ERK) kinase (MEK) pathway by the autocrine stimulation of fibroblast growth factor-4 (FGF4)^[18]. Ying *et al.*^[16] revealed that LIF, serum and BMP affect the downstream of ERK, and demonstrated that the inhibition of MEK and the FGF receptor (FGFR) maintains the pluripotency of mouse ES cells under serum-free conditions. Moreover, the self-renewal capacity of mouse ES cells was promoted by an additional inhibition of glycogen synthase kinase 3 (GSK3), as the GSK3 pathway is involved in the maintenance of ES cells in the undifferentiated state *via* β -catenin/Wnt signaling^[19-25]. These findings revealed that two types of culture conditions, a combination of the MEK inhibitor and GSK3 inhibitor (2i condition), and a combination of the MEK inhibitor, FGFR inhibitor, and GSK3 inhibitor (3i condition), enable not only the maintenance of mouse ES cells but also the "robust" and "effective" establishment of rat ES cells displaying all the defining properties of ES cells^[9,10,26-31]. The morphology of rat ES cells is rounded and loosely attached on feeder cells, unlike mouse ES cells; however, the rat ES cells express the same undifferentiated markers as mouse ES cells: Oct4, Nanog, Sox2, Rex1, FGF4, and SSEA1. Kawamata *et al.*^[26] succeeded in the establishment of rat ES cells even in FBS-containing medium using Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor and activin receptor-like kinase (ALK5) inhibitor in addition to the MEK inhibitor and GSK3 inhibitor, which cells showed the defining properties such as pluripotent markers, embryonic body formation, and normal karyotype (Figure 2; our unpublished data). Table 1 shows various attempts to establish rat ES cells, and the properties of the resultant cells.

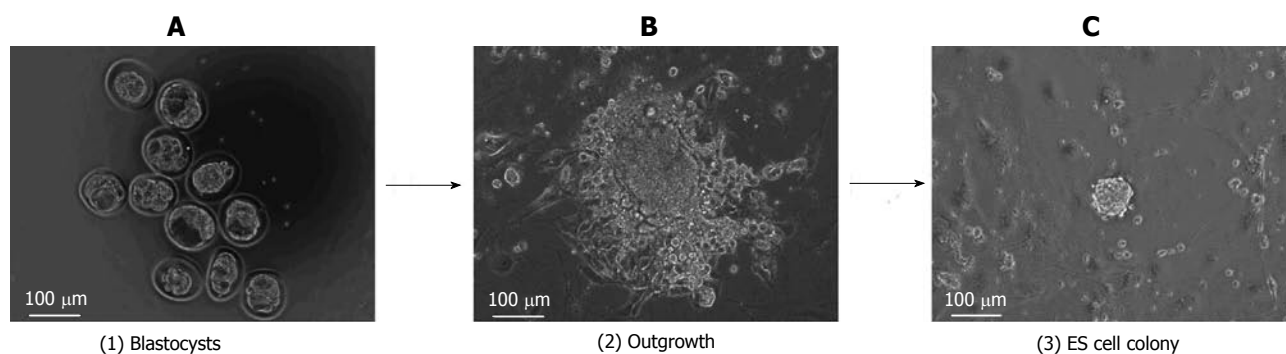
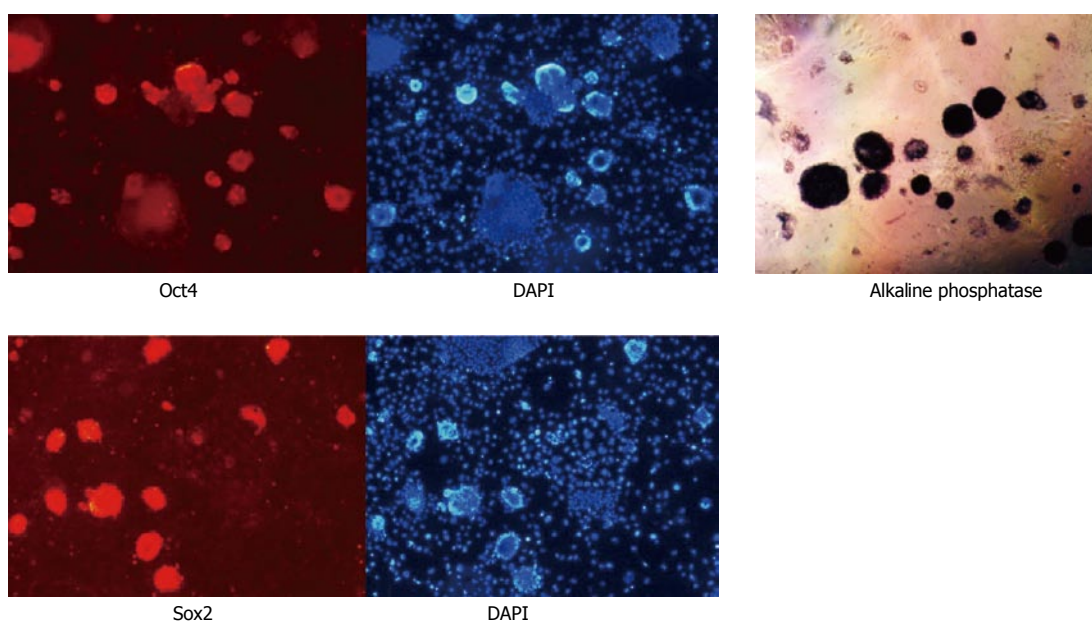


Figure 1 Establishment of rat embryonic stem cells. A: E4.5 Blastocysts are isolated from Sprague-Dawley rats, and the blastocysts dissolved zona pellucida are put on mouse embryonic fibroblasts (MEF) feeders; B: After 7-10 d, the outgrowth formed from the blastocysts are dispersed, and transferred on MEF feeders; C: Approximately 7 d after culture, rat embryonic stem (ES) cells are appeared (our unpublished data).

A Pluripotent markers



B Embryonic bodies



C karyotype

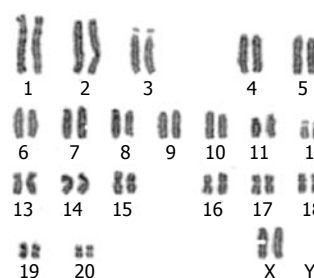


Figure 2 Properties of the rat embryonic stem cells. The rat embryonic stem cells are established using the combination of four inhibitors and serum: mitogen-associated protein kinase/extracellular signal-related kinase kinase (MEK) inhibitor, glycogen synthase kinase 3 inhibitor, activin receptor-like kinase (ALK5) inhibitor, Rho-associated, coiled-coil containing protein kinase inhibitor and FBS. A: Expression of the pluripotent markers Oct4 and Sox2, and alkaline phosphatase staining; B: Embryonic body formation; C: Karyotype analysis by G-band staining (our unpublished data). DAPI: 4',6'-diamidino-2-phenylindole; FBS: Fetal bovine serum.

Improvement of rat embryonic stem cell culture efficiency

Numerous approaches have been made to efficiently propagate rat ES cells. Soon after the establishment of rat ES cells^[9,10], rat induced pluripotent stem (iPS) cells were established using the 2i culture conditions^[32,33].

The additional inhibition of ALK5 allowed to propagate rat iPS cells as a homogeneous population, with less spontaneously differentiated colonies as compared to the 2i condition without ALK5 inhibition^[32]. Furthermore, the inhibition of ROCK prevents from apoptosis in human ES/iPS cells and enhances their attachment on

Table 1 Summary of components for the establishment of rat embryonic stem cells and the properties of the resultant cells

Ref.	Additives	Pluripotent markers	Karyotype stability	EB/teratocarcinoma	Germline transmission
Iannaccone <i>et al</i> ^[13]	FBS LIF	AP SSEA1	Unconfirmed	Failed	Failed
Takahama <i>et al</i> ^[14]	FBS LIF	AP Oct3/4 SSEA1	Unconfirmed	Unconfirmed	Unconfirmed
Vassilieva <i>et al</i> ^[15]	FBS LIF	AP Oct3/4 SSEA1	Unconfirmed	Unconfirmed	Unconfirmed
Buehr <i>et al</i> ^[9]	(A) N2B27 FGFRi MEKi GSK3i (B) N2B27 MEKi GSK3i LIF	AP Oct3/4 SSEA1 Sox2 Nanog Fgf4 Rex1	Confirmed	Confirmed	Confirmed
Li <i>et al</i> ^[10]	(A) N2B27 MEKi GSK3i (B) N2B27 MEKi GSK3i LIF	AP Oct3/4 SSEA1 Sox2 Nanog	Confirmed	Confirmed	Confirmed
Kawamata <i>et al</i> ^[26]	FBS MEKi GSK3i ALK5i ROCKi	AP Oct3/4 Sox2 Nanog Rex1	Confirmed	Confirmed	Confirmed

EB: Embryo culture media; FBS: Fetal bovine serum; LIF: Leukemia inhibitory factor; MEK: Mitogen-activated protein kinase/ERK kinase; GSK3: Glycogen synthase kinase 3; FGFR: Fibroblast growth factor-receptor; ALK5: Activin receptor-like kinase; ROCK: Rho-associated, coiled-coil containing protein kinase; AP: Alkaline phosphatase.

feeder cells after enzymatic dissociation^[34,35]. These studies suggested that the addition of the ALK5 inhibitor and ROCK inhibitor to either the 2i or 3i condition promotes the efficient culture of rat ES cells. Kawamata *et al*^[26] confirmed the effect of the ROCK inhibitor on the self-renewal of rat ES cells by using Oct4-Venus-transgenic rat ES cells, in which cells were derived from the transgenic rats expressing the fluorescent Venus protein under the Oct4 promoter/enhancer. In a condition containing all the four inhibitors (the ROCK inhibitor Y-27632, the MEK inhibitor PD0325901, the ALK5 inhibitor A-83-01, and the GSK3 inhibitor CHIR99021) referred to as YPAC, a great number of colonies expressing the pluripotent markers Oct4 and alkaline phosphatase were appeared after single-cell enzymatic dissociation. In contrast, the rat ES cell colonies maintained withdrawal of the ROCK inhibitor from the YPAC condition expressed the pluripotent markers, but the colonies were sparse under the three-inhibitor condition compared to the YPAC condition^[26]. Kawamata *et al*^[26] concluded that the ROCK inhibitor promotes the attachment of rat ES cells on feeder cells, which leads to efficient culture expansion. Li *et al*^[36] compared the 2i condition with the YPAC condition, and concluded that rat ES cells could expand approximately

twice more under the YPAC condition than under the 2i condition, while maintaining the undifferentiated state. A combination of the serum and the 2i condition was also attempted for the culture of rat ES cells, and as a result, the addition of the serum enhanced the attachment of rat ES cells to feeder cells^[29]. However, the addition of serum triggers differentiation in the cultured ES cells, and may introduce unexpected side effects due to unknown factors within the serum^[37]. Actually, the expression level of a trophectoderm marker Cdx2 was three times higher in the rat ES cells cultured in the serum with YPAC than in the cells cultured in the serum-free 2i condition^[38].

Regulation of Cdx2 expression in rat embryonic stem cells

Cdx2 is the marker of trophectoderm cells, which is not expressed in mouse ES cells; hence, some studies attempted to find culture condition to repress the expression of Cdx2^[39,40]. It was reported that the expression of Cdx2 was related to the concentration of the GSK3 inhibitor. The low concentration of the GSK3 inhibitor 1.5 $\mu\text{mol/L}$ was found to maintain pluripotency and reduce Cdx2 expression; however, the higher concentration of the GSK3 inhibitor 3 $\mu\text{mol/L}$, which has

generally been used to establish and maintain rat ES cells, promotes the expression of Cdx2^[41]. In addition, a decrease in Cdx2 expression was reported by maintaining rat ES cells on Matrigel® or in suspension^[29].

Maintenance of chromosomal states in rat embryonic stem cells

One of the defining properties of ES cells is the retention of a normal karyotype after prolonged culture. However, the rat ES cells cultured under the 2i condition retained normal karyotypes fewer than 40%, which were lower than that found in mouse ES cells^[42]. It is known that the low germline-competence of mouse ES cells results from abnormal karyotypes^[43], and hence, the instability of karyotypes in rat ES cells would make germline transmission difficult. It is reported that the combination of the serum and YPAC improves the karyotype stability, which retains normal karyotypes with over 70%^[26]. The effect of YPAC on karyotype stability was clarified by Li *et al.*^[36] by the successful generation of germline-competent chimeric rats by using the highly passaged rat ES cells maintained under the serum-free YPAC condition, while the rat ES cells maintained under the 2i condition failed to contribute germline. These results suggest that the inhibition of ROCK and/or ALK5 in addition to the 2i condition can stabilize the karyotypes of rat ES cells.

DIFFERENTIATION OF RAT EMBRYONIC STEM CELLS INTO FUNCTIONAL CELLS

Only a few studies have reported a stable induction method to differentiate rat ES cells into functional cells *in vitro*. Cao *et al.*^[44] supplied a protocol to differentiate rat ES cells into cardiomyocytes, and showed that the functional properties of the differentiated cells were similar to those of rat fetal cardiomyocytes and mouse ES cell-derived cardiomyocytes. However, the efficiency of the cardiac differentiation of rat ES cells was approximately 40%; this was lower than that of mouse ES cells, which were differentiated with over 80% efficiency^[45]. Normally, the first step for *in vitro* differentiation of mouse ES cells is to induce cell aggregation into embryonic bodies (EBs), which can be accomplished in a relatively simple procedure using a differentiation medium containing serum. However, apoptosis was observed in rat ES cells during the formation of EBs in the differentiation medium^[26,44]. On the other hand, Peng *et al.*^[46] succeeded in efficient differentiation of neural precursors from rat ES cells. They demonstrated that the ROCK inhibitor facilitates the neural differentiation and the GSK3 inhibitor maintains the survivability of the differentiated cells, and high-efficiency neural precursor differentiation (90%) was achieved using the two inhibitors. For the survival of neural precursors differentiated from mouse ES cells, it is sufficient only in the ROCK inhibitor^[47]. Thus, optimized differentiation media for rat ES cells

are necessary to induce rat ES cells into functional cells efficiently. These media may facilitate not only *in vitro* studies but also *in vivo* studies such as the transplantation of rat ES cell-derived differentiated cells or grafts to animal models, which *in vivo* studies were not reported even though rats are more relevant to humans than mice.

GENERATION OF KNOCK-IN AND KNOCK-OUT RATS USING RAT EMBRYONIC STEM CELLS

Gene-targeting to rat embryonic stem cells

Gene targeting to rat ES cells has been explored in some previous studies. Tong *et al.*^[11] and Yamamoto *et al.*^[48] reported that the efficiency of homologous recombination was 1.12%–3.70% and 0.9%, respectively, while Meek *et al.*^[28] reported that the efficiency was similar to that of mouse and human ES cells. Moreover, the gene-targeted rat ES cells express the pluripotent markers to the same extent as the parent ES cells, and retain the ability to differentiate into all three germ layers^[28]. It was noted that serum was effective at minimizing the damage caused when electroporation was performed^[49]. In fact, Yamamoto *et al.*^[48] used 1% serum during their electroporation procedure. Such efforts are not necessary for the rat ES cells maintained in the condition developed by Kawamata *et al.*^[26], because the culture condition already contains 20% FBS.

Embryonic stem cell maintenance for creating knock-out rats

Soon after the generation of transgenic rats using rat ES cells^[26,50], the first study of targeted gene disruption in rats *via* homologous recombination in rat ES cells were reported by Tong *et al.*^[11]. Figure 3 shows a process of generating genetically-modified rats. Tong *et al.*^[11] succeeded in generating germline chimeric rats by selecting the colonies that formed a small, round, compact shape, and loose attachment on feeder cells, not the large or strongly adherent colonies. This finding provides a guideline to choose rat ES cell colonies for microinjection. Tong *et al.*^[11] found that over 65% of the large and strongly adherent colonies were polyploid, and concluded that such chromosomal abnormalities caused the failure of germline transmission, as the same as the case in mouse ES cells. Hence, the likelihood of successful germline transmission could be increased by the selection of small, loosely adherent rat ES cell colonies for the purpose of microinjection.

An unstable state of rat ES cells in recipient blastocysts after microinjection was also considered as a possible cause for failure to produce germline-competent chimeric rats. In order to study the fate of rat ES cells in recipient blastocysts, Kawamata *et al.*^[26] delivered CAG-AmCyan1-transgenic rat ES cells into recipient

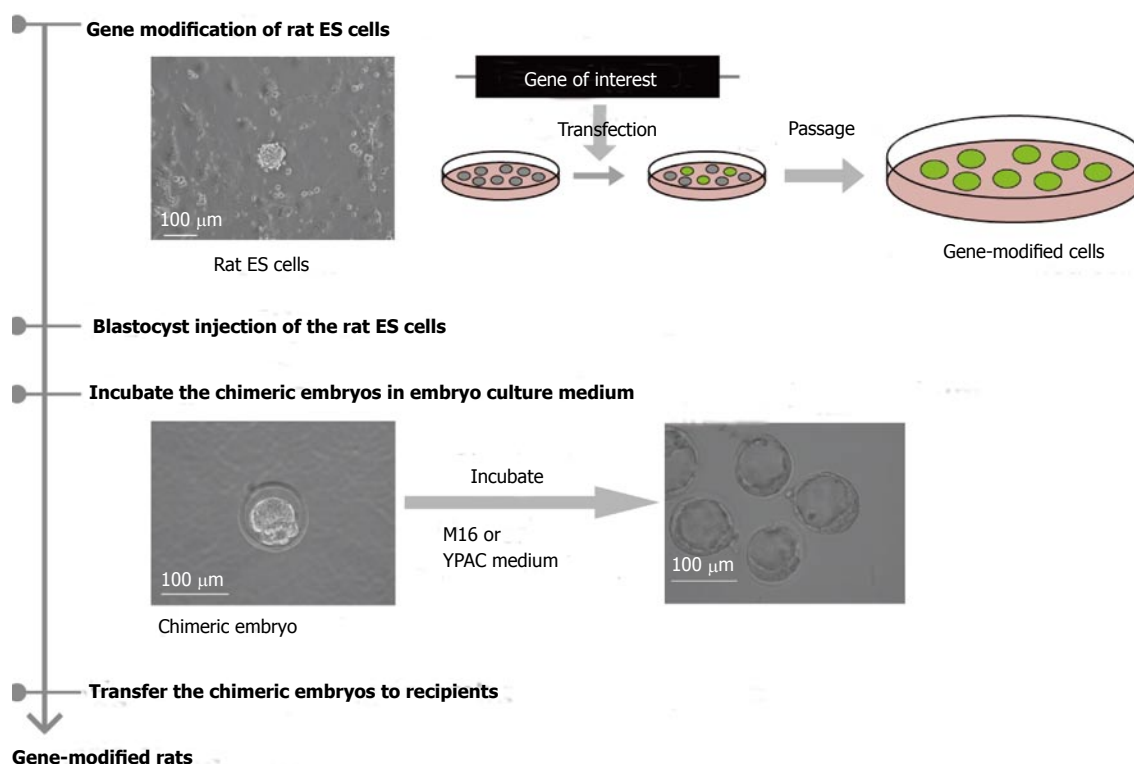


Figure 3 Generation of embryonic stem cell-derived gene-modified rats. First, a gene of interest is introduced into rat embryonic stem (ES) cells. Next, the gene-targeted rat ES cells are injected into blastocysts and are incubated in embryo culture medium or YPAC medium. Then, the chimeric rat embryos are transferred into recipient rats. After that, gene-modified rats will be generated following breeding of the chimeric rats obtained from chimeric embryos.

blastocysts by microinjection. In the absence of YPAC, few cyan-positive cells were present in the blastocysts after 30 h incubation, whereas cyan-positive rat ES cells remained on the surface of the ICM when YPAC was used in both the injection and incubation media. It is reasonable that the addition of the ROCK inhibitor not only suppresses apoptosis, but also promotes adherence to the ICM. Some embryo culture media such as M2, M16, and KSOM have been generally used for the microinjection of mouse ES cells. Similarly, the use of the embryo culture media is also proposed for microinjection of rat ES cells^[51]. Specifically, embryos are washed in M2 medium after collection from pregnant rats, and the chimeric embryos are incubated in M16 medium after microinjection to allow recovery. However, the use of the YPAC medium might offer a superior microinjection method to the conventional methods.

Embryonic stem cell-based knock-out rats and their phenotypes

Two kinds of gene-targeted rats - p53-knock-out rats and protease-activated receptor-2 (Par-2) knock-out rats - have been developed using rat ES cell technology, by three groups^[11,48,52]. The p53-knock-out rats and p53-knock-out mice revealed differing phenotypes: only 4% of female rats homozygous for the p53 knock-out could survive, whereas the female mice homozygous for the p53 knock-out were normally present at weaning^[52]; the major tumor type of the p53-knock-out rats is hemangiosarcoma, while that of the p53-

knock-out mice is lymphoma^[53]; and developmental abnormalities associated with neural tube defects occurred more frequently in the female p53-knock-out rats than in those female mice^[54]. Such sexual distortion associated with the neural tube defects also cause in human^[55,56].

A phenotype of Par-2-knock-out rats were reported by Yamamoto *et al.*^[48]. The Par-2-knock-out rats showed a lack of responsiveness to PAR-2 receptor activating peptides, which clearly indicated the deficiency of Par-2 protein.

Rat models in studying human diseases

The transgenic rat model of Huntington's disease reflects more typical adult patient pathologies in comparison to the transgenic mice^[57]. The transgenic rat model of Alzheimer's disease, harboring mutant human transgenes for amyloid precursor protein and presenilin 1, manifests a complete repertoire of Alzheimer's disease pathological features and demonstrates a markedly greater abundance of soluble oligomeric amyloid- β peptides than mice harboring the same human transgenes^[58]. Moreover, the usefulness of transgenic rat models was shown in autism and fragile X syndrome researches. Transgenic rat models lacking disease-associated genes such as fragile X mental retardation 1 gene and neuroligin-3, and these mutations display traits that may be analogous to the characteristics of in their respective human diseases^[59].

Although gene knock-out strategies are a promising

Table 2 List of knock-out rats generated using new gene-targeted technologies and rat embryonic stem cell-based technologies

Ref.	Cell type	Technology	Target
Ménoret <i>et al.</i> ^[69]	Embryo	Meganuclease	Rag1
Ménoret <i>et al.</i> ^[70]	Embryo	ZFN	Immunoglobulin heavy chain
Zschemisch <i>et al.</i> ^[71]	Embryo	ZFN	Rag1
Geurts <i>et al.</i> ^[7]	Embryo	ZFN	GFP
	Embryo	ZFN	IgM
	Embryo	ZFN	Rab38
Mashimo <i>et al.</i> ^[8]	Embryo	ZFN	IL-2 receptor gamma
Moreno <i>et al.</i> ^[72]	Embryo	ZFN	Renin
Chu <i>et al.</i> ^[73]	Embryo	ZFN	Mdr1
Vaira <i>et al.</i> ^[74]	Embryo	ZFN	Reptin
Tesson <i>et al.</i> ^[66]	Embryo	TALEN	IgM
Ferguson <i>et al.</i> ^[67]	Embryo	TALEN	Tlr4
Yamamoto <i>et al.</i> ^[48]	ES cells	Homologous Recombination	Par2
Tong <i>et al.</i> ^[11]	ES cells	Homologous Recombination	p53
Kawamata <i>et al.</i> ^[52]	ES cells	ZFN	p53

ES: Embryonic stem; ZFN: Zinc-finger nuclease; TALEN: Transcription activator-like effector nuclease.

tool to clarify the mechanism of human diseases such as those mentioned above, some human disease-related gene knock-outs are developmentally lethal. This problem can often be overcome through the use of conditional-knock-out animals, which enable site-specific and inducible gene deletion. Brown *et al.*^[60] first generated the conditional-knock-out rats *via* ZFN-mediated technologies. The system is based on Cre/loxP recombination system, and a target gene is modified by the insertion of two flanking loxP sites, enabling excision of the flanked (floxed) gene segment through Cre-mediated recombination. Conditional-knock-out mice are obtained by mating the floxed mouse line with a Cre-transgenic line, such that the target gene in the progeny becomes inactivated *in vivo* within the expression domain of Cre^[61,62]. To create the conditional-knock-out rats, Brown *et al.*^[60] mated the two strains: floxed allele-harboring rats and Cre allele-harboring rats, generated *via* pronuclear co-injection of a pair of ZFNs along with each donor plasmid.

PERSPECTIVES

Rats are used as the first-preferred animal species for *in vivo* tests of chemicals. In the meantime, *in vitro* alternative methods reflecting a response to chemicals have been demanded due to concerns about animal welfare. The use of the *in vitro* alternatives has many advantages: a decrease in the number of animals used, a reduced cost of animal maintenance, a smaller quantity of chemical needed for testing, and a shortening of the time essential to prepare for and conduct experiments^[63-65]. Rat ES cells could provide innovative *in vitro* screening models, as rat ES cells have an infinite proliferative capacity and can be

continuously supplied.

The generation of transgenic rats have been supported by not only rat ES cell-based gene-modification technology but also other new tools for gene modification such as ZFNs, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeat^[66-68]. Table 2 shows the knock-out rats generated by using of rat ES cells and new gene-modification technologies. However, the new technologies carry a risk of off-target effects by site-specific nuclease activity, and require screening to detect targeted events. In contrast, using ES cell-based technologies allows researchers to easily approach the generation of genetically modified rats with the knowledge obtained from experience in generating genetically modified mice. Therefore, rat ES cell-based gene targeting is still an essential tool for gene modifications.

CONCLUSION

Exploring small molecules for the optimum culture conditions for rat ES cells opened up avenues for the generation of genetically modified rats. Although ES cell-based knock-out/knock-in rats have not been widely generated due to the karyotype instability of rat ES cells, further exploration of new combinations of small molecules and growth factors will facilitate germline transmission of genetically modified rat ES cell clones. Additionally, human ES cells with a naïve state could be created by above effort^[75]. As the phenotypes of gene-knock-out animals can be different among species, rats represent a valuable tool in which phenotypes can be generated and compared to those of mice with relative ease. Studies using a combination of both mouse and rat disease models will provide beneficial information to clarify the mechanisms of human disease, leading to the development of new drugs. We hope that many researchers again choose to utilize rat models for research, and establish a new platform for basic and clinical applications using gene modification technology.

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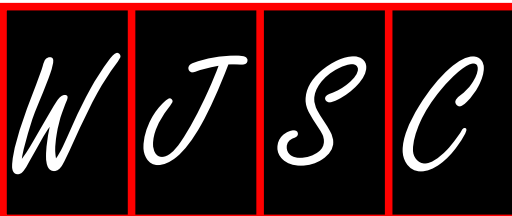
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Basic Study

Simplified three-dimensional culture system for long-term expansion of embryonic stem cells

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Abstract

AIM: To devise a simplified and efficient method for long-term culture and maintenance of embryonic stem cells requiring less frequent passaging.

METHODS: Mouse embryonic stem cells (ESCs) labeled with enhanced yellow fluorescent protein were cultured in three-dimensional (3-D) self-assembling scaffolds and compared with traditional two-dimensional (2-D) culture techniques requiring mouse embryonic fibroblast feeder layers or leukemia inhibitory factor. 3-D scaffolds encapsulating ESCs were prepared by mixing ESCs with polyethylene glycol tetra-acrylate (PEG-4-Acr) and thiol-functionalized dextran (Dex-SH). Distribution of ESCs in 3-D was monitored by confocal microscopy. Viability and proliferation of encapsulated cells during long-term culture were determined by propidium iodide as well as direct cell counts and PrestoBlue (PB) assays. Genetic expression of pluripotency markers (Oct4, Nanog, Klf4, and Sox2) in ESCs grown under 2-D and 3-D culture

conditions was examined by quantitative real-time polymerase chain reaction. Protein expression of selected stemness markers was determined by two different methods, immunofluorescence staining (Oct4 and Nanog) and western blot analysis (Oct4, Nanog, and Klf4). Pluripotency of 3-D scaffold grown ESCs was analyzed by *in vivo* teratoma assay and *in vitro* differentiation *via* embryoid bodies into cells of all three germ layers.

RESULTS: Self-assembling scaffolds encapsulating ESCs for 3-D culture without the loss of cell viability were prepared by mixing PEG-4-Acr and Dex-SH (1:1 v/v) to a final concentration of 5% (w/v). Scaffold integrity was dependent on the degree of thiol substitution of Dex-SH and cell concentration. Scaffolds prepared using Dex-SH with 7.5% and 33% thiol substitution and incubated in culture medium maintained their integrity for 11 and 13 d without cells and 22 ± 5 d and 37 ± 5 d with cells, respectively. ESCs formed compact colonies, which progressively increased in size over time due to cell proliferation as determined by confocal microscopy and PB staining. 3-D scaffold cultured ESCs expressed significantly higher levels ($P < 0.01$) of Oct4, Nanog, and Klf4, showing a 2.8, 3.0 and 1.8 fold increase, respectively, in comparison to 2-D grown cells. A similar increase in the protein expression levels of Oct4, Nanog, and Klf4 was observed in 3-D grown ESCs. However, when 3-D cultured ESCs were subsequently passaged in 2-D culture conditions, the level of these pluripotent markers was reduced to normal levels. 3-D grown ESCs produced teratomas and yielded cells of all three germ layers, expressing brachyury (mesoderm), NCAM (ectoderm), and GATA4 (endoderm) markers. Furthermore, these cells differentiated into osteogenic, chondrogenic, myogenic, and neural lineages expressing Col1, Col2, Myog, and Nestin, respectively.

CONCLUSION: This novel 3-D culture system demonstrated long-term maintenance of mouse ESCs without the routine passaging and manipulation necessary for traditional 2-D cell propagation.

Key words: Three-dimensional culture; Pluripotency; Embryonic stem cells; Self-assembling scaffold; Hydrogel

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Core tip: The pluripotent nature of embryonic stem cells (ESCs) makes them an ideal source for cell-based therapeutics and regenerative medicine. Efficient and reproducible expansion of ESCs *ex vivo* is critical for high quality cells for translational applications. However, propagation of ESCs is technically challenging, and often leads to differentiation due to inefficient two-dimensional culture techniques *in vitro*. To mimic the three-dimensional microenvironment *in vivo*, self-assembling scaffolds made from thiol-functionalized dextran and polyethylene glycol tetra-acrylate were designed to encapsulate and propagate mouse ESCs. This culture

system is simple, robust, efficient and reproducible, permitting long-term maintenance of ESCs without routine passaging and manipulation.

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INTRODUCTION

The pluripotent state of embryonic stem cells (ESCs) allows their use in a wide array of translational and clinical applications^[1]. Mouse ESCs have been used to investigate developmental and diseases processes, toxicology, cell-based therapeutics and regenerative medicine^[2,3]. Research performed using animal models^[4,5] and more recently human clinical trials^[6,7] have shown promising potential for ESCs in cell therapies, repair of damaged tissues and organs, and *in vitro* disease modeling. However, these applications require routine and efficient expansion of pluripotent ESCs and controlled differentiation to obtain a homogeneous population of cells. The pluripotency of ESCs is controlled by an intrinsic regulatory network^[8] and extrinsic factors including the microenvironment, organization and composition of the extracellular matrix (ECM), cell-cell signaling, and the temporal and spatial gradient of soluble factors^[9-12]. The complex relationship between stem cell fate and their native microenvironment results in a large discrepancy between *in vivo* and *in vitro* culture conditions effecting the quality of cultured cells^[13].

Conventionally, ESCs are grown in two-dimensional (2-D) plastic culture plates on mouse embryonic fibroblast (MEF) feeder layers or ECM components (such as gelatin and Matrigel)^[14]. Mouse ESCs can be maintained in their pluripotent state by the addition of soluble cytokines, such as leukemia inhibitory factor (LIF), to the culture media^[11,15]. However, reliance on MEF feeder layer, cytokines, and/or growth factors complicates maintenance of ESCs due to the potential transmission of xenogeneic pathogens and the fluctuation of lot-to-lot quality^[9]. Furthermore, the distribution of soluble factors in 2-D culture lacks the spatial gradient observed in three-dimensional (3-D) microenvironments, which can alter cell growth and fate determination^[16]. Studies have shown that the ECM composition and organization send mechanical signals for cell differentiation and the culture of ESCs in 2-D culture can signal differentiation into specific cell lineages^[17]. For these reasons, the maintenance of the self-renewing state of pluripotent ESCs and induced-pluripotent stem cells remains a challenge^[18]. In addition to strict culture media and growth conditions, ESCs require regular passaging (every 2 to 3 d).

Consequently, culturing of ESCs is laborious, expensive and requires a high level of expertise^[19].

In order to overcome the problems associated with 2-D culture, we hypothesized that 3-D culture may better mimic the *in vivo* environment supporting the growth and maintenance of ESC pluripotency. 3-D growth of ESCs can be facilitated by hydrogel scaffolds, composed of hydrophilic polymer networks, which emulate the fully hydrated native ECM and natural soft tissue^[20]. Hydrogel constructs incorporating drugs, cytokines, and growth factors have been shown to promote proliferation, directed differentiation, and integration of cells to regenerate target tissue^[21-24]. Recently, ESCs were cultured in 3-D hydrogel scaffolds but required routine passaging, much like 2-D cultures^[19,25].

Studies have utilized dextran-based hydrogels to promote neovascularization and differentiation of ESCs into endothelial cells^[24,26]. Whereas, thiol-functionalized dextran (Dex-SH) was combined with PEG functionalized with tetra-acrylate (PEG-4-Acr) to form chemically cross-linked hydrogels by a Michael-type addition for differentiation of chondrogenic progenitors^[27]. In this report, we developed a 3-D culture system for propagation and maintenance of mouse ESCs utilizing Dex-SH and PEG-4-Acr. Cells grown in the 3-D scaffolds proliferated for extended periods of time, and exhibited ESC characteristics including self-renewal and pluripotency. Interestingly, ESCs grown in 3-D scaffolds had upregulated expression of pluripotency genes. This novel culture system is efficient, reproducible and less cumbersome for long-term maintenance of ESCs without the routine passaging and manipulations associated with 2-D culture. These studies should help facilitate development of methods for expansion of high quality and homogenous populations of human ESCs, which are critically important for regenerative medicine and therapeutic applications.

MATERIALS AND METHODS

Maintenance and growth of ESCs

The mouse ESC line 7AC5, labeled with enhanced yellow fluorescent protein (EYFP/GFP), (ATCC, Manassas, VA) was cultured on irradiated MEF feeder layer with ESC medium containing high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Aleken Biologicals, Nashville, TX), 0.1 mmol/L 2-mercaptoethanol (Sigma, St. Louis, MO), 0.1 mmol/L nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Sigma), and 1000 U/mL of leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA) as previously described^[15].

Preparation of self-assembling scaffolds

Dextran (25 kDa, $M_w/M_n = 1.30$, Sigma) was functionalized with pendant SH groups at differing degrees of thiol substitution ranging from 4% to 34%, using a published method^[27]. Dex-SH was characterized by ¹HNMR spectroscopy using a 400 MHz Bruker Avance II

spectrometer (Bruker, Billerica, MA).

Hydrogel scaffolds were formed by mixing Dex-SH with PEG-4-Acr (20 kDa, Creative PEGWorks Winston Salem, NC) *via* a Michael addition reaction, as previously described^[22]. For this study, scaffolds were prepared with final polymer concentrations of 5% w/v. The molar ratio of thiol to acrylate groups used was 1:1.

Encapsulation of ESCs in self-assembling scaffolds

To encapsulate cells, Dex-SH and PEG-4-Acr were dissolved separately in culture medium and mixed with various concentrations (1×10^4 to 4×10^6 cells/mL) of ESCs, which were harvested at 70% confluency. Unless otherwise stated, ESCs were encapsulated at a concentration of 2×10^6 cells/mL. The resulting mixture was transferred to either a well of a 96-well plate or 1cc syringe for polymerization to produce fixed or floating scaffolds, respectively. Floating scaffolds were transferred from the syringes to 24-well plates. ESCs encapsulated in 3-D scaffolds were incubated the ESC medium and changed every 3 to 4 d or as needed. Cell growth was monitored by phase-contrast and confocal microscopy and analyzed by NIS Elements AR software (Nikon Instruments Inc., Melville, NY).

Swelling test

The degradation rate of floating scaffolds was determined by swelling tests performed under physiological conditions (37 °C). The initial dry weight (W_i) of the floating scaffolds was measured before incubation in ESC medium. At regular intervals, the scaffolds were removed from the medium to record the swollen weight (W_s) for analysis. The swelling ratio was defined as the difference between W_s and W_i divided by W_i ^[28,29]. The degradation time was determined by the time required to completely dissolve the hydrogel scaffolds of each condition prepared in triplicate.

Cell viability and proliferation assays

Cell viability was determined qualitatively by propidium iodide (PI, 1 mg/mL) (Fisher Scientific, Pittsburgh, PA) staining in triplicate experiments, and was visualized using fluorescent microscopy.

The quantitative analysis of cell growth in the scaffolds was determined by direct microscopic count using hemocytometer and PrestoBlue (PB) assays (Invitrogen), following the manufacturer's instructions. The scaffolds were incubated in PB solution for 4 h, before measuring the absorption of the solution at 570 nm and normalized to the reference wavelength of 600 nm using the Epoch microplate reader (BioTek, Winooski, VT). PB, a resazurin-based solution, was reduced proportional to the number of metabolically active cells to fluorescent resorufin.

Teratoma formation assay

For teratoma formation, ESCs were harvested following trypsin treatment, washed and re-suspended in PBS, and mixed with an equal volume of Matrigel (BD

Table 1 Mouse primer sets used in quantitative real time polymerase chain reaction

Target-gene	Forward (5'-3')	Reverse (5'-3')	Product size (bp)
<i>Oct4</i>	TCTGTTCCCGTCACTGCTCT	TGTCCTACCTCCCTTGCCCTTG	96
<i>Nanog</i>	GCAAGCGGTGGCAGAAAAAC	GCAATGGATGCTGGGATACTCA	92
<i>Klf4</i>	AAGCCAAAGAGGGGAAGAAG	CAGTGGTAAGGTTTCTCGCC	146
<i>Sox2</i>	CGAACTGGAGAAGGGGAGAG	AAGCGTTAATTGGATGGGA	165
<i>Col1</i>	GCAGGTTACCTACTCTGTC	CTTGCCCCATTCAATTTGTCT	62
<i>Col2</i>	ACCCCGAGGTGCTAATGG	AACACCTTTGGGACCATCTTT	76
<i>Myog</i>	CCTAAAGTGGAGATCCTGCG	GTGGGAGTTGCATTCACTGG	147
<i>Nestin</i>	AGACAGTGAGGCAGATGAG	CTCTCAGCTGTGGTGGTGAA	224
<i>Brachyury (T)</i>	CACACCACTGACGCACAC	GAGGCTATGAGGAGGCTTTG	132
<i>FGF5</i>	GCTCAATGATCAGAAGGAGGA	TCAGCTGGTCTTGAATGAGG	175
<i>GATA4</i>	GATGGGACGGGACACTACCTG	ACCTGCTGGCGTCTAGATT	309
<i>Gapdh</i>	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA	151
<i>β-Actin</i>	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA	228

Biosciences, San Jose, CA)^[30]. Cells (1×10^6) were subcutaneously injected (20 μ L) using a Hamilton syringe into 4-wk-old male immune-compromised SCID (severe combined immunodeficient)-beige mice (Fox Chase SCID Beige, Charles River, Wilmington, MA). Animals were anesthetized by inhalation of isoflurane gas for the injection of cells, and monitored daily. All efforts were made to minimize discomfort. After teratoma formation (3 to 4 wk), the animals were humanely euthanized by CO₂ overdose. Teratoma tissue was explanted, and flash frozen in liquid nitrogen for isolation of RNA using the RNeasy Midi kit (Qiagen, Germantown, MD)^[31]. Teratoma assays were performed in triplicate. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Oakland University (IACUC protocol number: 14033).

Differentiation of ESCs

To differentiate ESCs, embryoid bodies (EBs) were prepared by the hanging drop method^[32]. EBs were cultured in ESC growth medium for differentiation into a myogenic phenotype. EBs treated with 10^{-7} mol/L trans-retinoic acid (RA) were cultured in β -glycerol phosphate (10 mmol/L; Sigma), and ascorbic acid (50 μ g/mL; Sigma) for osteogenic differentiation^[32]; TGF- β (10 ng/mL; Sigma), insulin (10 μ g/mL; Sigma) and ascorbic acid (50 μ g/mL; Sigma) for chondrogenic differentiation^[15]; and neurobasal medium (Invitrogen) supplemented with B-27 (10 μ L/mL; Invitrogen), L-glutamine (0.5 mmol/L; Sigma), penicillin/streptomycin (1 μ L/mL; Sigma) and bFGF (5 ng/mL) for neural differentiation^[33]. Cell morphology was monitored by light microscopy on a daily basis. Osteogenic cells were analyzed for calcium deposition by von Kossa staining^[32]. Proteoglycans produced by chondrogenic derivatives were visualized by alcian blue staining^[15]. Analysis of lineage specific markers was performed as described below.

Expression of genetic markers

Gene expression studies were performed using quantitative real time polymerase chain reaction (qRT-PCR). RNA was isolated from cells using the RNeasy Mini

kit (Qiagen). ESCs grown in 3-D scaffolds were flash frozen with liquid nitrogen, ground into a fine powder using a mortar and pestle, and homogenized using the QIAshredder column (Qiagen)^[34]. RNA was purified by treating with RNase-free DNase (Promega, Madison, WI) and cDNA was synthesized with the iScript kit (BioRad, Hercules, CA). PCR reactions were performed in a 10 μ L reaction volume using the BioRad CFX90 Real-Time PCR system and SsoAdvanced SYBR Green Supermix. The specific PCR conditions used were as follows: polymerase activation 3 min at 95 °C, 40 cycles of denaturation, 15 s at 95 °C; annealing, 20 s at 60 °C; and melt curve, 5 s/step at 60 °C-95 °C. The markers used in this study represent pluripotency, all three germ layers, as well as osteogenic, chondral, myogenic, and neural cell lineages. Primers (IDT Technologies, Coralville, IA) are listed in the supplemental material (Table 1). All reactions were prepared in triplicate and normalized to reference genes, Gapdh and β -Actin.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS, permeabilized with 0.5% Triton X-100 (Sigma) for 10 min, and then blocked with 2% BSA (Sigma) for 1 h at room temperature. Fixed cells were treated with primary antibodies (1:100 diluted in blocking buffer), Oct4 (ab19857, Abcam Inc., Cambridge, MA), Nanog (sc-33760, Santa Cruz Biotechnology, Santa Cruz, CA), brachyury (sc-20109, Santa Cruz), NCAM (sc-10735, Santa Cruz), and GATA4 (sc-25310) overnight at 4 °C. Primary antibody treated cells were washed, and then stained with 1:200 diluted secondary antibodies, anti-rabbit Alexa Fluor 568 (A-11011, Molecular Probes, Eugene, OR) or Cy3-labeled anti-mouse IgG (072-01-18-06, KPL, Gaithersburg, MD, United States). Nuclei were counterstained with 1 mg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes). Images were acquired using confocal microscopy.

Western blot assay

Cells were lysed in RIPA buffer with protease inhibitors (1 mmol/L PMSF) (Fisher Scientific), centrifuged at

12000 rpm for 20 min at 4 °C, and the supernatants were collected. Protein concentrations were determined by the Pierce 660 nm protein assay (Fisher Scientific,) using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), with BSA as a standard. Equal amounts of protein (10 µg) of 2-D and 3-D scaffold grown cells were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad). Membranes were incubated for 30 min at room temperature in blocking buffer [5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST)]. The blocked membranes were then probed with 1:200 diluted primary antibodies overnight at 4 °C against Oct4 (Abcam), Nanog (Santa Cruz), Klf4 (ab21949, Abcam) and β-Actin (sc-130656, Santa Cruz). After washing with PBST, membranes were incubated with 1:10000 diluted horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature (Santa Cruz). Proteins on the membranes were detected by using an ECL chemiluminescence kit (BioRad) and by exposing the membranes to X-ray film. Finally, protein bands were analyzed using ImageJ (NIH, Bethesda, MA), normalized to β-Actin and expressed in arbitrary densitometric units.

Statistical analysis

All quantitative data were expressed as mean ± SE. One-way ANOVA analysis was performed on natural log transformed data, and analyzed for unequal variances. Post hoc tests used for multiple comparisons include Games-Howell analysis for PB analysis as well as Tukey and Dunnett's test for relative gene expression in qRT-PCR studies. Results with a *P* value less than 0.05 were considered to be significant (^a*P* < 0.05 and ^b*P* < 0.01). All analyses were performed using SPSS version 11.5 (SPSS Inc, United States). The statistical methods of this study were reviewed by Dr. Harvey Qu from the Department of Statistics at Oakland University.

RESULTS

Viability and growth of ESCs in the 3-D scaffolds

The viability of 3-D grown ESCs was not affected by encapsulation or the process of scaffold self-assembly. Cell growth increased over a period of 3 wk, as evident by the increase in the intensity as well as the number of EYFP/GFP expressing cells in fixed scaffolds (Figure 1A-B). PI staining of the scaffolds 1 d after culturing showed that a majority of the cells were viable cells (green) and contained only a small fraction (< 5%) of dead cells (yellow). Presence of dead cells could be due to damage resulting from trypsinization of ESCs used for encapsulation. When trypsinized ESCs were tested by trypan blue staining, a similar fraction of dead cells (about 10%) was observed, indicating that encapsulation did not cause cell death. However, it is interesting to note that unlike in 2-D culture, encapsulated dead cells did not lyse during 3-D culture. We speculate that the scaffold microenvironment

Table 2 Effects of thiol substitutions of Dex-SH on the rate of scaffold degradation

Degree of thiol substitution of Dex-SH	Time in degradation (d)
4%	10 ± 2
6%	13 ± 4
7.5%	22 ± 5
12%	28 ± 6
30%	35 ± 5
33%	37 ± 5
34%	37 ± 5

limited proteolytic activity. In our preliminary studies, the colony size of viable cells was restricted due to the limited swelling of fixed scaffolds (Figure 1A and B). We reasoned that if the scaffolds were allowed to swell freely, ESC growth and colony size would increase. Indeed, when ESCs were cultured in the floating scaffolds, cell growth and colony size increased in a time dependent manner (Figure 1C and D). Growth of encapsulated cells in floating scaffolds was confirmed by direct cell counts as shown in Figure 1E. After a lag period, ESCs grew at a generation rate of 36 h. A similar growth pattern was observed using PB staining. The results depicted in Figure 1F showed a continuous and significant increase in proliferation at 2, 3 and 4 wk of culture following a period of acclimatization. The highest increase in cell growth was observed between week 3 and 4, following which, the scaffolds degraded rapidly. Evidently, cell growth and colony size increased concomitant with reduction in scaffold integrity. Conceivably, scaffolds that retain integrity beyond 4 wk would continue to support the undifferentiated growth of ESCs.

Effect of composition and cell concentration on the rate of degradation of scaffolds

The polymer concentration and MW of Dex-SH and PEG-4-Acr, as well as the degree of thiol substitution of Dex-SH influenced the polymerization and degradation rate of scaffolds (Table 2), similar to previous reports^[23,27]. After preliminary evaluation, we selected Dex-SH (MW = 25 kDa) with 7.5% and 33% thiol substitution and PEG-4-Acr (MW = 20 kDa) at 5% w/v for further studies for propagating cells for various culture periods; these preparations yielded scaffolds that degraded without cells in 11 and 13 d and degraded with cells in 22 ± 5 d and 37 ± 5 d, respectively.

Scaffolds prepared using 7.5% thiol substitution of Dex-SH and various cell concentrations, ranging from 1 × 10⁴ cells/mL to 10 × 10⁶ cells/mL, exhibited different swelling profiles, although the initial swelling capacity of the scaffolds was similar. Scaffolds containing various cell concentrations (0, 2 × 10⁶ cells/mL or 4 × 10⁶ cells/mL) as shown in Figure 2A, exhibited comparable swelling ratios until day 9; after which, control scaffolds without cells degraded rapidly, followed by scaffolds prepared with 4 × 10⁶ cells/mL and finally scaffolds with 2 × 10⁶ cells/mL. Scaffolds with the higher number of cells (4

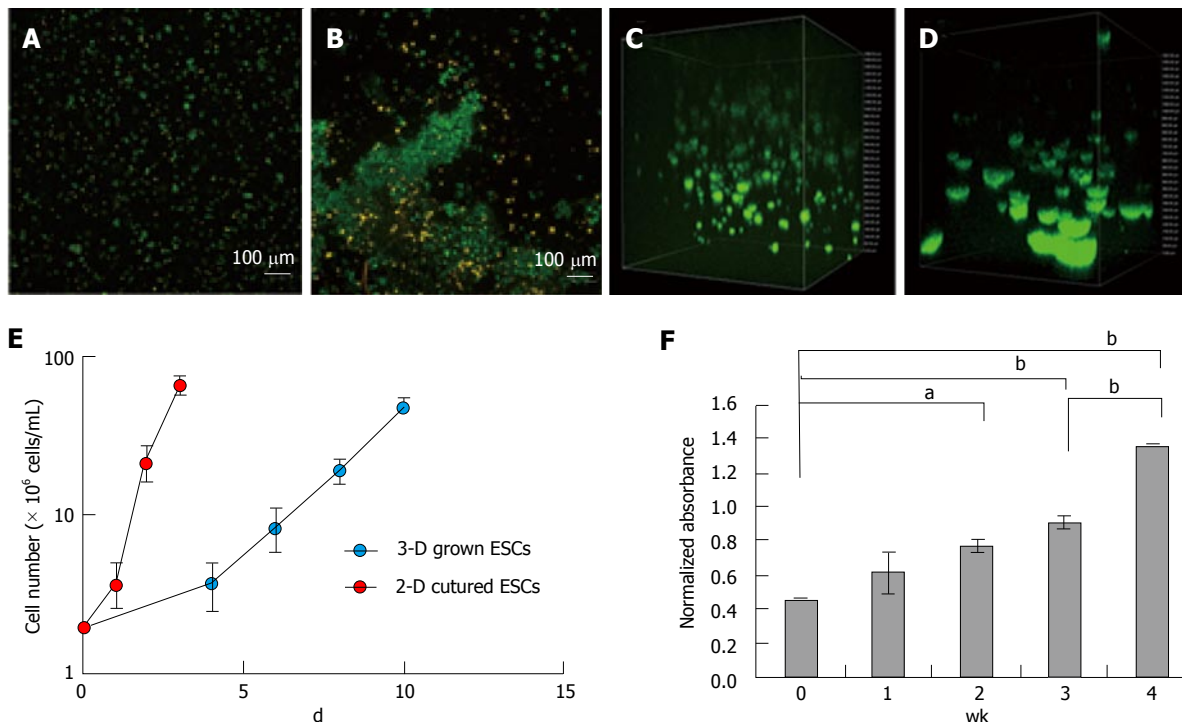


Figure 1 Cell viability and growth of embryonic stem cells encapsulated in self-assembling three-dimensional scaffolds. Enhanced yellow fluorescent protein-labeled ESCs were encapsulated (2×10^6 cells/mL) in fixed and floating scaffolds prepared using Dex-SH with 7.5% thiol substitution and PEG-4-Acr. Encapsulated cells were cultured in ESC medium and periodically cell growth was visualized using fluorescent microscopy. A and B: Live (green) and dead (yellow) cells in fixed scaffolds stained with PI at day 1 and week 3, respectively. While the viable cells increased with time, the number of dead cells remained constant. Scale bars = 100 μ m; C and D: Three-dimensional (3-D) confocal images of floating scaffolds representing ESC growth at day 3 and week 3, respectively; E: Growth of ESCs encapsulated in 3-D scaffolds was assayed by direct count using hemocytometer. Cells seeded in 3-D scaffolds (at 2×10^6 cells/mL) were incubated in ESC medium and counted at various time intervals. Data presented as cell number ($\times 10^6$ cells/mL) $\pm$ SE ($n = 3$) and plotted against the days of incubation; F: Quantitative determination of ESC proliferation in floating scaffolds at week 0, 1, 2, 3, and 4 of culture after staining with PB as analyzed by microplate reader. The results were expressed as the normalized absorbance $\pm$ SE ($n = 3$), with a significant increase in cell number, compared to initial ESC viability at time 0. $^aP < 0.05$ and $^bP < 0.01$ using one-way ANOVA with the non-equal variance hypothesis and Games-Howell multiple comparisons test. ESCs: Embryonic stem cells; Dex-SH: Thiol functionalized dextran; PEG-4-Acr: Polyethylene glycol tetra-acrylate.

Table 3 Cell concentration for optimal growth of embryonic stem cells under 2-D and 3-D culture conditions

Culture conditions	Cell concentration	
	(cells/mL)	(cells/cm ²)
2-D Culture	---	3×10^4 to 5×10^4 ^[33]
3-D Culture	1×10^4	1×10^3

3-D: Three-dimensional; 2-D: Two-dimensional.

$\times 10^6$ cells/mL) swelled to 2-fold of their initial weight and started to degrade on day 19 of culture. Whereas, scaffolds with a lower number of cells (2×10^6 cells/mL) swelled to nearly 3 times their initial weight and started to degrade on day 27 of culture. Furthermore, scaffolds prepared with the lower cell concentration (2×10^6 cells/mL) resulted in rapid clonal growth (Figure 2B; B1, B2) as compared to the higher cell concentration (4×10^6 cells/mL) (Figure 2B; B3, B4). Quantitative analysis of colony size showed a 2-fold increase in the mean diameter of colonies in scaffolds prepared with lower numbers of cells (Figure 2C). The scaffold supported the growth of encapsulated ESCs at concentrations as low as 1×10^4 cells/mL (or 10^3 /cm²), suggesting that low concentration had no effect on the cell viability (Figure 2D). Maintenance of ESCs at low concentrations

is more advantageous in 3-D culture compared to 2-D culture (Table 3), since low seeding densities in 2-D culture results in poor growth, decreased viability and differentiation of ESCs.

Expression of pluripotency markers in the 3-D scaffold cultured ESCs

To confirm whether 3-D grown ESCs maintained pluripotency, expression of selected ESC-specific markers was analyzed using qRT-PCR. The results depicted in Figure 3 indicate that expression of Oct4 and Nanog increased 2.8 and 3.0 fold, respectively in 3-D scaffold (prepared using 33% Dex-SH) grown ESCs as compared to cells grown under 2-D culture conditions. The expression of these markers significantly and successively increased in 3-D grown ESCs during 1, 2 and 3 wk. The expression of Klf4 also increased significantly (1.8 fold) above the level of 2-D grown ESCs but only during the second week of cell growth; whereas, the expression of Sox2 was not affected by 3-D culture. Concomitant with the start of degradation of the scaffolds at 6 wk of culture, expression of pluripotency markers gradually decreased to the levels of 2-D grown ESCs.

Immunofluorescence analysis of 3-D grown ESCs

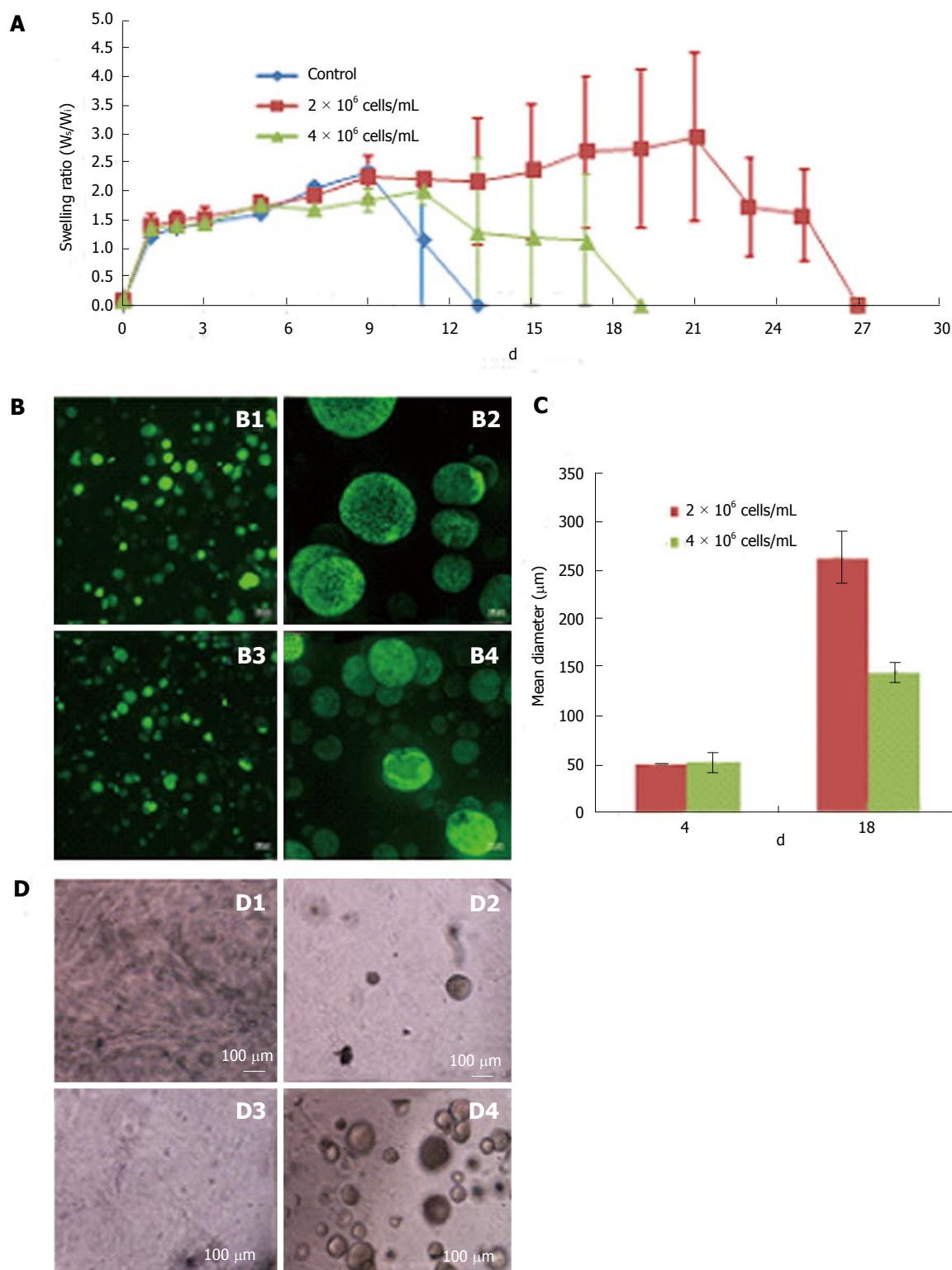


Figure 2 Effect of cell concentration on the rate of scaffold degradation and embryonic stem cells colony size. **A**: Swelling tests of floating scaffolds prepared with 7.5% thiol substitution were used to compare the rate of degradation of scaffolds prepared with various cell concentrations (0, 2×10^6 cells/mL and 4×10^6 cells/mL). Scaffolds with and without ESCs were incubated in ESC medium and weighed periodically. Data expressed as the mean swelling ratio (W_t/W_i) $\pm$ SE ($n = 3$); **B**: Representative composite confocal images of ESCs grown in floating scaffolds encapsulating 2×10^6 cells/mL (B1 and B2) and 4×10^6 cells/mL (B3 and B4) at day-4 and day-18, respectively. Scale bars = 100 μ m; **C**: Quantification of ESC colony size in floating scaffolds encapsulating 2×10^6 cells/mL or 4×10^6 cells/mL at day-4 and day-18 of culture. Cell colony size was expressed as mean diameter (μ m) $\pm$ SE ($n = 3$); **D**: Growth of low concentrations of ESCs in 3-D scaffolds. Light micrographs of the scaffolds encapsulating 1×10^4 cells/mL at day-0 and day-7 (D1 and D2, respectively) and 1×10^5 cells/mL at day-0 and day-7 (D3 and D4 respectively). Scale bars = 100 μ m. ESCs: Embryonic stem cells; 3-D: Three-dimensional.

showed upregulation of protein levels of Oct4 and Nanog as evident by the increase in the fluorescent

intensity of cells stained with the respective antibodies (Figure 4A). Furthermore, expression of these proteins

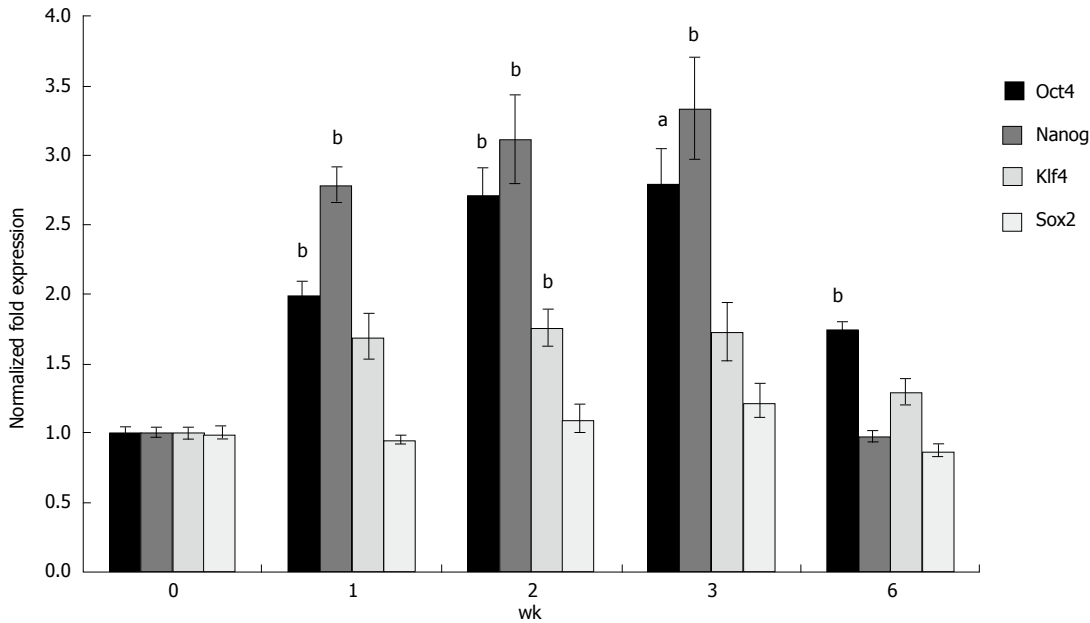


Figure 3 Expression of pluripotent markers in three-dimensional grown embryonic stem cells. ESCs in scaffolds prepared with Dex-SH with 33% thiol substitution were cultured and analyzed for the expression of selected markers, Oct4, Nanog, Klf4, and Sox2 using qRT-PCR at week 0, 1, 2, 3, and 6. Results of experiments were expressed as the fold expression $\pm$ SE ($n = 3$) normalized to reference genes Gapdh and β -Actin, where a significant (^a $P < 0.05$ and ^b $P < 0.01$) increase of pluripotent marker expression was compared to initial 2-D grown ESCs (day 0), using one-way ANOVA with the non-equal variance hypothesis and Dunnett's multiple comparisons test. Results depicted showed upregulation of Oct4, Nanog, and Klf4, but Sox2 was maintained at levels similar to the control. Expression of Oct4 and Nanog was successively and significantly higher at week 1, 2 and 3 until the onset of degradation (week 6), at which point their expression was decreased to initial levels. ESCs: Embryonic stem cells; Dex-SH: Thiol functionalized dextran; qRT-PCR: Quantitative real time polymerase chain reaction; 2-D: Two-dimensional.

was analyzed by western blot and results are shown in Figure 4B. Similarly, levels of Oct4, Nanog and Klf4 were visibly increased in the 3-D grown cells when compared with the control. Quantitative analysis of the western blot confirmed the increase in Oct4, Nanog and Klf4 (1.9, 3.9 and 1.3 fold, respectively) compared to the control (Figure 4C); this was well correlated with the observed transcription levels of these genes.

Maintenance of pluripotency of 3-D grown ESCs was further investigated by subculturing under 2-D culture conditions (Figure 5A, A1-A3). They displayed typical compact colony morphology (even upon passaging five times) in 2-D culture and were indistinguishable from the initial ESCs seeded and grown in 3-D scaffolds. Expression of pluripotent genes in ESCs first grown in 3-D scaffolds for 2 wk and then subcultured under 2-D conditions were analyzed. The results depicted in Figure 5B showed that the expression of Oct4, Nanog, and Klf4 returned to levels comparable to traditionally 2-D propagated ESCs (Figure 5B).

Pluripotency of 3-D scaffold grown ESCs was further validated by *in vivo* teratoma assays using immune-compromised mice. The animals injected with 1×10^6 cells formed teratomas within 4 wk (Figure 6A and B). Analysis of explanted teratoma tissue showed expression of Brachyury, FGF5, and GATA4, suggesting differentiation of injected ESCs into cell lineages of all three germ layers (Figure 6C). Conceivably, the 3-D scaffold provided a microenvironment for upregulation of pluripotent genes, maintenance of pluripotency and

self-renewal of ESCs.

Differentiation of the 3-D scaffolds grown ESCs into various cell lineages

Differentiation potential of the 3-D grown ESCs was also studied *via* EB formation. The EBs from 3-D grown ESCs were allowed to spontaneously differentiate into all three germ layers, mesoderm, ectoderm, and endoderm and expressed specific protein markers such as Brachyury, NCAM, and GATA4 (Figure 7A). Furthermore, 3-D grown ESCs were induced to differentiate into various cell lineages, including osteogenic, chondrogenic, myogenic, and neural cell types. The results of differentiation depicted osteogenic derivatives that had the cobblestone appearance of osteoblast cells and were positive for calcium deposition as determined by von Kossa staining (Figure 7B, B1). Chondrogenic derivatives of 3-D grown ESCs were analyzed by alcian blue staining showing positive proteoglycan production (Figure 7B, B2). Light micrographs of myogenic derivatives of 3-D grown ESCs had spindle shaped morphology (Figure 7B, B3), while neural derivatives of ESCs displayed neurofilaments (Figure 7B, B4). Further analysis of the 3-D grown ESC derivatives revealed expression of cell-specific markers (Figure 7C) as determined by qRT-PCR; osteogenic, chondrogenic, myogenic, and neural derivatives expressed Collagen type 1 (Col1), Collagen type 2 (Col2), Myogenin (Myog), and Nestin, respectively. The expression of these cell-specific markers signified that 3-D grown ESCs maintained differentiation potential.

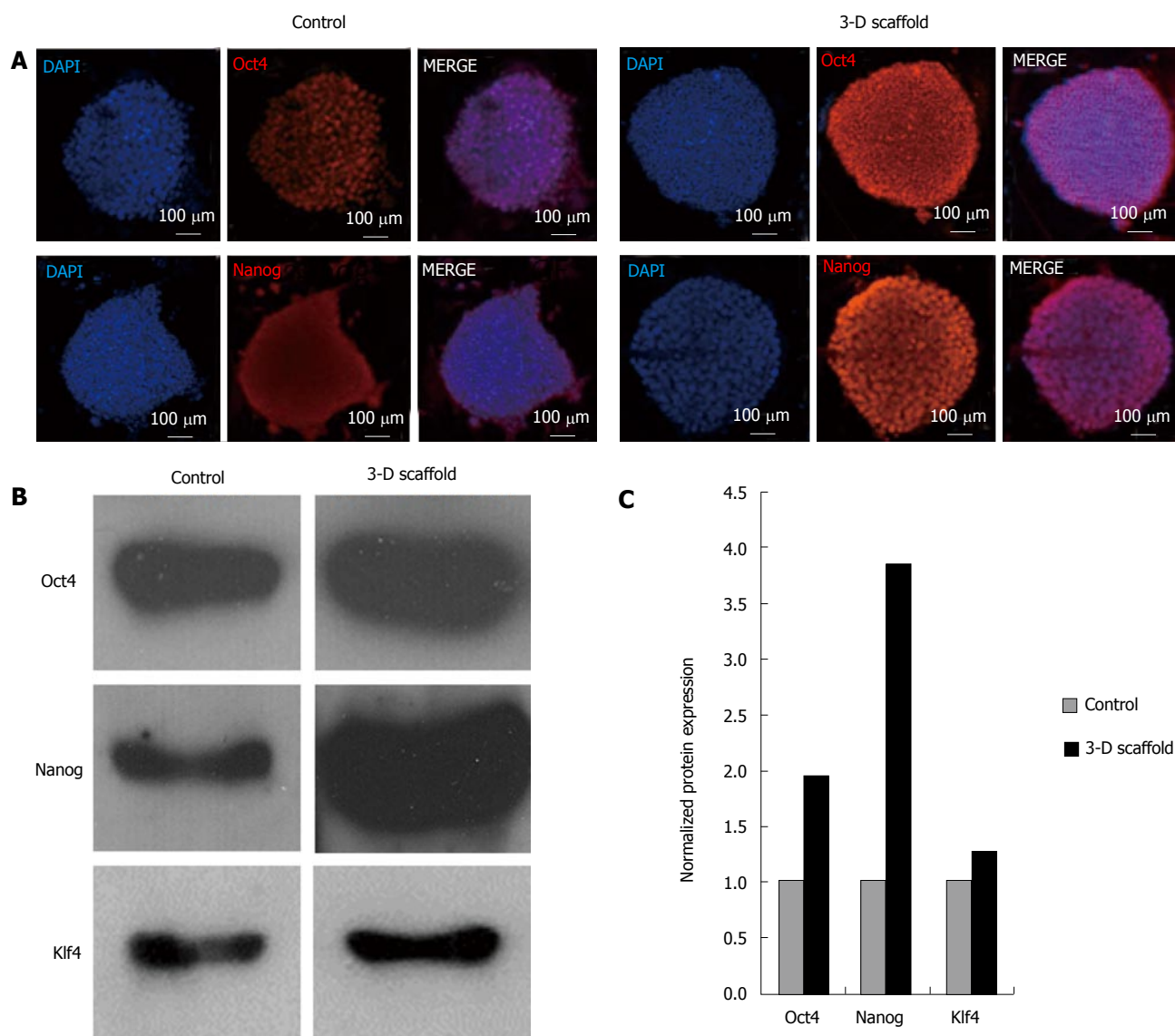


Figure 4 Protein expression of pluripotency markers in embryonic stem cells grown in two-dimensional and three-dimensional cultures. ESCs grown in two-dimensional (2-D) and in 3-D floating scaffolds (prepared with 33% thiol substitution) were analyzed by immunofluorescence staining and western blotting. A: Cells grown in 2-D culture and 3-D scaffolds for 2 wk were cultured on coverslips for 2 d and stained with primary antibodies, Oct4 and Nanog, treated with Alexa Fluor 568 conjugated secondary antibodies, and counterstained with DAPI. Confocal images depicted an increase in Oct4 and Nanog protein expression in 3-D scaffold grown ESCs compared to 2-D cultured cells; B: Western blot analysis showed increased expressions of Oct4, Nanog, and Klf4 proteins in ESCs grown in the 3-D scaffold for 2 wk compared to initial 2-D grown ESCs (day 0). Cell were lysed in RIPA buffer and aliquots containing an equal amount of protein (10 μ g) were subjected to 12% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies against Oct4, Nanog, and Klf4; C: Quantitative analysis of western blots (normalized to β -actin levels) showed that Oct4, Nanog and Klf4 protein levels in 3-D grown cells were increased by 1.9, 3.9 and 1.3 fold, respectively, as compared to 2-D cultured controls. Representative results are shown. ESCs: Embryonic stem cells. DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

DISCUSSION

In this report, we described an alternative to traditional 2-D culturing of ESCs without the need for a MEF feeder layer and routine passaging. This method was developed utilizing two components, Dex-SH and PEG-4-Acr, for self-assembling 3-D hydrogel scaffolds. Evidently, this hydrogel scaffold better emulated the fully hydrated native 3-D microenvironment and supported the pluripotent growth of ESCs. This method eliminates laborious and time-consuming manipulations, which often results in the loss of ESC lines due to contamination of cultures or differentiation of cells.

The polymerization of Dex-SH and PEG-4-Acr, *via* a

Michael addition reaction, facilitated the self-assembly of the 3-D scaffolds and encapsulation of ESCs. Polymerization was dependent upon several factors including concentration and ratio of polymers, degree of thiol substitution of Dex-SH, and cell concentration. Optimal conditions for scaffold polymerization, cell encapsulation, and cell viability were achieved by varying these parameters. The results showed that higher the degree of thiol substitution of Dex-SH, the faster the polymerization of hydrogel. Consistent with a previous report^[27], the ratio and amount of Dex-SH and PEG-4-Acr also affected the formation and swelling properties of the hydrogel scaffolds. In one study, 10% w/v polymer concentrations were used to prepare Dex-

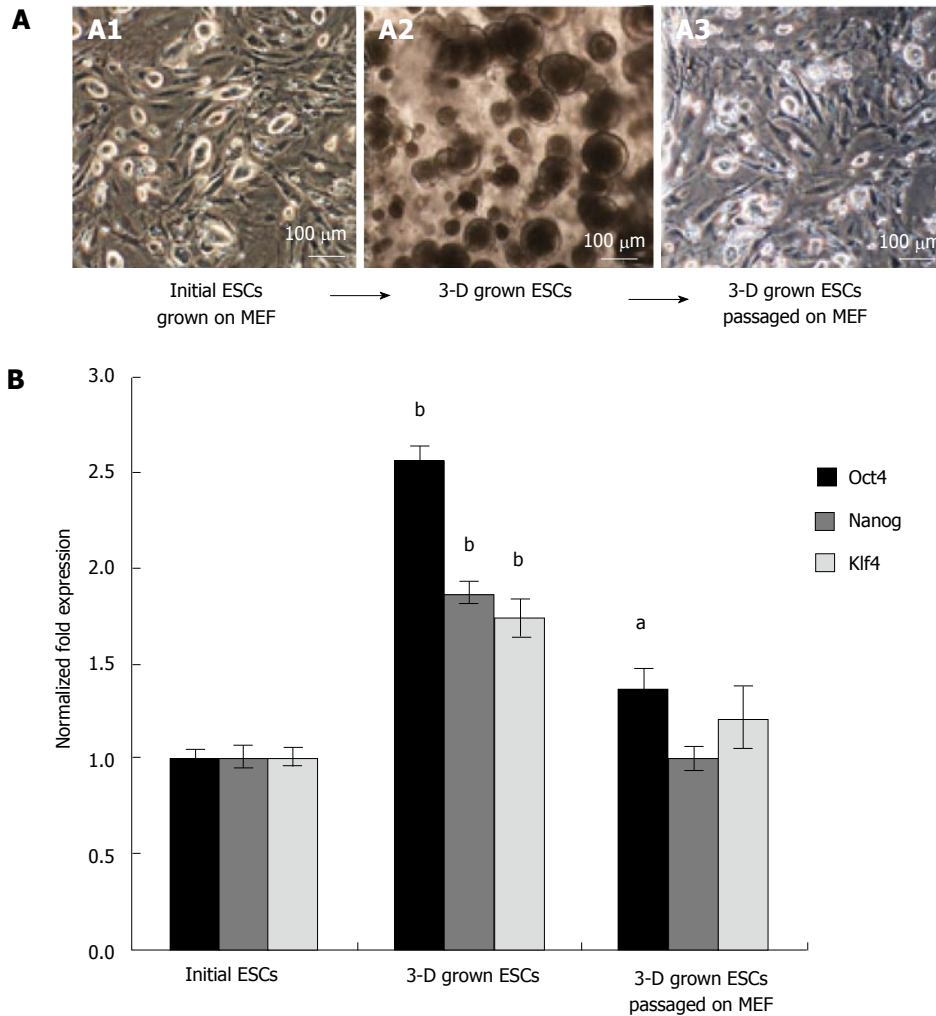


Figure 5 Three-dimensional grown embryonic stem cells were subcultured under two-dimensional culture conditions expressed normal levels of pluripotent markers. A: Light micrographs depicting cell morphology of the initial ESCs used for encapsulation (A1), cultured in three-dimensional (3-D) scaffolds for 2 wk (A2), and subsequently passaged 5 times in 2-D culture (A3); B: Expression of Oct4, Nanog, and Klf4 in ESCs grown in 2-D culture, 3-D grown ESCs, and 3-D grown ESCs, which were subsequently passaged 5 times in 2-D culture as determined by qRT-PCR. Results were expressed as the fold expression $\pm$ SE ($n = 3$) normalized to reference genes Gapdh and β -Actin where a significant (^a $P < 0.05$ and ^b $P < 0.01$) increase of marker expression was compared to initial 2-D grown ESCs, using one-way ANOVA and Tukey multiple comparisons test. ESCs: Embryonic stem cells; MEF: Mouse embryonic fibroblasts; qRT-PCR: Quantitative real time polymerase chain reaction; ANOVA: Analysis of variance.

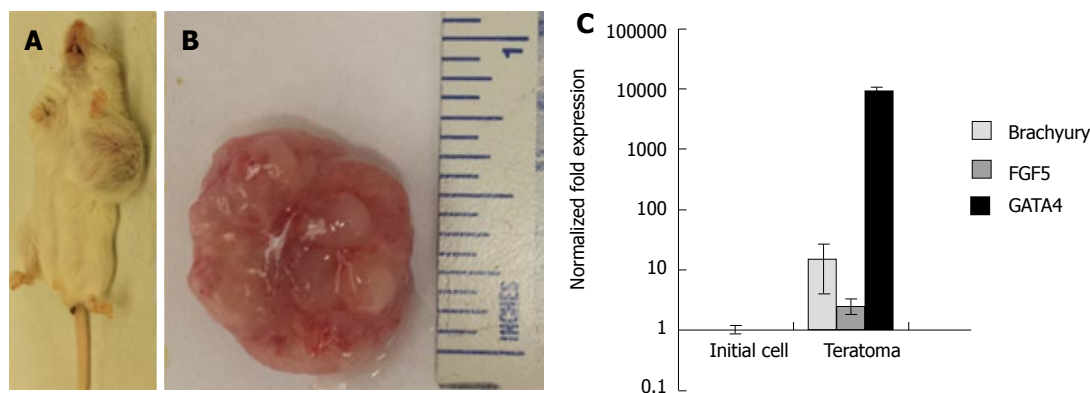


Figure 6 Three-dimensional grown embryonic stem cells formed teratomas in severe combined immunodeficient-beige mice. Explanted teratoma tissues were analyzed for the expression of three germ layer markers. A: Gross images of tumor growth resulting from injection of 3-D grown ESCs. Tumor growth was observed in all mice injected ($n = 3$); B: Explanted tumor at 4 wk showed encapsulated, lobular and well-circumscribed gross morphology consistent with teratoma growth; C: Expression of germ layer markers, Brachyury, FGF5, and GATA4 representing mesoderm, ectoderm, and endoderm was analyzed by qRT-PCR. Results of tumor explants were expressed as fold expression $\pm$ SE ($n = 3$) normalized to reference genes Gapdh and β -Actin, and compared to initial cells injected *in vivo*. ESCs: Embryonic stem cells; qRT-PCR: Quantitative real time polymerase chain reaction..

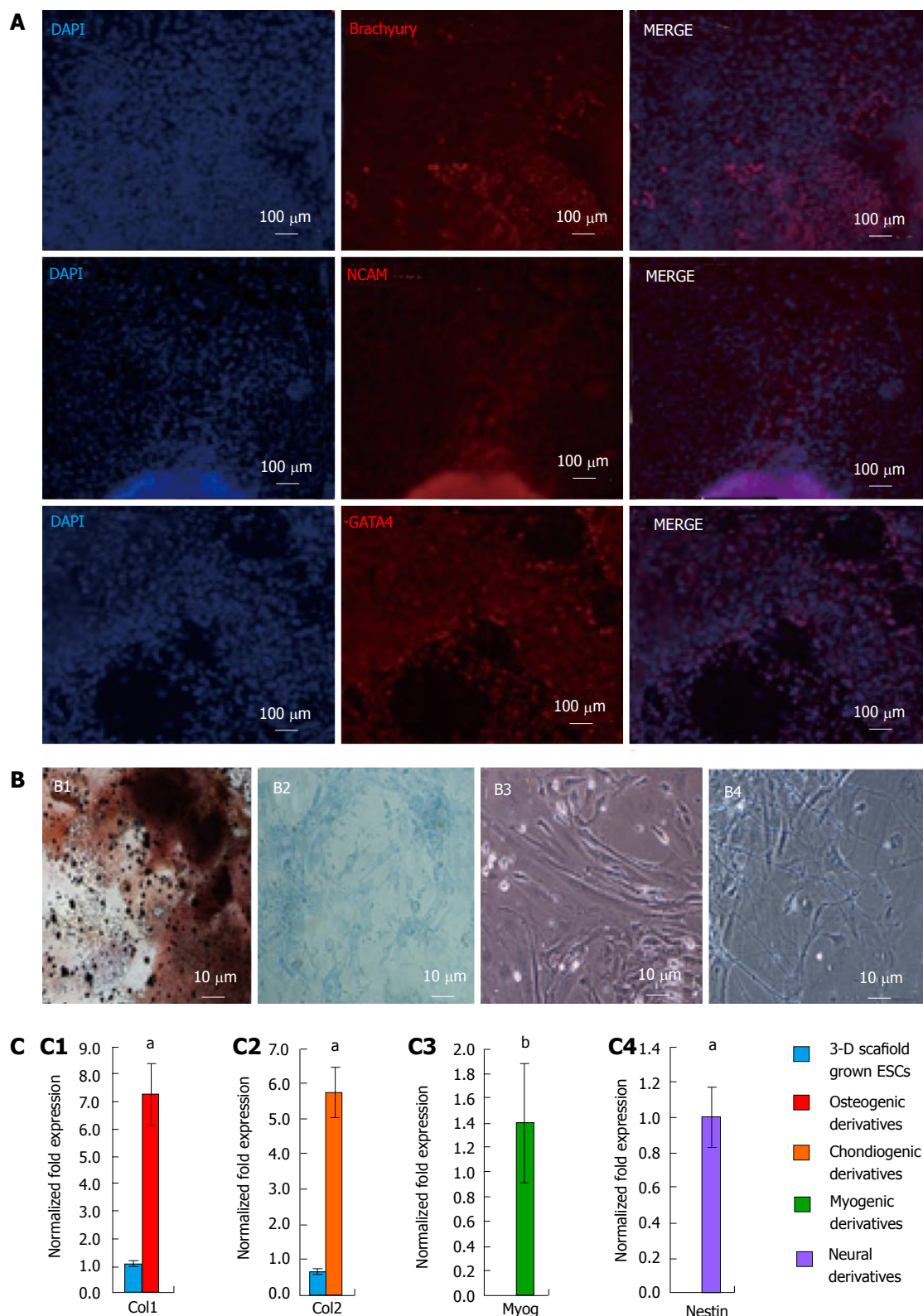


Figure 7 Differentiation potential of three-dimensional grown embryonic stem cells. A: Embryoid bodies derived from ESCs grown in the 3-D scaffolds were allowed to spontaneously differentiate and analyzed by immunofluorescence staining with antibodies against all three germ layers. Confocal images depicted the presence of Brachyury, NCAM, and GATA4 protein expression, showing the potential of ESCs to differentiate into mesoderm, ectoderm, and endoderm, respectively; B: 3-D scaffold grown ESCs were also directed to differentiate via EB formation and selective induction media. Shown are light micrographs depicting cell morphology of ESC derivatives, osteogenic, chondrogenic, myogenic, and neural (B1, B2, B3, and B4 respectively). Osteogenic and chondrogenic derivatives were cultured for 4 wk and analyzed by von Kossa, and alcian blue staining, respectively. Osteogenic cell-specific ECM stained dark brown for calcium deposition (B1). Proteoglycan produced by chondrogenic derivatives stained blue; C: Expression of cell-specific markers in differentiated ESC derivatives expressed Col1 (C1), Col2 (C2), Myog (C3), and Nestin (C4) markers for osteogenic, chondrogenic, myogenic and neural cells, respectively. Results represent the fold expression $\pm$ SE ($n = 3$) normalized to reference genes Gapdh and β -Actin where a significant (^a $P < 0.05$ and ^b $P < 0.01$) increase of marker expression was compared to initial 2-D grown ESCs (day 0), using one-way ANOVA with the non-equal variance hypothesis. ESCs: Embryonic stem cells; ANOVA: Analysis of variance..

SH and PEG-4-Acr scaffolds to promote differentiation of ESCs^[23]. However, we determined that 5% w/v polymer concentrations were ideal to provide a scaffold microenvironment with a greater degree of flexibility and suitability for ESC growth. Mixing of cells with PEG-4-Acr prior to scaffold molding yielded homogeneous distribution of cells in both fixed and floating scaffolds. The 3-D scaffold grown ESCs displayed undifferentiated compact round colony morphology, even upon prolonged culturing. Previously, Gerecht *et al.*^[26] reported the potential of hyaluronic acid-based hydrogels in maintaining human ESCs with an undifferentiated morphology for 20 d. However, polymerization of hyaluronic acid-based hydrogels was achieved with ultraviolet light, a potent carcinogen. Whereas, self-assembly of the Dex-SH and PEG-4-Acr scaffold does not require a mutagenic catalyst, and ESCs can be maintained for over 6 wk. They also reported that at least 5×10^6 cells/mL to 10×10^6 cells/mL were required for cell growth and colony formation in the hyaluronic acid-based scaffolds. In contrast, we found that use of Dex-SH and PEG-4-Acr scaffolds supported ESC growth at concentrations as low as 1×10^4 cells/mL or $1 \times 10^3/\text{cm}^2$ in 3-D scaffolds. At concentrations below 1×10^4 cells/mL successful encapsulation in the scaffold was difficult to assess due to low cell density. Furthermore, low concentration may also affect the viability of the cells. In traditional 2-D culture, optimal maintenance of ESCs requires a much higher cell density (3×10^4 to 5×10^4 cells/cm²)^[33].

ESC self-renewal was not affected by the duration of growth in 3-D scaffolds as long as the integrity of the scaffold was maintained. Scaffold integrity also influenced the cell proliferation rate and colony size. ESCs grew at a slower rate with smaller colony size in fixed scaffolds. However, cell growth rate and colony size was less restricted in floating scaffolds. In agreement with previous reports^[35,36], we found that mouse ESCs doubled nearly every 12 h in 2-D culture, whereas the generation time for ESCs in the 3-D culture system was approximately 36 h. It has been reported that the growth rate of stem cells *in vivo* vary from a few hours to months depending upon the niche^[37]. In 2-D culture, ESCs reached near confluency in 3 d and started to differentiate, whereas the growth rate in 3-D was slower; the cells did not differentiate and continued to grow for up to 6 wk, limited only by scaffold degradation. Using the exponential cell growth formula $N = N_0 \times 2^g$, where N is the final cell number, N_0 is the initial cell number (2×10^6), and g is the number generations (25), the expected total number of cells after 6 wk of growth would be 134×10^{12} cells. The fact that ESCs grew for an extended period without the loss of cell viability and their self-renewal potential suggested that the 3-D culture system described here was superior to 2-D culture, which requires routine passaging.

In addition to the concentration of the scaffold components and degree of thiol substitution, cell con-

centration significantly affected the rate of scaffold degradation. Consistent with previous studies^[23], scaffolds without cells degraded earlier compared to seeded scaffolds (13 d vs 27 d, respectively) suggesting that the addition of cells improved the stability of the scaffolds. Moreover, scaffolds encapsulating a higher concentration of ESCs degraded faster and swelled less than scaffolds with lower concentration of ESCs. Consequently, ESC colony size was smaller in scaffolds with a higher concentration of cells as compared to scaffolds prepared with a lower cell concentration. In addition, encapsulated ESCs in floating scaffolds grew rapidly with larger colony sizes as compared to fixed scaffolds of similar composition. These results suggested that the swelling plasticity of the self-assembling scaffolds favorably promoted the growth and maintenance of ESC colonies. Previous studies also indicated that cell encapsulation increased integrity of the scaffold as compared to scaffolds without cells^[23]. However, in this report the effect of cell concentrations on scaffold integrity was not reported. Our observations indicated that higher cell concentrations inversely effected scaffold integrity suggesting that the cells may be producing factors that de-stabilize the scaffolds. The nature of these factors remains unknown.

ESCs grown in the 3-D scaffolds for 6 wk maintained their pluripotency and differentiation potential, as determined by their ability to spontaneously differentiate into cell types comprising all three germ layers. The EBs differentiated into osteogenic, chondrogenic, myogenic, and neural cell types, expressing cell-specific markers. These results indicated that the self-assembling scaffolds support the self-renewal and pluripotency of ESCs, even after prolonged periods of culturing. The pluripotency of 3-D grown ESCs was further confirmed by teratoma formation *in vivo*. Teratomas explanted from immune-compromised mice following injection of 3-D grown ESCs showed specific gene expression representing all three germ layers.

Interestingly, the expression of three pluripotency markers, Oct4, Nanog and Klf4, was higher in 3-D cultured ESCs as compared to 2-D grown ESCs both at a transcriptional and translational level. The expression of these genes remained at higher levels throughout the extended growth period (up to 6 wk) until the onset of scaffold degradation. In contrast, Sox2 expression levels in 3-D grown ESCs was found to be similar to the control (2-D grown ESCs). Although high expression of Nanog and Klf4 has been reported to enhance self-renewal and pluripotency^[38], overexpression of Oct4 has been implicated in spontaneous ESC differentiation^[39]. Contrary to this, we did not observe differentiation of ESCs grown in 3-D scaffolds. When 3-D grown ESCs were subcultured under 2-D culture conditions, expression of pluripotent markers returned to levels similar to 2-D grown cells. Previous reports^[40] also described differential regulation of stemness genes, with an upregulation of Oct4 but downregulation of Sox2 in ESCs cultured in three different scaffolds prepared

using PGLA, collagen and chitosan; whereas Nanog was only highly upregulated in chitosan scaffolds. Taken together with our findings, it can be argued that the scaffold microenvironments play an important role in influencing cell-matrix communication, thus effecting gene expression. It is also possible that cellular proteins may have interacted with thiol groups during the encapsulation step of self-assembly of the scaffolds resulting in alteration of their activities. Nevertheless, the underlying molecular mechanism responsible for the upregulation of pluripotent genes in ESCs grown in 3-D Dex-SH and PEG-4-Acr scaffolds remain unknown and warrants further investigation.

The findings reported here demonstrate a novel, efficient, reproducible, and simple approach to ESC cultivation. However, these results were obtained using mouse ESCs as a model, further studies are required to expand these results to human pluripotent stem cells. Previous attempts at 3-D cultivation of ESCs have required routine passaging^[19,25,26]. To our knowledge this is the first report that described a robust system for 3-D culturing of ESCs for extended periods without passaging or manipulation. These improvements in the maintenance of ESCs will help their use in translational research, disease modeling, stem cell therapies, and regenerative medicine.

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COMMENTS

Background

Embryonic stem cells (ESCs) are capable of differentiating into any of the over 200 cell types found in the body, which makes them an ideal source for cell-based therapies and regenerative medicine. These applications require efficient and reproducible expansion of ESCs *ex vivo* for high quality cells, which then can be uniformly differentiated for clinical use. However, propagation of ESCs is technically challenging, laborious and expensive. Traditional ESC culture techniques are heavily dependent on cell adherence to two-dimensional (2-D) plastic culture plates, regular passaging (every two to three days) and xenogeneic feeder layers. This often results in differentiation and loss of cell lines.

Research frontiers

In order to overcome the problems associated with 2-D cell culture, few studies have reported the use of three-dimensional (3-D) scaffolds to propagate ESCs. However, these methods are not efficient and require the use of carcinogenic catalysts for polymerization of the scaffolds. Moreover, these techniques required routine passaging in order to maintain the pluripotency of ESCs.

Innovations and breakthroughs

A novel 3-D culture system was developed for propagation of ESCs for an extended period of time. In this system, ESCs were encapsulated upon self-assembly of 3-D scaffolds prepared with thiol-functionalized dextran and polyethylene glycol tetra-acrylate. The 3-D scaffold microenvironment supported the growth of ESCs while maintaining their pluripotency and self-renewal potential

for extended periods without passaging and extensive manipulation, which is required for 2-D culture of ESCs.

Applications

This new culture system should help develop methods of expansion for high quality and homogenous populations of ESCs, which are critically important for regenerative medicine and therapeutic applications.

Terminology

A scaffold is an engineered matrix formed from biomaterials mimicking the microenvironment and capable of supporting cell growth. In this study, self-assembling scaffolds were formed upon combination of two polymers, polyethylene glycol tetra-acrylate and thiol-functionalized dextran.

Peer-review

The authors designed a simplified and efficient system, which mimics the microenvironment *in vivo* for long-term proliferation and maintenance of ESCs. Furthermore, the culture system is efficient and reproducible and is expected to lead to further innovations.

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**EDITORIAL**

- 1078** Emerging role of microRNAs in cancer stem cells: Implications in cancer therapy
Garg M

REVIEW

- 1090** Complement activation in the context of stem cells and tissue repair
Schraufstatter IU, Khaldoyanidi SK, DiScipio RG

MINIREVIEWS

- 1109** From cellular to chemical approach for acute neural and alternative options for age-induced functional diseases
Bukovsky A
- 1118** Stem cells: An eventual treatment option for heart diseases
Bilgimol JC, Ragupathi S, Vengadassalapathy L, Senthil NS, Selvakumar K, Ganesan M, Manjunath SR

ORIGINAL ARTICLE**Basic Study**

- 1127** Autologous tissue patch rich in stem cells created in the subcutaneous tissue
Garcia-Gomez I, Gudehiithlu KP, Arruda JAL, Singh AK
- 1137** Easily-handled method to isolate mesenchymal stem cells from coagulated human bone marrow samples
Wang HX, Li ZY, Guo ZK, Guo ZK

Contents

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ABOUT COVER

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Emerging role of microRNAs in cancer stem cells: Implications in cancer therapy

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Abstract

A small subset of cancer cells that act as tumor initiating cells or cancer stem cells (CSCs) maintain self-renewal and growth promoting capabilities of cancer and are responsible for drug/treatment resistance, tumor recurrence and metastasis. Due to their potential clinical importance, many researchers have put their efforts over decades to unravel the molecular mechanisms that regulate CSCs functions. MicroRNAs (miRNAs) which are 21-23 nucleotide long, endogenous non-

coding RNAs, regulate gene expression through gene silencing at post-transcriptional level by binding to the 3'-untranslated regions or the open reading frames of target genes, thereby result in target mRNA degradation or its translational repression and serve important role in several cellular, physiological and developmental processes. Aberrant miRNAs expression and their implication in CSCs regulation by controlling asymmetric cell division, drug/treatment resistance and metastasis make miRNAs a tool of great therapeutic potential against cancer. Recent advancements on the biological complexities of CSCs, modulation in CSCs properties by miRNA network and development of miRNA based treatment strategies specifically targeting the CSCs as an attractive therapeutic targets for clinical application are being critically analysed.

Key words: Cancer stem cells; Drug resistance; Tumor recurrence; MicroRNAs; Cancer therapy

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Core tip: Cancer stem cells (CSCs) which are believed to be the prerequisite for metastasis and tumor recurrence, are endowed with ability to undergo symmetric cell division, capacity for self-renewability, long term proliferation and resistance to anti-neoplastic therapeutic drugs. Regulatory characteristics of microRNAs (miRNAs) which are the clusters of non-coding RNA molecules, include widespread changes in gene expression through gene silencing at post-transcriptional level and are dysregulated in human cancer. Over the past two decades, miRNAs have gained widespread attention due to their involvement in acquisition of stem cell-like properties, regulation and reprogramming by cancer cells during cancer progression. Many studies are coming up which document miRNAs as novel therapeutic tool in targeting CSCs functions, sensitizing them to apoptotic effects of anti-cancer drugs, and reducing tumor burden with no relapse in current clinical settings.

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INTRODUCTION

Despite high response rates to initial treatments including chemotherapy, radiotherapy or sometimes even after combinational chemotherapies, cancer remains the most lethal disease. Recent technological advancements and animal studies have validated the presence of small subset of cancer cells among the heterogeneous population of tumor cells and are known as cancer stem cells (CSCs) or tumor initiating cells. They have different biological properties, increased tumorigenic potential and are supposed to serve important role in drug resistance, tumor development and its recurrence. Anti-cancer therapies are being employed on the basis of their ability to shrink tumor cells, but CSCs survive and proliferate owing to an increased intrinsic (*de novo*) or acquired resistance to anti-cancer drugs. This makes the clinical therapy ineffective, leads to tumor recurrence and results in high mortality rates in cancer patients. Decreased expression of energy dependent transporters to retain drugs inside the cancer cells and increased efficacy of drug response by sensitizing these subset of cancer cells to drug induced apoptosis could be key mechanisms towards improved treatment of cancer. Therefore, there is an urgent need to understand the molecular mechanisms controlling CSCs population and functions to develop effective therapies to eradicate recurrence which is a real threat to complete cancer cure. Emerging evidences have suggested the critical involvement of protein-coding genes as well as non-coding RNAs (ncRNAs) including microRNAs (miRNAs) in various types of cancers and serve important role in CSCs functions. Recent findings on the miRNAs, responsible for the maintenance and regulation of CSCs properties like tumorigenicity and drug resistance, are briefly summarized in the later sections. Gaining further insight into the potential and therapeutic approaches by targeting CSCs using miRNAs as a tool would be helpful for deciphering and designing future strategies for human cancer treatment with better outcome by preventing metastatic progression and tumor recurrence.

CSCS

CSCs with tumor initiating potential constitute only a fraction of neoplastic cells and were first reported by Lapidot *et al*^[1]. These cells are endowed with the capacity of long term proliferation, ability to undergo symmetric cell division and seed new tumors by virtue of their intrinsic self-protection and self-renewal

capabilities. Capacity for self-renewal, propagation of differentiated progenitors and the expression of specific stem cell genes are some of the biological properties that are shared by both normal and CSCs. Accumulation of genetic and epigenetic alterations deregulate the basic stem cell biology and distinguish CSCs from the normal stem cells in their chemoresistance, enhanced tumorigenic and metastatic activities^[2]. Recent experimental studies have identified the activation of oncogenes and inactivation of tumor suppressor genes to be responsible for self-renewal ability and pluripotency of CSCs^[3]. Concept of heterogeneity among CSCs as stationary and migrating CSCs came into existence with the discovery of process of epithelial-to-mesenchymal transition (EMT). EMT is a crucial event which allows the stationary CSCs at the primary site to lose their epithelial character, morphological transition, and loss of cell-cell adherence. This transition imparts high motility to the stationary CSCs, which is a necessary requirement by migrating CSCs for local invasion and metastatic dissemination. The concept of migrating CSCs and especially EMT as a widespread mechanism of stem cell generation validates the dynamic nature of CSCs and strongly supports the current CSC theory^[4,5].

Over the past few years, number of studies have reported the isolation and characterization of CSCs for hematological malignancies including acute lymphoblastic leukemia, chronic myeloid leukemia, acute myelogenous leukemia, multiple myeloma and also for solid tumors of breast, colon, stomach, brain, lung, liver, skin, prostate, testis, ovary and pancreas^[6]. The subpopulation of CSCs are identified, isolated and characterized on the basis of capacity to form sphere-clusters with a high clonogenic efficiency, a stronger self-renewal ability and much higher levels of cell cycle markers which account for G1-S/G2-M phases progression, side population profile and stemness gene expression.

Stem cell maintenance, survival, self-renewal and differentiation of CSCs are known to be regulated by several signaling pathways and molecules. Notch, Wnt/catenin, sonic hedgehog, bone morphogenetic protein, receptor tyrosine kinase and TGF- β signaling pathways are studied to be aberrantly active in CSCs and regulate self-renewal activity, thereby play significant role in tumor initiation and development in various malignancies including breast, colorectal, prostate, pancreatic, glioma, leukemia and colon cancers^[7-11].

CSCs are not only the source of initial tumor formation, postoperative recurrence and metastasis but also contribute to chemo/radioresistance. High invasiveness and frequent recurrence due to the presence of inefficient DNA repair mechanisms, which render CSCs highly resistant to chemotherapeutic and ionizing radiations, are the main reasons for treatment failure and recurrent disease^[12,13]. Targeting the pathways and mechanisms behind the development of chemo/radioresistance, modifying the niche of cancer

cells, regulating the cellular response to damage by modulating apoptosis, cell cycle proliferation, DNA repair, invasion and differentiation functions might help in eliminating CSCs subpopulation and developing therapeutic strategies for complete cancer treatment with no relapse.

MiRNAs have gained widespread attention in modulating the CSCs functions through affecting the expression level of target genes and proteins that are involved in signaling pathways including cell proliferation and cell death. Recent advancements on the role of miRNAs in regulation and maintenance of tumor cells with stem cell properties are discussed in later sections.

MIRNAS AND THEIR REGULATORY FUNCTIONS IN CSCS

MiRNAs and its biogenesis

The most important advancement over the past recent years is the discovery of non-coding RNA families which are actively transcribed from the genome of many organisms and generate widespread changes by regulating vital biological processes. Among them, miRNAs are the novel small non-protein coding, evolutionarily conserved, 21-23 nucleotide long single-stranded RNA molecules. Many important diverse functions of miRNAs include the regulation of cellular differentiation, proliferation and apoptosis by regulating the stability or translational efficiency of target messenger RNAs which are expressed in tissue-specific or developmental stage-specific manner.

MiRNA-mRNA target recognition due to sequence complementarity of the *lin-4* miRNAs to multiple conserved sites within the *lin-14* 3' untranslated region (3'UTR) leads to the discovery of miRNAs way back in 1993 during genome study in *Caenorhabditis elegans* development^[13,14]. MiRNAs are randomly located in mammalian genome and the complex process of biogenesis begins in nucleus with the transcription of polycistronic primary-miRNA (pri-miRNA) transcript by RNA polymerases II and III^[15,16]. Hundreds or thousands of nucleotides long pri-miRNA transcripts with one or many stem loops, 5' capping and 3' polyadenylation, undergo further cleavage by nuclear microprocessor complex containing enzymes Drosha, RNA III endonuclease and double stranded RNA binding protein DiGeorge syndrome critical region 8 (DGCR8)^[17,18]. The resulting 70 nucleotide long pre-miRNAs are exported to cytoplasm by exportin 5 and Ran-GTP^[19]. Another enzyme, RNA III endonuclease known as Dicer, cleaves hairpin-like pre-miRNA into two complementary fragments and one of which is mature miRNA^[20]. Mature miRNA strand is then incorporated into the members of Agronaute protein family, which constitutes the catalytic portion of the multi-protein RNA-induced silencing complex (RISC). MiRNAs then direct RISC to target mRNAs which share sequence complementation in seed region that consists of nucleotides at position

2-8 of 5' end of mature miRNA. Complementation between the seed sequence and 3'UTR of target mRNA results in mRNA transcript degradation while imperfect complementation results translational repression^[21]. Nuclear pri-miRNA and cytoplasmic pre-miRNA cleavage steps and mature miRNA-target mRNA recognition sites are the potential therapeutic points against cancer by regulating miRNA processing, its biogenesis and miRNA functions (Figure 1).

Statistical methods and profiling studies validate the presence of miRNAs in close proximity to chromosomal breakpoints, cancer associated genomic regions and fragile sites where mutations/deletions can occur. Loss of tumor suppressive miRNAs and increased expression of oncogenic miRNAs enhance the expression of target oncogenes and repress the target tumor suppressor genes respectively. Deregulation of novel miRNAs expression leads to induction of anti-apoptotic activity, tumor invasion, drug resistance and metastasis and has been correlated with the pathogenesis of cancer. MiRNAs are present in complex regulatory circuits to regulate stem cells function and are being examined to play important role in generation of CSCs, maintenance of enhanced self-renewal capacities of CSCs, pluripotency and their neoplastic transformation into tumors (Figure 2).

MiRNAs in maintenance and regulation of CSCs properties

Many important functions of miRNAs in embryonic development and stem cells regulation in mammals are investigated. Dynamic expression profile of miRNAs in CSCs validates its significant role in controlling the self-renewal ability, pluripotency, differentiation of progenitor cells, prosurvival and antistress mechanisms (Table 1).

MiRNAs playing role in differentiation processes can either directly suppress the self-renewal state by suppressing the markers of pluripotency including Nanog, POU class 5 homoeobox 1 (POU5f1) also known as Oct4, Kruppel like factor 4 and sex-determining region Y-box containing gene 2 [Sox2, a crucial transcription factor for the maintenance of embryonic stem cell (ESC) pluripotency and the determination of cell fate], or stabilize the differentiated cell fate by targeting the transcripts that are regulated by the pluripotency transcription factors including *Oct4*, *Sox2*, *Nanog* and *Tcf3*^[22]. The transfection of let-7c, member of Let-7 family has been shown to rescue the differentiation defects in DGCR8^{-/-} cells by downregulating the stemness genes including *Oct4*, *Sox2* and *Nanog*^[22]. Negative feedback regulation between Lin-28, a marker of undifferentiated ESCs and Let-7 family members has been reported in mouse differentiated cells and embryonic carcinoma cells^[23]. Reduced patient survival and tumor relapse in human colon adenocarcinomas has been correlated with increased expression of Lin-28^[24]. Studies on inhibition of Sox2 and placenta-specific 1 gene by miR-126 has been reported in gastric

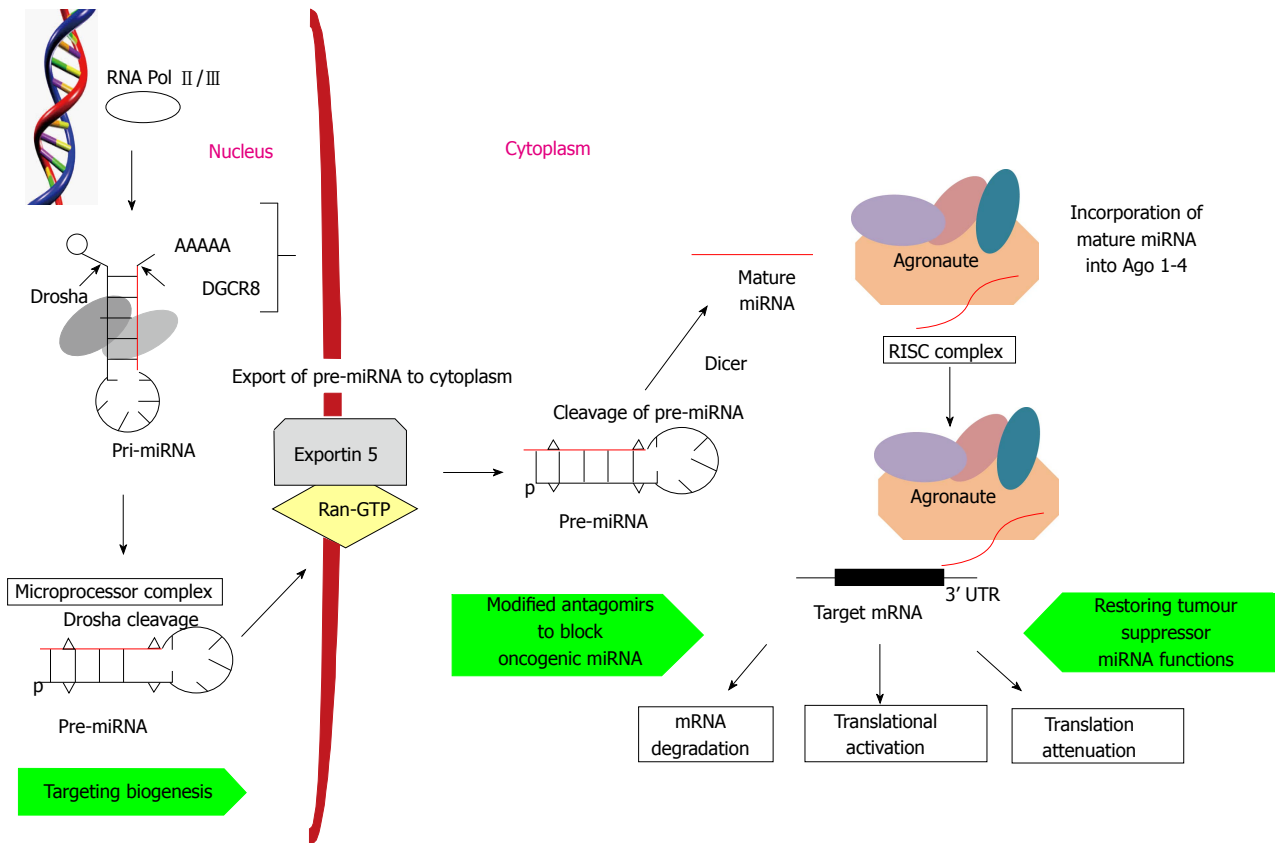


Figure 1 MicroRNA biogenesis, activity and potential therapeutic targets. Mature miRNAs are generated from hairpin-like longer primary transcripts by two sequential processing steps mediated by a nuclear (Drosha) and a cytoplasmic (Dicer) RNAase III endonuclease. MiRNA then directs RISC to target mRNAs which share sequence complementation between seed region at 5' end of mature miRNA and 3'UTR of target mRNA. Stability of miRNA:mRNA recognition and degree of complementation leads to translational activation but more commonly mRNA degradation and translational repression. Potential therapeutic target sites are shown which include steps during miRNA biogenesis and target mRNA recognition. 3'UTR: 3' untranslated region; RISC: RNA-induced silencing complex; miRNA: MicroRNAs; Ago: Agronaute.

carcinogenesis^[25].

First oncomiR reported as miR-17-92 polycistron has been examined to regulate c-Myc expression and accelerate tumor development in stomach, prostate, pancreatic, colon, lung and breast cancers. Novel stem cell specific miRNAs including miR-290, miR-302/367 and miR-371 clusters are identified in human ESCs and exhibit altered cell cycle profile and inhibit ESCs transition from self-renewal to differentiated state. Members of the miR-302 family has been shown to reprogram human skin carcinoma cells into pluripotent ESC-like state^[26].

Inhibition of tumor sphere growth *in vitro* and tumor formation *in vivo* upon restoration of miR-34 has been concluded to be associated with the suppression of growth of CSCs with biomarkers of CD44⁺ and CD133⁺ in human pancreatic cancer cells. These CSCs are deficient in miR-34 and show higher expression of Bcl-2 and Notch, which are the target genes for tumor suppressor like p53, and are involved in survival and self-renewal of CSCs. MiR-34 has been shown to inhibit the reprogramming through repressing pluripotency genes like *Nanog*, *Sox2* and *N-Myc*^[27]. Further research has confirmed that restoration of miR-34 directly regulates Bcl-2 and Notch target genes, activates caspase-3,

induces apoptosis, thereby could increase chemotherapeutic and radiotherapeutic sensitivities in pancreatic cancer cells^[28,29]. Suppression of tumorigenesis *in vitro* has been found to be regulated by miR-134 by downregulating the Notch target proteins and affecting G2/M phase of cells in human endometrial CSCs^[30]. Independent study by Park *et al*^[31], 2013 revealed the differential expression of 43 miRNAs and their putative target genes such as *p53*, *ErbB1*, *Notch*, *Wnt*, and TGF- β signaling pathways which are the key regulators for stem cells and are mainly involved in cell death, cellular development, cellular growth, proliferation and maintenance of cancer and stem cell in glioblastoma multiforme (GBM) which is the most aggressive primary brain tumor, and notorious for resistance to chemo/radiotherapy.

Owing to high degree of biochemical specificity and potency shown by miRNAs in regulating the multiple vital pathways that can significantly affect the cancer progression, development of miRNAs as therapeutic molecule in association with anti-cancer drugs provides immense opportunities to counteract chemo/radioresistance and improve treatment outcome in various human cancers. Recent advances in the mechanisms, co-delivery of therapeutic molecules using

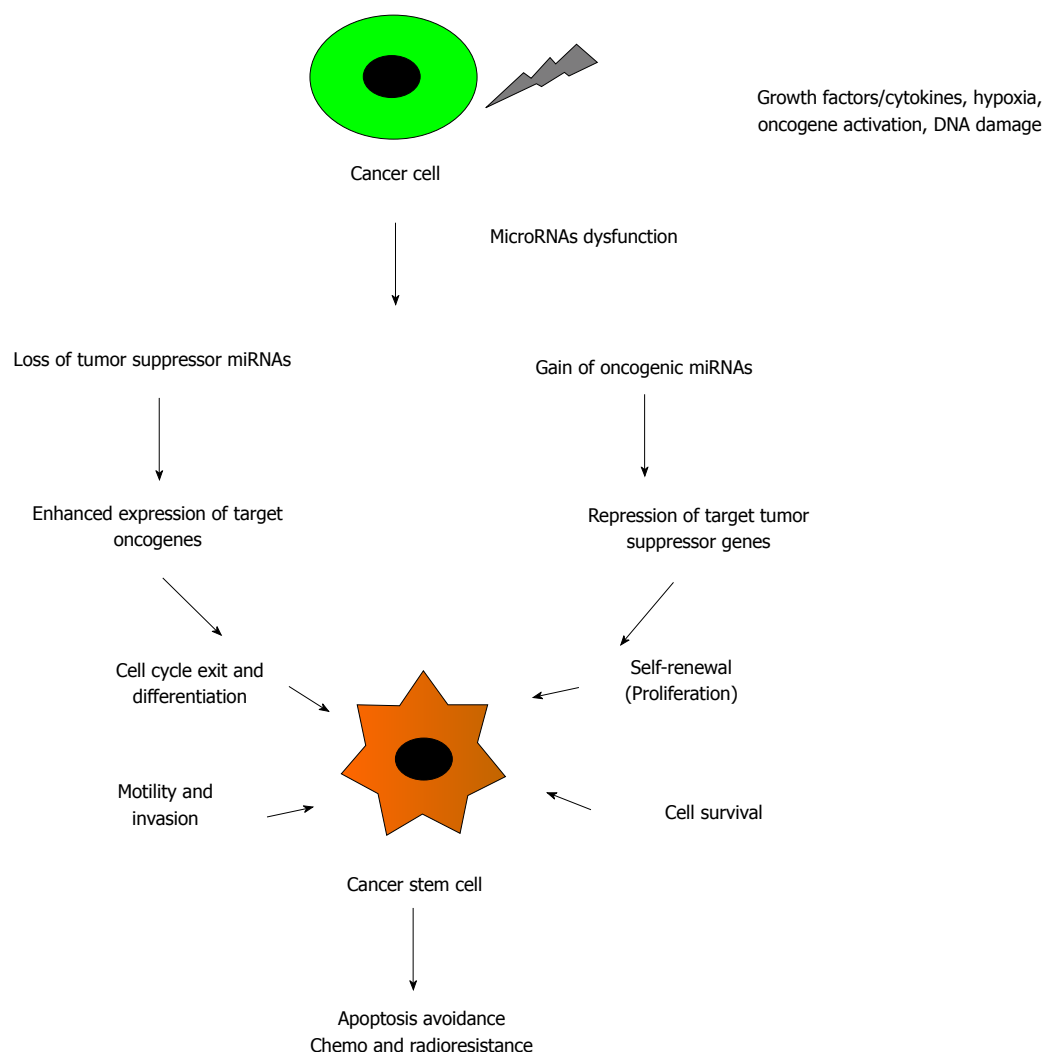


Figure 2 MicroRNAs affect and regulate the key properties of cancer stem cells. Tumor microenvironment influences the maintenance and properties of CSCs. Secreted growth factors and cytokines from cancer cells, hypoxia, oncogene activation, DNA damage, errors in DNA repair and aberrant microRNA expression modify cancer cell functions by increasing the self-renewability, cell cycle exit and differentiation, epithelial-to-mesenchymal transition, migration, invasion and survival rate in CSCs thus promote cancer progression and or tumor recurrence. CSCs: Cancer stem cells.

nano-carriers and various advantages of combinational therapy in elimination of CSCs population and in complete cure of human cancer with no recurrence or metastasis are discussed in the subsequent section.

COMBINATIONAL THERAPEUTIC APPROACHES TO TARGET CSCS

MiRNAs involved in acquisition of stem cell-like properties, regulation and reprogramming by cancer cells during cancer progression are not only exploited as molecular biomarkers to predict the risk of metastasis, systemic treatment resistance, and disease relapse of patients with cancer but are also an important novel therapeutic tools to improve patient-tailored treatments based on the unique signatures of a patient disease. MiRNAs can function as tumor suppressors or oncogenes and thereby either silence or express hundreds of genes and at the same time, where one gene can be targeted by multiple miRNAs. Local administration

of miRNA-sponges which are the transcripts having multiple, tandem binding sites to the miRNAs of interest; antagomiRNAs/anti-miRNAs (highly chemically modified miRNA passenger strand) which include strong RNA binding analog locked nucleic acid in mixers with DNA, charged neutral anti-miRNA oligonucleotides like peptide nucleic acid, phosphorodiamidate morpholino oligonucleotides, 2'-modified anti-miRNA oligonucleotides such as 2'-O-methyl, 2'-O-methoxyethyl (MOE), 2'-fluoro/2'-methoxyethyl mixers (2'F/MOE) enhance binding affinity for their RNA targets, steric-block potency and biostability and induce inhibition of endogenous miRNA functions following miRNA- anti-miRNA oligonucleotide binding in a chemistry dependent manner^[32,33]. Expression levels of related miRNA families that share the same seed sequence can be effectively reduced by using synthetic mRNAs, which contain multiple binding sites for endogenous miRNAs^[34]. MiRNA replacement therapy is another important therapeutic intervention which is based on the systematic delivery of

Table 1 Some of the potential microRNAs involved in regulation of cancer stem cells

MicroRNAs	Transcript target	Relevance to cancer progression and its biological functions	Ref.
Upregulated microRNAs			
miR-1246	CCNG2	Pancreatic cancer: Induces chemoresistance and CSC-like properties	[53]
miR-495	E-cadherin and REDD1	Breast cancer: Promotes oncogenesis and hypoxia resistance	[54]
miR-371-373 cluster	Wnt/ β -catenin, DKK1	Many cancers: Promotes cell growth and invasive activity	[55,56]
miR-216a/217	Myc	Liver cancer: Regulates the properties of CSCs	[57]
	PTEN and SMAD7	Hepatocellular carcinoma: Increased proliferation, migration and metastatic ability	
miR-210	Nanog, Oct4 and EZH2	Pancreatic cancer: Increased cell migration and invasion	[58]
miR-191	BASP1, Wnt/ β -catenin	Lung cancer: Increased migratory potential and neoplastic properties	[59]
miR-130b	P53-induced nuclear protein 1	Acute myeloid leukemia: Regulates hematopoietic stem cells	[60]
miR-29a	P53-induced nuclear protein 1	Acute myeloid leukemia: Regulates hematopoietic stem cells	[61]
miR-21	Nanog, Oct4 and EZH2	Pancreatic cancer: Increased cell migration and invasion	[58]
miR-18	DLL4, inhibitor of Notch signaling	Glioma: Promotes tumorigenic potential of GSCs	[62]
Downregulated microRNAs			
Let-7	Lin-28	Colon adenocarcinomas: Promotes cell migration, invasion and transforms immortalized colonic epithelial cells	[24,63]
miR-487b	SUZ12, BMI1, WNT5A, MYC, and KRAS	Pancreatic cancer: Increased pluripotency	[64]
miR-451	SMAD 3 and 4	Lung cancer: Increased proliferation and invasion	
miR-326	Hh smoothened signal transducer	GBM: Controls GBM stem cells differentiation	[41]
miR-204	Sox4 and Ephrin receptor EphB2	Chronic myeloid leukemia: Increased cell proliferation and decreased apoptosis	[65]
miR-200 family	VEGFR1, VEGFR2 and EMT-related transcription factors	Glioma: Involved in GSCs self-renewal and invasion	[66]
miR-200a	ZEB1, ZEB2, SNAIL and SLUG	Pancreatic cancer: Regulates CSCs properties	[63]
miR-181	N-cadherin, ZEB1, vimentin, E-cadherin	Pancreatic cancer: Increased cell migration and invasion	[67]
	ATM	Breast cancer: Regulates the properties of CSCs	
	MYb	Acute myeloid leukemia: Blocking of myeloid differentiation	
miR-150			[69]
miR-143/145 cluster	KRAS2 and its downstream effector RREB1	Pancreatic cancer: Regulates CSCs survival	[70]
miR-145	Oct4, Sox2, Nanog, Klf4 as well as Kras and Rreb1	Pancreatic cancer: Increased pluripotency	[63]
miR-128	Histone methylation [H3K27me(3)], Akt phosphorylation, p21(CIP1) Bmi-1	Glioma: Increased self-renewal and proliferation	[71]
miR-107	Nanog, Oct3/4, and Sox2	Head and neck squamous cell carcinoma: Increased CSC proliferation	[72]
miR-100/let-7a-2/miR-125b-1 cluster	Myc	Liver cancer: Regulates the properties of CSCs	[56]
miR-34 family	Notch and Bcl-2	Pancreatic cancer: Involved in self-renewal of CSCs	[28,29]
miR-29b-1	CD133, N-Myc, CCND2, E2F1 and E2F2, Bcl-2, IAP-2, Oct3/4, Sox2 and Nanog	Osteosarcoma: Increased proliferation, self-renewal and chemoresistance	[73]
miR-27a	14-3-3 β , Bax and Bad	Acute leukemia: Regulate apoptosis	[74]
miR-23b	Cell cycle arrest	Glioma: Inhibits proliferation	[75]

CCNG2: Cyclin-G2; DKK1: Dickkopf-1; PTEN: Phosphatase and tensin homolog; BASP 1: Brain abundant, membrane attached signal protein 1; ATM: Ataxia telangiectasia mutated; Myb: Myeloblastosis protooncogene protein; RREB1: Ras-responsive element binding protein-1; IAP-2: Inhibitor of apoptosis 2; CCND2: Cyclin D2; GSC: Glioma stem cell; CSC: Cancer stem cell; REDD1: Regulated in development and DNA damage responses 1; Myc: Myelocytomatosis viral oncogene; SMAD7: Homologs of caenorhabditis elegans protein small body size and drosophila protein mothers against decapentaplegic family member 7; Oct 4: Octamer-binding transcription factor 4; EZH2: Enhancer of zeste homolog 2; P53: Tumor protein 53; DLL4: Delta like ligand 4; SUZ12: Polycomb repressive complex 2 subunit; BMI1/Bmi-1: BMI1 proto-oncogene, polycomb ring finger; WNT 5A: Wingless-type MMTV integration site family, member 5A; KRAS: Kirsten rat sarcoma viral oncogene homolog; SMAD3 and 4: Homologs of caenorhabditis elegans protein small body size and drosophila protein mothers against decapentaplegic family member 3 and 4; Hh: Hedgehog; Sox 4: Sex-determining region Y-box containing gene 4; EphB2: Ephrin type-B receptor 2; VEGFR1: Vascular endothelial growth factor receptor 1; VEGFR2: Vascular endothelial growth factor receptor 2; EMT: Epithelial-to-mesenchymal transition; ZEB1: Zinc finger e-box binding homeobox 1; ZEB2: Zinc finger e-box binding homeobox 2; E-cadherin: Epithelial-cadherin; Klf4: Kruppel like factor 4; H3K27 me (3): Histone H3K27me3 (trimethyl Lys27); Akt: Protein kinase B; Bcl-2: B-cell lymphoma 2; E2F1: E2F transcription factor 1; E2F2: E2F transcription factor 2; Bad: Bcl-2-associated death promoter; Bax: Bcl-2-associated X protein; P21 (CIP1): Cyclin-dependent kinase inhibitor 1A; GBM: Glioblastoma multiforme.

miRNA mimics into the cytoplasm of target cells to gain tumor suppressor functions. Inducing miRNA-mediated biological processes or blocking specific targets either could reverse the development of cancer or delay tumor growth^[35]. Besides this, based on the relationship between expression of miRNA-regulated target genes

and their reaction to drug therapies, some other miRNAs are explored which can mediate molecular target drugs and regulate chemosensitivities and chemoresistances of cancer. Experimental evidences document that the co-administration of CSCs with either antagomiRNAs/anti-miRNAs or miRNA mimics along with potential

Table 2 Targeting cancer stem cells with specific microRNAs as a potential combinational therapeutic strategy in human cancer

Co-delivery of microRNAs/anti-cancer drug	Biological functions	Cancer type	Ref.
Oncogenic microRNAs			
miR-9/temozolomide	Inhibit the expression of drug efflux transporter, P-glycoprotein	Glioblastoma multiforme	[39]
miR-21/metformin, 5-Fluorouracil and Oxaliplatin	Target Wnt/ β -catenin	Colon cancer	[38]
miR-125b/temozolomide	Target PIAS3, which contributes to reduced STAT3 transcriptional activity and subsequent decreased expression of MMP-2 and -9	Glioblastoma	[40]
miR-125b/temozolomide	Target pro-apoptotic Bcl-2 antagonist killer 1 and sensitize CSCs to temozolomide induced apoptosis	Glioblastoma	[76]
Tumor suppressor microRNAs			
miR-612/cisplatin, 5-Fluorouracil	Target Wnt/ β -catenin, regulate EMT and inhibit cell proliferation, migration, invasion, and metastasis	Liver cancer	[48]
miR-205/gemcitabine	Decreased tumor cell population and increased apoptosis	Pancreatic cancer	[42]
miR-200c/docetaxel	Reduced TUBB3 level, and reversed EMT	Gastric cancer	[43]
miR-146a/cetuximab	Target Numb to stabilize β -catenin, regulate EMT, direct ACD-to-SCD switch	Colorectal cancer	[77]
miR-145/metformin, 5-Fluorouracil and Oxaliplatin	Target Wnt/ β -catenin	Colon cancer	[38]
miR-34 family/5-Fluorouracil	Repression of c-Kit by p53	Colorectal cancer	[45]
miR-34a/doxorubicin	Target Notch1 and reduce cancer stem cell properties	Breast cancer	[44]

STAT: Signal transducer and activator of transcription; PIAS3: Protein inhibitor of activated STAT; MMP: Matrix metalloproteinase; ACD: Asymmetrical cell division; SCD: Symmetrical cell division; EMT: Epithelial-to-mesenchymal transition; CSC: Cancer stem cell; MMP: Matrix metalloproteinase; TUBB3: Tubulin beta class III.

anti-cancer drugs can better sensitize cancer cells to promote apoptosis and autophagy, downregulate efflux transporters, revert EMT and inhibit tumor angiogenesis and could be employed as a more popular therapeutic approach for better clinical outcome in the treatment of human malignancies (Figure 3).

Viral vectors like lentiviral/adenovirus vectors are efficient delivery systems for miRNAs but toxicity and immunogenicity are the major hurdles for their clinical use. MiRNAs enclosed into non-viral particles, nanoparticles (NP) to form micelle-like structures for their effective delivery and enhanced stability in circulation. Some of the commonly used NPs for miRNAs delivery include polycationic-liposome hyaluronic acid, neutral lipid 1,2 dioleoyl-sn-glycero-3 phosphatidylcholine, polyethyleneimine, dendrimers, protamine, atelocollagen, poly(lactide-co-glycolide) particles, gold- or silica-based inorganic NPs. Binding of tumor specific ligands to NPs or conjugation with different compounds that have specific affinity with tumor cells ensure tissue-specific targeted delivery of miRNAs^[36]. Some of the significant recent work being conducted in cancer cell lines and or animal models to understand the cellular processes that regulate CSCs functions and investigate the therapeutic effects of miRNAs and add new dimensions in cancer treatment, are being discussed here (Table 2).

The effects of knockdown of miR-21 expression include induced apoptosis, reduced cell proliferation, invasion, and colony formation ability of colon tumor cells, inhibited G1/S cell cycle transition and increased sensitivity of cancer cells to 5-Fluorouracil (5-FU) chemotherapy. Direct targeting of human mut S homolog 2 and indirect regulation of the expression of thymidine phosphorylase and dihydropyrimidine dehydrogenase

by miR-21 confirms its important role in enhancing the sensitivity of 5-FU resistance of colon cancer cells. Re-emergence of chemotherapy-resistant CSCs could be one of the possible reasons for recurrence in colon cancer^[37]. The synergistic effect of increased miR-145 and reduced miR-21 expression which target Wnt/ β -catenin signaling pathway along with co-delivery of metformin, in combination with 5-FU and oxaliplatin, has been examined inducing cell death in chemoresistant colon cancer cells, inhibiting colonospheres formation and enhancing colonospheres disintegration in the treatment of recurring colon cancer^[38]. The delivery of synthetic Cy5-tagged anti-miR-9 to the resistant GBM cells has been observed to reverse the expression of the drug efflux transporter, P-glycoprotein and stimulate the sensitization of GBM cells to temozolomide, as shown by increased cell death and caspase activity^[39]. Experimental studies by Shi *et al*^[40], 2014 demonstrate the therapeutic effects of miR-125b inhibitor in enhancing the invasion-prevention activity of temozolomide in glioblastoma stem cells through targeting PIAS3 [protein inhibitor of activated signal transducer and activator of transcription (STAT)], which contributes to reduced STAT3 transcriptional activity and subsequent decreased expression of matrix metalloproteinase-2 (MMP-2) and MMP-9.

The cooperative effect of miR-451 (having a target site for SMAD in its promoter region) in combination with imatinib mesylate treatment in dispersal of GBM neurospheres and reduced tumorigenicity in glioblastoma validates this co-treatment as a new potential drug against the stem-cell like characteristics of glioblastoma^[41]. The delivery of combinational formulations of gemcitabine and miR-205 conjugated

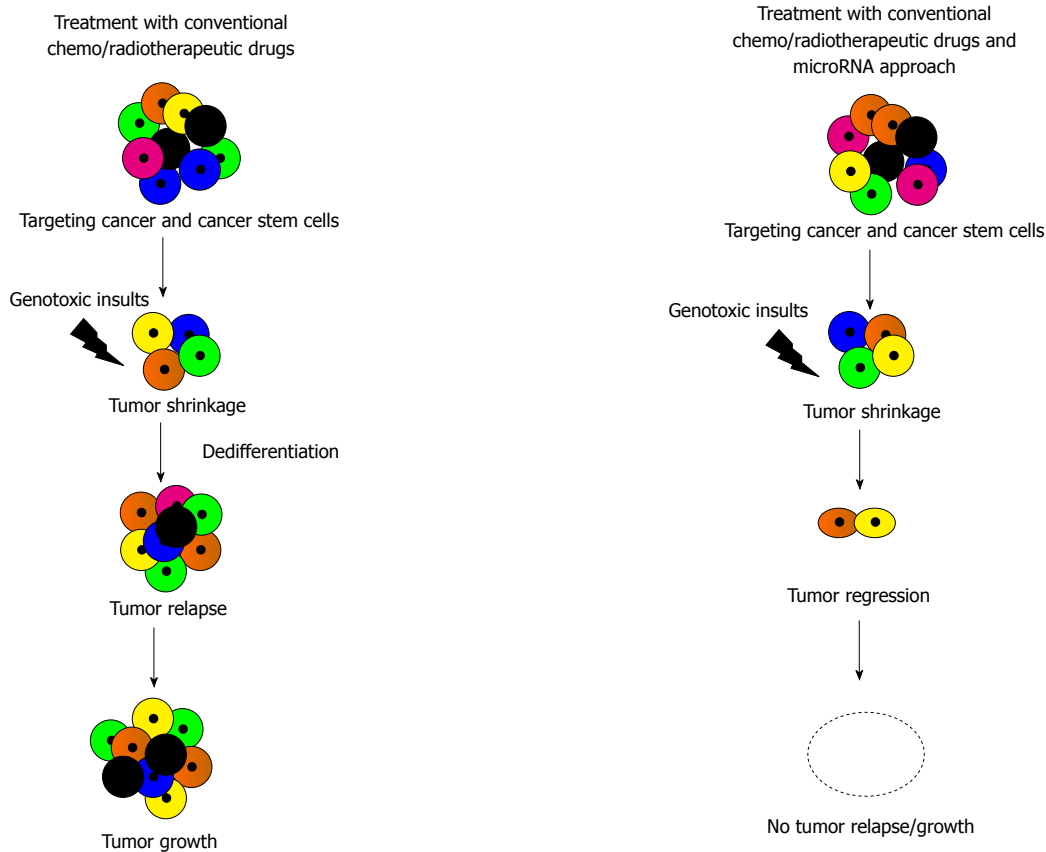


Figure 3 Effective novel tumor therapies. Conventional chemo- and radio-therapeutic drugs target the bulk of tumor cell population and lead to tumor shrinkage. Cancer stem cells however develop resistance to these drugs and as a result of genotoxic insults, tumor cells relapse. MicroRNA approach along with the chemo and radiotherapeutic drugs as a novel treatment strategy target transit amplifying cells as well as differentiated cancer cells and induce differentiation in cancer stem cells thereby remove the bulk of tumor and also the source of cancer.

copolymers in pancreatic cancer cells effectively reversed chemoresistance, invasion and migration by inducing apoptosis and inhibiting the growth and proliferation of CSCs^[42]. In another study, synergetic effects of targeted delivery of miR-200c and docetaxel (DOC) by gelatinases-stimuli NPs on inhibition of CSCs and non-CSC cancer cells in gastric cancer has been verified. The treatment of cancer cells with miR-200c/DOC NPs has been shown to significantly enhance the cytotoxicity of DOC, possibly by decreasing tubulin beta 3 Class III (TUBB3) level and reversing EMT, thereby affect tumor cell viability, migration and invasion^[43]. Ectopic expression of another tumor suppressor, miR34a has been studied to reduce CSCs properties and increase sensitivity to doxorubicin treatment by directly targeting Notch1 in chemoresistant breast cancer cells^[44]. c-Kit has been established as a new direct target of miR-34 where p53-induced miR-34 microRNA family mediated repression of c-Kit *via* a conserved seed-matching sequence in the c-Kit 3'-UTR, result reduced chemoresistance to 5-FU, reduced migration/invasion and stemness, further proves the hypothesis that this regulation interferes with several c-Kit-mediated effects in colorectal cancer cells^[45]. Recent preliminary studies document the tumor growth inhibition in human non-small cell lung cancer xenografts and KRAS-G12D

transgenic mouse model or the elimination of self-renewing breast CSCs upon therapeutic delivery of Let-7 mimics^[46,47]. Up-regulation of miR-30e with tumor suppressor functions by downregulating BCR-ABL (Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1) expression in chronic myeloid leukemic cells has been examined to render therapeutic efficacy against this disease. Owing to presence of putative target site for miR-30e in the 3' UTR of the *ABL* gene, enforced expression of miR-30e in K562 cells has been shown to suppress proliferation and induce apoptosis of these cells and sensitize them to imatinib treatment^[48]. Attenuation of EMT by retaining epithelial cell morphology, reducing the levels of α -smooth muscle actin and increasing the levels of E-cadherin in human kidney proximal tubular epithelial cells has been observed after enforced expression of all the three members of miR-106b-25 cluster (miR-106b, miR-93 and miR-25) in salivarnolic acid B^[49].

Protective functions of decreased expression of oncogenic miR-21 and increased expression oftumor suppressor miR-138 regulated by α -solanine has been shown in prostate cancer. These therapeutic effects have been experimentally examined to be mediated by suppressing extracellular signal-regulated kinases and phosphatidylinositol-3 kinase - serine/threonine kinase

signaling pathways, inhibiting EMT, reducing proliferation and inducing apoptosis^[50].

Systemic administration of cationic lipid NP/pre-miR-107 significantly has been shown to retard tumor growth by 45.2% compared to NP/pre-miR-control by decreasing the expression of miR-107 targets including protein kinase C ϵ , cyclin-dependent kinase 6, and hypoxia-inducible factor 1- β . NP/pre-miR-107 further has been shown to reduce the cancer-initiating cell population and dampen the expression of the core embryonic stem cell transcription factors, Nanog, Oct3/4, and Sox2 and inhibit the clonogenic survival, cell invasion, and cell migration of head and neck squamous cell carcinoma cells^[51].

Overexpression of miR-612 has been shown to suppress the stemness of hepatocellular carcinoma by reducing the number and size of tumorspheres, clone formation in soft agar and relieve the cancer cells from drug resistance to cisplatin and 5-FU by targeting Wnt/ β -catenin signaling, thereby control EMT-associated stem cell-like traits^[52].

Diverse cellular pathways that modulate CSCs functions act in concert with various molecular events and promote tumor initiation, progression and metastasis. Over the past recent years, emergence of miRNAs and molecular-targeted therapies has intensified research in the development of miRNA based therapeutic agents for cancer treatment with improved clinical outcome. Preclinical therapeutic data on mechanistic regulation of metastasis by miRNAs from *in vitro* studies need to be reevaluated in genetically engineered animal models which could further help us to provide new insights into clinical applications of miRNAs. Understanding the complex network of biological properties of CSCs, their control by multifunctional miRNAs and potential involvement of miRNAs in the development of anti-cancer drug resistance, reversion of cancer stemness and thereby rendering advanced cancers more susceptible to long term control are some of the open challenges to design novel strategies that can be used in preventive and treatment settings as an adjuvant to current cancer therapeutics.

CONCLUSION

CSCs which reside as a subset in many cancers are capable of self-renewal, tumor initiation, recurrence, metastasis, conferring resistance to anti-neoplastic drugs are still considered as huge obstacles on the way to cure cancer. This highlights the need to design strategies and therapeutics that specifically target and kill CSCs to eliminate cancer. Over the past few years, small ncRNAs, called miRNAs, have gained widespread attention in molecular biology due to their involvement in DNA translational control, their impression on mRNA and protein expression levels, and their ability to reprogram molecular signaling pathways in cancer. Differential miRNA profile in CSCs make them as potential biomarkers for aggressive tumor biology and

therapeutic resistance and their biological specificity in targeting the various properties of CSCs make them strong targets for improving the response to anti-cancer drugs by sensitizing these cells to enhanced apoptotic effects of drugs. Novel anticancer therapies are based on the manipulation of oncogenic or tumor suppressor miRNAs by reducing or increasing their expression levels respectively. Success of using miRNAs as specific drug target in clinically relevant animal models as well as in preclinical development has been tested and determined by normal organ morphology, blood chemistries, serum cytokine levels, significant tumor regression and prolonged survival.

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Complement activation in the context of stem cells and tissue repair

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Abstract

The complement pathway is best known for its role in

immune surveillance and inflammation. However, its ability of opsonizing and removing not only pathogens, but also necrotic and apoptotic cells, is a phylogenetically ancient means of initiating tissue repair. The means and mechanisms of complement-mediated tissue repair are discussed in this review. There is increasing evidence that complement activation contributes to tissue repair at several levels. These range from the chemo-attraction of stem and progenitor cells to areas of complement activation, to increased survival of various cell types in the presence of split products of complement, and to the production of trophic factors by cells activated by the anaphylatoxins C3a and C5a. This repair aspect of complement biology has not found sufficient appreciation until recently. The following will examine this aspect of complement biology with an emphasis on the anaphylatoxins C3a and C5a.

Key words: Complement activation; Embryonic and adult stem cells; C3a; C5a; Tissue repair

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Core tip: This review article provides an overview over the scenarios, where complement activation contributes to tissue repair and regeneration through its effect on stem and progenitor cells, which is an area that needs further investigation.

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INTRODUCTION

Complement activation cascade

Complement is an effector system present in blood

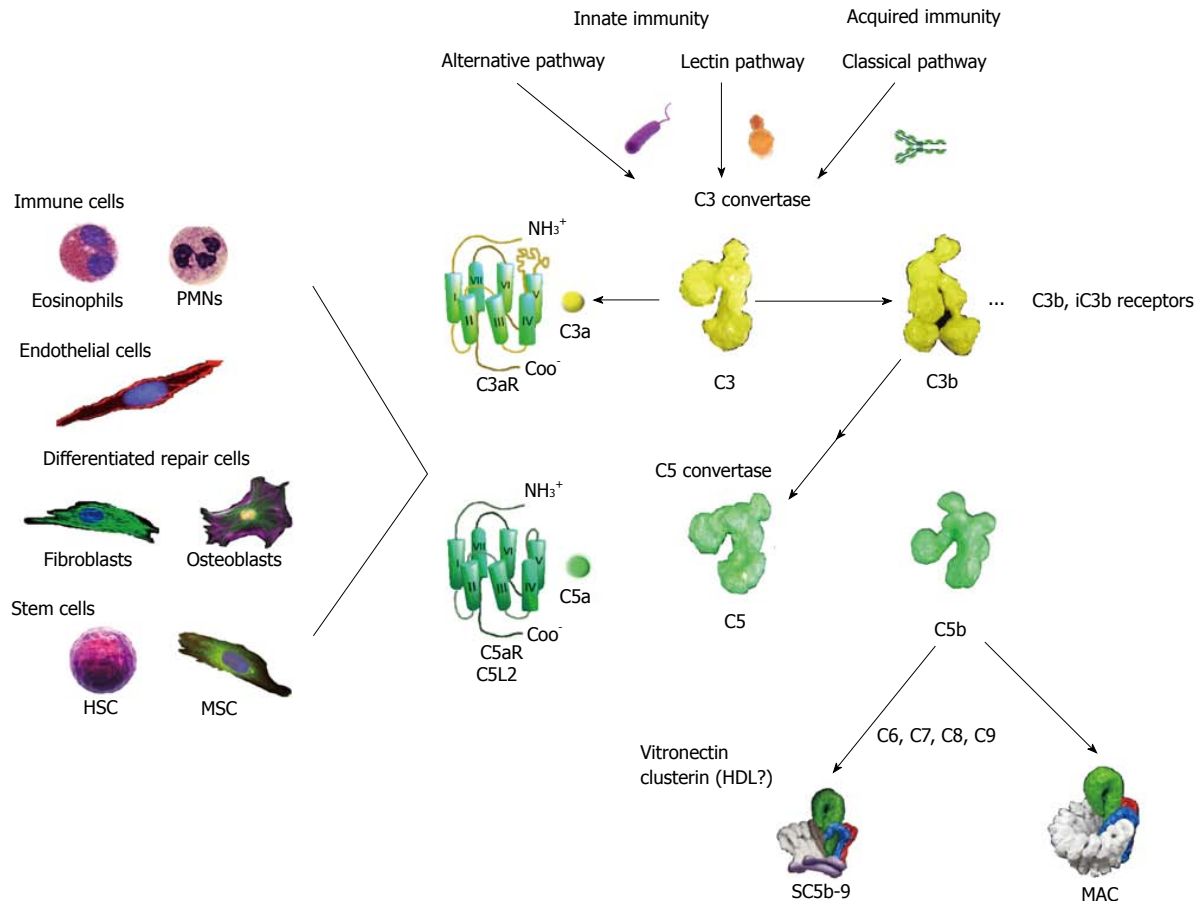


Figure 1 Schematic presentation of the complement pathways with emphasis on outcomes relevant to tissue repair and regeneration. The complement system can be activated by three pathways, two of which are part of innate immunity, the alternative and lectin pathways, whereas the classical pathway is normally initiated by immunoglobulins. All routes converge on the cleavage by complex enzymes referred to as C3 convertases of component C3 (Mr: about 195000) to C3a (Mr: about 8500) and C3b (Mr: about 185000). As a consequence of C3b deposition on C3 convertase, C5 convertase is created that acts similarly producing from component C5 (Mr: about 196000) the activation peptide C5a (Mr: about 11000) and C5b (Mr: about 185000). The anaphylatoxins, C3a and C5a, are important for elaboration of mechanisms of wound healing and regeneration. These small mediators are recognized by their cognate receptors: C3aR and C5aR/C5L2 that are GPCRs found on a diversity of cells inclusive of immune cells, endothelial cells, differentiated repair cells, and stem cells. In addition C3b and its split product iC3b, C3d are recognized by receptors inclusive of CR1-4 that assist clearance of microorganisms, cellular debris, immune complexes, and apoptotic cells. The ultimate outcome of complement activation is the formation of the MAC that is a transmembrane pore (100 Å) assembly that embeds in target cell membranes. In the absence of proximal phospholipid membranes, the terminal components of complement associate into complexes referred to as SC5b-9. These are probable heterogeneous and contain multiple copies of vitronectin and clusterin (apolipoprotein J). Because vitronectin in an oligomeric state can present the canonical tripeptide, Arg-Gly-Asp, to integrins on a variety of restorative cells, such as fibroblasts and keratinocytes, SC5b-9 may have a wound healing function. MAC: Membrane attack complex; MSC: Mesenchymal stem cells; HDL: High density lipo-protein; HSC: Hematopoietic stem cells; PMNs: Polymorphonuclear cells.

consisting of about 30 soluble proteins and 15 cellular receptors. Although it has been known for over a century that complement is a participant in host immunity, it has recently become generally realized that complement is a contributor to a variety of non-immune functions inclusive of resolution of inflammation, clearance of apoptotic cells, angiogenesis, wound healing, stem cell recruitment and activation, as well as repair processes^[1-6].

There are three routes of complement activation, the alternative pathway, the lectin pathway, and the classical pathway (Figure 1). All of these converge on the specific cleavage of component C3 (Mr: about 195000) by C3 convertase to yield the split products C3a (Mr: about 9000) and C3b (Mr: about 185000).

The alternative pathway activation is brought about by contact with large complex polysaccharides such as those found on microbial cell walls. This pathway is commenced by a diminished capacity to inactivate C3

convertase on a carbohydrate surface by the control factor H as well as pattern recognition by properdin and possibly contact activation by C3^[7-11]. Factor B combines with initially deposited C3b along with the stabilizer properdin to compose this pathway's C3 convertase, which consists of properdin, C3b, and Bb^[12].

The lectin pathway is started by special collagen containing C-type lectins (collectins), namely mannan binding lectin (MBL) and ficolins, which recognize carbohydrate patterns typically characterized by high mannose content, for example mannan that is a component of the coats of a variety of yeast, fungi, and other microorganisms^[13]. Proteases, referred to as mannan binding protein associated serine proteases link to fixed MBL to cleave C4 and C2 generating the complex enzyme C3 convertase (C4b, C2a)^[14].

The classical pathway can be initiated by IgG subclasses 1, 2, 3 as well as by IgM. Once tagged by

immunoglobulins, the collectin, C1q, links to these, along with C1r and C1s to evoke the cleavage of C4 and C2 resulting in the assembly of a C3 convertase (C4b, C2a), which has the same composition as that formed by the lectin pathway^[15].

Additional C3b deposition onto either the alternative or classical pathway C3 convertases changes these into C5 convertases (C3b₂, Bb, P or C4b, C3b, C2a)^[16,17]. These complex enzymes are now competent to process component C5 (Mr: about 196000) into C5a (Mr: about 11000) and C5b (Mr: about 185000)^[18].

Basic biology of C3a and C5a

The small activation peptides, C3a and C5a, Figure 1 are in inflammation and germane to this review in wound healing and regeneration^[19]. Both C3a and C5a, collectively referred to as anaphylatoxins, cause vasodilation, smooth muscle contraction, and increase vascular permeability^[20-22]. Although C3a can be generated in greater abundance than C5a, the latter has greater specific inflammatory potential^[21,23]. C5a especially is known for its ability to evoke chemotaxis of immune cells such as neutrophils and eosinophils^[24,25]. Both C3a^[23,26-28] and C5a^[21,24,26,29-31] can stimulate an oxidant burst in granulocytes, but the response of these cells to C3a is considerably weaker and more transient than that to C5a^[24,25,30,31]. In particular C3a fails to chemo-attract circulating leukocytes *in vivo*^[25]. Apart from the weak response of leukocytic C3aRs, the response to C3a *in vivo* would be expected to be limited largely to the interstitial space, since C3a is inactivated by serum carboxypeptidase N (CPN)^[32].

The anaphylatoxins are recognized on target cells by G-protein coupled receptors (GPCRs)^[33-35] coupled primarily to Gi. Unusually, C3aR has a long second extracellular loop that is important for binding C3a^[33,34].

C5a is recognized by two distinct GPCRs, C5aR (CD88) and C5L2, but only the former is coupled to Gi proteins, whereas the latter is enigmatic because it is not connected to a signal transduction pathway, and its biological role has not been established^[36]. Several investigations have assigned roles for C5L2 inclusive of an anti-inflammatory function^[37] and as a decoy-scavenger receptor^[38], but it has also been argued from studies using C5L2 knockout mice that this receptor is important for C5a-mediated signal transduction in neutrophils, macrophages and fibroblasts^[39]. Thus the true biological roles of C5L2 to date are not established^[40].

The anaphylatoxins are inactivated by plasma CPN (EC 3.4.17.3), a tetrameric protein (Mr: about 260000) that can excise basic amino acids from the carboxyl-termini of C3a, C5a, as well as bradykinin and other polypeptides^[32,41,42].

Whereas C3a desArg completely loses its activity^[43], C5a desArg retains a small fraction of its specific activity for neutrophil chemotaxis^[24,32].

The receptors for the anaphylatoxins are not restricted to immune cells as C3aR and C5aR are found

on a variety of non-immune cells^[44]. These include differentiated cells that can be important for wound healing and regeneration: mast cells^[45], tenocytes^[46,47], chondrocytes^[48,49], synoviocytes^[50], smooth muscle cells^[51], endothelial cells^[52-54], alveolar epithelial cells^[55], mesangial cells^[56,57], and regenerating hepatocytes^[58]. In addition various stem and progenitor cells express the C3aR and C5aR^[2,59-61] including HSC, mesenchymal stem cells (MSC)^[61], NSC^[2], and dental pulp progenitor cells^[62]. Table 1 shows a list of the cell types that express C3aR and C5aR and their function.

Late-acting components of the complement system

While the C3b portion of C3 binds to the surface of pathogens leading to greater internalization by phagocytic cells, C5b, the remaining split product of C5, assembles with complement C6, C7, C8, and polymeric C9 to form the membrane-spanning membrane attack complex (MAC), which lyses bacteria, but which can also damage eukaryotic cells. Finally, a C3b cleavage product, iC3b can bind to the β 2-integrins CR3 (CD11b/CD18) and CR4 (CD11C/CD18) on phagocytic cells facilitates the clearance of apoptotic cells.

There are multiple modalities which inhibit complement activation or the formation of the MAC; these include the plasma proteins factor H and C4b-binding protein and the membrane-anchored complement receptor 1 (CR1/CD35), membrane cofactor protein (CD46), decay accelerating factor or CD55, and MAC-inhibitory protein (CD59). As the plethora of inhibiting factors indicates complement activation has to be fine-tuned to provide optimal protection from infection without causing inflammatory tissue injury.

Sites of complement synthesis and activation

While complement proteins in the circulation are primarily produced by the liver except for the late acting complement components in particular C7 which are produced by monocytes/macrophages^[63,64], it has become apparent that production and activation of complement proteins can happen in a localized fashion in many different parts of the body^[65-68], and one would expect prolonged activation by the anaphylatoxins C3a and C5a under such conditions because of the absence of CPN in the interstitial space.

Role of complement activation in inflammation

The important role of complement in the defense against infection comes, however at a price: excessive complement activation plays a role in numerous disease processes ranging from ischemic reperfusion injury^[69-71] to asthma^[72], acute lung injury^[73,74], glomerulonephritis^[75], rheumatoid arthritis^[76], Alzheimer's disease^[77], multiple sclerosis and demyelination in general^[78,79], and age-related and genetic macular degeneration^[80-83]. In some instances the specific injurious complement pathway components have not been distinguished^[75,76], in others C5a^[69-71,77,80] or the MAC are the clear culprits^[79]. A role

Table 1 Cell types expressing the C3aR and the C5aR and their function

Cells expressing C3aR	Function of C3aR	Cells expressing C5aR	Function of C5aR
Neutrophils ^[244]	Respiratory burst ^[26] , bone marrow retention <i>in vivo</i> ^[189]	Neutrophils ^[245]	Respiratory burst ^[28] , chemotaxis ^[24] , enzyme release ^[127]
Eosinophils ^[30]	Chemotaxis ^[30] , <i>in vitro</i> but not <i>in vivo</i> ^[25]	Eosinophils ^[246]	Respiratory burst ^[27] , chemotaxis
Monocytes/macrophages ^[31]	Chemotaxis ^[247] , cytokine/chemokine production ^[164]	Monocytes/macrophages	Chemotaxis ^[104,248] , cytokine/chemokine production ^[164]
Mast cell	Mediator release ^[102] , chemokine production ^[249] , chemotaxis ^[100,101]	Mast cell	Mediator release ^[102] , chemokine production ^[249] , chemotaxis ^[100,101]
Small fraction of lymphocytes ^[250,251]	Complex <i>in vivo</i> functions ^[252]	Small fraction of lymphocytes ^[251,253]	Complex <i>in vivo</i> functions
Osteoblasts ^[155,173,254]	Chemotaxis, accelerated osteogenesis, improved bone healing <i>in vivo</i> ^[174]	Osteoblasts ^[97,173]	Chemotaxis ^[97] , accelerated osteogenesis ^[175] , improved bone healing <i>in vivo</i> ^[174]
Chondrocytes ^[172]	Osteogenic differentiation (?)	Chondrocytes ^[172]	Osteogenic differentiation (?)
Tenocytes ^[46]	Not clear	Tenocytes ^[46]	Not clear
Smooth muscle cells ^[51]	Increased mediator release from mast cells ^[255]	Smooth muscle cells ^[44,51]	Not clear
Endothelial cells ^[52]	Transient ERK and rho activation ^[52] , cytokine production ^[53]	Endothelial cells ^[52]	Chemotaxis ^[52] , increased permeability ^[52] cytokine production ^[53] , proliferation ^[128]
Hepatocytes ^[88]	Protection from apoptosis ^[88] , liver regeneration <i>in vivo</i> ^[87,88]	Hepatocytes ^[44]	Proliferation ^[58] , protection from apoptosis liver regeneration <i>in vivo</i> ^[87,94]
Renal epithelial cells ^[256]	Chemokine production ^[257] , EMT under stress conditions ^[258]	Renal epithelial cells	EMT under stress conditions ^[259]
Neurons ^[193]	Protection from cell death ^[193,199]	Neurons ^[194]	Protection from cell death ^[193-195]
Astrocytes ^[260]	Indirect neuroprotection ^[198] , NGF expression ^[200]	Astrocytes ^[261]	Cytokine and NGF expression ^[200,262]
MSC ^[61,90]	Chemotaxis ^[61] , protection from apoptosis ^[61] , production of angiogenic factors ^[91]	MSC ^[61,90]	Chemotaxis ^[61] , protection from apoptosis ^[61] , production of angiogenic factors ^[91]
HSPC ^[59]	Enhanced effects of SDF-1 ^[263] , improved bone marrow engraftment ^[60,188]	Not expressed	Indirect: decreased mobilization ^[192] , indirect: improved bone marrow engraftment ^[191]
CSPC ^[182]	Chemotaxis ^[182] , proliferation ^[182]	CSPS ^[182]	Chemotaxis ^[182] , proliferation ^[182] cardiac dysfunction in C5/C5aR -/- mice ^[180]
NSPC ^[2]	Increased neurogenesis ^[2] , chemotaxis and differentiation ^[89]	NSPC ^[2]	Increased neurogenesis ^[2]
ESC	Not expressed	ESC	Prevents differentiation ^[168]

ERK: Extracellular signal-regulated kinase; EMT: Epithelial-to-mesenchymal transformation; NGF: Nerve growth factor; MSC: Mesenchymal stem cells; SDF: Stromal-cell derived factor; HSPC: Hematopoietic stem and progenitor cells; CSPC: Cardiac stem and progenitor cells; NSPC: Neural stem and progenitor cells; ESC: Embryonic stem cells; MAC: Membrane attack complex.

for C3a was only seen in a mouse asthma model^[72] and a mouse model of laser-induced macular degeneration, where the presence of the C3aR was associated with increased angiogenesis^[80], which is detrimental in the retina, but which could support repair following ischemic insults in other tissues.

It should be noted here that C5a appears the major culprit responsible for most of the observed pathologies, and that specific C5/C5a inhibition preserving the early steps of complement activation could be highly advantageous in some circumstances.

ROLE OF COMPLEMENT ACTIVATION IN TISSUE REPAIR

Complement mediated inflammation leading to resolution and clearance

While the inflammatory aspect of complement activation has long been emphasized, it has been largely ignored that complement activation contributes also to resolution of inflammation and tissue repair with few

reviews covering this aspect^[4,84-86].

In particular, C3a has anti-inflammatory and regenerative effects^[2,61,87-91]. In fact the regenerative potential of C3/C3a dates way back phylogenetically, as its expression is prominently up-regulated in mesenchymal cells in the regeneration zone in amphibians undergoing limb regeneration^[92]. Furthermore, recent findings indicate that the C3aR on mesenchymal cells plays an important migration-directing role during early vertebrate development in zebra-fish^[93]: Neural crest cells mutually attract each other *via* C3a and the C3aR forming clusters of migratory mesenchymal cells. Such collective cell migration is a phenomenon crucial for morphogenesis. It remains to be seen, whether C3a and the C3aR play the same role during mammalian embryonic development.

While C5a also has regenerative effects for instance by its effects on the liver^[94,95], neurons^[96], osteoblasts^[97], and dental pulp progenitors^[62], these properties are often overshadowed by the strong inflammatory reaction caused by the activation of leukocytic C5a receptors,

which are involved in most of the pathologic conditions described above.

However, it should also be considered that inflammation itself constitutes a first step in wound healing. C3a and C5a can lead to an increase in vascular permeability^[21,98], which is important for wound healing as it aids the flow of chemical and cellular entities necessary for repair and regeneration while facilitating waste removal^[99].

Although swelling is traditionally seen as a characteristic of inflammation, edema is also necessary for the resolution of inflammation and restoration of functional tissue because an increase in vascular permeability facilitates entry of repair and restorative cells. Specific to this theme is the function of histamine. C3a and C5a both are chemotactic for mast cells and both are inducers from these cells of histamine release^[100-102]. Histamine due to its potent vasodilation activity can induce swelling, but histamine is also required for skin wound healing as demonstrated using Kit mutant mice that are mast cell deficient. These animals are unable to secrete mast cell derived histamine, and the animals were found to have a defective response to cutaneous wound healing^[103].

The increase in vascular permeability facilitates the recruitment of monocytes that can respond to C5a mediated chemotaxis gradients^[104], and these cells are crucial for "cleanup" functions. Today it is understood that clearance of debris and apoptotic cells is an important activity necessary for subsequent wound healing, and complement along with pentraxins have been shown to participate in this activity^[105,106]. Indeed the clearance function was probably the original function of the complement system dating all the way back to metazoans^[107].

The collectins C1q and MBL are important for enhanced phagocytosis by monocytes and macrophages of modified lipoprotein complexes, immune complexes, and apoptotic cells^[108-111]. Apoptotic cells present exteriorized phosphatidyl serine that can be recognized at an early stage by the lectin domains of members of the collectin family^[112-114]. Apoptotic cells, debris or immune complexes tagged by C1q or MBL are identified by monocytes and macrophages bearing CD91 that can be in complex with a collectin receptor, calreticulin^[115-117]. The facilitated uptake of these "disposables" has been referred to as macropinocytosis^[118].

In addition to recognition of pathogens, debris and dead cells by members of the collectin family, fragments of C3 are important for clearance functions. C3b is susceptible to processing by Factor H and I to iC3b that can be cleaved further into C3d and C3c^[119]. C3 fragments are recognized by receptors such as CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18), CR4 (CD11c/CD18), and CRIg found on Kupffer cells, monocytes and macrophages, which are immune adherence receptors that facilitate removal of opsonized microorganisms, immune complexes and apoptotic cells^[120,121].

Complement and angiogenesis

The importance of angiogenesis in wound healing and regeneration has been clearly understood^[122]. The process has been categorized in three continuous overlapping phases: inflammatory, proliferative, and remodeling^[122].

Some aspects of participation in inflammation inclusive of increase in vascular permeability induced by C3a and C5a have already been discussed, but these mediators have additional functions that indirectly support angiogenesis. C5a but not C3a has been shown to induce an upregulation of gene expression on endothelial cells for adhesion molecules E-selectin, ICAM-1, and VCAM-1^[123,124]; the upregulation of these adhesion molecules facilitates extravasation of immune cells inclusive of monocytes that are important for debridement, remodeling and angiogenic mediator secretion^[125]. Angiogenesis requires restructuring of the extracellular matrix by controlled proteolysis, and the anaphylatoxins were reported to increase the levels of MMP-1 and MMP-9 in monocytes^[126] and to be secretagogues of MMP-9 from granulocytes^[127].

Both C3aR and C5aR are found on cultured endothelial cells, but these mediators use different signal transduction pathways and the response to C3a is more transient^[52]. Both the anaphylatoxins up-regulates chemokine production in endothelial cells^[53], but only C5a is chemotactic for human umbilical vein endothelial cells (HUVECs)^[52] and microvascular endothelial cells^[54]. Moreover, it was reported that C5a could induce not only migration of cultured microvascular endothelial cells but proliferation and ring formation as well^[128].

C3a and C5a were found to increase vascular endothelial cell growth factor (VEGF) in human culture retinal pigment epithelial cells, and when the anaphylatoxins were injected intravitreally into normal mice, an increase in VEGF within the retinal pigment epithelial-choroid layer of the retina was observed^[80]. Others found that C5a but not C3a induced VEGF synthesis and secretion from a retinal pigment epithelial cell line^[129]. Furthermore, both C3a and C5a were reported to induce production and secretion of VEGF from MSC^[91]. Although there is no *in vitro* evidence that C3a and C5a are directly angiogenic, they have been shown to be angiogenic in *in vivo* situations^[80,130,131], perhaps in response to angiogenic factors that the anaphylatoxins induce in cells in the proximity as just described.

In summary, C3a and C5a can contribute to the inflammatory and proliferative phases of angiogenesis, and thus the anaphylatoxins can be viewed as factors with indirect angiogenic potential; however, it is necessary to mention that one publication is in apparent contradiction to this view, namely investigators studying experimental retinal neovascularization published that C5a is anti-angiogenic^[132]; however, these investigators were examining murine models of retinopathy of prematurity and hypoxia induced retinal vascularization, and these observations though correct may not be of a

general nature.

ROLE OF COMPLEMENT ACTIVATION IN SPECIFIC REPAIR PROCESSES

Role of complement activation in liver regeneration

Although tissue regeneration is very limited in mammals, the mammalian liver has retained an amazing capacity for regeneration following viral infection, exposure to toxins or surgical resection. This regeneration can occur at the hepatocyte level in cases of acute liver injury, although liver stem and progenitor cells appear to contribute in more chronic conditions.

The complement activation products C3a and C5a play an essential role in regeneration of the liver parenchyma^[87,95]. After experimental CCl₄ induced liver toxicity or partial hepatectomy, mice deficient in C3 or C5 exhibited defective regeneration and a higher frequency of mortality^[87]. Furthermore, C5a was demonstrated to be a growth factor for regenerating hepatocytes, and blockage of the C5aR in experimental liver regeneration experiments resulted in the inability of hepatocytes to proliferate leading to defective liver restoration^[58,94].

However, the role of complement activation is a double-sided sword in hepatic regeneration and the MAC was found to be the principle mediator of hepatic ischemia reperfusion injury^[133], which creates a dilemma, since the early components of complement activation, C3a, and C5a are necessary for liver regeneration. However, targeted inhibition of MAC formation with CR2-CD59 significantly improved survival after partial hepatectomy in mice^[133], while retaining the benefit of complement activation and anaphylatoxin production.

EFFECTS OF COMPLEMENT ACTIVATION ON MSC AND OTHER MESENCHYMAL CELLS

MSC and tissue repair

MSC are rare, often perivascular cells found in all tissues that are able to differentiate into all types of connective tissue lineages including osteoblasts, adipocytes and chondrocytes. Furthermore, these cells produce a variety of angiogenic and trophic factors^[134,135] and possess anti-inflammatory properties^[136-138]. Owing to the immune-evasive properties of MSC, allogeneic MSC transplantation is generally accepted. Because of all these properties MSC have started to find clinical application in a variety of diseases ranging from myocardial infarction^[139] to graft vs host disease^[140] and have found attention in the context of acute lung injury^[141].

Limitations of MSC therapies as used today

However, in the rush to the clinic, survival of the transplanted MSC has not been sufficiently considered, and there have been failed clinical trials using MSC - in spite of promising results in animal models^[142-146],

and the full regenerative potential of these cells has not been harnessed due to poor tissue homing and limited cell survival following transplantation. Successful clinical trials will require additional information about the mechanisms by which MSC repair injured tissues, about the optimal route of administration, and about means of increasing their survival at a site of tissue injury. It is surprising, how little there is known about MSC recruitment and survival *in vivo* for a cell type that is being investigated in numerous clinical trials. Various means of improving MSC homing^[147], growth factor production^[148,149] and survival^[150] are being pursued as ways to improve the therapeutic efficacy of MSC, but usually different means are used to achieve each one of these goals. It is hypothesized here that C3a can improve all of these functions, since we postulate that the C3a-dependent regenerative capacity of MSC seen in amphibians^[92] has been preserved in mammalian tissue repair.

MSC and complement activation

Although MSC have various anti-inflammatory and immune-evasive properties^[151] - including the ability to inhibit the proliferation of allogeneic T cells, low levels of expression of MHC class I and II proteins, the ability to convert inflammatory M1-type macrophages to repair-type M2 macrophages, and secretion of the complement-inhibitory factor H^[152], - they are not fully protected from complement induced injury themselves, and complement activation appears to be involved in the demise of MSC following allogeneic transplantation^[151,153]. One would wish that such basic complement biology had been considered before using allogeneic MSC in clinical trials. Incubation of MSC with complement active human plasma resulted in the deposition of C3c and iC3b on the cell surface of the MSC and C3a and soluble C5b-9 detection in the supernatant^[90], indicative of complement activation, which could be prevented by various means of complement inhibition.

In addition, MSC as well as osteoblasts express components of the complement cascade themselves^[154] including C3, C5^[155], the C3aR and C5aR^[61] and the cell surface complement regulators CD46, CD55, and CD59^[155]. Furthermore, MSC engineered to up-regulate CD46, CD55, and CD59 protected these cells from complement-mediated cell lysis *in vitro* and *in vivo*^[156].

Effect of C3a and C5a on MSCs

MSC show tropism for areas of tissue damage^[157,158], but it is controversial which chemotactic factors are responsible for this. In leukocytes a large degree of cell recruitment to an area of tissue injury depends on chemokines and C5a, but the role of chemokines in trafficking of MSC is unclear with widely contradictory findings^[158-162]. Since MSC are chemo-attracted by C3a and C5a *in vitro*^[61], we hypothesize that complement activation is an important player in attracting MSC to an area of tissue damage *in vivo*. C3a and C5a can be locally generated at the surface of MSC which contact serum^[90]

in close proximity of C3aRs and C5aRs expressed by MSC; it is possible that this may circumvent access to CPN-mediated inactivation of the anaphylatoxins C3a and C5a. In addition to being potent chemoattractants for MSC^[61], C3a and C5a increase the survival of MSC under conditions of oxidative stress^[61], which would be encountered in an area of tissue injury. Indeed C3/C3a may be a survival factor for MSC^[163]. Furthermore, C3a, - and to a lesser degree C5a, - induce the production of trophic and angiogenic factors by MSC including VEGF, basic fibroblast growth factor (bFGF), platelet derived growth factor, IL-6, and IL-8^[91], and supernatants from C3a-stimulated MSC are angiogenic for HUVECs *in vitro*^[91]. The increased production of growth factors by MSC stimulated with C3a or C5a was largely due to activation of NF κ B^[91], but in contrast to other cell types, in which C3a and C5a cause NF κ B activation such as monocytes/macrophages^[164], this does not lead to the concomitant release of the inflammatory cytokines TNF- α and IL-1 β , thus converting a normally inflammatory pathway into one that supports regenerative processes. For TNF- α this occurs through promoter inactivation^[165], while IL-1 β production in MSC appears to be blocked at the level of protein processing.

We propose that C3a and C5a play a physiological role in MSC-dependent tissue repair by recruiting MSC to an area of tissue injury, by increasing MSC survival under challenging conditions, and by increasing the production of trophic, angiogenic and anti-inflammatory factors by these cells. It is also suggested that pretreatment of MSC with C3a, -C5a is considered too inflammatory - prior to transplantation may increase the repair capacity of MSC by augmenting the ability of the MSC to survive in an area of tissue damage and by inducing increased production of angiogenic and anti-inflammatory factors.

In addition, it has also been reported that complement C1q is a chemoattractant for MSC^[166].

It is also worth mentioning that C3a and C5a cause prolonged activation of the ERK^[61], Akt^[61], NF κ B^[91], and Stat3^[167] pathways in MSC and other stem cells, which are the same pathways that are activated by bFGF albeit with differing routes of activation, and it will remain to be seen whether C3a or C5a have a similar effect as bFGF in maintaining stem cells in the undifferentiated state as has been suggested for C5a in embryonic stem cells (ESC)^[168].

MSC are not the only mesenchymal cells expressing complement components and responding to complement activation. Myoblasts express the complement components of both the alternative and classical pathways (C1q, C1r, C1s, C2, C3, C4, factor B, factor H, factor I^[169,170], as well as the C3aR, and they spontaneously activate allogeneic complement, but are themselves protected from self-killing due to expression of high levels of CD46 and CD59^[171]. Finally, scratch-injured tenocytes showed increased proliferation and survival in the presence of C3a^[46].

Role of complement activation on bone formation

Consistent with the role of complement activation during limb regeneration in amphibians^[92] described above, it has been suggested some time ago that complement activation may be important in cartilage-bone transformation during fracture healing and that the alternate complement activation pathway may be involved^[172]. Like their MSC precursors, osteoblasts are able to express the key complement proteins C3 and C5^[155] and express the C3aR and C5aR, which both mediate osteoblast migration^[97]. Expression of the C5aR was highly up-regulated during osteogenic differentiation^[97], but later during osteoblast to osteocyte differentiation complement genes were greatly down-regulated^[173].

Although osteogenic differentiation of MSC can occur in the absence of C3a or C5a, it is accelerated in the presence of C3a or C5a in a C3aR and C5aR-specific fashion as shown with receptor-specific inhibitors in Figure 2A: After two weeks of osteogenic differentiation in the presence of fetal calf serum (FCS) that was not heat-inactivated, *i.e.*, complement proteins had not been inactivated, Alizarin red staining of calcium salt deposits indicated moderate staining in FCS, which was significantly augmented, when C3a or C5a had been added to the media. However, by 3 wk the difference between these groups was largely diminished (results not shown). If heat-inactivated FCS (FCS) replaced the FCS, osteogenic differentiation was still further delayed but the addition of C3 or C5 partially substituted for the presence of serum complement components (Figure 2B) indicating that the differentiating cells themselves must have provided the necessary complement components.

Consistent with these *in vitro* findings, delayed fracture healing was observed in C3 or C5-deficient mice which received a standardized femur osteotomy^[174]. C5-deficiency also resulted in poor quality bone^[174], indicating that complement activation plays an important role in fracture healing. However, under chronic conditions the osteogenic effect of complement activation is a double-edged sword, because it can also result in vascular calcification during the atherosclerotic process, where MSC-derived C5aR participation has been shown recently^[175].

Role of complement activation in cardiac repair

Following cardiac infarction extensive necrosis of ischemic cardiomyocytes activates complement. The ensuing infiltration of the infarct zone with neutrophils and monocytes serves to clear the injured site from dead cells and debris, and initiates reparative pathways.

However, there is little doubt that complement activation plays an injurious role in the acute phase of myocardial infarction mostly in the context of C5a-mediated reperfusion injury and neutrophil influx^[176-178], but clinical trials inhibiting at the level of C5 have been unsuccessful^[179] indicating that even in this early phase, complement activation is not all deleterious.

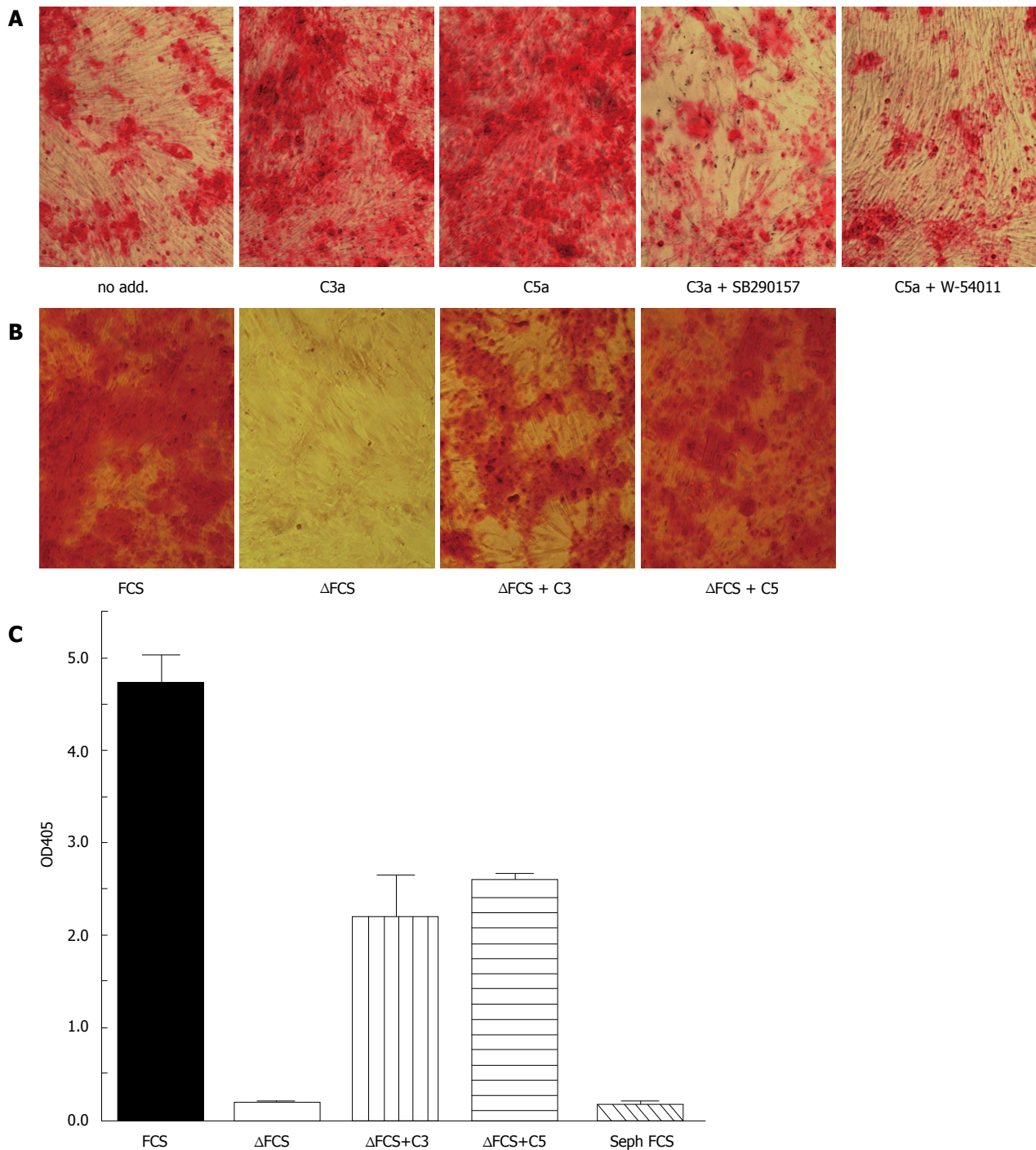


Figure 2 Complement activation accelerates osteogenic differentiation of bone marrow mesenchymal stem cells. MSC were cultured in osteogenic media (α -MEM containing 16.5% FCS, not heat inactivated, 10 nmol/L dexamethasone, 20 mmol/L β -glycerolphosphate, and 50 μ mol/L ascorbic acid 2-phosphate) for the indicated time. Osteogenesis was detected by alizarin red S staining. A: All cells were cultured in the presence of the carboxypeptidase inhibitor 2-mercaptomethyl-3-guanidinoethylthiopropionic acid (80 nmol/L) to maintain C3a and C5a activity. C3a (100 nmol/L) or C5a (10 nmol/L) were added during the first 3 d of cultures in the presence or absence of the C3aR inhibitor SB290157 (1 nmol/L) or the C5aR inhibitor W-54001 (1 nmol/L). Both C3a and C5a accelerated calcification in a C3aR and C5aR specific fashion as detected by Alizarin red staining on day 14; B: Osteogenesis was considerably delayed in heat-inactivated FCS (FCS), in which complement components are inactivated. Addition of either C3 or C5 partially reconstituted the effect of FCS. Alizarin staining on day 21; C: Quantitation of the alizarin staining of figure 2B following solubilizing in acid SDS solution. FCS: Fetal calf serum; MSC: Mesenchymal stem cells.

Furthermore, even C5a appears to be protective in several models of cardiac hypertrophy, where C5/C5aR knockout mice fared worse than wild type mice^[180].

The beneficial effect of complement activation becomes more apparent in the more chronic situation, where complement activation contributes to tissue

repair^[181]: C3-deficiency in C3 knockout mice exacerbated myocardial dysfunction four weeks after coronary artery ligation showing more scar tissue, and decreased cardiac stem/progenitor cells (CSPC) in the infarct zone^[181]. Both murine and human CSPC express C3aR and C5aR, are chemo-attracted by C3a

and C5a, and show greater proliferation in the presence of the anaphylatoxins^[182]. It remains to be seen, whether they also produce more angiogenic factors as described above for MSC stimulated with C3a or C5a, which would be a further advantage in the context of cardiac repair. In CSPC C3a or C5a also induced several genes associated with - unwanted - myofibroblast differentiation *in vitro*^[182], but it remains to be seen, whether this is relevant *in vivo*.

Effect of complement activation on HSC

Like any tissue damage, myeloablation by radiation or chemotherapy activates complement resulting in the generation of the complement activation peptides C3a and C5a^[59,60]. Following bone marrow transplantation fast and efficient homing to and engraftment in the bone marrow is important. In this scenario SDF-1 is the most important chemotactic factor, which chemo-attracts hematopoietic stem and progenitor cells (HSPC) to the bone marrow and retains them there through the CXC chemokine receptor 4 on these cells^[183,184].

While HSPC express the C3aR, C3a itself does not appear to be a direct chemo-attractant, but it augments the chemotactic responsiveness of HSPC to gradients of SDF-1 as well as to sphingosine-1-phosphate and ceramide-1-phosphate^[59,60,185,186]. *In vivo*, mice deficient in complement C3 exhibited delayed engraftment of HSPC^[60]. This effect was specifically mediated by the C3aR as shown when HSPC from C3aR^{-/-} mice were injected into irradiated wild type mice, which resulted in a significant delay in recovery of leukocytes and platelets and decreased committed progenitors in the bone marrow^[187]. Similarly, engraftment of human CD34⁺ cells treated with a C3aR inhibitor showed impeded engraftment in nonobese diabetic/severe combined immune deficiency mice^[187].

C3a also contributes to the retention of HSPC in the bone-marrow as C3^{-/-} or C3aR^{-/-} mice showed accelerated mobilization of HSPC into the peripheral blood following administration of granulocyte colony-stimulating factor (G-CSF)^[188]. This retention mechanism is not limited to HSPC, but also applies to their neutrophil progeny, and indeed the C3aR protects from ischemic intestinal injury due to reduced neutrophil mobilization, and increased neutrophil accumulation causes exacerbated injury in C3aR deficient mice^[189]. Indeed, decreased neutrophil mobilization in wild type vs C3aR^{-/-} mice may explain the increased mortality observed in C3aR^{-/-} mice in an endotoxin shock model^[190], although the mechanism was not reported for this model.

C5-deficient mice also exhibited impaired HSPC engraftment: In this scenario the role of C5 cleavage leading to the formation of soluble MAC resulted in increased adhesion of HSPC to bone marrow stromal cells and augmented secretion of SDF-1 by the bone marrow stroma^[191]. However, HSPC do not express the C5aR themselves, and C5 deficient mice show

reduced HSPC mobilization following the administration of G-CSF^[192], which causes complement activation. Apparently, granulocytes, which are released into the circulation in response to C5a formation, pave the way for HSPC to egress from the bone marrow perhaps due to MMP9 release, which facilitates HSPC mobilization^[192].

Effect of complement activation on neurons, neural stem and progenitor cells

It has been known for some time that neurons express both C3aR^[193] and C5aR^[194], and that these two receptors protect from neural cell death^[193-195]. This protective effect is not limited to differentiated neurons, but already functions in neural stem and progenitor cells, which express both C3aR and C5aR. C3-deficient mice showed deficits in both basal and ischemia-induced neurogenesis^[2], and C3aR expression was essential for basal neurogenesis^[2], while C5aR expression made no difference in this respect^[196]. Consistent with these results, C3a protected from ischemic insult-induced memory impairment in neonatal mice^[197].

In vitro, C3a could induce neuronal differentiation of neural progenitor cells^[89], and increased the chemotactic response to low concentrations of SDF-1^[89] similar to the situation with HSPC. In addition, C3a protected from NMDA neurotoxicity, but only in the presence of astrocytes^[198], which suggests that C3a-stimulated astrocytes, which express the C3aR^[199], were the primary target, and that they in turn protected through the production of NGF and other neurotrophic factors^[200]. However, in a mouse model of ischemic reperfusion injury, C3aR inhibition had the opposite effect resulting in increased neuroprogenitor proliferation and suppressed T cell infiltration^[201]. The reasons for such opposing results are not clear, although it is possible that the last model includes a larger inflammatory response that may cancel out any direct effect of C3a on neuronal progenitors and/or astrocytes. Specific pathways by which complement activation protect neural stem and progenitor cells await further elucidation.

Interestingly complement C1q, - in the absence of other components of the complement cascade increased neuron viability and neurite outgrowth and prevented α -amyloid-induced neuronal death *in vitro*^[202] and *in vivo*^[203]. Neuroprotection was promoted by activation of the transcription factor cAMP responsive element binding protein and by increasing LRP1B and GPR6 expression^[203]. Furthermore, in retinal neurons, TGF- β signaling regulates C1q expression, which in turn is necessary for synaptic pruning^[204]. Indeed, complement activation plays a role during a process called synaptic elimination in new-born mice^[205], where either C1q or C3 deficiency resulted in failure of synaptic elimination^[205], implying the classical complement cascade in this process. Interestingly, C1q^{-/-} mice presented with signs of epilepsy due to increased excitatory synaptic connectivity^[206].

Complement involvement during embryonic development

ESC only express a limited number of proteins of the complement cascade including C6, C7, C8, C9, factor I, H, properdin/factor D, and complement component 1r, s and q receptor, and beta polypeptide^[207,208]. However, a recent report indicates that they may also express C5 and the C5a receptor^[168] and more importantly that C5a promotes survival and maintenance of the pluripotent state of ESC in the absence of bFGF^[168], the standard addition to maintain human ESC in the undifferentiated state. While this report awaits further validation, it highly suggests that complement activation presumably with the support of maternal complement components plays a role in embryonic development from the very beginning.

It is known that the maternal complement system plays a crucial role starting early on during fetal development and that it is essential for the maintenance of fetomaternal tolerance. In mice Cr1/Crry (complement regulatory protein) deficiency is embryonically lethal, but the embryos are rescued in C3-/- mothers^[209]. Indeed, ESC are more susceptible to complement mediated cell lysis than differentiated cells, and this pathway may contribute protection from teratocarcinoma formation during pregnancy^[210]. Complement activation has, however to be finely regulated during pregnancy, since excessive activation of this pathway in later pregnancy is associated with miscarriage^[211] and preeclampsia^[212-215].

There is limited knowledge about the role of complement in early vertebrate development with much of the information derived from lower vertebrates. While further investigation using mammalian models is surely required, the existence of these complement pathways during amphibian development indicates that complement activation is a phylogenetically preserved ancient process during embryogenesis. In xenopus complement components are extensively expressed during development starting during the gastrula/early neurula stage^[216] with organ-specific expression patterns during early organogenesis. C1qA, C3 and C9 are strongly expressed in the early neural plate, while C1qR and C6 are expressed at the periphery of the neural plate presumably in the neural crest^[216,217] preceding the development of hematopoiesis. At this point C3 and C3aR show a predominantly mesodermal expression. Interestingly, neural crest cells, a multipotent embryonic cell population undergo epithelial to mesenchymal transition (EMT) in xenopus and zebrafish in a fashion reminiscent of metastasizing cancer cells and it is following this EMT transition that they express both C3 and the C3aR^[93]. These cells form cohesive clusters of migrating cells that are co-attracted *via* C3a and the C3aR and this process is necessary for collective migration of these cells^[93] suggesting that C3aR/C3a contribute to the intricate mass cell movements of the developing embryo.

In rats C3 derived from the visceral yolk sac is an embryotrophic factor between days 9.5 to 11.5 post

conception^[218], - however no further details have been elucidated.

Evidence for a role of C3a in fetal tissue regeneration comes from studies on embryonic chick retina regeneration. In this model C3a can induce complete regeneration of the ablated chick retina from stem/progenitor cells *via* Stat3 mediated up-regulation of IL-6, IL-8, and TNF- α ^[219]. However, there was an optimal concentration of C3a that induced regeneration, while very high concentrations caused apoptosis, indicating that fine-tuning of the C3a/C3aR axis is necessary, perhaps not surprising since the cytokines produced by C3a stimulation may serve as growth factors at low concentrations, but become highly inflammatory at higher concentrations.

Beyond the early effect of C5a stimulation on ESCs mentioned above, C5a and the C5aR play a continued important role during mammalian development: They are both expressed during the period of neurogenesis in mice and humans^[220], and while C5aR knockout mice show no congenital defects under normal pregnancy conditions, they present with a wide variety of congenital malfunctions due to neural tube defects ranging from anencephaly to scoliosis and anophthalmia, if the mothers are folate deficient^[220].

Soluble complement C5b to 9: Possible roles for vitronectin and clusterin in wound healing and stem cell biology

Complement evolved to destroy microorganisms, and one effector outcome of complement activation is the assembly within target cell membranes of a multiprotein complex referred to as the MAC. This consists of one molecule each of C5b, C6, C7, C8 and multiple copies of C9 (6 or more). In its complete form the MAC creates a transmembrane pore of 100 Å that destroys the functional integrity of cellular membranes^[221,222].

In the absence of proximal phospholipid membranes the terminal components of complement form a soluble complex referred to as Soluble complement C5b to 9 (SC5b-9), which was initially described as having a composition of one molecule each of C5b through C8 and three units of C9 and vitronectin^[223]. Later it was also shown to contain clusterin (apolipoprotein J)^[224], which is known to be a component of a subclass of high-density lipo-protein (HDL) particles^[225]. Although the term "SC5b-9", as originally conceived designated a soluble form of the terminal complement complexes, it is probable that these assemblies are heterogeneous with some containing vitronectin and others clusterin presumably associated with HDLs. Whether heterogeneous or not, indications exist that these macromolecular composites may be adaptive for recovery from injury.

Vitronectin, a known matrix and adhesive protein, circulates in human plasma in an inactive state in which its heparin linkage region and integrin binding site, containing the canonical Arg-Gly-Asp sequence, are buried^[226,227]; however, as a consequence of

oligomerization and conformational change these regions on the protein can interact with glycosaminoglycans (GAG) and integrins^[228,229]. GAGs are a fundamental constituent of the extracellular matrix that will necessarily become exposed upon tissue damage. Furthermore, vitronectin binding integrins, $\alpha\beta_{1,3,5,8}$ and $\text{IIb}\beta_3$, are found on a variety of cells responsive to injury inclusive of platelets, fibroblasts, myoblasts, vascular smooth muscle cells, and endothelial cells^[230-232].

Thus incorporation of plasma derived vitronectin into damaged ECM can be seen as a beneficial response that facilitates wound healing because this arrangement can help dock and anchor restorative cells. Furthermore, because vitronectin is known to bind growth factors such as insulin like growth factor^[233], it may be speculated that vitronectin in context of SC5b-9 could deliver the growth mediators to a wound site.

It is also conceivable that complexes of SC5b-9 containing clusterin may also contribute to host recovery from injury. Clusterin is found in HDL containing apolipoprotein A-I but not apolipoprotein A-II^[224,234-237]. HDL particles are highly heterogeneous, and whereas HDLs were originally ascribed to function for reverse cholesterol transport, it is now realized that these operate for a diversity of biological roles inclusive of transport of hormones and bioactive lipids, inflammation regulation, clearance, and immune defense against parasites and microorganisms^[238-241].

Although investigations about the interface of HDLs and stem/progenitor cell biology are just commencing, a few publications suggest that this will be a fruitful topic for future research. For example HDL can promote MSC proliferation by interaction with Scavenger receptor class B member 1^[242]. Also HDL have been shown to advance endothelial cell precursor migration and proliferation^[243].

We leave it an open question as to whether HDL-associated SC5b-9 can facilitate wound healing through influence on stem and progenitor cells.

CONCLUSION

Although complement is best known for its role in inflammation, increasing evidence has accumulated that emphasizes that complement activation and in particular the complement split products C3a and C5a play a role in many scenarios of tissue repair. Table 1 shows a compilation of cell types expressing C3aR and C5aR and the function of these receptors on any particular cell. However there are still many gaps in our understanding of the role of complement activation outside the inflammatory axis. A more complete understanding of the effects of complement activation in stem cell biology will contribute to improve the therapeutic potential of these cells.

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From cellular to chemical approach for acute neural and alternative options for age-induced functional diseases

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Abstract

Endogenous "stem cell niche" (SCN) accompanying vessels contains immune system components which *in vivo* determine differentiation of multi potent stem cells toward proper cell types in given tissue. Combinations of sex steroids may represent novel chemical approach

for neuronal areas of regenerative medicine, since they cause transformation of vascular smooth muscle stem cells into differentiating neuronal cells. Circulating sex steroids are present during pregnancy and can be utilized where needed, when various embryonic/fetal tissues develop from their stem cells. Utilization of induced regeneration of tissues (regenerative medicine) is expected being more effective in sudden failures of younger individuals carrying intact SCN, as compared to established chronic disorders caused by SCN alteration. An essential component of SCN are monocyte-derived cells exhibiting tissue-specific "stop effect" (SE) preventing, for instance, an aging of neuronal cells. Its alteration causes that implantation of neuronal stem cells will also result in their differentiation toward aging cells. When we repair the SE by supply of circulating mononuclear cells from young healthy individuals, we may be able to provide novel regenerative treatments of age-induced neural diseases by sex steroid combinations. Questions regarding some age-induced body alterations are also addressed.

Key words: Cellular differentiation; Endogenous stem cells; Neurological disorders; Regenerative medicine; Sex steroid combinations; Stem cell niche; Treatment of aging diseases

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Core tip: Sex steroid combinations have been found to transdifferentiate *in vitro* the vascular smooth muscle cells into neurons. Therefore, utilization of sex steroid combinations may enable regeneration of neural tissues affected by acute/traumatic disorders. In aging individuals, however, an altered immune system components in the stem cell niche may be unable to preserve regenerating cells in the functional stage due to the immune system regression with age advancement. This could be improved by transfer of blood mononuclear cells from young healthy individuals. Beside that, the local and systemic utilization of honey

bee propolis alone has been found to ameliorate some age-induced disorders.

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INTRODUCTION

To deal with regeneration of tissues, we need to better understand their preservation in the appropriate functional state. Under normal embryonic and fetal development, the normal tissue function is established and programmed to last for certain period of subsequent life. In other words, even in normally developed individuals, functional longevity in distinct tissues is different. The most extended functional tissue life is established for such tissues that evolved functionally as earliest, and the opposite applies for those which functionally developed as the latest, with variations between these states. For instance early developing functional human heart can work for one hundred years, while the proper function of late developing fetal ovary will last for a much shorter period. In reality, the ovarian dysfunction is initiated between 35 and 40 years of age, due to the age-induced alteration of the ovarian stem cell niche (SCN). Due to the absence of corpora lutea during fetal immune adaptation toward self tissues, they are considered to be a "graft", which results in their cyclical rejection during menstrual cycles in adulthood (reviewed in^[1]). The corpus luteum rescue during pregnancy accompanies immune tolerance of fetal allograft, and both effects required for successful pregnancy are considered to be caused by trophoblast-derived chorionic gonadotropin and other endocrine factors^[2,3].

The perivascular tissue-specific SCN enables regeneration of tissues from endogenous stem cells. We still do not have a detailed information regarding role of SCN in distinct tissues. The intent of the current regenerative medicine is to deal with a replacement of dysfunctional cells with various types of exogenous stem cells, utilizing for instance induced pluripotent stem cells (iPSCs), embryonic stem cells (ESC), or somatic stem/progenitor cells, which are able to differentiate into functional cells of particular tissue^[4]. Such an approach, however, carries a number of complications, including ethical concerns, immune responses toward implanted cells, possibility of teratoma development, lack of ability of implanted cells to regenerate, failure of their cell cycle, lack of their preservation in functional stage, and apoptosis^[5-7]. Moreover, most attempts to establish ESC from large mammals have failed^[8] and human iPSCs develop into teratomas when tested in immunosuppressed animals^[9]. Recent observation also indicates

that iPSCs carry a genome methylation memory of their former differentiation history, which might alter their therapeutic utilization^[10].

Another possibility is to stimulate autologous pluripotent stem cells to develop *in situ* into functional cells. Such approaches eliminate some of the hurdles mentioned above but don't deal with concerns about the success of the return of regular tissue function^[5]. Chronic disorders are expected to be caused by an altered tissue-specific SCN which causes permanent tissue damage. Of particular interest are SCN alterations stimulating degeneration of functional tissue cells toward apoptosis in degenerative diseases.

An essential role in the maintenance of tissues by SCN plays so called "stop effect" (SE) encoded at monocyte derived cells (MDC) in tissue-specific manner. The SE enables tissue cells to differentiate into the proper stage for their function, but not beyond. Stage of cellular differentiation needed for proper tissue function differs among the tissues. For instance brain neural cells should be prevented to undergo advanced differentiation toward apoptosis, while superficial cells of epidermal stratified epithelium should differentiate into apoptotic squamous cells for the proper epidermal function and protection. The SE is established epigenetically, during the fetal immune adaptation. Lower or higher cellular differentiation during that period than required for proper tissue function will cause dysfunction of that tissue during subsequent life (reviewed in^[1]). In aging individuals, there is a "shift up" of the SE, causing age-induced functional diseases, *e.g.*, Alzheimer's disease.

In other words, improper function of tissues is caused by altered tissue-specific SCN, and may result in either functional immaturity or aging of such tissues.

Utilization of stem cells alone will not improve altered tissue function unless we will be able to induce the SCN repair^[1] either endogenously or by allogeneic body rejuvenation (reviewed in^[11]).

Utilization of animal models for the treatment of degenerative disorders was slightly encouraging as compared to the human experimental trials^[6], possibly due to the persistence of an unaltered tissue-specific SCN after initiation of a disorder in otherwise normal healthy animals.

WHAT IS THE SCN?

Stem cells in distinct tissues are present in a perivascular location of microvessels as pericytes that function as mural vascular cells^[12]. They secrete growth factors which stimulate constitutional tissue progenitor cells^[12]. Some pericytes exhibit properties of mesenchymal stem cell and exhibit nuclear reprogramming into such cells as testicular Leydig cells^[13], cardiomyocytes^[14], cartilage and bone^[15], and neural and neuronal type cells^[16]. Pericytes and neural cells originate from the neural crest^[17], but from different germ layers. The functional behavior of SCN is modulated by autonomic innervation^[18], which influences numbers of

pericytes and inhibits or enables their activity^[19,20].

Another essential component of the perivascular SCN are MDC accompanying microvasculature. Depletion of MDC in one testis alters differentiation of Leydig cells from their precursors^[21] represented by vascular pericytes^[13]. Circulating CD14+ primitive MDC (pMDC) play important roles in tissue morphostasis, in addition to their roles in the immune responses^[22]. The pMDC enable division of progenitor cells and influence by SE the persistence of tissue-specific cells in the epigenetically encoded particular differentiation state^[1]. Hence, the MDC exhibit several roles in the perivascular SCN.

The SCNs accompanying body microvasculatures represent basic "tissue control units" incorporated into a more complex tissue control system (TCS)^[1], which shows distinct diversity between the tissue types. The tissues differ in their proper stage of cellular differentiation, ranging between stem cells and cellular apoptosis. Extent of cellular differentiation of certain tissues ceases relatively early, e.g., skeletal muscle or brain. A moderate stage of proper cellular differentiation is present in gut epithelium, where intraepithelial lymphocytes are involved, and cellular apoptosis with binding of immunoglobulin is required for the proper function of stratified epithelium of epidermis and uterine ectocervix (reviewed in^[1]).

Developmental tissue alteration prior reaching its functional stage will cause its permanent immaturity, e.g., muscular dystrophy. On the other hand, when differentiation of tissue cells is enhanced toward higher stage then required for their proper function, the autoimmune and degenerative diseases, e.g., rheumatic diseases, atherosclerosis, or Alzheimer's disease, will occur^[23,24].

17 BETA ESTRADIOL IN VASCULAR SMOOTH MUSCLE CELL CULTURES AFTER TESTOSTERONE, PROGESTERONE AND TESTOSTERONE + PROGESTERONE TREATMENT

Our former research^[25] has shown that initial utilization of estradiol (E2) is necessary to induce transdifferentiation of ovarian stem cells (OSCs) and granulosa cells to the neural stem cells (NSC) and neurons following subsequent progesterone (PG) + testosterone (TS) application. However the transdifferentiation was also sometimes observed after PG + TS alone, because of the PG and TS transformation into E2 by aromatase. This possibility was investigated in smooth muscle cell (SMC) cultures. Fresh and spent control medium exhibited E2 1050 ± 150 pg/mL and 1477 ± 742 pg/mL SEM only. However TS pre-treatment caused significant E2 increase (22086 ± 4650 pg/mL) and PG pre-treatment was even three times more efficient (71920 ± 9090 pg/mL). An addition marked E2 increase was

observed after pretreatment with PG + TS (198202 ± 40088 pg/mL)^[16]. This observation indicates that cultures with PG and TS also contain significant E2 levels. It is apparent that vascular SMC contain aromatase capable to cause conversion of PG and TS into E2.

Estradiol is also produced in extragonadal localities, such as vascular SMC and brain^[26]. The SMC associated with vessels exhibit aromatase and 17 beta-hydroxysteroid dehydrogenase type I, which transform estrone produced by aromatase into the final E2^[27]. This indicates that, similarly to *in vitro* experiments, *in vivo* treatment with PG and TS may cause a transdifferentiation of vascular SMC into pluripotent stem cells and induce a differentiation of neural/neuronal cells in those locations, which require that.

THE PG AND TS COMBINATION CAN BE SUFFICIENT

The high PG + TS combination is virtually absent in adult human females or males. The combinations of E2 + PG or E2 + TS we not found to transdifferentiate OSCs or vascular SMC into vascular stem cells and neurons. Suitable sex steroid combinations may, however, be present in the brain as neurosteroids^[28]. Our observations^[16,25] indicate that E2 + PG + TS or PG + TS combinations (assuming E2 is created by aromatase of target cells) could be considered.

During human prenatal development the estrogens, progestogens, and androgens are physiologically present in the circulation of developing fetus^[29]. This indicates that such sex steroid combination is not harmful for the fetal development and it may act where actually needed, e.g., for the proper brain development.

Some studies indicate that utilization of PG limits central nervous system damage after injury, inhibits a loss of neural cells (CNS), and stimulates brain function (reviewed in^[30]). This may be caused by an effect of supplied PG and *in situ* aromatase-created E2. Yet, utilization of PG + TS combination can accelerate levels of E2 and cause vascular SMC differentiation into neural cells. This may work well for injured brain or spinal cord in younger individuals.

VASCULAR SMC

Since vascular SMC accompany as pericytes all vessels, including the CNS, their ability to differentiate into neural cells is of particular interest. Utilization of all three sex steroid combination can cause a fast conversion into pluripotent stem cells. Such cells can thereafter differentiate into mature vascular SMC. These cells, known as vascular pericytes, influence properties of endothelial cells and participate in the functional ability of blood vessels. Pericytes can serve for differentiation of other types of cells^[31]. Collectively, the vascular SMC accompanying capillaries and post-capillary venules^[32] can serve in various sites (brain, muscle, bone marrow, kidney, lung, vessels, liver, thymus and spleen) as

mesenchymal stem cells^[33-36]. The mesenchymal stem cell populations in distinct tissues showed alpha smooth muscle actin expression, which suggests their origin from alpha smooth muscle actin positive pericytes^[37].

Taken together, systemic or local (spinal cord injury) utilization of sex steroid combinations alone can represent a regenerative chemical approach with a perspective of improvement of neurological and vascular diseases, without a need to develop and implant particular stem cells *in vitro*.

A preservation of normal tissue functions continues to decline with age advancement, in association with a decline of the immune system function. For instance it has been shown that the immune system components play an essential role in the renewal of ovarian follicles during the prime reproductive period (till 38 ± 2 years of age), when the human female fertility is well preserved. After that period, however, women's fertility begins to decline along with age-induced alterations of immune system functions, beginning from the age of 35 years^[38]. Due to the cessation of ovarian follicular renewal the ovaries begin to preserve aging oocytes unsuitable for regular or assisted reproduction (reviewed in^[11]).

Simultaneously, additional alterations of certain tissue functions, like type 2 diabetes mellitus accompanied a pathology of pericytes^[39] and degenerative neuronal diseases associated with apoptosis of neurons^[40] may occur due to the age-induced shift up or lost SE of MDC. This indicates that regenerative medicine could be more successful in the treatment of acute tissue alterations in younger individuals with preserved appropriate SE of MDC than in chronic or age induced tissue disorders^[41], unless there is an improvement of altered SE, for instance by a transfer of circulating mononuclear cells from young healthy individuals, as recently suggested^[11].

CELLULAR REGENERATION WITHOUT CELLS

The regenerative medicine of neurologic disorders is currently expected to utilize *in vitro* developed NSC implanted into the CNS with expectation of renewal and repair of altered or degenerated mature neuronal cells. Such cells may be collected from CNS and expanded by growth factors with mitogenic effects^[42,43]. Another origins of NSC are represented by ESC, blood from umbilical cord, amnion cells, stem cells from bone marrow, and blood-derived CD133+ cells^[44-47]. After some passages, such cells can be differentiated into NSC by fibroblast growth factor-1, 12-O-tetradecanoylphorbol-13-acetate, which is an activator of protein kinase C, isobutylmethylxanthine, which is a non-specific inhibitor of phosphodiesterases, and either by forskolin, representing protein kinase A activator, or by all-trans-retinoic acid and 2-mercaptoethanol^[48]. Such substances, however, can't be utilized for an *in vivo* treatment.

Another possibility is a "systemic regenerative therapy" or "*in situ* regenerative treatment" by substances capable to cross blood-tissue barrier, e.g., the blood-brain barrier, due to their low molecular weight.

We have also realized that combinations of sex steroids are capable to transdifferentiate directly vascular SMC cultures, which have been shown to exhibit receptors for sex steroids^[49-51], into the neural and neuronal cells^[16], as pericytes accompany microvasculature of all tissues, including CNS.

It was also observed that appearance of vascular SMC stem cells can be induced by combinations of sex steroids, and such stem cells within few days develop back into mature SMC^[16]. Sex steroids may stimulate renewal of vascular pericytes, which play by themselves an important role in the physiology of vessels. They influence endothelial cell properties and contributions to the proper vascular functions. Moreover, pericytes were considered to represent precursors for certain types of cells^[31], e.g., cardiomyocytes^[14]. Therefore, systemic regenerative treatment may also improve vascular disorders, e.g., type 2 diabetes, which is closely twisted together with microvascular pericytes throughout the body^[52], improve functions of vessels affected by atherosclerosis, and induce regeneration of cardiomyocytes affected by myocardial infarction. Therefore, systemic and *in situ* treatment with sex steroid combinations may represent a novel regenerative approach for the stem cell therapy without the cells.

SEX STEROID ROLE IN THE NEURAL SCN

Depletion of androgens with age advancement in men is accompanied by depression and memory alteration. Decline of TS concentrations several years precede the diagnosis of Alzheimer's disease. The therapy with androgens, however, did not show any improvement of cognitive memory in altered aging individuals. Beside gonads and adrenals, some sex steroids are also produced within the brain. Such sex steroids are eventually called neurosteroids (reviewed in^[28]).

Sex steroids could stimulate neuronal regeneration from persisting NSC, since they cross the blood-brain barrier and associate with their receptors important for the regulation of emotions, cognition, and behavior^[53]. It appears, however, that utilization single sex steroid, such as androgen, does not prevent or treat neuronal degeneration (see above).

COMBINATIONS OF SEX STEROIDS AND REGENERATIVE STEM CELL THERAPY WITHOUT THE CELLS

The consideration of sex steroid treatment provided below is based on the sex steroid doses utilized for other clinical applications. It is recommended that, before any clinical trial, relevant doses of sex steroid combinations are tested in animal studies.

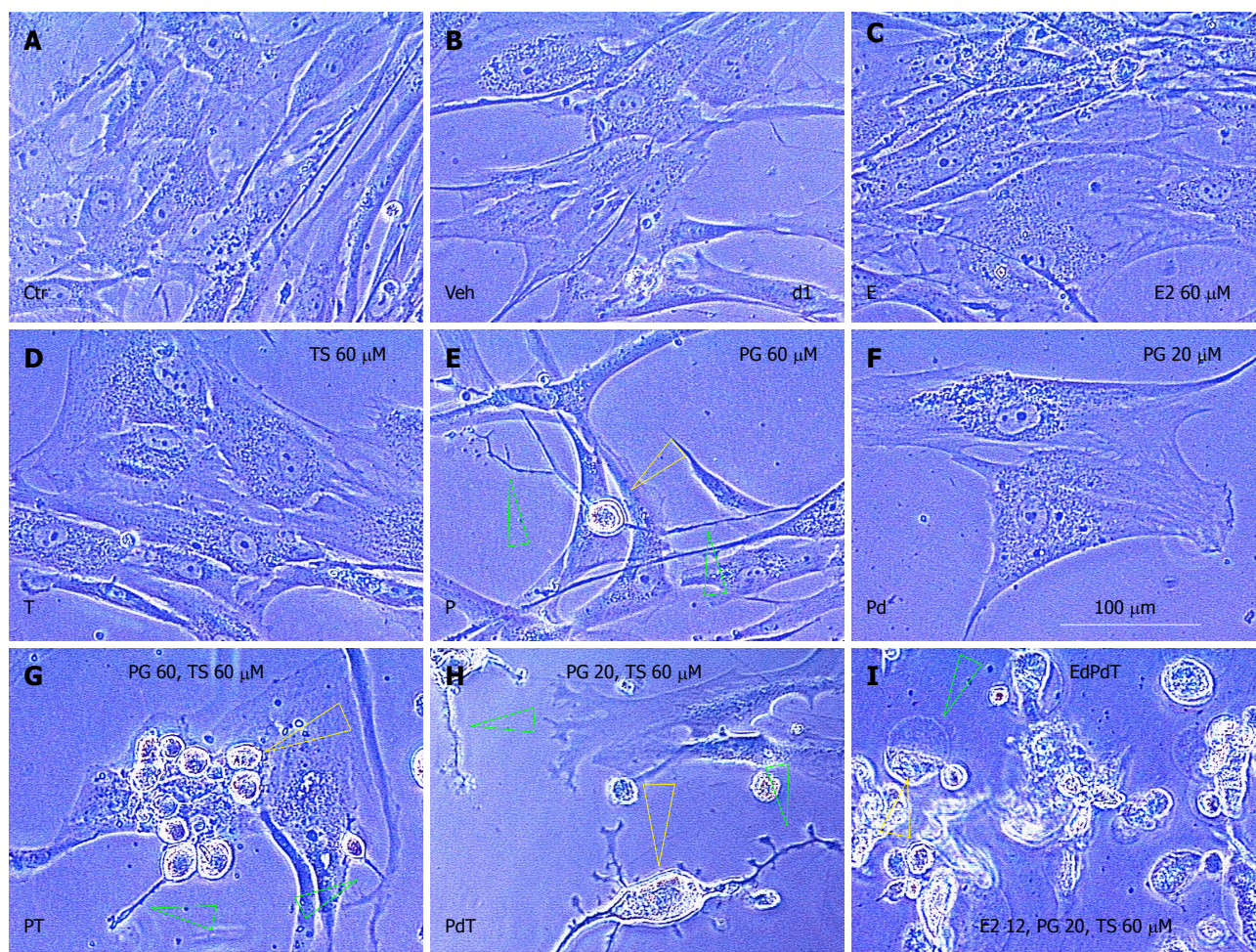


Figure 1 Sex steroid doses and combinations in vascular smooth muscle cell cultures. Reprinted from^[68] with permission, ©Page Press Publications. A-D: Control (A), vehicle (B), high estrogen (E2 60 microM) (C), and high testosterone (TS 60 microM)-treated cultures of vascular SMC showed no morphological changes (D); E: High progesterone treatment (PG 60 microM) was, however, associated with occasional neuronal type cells; F: This was not observed in cultures with diluted progesterone (PG 20 microM); G: Cultures with high PG and high TS showed numerous neural type cells; H: Cultures with diluted PG and high TS showed direct nuclear transdifferentiation into neuronal type cells with branching extensions; I: Diluted estradiol and PG and high TS caused formation of undifferentiated stem type cells. Open yellow arrowheads indicate neural/neuronal type cell bodies, green arrowheads indicate extensions. Green arrowhead in panel I indicates bubble-like anchor. Ctr: Control; E: Estradiol; Ed: Estradiol diluted; P: Progesterone; Pd: Progesterone diluted; PT: Progesterone + testosterone; Veh: Vehicle; TS: Testosterone; PG: Progesterone.

The *in vitro* studies were performed in order to approach a question on which sex steroid combinations and their doses could be effective for the regenerative treatment. Figure 1A and B show that control cultures and media with a vehicle alone exhibited no transdifferentiation into neuronal type cells. When 60 microM E2 (Figure 1C) or 60 microM TS (Figure 1D) was applied, no changes were observed next day after the treatment. The 60 microM PG, however, showed sporadic emergence onset of neuronal cells as indicated by yellow arrowhead in Figure 1E. They exhibited typical neuronal extensions (green arrowheads). Such concentration of PG in tissue culture is relevant to the 16 mg/kg PG density, which was reported to diminish death, gliosis, and functional defects following trauma-induced rat brain alterations^[54]. Therefore, the PG alone could exhibit certain protective effect *in vivo*. Decrease of PG to 20 microM, however, exhibited lack of the *in vitro* effect for an emergence of neural cells - see Figure

1F.

Combination of 60 microM PG with 60 microM TS, caused frequent appearance of small NSC shown by yellow arrowhead in Figure 1G, exhibiting some extensions (see green arrowheads). Therefore, many new NSC could develop using PG along with TS. In other words, the TS significantly potentiated a small PG neuronal effect.

When a reduced dose of PG (20 microM) was utilized with 60 microM TS, the cultured SMC exhibited a transdifferentiation to the well differentiated cells of neuronal type (see yellow arrowhead in Figure 1H) - note the branching extensions indicated by green arrowheads.

The dose of *in vitro* used PG is relevant to PG suppositories (200 mg), utilized 2 times a day to prevent a miscarriage or a preterm labor^[55,56]. Relating the TS dose, it corresponds with weekly intramuscular androgen 600 mg injections, which were tested in human males^[57,58]. Therefore, 400 mg PG daily with weekly 600

mg TS could be considered to treat dysfunctions of CNS.

In vitro utilization of a small concentration of E2 (12 microM) along with 20 microM PG and 60 microM TS, caused a development of many stem type cells in vascular SMC cultures (see yellow arrowhead in Figure 1I) exhibiting bubble-like anchors (green arrowhead). Within few days such stem type cells differentiated backward into typical mature SMC^[16]. Therefore, it is possible to consider that vascular disorders might be treated with such therapy. The E2 12 microM dose used *in vitro* is relevant to 120 mg in humans. For hormone-replacement therapy a transdermal 17 beta-estradiol 50 mg was reported to be used twice a week^[59].

As mentioned above, the pericytes were proposed to represent pluripotential stem cells for other cell types^[31], incorporating cardiomyocytes^[14]. Utilization of all three sex steroids, *i.e.*, PG daily + TS weekly + 50 mg of E2 twice a week during about four weeks could contribute to vascular regeneration after the stroke occurrence or for cardiomyocyte regeneration after the heart infarction.

It is considered that aging humans, and those having a possibility of inherited CNS or heart and vascular alterations, could be prevented by annual PG + TS or E2 + PG + TS combinations used about four weeks to prevent such disorders. Because the E2 could be derived from other two sex steroids, the PG and TS treatment might be sufficient. Such sex hormone supplementation might also be effective for the acute trauma of spinal cord, stroke, or myocardial infarction.

WHAT THE REGENERATIVE MEDICINE OPTIONS MIGHT BE

It is currently expected that the regenerative medicine should replace altered functional cells with newly developed mature cells formed from implanted stem cells or by *in vitro* reprogramming of already differentiated cells from one cell type into a needed distinct one.

In some instances, it might be possible to use the therapy resulting in the *in situ* transdifferentiation of *in vivo* existing autologous progenitor cells, *e.g.*, by substances crossing the blood barrier for the treatment of neurological diseases.

Utilization of exogenous sex steroids could be a preferred option since they are naturally occurring during the life vs other possible chemical approaches for regenerative medicine without the cells. Since regeneration of tissues is dependent on the homeostatic function of the immune system components^[1,60], and the immune system exhibits progressive regression since 35 years of age^[38], the effect of sex steroid combination is expected to be more effective in younger individuals, whose tissues exhibit normal ability to regenerate. Therefore, such treatment could be more effective in sudden traumatic and ischemic disorders of younger individuals exhibiting preserved SCN and TCS exhibiting regular function. In contrast, the degenerative

diseases caused by an altered function of the TCS will not be treatable, unless its normal components are restored, for instance by a blood transfusion from a young healthy individuals (see below).

IS THERE A POSSIBILITY TO AMELIORATE AGING?

Heterochronic parabiosis between aged and young mice was reported to improve the activity of progenitor tissue cells by the blood serum^[61]. Subsequent studies supported this proposal (reviewed in^[11]). The issue of rejuvenation by young blood is, however, more complex than the serum content alone. Blood transfusion, beside serum with immunoglobulins, polynuclear leucocytes and erythrocytes, contains immune system-related mononuclear cells, which are important for the morphostasis of various tissues as the components of the complex TCS^[62]. Numerous studies have shown that the cells of the immune system, beside immunity, participate in regeneration of tissues and healing of the wounds. The TCS is involved in the regulation of tissue-specific cellular regeneration, preservation of developed cells in the proper functional state, and in the maintenance of each tissue in the proper quantity of the cells (reviewed in^[1]).

There are several problems with heterochronic parabiosis. Firstly, this is an animal approach which has no therapeutic use in human beings *per se*. Secondly, long lasting heterochronic parabiosis has been shown to alter the young animal by the old animal blood components^[63]. Thirdly, and most importantly, the utilization of a single dose of blood serum from young to older individual can not be expected to exhibit any long lasting rejuvenation of the recipient's tissues, and the same can be expected after the whole blood transfusion. Regarding the blood transfusion, it can only be utilized as a partial blood volume replacement for two reasons - to prevent an overdose of erythrocytes and to improve a proportionality between the young and aged blood.

Yet, the partial blood replacement with a temporary rejuvenation may have some therapeutic utilization, for instance to temporarily enable gonadal function in order to produce new gametes for the treatment of infertility. Longer lasting tissue rejuvenation after partial blood replacement will require an accompanying stimulation of the immune system morphostatic functions by the honey bee propolis or drugs with similar effects - reviewed in Ref.^[11].

Regarding the role of the immune system related cells in tissue morphostasis, it has been shown that after a partial hepatectomy or unilateral nephrectomy the immune system is specifically activated to repair the liver or kidney tissue depletion. The T lymphocytes initiate and then stop the growth of the liver or kidney until the morphostasis reappears^[64]. Our observations from the ovarian physiology indicate that the active

tissue growth is dependent on pericytes releasing Thy-1 differentiation glycoprotein, the most primitive member of the immunoglobulin gene superfamily^[65]. Activity of pericytes for Thy-1 release is inhibited by vegetative innervation in the sites, where regeneration is not needed. In other words, the neural system regulates proper tissue quantity, and immune system cells stimulate the regeneration when asked to do so by the Thy-1 release from pericytes.

In addition, the incidence of malignant diseases progressively increases with age advancement. Albeit a single accidental consumption of an unprocessed shiitake mushroom caused a common severe skin erythema with pruritus^[66] lasting for a couple of weeks. This immune system systemic hypersensitivity, however, successfully eradicated a rectal cancer. The subsequent weekly consumption of small amounts of unprocessed shiitake mushrooms in salads was without skin reaction and prevented cancer relapse and any occurrence of malignancy for many years thereafter^[67].

An elaborated immunotherapy, consisting of human gammaglobulin to unblock antibodies against alloantigens, a single dose of cyclophosphamide to eliminate immune system support of cancer progression (see Ref.^[1]), followed by two separate blood transfusions to enhance immune system reactivity against cancer alloantigens, and weekly intradermal applications of Bacterinum adnexitidicum to booster a reversion of immunosuppression, was capable to induce within few months a gradual regression of advanced human ovarian cancer, including complete regression of ascites and severe liver metastases, accompanied by a regeneration to the regular liver size (for details see Ref.^[67]).

CONCLUSION

Available data indicate that sex steroid combinations will represent a novel approach of regenerative medicine enabling stem cell therapy of neuronal alterations without the cells. In other words, this approach can enable utilization of endogenous pluripotent cells to differentiate into new neurons where needed. Sex steroid levels are high during pregnancy in both the mother and the fetus, and do not alter their bodies in general, but contribute to the fetal development and its immune tolerance by the mother. Utilization of sex steroids in aging individuals exhibiting alteration of neuronal tissues may, however, not be effective, unless its morphostasis mediated by the immune system, is improved. A combined treatment with sex steroid combinations to enable a transdifferentiation of vascular pericytes into neuronal cells, and with a partial blood replacement (500 mL blood withdrawal with immediate replacement of the same fresh blood volume from the young healthy individual) can cause a temporary brain tissue rejuvenation, possibly including improvement of the Alzheimer's disease. To preserve the achieved regeneration will require a permanent utilization of drugs stimulating immune system role in normal tissue

morphostasis, as reported for the honeybee propolis.

The age advancement is also accompanied by malignancy development. Early cancer development in untreated individuals can be eradicated by a consumption of unprocessed (rough) shiitake mushrooms. The regression of advanced and metastasizing cancers in otherwise previously untreated patients could be achieved by an elaborated immunotherapy. The patients who had undergone a prolonged and/or high dose or long lasting cytostatic therapy, high dose irradiation, excessive surgery, and exhausted cases close to death, are unlikely to respond the elaborated immunotherapy of cancer.

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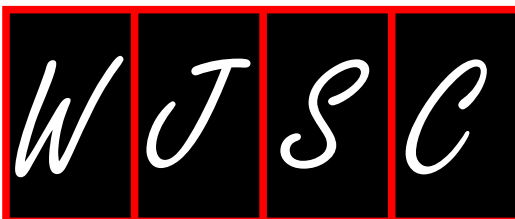
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Stem cells: An eventual treatment option for heart diseases

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Abstract

Stem cells are of global excitement for various diseases including heart diseases. It is worth to understand the mechanism or role of stem cells in the treatment of heart failure. Bone marrow derived stem cells are commonly practiced with an aim to improve the function of the heart. The majority of studies have been conducted with acute myocardial infarction and a few has been investigated with the use of stem cells for treating chronic or dilated cardiomyopathy. Heterogeneity in the treated group using stem cells has greatly emerged. Ever increasing demand for any alternative made is of at most priority for cardiomyopathy. Stem cells are of top priority with the current impact that has generated among physicians. However, meticulous selection of proper source is required since redundancy is clearly evident with the present survey. This review focuses on the methods adopted using stem cells for heart diseases and outcomes that are generated so far with an idea to determine the best therapeutic possibility in order to fulfill the present demand.

Key words: Bone marrow stem cells; Mesenchymal stem cells; Induced pluripotent stem cells; Heart diseases; Progenitor cells; Stem cells

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Core tip: Heart diseases are most common all over the world and any form of treatment would be well appreciated to enable successful well-being in the patients. Though there are enormous efforts attempted to handle heart diseases, there has been no propounded evidence so far to utilize a particular source of cells globally. In this regard, if the responses all over the

world are collectively segregated to a global database, there may be options to identify the best suitable cell source that provides effective cure.

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INTRODUCTION

Cardiac diseases have become a major cause of death worldwide^[1-4]. Common causes of heart failure are ischemia, hypertension, coronary artery disease and idiopathic dilated cardiomyopathy. Up to 40% of cardiac patients are predominantly affected by diastolic dysfunction, although the left ventricular (LV) systolic function is mostly unaffected^[5]. There are established medical and surgical procedures available for cardiac patients but these standard therapeutic modalities have not been much responded^[1,6,7]. Cardiac risks are also associated with other complications^[8-10], which thus gives an steady increase in the number of patients looking for an effective treatment option that can sustain their normal well-being. There is an urge for alternative therapies that can fulfill the present overwhelming demand^[6,11]. Cardiac diseases trigger damage to cardiomyocytes that affects the heart muscles and there is a possibility of scar formation that will overload the blood flow and eventually stretch the cardiac cells. This ultimately leads to heart failure and commonly, deaths^[12].

The current situation is therefore mostly favorable for alternative treatment possibilities that can improve the disease considerably at least with respect to the rate of survival or quality of life^[13-15]. Recent evidence on cell based therapies has suggested improvement in cardiac function and regeneration of the damaged tissue^[16]. It is stated that cell therapy has got potentials to do what standard drugs cannot do by replacing the damaged tissues with healthy tissue. Drugs have been demonstrated to slow the progression of heart failure, but none have shown their potential in reversing the process. However, the physiological perspectives have not shown any clear picture with cell therapies as it is difficult to estimate the cell survival and monitor cells *in vivo*^[17,18] along with their low engraftment rate^[18]. Valid doubts and concerns are raised for the existing use of stem cells for heart diseases and it is elaborated that although regenerative medicine has lots of hopes, results have not substantiated their importance in the in-house tentative studies^[19]. There are some important factors that need to be considered such as appropriate selection of the cell type, number of cells required and appropriate route of administr-

ation that can help regeneration. Routes of injection generally include catheter-guided transendocardial, intracoronary, transvenous, and transepicardial injections^[20]. The selected cells should have a control over their migration, proliferation and differentiation *ex vivo* and also *in vivo*^[21]. Even though the mechanisms of action pertaining to cell therapies are not very clear, studies in comparison with routine therapies have shown promising results by significantly improving vessel density, inhibiting apoptosis of myocytes, and decreasing fibrosis and increasing survival rate even in non-ischemic heart failures^[22,23]. There are other reports available with similar strategy to improve the survival of transplanted cardiac progenitor cells^[24-27].

The progress of cell therapy is reported in Mayo Clinic proceedings as breakthroughs that have reached certain milestones and expectations that have a high prospect towards imminent benefits. The six major breakthroughs are summarized as: (1) safety establishment in intracoronary delivery; (2) possibility of therapeutic regeneration; (3) allogeneic cell therapy; (4) increasing mechanistic insights; (5) sparkling clinical efficacy; and (6) progress to phase 2 and 3 studies^[28]. Cell therapy has shown enormous progress since its application in 1998 and this lightning speed is progressing towards eliminating the underlying cause of heart failures and handling the control over the damage^[29]. Different cell types have been used to treat heart diseases^[30-32]. There are potential benefits with the application of cardiac progenitor cells and induced pluripotent stem cells (iPSCs) as a promising approach to bring true myocardial regeneration^[30].

The advent of iPSCs is also under remarkable consideration to model heart disease^[33]. Stem cells have been suggested to be promising new therapeutic usage for heart diseases because of their remarkable proliferative and differentiation capacity that can supply specific cell types along with the viable functioning of heart muscles^[12]. Evidence has shown that resident cardiac stem cells have got potentials to turn into cardiomyocytes *in vivo*^[31]. There are other well-known and commonly available sources of progenitor cells from bone marrow, adipose tissue, and skeletal muscle or cells circulating in blood that are capable of improving myocardial function^[11,34].

Although there are waves of research undertaken with stem cells or any cell therapy applications, *in vitro* studies are required to explore which is of high priority to advance with the strategies to tailor clinical applications and the task is highly challenging^[35]. Appropriate selection of patients, informed consent and periodic patient monitoring are important factors to be reviewed by expert panel independent of investigators while opting for clinical trials^[36,37]. While manufacturing stem cells, parameters like screening and testing for pathogens have to be carried out with utmost precision. Generation of consistent batches while manufacturing is highly recommended for therapeutic derivative of stem cells and this has to be carried out in aseptic conditions

withstanding robust quality programs that qualify regulatory expectations which are suitable for clinical use in patients. It is also necessary to validate the procedures for transplant and confirm the regeneration of infarcted heart^[38,39]. Overall, it is expected that the strategies adopted from routine therapies into stem cell-based therapies, have glorious potentials to increase the efficacy of treatment^[40]. This review thus focuses on the studies attempting to assess various alternative therapeutic applications for heart diseases and to suggest the best suitable and effective source that has produced better success rate.

CURRENT PROGRESS IN ROUTINE THERAPIES

Heart disease continues to be the main cause of death though there is a considerable decline in the death rate with the advancement in medicine since 1999. Nonetheless, medical care has not been successful in reversing the loss of functional cardiomyocytes^[41]. The surgeries being attempted for diagnostic cardiac catheterizations, coronary artery bypass graft procedures, cardiac pacemaker procedures, percutaneous transluminal coronary angioplasty procedures and heart valve procedures are all very expensive since they require hospitalizations, post-surgical care, etc. They consume enormous time to handle the recovery process. Still, evidence suggests that cardiovascular diseases are not prevented though the lethality has been reduced considerably^[42]. Presently, the treatment options vastly carried out for cardiovascular function include organ transplantation, surgical reconstruction, and usage of mechanical or artificial devices or administration of metabolically derived products^[43]. Benefits have been associated with inotropic therapy, ventricular assist devices and hospice care. Recommendations are given towards the use of angiotensin-converting enzyme inhibitors as a first line of treatment in patients with reduced LV systolic function with ejection fraction $\leq 35\%$ -40%^[44]. For normal ventricular ejection fraction, beta-blockers, isosorbide dinitrate plus hydralazine are being considered^[45].

In this context, researchers have been encouraged to identify better strategies to image the detection and monitoring of cardiovascular disease progression. Biotech industries have also been progressing in different scenarios with other therapeutic possibilities which with mutual understanding between the pharmaceutical industries and medical specialists, may aim to bring options that provide immediate recovery along with cost effective strategies^[3,46]. Interest relies on the targeted delivery of alternatives such as stem and progenitor cell populations that has also kindled the combinatorial use of genes to treat cardiovascular diseases. However, challenge depends on the host environment where it has poorly retained the survival of implanted cells and thus it requires the delivery of

cells transfected or transduced *ex vivo* with genes that can enhance resistance to apoptosis in order to provide protection from the stressed microenvironment that can enable the improved function of the engrafted cells to appreciate better therapeutic outcome^[47].

ROLE OF ALTERNATIVE MODALITIES IN RESTORATION OF HEART FUNCTION

Mesenchymal stem cells

Even after maturation, cardiomyocytes have limited flexibility. The recovery of heart function after structural damage is less possible. Mesenchymal stem cell (MSC) therapy is predicted to be a promising area of research for regenerating damaged cardiac tissues. MSCs lack both major histocompatibility II and T-cell co-stimulatory signal expression and thus are immune-privileged. When MSCs are delivered systematically, they own a unique ability to enter into the specific sites of myocardial damage. There are mechanisms by which MSCs promote cardiac tissue regeneration. Data generated from several studies reveal that MSCs are therapeutically valuable and play a great role in cardiac diseases. In future, MSCs could be used for cell-based therapy for cardiac diseases.

MSCs are generally derived from any tissue sources. In the translational research MSCs used are mainly derived from bone marrow. Specifically, for cardiac repair or cardiac disorders, the MSCs used are derived from bone marrow^[48]. Several studies are carried out to understand the mechanism of action of MSCs in cardiac repair. One of the studies conducted by Yang *et al.*^[49] demonstrated that a combination of statin along with bone marrow derived MSCs intramyocardial injections improved the efficiency of cardiomyocyte differentiation by 4-fold in a swine model.

Numerous preclinical studies are carried out using large animal models to understand the efficacy of MSC therapy for cardiac diseases. In these studies, stem cells are delivered to the heart *via* peripheral intravenous infusion during the open heart surgery. It can also be administered through catheter-based intracoronary infusion. The studies carried out after delivery of MSCs showed that intramyocardial injection had the highest retention rate of cells^[50]. Among all the preclinical studies carried out with MSCs for cardiac diseases, surgical injection was the most commonly used method to deliver MSCs^[51-53].

Though there are varieties of sources of MSCs, there has been particular interest in adipose lineage MSCs which were found to be easily available and successful in several preclinical studies. Several studies were carried out by the administration of adipose derived MSCs for myocardial infarction (MI), which were found to be successful^[54].

Hematopoietic stem cells

Many clinical trials are carried out to improve the re-

covery of cardiac function. There were several favorable and unfavorable results during the course of time. In many of the studies, bone marrow mononuclear cells were injected. Nevertheless, detailed studies are to be carried out to understand the fate and effects of transplanted cells^[55-58]. It is understood that bone marrow contains heterogeneous populations of cells, generally Hematopoietic stem cells (HSCs) and MSCs^[59].

Apart from these, bone marrow also has different progenitors of myeloid and lymphoid lineages. Several studies indicate that both HSCs and MSCs are reported to acquire the phenotype of cardiomyocytes in syngeneic or xenogeneic recipients. Even though various studies were carried out, none of them quantitatively analyzed the regenerative capacity. Nevertheless, there is evidence shown by Norol *et al.*^[60] in nonhuman primate models that HSCs do not differentiate into cardiomyocytes or into endothelial cells in the infarcted myocardium. Apparently, the growth factors produced locally does not have any positive effect on the regeneration of myocardium^[60]. It is well known that HSCs are a unique source of adult stem cells that are easily accessible. It is documented that HSCs were used to induce neoangiogenesis to treat ischemic vascular diseases. HSCs subsets also contain endothelial precursors that are AC133+, which are considered to be major requirements in patients with ischemic and refractory peripheral vascular or coronary artery diseases^[61].

Endothelial progenitor cells

Endothelial progenitor cells (EPCs) consist of a heterogeneous group of cells which originates from multiple precursors within the bone marrow and are present in different stages of endothelial differentiation. The existence of endothelial dysfunction forecasts the consequences in patients who suffer with cardiovascular risk factors and persons with coronary artery disease. EPCs possess the ability to home to sites of vascular injury. Thus, EPCs could be easily adopted for therapeutic uses related to angiogenesis. Several studies conducted in coronary artery disease (CAD) patients revealed that they had reduced number of EPCs. They also had reduced number of circulating EPCs^[62-65].

Bone marrow derived EPCs will be mobilized to stimulate angiogenesis and will attenuate tissue ischemia for CAD and peripheral arterial disease (PAD). Further to this, clinical trials carried out with different animal models favor the application of EPCs as a potential therapeutic agent in cardiovascular diseases^[66,67].

Usage of EPC capture stent is also in practice. EPC capture stent is a device which uses the ability of bone marrow derived EPCs to repair damaged arterial segments. This instrument is manufactured in such a way that the surface of EPC antibody contains a covalently coupled polysaccharide intermediate coating with anti-human CD34 antibody and it is attached to a stainless steel stent. Once the stent is placed, the circulating EPCs will be attracted to CD34 antibodies and

will be directed to differentiate into mature endothelial cells to form functional endothelium layer^[68].

There are several studies ongoing to understand the effects of pharmacotherapies with EPCs in cardiac patients. EPC behavior and mechanisms are also elucidated in several studies conducted in patients with cardiovascular disease (CVD), CAD, heart failure and PAD. There are several promising studies showing that EPCs could be used as a novel therapy for CVD^[69]. However, there are several limitations with EPCs due to its paucity of circulating cells. The efficacy of the treatment using EPCs can be obtained by increasing the cell number by concentrating and transplanting or by functional improvement using *ex vivo* augmentation.

iPSCs

Usage of iPSCs emerged as a novel therapy for the treatment of cardiac diseases. It has also attracted much attention and is considered to be the promising cell source for patient specific cell therapy^[70]. Major advantage of iPSCs are that the clinically relevant number of cells could be generated easily when compared to adult stem cells and that they are associated with a less risk of immune rejection when compared to embryonic stem cells^[71].

iPSCs are generally engendered from adult somatic tissues with precise reprogramming. Studies prove that iPSCs have many similarities to human embryonic stem cells especially in terms of morphology, proliferation, feeder dependence, surface markers, gene expression, epigenetic status, promoter activities, telomerase activities, *etc.*^[72]. A study was conducted in a mouse model of MI, and human iPSCs were induced in this model. The results disclosed that the mice had regenerated myocardium, smooth muscles and endothelial tissue, restoring post ischaemic contractility performance and electric stability^[73]. Even though, several studies and clinical trials were carried out to understand the efficacy of iPSCs in cardiac repair. When it comes to gene therapy, the integration of viral vectors in the genome remains unanswered. These viral vectors in the genome might promote malignancy. Apart from that, these virus-encoded genes can act as oncogenes. Thus, the clinical application of iPSC derived cardiomyocytes appears to be a distant possibility at present. However, the advent of different study models will resolve the issues in the future.

Bone marrow derived mononuclear cells

Although many cell types have been attempted, there have been promising outcomes with crude extracts of bone marrow derived cells. Utilization of such resources not only minimizes time but also may be useful in reducing the cost of expenses of application^[74]. Recent use of bone marrow derived mononuclear cells (BMMNCs) in heart diseases has been able to improve LV function, and increase the myocardial perfusion, but has not produced any significant change in the

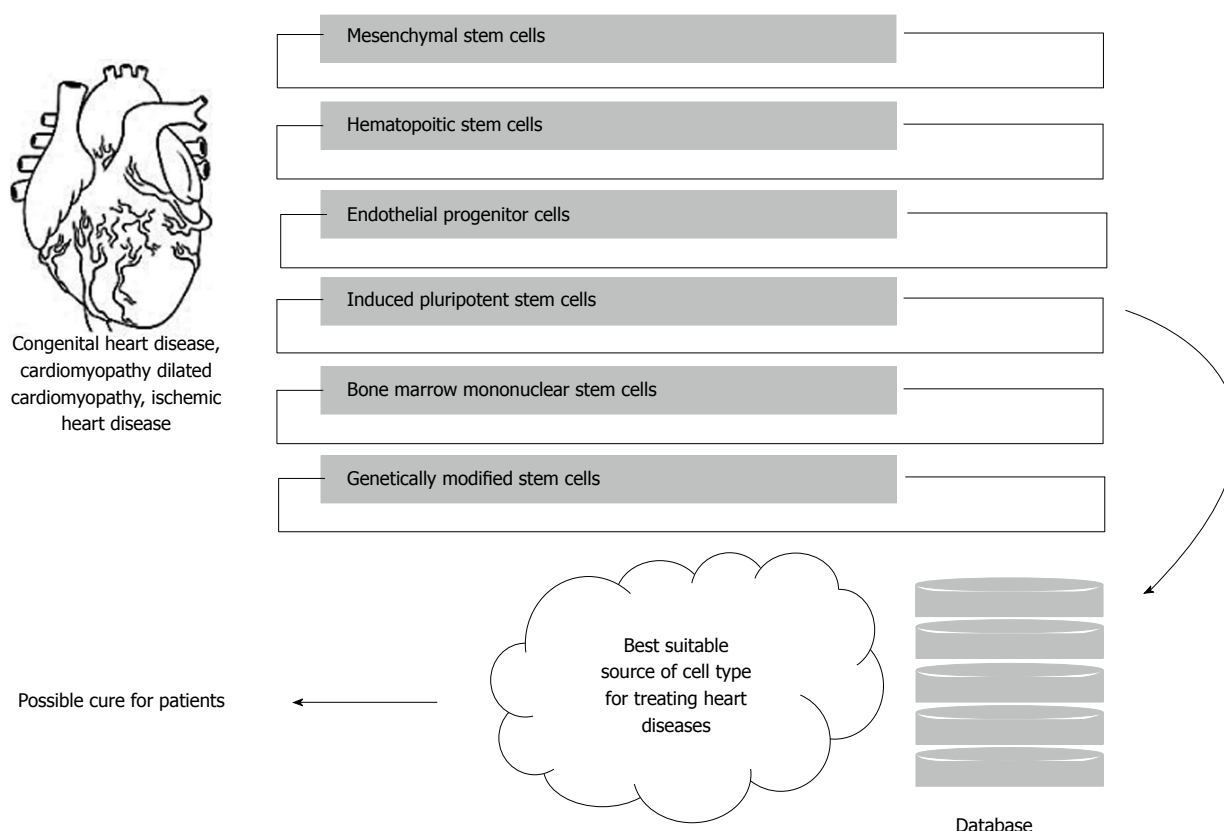


Figure 1 Illustration to show the requirement of a database for identifying alternative cell therapy for cure of heart diseases.

scar tissue^[75,76]. A double blind study of a total of 16 patients after previous placebo injection has described the improvement in angina symptoms and myocardial perfusion with the infusion of BMMNCs in chronic myocardial ischemia patients^[77]. However, Heesch *et al.*^[78] have shown that the application of BMMNCs to ischemic cardiomyopathy patients when injected, has profoundly reduced the neovascularization and thus limited its therapeutic potential for cell therapy. However, the reduced neovascularization is mostly reported to be impaired with aging, which is largely attributed to aged BMMNCs, but ageing does not affect the revitalization of neovascularization in the murine host with the transplantation of young BMMNCs^[79]. Although the results have been more commented, scientists overall are not in favor of using BMMNCs because the small number of undifferentiated cells is unlikely to be effective^[74].

Genetically modified cells

Genetic modifications of stem cells will result in enhanced efficacy. Genetically modified stem cells can be utilized for different diseases. A number of diseases can be treated now with them. Various strategies like pre-treatment with growth differentiation factors or cytokines, pre-conditioning like hypoxia and genetic modifications to overexpress anti-death signals are developed. It was Mangi *et al.*^[80] who first genetically modified MSCs to overexpress Akt which are resistant

to apoptosis *in vitro* and *in vivo*. They proved that there was a drastic improvement in cardiac function in an MI rodent model^[80]. Further work on this showed that Akt transduction stimulates MSCs to produce paracrine factors which are advantageous to infarcted heart post-engraftment^[81,82]. In another experiment transfected MSCs with bFGF showed an increased survival and cytoprotection in hypoxic condition^[83] (Figure 1).

CHALLENGES TOWARDS FUTURE DEVELOPMENT

It is clearly understood that there are underlying challenges for the identification of well-deserved type of regenerative therapy. The majority of cell types are under clinical trials but the results have been heterogeneous. Causes have been predicted to be poor survival, proliferation, engraftment and differentiation of engrafted cells. Ultimately, the objective is to increase the functional ability of stem cell before and after transplantation^[4,84]. The concern thus lies in the knowledge on structural and functional characteristics of stem cell-derived cardiomyocytes, and their capability to integrate with host tissue^[85,86]. Concerns need to be imparted for appropriate selection of patients with clear understanding about the variability of disease condition. Prospective outcomes have to be confirmed by the anatomical and histological examinations with

a large number of studied patients in order to analyze significant observations^[87]. With the prevailing methods for achieving an alternative approach to deal with heart diseases, the approach that would in future be available for the treatment is believed to possess more effective delivery methods and growth and storage methods^[17].

Researchers are working on to effective management of the challenges for the utilization of stem cell therapy wherein, newer strategies are being adopted for the combination of cells with bioengineering techniques that may improve the efficacy considerably^[88]. However, approaches dealing with the utilization of epicardium-derived cells and cardiomyocyte progenitor cells have synergistically shown better impact in cardiac function which is believed to be because of complementary paracrine actions^[89]. Whatever the sources have been utilized or approached such as embryonic stem cells, iPSCs, adult stem cell lineages, bone marrow derived stem cells, or MSCs, it is widely acceptable that there is enormous hope underlying the usage of stem cell therapy as a clinically viable alternative for heart diseases^[90] and the direction that the research is progressing is believed to be perfect which requires clear understanding and revival of the attempted trails to progress with newer ideas and accomplish the ultimate goal.

Challenges are still prevailing for the application of cell therapy to the patient. Considering the complications in delivery of the stem cells to the patient, three factors are to be taken into consideration: the type and nature of injury, timing of the therapy, and the ability of the cells to engraft the host myocardium^[82]. There is a huge dilemma regarding the best time for stem cell delivery to patients. Several studies carried out in this field prove that early time points will be ideal for stem cell transplantation^[91]. Choosing of best stem cell type also plays a crucial role in transplantation and engraftment ability to host myocardium. Recent studies indicated that transplanted cells are vulnerable to hostile ischemic environment. Thus, they will tend to disappear in a few days^[92]. Several investigations are going on to improve the microenvironment and engraftment followed by survival.

CONCLUSION

Even though the mechanisms of action for all these stem cell based therapies are not well established, it remains a promising field of research. However, a major obstacle in this arena is the poor viability of implanted stem cells for cardiac repair. The strategies reviewed here are for the augmentation and development of appropriate approaches for the treatment of cardiac disorders. Attempts performed using a specific kind of cell type by any organization are claimed to provide efficient results in comparison to the other. This has created disputes in pursuing cell types that can generate positive outcomes and thus has deliberately given poor understanding amongst the physicians.

Involvement of cell therapy possibilities has therefore anonymously diminished though it is under immediate purview. Future challenges pertaining to the application hence depend on the clear validation of the cell therapy with respect to source, mode of application, dosage and expected outcome. Reciprocation of these results is vital in order to pursue the technology.

Ethical constraints are also major hurdles to circumvent the use of alternative strategies which needs to be clearly framed considering the benefits that medical arena would acquire. Adult stem cells in this regard, have proven safe and generated decent outcomes. We suggest that, instead of exploiting research knowledge to fewer more decades, concentration towards the ultimate requirement is very important. The studies generated so far with various alternative treatment modalities for heart diseases worldwide can be collectively segregated in a common database and significance with the available data can be generated to find the best and viable source of approach that proves effective for heart diseases. In case of any discrepancy with the results, the reciprocation of the data can be attempted in order to obtain a solution.

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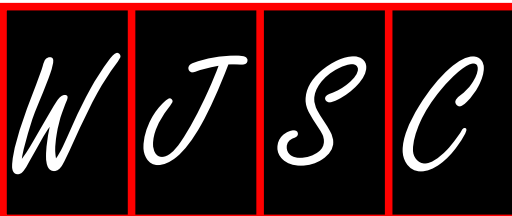
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Basic Study

Autologous tissue patch rich in stem cells created in the subcutaneous tissue

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Abstract

AIM: To investigate whether we could create natural autologous tissue patches in the subcutaneous space for organ repair.

METHODS: We implanted the following three types of inert foreign bodies in the subcutaneous tissue of rats to produce autologous tissue patches of different geometries: (1) a large-sized polyvinyl tube (L = 25 mm, internal diameter = 7 mm) sealed at both ends by heat application for obtaining a large flat piece of tissue patch for organ repair; (2) a fine polyvinyl tubing (L = 25 mm, internal diameter = 3 mm) for creating cylindrically shaped grafts for vascular or nerve repair; and (3) a slurry of polydextran particle gel for inducing a bladder-like tissue. Implantation of inert materials was carried out by making a small incision on one or either side of the thoracic-lumbar region of rats. Subcutaneous pockets were created by blunt dissection around the incision into which the inert bodies were inserted (1 or 2 per rat). The incisions were closed with silk sutures, and the animals were allowed to recover. In case of the polydextran gel slurry 5 mL of the slurry was injected in the subcutaneous space using an 18 gauge needle. After implanting the foreign bodies a newly regenerated encapsulating tissue developed around the foreign bodies. The tissues were harvested after 4-42 d of implantation and studied by gross examination, histology, and histochemistry for organization, vascu-

larity, and presence of mesenchymal stem cells (MSCs) (CD271+CD34+ cells).

RESULTS: Implanting a large cylindrically shaped polyvinyl tube resulted in a large flat sheet of tissue that could be tailored to a specific size and shape for use as a tissue patch for repairing large organs. Implanting a smaller sized polyvinyl tube yielded a cylindrical tissue that could be useful for repairing nerves and blood vessels. This type of patch could be obtained in different lengths by varying the length of the implanted tube. Implanting a suspension of inert polydextran suspension gave rise to a bladder-like tissue that could be potentially used for repairing heart valves. Histologically, the three different types of tissue patches generated were organized similarly, consisting of three layers, increasing in thickness until day 14. The inner layer in contact with the inert material was avascular; a middle layer that was highly vascular and filled with matrix, and an outer layer consisting of loose connective tissue. MSCs identified as CD271+CD34+ cells were present in the medial layer and around major blood vessels at day 4 but absent at later time points. The early-harvested tissues, endowed with MSCs, could be used for tissue repair, while the later-harvested tissues, being less vascular but thicker and tougher, could be used as filler tissue for cosmetic purposes.

CONCLUSION: An autologous, vascularized tissue patch of desired shape and size can be created in the subcutaneous space by implanting different types of inert bodies.

Key words: Autologous tissue patch; Foreign body; Subcutaneous implantation; Mesenchymal stem cells; CD271; CD34

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Core tip: While much progress has been made in man-made tissue patches for organ repair, their clinical success has been bogged down by their inability to get promptly vascularized *in vivo*. We describe a methodology for creating tissue patches of several possible shapes/sizes in the subcutaneous tissue utilizing the foreign body reaction. These patches are endowed with a rich capillary network, abundant mesenchymal stem cells, and other accessory cells that support the viability of the tissue. Such *in vivo* generated patches for tissue repair promise to be safer, more readily vascularized, and stable for longer period in the body.

Garcia-Gomez I, Gudehithlu KP, Arruda JAL, Singh AK. Autologous tissue patch rich in stem cells created in the subcutaneous tissue. *World J Stem Cells* 2015; 7(8): 1127-1136 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i8/1127.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i8.1127>

INTRODUCTION

There is an unmet need to develop biocompatible tissue patches for repair of various diseased organs such as ischemic heart, large open wounds, vascular defects, damaged nerves and many others. Much progress has been made in creating engineered tissue patches using polymer scaffolds (which mimic tissue matrix) or processed xenogeneic biomaterials seeded with functional cells, endothelial cells, paracrine growth factors, and fetal or adult stem cells^[1-7]. The complexity of these constructs is necessitated by the need to have an immediate blood supply to the patch for survival of the seeded endothelial and functional cells. A myriad of growth factors (from stem cells) should also be available in the patch to rapidly form the new vasculature that would connect with the host blood vessels. Despite such advances the constructs have proven to be sub-optimal in performance. There is the problem of core degradation of the construct and the lack of integration of the seeded functional cells with the host tissue cells, probably arising from lack of adequate blood supply in the construct^[3,8]. The failing patches could occasionally end up being a source of dying cells and thus causing adverse reactions rather than benefit. A multitude of accessory supporting cells other than endothelial cells are needed to form the desired new blood vessels and maintain the integrity of an avascular patch^[9-14]. Because many of these cells are not readily available and several others remain unknown, these patches remain poorly perfused *in vivo*. A natural autologous tissue patch containing stem cells, other supporting cells, and more importantly, a functional vasculature, would be a good solution to the problem.

We present here a methodology for creating tissue patches in the subcutaneous space of different shapes and sizes that are richly endowed with mesenchymal stem cells (MSCs), microvessels and interstitial cells. The principle behind the technique is to implant an inert material in the subcutaneous space which elicits a "foreign-body reaction" leading to the formation of a highly vascular, newly regenerated tissue surrounding the foreign body. Using this approach we have created vascularized tissue patches of various shapes and identified the time after implantation when the MSC population is highest in the new tissue for the purpose of using them as grafts for tissue repair.

MATERIALS AND METHODS

Preparation of inert materials for subcutaneous implantation

The following types of inert materials were implanted in the rat subcutaneous space: (1) large-sized polyvinyl tube (Polyvinyl chloride 180; Nalgene Nunc International, Rochester, New York) (L = 25 mm, internal diameter = 7 mm) sealed at both ends by heat application for obtaining a large flat piece of tissue patch

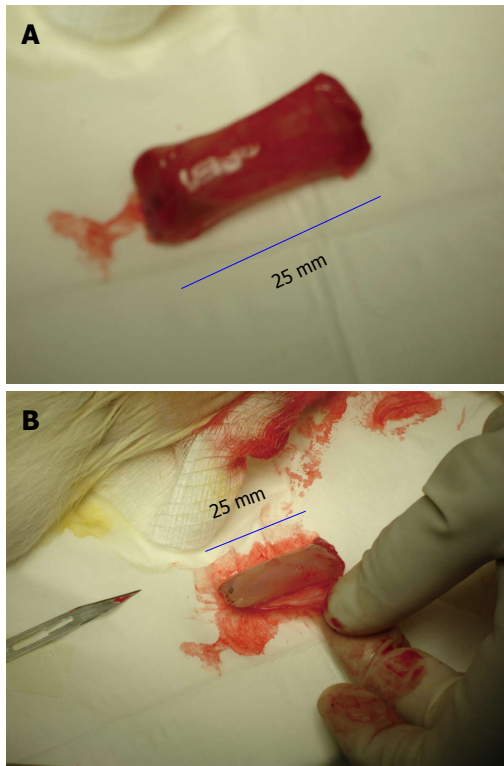


Figure 1 Gross appearance of tissue patch obtained by implanting a large-sized polyvinyl tube (L = 25 mm; ID = 7 mm) in the subcutaneous space of a rat and extracted after 4 d. The implant was completely encapsulated by a *de novo* tissue (A) after tissue that could be conveniently stripped off the implant to obtain a flat piece of tissue patch (B) after patch. Note the high vascularity of the encapsulating tissue.

for organ repair (Figure 1); (2) fine polyvinyl tubing (L = 25 mm, internal diameter = 3 mm) for creating cylindrically shaped grafts for vascular or nerve repair (Figure 2); and (3) a slurry of polydextran particle gel for inducing a bladder-like tissue. Polyvinyl chloride after polyvinyl bodies were sterilized by immersing them in 70% ethanol, then washed vigorously with sterile saline and air-dried before implantation in the rat. Polydextran slurry was made from Sephadex-25 (course; particle size 100-300 μm diameter; General Electric Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, United States). The polydextran particles were pre-swollen in water and sterilized by suspending them in 70% ethanol for 2-3 h followed by removal of ethanol and re-suspension in 10 volumes of sterile saline. After allowing the particles to re-swell for several days in sterile saline, excess saline was removed and the slurry adjusted to a particle: saline ratio of 1:1 (vol/vol) for use.

Surgical placement of the inert materials in rats

All the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the John H. Stroger, Jr. Hospital of Cook County. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to the laboratory conditions for 2 wk prior to experimentation and given food and water *ad libitum*.



Figure 2 Gross appearance of the tissue patch obtained by implanting a fine polyvinyl tube (L = 25 mm; ID = 3 mm) in the subcutaneous space of a rat after 4 d. A: Shows the construction of the polyvinyl tube; B: Shows the *de novo* tissue encapsulating the fine polyvinyl tube implant. The tissue could be gently slipped off the tube for creating a cylindrically shaped graft for tissue repair. Note the high vascularity of the tissue in (B). ID: Internal diameter.

All animals were euthanized by barbiturate overdose (150 mg/kg body weight pentobarbital).

Sprague-Dawley males rats (250-300 g) were anesthetized with intraperitoneal sodium nembutal (5 mg/100 g wt) and their thoracic-lumbar region was shaved and cleaned with alcohol/povidone. For implantation of inert materials a small incision was made on one or either side of the region. Subcutaneous pockets were created by blunt dissection around the incision into which the inert materials were inserted (1 or 2 per rat). The incisions were closed with silk sutures, and the animals were allowed to recover. In case of the polydextran gel slurry 5 mL of the slurry was injected in the subcutaneous space using an 18 gauge needle.

Rats were sacrificed at day 4, 7, 14, 21, 35 and 42 after implantation of the three types of inert materials ($n = 3$ rats at each time point for large and fine polyvinyl tube and $n = 2$ at each time point for polydextran; total rats used = 48).

Harvesting and processing of the subcutaneous tissue patch for gross and histological examination

At day 4, 7, 14, 21, 35 and 42 after placement in the subcutaneous space the inert bodies were harvested aseptically. The tissue encapsulating the materials was removed according to the type of tissue required. For obtaining a flat piece of tissue, the tissue encapsulating the large polyvinyl tube was removed by cutting along the length of the tube as shown in Figure 1. For obtaining a cylindrical tissue as for a vascular graft, the encapsulating tissue from the finer polyvinyl tube was gently slipped off the tube as shown in Figure 2. The bladder-like tissue from the polydextran slurry injected rats was separated from the adhering connective tissue using blunt dissection (Figure 3).

For histological examination samples of the three tissues were fixed in 4% para-formaldehyde for 24 h with vigorous shaking. The fixed tissues were then paraffin embedded in the manner that the inner and

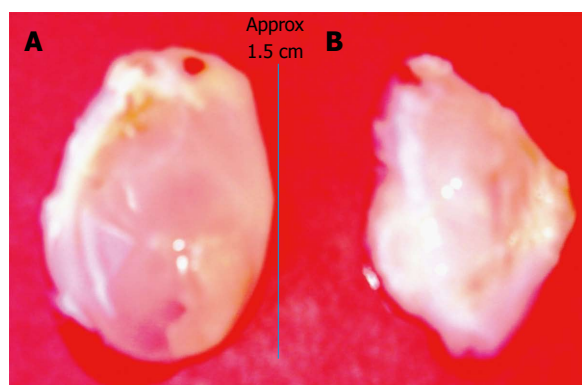


Figure 3 Bladder-like *de novo* tissue obtained 14 d after injecting a slurry of poldextran gel particles in the subcutaneous space of a rat. A: The bladder-like tissue enclosed the injected gel particles; B: By piercing the balloon-like tissue the polydextran particles could be removed from the tissue to deflate the bladder. The tissue could then be used in a variety of ways including as a cusp for cardiac valve repair after cutting it appropriately to the required curvature.

outer aspects of the tissues were histologically recognizable. Approximately 5 μm thick sections were obtained on glass slides for routine histological staining (trichrome) and histochemistry described below.

Histochemical staining

Indirect immunofluorescence, double immunofluorescence or immune-phosphatase techniques were performed on 4–6 μm thickness sections of paraffin embedded tissue patches. Sections were deparaffinized before staining.

For indirect immunofluorescence, the sections were treated with citrate buffer (pH = 6) at 100 °C for 10 min for antigen retrieval followed by 10 min incubation with sodium borohydride (4 mg/mL) to minimize auto-fluorescence. Sections were incubated with the primary antibody (mouse anti-proliferating cell nuclear antigen (PCNA) (Millipore, Temecula, CA) or mouse anti- α -smooth muscle actin (α -SMA) (Sigma, Saint Louis, MO) at 4 °C overnight. Next day the sections were incubated for 1 h at room temperature with the appropriate secondary antibody [anti-mouse IgG linked to Alexa Fluor 633 (Invitrogen, Eugene, OR)] and mounted with Prolong Gold anti-fade reagent containing 4',6-diamidino-2-phenylindole (DAPI) to counterstain nuclei with blue fluorescence (Invitrogen, Eugene, OR).

For double immunofluorescence, the protocol followed was as described above with the difference that the overnight incubation was with both primary antibodies [goat anti-CD34 (R and D, Minneapolis, MN) and mouse the anti-CD271 (Abcam, Cambridge, MA)] at the same time. Next day, the sections were incubated with the secondary antibodies [anti-mouse IgG linked to Alexa Fluor 633 and also anti-goat IgG linked to Alexa Fluor 488 (Invitrogen, Eugene, OR)] at the same time for 1 h at room temperature followed by mounting with Prolong Gold anti-fade reagent containing DAPI (Invitrogen, Eugene, OR).

For immune-phosphatase, the sections were treated with 75 mmol/L borate buffer (pH = 10.0) at 121 °C for 10 min for antigen retrieval. Sections were incubation with goat anti-collagen type IV (Southern Biotechnology Associates, Birmingham, AL) overnight, followed by incubation with corresponding alkaline phosphatase conjugated secondary antibody. The reaction was detected with alkaline phosphatase substrate (red) (Vector Laboratories Burlingham, CA) and finally mounted in 1:1 water: glycerol solution.

Negative controls consisted of sections in which the primary antibodies were omitted. All slides were examined and digitally photographed using a fluorescent/optical microscope (Nikon, New York, NY). Merge panels for immunofluorescence and double immunofluorescence were performed with Adobe Photoshop CS6 software (Adobe Systems, San Jose, CA).

RESULTS

Tissue patches obtained after implanting various inert materials in the subcutaneous space

When a large-sized polyvinyl tube implant was placed in the subcutaneous space and extracted after 4 d, the implant was completely encapsulated by a *de novo* tissue (Figure 1A). The *de novo* tissue could be conveniently stripped off the implant as shown in Figure 1B to obtain a flat piece of tissue patch. The patch could be tailored to the size and shape required. The size of the flat tissue patch depended on the size of the original inert material implanted, as long as the size and shape of the implant conformed to the available subcutaneous space.

A cylindrically shaped tissue patch could be obtained by implanting a fine polyvinyl tubing (Figure 2A) and removing the cylindrical tissue by slipping off the tissue from the implant like removing a “sock” (Figure 2B). This type of patch could be obtained in different lengths by varying the length of the implanted tube (not shown).

Injecting a slurry of poldextran gel particles in the subcutaneous space induced an unusual tissue patch in the form of a sealed bladder or pouch. While the day 4–7 bladder tissues were too delicate to handle, the day 14 tissue was strong enough to be obtained as an integral bladder (Figure 3A). The bladder-like tissue enclosed the injected gel particles. By piercing the balloon-like tissue the polydextran particles could be removed from the tissue to deflate the bladder (Figure 3B). The tissue could potentially be used in a variety of ways including as a cusp of a required curvature for repair of solid tissues or heart valves.

Gross appearance and histology of the de novo tissue surrounding the inert material

All the three types of tissue showed similar histology with regard to the inner and outer aspect of the tissue. The inner aspect of the tissue represented the surface

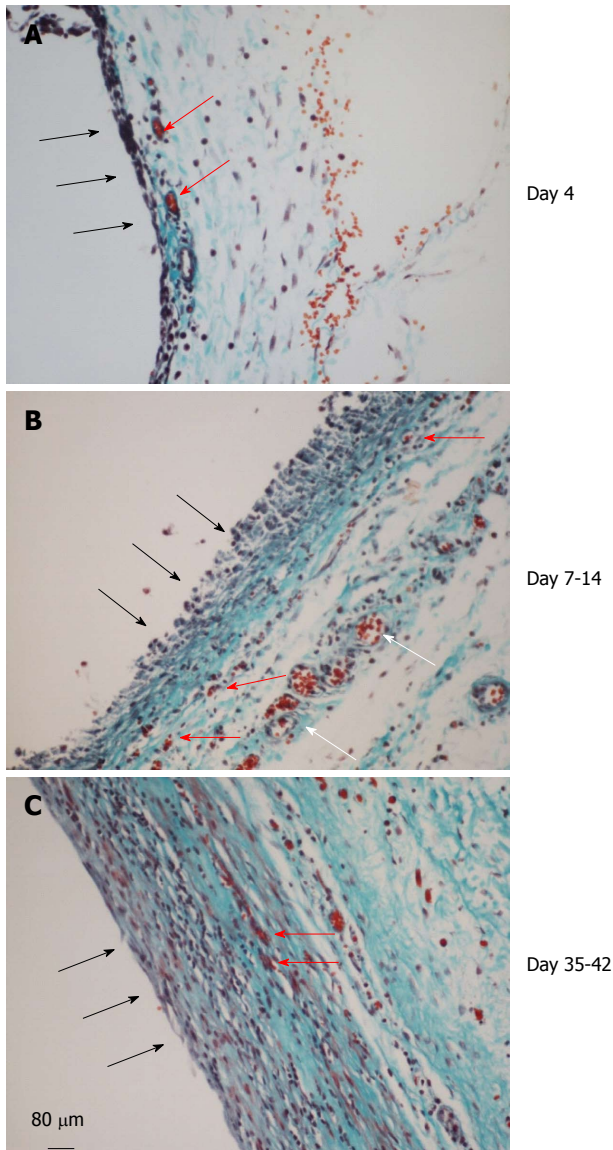


Figure 4 Histology of the *de novo* tissue patch at various times after implantation of the inert body in the subcutaneous space (trichrome stained). Black arrows show the inner aspect of the tissue in contact with the inert body. A: The tissue at day 4 showed an inner layer consisting of one or two-cell thick mononuclear cells but devoid of blood vessels. The medial layer was 4-6 cell thick and contained microvessels (red arrows) and fibrous matrix (blue stain). The outer layer consisted of loose connective tissue; B: At day 7-14 the tissue was histologically better organized into inner, medial and outer layers. The inner layer in contact with the inert material was thicker, consisted of mononuclear cells but devoid of extracellular matrix and blood vessels. The medial layer was thicker than at day 4, consisting of fibroblastic cells, rich network of microvessels (shown in Figure 5) and extracellular matrix. The outer layer remained loose but contained large blood vessels (white arrows); C: Between day 35-42 the granulation tissue appeared slightly thicker and more compact than day 7-14 tissue with the inner layer becoming less distinguished than at day 7-14 and merging with the medial layer. Also, this layer showed reduced number of blood vessels and increased amounts of extracellular matrix arranged in parallel sheets. The outer layer remained unchanged.

in contact with the inert material and the outer aspect represented the surface away from the inert material and in contact with the surrounding connective tissue. We have chosen to describe the histology of the tissue obtained from the large-sized polyvinyl tube as

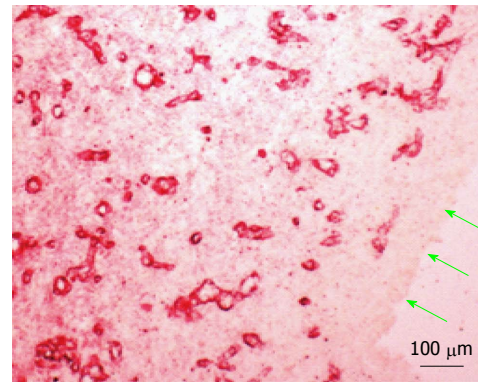


Figure 5 Vascularity of the medial layer of the subcutaneous tissue patch 14 d after implantation of the inert body. Microvessels were revealed by immune-staining for collagen type IV, shown earlier to stain newly formed blood vessels^[15]. Green arrows point to the inside aspect of the tissue patch in contact with the inert body. Note the absence of blood vessels in the inner layer.

representative of all others.

Implanting any of the three types of inert material in the subcutaneous tissue induced a *de novo* tissue which completely surrounded the foreign body as a capsule by day 4. The tissue at day 4 grossly appeared thin and filmy but well vascularized (Figure 4A). The inner layer consisted of one or two-cell thick mononuclear cells (20-40 μm) but devoid of blood vessels. The medial layer was 4-6 cell thick (100-150 μm) and contained microvessels and fibrous matrix. The outer layer (300-400 μm) consisted of loose connective tissue.

At day 7-14 the *de novo* tissue appeared thicker and stronger than at day 4 and histologically better organized into inner, medial and outer layers (Figure 4B). The inner layer in contact with the inert material was approx. 100 μm thick, consisted of mononuclear cells but devoid of extracellular matrix and blood vessels. The medial layer was thicker (200-300 μm), consisting of fibroblastic cells, rich network of microvessels and extracellular matrix. The outer layer remained loose but contained large blood vessels. The high vascularity of the medial layer at this time point is shown in Figure 5 by immune-staining sections for collagen type IV, shown in our previous study to reveal newly formed blood vessels^[15].

Between day 35-42 the granulation tissue appeared slightly thicker than day 7-14 tissue but was tougher and more fibrous to touch. Histologically this tissue appeared more compact with the inner layer becoming less distinguished than at day 7-14 and merging with the medial layer. Also, this layer showed reduced number of blood vessels and increased amounts of extracellular matrix arranged in parallel sheets. The outer layer remained unchanged (Figure 4C).

Proliferating layer and myofibroblasts in the *de novo* tissue patch

Because the tissue patch was found to be a dynamic tissue between day 4-42 after implantation of the inert body, it was important to determine the growing edge

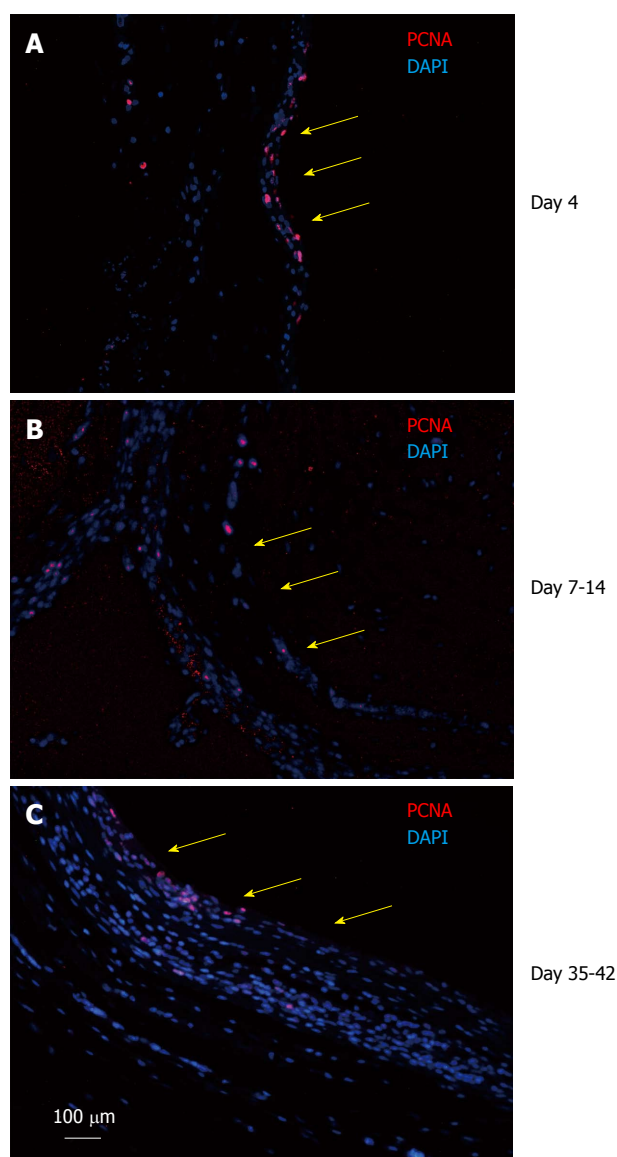


Figure 6 Tissue patch at various times immune-stained for proliferating cell nuclear antigen to determine the proliferating edge of the tissue. We found that the patch was a continually growing tissue with the inner aspect (side in contact with the inert body shown by yellow arrows) and the medial layer staining positive for PCNA (red) at all times tested (A: Day 4; B: Day 7-14; C: Day 35-42), suggesting that the patch was growing from inside to outside. Sections were counterstained with DAPI to counterstain nuclei blue. PCNA: Proliferating cell nuclear antigen; DAPI: 4',6-diamidino-2-phenylindole.

of the tissue. The tissues were immune-stained for PCNA to identify the proliferating cells in the patch. We found that the patch was a continually growing tissue with the inner aspect (side in contact with the inert body) and the medial layer staining positive for PCNA at all times tested (Figure 6), suggesting that the patch was growing from inside to outside.

The tissues were immune-stained for α -SMA, a marker of myofibroblasts, to identify the source and organization of the fibrous material identified in the medial layer of the tissue patch. At day 4-7, α -SMA was found to be associated with blood vessels as expected, but little extracellular α -SMA was observed at these

times (Figure 7). At later time points there was more and more of α -SMA observed in the extracellular areas (not shown), which by day 35-42 had stratified and compacted itself in parallel to the length of the implants (Figure 7), consistent with the tissue being tougher and fibrous to touch.

Location of MSCs in the *de novo* tissue

It has been recently recognized that CD34+CD271+ cells mark the presence of MSCs in a tissue^[16-20]. Tissue patches from days 4-42 were immune-stained for CD34 and CD271 to identify the location of MSCs. We found that MSCs were clearly identifiable in the stroma of the medial layer of the tissue patch at day 4 after the implantation of the inert body but rarely seen at day 7 or after (Figure 8). Also, MSCs were found to be concentrated in the vicinity of major blood vessels in the medial layer at day 4 but never at day 7 or after (Figure 9).

DISCUSSION

We demonstrate that the subcutaneous space is an effective and convenient compartment to create an autologous tissue patch of desired shape and size for the purpose of repairing organs or tissues. By implanting inert material of different shapes and sizes we obtained newly regenerated tissues of corresponding shapes and sizes containing abundant blood vessels, multitude of other functional cells, and importantly, also MSCs in a matter of 4 d. These early-harvested tissues, which were highly vascular and abundant in MSCs, could potentially be used for tissue repair. When the *de novo* tissues were allowed to grow for longer times the tissues obtained were less vascular but thicker and tougher. Such tissues could be applied for structural support or as a filler tissue for cosmetic purposes.

There are several advantages of using subcutaneous tissue patches as described in this report compared to patches made of synthetic materials and seeded with cultured cells^[1-7]. First and foremost is the autologous nature of the subcutaneous patch which can be created in the patient's own body. Clearly, such patches will be safer because they will be better tolerated immunologically in the patient body than patches made from xenogenic or synthetic materials^[21]. Second, the subcutaneous patches will have a ready-made vascular system for the perfusion of blood through the entire body of the patch. Man-made patches have the problem of core degeneration because of lack of vascularity which results in patch failure and the possibility of adverse reactions because of necrotic foci in the patch. Thirdly, these *in vivo* created patches contain other important accessory cells including interstitial cells which create matrix to provide body and integrity to the patch, and pericytes which provide support to the developing blood vessels during their integration with the host blood vessels.

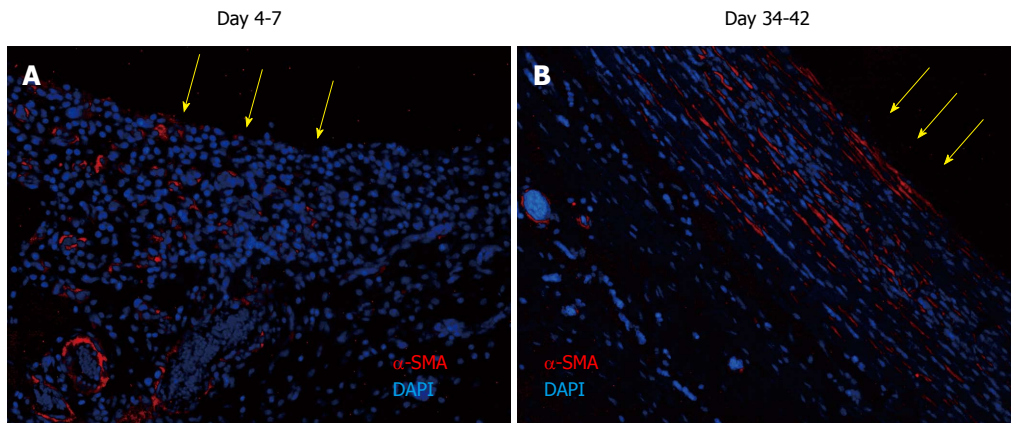


Figure 7 Tissue patch at various times immune-stained for α -smooth muscle antigen to determine the organization of the extracellular matrix in the patch. At day 4-7, α -SMA was found to be associated with blood vessels as expected, but little extracellular α -SMA was observed at these times. At later time points there was more and more of α -SMA observed in the extracellular areas (not shown), which by day 35-42 had stratified and compacted itself in parallel to the length of the implant, consistent with the tissue being tougher and fibrous to touch. Sections were counterstained with DAPI to counterstain nuclei blue. Side in contact with the inert body is shown by yellow arrows. α -SMA: α -smooth muscle antigen; DAPI: 4',6-diamidino-2-phenylindole.

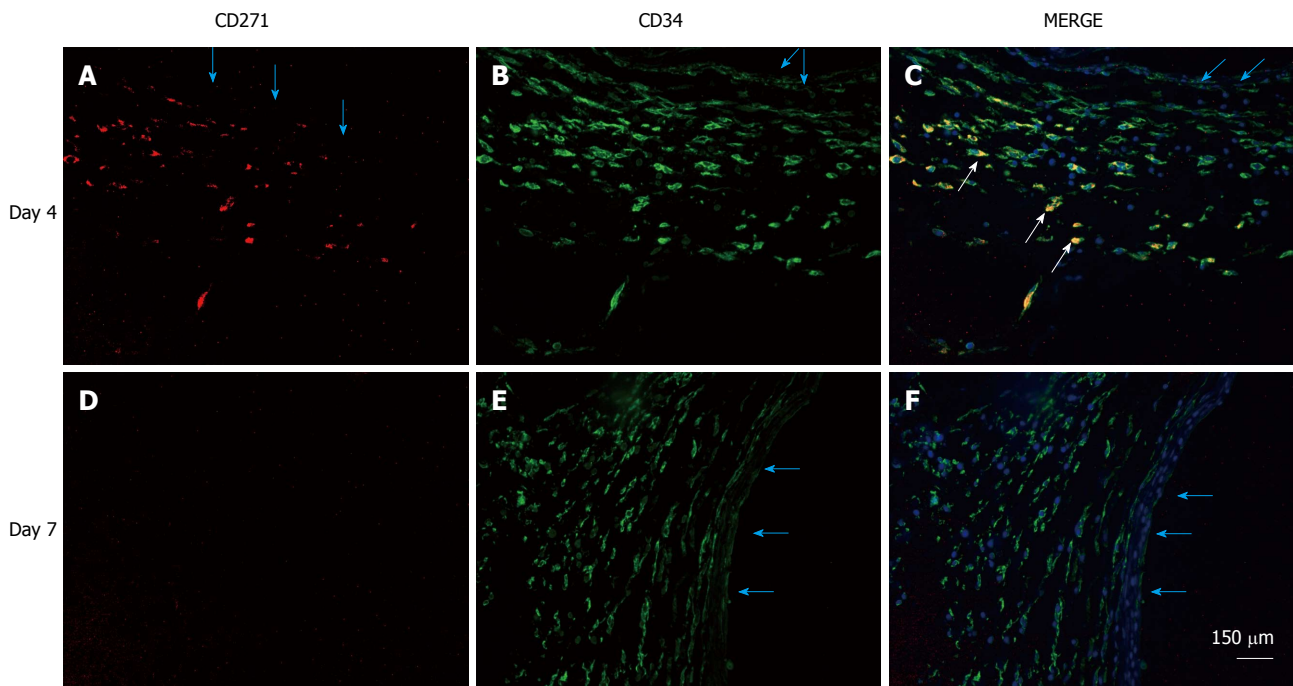


Figure 8 Localization of mesenchymal stem cells in the *de novo* tissue patch induced in the subcutaneous space by an inert body. CD271 cells were immune-stained by a red fluorescent probe, CD34 cells by a green fluorescent probe and all nuclei (counterstained in the merge picture only) by DAPI. MSCs identified as CD271+CD34+ were clearly identified in the medial layer of the tissue patch at day 4 after the implantation of the inert body (white arrows in the merged picture), but rarely seen at day 7 or after. Light blue arrows indicate the inner aspect of the tissue patch. MSCs: Mesenchymal stem cells; DAPI: 4',6-diamidino-2-phenylindole.

The efficacy of autologously created *in vivo* patches in repairing functional organs is highlighted by the omentum which, either as an attached (with vascular connection intact) or detached tissue (severed vascular connection), has proven to be successful in repairing ischemic heart, spinal cord, and bony fractures^[22]. More recently, we showed that the omentum is capable of being activated to a state in which its content of MSCs and blood vessels become several fold higher^[23,24]. In this state it could be used for regenerating endocrine damaged pancreas, liver and kidney^[25-27]. Notwithstan-

ding the utility of the activated omentum in regenerating tissues, its use has been greatly restricted by the difficulty of obtaining the tissue from the abdominal cavity which necessitates laparotomy, a risky surgical procedure. A similar patch created in the subcutaneous tissue, as described here, is clearly less risky to the patient.

The presence of an intact and functional vascular system in a tissue implant is clearly a factor which favors its success *in vivo* with respect to survival and integration with the host tissue. Consistent with this

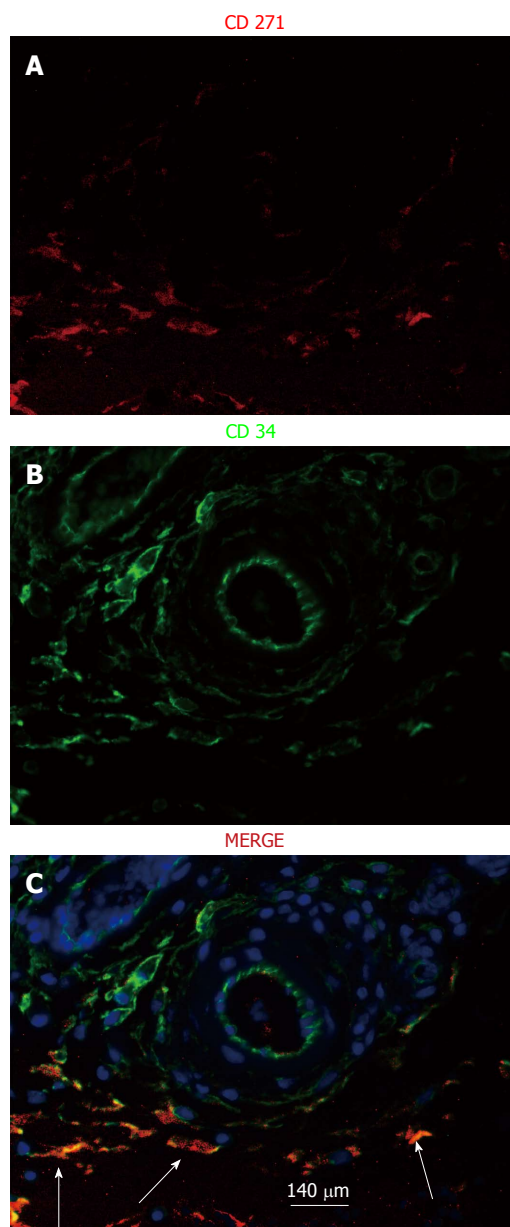


Figure 9 Localization of mesenchymal stem cells in the vicinity of major vessels at day 4 after implantation of an inert body in the subcutaneous space. MSCs, identified as CD34+CD271+ cells (white arrows in the merge picture), were found to be concentrated in the vicinity of major blood vessels in the medial layer at day 4 but never at day 7 or after (not shown). The merge section was counterstained with DAPI to counterstain nuclei blue. MSCs: Mesenchymal stem cells; DAPI: 4',6-diamidino-2-phenylindole.

premise, pre-vascularized man-made tissue patches for later implantation have proven to be longer-lasting than un-prevascularized patches^[28,29]. Because of the preformed capillary network in such constructs, whether created *in vitro* or *in vivo*, they were able to anastomose with the host vasculature and thereby perfuse the construct promptly after *in vivo* implantation^[12,28,30,31]. The strategies used for prevascularization of synthetic scaffolds involve the sandwiching of pro-endothelial cells and myoblasts on the scaffolds which are subsequently placed in tissue culture, resulting in capillary network formation^[32]. Another alternative is to pre-implant the

construct on the omentum or in the renal pouch^[29,30], a tissue known to induce angiogenesis in tissue grafts, resulting in a rich network of capillaries penetrating the construct. However, there are drawbacks to these approaches which need to be overcome for better outcomes: (1) the technology to create such capillary network *in vitro* is labor intensive, expensive, and difficult in terms of controlling quality; (2) pre-implanting the construct on the omentum (or renal pouch) for pre-vascularization, is unsafe due to the risk of infections by the surgical procedures required, as mentioned above; and (3) such patches have yet the potential problem of safety because of the use of allogeneic cells and materials for their production. In light of the above considerations a tissue patch created in the subcutaneous tissue overcomes many of the problems inherent in man-made patches.

The finding of MSCs in the subcutaneous patch we describe herein is not surprising considering that the patch is a newly regenerated tissue and must involve such cells to create an organized tissue. Earlier we had shown that the subcutaneous patch also contained cells which expressed CXC chemokine receptor 4 (CXCR4), the receptor for stromal cell derived factor-1 (SDF-1), a protein which is released by injured tissues^[15]. The presence of CXCR4 has been considered to be a stem cell marker based on the rationale that an injured tissue releasing SDF-1 would attract the CXCR4 bearing bone marrow cells for repair. One outstanding problem in the field of MSCs is the lack of validated markers to identify them, especially in an intact tissue. While many cell markers in blood cells have been described as representing MSCs there is a dearth of knowledge on such markers to identify MSCs in a solid tissue. We therefore believe there may be many other cell-specific stem cells in addition to the CD34+CD271+ we found in this tissue.

In summary, we demonstrate here that it is convenient to create a tissue patch of preferred shape and size in the subcutaneous space. A patch created in the subcutaneous tissue was highly vascularized, and contained MSCs and other accessory cells, which should endow it with the ability to successfully graft to an injured tissue and augment its healing.

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COMMENTS

Background

Despite much progress in the development of engineered tissue patches for organ repair these constructs often fail from lack of adequate blood supply. A

natural autologous tissue patch containing a functional vasculature and stem cells would overcome such problems. The authors created such autologous tissue patches of different geometries by implanting three types of inert foreign bodies in the subcutaneous tissue of rats.

Research frontiers

There is an unmet need to develop biocompatible tissue patches for repair of various diseased organs such as ischemic heart, large open wounds, vascular defects, damaged nerves and many others. Much progress has been made in creating engineered tissue patches using polymer scaffolds or processed xenogeneic biomaterials seeded with functional cells, endothelial cells, paracrine growth factors, and fetal or adult stem cells. Despite such advances the constructs have proven to be sub-optimal in performance. There is the problem of core degradation of the construct and the lack of integration of the seeded functional cells with the host tissue cells, probably arising from lack of adequate blood supply in the construct. A natural autologous tissue patch containing stem cells, other supporting cells, and more importantly, a functional vasculature, would be a good solution to the problem.

Innovations and breakthroughs

The authors demonstrate in this manuscript that it is possible to create an autologous tissue patch of preferred shape and size in the subcutaneous space. A patch created in the subcutaneous tissue is highly vascularized, contains mesenchymal stem cells (MSCs), and other accessory cells, which should endow it with the ability to successfully graft to an injured tissue and augment its healing.

Applications

Availability of an autologous tissue patch containing MSCs, as described herein, will open up the possibility of repairing diseased or injured organs of patients in a safe and effective manner.

Terminology

Although creating man-made patches for tissue repair have been described, they contain synthetic materials and cells grown outside the body, which make such patches bio-incompatible and unsafe for implanting in patients. An autologous tissue patch on the other hand is one which is created in the patient itself making them completely bio-compatible and safer to use in the patient. A synthetic or natural tissue patch implanted in the patient's body must be sustained by blood. If the patch lacks built-in blood vessels the patch will quickly die and fail. On the other hand if the patch already has blood vessels in it (functional vasculature) it has a better chance of survival and functionality.

Peer-review

Authors reported their successful challenge to create a vascularized, ready-to-use patch by an extremely feasible method of subcutaneous implantation of polyvinyl tubes. Their technique will contribute to therapeutic development for the treatment of acute tissue injuries. The only one regrettable point is that they did not present a case where the implanted patch eventually improved the repair of injured tissues. It is best for the authors to prove the efficacy of their patches in their future works.

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Basic Study

Easily-handled method to isolate mesenchymal stem cells from coagulated human bone marrow samples

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Abstract

AIM: To establish an easily-handled method to isolate mesenchymal stem cells (MSCs) from coagulated human bone marrow samples.

METHODS: Thrombin was added to aliquots of seven heparinized human bone marrow samples to mimic marrow coagulation. The clots were untreated, treated with urokinase or mechanically cut into pieces before culture for MSCs. The un-coagulated samples and the clots were also stored at 4 °C for 8 or 16 h before the treatment. The numbers of colony-forming unit-fibroblast (CFU-F) in the different samples were determined. The adherent cells from different groups were passaged and their surface profile was analyzed with flow cytometry. Their capacities of *in vitro* osteogenesis and adipogenesis were observed after the cells were exposed to specific inductive agents.

RESULTS: The average CFU-F number of urokinase-treated samples ($16.85 \pm 11.77/10^6$) was comparable to that of un-coagulated control samples ($20.22 \pm 10.65/10^6$, $P = 0.293$), which was significantly higher than those of mechanically-cut clots ($6.5 \pm 5.32/10^6$, $P < 0.01$) and untreated clots ($1.95 \pm 1.86/10^6$, $P < 0.01$). The CFU-F numbers decreased after samples were stored, but those of control and urokinase-treated clots remained higher than the other two groups. Consistently, the numbers of the attached cells at passage 0 were higher in control and urokinase-treated clots than those of mechanically-cut clots and untreated clots.

The attached cells were fibroblast-like in morphology and homogeneously positive for CD44, CD73 and CD90, and negative for CD31 and CD45. Also, they could be induced to differentiate into osteoblasts and adipocytes *in vitro*.

CONCLUSION: Urokinase pretreatment is an optimal strategy to isolate MSCs from human bone marrow samples that are poorly aspirated and clotted.

Key words: Coagulated marrow sample; Mesenchymal stem cells; Isolation; Urokinase

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Core tip: It is usually difficult to isolate mesenchymal stem cells (MSCs) from bone marrow samples that have been coagulated due to inappropriate marrow aspirations or in the setting of pathologically hypercoagulable state. In this study, the authors found that urokinase pretreatment could dissolve stem cells from the clots. The MSCs were fibroblast-like in morphology and homogeneously positive for CD44, CD73 and CD90, and negative for CD31 and CD45. Also, they could be induced to differentiate into osteoblasts and adipocytes *in vitro*. Urokinase pretreatment is an optimal strategy to isolate MSCs from poorly aspirated and clotted human bone marrow samples.

Wang HX, Li ZY, Guo ZK, Guo ZK. Easily-handled method to isolate mesenchymal stem cells from coagulated human bone marrow samples. *World J Stem Cells* 2015; 7(8): 1137-1144 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i8/1137.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i8.1137>

INTRODUCTION

Mesenchymal stromal cells (MSCs) are a kind of adult stem cells characterized by their hematopoiesis-supporting and immune regulatory activities, and have been clinically trialed in the treatment of various kinds of diseases including graft-versus-host disease, musculoskeletal defects, ischemic disorders, and neurological diseases^[1-3]. Though MSCs are low in immunogenicity and allogeneic transplantation has been widely used, autologous MSCs still remain an ideal option in some cases, such as in the repair of bone and cartilage defects. And in these settings, bone marrow is usually aspirated from the patients though adipose tissue might be another choice for MSC isolation^[4]. In some cases, however, bone marrow samples are poorly aspirated and clotted promptly after the procedure, especially when the patients are in a hypercoagulable state^[5-7]. This usually leads to the decrease of MSC output or even failure to culture-expand MSCs. To solve this practical problem, we have developed an easily-handled method to isolate MSCs from human marrow

clots.

MATERIALS AND METHODS

Marrow sample collection and treatment

Heparinized bone marrow samples were aspirated from seven healthy adult donors after informed consent in accordance to the Ethical Guidelines for Research Involving Human Subjects or Human Tissue from General Hospital of Air Force. Nucleated cells were counted by trypan blue exclusion assay and the samples were diluted with PBS to a concentration of 2×10^6 or 2×10^7 /mL. The samples were then treated as depicted in Figure 1. Briefly, one sample was divided into 12 aliquots containing 2×10^6 nucleated cells and 12 aliquots containing 2×10^7 nucleated cells. To mimic coagulation, eight aliquots of each group were treated with thrombin (Sigma) at a final concentration of 1 U/mL and maintained at 37 °C for 1 h. The thrombin-treated and untreated samples were stored at 4 °C for 0 (subgroup 0 h), 8 (subgroup 8 h) and 16 h (subgroup 16 h) for further treatment. Each subgroup contained 3 thrombin-treated samples and one un-coagulated sample. The three clotted samples were either untreated, mechanically minced into pieces or treated with urokinase. For urokinase treatment, urokinase (Urokinase for injection, Lizon Pharmaceutical Factory, Zhuhai, Guangdong) was added into the samples at a final concentration of 50000 U/mL and maintained at 37 °C with a horizontal rotation of 120 rpm for 20 min. All the samples were further treated with NH₄Cl buffer to lyse the contaminated red blood cells, washed twice in PBS, and suspended in alpha-MEM containing 10% fetal bovine serum from selected lots (Stemcell Co. Canada)^[8]. The cells were used in the experiments described below.

Colony-forming unit-fibroblast assay

The samples which contained 2×10^6 nucleated cells each and stored at different times were separately seeded into culture dishes with a diameter of 100 mm. The culture was maintained for 12 d. The medium was then removed, and the cells were washed twice with PBS. Wright-Giemsa staining was performed to reveal the colonies that were more than 0.5 mm in diameter. The number of colonies was calculated with the software of Quantity One 4.4 as described previously^[9].

Cell passage culture

When the confluence of the attached cells cultured from un-clotted samples reached to around 80%, the cells from all groups were harvested by trypsin digestion, counted and re-seeded into culture dishes at a density around 6000 cells/cm². When the cell attachment from the control samples reached around 80%, the cells from all the counterpart groups were detached, the cell numbers were counted and the cells were plated for passage culture. The cells at passage 2 were also collected for phenotype analysis and *in vitro*

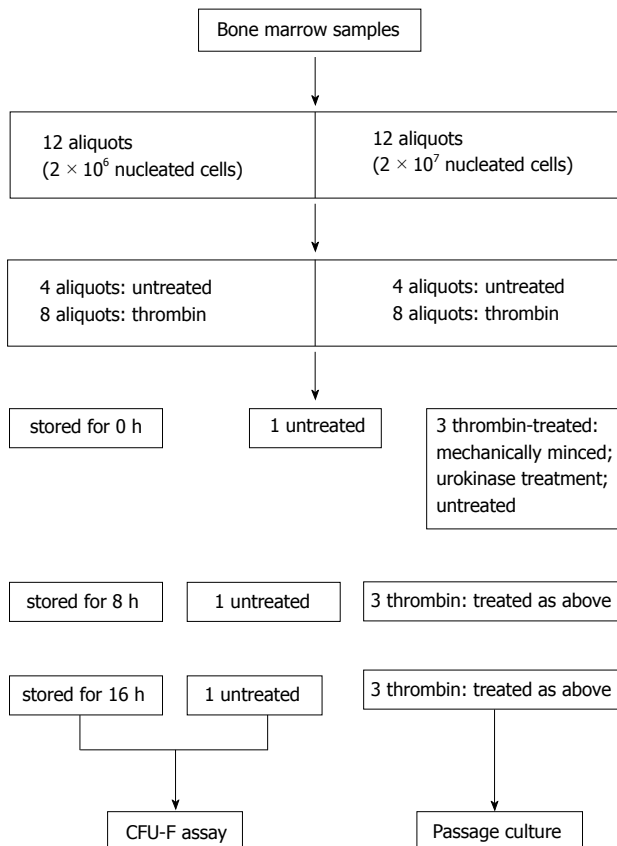


Figure 1 Study design. Bone marrow samples from 7 healthy volunteers were aliquoted and treated by different protocols. The cells were plated for passage culture or CFU-F assay. CFU-F: Colony-forming unit-fibroblast.

differentiation assay as reported previously^[9,10]. Colony-forming unit-fibroblast (CFU-F) was stained with Giemsa solution and counted as routinely described^[9].

Flow cytometry

The attached cells from passage culture were detached by trypsin digestion, washed in PBS and reacted with PE-conjugated monoclonal antibodies (BD, United States) against human CD31 (endothelial cell marker), CD45 (hematopoietic cell marker), CD73 (positive on mesenchymal cell lineage) and CD90 (stem cell marker) for 30 min in dark at room temperature. The cells were washed twice in PBS and at least 10000 events were collected with FACSCalibur (BD, United States). The data were analyzed with WinMdi 2.9 software after the targeted events were gated according to the cell size and granularity.

Differentiation assays

For *in vitro* osteogenic differentiation, the attached cells for passage culture were digested with 0.05% trypsin, washed in PBS, and seeded into 24-well plates at a density of 5000 cells per well. The cells were allowed to attach overnight and then exposed to 0.1 $\mu\text{mol/L}$ dexamethasone, 50 $\mu\text{mol/L}$ ascorbic acid 2-phosphate, and 10 mmol/L β -glycerophosphate as previously described with a little modification^[10,11]. After 12 d,

cellular activity of alkaline phosphatase (ALP) was revealed with ALP immunohistochemistry kit (Sigma, United States) following the manufacture's protocol.

For adipogenic differentiation, the cells were seeded a 24-well plate at a density of 10000 cells per well. Inductive agents containing 1 $\mu\text{mol/L}$ dexamethasone, 0.5 mmol/L isobutylmethylxanthine (IBMX, Sigma, United States), and 100 $\mu\text{mol/L}$ indomethacin (IDM, Sigma, United States) were supplemented. The culture was maintained for 7 d and intracellular lipid droplets were revealed by Oil-Red staining^[9-11].

Statistical analysis

The data were expressed as mean values incorporating the standard deviation. Statistical significance was analyzed by Student's *t* test. A *P*-value less than 0.05 was considered significant. The statistical analysis has been reviewed by Dr. Qing-Yi Zhang from Beijing Institute of Basic Medicine.

RESULTS

Urokinase treatment could release MSCs from clotted marrow samples

To establish a ready-use method to isolate MSCs from clots of human bone marrow aspirates, heparinized marrow samples were aliquoted according to the nucleated cell numbers and treated with thrombin to coagulate the samples. Then, the clots were treated with urokinase digestion, mechanically minced or untreated. The cells were plated to allow MSCs to attach and expand. The numbers of CFU-F and the attached cells were counted separately.

As expected, bone marrow coagulation did affect the output of MSCs. The numbers of CFU-F were greatly decreased from clotted samples compared with those from un-clotted samples (Figure 2A). However, the average CFU-F number of urokinase-treated samples ($16.85 \pm 11.77/10^6$) was comparable to that of un-coagulated control samples ($20.22 \pm 10.65/10^6$, $P = 0.293$), which was significantly higher than those of mechanically-cut clots ($6.5 \pm 5.32/10^6$, $P < 0.01$) and untreated clots ($1.95 \pm 1.86/10^6$, $P < 0.01$). Statistical analysis showed that the CFU-F numbers decreased after samples were stored for 8 h, and those were greatly decreased after the samples were stored for further 8 h compared with those from the same groups. However, the CUF-F numbers of control and urokinase-treated clots from stored samples were higher than those in the stored samples of the other two groups (Figure 2A).

Consistent with the results above, as shown in Figure 2B, the numbers of the attached cells at passage 0 were higher in control (average: $38.86 \pm 17.4 \times 10^4$, $n = 7$) and urokinase-treated clots ($38.57 \pm 16.78 \times 10^4$, $n = 7$) than those of mechanically-cut clots ($18.57 \pm 13.69 \times 10^4$, $n = 7$, $P = 0.016$ compared with control) and untreated clots ($13.14 \pm 8.39 \times 10^4$, $n =$

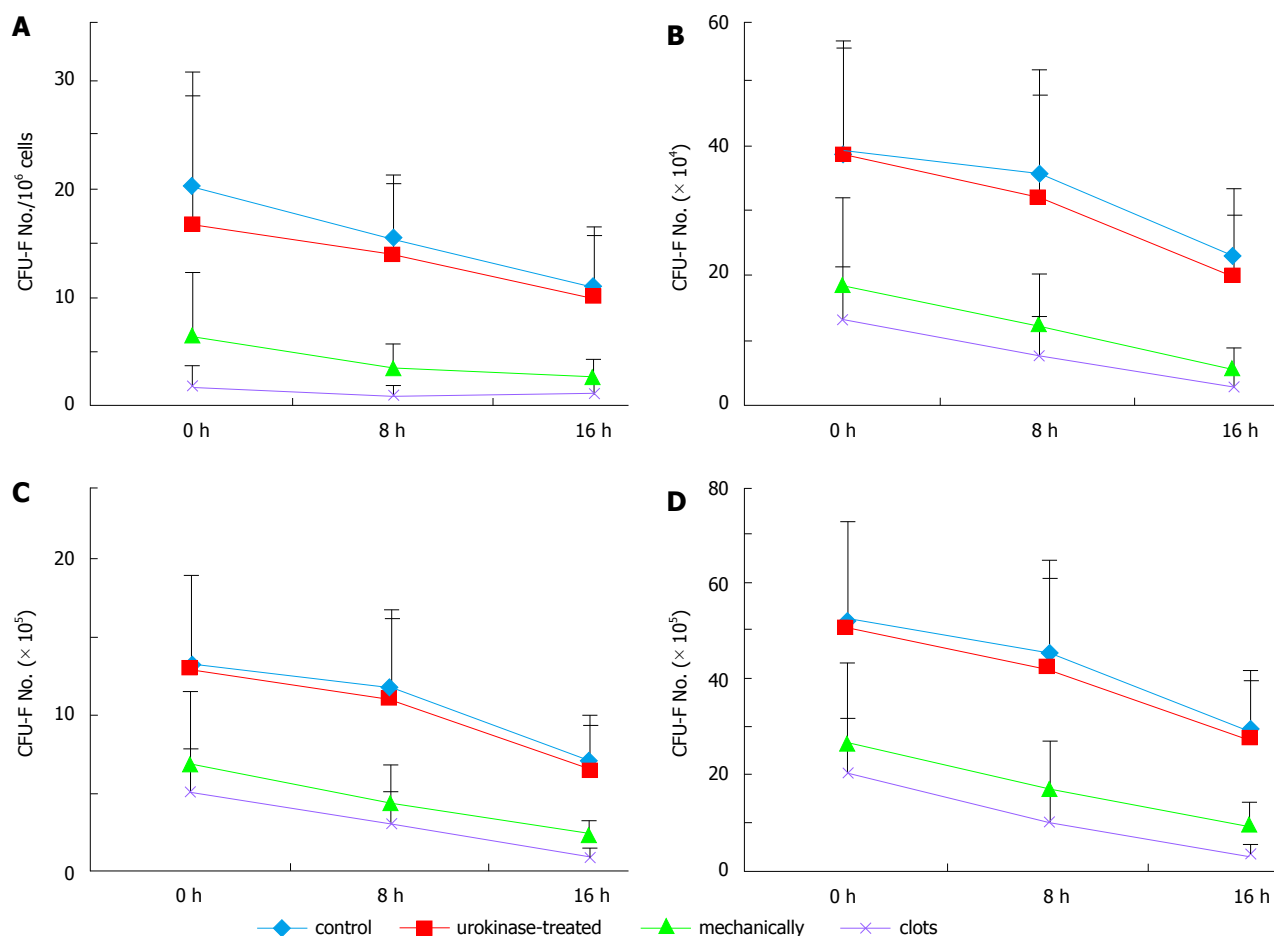


Figure 2 Colony-forming unit-fibroblast and mesenchymal stromal cell numbers of different groups. A: CFU-F numbers per 10⁶ nucleated cells from seven bone marrow samples; B-D: Bone marrow samples were diluted and split into 12 aliquots containing 10⁷ nucleated cells. The attached cells at passage 0 (B: × 10⁴), passage 1 (C: × 10⁵) and passage 2 (D: × 10⁵) were counted and summed up. The average and standard errors are shown. CFU-F: Colony-forming unit-fibroblast.

7, $P = 0.002$ compared with control). These differences were also observed when cell counting was performed at passage 1 and 2 (Figure 2C and D). The results further confirm the advantages of urokinase treatment on bone marrow coagulated clots.

The attached cells were MSCs *per se*

Marrow samples were treated by different protocols and the nucleated cells were cultured in media traditionally for MSC expansion after the contaminated red cells were lysed in NH₄Cl. After 72 h, cells attached to the plastic were observed and colonies were evident after 12 d of culture. As shown in Figure 3, the attached cells appeared in all of the four groups and were fibroblast-like in morphology, suggestive of the features of the cells as MSCs.

To further confirm that the attached cells from differently treated bone marrow samples were MSCs *per se*, some of the surface markers on several kinds of fibroblast-like cells were analyzed with flow cytometry. The results showed that the cells from the four groups were homogeneously negative for CD31 and CD45, proving that the cells were devoid of endothelial and hematopoietic cells, two cell types that are commonly observed when bone marrow cells are cultured (Figure

4). Meanwhile, the cells were positive for CD73, a mesenchymal marker^[12], and for CD90, a pan-marker for stem cells including MSCs^[13].

Furthermore, when the attached cells were exposed to specific inductive conditions, they became ALP-positive and intracellular lipid droplets were observed (Figure 5). The results prove that the cells had the capacity to differentiate into osteoblasts and adipocytes. The fibroblast-like in morphology, the expression of unique surface markers and the differentiation capacity support the conclusion that the attached cells were MSCs *per se*^[14].

DISCUSSION

Bone marrow sample coagulation is a common phenomenon in clinical practice and, it is indeed a problematic issue to isolate MSCs from the samples. To overcome this problem and establish an easily handled method, urokinase was used in this study to dissolve the clots and subsequently, the contaminant red blood cells in the pre-treated bone marrow samples were lysed with ammonium chloride, and the nucleated cells were seeded. The procedures described here seem practical in isolating MSCs from the bone marrow clots

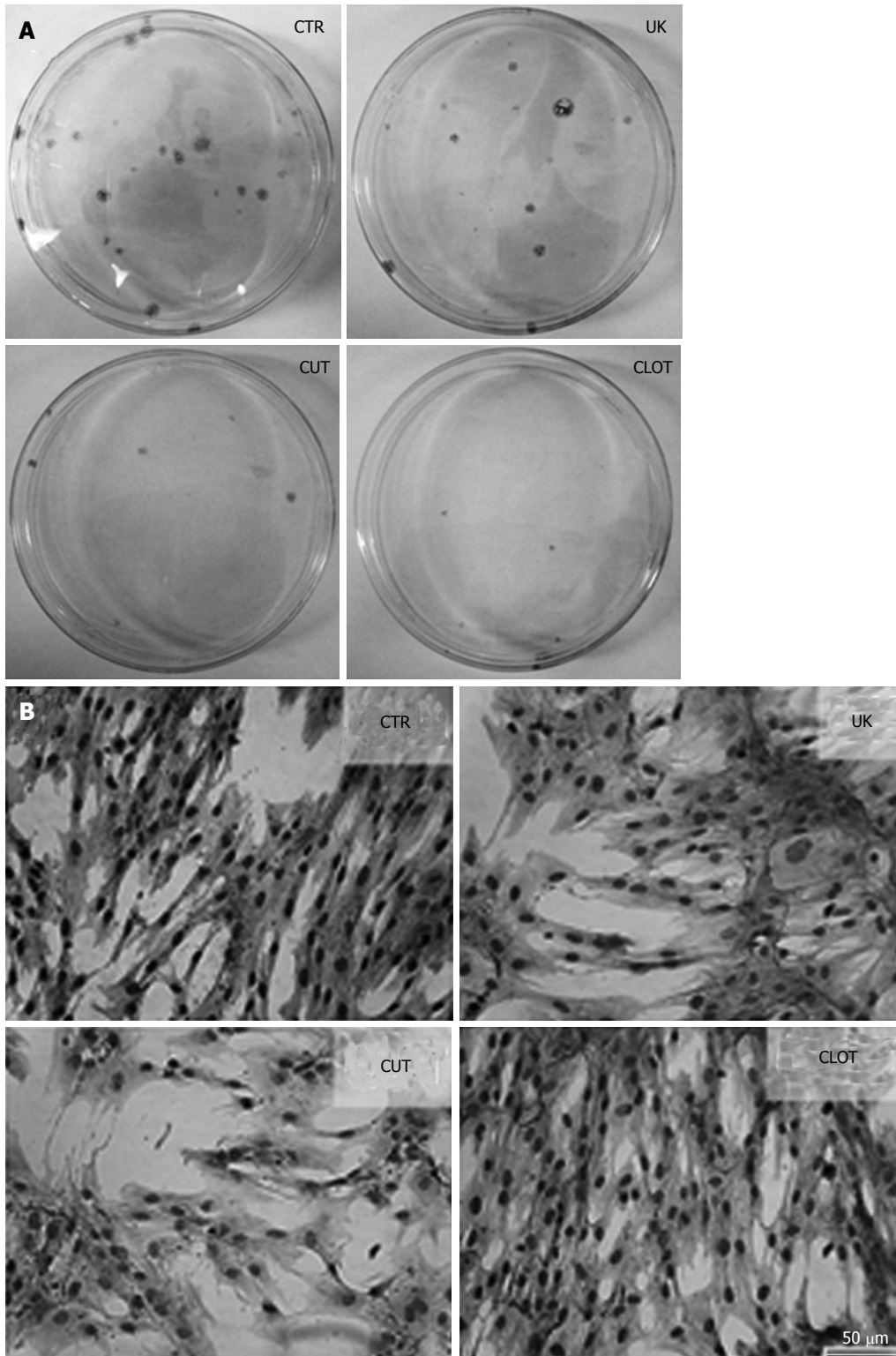


Figure 3 Representative morphological features of the colonies and the attached cells. Bone marrow samples were treated by different protocols and the cells were maintained for 12 d. Colonies of fibroblast-like cells were stained with Wright-Gimsa solution and observed under a microscope with a lower (A) and a higher magnification field (B). CTR: Un-coagulated control; UK: Urokinase-treated clots; CUT: Mechanically-cut clots; CLOT: Untreated clots.

as the treated cells could form CFU-F and the attached cells had the capacity to differentiate osteoblasts and adipoblasts.

CFU-F is a well-recognized surrogate for MSCs^[15-18]. The numbers of CFU-F in urokinase-treated clots were

comparable to those in uncoagulated controls at three time points, suggesting that urokinase treatment could optimally release MSCs in the clots. Urokinase is not only able to dissolve the coagulated clots but also could migrate MSCs from the potentially existed undissolved

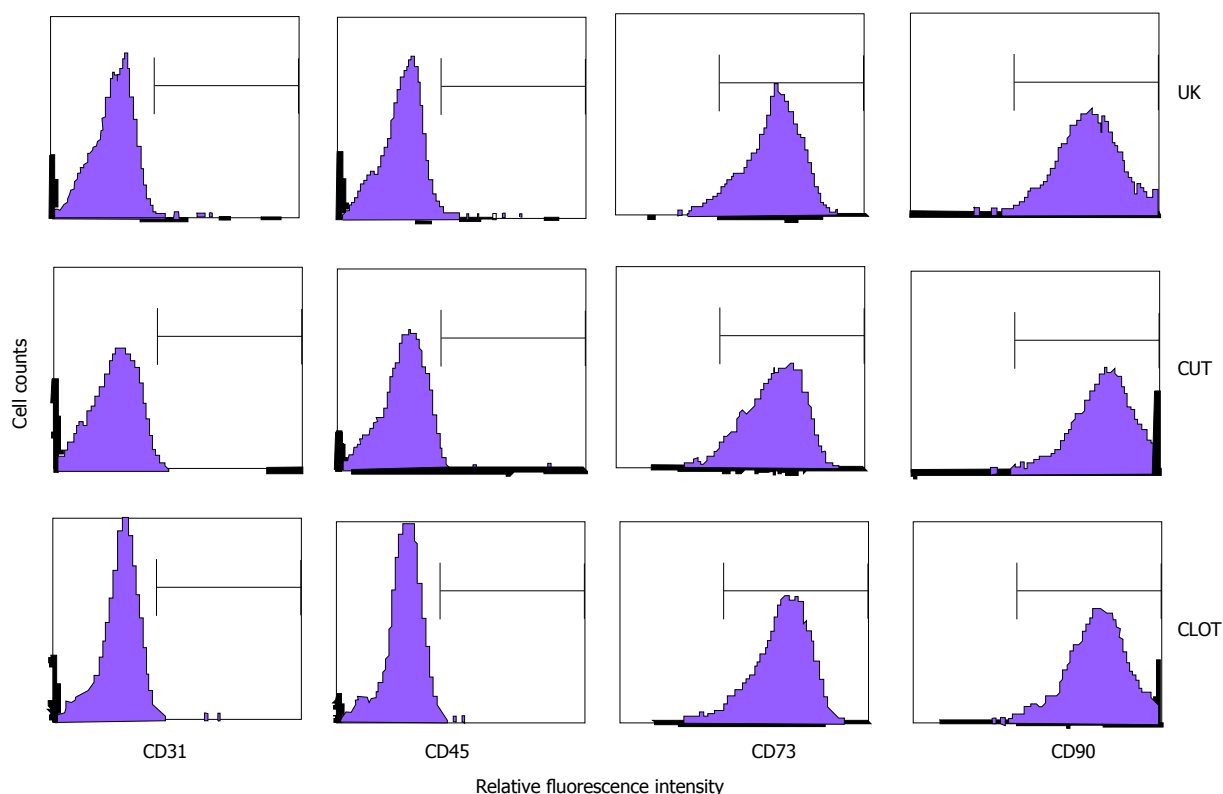


Figure 4 Surface marker analysis of the attached cells by flow cytometry. The attached cells at passage 2 were collected for flow cytometric analysis. The antigenic markers are indicated. UK: Urokinase-treated clots; CUT: Mechanically-cut clots; CLOT: Untreated clots. The results are representative of 7 individual experiments.

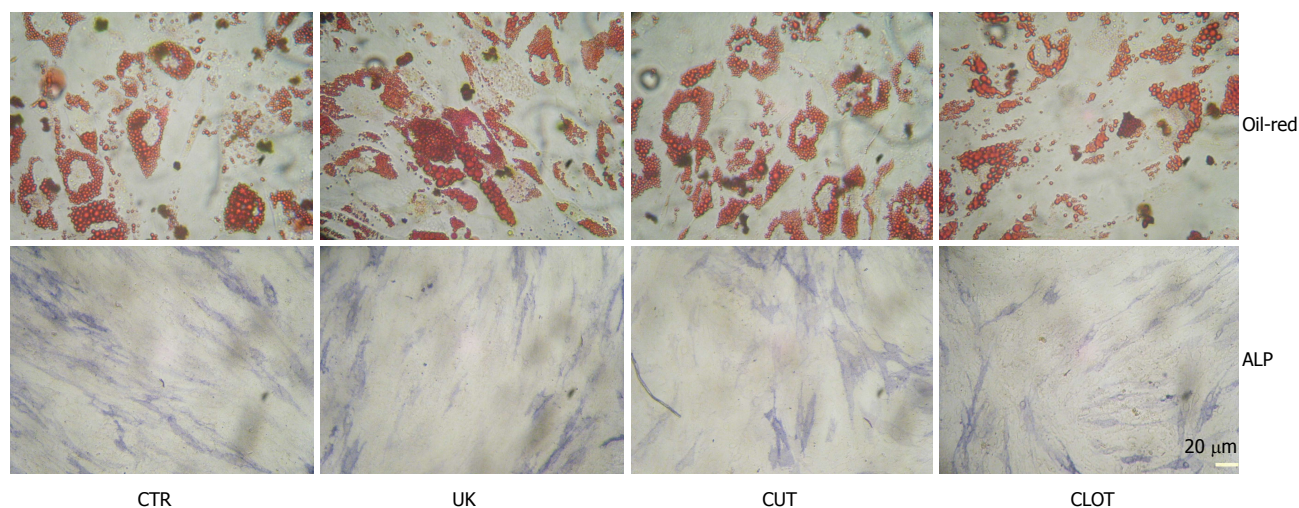


Figure 5 Identification of *in vitro* osteogenesis and adipogenesis. The attached cells at passage 2 were exposed to specific inductive conditions and stained with oil-red (upper panel) or ALP solutions. The results are representative of 7 individual experiments. ALP: Alkaline phosphatase. CTR: Un-coagulated control; UK: Urokinase-treated clots; CUT: Mechanically-cut clots; CLOT: Untreated clots.

small ones^[19], resulting in the “liberation” of MSCs from the dense fibrin arrangement. Further, it is interesting that the numbers of CFU-F decreased sharply when the samples, pre-coagulated or uncoagulated, were stored at 4 °C for 8 or 16 h. It has been proven that prolonged storage time does decrease the viable cell numbers in bone marrow aspirates^[20], which is also observed in this study. Meanwhile, the temperature is also an important issue for preservation of marrow samples. It is clear that

room temperature is an optimal condition for marrow sample storage when the sample is designed to be used shortly afterwards^[21]. Therefore, it is recommended that human bone marrow samples be used as soon as possible or be stored at room temperature when necessary.

The results above suggest that urokinase pretreatment is an optimal strategy to culture MSCs when human bone marrow are poorly aspirated and clotted.

Urokinase might not only dissolve the coagulated clots but also mobilize MSCs wrapped in the fibrin structure^[19]. Previous data have also shown that urokinase might affect the migration and differentiation^[19,22]. However, urokinase treatment described in this protocol needs only four hours and the potential ligation of urokinase to the receptor on MSCs is transient. The differentiation properties of MSCs might be unaffected. Therefore, urokinase treatment should be a practical method to release MSCs in the coagulated clots.

COMMENTS

Background

Bone marrow is a commonly used origin for the isolation of mesenchymal stem cells (MSCs); however, in some cases, marrow samples are poorly collected and clotted promptly after aspiration, leading to the decrease of MSC output or even failure to culture-expand MSCs.

Research frontiers

MSCs are adult stem cells with the capacities to differentiate several connective tissues, support hematopoiesis, promote angiogenesis, and regulate immune reactions. Therefore, several hundreds of clinical trials with MSCs are on-going and MSCs have officially approved to treat ischemic diseases and immune-associated disorders.

Innovations and breakthroughs

In this study, the authors found that urokinase treatment could release MSCs from clotted marrow samples and maintain the number of colony-forming units compared with the uncoagulated control. The isolated MSCs exhibited fibroblast in morphology, expressed the specific markers and could be induced to osteoblast and adipoblast.

Applications

Coagulation of human bone marrow samples is a common problem in practice. The study strongly suggests urokinase digestion is a good method to treat the clots to isolate MSCs in this setting.

Terminology

Mesenchymal stromal cells: MSCs are adult stem cells characterized by their hematopoiesis-supporting, angiogenesis-promoting and immune regulatory activities, and have been officially approved by several countries to treat various kinds of diseases including graft-versus-host disease, musculoskeletal defects and ischemic heart disorders. Colony-forming unit-fibroblast: Colony-forming unit (CFU) is a unit commonly used to estimate the number of viable targeted cells in a sample. CFU-fibroblast (CFU-F) expresses the frequency of cells in a sample that can form colonies consisting of fibroblasts or fibroblast-like cells after culture for several days. The visual appearance of a colony in a cell culture requires significant growth, and when counting colonies it is uncertain if the colony arose from one cell or a group of cells. Expressing results as colony-forming units reflects this uncertainty. CFU-F is a well-recognized surrogate for MSCs and used to evaluate the potential proportion of MSCs in a sample. Urokinase: Urokinase is a serine protease that was originally isolated from human urine. The primary physiological substrate of urokinase is plasminogen. Clinically, urokinase is used in the treatment of deep venous thrombosis, pulmonary embolism, and myocardial infarction.

Peer-review

This is a well-designed study that clearly shows that urokinase-treated samples provides a superior method for obtaining useable mesenchymal stem cells. The methods of this study were sound and the conclusions followed logically from the data obtained. The study has significant relevance for stem cell therapies.

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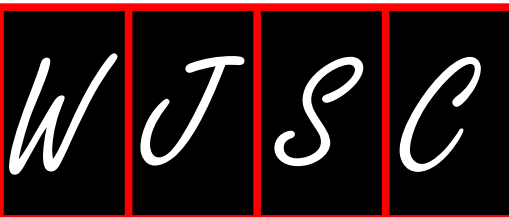
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EDITORIAL

- 1145 Understanding leukemic hematopoiesis as a complex adaptive system

Thomas X

REVIEW

- 1150 Common stemness regulators of embryonic and cancer stem cells

Hadjimichael C, Chanoumidou K, Papadopoulou N, Arampatzi P, Papamatheakis J, Kretsovali A

- 1185 Therapies targeting cancer stem cells: Current trends and future challenges

Dragu DL, Necula LG, Bleotu C, Diaconu CC, Chivu-Economescu M

Contents

World Journal of Stem Cells
Volume 7 Number 9 October 26, 2015

ABOUT COVER

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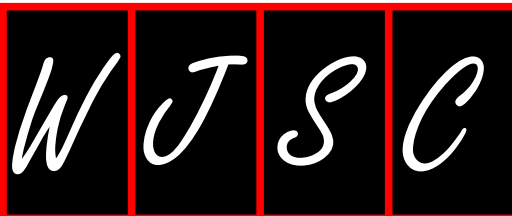
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Understanding leukemic hematopoiesis as a complex adaptive system

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Abstract

Normal and abnormal hematopoiesis is working as a complex adaptive system. From this perspective, the development and the behavior of hematopoietic cell lineages appear as a balance between normal and abnormal hematopoiesis in the setting of a functioning or malfunctioning microenvironment under the control

of the immune system and the influence of hereditary and environmental events.

Key words: Acute leukemia; Complex adaptive system; Stem cells; Hematopoiesis; Treatment

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Core tip: Complex adaptive systems are self-organizing systems involved everywhere. In bone marrow, cell lineages are working as a complex adaptive system. While the standard leukemia stem cell theory postulates that rare stem cells with self-renewal capacities are able to give rise to partially differentiated progenies, leukemia appears as a breakdown of the existing biological order under the influence of genetic and environmental factors, more than only the presence of abnormal cells.

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Complex adaptive systems are self-organizing systems involved everywhere in all disciplines^[1]. They have in common the emergence of self-organization on the macro-scale from micro-scale interactions of the agents contributing to the system. Complex adaptive systems share common traits of which: (1) simple rules of interaction potentially leading to self-organization when a group of individuals achieve a certain size; (2) the complexity is only at a macro level, individuals are ignorant of the overall organization since simple rules regulate local interactions between individuals and their environment; (3) local self-organization fails to emerge; and (4) interactions between agents or agents and their

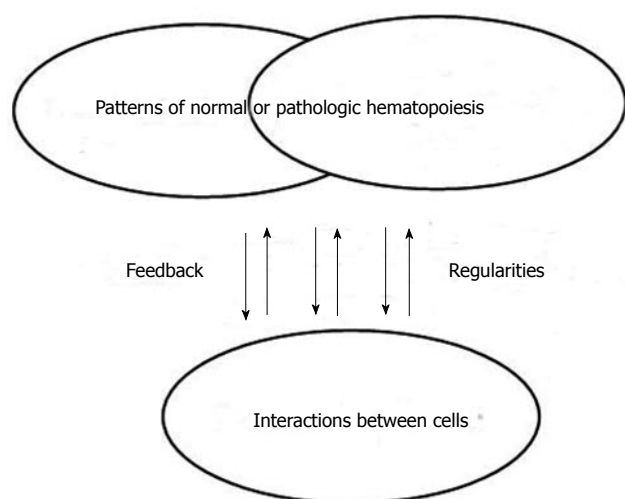


Figure 1 Normal and pathologic hematopoiesis as complex adaptive system.

environment form negative feedback loops leading to adapted responses, maintaining the complexity of the system. Although hematopoiesis is classically regarded as a linear process, stem cell processes and cell lineages that arise from them, are working within the framework of an adaptive complex network^[2,3]. Bone marrow cells share many of the above defining characteristics. They only respond to local signals and interactions through feedback loops, have no action on the entire organism and can work independently of the whole hematopoietic system.

Despite intensive studies and analyses to complete the picture, malignant hemopathies did not always behave as expected. It is apparent that pathologic hematopoiesis (like normal hematopoiesis) is working according to a very different set of rules to cause and effect than those commonly admitted. Complexity theory, which has emerged in all disciplines and can be also considered for hematopoiesis, is based on relationships, emergence, patterns and iterations (Figure 1). All cells present in the hematopoietic system should be considered as involved in that system. All interactions and connections between those cells are not predictable and not planned. A pattern will emerge from cell interactions, will feedback on the hematopoietic system and will inform the interactions of the various agents leading to the establishment of a new balance.

Our understanding of the pathogenesis of acute leukemia has evolved over the years. Leukemic hematopoiesis appears to retain some semblance of its normal counterpart, and also works as a complex adaptive system. Actually, the first one derives from the second one, and both normal and abnormal systems can coexist at variable functional, qualitative, and quantitative intensities under the influence of inner and outer factors (Figure 2). Most systems are not unique, but are within other systems and participate to those bigger systems. In the standard leukemia stem cell theory, rare stem cells with self-renewal capacities

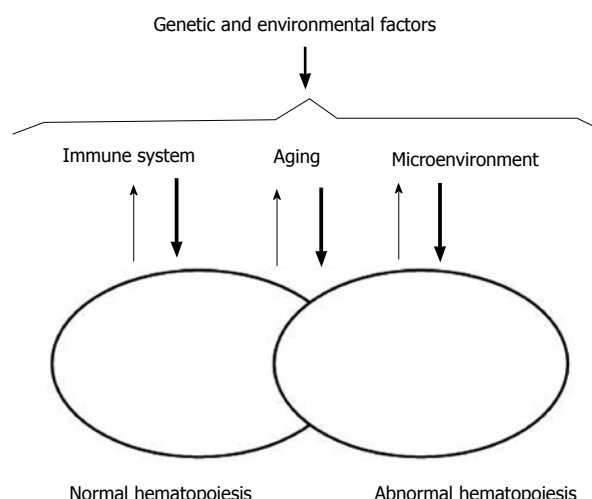


Figure 2 Factors influencing interactions between normal and abnormal hematopoiesis.

can give rise to partially differentiated progenies which will constitute the bulk of leukemia^[4,5]. However, this view of leukemia hematopoiesis reveals only dominant pathways and do not take into account the alternate pathways representative of a complex adaptive system. It is based on linear relationships which imply precise solutions. Actually, a hierarchy of command and control does not exist. Nothing is planned or managed. The hematopoietic system is continually self-organizing to find the best fit with the microenvironment. Cells interact through the process of emergence and feedback apparently at random with non-linear relationships and only approximate solutions. Patterns emerge from these interactions and will influence the behaviour of these cells within the system and the hematopoietic system itself.

The ways in which the cells interact to one another is critical to the survival of hematopoietic cell lineages. Patterns are formed from these connections as well as the feedback. Connections between the cells appear generally more important than the cells themselves. The bulk leukemia cells are supposed to possess limited proliferative potential and to have few capacities for initiating or maintaining the disease. Eradicating these cells is therefore not enough to cure the disease, while drug-resistant leukemia stem cells will re-populate the bone marrow and cause leukemia relapse^[6,7]. The potential emerging patterns are numerous, even if the rules governing the overall function of the system remain quite simple. The total number of mutations potentially found in leukemia cells is quite large. However, few mutations commonly occur in coding regions and are relevant to disease pathogenesis. Any leukemic cell has relatively few of these recurring mutations^[8,9]. Small changes in an initial condition can have significant effects. In a hematopoietic stem cell, clonal expansion starts with the occurrence of an "initiating" mutation. Such a mutation might be a translocation or a coding sequence mutation. Within the expanding clone, new

mutations can occur, completing the transformation process. Different sub-clones may develop within this founding clone. The situation may also evolve under the influence of disease evolution. New mutations may randomly occur as a matter of time. They can also occur as a result of the treatment mutagenic potential. Clonal evolution is common at the time of relapse and occurs through the acquisition of new mutations, either in the dominant primitive clone or in a subclone of the founder clone^[10]. Therapy itself can contribute to the acquisition of therapy-resistant mutations. A dominant sub-clone may evolve, possibly because of varying sensitivities to therapy or the influence of therapy on the microenvironment and on residual normal hematopoiesis^[10].

Other factors may influence the balance between normal and abnormal hematopoiesis.

Hematopoietic cells are part of their microenvironment. Cell populations present in the bone marrow niche are able to regulate stem cells. Leukemia stem cells depend on the same signals as their normal counterpart^[11]. In response to infection or bone marrow stress, the cellular composition of the microenvironment and therefore the production of cytokines are able to change fundamentally^[12]. Hematopoietic cells will have to adapt and to respond to all changes susceptible to occur in this environment to ensure the best fit. They will also modify their microenvironment by causing inflammation and by inducing an adaptive and innate immune response in the bone marrow. Furthermore, as the environment has already changed they will need to change again as a constant process. Leukemia-specific effector T cells infiltrating bone marrow may potentially target leukemia stem cells and participate to their eradication. However, mechanisms that evolved to protect normal hematopoietic stem cells and to regulate demand-adapted responses can protect leukemia stem cells against an attack from the immune system and favor their progression. Quiescence warrants the genetic integrity of stem cells as frequent chromosomal replications may introduce oncogenic DNA mutations and also protect stem cells from uncontrolled proliferation. Hematopoietic stem cells can undergo asymmetrical division. This non-exhaustive proliferation capacity can be initiated by all stress situations, of which infections and cytotoxic chemotherapy, and respond to signals from immune cells^[13]. Self-renewing hematopoietic stem cells persist for long periods of time, allowing the accumulation of genetic damage and malignant transformation. They can therefore serve as the cell-of-origin for leukemia stem cells^[14]. However, more mature progenitor cell types can serve as leukemia-initiating cells in some cases^[15]. Although not excluding a hierarchical model, this sustains the stochastic model hypothesis. Here, every malignant cell, given that it enters a permissive situation, has the ability to self-renew and to recapitulate the disease phenotype^[16].

During leukemogenesis, leukemia stem cells can "hijack" the niche and the signaling molecules from

normal hematopoietic cells. Molecular changes in the bone marrow niche contribute to leukemia development. In the niche, leukemia stem cells may be more therapy resistant and may therefore potentially provide important cellular markers of clinically relevant minimal residual disease^[17]. Immune cells are involved in the regulation of hematopoietic stem cell homeostasis and emergency hematopoiesis^[18]. CD4⁺ T cells are particularly important in maintaining hematopoietic stem cell function^[19]. T regulators represent one-third of all CD4⁺ T cells in the bone marrow. They provide an immune-privileged niche, protecting hematopoietic stem cells from immune destruction^[20]. Monocytes and macrophages are also important cells serving as regulators of hematopoietic stem cell egress from the bone marrow^[21]. The immune system interacts with hematopoietic stem cells *via* direct cell-cell interactions and *via* soluble factors. Leukemic cells express can interact with T cells through the molecular repertoire of major histocompatibility molecules and co-stimulatory ligands. Cytotoxic T lymphocytes directed against leukemia antigens have been detected^[22]. However, an activated immune system can also contribute to the leukemia progression by CD27 signaling on leukemia stem cells.

Hematopoietic stem cells are not spared by the aging process, in which DNA damage, telomerase shortening, oxidative stress, and poor homing efficiency have been reported. This could explain some of the differences between childhood and adult leukemias. Such genetic and epigenetic damage together with decreased immune system efficacy, vulnerability of older patients with a decreased function of various organs, and environmental factors can result in the development of malignant hemopathies in elderly patients^[23,24]. Furthermore, two obvious contributory factors are the dominant determinant of the country-to-country variability of leukemia incidence: heredity and environment.

Hematopoiesis permanently fluctuates between equilibrium and chaos. In a state of equilibrium, the hematopoietic system will slowly fail because its internal dynamics is not able to allow enough responses to its microenvironment. Similarly, a system in chaos will also fail, but that is because it will cease to function as a system. The most productive state always remains at the edge of chaos. This situation is the one that generates a maximum of variety and creativity, and a maximum of new possibilities for the evolution of the system. Leukemia is a disease of chaos, a breakdown of the existing biological order within the bone marrow, more than only the presence of abnormal cells. The disorder derives directly from malfunctioning of the controls that are normally present. We are still far from being able to understand with any precision how any single human leukemia arises. The process of leukemia formation is a complex one of multiple steps involving multiple alterations of cells and their physiologic control mechanisms. These steps may occur rapidly and

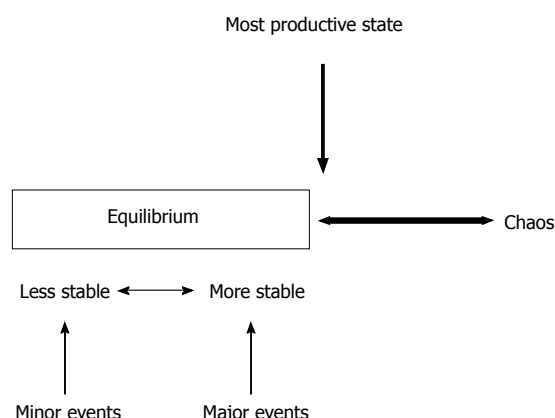


Figure 3 Hematopoiesis between equilibrium and chaos.

therefore may not be “rate-limiting”, while others may require more time to complete. Multistep leukemia progression can be depicted as a form of Darwinian evolution. Darwinian evolution involves expansions of cells that are endowed with advantageous genotypes and thus phenotypes. Recent whole genome and exome sequencing studies have begun to illustrate the extent of molecular heterogeneity in acute leukemia. The biologic behavior of leukemia cells is the sum of all the acquired genetic changes. Leukemia stem cells, rather than the bulk leukemia cell population, may be the objects of genetic alteration and clonal selection. The clonal descendants of the “favourable” mutated cell dominate by the cells that lack this mutation, resulting in a clonal expansion.

The development and the behavior of hematopoietic cell lineages appear therefore as a balance between normal and abnormal hematopoiesis in the setting of a functioning or malfunctioning microenvironment under the control of the immune system and the influence of hereditary and environmental events. Multiple equilibrium states may exist. Having leukemia cells does not mean having leukemia. All states seem possible, either stable or transitory, going from normal and functional hematopoiesis to the presence of abnormal cells easily controlled by the immune system, or to pre-leukemic stages without any consequences on normal hematopoiesis, or pre-leukemic stages with inhibition of normal cell lineages without major expansion of abnormal cells, or to the development of an abnormal clone without major infiltrative and proliferative abilities, or to the development of an aggressive leukemia clone taking absolute control of the bone marrow leading to an overt disease. The number of events necessary to affect the equilibrium depends on its stability. In this setting, massive events are required to modify a stable state, while a less stable equilibrium will be changed by minor events. The system, that will emerge, will be the one that will be better than its competitors. Then, the system will trade off increased efficiency every time in favor of greater effectiveness. The strength of a system is proportional to the variety within the system.

Contradictions are used by the hematopoietic cells, as by all complex adaptive systems. They are required to create new possibilities to co-evolve with their microenvironment (Figure 3).

Better understanding pathologic hematopoiesis as a complex adaptive system should have consequences in a therapeutic point of view and should modify our vision to consider disease control. Current therapeutic successes using aggressive treatment approaches, such as intensive chemotherapy or stem cell transplantation, have been conceived based upon the hierarchical concept of hematopoiesis with a “black or white” initial response to therapy and long-term survival defining “cure”, relying on a definitively fixed organization of hematopoiesis. Instead of this traditional reductionist approach, a moving status with a permanent adapting condition of hematopoietic cells according to inner and outer factors as suggested in a complex adaptive system is probably more realistic and could represent the common rule. Indeed, in real life, the concept of morphologic complete remission appears not anymore as an absolute requisite for therapeutic decision-making. The development of more accurate techniques to evaluate residual disease and a better knowledge of intra-cellular mechanisms leading to the introduction of targeted therapies should help the change our way to conceive leukemia therapy. Novel therapeutics should authorize more individualized therapeutic approaches. As it has already been the case in chronic leukemias since the introduction of tyrosine kinase inhibitors, a concept of acute leukemia therapy moving to an effective control of the disease compatible with a normal life instead of a supposed “complete” eradication of malignant cells seems a more accurate and more realistic goal.

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Common stemness regulators of embryonic and cancer stem cells

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Abstract

Pluripotency of embryonic stem cells (ESCs) and induced pluripotent stem cells is regulated by a well characterized gene transcription circuitry. The circuitry is assembled by ESC specific transcription factors, signal transducing molecules and epigenetic regulators. Growing understanding of stem-like cells, albeit of more complex phenotypes, present in tumors (cancer stem cells), provides a common conceptual and research framework for basic and applied stem cell biology. In this review, we highlight current results on biomarkers, gene signatures, signaling pathways and epigenetic regulators that are common in embryonic and cancer stem cells. We discuss their role in determining the cell phenotype and finally, their potential use to design next generation biological and pharmaceutical approaches for regenerative medicine and cancer therapies.

Key words: Embryonic stem cells; Cancer stem cells; Pluripotency; Self-renewal; Tumorigenicity

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Core tip: Accumulating experimental evidence has revealed the existence of common stemness regulators for embryonic and cancer stem cells. In this review, we highlight current results on biomarkers, gene signatures, signaling pathways and epigenetic regulators that determine the phenotype of these two types of stem cells. We also discuss how this knowledge may promote the design of next generation biological and pharmaceutical tools for regenerative medicine and cancer therapies.

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INTRODUCTION

Embryonic stem cells (ESCs) have two unique properties, self-renewal and pluripotency^[1]. Mouse ESCs (mESCs) are isolated from day 3.5 blastocyst and possess ground state pluripotency whereas human ESCs (hESCs) are isolated from late blastocyst and correspond to the epiblast stem cells of the mouse^[2,3]. The pluripotency of ESCs is determined by the concerted action of signaling pathways that respond to external stimuli, intrinsically expressed transcription factors and complexes that govern the epigenetic state. The extended transcriptional network of ESCs is centered on the triad of master regulators of pluripotency Oct4, Sox2 and Nanog^[4]. In the last decade the introduction in somatic cells of transcription factors (Oct4, Sox2, Klf4, c-Myc), microRNAs and small molecules allowed the generation of induced pluripotent stem (iPS) cells^[5]. Due to their ability to give rise to any type of differentiated cells and tissues, both ES and iPS cells offer many opportunities for modeling human diseases and development of regenerative medicine^[6].

ESCs and tumor cells share many common properties exemplified by rapid proliferation, similar metabolic requirements and inhibition of differentiation. Pluripotent ESCs have inherent tumorigenic potential and they generate benign tumors and teratomas when injected in immunodeficient mice^[7]. Reprogramming of somatic cells into pluripotency by oncogenes like Myc and Klf4 suggest a strong link between pluripotency and tumorigenicity^[8,9]. Currently, growing experimental evidence has revealed that tumors contain a variable number of cells that have self-renewal and partial differentiation capacities^[10-12]. Because these cells share these properties with the adult tissue stem cells from which they are likely derived they were termed cancer stem cells (CSCs)^[10-12]. The procedures of somatic cell reprogramming and CSC establishment are both dependent on transitions between epithelial and mesenchymal states (EMT/MET)^[13,14]. In addition, CSCs from epithelial tumors also exhibit ESC-like signatures^[15,16] that include the oncogene c-Myc and factors important for pluripotency such as Sox2, Dnmt1, Cbx3 and HDAC1^[16].

The CSC model^[10-12] ultimately links Cancer with Stem cell biology and provides a common framework that is proposed to account for all the properties of stem cells, regardless of their early or late developmental origin in normal or pathological states. In this review, we analyze common regulatory mechanisms of embryonic

and CSCs focusing on biomarkers, signaling pathways, transcription factors and epigenetic complexes. This information can elucidate the risks stemming from the tumorigenic potential of pluripotent ESC and iPS cells used in tissue regeneration therapies. Additionally, knowledge about their stem cell properties is valuable for the eradication of CSCs that are responsible for therapy resistance, tumor invasion and metastasis.

GENERAL PROPERTIES AND MARKERS FOR EMBRYONIC/PLURIPOTENT AND CSC

Three types of markers for the identification of either ESCs or CSCs are utilized: cell surface molecules, signaling pathway markers and transcription factors^[17]. However, cell surface molecules are mostly used as biomarkers since they can be assessed on intact living cells.

ESC biomarkers

All pluripotent stem cells express on their surface glycan epitopes that show species-specific and differentiation stage-specific expression, the stage specific embryonic antigens (SSEA-1, 3 and 4). SSEA-1 (CD15/Lewis x) is expressed on mESCs and embryonic carcinoma cells (ECCs) but is absent from hESCs^[18,19]. Upon mESC differentiation SSEA-1 expression decreases whereas SSEA-4 expression is induced. Undifferentiated hESCs express SSEA-3/4 but following differentiation they are both silenced and SSEA-1 is induced^[20-22] (Table 1).

Human ESC, embryonic carcinoma and germ tumors are characterized by the expression of tumor rejection antigens (TRA-1-60 and TRA-1-81). These are proteoglycan epitopes that reside on the 200 kDa form of podocalyxin (SC-podocalyxin)^[23,24]. Because they are not expressed on somatic cells, TRAs are useful markers for the isolation of human iPS cells during reprogramming^[25].

Cluster of differentiation antigens (CDs) are membrane proteins that function in diverse processes such as cell adhesion, communication and differentiation. Various family members are expressed in mESCs, hESCs and ECCs. Their expression usually changes following differentiation^[26]. CD324 (E-cadherin), CD31 (PECAM-1), CD24, CD90 (Thy-1), CD9, CD59, CD133 and CD326 (EpCAM) are present on the membrane of ESCs and ECCs although their expression levels can vary depending on the cell line and culture conditions^[17]. Among CDs the epithelial marker CD326 (EpCAM) is the more closely correlated with the undifferentiated state and is rapidly lost upon differentiation^[27] (Table 1). Interestingly, some of the above CDs are also used as CSC markers, as will be discussed below.

Among these markers of stemness, Cripto-1 (CR1, TDGF-1) represents an important component of a critical core pathway that is used by ESCs (Table

Table 1 Biomarkers of pluripotency in mouse and human embryonic stem cells

Biomarker	Role in mESC	Role in hESC
Oct4	Pluripotency ^[200]	Pluripotency ^[4]
Sox2	Pluripotency ^[215]	Pluripotency ^[4]
Nanog	Pluripotency ^[227]	Pluripotency ^[4]
Klf4	Pluripotency ^[253,255]	Pluripotency ^[253]
c-Myc	Pluripotency ^[190]	Pluripotency ^[190]
SSEA1/CD15	Pluripotency ^[118,19]	Not expressed
SSEA 3, 4	Not expressed	Pluripotency ^[20,22]
TRA-1-68	Not expressed	Pluripotency ^[24]
TRA-1-81	Not expressed	Pluripotency ^[24]
Cripto-1	Pluripotency ^[28,474]	Pluripotency ^[28,474]

hESC: Human embryonic stem cell; mESCs: Mouse embryonic stem cells; SSEA: Stage specific embryonic antigens; TRA: Tumor rejection antigens.

1). This growth factor acts during embryogenesis as a TGF- β ligand, co-receptor and as an oncogene. Moreover, Cripto-1 is involved in PI3K/Akt and MAPK pathways in a SMAD-independent manner and it enhances the Wnt and Notch pathways acting as a chaperone for low-density lipoprotein receptor-related protein 5 (LRP5) and Notch respectively^[28]. Cripto-1 is crucial for early embryonic development and is expressed in both mouse and human ESCs resulting in maintenance of stem cell pluripotency. Additionally, Cripto-1 regulates ESC fate choices by repressing the neural and enhancing the cardiomyocytic differentiation^[29]. Recently, it was shown that Cripto-1 performs an essential role in the etiology and progression of several types of human tumors, where it is expressed in a CSC subpopulation and facilitates epithelial-mesenchymal transition (EMT)^[28]. Intracellular transcription factors such as Oct4, Sox2, Nanog, Klf4 that are specifically expressed in undifferentiated stem cells are also important biomarkers. Their importance for pluripotency is examined in detail below.

CSC biomarkers in solid tumors

Breast cancer was the first type of solid tumor where CSCs were identified. Cells exhibiting the EPCAM⁺ ESA⁺CD44⁺CD24^{-/low}Lin⁻ phenotype were able to propagate breast tumors when injected even in a low concentration into the mammary fat pad of immunodeficient mice^[30]. Further studies identified a subpopulation of CD44⁺CD24⁻ cells expressing Aldehyde dehydrogenase (ALDH) capable of propagating tumor, by injection of as little as 20 cells in immunodeficient mice^[31]. CD44 is a cell membrane protein that binds hyaluronan (HA) and has a role in cell-cell and cell-extracellular matrix (ECM) interactions. Studies have implicated CD44 in breast cancer cell adhesion, migration, invasion and metastasis^[32]. Additionally, it protects cells from apoptosis which is an important characteristic of CSCs^[33]. HA-CD44 interaction promotes multiple cascades, activating gene transcription of stem cell-related factors in many different tumors, such as ovarian, breast, head and

neck cancer^[33]. Like CD44, CD24 is a widely expressed glycosylated cellular adhesion protein. Low expression of CD24 in breast CSCs was shown to enhance their growth ability and metastatic potential, through a chemokine receptor CXCR4 response^[34]. Additional surface markers for breast CSCs are: CD13, also a marker for brain and colon CSCs, α 6-integrin, CD61, CD29 and CD49.

The ALDH family of enzymes catalyzes the oxidation of aldehydes into carboxylic acids in a NADP⁺ dependent manner^[35]. ALDHs play a crucial role in retinoic acid biosynthesis, metabolism of cyclophosphamides and clearing toxic byproducts of reactive oxygen species^[36]. High ALDH activity was measured in human adult hematopoietic and breast stem cells, murine neural stem cells, as well as leukemia, breast, colon, head and neck CSCs^[35]. Emerging evidence supports the significance of ALDH as a biomarker for adult and CSCs of different origin, including pancreatic, prostate, ovarian, lung, head and neck squamous cell carcinoma^[37].

The first evidence for the existence of CSCs in brain tumors was provided by Singh *et al.*^[38]. Brain CSCs express the cell surface marker CD133 and lack the expression of neural differentiation markers. CD133 is a transmembrane glycoprotein expressed in mouse and human ESCs, as well as in different types of adult stem/progenitor cells, including hematopoietic and neural stem cells, endothelial precursors, mesenchymal progenitors, kidney, mammary glands and pancreatic, colorectal, testis, prostate, ovarian, lung and melanoma CSCs^[39]. The exact function of this protein remains unknown, but it was proposed to act as an organizer of the cell membrane topology^[40]. Limitations on the exclusive use of CD133 as a brain CSC marker arise from a study by Beier *et al.*^[41] who have reported that CD133 negative cells from glioblastoma sphere cultures are able to propagate tumors in immunodeficient mice. Moreover, CD133 expression was shown to depend on culture conditions and hypoxia levels. Additional markers shared by normal and cancer brain stem cells are nestin, Sox2, Musashi-1 and Bmi-1^[42]. Several studies have indicated that multidrug resistance transporters (MDR) such as ATP binding cassette (ABC) transporters and breast cancer resistance protein (BCRP/ABCG2) increase drug efflux from the cells^[43,44].

Stage-specific embryonic antigen 1 (SSEA1), also known as CD15, is a well characterized marker of undifferentiated mouse and differentiated human ESCs. It was first identified in neural embryonic progenitors and represents a putative brain CSC marker. SSEA1 expression correlates with increased cell proliferation, decreased differentiation and apoptosis^[45]. CD15⁺ cells exhibit high tumorigenicity. Another potential brain CSC marker is Nestin, an intermediate filament protein that was first identified as a neural stem cell marker^[46]. It is expressed in many different brain tumors and is involved in stemness, cell growth, invasion and migration^[47]. Finally, brain, like other CSCs

from breast and lung are resistant to chemotherapy and radiotherapy because of the expression of MDR1 transporter on their cell surface^[48].

Colorectal CSCs also express CD133^[49] and the surface molecule Epithelial Specific Antigen (ESA/EpCAM), while lacking expression of intestinal differentiation markers such as cytokeratin 20 (CK20). EpCAM is an epithelial adhesion molecule involved in proliferation, differentiation, migration and signaling^[50,51]. Another adhesion protein, CD166, was proposed as a biomarker in colorectal CSCs^[51]. CD166 is present in a wide variety of normal tissues, as well as in different cancers including breast, lung, prostate and melanoma^[52]. CD166 is used as a positive prognostic marker for survival in colorectal cancer despite the contradictory studies regarding its timeframe of expression during tumorigenesis. Other recently identified potential markers include CD24, CD29 and Lgr5. CD29 (β 1-integrin), a transmembrane receptor for extracellular proteins activates signaling cascades responsible for proliferation, differentiation, migration, survival or death^[53]. Finally, Leucine-rich repeat-containing G protein coupled receptor 5 (Lgr5), a receptor for R-spondins is characterized by a large extracellular and seven transmembrane domains. It binds R-spondin proteins which activate Wnt/ β -catenin signaling as a Wnt pathway co-receptor^[54,55]. Lgr5, a Wnt pathway target itself, is expressed in various stem cell types. High Lgr5 expression levels are associated with high vimentin and low miR-200c expression followed by increased invasiveness and lymph node metastasis^[56].

Pancreatic CSCs are characterized as CD44⁺CD24⁺-EpCAM⁺^[57], like CSCs from other solid tumors, such as ovarian cancer^[46]. On the contrary, breast CSCs exhibit low CD24 expression. Tyrosine kinase c-Met and CD133 have emerged as additional pancreatic CSC markers^[58,59]. Indeed, solely CD133⁺ cells, were shown to induce tumor formation in high frequency^[59]. Moreover, concomitant expression of CXCR4 promotes metastasis and represents a useful target for antitumor drugs^[59,60].

Prostate CSCs are characterized by high expression of CD44 and CD133, as well as the existence of ALDH1A1 and ABCG2 transporter on their cell surface, which confer chemoresistance. A2 β 1 integrin was also proposed as a prostate CSC marker, in addition to the lack of differentiation markers, such as PSA^[61].

Ovarian CSC markers are CD133, CD44 and CD24^[62]. Additional markers are EpCAM, ALDH1 and CD117 or c-kit proto-oncogene^[62]. The c-KIT receptor is activated by autophosphorylation upon stem cell factor (SCF) binding and is involved in cell proliferation, apoptosis, differentiation and adhesion^[46]. Moreover, CD117 was associated with chemotherapy resistance^[63]. Finally, other molecules that are significant for ovarian CSCs are MyD88 for chemoresistance, Lin28 and Oct4 for cancer stemness and dedifferentiation^[35].

Melanoma CSCs were recently found to be positive for CD20, CD133 and the ABC transporters, ABCG2 and

ABCB5^[64]. Additional markers are CD133 and CD166^[64]. CD133⁺ melanoma cells, which overexpress CD166 and nestin, exhibit tumor-propagating ability and high expression levels of genes responsible for tumor initiation and metastasis^[65]. CD271, which was proven to be important for maintenance of stem-like properties and tumorigenicity of melanoma cells^[66] is considered as the most convincing biomarker for melanoma CSCs. In addition, CXCR6 is implicated in their asymmetric division^[67] and Oct4 that is induced upon hypoxia is capable of promoting melanoma cell dedifferentiation into CSCs^[68].

Lung CSCs express CD133 as well as CD24, CD34, CD44, CD87 and ALDH1^[35,69]. CD133⁺ cells show increased expression of Oct4 protein and the ABCG2 transporter^[70]. Finally, Bmi-1 which is expressed in human small cell lung cancers could be applied as a potential lung CSC marker due to its role in self-renewal^[71].

Hepatocellular CSCs express CD133^[72]. CD133⁺ cells exhibited increased expression of Oct4, Notch, Wnt/ β -catenin, Hedgehog and Bmi-1, genes implicated in self-renewal, pluripotency, proliferation and differentiation^[73]. EpCAM, CD90 (Thy-1), CD44, CD13, ALDH1, ABCG2, CD117 and AFP represent additional hepatic CSC markers^[74].

The head and neck squamous cell carcinoma share the same biomarkers as most of the above described CSCs, including CD44, CD133, ABCG2, ALDH1, c-Met, Bmi-1 and Lgr5^[75-77].

In conclusion, putative CSCs have been characterized and enriched from many types of solid tumors using various cell surface markers. These CSC biomarkers offer important biological diagnostic and therapeutic tools (Table 2).

CONVERGENCE OF SIGNALING PATHWAYS IN EMBRYONIC AND CSC

Pluripotency of ESCs is regulated by core transcription factors as well as key signaling pathways, including LIF/Stat3 in the case of mESCs, Wnt, Hedgehog, Notch, FGF and TGF- β for both mESCs and hESCs^[78]. One hallmark of CSCs is their self-renewal capacity driven by developmental pathways^[13,79,80]. Below, we will outline the common signaling mechanisms in self-renewal, differentiation and pluripotency of mES, hES and CS cells in solid tumors.

Jak/Stat signaling

Binding of cytokines to their cognate receptors induces the activation of Jak kinases and the phosphorylation, dimerization and nuclear shuttling of signal transducers and activators of transcription (STATs). The Jak/Stat signaling pathway is activated by the cytokine LIF (leukemia inhibitory factor) in mESCs and is required for their self-renewal and pluripotency^[81]. In contrast, LIF does not support the pluripotency of human ESC.

Table 2 Biomarkers of cancer stem cells

Cancer type	Biomarkers
Breast	CD44 ^[32,33] ALDH ^[31,35] EpCAM ^[30] CD13 ^[35] A6-integrin ^[35] CD61 ^[35] CD29 ^[35] CD49 ^[35] ABCG2 ^[43,44] Nanog ^[204] Klf4 ^[263,264]
Brain	CD133 ^[39] Nestin ^[42] Sox2 ^[42] Musashi-1 ^[42] Bmi-1 ^[42] ABCB1 ^[48] ABCG2 ^[48] ABCC1 ^[48] SSEA1 ^[45] Nanog ^[236] Sox2 ^[42,225] Klf4 ^[269]
Colorectal	CD133 ^[49] EpCAM ^[50,51] CD166 ^[51] CD24 ^[51] CD29 ^[51] Lgr5 ^[56] Klf4 ^[266]
Pancreatic	CD44 ^[57] CD24 ^[57] EpCAM ^[57] c-Met ^[58] CD133 ^[59] CXCR4 ^[59,60] Sox2 ^[221] CD44 ^[61] CD133 ^[61]
Prostate	ALDH1A1 ^[61] ABCG2 ^[61] A2β1 ^[61] Sox2 ^[61] CD133 ^[62] CD44 ^[62] CD24 ^[62] EpCAM ^[62] ALDH1 ^[62] CD117 ^[62] c-kit ^[62] CD117 ^[63] MyD88 ^[35] Lin28 ^[35] Oct4 ^[35]
Ovarian	Nanog ^[232,238] Sox2 ^[219] CD20 ^[64] CD133 ^[64] ABCG2 ^[64] ABCB5 ^[64] CD133 ^[64] CD166 ^[64] CD271 ^[66] CXCR6 ^[67] Oct4 ^[68] Sox2 ^[224]
Melanoma	

Lung	CD133 ^[69] CD24 ^[35] CD34 ^[35] CD44 ^[35] CD87 ^[35] ALDH1 ^[35] ABCG2 ^[70] Bmi-1 ^[71] Nanog ^[234] Sox2 ^[223]
Hepatocellular	CD133 ^[72] EpCAM ^[74] CD90 ^[74] CD44 ^[74] CC13 ^[74] ALDH1 ^[74] ABCG2 ^[74] CD117 ^[74] AFP ^[74]
Head and neck (HNSCC)	CD44 ^[75] CD133 ^[76] ABCG2 ^[76,77] ALDH1 ^[77] c-Met ^[77] Bmi-1 ^[77] Lgr5 ^[77]

Numerous studies have shown the ability of STAT3 to promote tumorigenesis when it is aberrantly activated^[82]. Most importantly Stat3 has been recently shown to regulate the survival and proliferation of colon^[83] prostate^[84] and breast^[85] CSCs.

Wnt/β-catenin signaling

The Wnt/β-catenin branch of Wnt signaling-also referred to as the “canonical” Wnt-pathway- is important for proper embryonic development and adult tissue homeostasis. There are more than 30 extracellular Wnt-ligands, which bind to the receptor complex Frizzled and LRP5/6 (member of the LDL receptor family)^[86]. In the absence of Wnt signals, the scaffolding proteins Axin and adenomatous polyposis coli (APC) anchor the intracellular signaling protein β-catenin to a “destruction” complex involving Glycogen-activated kinase-3 (GSK-3). Wnt ligand-receptor binding initiates a series of events resulting in inhibition of the destruction complex and β-catenin cytoplasmic accumulation^[87]. Its concentration-dependent nuclear translocation and interaction with the T-cell factor/lymphocyte enhancer binding factor (Tcf/Lef) family leads to transcription of proliferative genes such as c-Myc and cyclinD1^[88,89].

Many members of the Wnt signaling pathway are implicated in stem-cell proliferation and activity. In mESCs, Wnt promotes self-renewal^[90]. Studies using small-molecule inhibitors have highlighted GSK-3 as a critical regulator of pluripotency for both mouse and human ESCs^[91]. β-catenin is dispensable for mESC maintenance, however, in its absence, the positive effect of GSK3 inhibition on self-renewal is abolished^[92] and β-catenin mutant mice display embryonic neural progenitor defects^[93]. Double mutants of GSK3 and β-catenin promote exit from pluripotency and

induction of neuroectoderm differentiation^[94]. From the mechanistic point of view, accumulating evidence indicates that the key pathway effector Tcf3 is acting as a repressor of Oct3/4, Sox2 and Nanog, whereas β -catenin inhibits this repression by converting Tcf3 into activator^[95]. Additionally, active β -catenin interacts with Oct4 and enhances its activity in a Tcf-3 independent manner^[96]. In hESCs, there is an ongoing debate about the contribution of Wnt signaling in self-renewal or differentiation^[91,97]. Long-term inhibition of Wnt did not affect survival of hESCs, whereas activation of Wnt signaling resulted in activation of mesoderm differentiation. In fact, differential activity of Wnt signaling associates with distinct lineage-specific differentiation potential of hESCs^[98].

Wnt signaling also regulates the activity of somatic SCs including those in the skin, blood, brain and the mammary gland, whereas aberrant signaling results in neoplasia^[94]. Mutations in key mediators of the Wnt pathway have been observed in approximately 90% of all colon cancers^[78]. Wnt activity was found to be enhanced in the CD133⁺ stem-like cell population of colorectal cancer and in Lgr5⁺ (a Wnt family member) intestinal crypt stem cells, which were identified as the origin of adenocarcinomas^[94,99]. Following transplantation in a mouse model for lineage tracing, Lgr5⁺ cells expand clonally repopulating all other adenomas^[100]. Myofibroblast-secreted factors were linked through β -catenin-dependent transcription to colon CSC clonogenicity and were shown to restore the CSC phenotype in more differentiated tumor cells, both *in vitro* and *in vivo*^[101]. These findings indicate a Wnt-dependent role of the tumor microenvironment in colon carcinogenesis.

Wnt is also involved in mammary gland tumorigenesis, since Wnt signaling expands mammary gland stem cells in early tumorigenic lesions of MMTV-Wnt1 transgenic mice^[102]. The mammary gland CSCs pool is sustained through recruitment of Wnt ligands by periostin, a component of the extracellular matrix of fibroblasts. Infiltrating tumor cells induce its expression in the stroma of secondary organs, such as lung, to allow colonization. Inhibition of its function prevents metastasis^[103].

Finally, although the role of Wnt in brain CSCs is still unclear, autocrine activation of Wnt/ β -catenin signaling by the orphan nuclear receptor Tlx, that induces glioma formation when overexpressed, was shown to enhance the proliferation of murine neural stem cells^[104]. Moreover, a recent study linked low oxygen levels in the hippocampus with neurogenesis through HIF-1 α enhanced Wnt signaling^[105]. Interestingly, brain tumor formation is promoted by hypoxia^[94] and HIFs were identified as key players in stemness and malignancy maintenance of colon cancer cells^[106].

Hedgehog and Notch signaling

Hedgehog (Hh) signaling is implicated in many developmental processes, such as in proliferation

and cell-fate specification of neural stem and neural crest stem cells. In lack of Hh pathway activity, many target genes like the Hh receptor Patched (PTC) and the Gli family of transcription factors are actively repressed. Secreted Hh-transmembrane PTC binding results in releasing PTC-repression on Smoothed (SMO), a G-protein-coupled receptor that activates downstream intracellular components^[107]. Despite the presence of functional pathway components, SHh (Sonic Hedgehog) ligand appears to be dispensable for maintaining pluripotency and promoting proliferation of undifferentiated hESCs. However, addition of exogenous SHh to embryoid bodies generated by hESCs promotes differentiation towards neuroectodermal^[108]. On the contrary, SHh was reported to stimulate mESC proliferation through the concerted effect of activated Gli1, increased intracellular Ca²⁺ levels, PKC (protein kinase C) and epidermal growth factor receptor (EGFR) activation^[109].

In adults the Hh pathway is mainly inactive, but it has been reported to participate in tissue maintenance and repair. Its deregulated activity has been linked to cancer development, however little is known about its role in CSCs^[110]. Hh signaling downstream effectors Gli1/2 and Bmi-1, a transcriptional repressor of the polycomb group and central regulator of self-renewal in normal stem cells, were recently shown to control proliferation and pluripotency of breast CSCs and normal human mammary stem/progenitor cells^[111]. Active Hh signaling has also been identified in glioblastoma CSCs. Inhibition of the pathway by chemical molecules or siRNAs leads to loss of their tumorigenic potential^[112,113]. Hh signaling is furthermore preferentially activated in colon carcinoma CSCs derived from primary clinical specimens, whereas its hindrance negatively regulates cancer cell proliferation and induced apoptosis of colon CSCs^[114]. Finally, a number of recent studies have implicated Hh signaling in EMT and metastasis, for example in pancreatic cancer cell lines inhibition of Hh results in EMT-inhibition and blocks metastasis^[115].

Notch signaling is a crucial regulator of cell to cell communication during embryogenesis, cellular proliferation, differentiation, and apoptosis^[79]. The Notch pathway consists of a membrane-tethered receptor (Notch 1-4), that undergoes proteolytic cleavage by ADAM-type metalloproteases and γ -secretase upon ligand binding (membrane-associated Delta-like, Jagged in mammals). Following cleavage, the released intracellular Notch domain shuttles to the nucleus to form a transcriptional complex with recombining binding protein suppressor of hairless (RBPJ, also known as CBF1) and mastermind-like proteins, ultimately activating genes of the hairy and enhancer of split-related family^[78,79].

Despite the presence of functional pathway components during early embryonic development, Notch signaling is dispensable for the maintenance of hESC or mESC pluripotency and constitutively activated Notch does not alter the stem cell phenotype^[116-118].

Inhibition of pathway components by siRNAs or by a chemical compound against γ -secretase activity (GSI) interfered with hESC proliferation without driving their differentiation^[119]. However, a number of studies indicate a decisive role for Notch in ESC fate determination. Activated Notch signaling was shown to promote neural commitment of both hESCs and mESCs^[117]. In undifferentiated hESCs it is required to form the progeny of all three embryonic germ layers except for trophoblast cells^[120]. In mESCs, there appears to be no *in vivo* requirement for the Notch pathway, until after all three germ layers have formed^[121].

Notch signaling is important for tissue maintenance in many organs, including the skin, blood, intestine, liver, kidney, central nervous system, bone and muscle^[121]. It promotes the maintenance of the neural, myogenic and intestinal stem cell pool in both *Drosophila* and mouse^[122].

Deregulation of Notch has been reported in several cancer types and is progressively linked to CSC self-renewal^[78]. Notch pathway components are characterized by higher expression level in pancreatic CSCs. Their inhibition using either GSI or Hes1 shRNA reduced CSC numbers and tumorsphere formation. Conversely, Notch activation increased pancreatic CSC self-renewal. *In vivo* treatment of orthotopic pancreatic tumors in NOD/SCID mice with GSI blocked tumor proliferation and reduced the CSC population^[123]. Notch signaling is also activated and plays a crucial role in promoting CSC survival, proliferation and tumor initiation (but not progression) in colon cancer. An antibody against Notch ligand DLL4 inhibited tumor growth in a xenograft mouse model^[124,125].

In medulloblastoma, increased Notch and Hh signaling have been linked to the maintenance of a stem-like cell population. Pharmacological depletion of Notch signaling inhibits medulloblastoma growth in mouse xenografts^[126]. In this context, Notch was proposed to interact with Hh signaling to promote oncogenesis^[127]. Additional pathway interactions were found in human breast epithelial cells, where oncogenic conversion is driven by increased Wnt signaling *via* Notch-dependent mechanism^[128]. Deregulation of Notch signaling is an early event in pre-invasive ductal carcinomas. Reduced mammosphere forming efficiency of *in situ* ductal carcinoma in the presence of Notch inhibitors suggested that Notch regulates breast CSC self-renewal^[129]. In normal breast tissue, Notch1 was proposed to regulate progenitor-to-luminal differentiation, whereas Notch4 stem-to-progenitor cell transitions. Interestingly, inhibition of Notch4 and, to a lesser extent, Notch1 signaling results in decrease of the stem-like cell population and of tumorsphere formation in primary breast cancer samples and cell lines and in limited tumor formation *in vivo*^[130]. Furthermore, Notch4 (but not Notch1) activation inhibits mammary epithelial cell differentiation and promotes mammary carcinogenesis in mice^[80,131].

TGF- β signaling

The TGF- β pathway plays a major role in development. Depending on the downstream effector molecules, it can be classified into the Smad1/5/8, the Smad2/3 and the Tab/Tak pathways. Secreted TGF- β ligands bind to the extracellular domain of Ser/Thr kinase type I and type II TGF- β trans-membrane receptors (TGF- β R) thereby phosphorylating and activating latent cytoplasmic SMAD transcription factors^[132]. Among 42 known ligands in humans, bone morphogenetic proteins (BMPs) and growth differentiating factors bind type I receptors (GDFs) activate Smad1/5. Activin and Nodal trigger phosphorylation of Smad2/3 through TGF- β R I/II. Activated Smads form a higher-order protein complex with Smad4, which then translocates to the nucleus to modify gene transcription. Inhibitory cytoplasmic Smad6/7, as well as molecules secreted by neighboring cells like Lefty, further increase the regulatory complexity of the pathway^[78].

During embryonic development cell fate determination, such as mesoderm and primitive streak formation in the mouse, as well as neural induction and mesoderm specification in *Xenopus* are affected by the TGF- β pathway^[133]. Both the Smad1/5/8 and the Smad2/3 branches are involved in ESC pluripotency/differentiation. Activin/Nodal/Smad2/3 signaling is important for sustaining self-renewal and pluripotency of mouse and human ESCs^[134-136], whereas BMP/Smad1/5/8 signaling promotes self-renewal in mESCs^[137,138] and differentiation in hESCs^[139-141]. The partly divergent signaling outcomes observed in mouse vs human ESCs are most likely due to the different developmental stages from which they are derived, hESCs being more similar to mouse epiblast stem cells (EpiSCs)^[3,142].

In mESC culture, concerted BMP/LIF signaling sustains pluripotency through the induction of inhibitor of differentiation (Id) proteins, and by inhibiting two major differentiation pathways, namely extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) at the same time^[137,138,143]. Furthermore, it was recently reported that mESC self-renewal is endogenously activated by autocrine loops of Activin/Nodal^[135].

In hESC culture, Activin A, which is secreted by mouse embryonic fibroblast feeder layers, suppresses BMP signaling and hESC differentiation, while stimulating the expression of pluripotency factors (e.g., NANOG, OCT4, FGF2/8, NODAL)^[144,145]. Nodal is secreted by hESCs themselves, reinforcing their pluripotent state by an autocrine mechanism. A regulatory loop is formed by simultaneous secretion of the Nodal inhibitor Lefty. Upon differentiation Nodal expression is rapidly down-regulated^[134,136,146]. Pluripotency is further sustained by downstream pathway effectors Smad2/3 which bind to and trans-activate Nanog expression in undifferentiated hESCs^[147,148]. Smad2/3 phosphorylation levels decrease upon early hESC differentiation^[134]. Smad3 alone was

also shown to form a complex with Oct4 and directly regulate many Oct4 targets^[149]. On the contrary, SMAD1/5 phosphorylation levels increase upon hESC differentiation^[134,150], a finding consistent with the ability of BMP4 to initiate differentiation to trophoblasts *in vitro*^[139].

The TGF- β pathway is also involved in EMT during embryonic morphogenesis. Interestingly, some epithelial cells acquire thereby self-renewing characteristics reminiscent of stem cells^[151]. In carcinomas, EMT can lead to metastasis and high-grade malignancy^[152].

TGF- β signaling plays a complex, context-dependent and tissue-specific role in cancer development and CSC proliferation. While inhibiting the onset of carcinogenesis the pathway may promote invasion and metastasis at later disease stages^[153].

Skin epithelia lacking TGF- β II exhibiting enhanced integrin/focal adhesion kinase (FAK) signaling were prone to age-dependent squamous cell carcinoma development^[154]. Moreover, tumor regression occurred in cancer cells lacking integrin/FAK signaling^[155]. CSCs isolated from the tumor/stromal interface of TGF- β II null squamous carcinoma formed less differentiated, highly aggressive and metastatic skin cancers. Interestingly, FAK depletion counterbalanced the TGF- β II-null phenotype^[156]. These data support an important role for TGF- β in counterbalancing the integrin/FAK-dependent tumorigenic effects in squamous cell carcinoma by down-regulating CSC proliferation and expansion^[133].

Breast cancer cells respond to TGF- β by exhibiting stem-like properties. A recent study revealed that chemotherapy relapse of triple-negative breast cancer (TNBC) might involve expansion of CSCs caused by activated TGF- β and IL-8 signaling in The resistant CSCs are prone to TGF- β pathway inhibitors^[157].

In malignant gliomas, TGF- β signaling also appears to exert agonistic effects on tumorigenesis through the Nodal/Activin branch of the pathway, increasing self-renewal of glioma stem cells (GSCs) by enhancing LIF/STAT signaling^[158]. Furthermore, TGF- β -Sox4-Sox2 signaling appears to be important for the maintenance of stemness of GSCs^[159]. On the other hand, the activation of the BMP branch of the pathway by BMP4, initiated neural differentiation and blocked tumor growth in a mouse xenograft model^[160]. In addition, epigenetically silenced BMP signaling was proposed to desensitize glioblastoma stem-like cells to normal differentiation cues and to promote their proliferation^[161].

The differential CSC responses to TGF- β cues underlie a serious dilemma over the clinical use of TGF- β agonists/antagonists^[133].

Fibroblast growth factor signaling

Most fibroblast growth factor ligands (FGF1-22) function in a classical autocrine or paracrine manner. Ligand-receptor binding results in autophosphorylation and dimerization of the intracellular region of a tyrosine kinase trans-membrane receptor (FGFR1-4)^[162]. The signal is further relayed through four main pathways:

RAS-RAF-MAPK (ERK), PLC γ -PKC, PI3K-AKT and JAK/STAT. Emerging evidence suggests that FGFRs also traffic to the nucleus, activating entirely different downstream molecules^[163].

Mutations of FGF pathway components result in pre-implantation lethality of the early mouse embryo^[162]. Autocrine FGF-induced ERK1/2 signaling is dispensable for mESC pluripotency but requisite for their differentiation into neural and mesendodermal lineages^[164,165]. Ying *et al.*^[166] further suggested that addition of LIF and BMP to culture media promotes mESC self-renewal solely by compensating for the pro-differentiation effects of FGF4. mESCs are comprised of heterogeneous populations. Cells primed for differentiation towards the primitive endoderm express FGF5 and Brachyury. FGF4 signaling on the other hand additionally maintains the primed state towards germ layer differentiation^[162,167].

In stark contrast to mESCs, hESCs require exogenous FGF2 to sustain self-renewal and the capacity to give rise to somatic lineages^[168,169]. The combined use of FGF2 and Activin is the most effective in maintaining hESCs and EpiSCs self-renewal. Recent studies suggest that spontaneous extra-embryonic differentiation, to which both hESCs and EpiSCs are prone, may be blocked by FGF^[3,162]. FGF2 seems to influence the pluripotent state of hESCs on several levels. It activates NANOG expression in cooperation with Activin signaling through SMAD2/3^[147] and synergizes with Noggin to repress trophoblast-inducing BMP signaling^[140,170]. FGF/ERK signaling leads to phosphorylation of c-Myc, c-Jun and c-Fos. Its inhibition leads to a decrease in the expression of core pluripotency factors Nanog, and Oct4^[171-173]. Interestingly, ERK and GSK-3 inhibition have successfully supported the reprogramming procedure for the generation of human iPS cells^[174]. Furthermore, conversion of hESCs to an mESC-like phenotype was achieved by the concerted action of ERK and p38 inhibitors with LIF^[175]. A number of recent studies raise the possibility of differential and sometimes opposing functions of FGF in hESCs, depending on the downstream effector signaling cascades^[172,176].

Exogenous FGF2 is also required for growth and maintenance of CSCs isolated from different human carcinomas (*e.g.*, brain, breast) in tumorspheres, but the mechanisms of FGF action remain to be elucidated^[177,178]. A recent study revealed that the expansion of a functional breast CSC pool in response to estrogens is induced through a paracrine FGF/FGFR/Tbx3 signaling cascade, which is also functional in epithelial stem cells from the normal mammary gland^[179]. Guthridge and colleagues transformed NIH3T3 cells with FGF4 and found out, that HSP-90, p63, LAMP-1 and CyclinD1 were massively activated^[180]. The obtained results suggest a link between FGF4, CSC expansion and tumorigenesis^[181]. Indeed, Cyclin D1 is considered to be a marker for cancer onset and progression, while in stratified epithelial tissues p63 is thought to regulate stem cell characteristics^[182,183].

In conclusion, emerging evidence indicates a complex crosstalk between signaling pathways in development, adult tissue homeostasis and cancer. A better understanding of the pathway interplay and how it controls the biology of ESCs, SCs and CSCs will be essential for the advance of regenerative medicine and for developing effective cancer therapies.

COMMON TRANSCRIPTIONAL REGULATORS OF EMBRYONIC/PLURIPOTENT AND CSC

Regulatory networks in pluripotency

Transcription factors Oct3/4 (Pou5f1), Sox2 and Nanog constitute the “core pluripotency network” that regulates pluripotency of both mouse and human ESCs. They bind synergistically to their own promoter/enhancer elements establishing an auto-regulatory circuit^[4,184]. This master pluripotency network, including additional factors such as Sall4, Klf4, and Stat3 binds to and regulates the expression of two distinct groups of genes in ESCs: genes related to self-renewal (active) and genes related to differentiation (silenced)^[185]. The latter group of genes is co-occupied by the epigenetic silencing complexes Polycomb (PRC1 and PRC2)^[186]. A second important multi-protein complex is centered on the oncoprotein Myc (Myc network). Both networks are mutually regulating each other and interact physically and functionally with chromatin remodeling and modification complexes^[187,188]. The Myc complex binds near the transcription start site (TSS), whereas the core pluripotency complex binds to upstream promoters and enhancers^[189-191]. The master pluripotency factors (Oct4, Sox2, Nanog) form super-enhancers, clusters of enhancer elements that recruit Mediator and determine cell identity^[192,193].

Oct4, Sox2, Klf4 and Myc were the initial factors with the potential to reprogram somatic cells into pluripotency^[188,194]. The individual role of these factors in pluripotent and CSCs will be examined below. Oct4 in ESCs and CSCs Oct4 belongs to the POU family of homeodomain proteins and is encoded by the *Pou5f1* gene. Its expression has been identified in undifferentiated ESCs, embryonic carcinoma cells (ECCs), pluripotent epiblast and embryonic germ cells (EGCs)^[195-197]. Nichols *et al.*^[198] reported that Oct4 expression is essential for the maintenance of ESC properties. They showed that Oct4-deficient embryos did not form a pluripotent inner cell mass and differentiated to trophoblast^[199]. Moreover, inhibition of Oct4 in mESCs led to the upregulation of trophoblast genes (*Cdx2*), while its overexpression caused differentiation into primitive endoderm and mesoderm^[200]. Under serum free culture conditions Oct4 overexpression in ESCs promoted neuroectoderm formation and subsequent neuronal differentiation^[201]. Oct4 cooperates with Sox2 to regulate the expression level of genes important for self-renewal and pluripotent

phenotype of ESCs (e.g., Nanog). On the other hand, when the Oct4 or Sox17 expression levels increased, Sox2 was replaced by Sox17 and targeted genes that trigger the endodermal expression program^[202]. These results indicated that the precise levels of Oct4 determined the ESC fate and that Oct4 is a master player in sustaining stem cell self-renewal.

Numerous studies have indicated that Oct4 plays a crucial role in tumorigenesis and tumor metastasis. It was shown to be upregulated in many human cancers such as bladder, seminoma, prostate and breast cancer^[15,203-206]. Hu *et al.*^[207] reported that in murine lung carcinoma cells and human breast cancer MCF7 cells, ablation of Oct4 expression leads to apoptosis of CSC-like cells through the Oct4/Tcl1/Akt1 pathway and inhibition of tumor growth. Another study confirmed that the reduction of Oct4 in lung cancer cells blocked the clonogenicity and tumor invasion^[70]. Chiou *et al.*^[208] enriched oral CSCs by sphere formation and concluded that these cells highly expressed Oct4 and had similar characteristics of stem cells and malignant tumors. A year later, Rentala *et al.*^[209] reported that the expression of Oct4 in prostate CSCs maintained their stem cells properties. Moreover, it has been revealed that ectopic expression of Oct4 into normal primary breast epithelial preparations generated cell lines which form triple-negative breast carcinomas in nude mice^[210]. Recently, Wang *et al.*^[211] demonstrated that cervical cancer cells expressed higher Oct4 levels than normal cervix cells. They proposed that Oct4 promotes tumor formation *in vivo* and inhibits apoptosis by the activation of miR-125b expression^[211]. In addition, Oct4 has been suggested to regulate stemness of head and neck squamous carcinoma CSCs. The overexpression of Oct4 activated Cyclin E leading to tumor growth and tumor invasion through slug expression^[212].

Sox2 in ESCs and CSCs

Sox2 is a member of the Sox (SRY-related HMG box) family that consists of transcription factors with a single high-mobility group box DNA-binding domain and also belongs to the SOXB1 subgroup^[213]. Sox2 is expressed in the inner cell mass (ICM) and extraembryonic ectoderm of pre-implantation blastocysts^[214]. Sox2 deficient blastocysts could not form a pluripotent ICM. Moreover, Sox2-deficient mESCs differentiated primarily into trophoblast, while the Oct4 overexpression rescued the pluripotency of Sox2-null mESCs^[215]. As a result, Sox2 is critical for the maintenance of Oct4 expression and hence the stem cells' properties. Furthermore, Masui *et al.*^[215] identified a synergistic function of Sox2 and Oct4 for the activation of Oct-Sox enhancers, leading to the regulation of various pluripotency genes, including Nanog, Oct4 and Sox2. Overexpression of Sox2 in ESCs led to their differentiation^[216,217]. This effect was due to the repression of pluripotency genes *Sox2*, *Oct4*, *Nanog*, *Fgf4* and *Utf1*^[216] and the induction of neuroectoderm, mesoderm and trophoblast^[217].

To date, many reports have demonstrated the

involvement of Sox2 in cancer biology and especially in CSCs. Sox2 is critical for osteosarcoma cell self-renewal and antagonizes the pro-differentiation Wnt pathway which can also affect negatively the expression of Sox2^[218]. In addition, it is implicated in the promotion of cell migration and invasion in ovarian cancer, through regulating fibronectin 1^[219]. Studies in gastric cancer showed that inhibition of Sox2 results in reduction of spheres formation and in increase of apoptotic sphere cells^[220]. The contribution of Sox2 in pancreatic CSCs was suggested by the fact that it regulates stemness *via* the control of genes of G1/S transition and EMT^[221]. In prostate CSCs, the inhibition of EGFR signaling led to the decrease of Sox2 expression and self-renewal of prostate CSCs. Moreover, knockdown of Sox2 reduces the ability of prostate CSCs to grow under anchorage-independent conditions^[222]. Similar findings have been extracted from non-small cell lung cancer studies. Singh and colleagues inhibited the expression of Sox2 and noticed a 2.5-fold reduction in sphere formation^[223]. Additionally, EGFR/Src/Akt signaling influenced Sox2 protein expression, due to the decreased levels of Sox2 during the EGFR or SRC inhibition^[223]. In melanoma CSCs, Sox2 is highly expressed and interact with Hedgehog-GLI (HH-GLI) signaling^[224]. In more detail, Santini *et al.*^[224] showed that knockdown of Sox2 decreases the melanoma sphere formation and self-renewal of melanoma CSCs. Two HH-GLI signaling transcription factors, GLI1 and GLI2, have the ability to bind the proximal promoter of Sox2 and thus the HH-GLI signaling regulates Sox2. Finally, Favaro *et al.*^[225] proved that Sox2 is required for CSC maintenance in a high-grade oligodendroglioma mouse model.

Nanog in ESCs and CSCs

Nanog is the third member of the core pluripotency network in undifferentiated ESCs^[226-228]. It is a homeodomain containing transcription factor, which was discovered through a functional screening for pluripotency factors which allowed the maintenance of ESC properties, in the absence of the LIF-STAT3 pathway^[227,228]. Chambers *et al.*^[228] also added that Nanog expression is high in Oct4-null embryos, whereas its overexpression does not counteract the differentiation program of ESCs prompted by Oct4 deletion. In the absence of Nanog, embryos do not form a pluripotent ICM^[227,229], although Nanog-null mESCs can be established^[227,228]. Intriguingly, these Nanog-deficient mESCs although disposed to differentiation, could still be maintained in the undifferentiated state^[227,228]. In 2005, Hyslop *et al.*^[230] showed that Nanog down-regulation in human ESCs promotes differentiation towards extraembryonic lineage, as shown by the upregulation of endodermal- and trophoblastic-characteristic genes. This suggests a pivotal role for Nanog in the maintenance of pluripotency in human embryonic development. Oct4/Sox2 heterodimers bind to the octamer/Sox elements within the Nanog proximal promoter and regulate

Nanog expression in ESCs^[231]. Moreover, Nanog, Oct4 and Sox2 cooperate with signaling pathways mediators resulting in delivering signals directly to the genes regulated by the core factors^[191]. Sites co-occupied by the three core regulators generally have enhancer activity, while the transcription of the respective genes requires the recruitment of at least one of the trio^[191].

Nanog expression was investigated in several types of cancer, including lung, breast, oral, kidney, gastric, cervix, brain, ovarian and prostate cancer^[232-237]. In particular, high expression levels of Nanog are related to a poor prognosis for ovarian serous carcinoma, colorectal, and breast cancer patients^[238-240]. In oral squamous cell and lung adenocarcinoma, Nanog and Oct4 high levels were linked to advanced cancer stage and shorter patient survival^[208,234]. Several groups demonstrated that Nanog expression is much higher in CSCs than in non-stem cancer cells in many types of cancer^[233,241-246]. In colorectal cancer, Nanog-positive CSCs constitute approximately 2% of the total cancer cell population^[241]. In addition, a direct connection between the surface markers of CSC and Nanog has not been clarified yet, but there are many studies which demonstrated that cancer cells expressing these markers would have higher levels of pluripotency genes^[247]. For instance, CD133⁻ or CD44⁻ cancer cells express significant lower levels of Nanog compared to CD133⁺ or CD44⁺ cells, respectively^[248,249]. Moreover, functional studies in various cancer types showed that Nanog induces CSC-like characteristics. Jeter *et al.*^[242] demonstrated that NANOGP8 overexpression in prostate cancer increased clonogenicity and tumor regenerative ability^[247]. Nanog activation leads a small population of colorectal cancer cells to acquire a stem-cell like phenotype^[247]. Furthermore, Han *et al.*^[250] proved that Nanog binds to Cyclin D1 promoter region and regulates proliferation and cell cycle of breast cancer cells^[247,250]. Recently, Siu *et al.*^[251] reported that increased expression of Nanog in ovarian cancer controls cell proliferation, migration and invasion through E-cadherin and FoxJ1 deregulation^[247,251]. These results propose that Nanog may constitute a CSC marker and play a vital role in cancer progression^[251].

Klf4 in ESCs and CSCs

Following the identification of KLF4 as a critical transcription factor for reprogramming, more attention was given to its actions. Klf4 belongs to the Kruppel-like transcription factor family and has a central role in cell cycle regulation, somatic cell reprogramming and pluripotency^[194,252-254]. Klf4 is highly expressed in mESCs and its expression decreases strongly upon differentiation^[255]. The inhibition of Klf4, using RNAi, leads to the differentiation of ESCs^[252,254], while Klf4 ectopic expression postpones differentiation, enhances the expression of Oct4 and promotes self-renewal^[256]. Klf4, in conjunction with Oct4 and Sox2 drives the expression of Lefty1^[257] and Nanog^[258]. In addition, the expression of Klf4 is regulated by STAT3 and Nanog^[252].

being a direct target of both transcription factors^[252,259]. In a recent report, Aksoy *et al.*^[260] described that Klf4 reduction induces differentiation towards visceral and definitive endoderm, concluding that Klf4 inhibits endoderm differentiation in mESCs.

It is not surprising that Klf4 plays a key role in maintaining CSC populations. It is known that telomerase activity is sustained by Klf4 *via* telomerase reverse transcriptase in both CSCs and hESCs, suggesting that Klf4 is important for the long-term proliferative potential of these cells^[261]. Moreover, Hoffmeyer *et al.*^[262] reported that β -catenin regulates Tert expression *via* the interaction with Klf4, supporting a connection between stem cells and oncogenesis. In 2011, Yu *et al.*^[263] reported for the first time that Klf4 is crucial in maintaining breast CSCs and inducing cell migration and invasion. Klf4 is expressed at high levels in CSC populations in mouse primary mammary tumor and human breast cancer cell lines. The inhibition of Klf4 in MCF-7 and MDA-MB-231 decreases the ability of breast CSCs to self-renew and form mammospheres and tumors *in vivo*^[264]. On the other hand, it was shown that Klf4 suppresses metastasis in MDA-MB-231 cells by maintaining the expression of E-cadherin and inhibiting EMT^[265]. Klf4 is highly expressed in colorectal CSCs and its knockdown leads to the decrease of spheres formation, migration, invasion and EMT. These results prove the essential role of Klf4 for maintaining colorectal CSCs^[266]. Furthermore, Wellner *et al.*^[267] reported that Klf4 is induced by ZEB1 through the repression of stemness inhibitor miR-203, and controls/enhances pancreatic and colorectal cancer cells ability to initiate tumor development. Moreover, repression of Klf4 by miR-7 inhibits metastasis of human breast CSCs in nude mice^[268] whereas inhibition of Klf4 by mir-152 suppresses the generation of glioblastoma SCs^[269].

These results propose that Klf4 has important roles not only in stem cell self-renewal and cell motility, but also in CSC and carcinoma cell invasion and metastasis.

Myc in ESCs and CSCs

Myc family which includes three important members - c-Myc, N-Myc and L-Myc - acts as an essential regulator in cell growth, proliferation, differentiation and apoptosis and it is thought to be crucial for stem cell pluripotency and proliferation^[270-272]. Myc is directly regulated by LIF/STAT3 signaling and its constitutive activity renders ESC self-renewal independent of LIF. In contrast, the overexpression of Myc dominant negative form induces differentiation^[273]. Although the individual inactivation of c-Myc and N-Myc has no effect on pluripotency, their simultaneous deletion destabilizes the pluripotent state leading to primitive endoderm and mesoderm differentiation^[274]. Moreover, the overexpression of either c-Myc or N-Myc restore pluripotency of ESCs^[274], supporting the idea that c-Myc and N-Myc perform redundant roles in maintaining pluripotent stem cell identity. Recently, Chappell *et al.*^[275] showed that Myc represses MAPK signaling and results in inhibiting

differentiation.

Several genome-wide analyses have been performed in order to determine how Myc regulates ESC pluripotency. These studies showed that Myc binds to and possibly regulates the transcription of at least 8000 genes in ESCs^[191,276-278]. The Myc-centered complex in ESCs is binding to the TSS and includes also E2F Max and NuA4 HAT complex^[189].

Myc deregulation and elevation have been observed in a wide range of human malignancies, associated with aggressive and poorly differentiated tumors^[279]. It is well-known that Myc is involved in the regulation of 15% of genes in the human genome^[280] and regulates important pro-tumorigenic factors including KRAS and AKT, and tumor-suppressors PTEN and p53^[281,282]. Some reports also showed that Myc-centered protein interaction networks in ESCs are enriched in some cancers, especially in the CSCs, conferring metastatic potential and poor outcome^[16,183]. These findings suggest that the Myc network is responsible for the similarities between ESCs and cancer cells. Wang *et al.*^[283] determined that glioma CSCs expressed high levels of Myc, which is crucial for growth, proliferation and survival. Furthermore, glioma CSCs with low levels of Myc did not generate neurospheres *in vitro* or tumors after xenotransplantation in the brains of immunodeficient mice^[283]. Salcido *et al.*^[284] found that Myc is expressed at high levels in CSC population of small-cell lung cancer. Additionally, it has been demonstrated that Myc expression is significantly upregulated in tumor spheres formed by rhabdomyosarcoma cell lines^[285]. In a recent study, it has been indicated that silencing of Myc using promoter targeting siRNA, decreased prostate CSC maintenance and tumorigenicity and induced senescence in the prostate CSC subpopulation^[286]. The clarification of the role of Myc in hepatic CSCs biology came from Akita work in 2014. They revealed a direct link between c-Myc expression levels and CSC properties and they have further mechanistically demonstrated that c-Myc modulates the hepatic CSC phenotype in a p53-dependent manner^[287].

Collectively the result of accumulating research suggests that ESCs and CSCs share critical transcription factors (Table 3). It is clear that pluripotency factors Oct4, Nanog, Sox2, Klf4 and Myc play a crucial role in cancer development and contribute to cancer treatment. However, further investigation of their role in determining the CSC phenotypes, will provide the exact regulatory mechanisms and possibly new regulatory factors relating to tumorigenesis and metastasis.

COMMON EPIGENETIC REGULATORS IN ESCS AND CSC

Cell epigenetic state has been recognized as an important factor in diverse developmental and differentiation processes *via* global or gene specific regulatory mechanisms. Genome wide analyses and

Table 3 Common signaling pathways, transcription factors, non-coding RNAs and epigenetic regulators of embryonic stem cells and cancer stem cells in solid tumors

ESCs		CSC type
Signaling pathways		
Wnt/ β -catenin	Self-renewal in mESCs/hESCs ^[91] Differentiation in hESCs ^[98]	Brain ^[104] Breast ^[103] Colon ^[99] Lung ^[475] Prostate ^[476]
Hedgehog	Self-renewal in mESCs ^[109] Differentiation in hESCs ^[108]	Brain ^[112] Breast ^[111] Pancreas ^[115]
Notch	Differentiation in mESCs and hESCs ^[117]	Brain ^[126] Breast ^[128] Colon ^[125] Pancreas ^[123]
TGF- β	Activin/Nodal promote self-renewal in mESCs and hESCs ^[134,135] BMP promotes self-renewal in mESCs ^[138] and differentiation in hESCs ^[141]	Brain ^[158] Breast ^[157] Skin ^[133]
FGF	Differentiation in mESCs ^[164] Self-renewal in hESCs ^[169]	Bladder ^[477] Brain ^[177] Breast ^[178]
Transcription factors		
4-Oct	Self-renewal and pluripotency ^[198]	Breast ^[207,210] Lung ^[70,207] Oral ^[208] Prostate ^[209] Cervical ^[211] Head and neck ^[212]
Sox2	Self-renewal and pluripotency ^[215]	Osteosarcoma ^[218] Ovarian ^[219] Gastric ^[220] Pancreatic ^[221] Prostate ^[222] Lung ^[223] Melanoma ^[224]
Nanog	Self-renewal and pluripotency ^[226-228]	Breast ^[250] Oral ^[208] Lung ^[234] Colorectal ^[238,241] Prostate ^[242] Ovarian ^[251]
Klf4	Self-renewal and pluripotency ^[254,256]	Breast ^[263-265,268] Colorectal ^[266,267] Pancreatic ^[267] Brain ^[269]
c-Myc	Self-renewal and pluripotency ^[189,191,270]	Brain ^[283] Lung ^[284] Rhabdomyosarcoma ^[285] Prostate ^[286] Hepatic ^[287]
DNA methylation regulators		
DNMT1	Differentiation ^[293]	Colon ^[336] Breast ^[338]
TET2	Differentiation ^[315,322]	Breast ^[344]
Chromatin modifications regulators		
EZH2	Self-renewal and pluripotency ^[186]	Breast ^[393] Pancreas ^[393] Brain ^[395] Prostate ^[396] Bone ^[397]

BMI-1	Self-renewal and pluripotency ^[186]	Prostate ^[400] Esophageal ^[401] Head and neck ^[402] Cervical ^[403] Colorectal ^[404] Laryngeal ^[405] Ovarian ^[406] Salivary adenoid cystic carcinoma ^[399]
Suz12	Self-renewal and pluripotency ^[186]	Breast ^[407] Colon ^[408]
MLL1	Self-renewal and pluripotency ^[186]	Brain ^[409]
MicroRNAs		
Let-7	Differentiation ^[430,431]	Breast ^[434] Prostate ^[448] Breast ^[437]
MiR-200 family	Differentiation ^[267]	Brain ^[444] Prostate ^[448] Pancreatic ^[451] Gastric ^[450] Colon ^[452]
MiR-34a	Differentiation ^[429]	Brain ^[444] Prostate ^[448] Pancreatic ^[451] Gastric ^[450] Colon ^[452]
MiR-145	Differentiation ^[424]	Brain ^[445] Breast ^[440]
Long non-coding RNAs		
LncRNA-RoR	Self-renewal ^[418]	Breast ^[461]

CSC: Cancer stem cell; ESCs: Embryonic stem cells; mESCs: Mouse ESCs; hESCs: Human ESCs.

knockout studies provide new information about the role of epigenetic processes in self-renewal and cancer initiation and permit the development of "epigenetic" therapy as a cancer treatment option^[288,289].

This part of the review highlights our current view of the most important common epigenetic regulators associated with DNA methylation, histone modifications as well as long-non coding RNAs and miRNAs in ESCs and CSCs, their significance in normal development and their deregulation in tumorigenesis.

DNA methylation regulators in ESCs and CSCs

DNA methylation is generated by DNA methyltransferases (DNMTs), enzymes that add methyl groups on cytosines. The most studied members of the mammalian DNMT family include DNMT1, DNMT3a and DNMT3b. DNMT1 is thought to be responsible for the replicative maintenance of the DNA methylation, while DNMT3A and DNMT3B function as *de novo* methyltransferases^[290]. Nevertheless, recent evidence shows that DNMT1 may also be required for *de novo* DNA methylation^[291] and that DNMT3a and DNMT3b can also participate in the maintenance of the DNA methylome^[292]. These findings emphasize the need to clarify the exact role of each DNMT and their potential crosstalk.

Restriction enzyme digestion-mediated and Methylation DNA Immunoprecipitation-ChIP (MeDIP-ChIP) analyses of global DNA methylation show that the DNA methylation levels are reduced in mouse ESC compared to the somatic cells and that methylations on promoter regions lie primarily outside of CpG islands^[293-295]. A methylation analysis of CpGs by Bibikova *et al.*^[296]

reported that the methylation levels of over 370 genes in 14 hESC lines were lower than those of mESCs^[294].

Two groups, Lister *et al.*^[297] and Laurent *et al.*^[298] have compared the methylation maps of hESCs and human fibroblast cell lines and observed significantly higher levels of non-CpG methylation present in hESCs that may be due to differences in methylation regulatory mechanisms between un- and differentiated cell types. Laurent *et al.*^[298] observed that CpA methylation was the most frequent type of non-CpG methylation in hESCs, and that this modification was lost upon differentiation. Non-CpG methylation is also present in mESCs and is reduced from 8% to 4.3%, six days after induction of differentiation^[299]. This non-CpG methylation is more abundant within gene bodies than promoter regions and is catalyzed by DNMT3a and DNMT3b requiring also the presence of DNMT3L^[300]. Methylation profiles of iPS cells are highly similar to the ones of ESC, showing that this is a unique characteristic of pluripotent cells^[297,301]. However its functional role remains unclear.

Deletion of Dnmt1 or 3b in mice results in embryonic lethality^[302], while Dnmt3a^{-/-} mice die within 4 wk after birth showing that these enzymes are essential for normal development^[303]. DNMT1 overexpressing ESCs when injected in blastocysts resulted in embryonic lethality, resembling the effect of DNMT1 deficiency^[304]. DNMT1, DNMT3a and DNMT3b are expressed in hESCs^[305]. Mouse knockout experiments have shown that deletion of DNMTs does not affect ESCs self-renewal but deregulates cell specification, suggesting that global methylation may be dispensable for the undifferentiated state but is critical for differentiation. More specifically, DNMT1^{-/-} EBs contain a large number of Oct4 positive pluripotent cells indicating that methylation is required for proper cell differentiation^[293]. In Dnmt3a^{-/-} Dnmt3b^{-/-} mouse ESCs, only 0.6% of CpGs are demethylated^[293], suggesting that these molecules have limited contribution on global DNA methylation. Furthermore, Pawlak *et al.*^[306] showed that Dnmt3a and Dnmt3b are dispensable for nuclear reprogramming.

Although many observations implied the reversibility of DNA methylation, only recently were identified the TET (ten eleven translocation) enzymes that actively demethylate DNA. The mammalian TET family has three members, TET1, TET2 and TET3 that catalyze 5mC oxidation and generate the 5mC derivatives 5hmC, 5fC or 5caC (5-hydroxymethylcytosine, 5-formylcytosine, 5-carboxylcytosine)^[307-310]. 5hmC is the first intermediate toward DNA demethylation and its variable amounts in different cells and tissues implies a distinct regulatory role^[311]. Recent reports, studying the genomic distribution of 5hmC in mouse and human ESCs, provide evidence that this modification may function as a specific epigenetic mark in gene expression regulation^[312-314].

The expression levels of TET1 and 2 are high in undifferentiated ESCs and decline upon differentiation, in parallel with an increase of TET3^[315]. Further studies have shown that TET1 adds 5hmC on promoter regions and TSSs whereas TET2 activity is detected

in the coding regions^[316]. TET1 activity in ESCs is associated with the demethylation and expression of pluripotency related genes as well as repression of Polycomb targeted developmental regulators^[317]. Moreover, TET proteins were found to participate in various gene expression regulatory complexes *via* interaction with Sin3A co-repressor^[318], Polycomb Repressing Complex 2 (PRC2)^[317] and the O-linked N-acetylglucosaminetransferase, (Ogt)^[319,320].

Although earlier studies have suggested a role for TET1 and TET2 in ESC self-renewal^[321], it was recently clarified that these enzymes are in fact required for proper differentiation of ESCs^[322]. TET1 and TET2 are regulated by the Oct4-Sox2 complex in ESCs and TET1 knock down promotes the differentiation toward endoderm/mesoderm and trophoblast pathways^[315]. A Tet1/2/3 triple knock-out mouse ESC line was unable to generate embryoid bodies, teratomas and could not give rise to healthy chimeras^[322].

A current model proposes that the accumulation of epigenetic and/or genetic changes in a normal adult stem cell generates eventually a heterogeneous tumor population that contains a subset of "CSCs" that are responsible for its long term maintenance^[323]. Regardless of the hierarchical or stochastic nature of the CSCs, studying their epigenome is of paramount importance both for understanding the origin and evolution of cancer as well as applying novel epigenetic therapies. As a result, a growing number of studies has recently been directed in the elucidation of common or distinct epigenetic mechanisms that govern the self-renewal program of ESCs and CSCs.

Cancer epigenome exhibits global DNA hypomethylation and specific promoter hypermethylation^[324,325]. DNA hypomethylation promotes cancer development by increasing genomic instability and activating growth-promoting genes such as R-Ras^[326]. On the contrary site-specific hypermethylation favors oncogenesis by repressing tumor suppressor genes, other genes encoding transcription and DNA repair factors as well as PRC target genes^[327,328]. The above studies provide compelling evidence for deregulated DNA methylation in cancer but do not address distinct cell subpopulations. In this line, Yasuda *et al.*^[329] studied 10 tumor suppressor genes (TSGs) in bulk and CSC enriched MCF7 breast cancer cells based on their ability to form tumor-spheres and found lower DNA methylation levels and H3K27m3 marks in the latter population. Ikegaki *et al.*^[330] proved that epigenetic modifiers can affect the expression of stemness genes and contribute to the establishment of CSCs. They showed that short-term treatment of Neuroblastoma cell lines with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5AdC) and/or the histone deacetylase inhibitor 4-phenylbutyrate, enhances their CSC phenotype^[330].

The expression levels of DNMTs are elevated in several cancer types^[331-333]. DNMT3b has been shown to play a crucial role in *de novo* hypermethylation of promoter CpG islands. In line with the role of DNMTs in

cancer development, sustained overexpression of the murine *dnmt1* gene in NIH3T3 cells results in cellular transformation^[334]. Conversely, reduction of DNMT1 by an antisense DNMT-RNA reversed the transformed phenotype of the Y1 tumor cell line^[335].

Morita *et al.*^[336] compared colorectal HCT116 WT and its *dnmt1* knockout derivative and showed that the latter has reduced stem cell markers and contained less CSCs, as assessed by tumor formation following xenotransplantation. Again no difference in DNMT3a and DNMT3b was detected^[336]. The importance of DNMT1 in CSC self-renewal was further confirmed by Trowbridge *et al.*^[337] using MLL-AF9 induced mouse leukemia. Additionally, a recent paper about the lyotropic reagent chloroquine reports that it can eliminate CSCs in a TNBC population through reduction of DNMT1 and Jak2 expression^[338]. The above studies demonstrate the role of DNMT1 in the maintenance of the CSC properties and *in vivo* tumorigenicity.

Concerning the effects of DNA demethylase, 5hmC levels are decreased in a broad range of cancer cells^[339]. TET1 has been found as a fusion partner of MLL in a subset of patients with acute myeloid leukemia^[340,341]. TET2 has been reported as one of the most frequently mutated genes in hematopoietic cancer types, but many mutations appear in sites that don't affect its enzymatic activity^[342]. In agreement with this, it was recently found that enzymatically inactive TET1, acting as a transcriptional co-activator for Hif1a, is required for EMT^[343]. Additionally, work by Song *et al.*^[344] has shown that TET2 is involved in breast cancer stemness and metastases due to the silencing of miR-200.

Investigating the methylation signature of CSCs permits identification of modifiers that can target their stemness properties, leading to increased tumor sensitivity to chemotherapy. Current DNMT inhibitors used in cancer therapy, such as Decitabine (5'-aza-2' deoxycytidine) act through incorporation into DNA therefore causing adverse side effects^[345]. Less hazardous alternatives include use of small molecule inhibitors such as SGI-1027^[346] and dietary phytochemicals^[342].

Chromatin modifiers in ESCs and CSCs

It is the complex interplay of DNA methylation with the posttranslational modifications of the histone tails that determines the transcriptional activity of a particular locus^[347]. The effect of histone modifications on diverse cellular functions, has caused intense interest in studying the chromatin modifying enzymes. These epigenetic modifiers include a variety of factors, such as histone methyltransferases (HMTs), demethylases (HDMs), acetyltransferases (HATs) and deacetylases (HDACs)^[348,349]. All the above mentioned factors participate in the regulation of chromatin structure that in turn governs gene transcription.

ESCs are characterized by permissive chromatin structure and consequently higher transcriptional activity compared to differentiated cells. Generally,

histone marks associated with active transcription are more abundant in ESCs and become reduced upon differentiation^[350,351], whereas repressive marks appear in higher levels in differentiated cells. Another interesting feature most commonly found in ESCs are bivalent domains, which are defined by the presence of the active H3K4me3 mark alongside the repressive H3K27me3 mark and is believed to hold genes in a "transcription-ready" state^[352,353]. However a recent study by Denissov *et al.*^[354] challenges the prevailing view by showing that not all bivalently marked genes in mESCs lose their differentiation responsiveness upon loss of H3K4me3. Thus, the role of bivalency and its association with pluripotency remains an open question.

Self-renewal in ESCs necessitates the action of chromatin repressive complexes in order to inhibit expression of differentiation-promoting genes. The best studied silencers of differentiation pathways in pluripotent cells are the PcG proteins, which are organized into two multiprotein complexes PRC1 and PRC2^[355]. PRCs are highly expressed in ESCs and bind mainly to CpG-rich promoters of developmentally regulated genes^[186,356].

The PRC2 complex has three core protein subunits: The enhancer of zeste homology (EZH2) component that catalyzes di- and trimethylation of H3k27, Embryonic ectoderm development (Eed) and suppressor of zeste (Suz12). PRC2 triggers gene silencing by recruiting PRC1, histone deacetylases and DNA methyltransferases^[357]. The PRC1 complex composition is highly variable and the canonical complexes include CBX (polycomb), polycomb group factor (PCGF), human polyhomeotic homolog (HPH) and RING, the E3-ligase that catalyzes the monoubiquitination of histone H2A on lysine 119^[358].

Depletion of PRC2 results in embryonic lethality in mice^[359], while mESCs lacking Eed, Suz12 or Ezh2 show loss of H3K27me2/3, retention of self-renewal capacity and *in vitro* differentiation defects^[360-363]. On the contrary, inactivation of PRC1 in mice leads to deficiencies concerning later developmental stages^[364]. RING1b (subunit of PRC1 complex) deficient mESCs show a slight deregulation of some genes and loss of differentiation potential^[365], whereas mESCs double mutated for Ring1a/Ring1b lose also the ability to self-renew^[366]. In summary, PcG proteins seem to be required for proper ESC cell fate transition but not for their self-renewal. More information is required about their partners to fully understand their regulatory role in ESCs^[367]. Moreover, it was shown that PRC1 and PRC2 can occupy distinct genomic sites and act independently^[368].

The maintenance of the expression program that determines pluripotency requires the presence of both repressive and activatory chromatin modifiers. A complex that counteracts the repressing effect of Polycomb is the Trithorax/MLL^[369]. Trithorax group (TrxG) complex contains a histone K4 tri-methyltransferase

(Set1a/b, MLL1-4), a subunit that recognizes the H3K4me3 mark, tryptophan-aspartate repeat protein 5 (WDR5), absent-small-homeotic-2-like (Ash2L), retinoblastoma-binding protein 5 (RbBP5) and dumpy-30 (DPY-30)^[369,370].

Both activating H3K4me3 and repressive H3K27me3 and H3K9me3 marks are removed by histone demethylases belonging to the Jumonji domain-containing protein family (Jmjd)^[371]. Jmjd proteins connect ESC core transcriptional network with chromatin modulation. More specifically, Jmjd2c participates in stem cell maintenance by reversing H3K9me3 marks at the Nanog promoter, consequently protecting it from silencing, whereas *Jmjd1a/2c* gene expression is positively regulated by Oct4^[372]. The diverse role of Jmjds is underlined by Pasini *et al.*^[362], who reported the involvement of JARID2 in the recruitment of PRC2 by differentiation-related genes in ES cells.

Another chromatin modifier called LSD1/KDM1 factor, is a histone demethylase that suppresses gene expression by removing methylation groups from H3K4. LSD1 has been found to colocalize with NuRD at the enhancer of pluripotency genes and down-regulates their expression upon differentiation^[373]. Finally, Suv39H1, Suv39H2 and G9a methylases, generate H3K9me3 repressive mark important in ESCs^[374].

Histone acetyltransferases (HATs) and the equivalent histone deacetylases (HDACs) constitute another critical category of chromatin modifiers acting as co-activators or co-repressors respectively^[375]. Although deacetylation is associated with gene silencing, ChIP-sequencing studies show that HDACs also colocalize with acetyltransferases at transcriptionally active loci, probably to reset acetylation levels after gene activation^[376].

HDAC1 and HDAC2, the most studied HDACs in ESCs, have been shown to be dispensable for mESCs self-renewal^[377]. However, HDAC1 knockout- ESCs show differentiation defects. HDAC1 and HDAC2 are usually part of large complexes with repressive action like NuRD, CoREST and Sin3^[378]. Alteration of the histone acetylation pattern interferes with stem cell pluripotency and differentiation^[376] as well as reprogramming^[379]. Most importantly inhibitors of HDACs are used as facilitators of reprogramming^[380]. Noteworthy, NuRD complex, which couples chromatin remodeling capacity and histone deacetylation activity^[381], has been shown to act as negative regulator of pluripotency associated genes by fine tuning their expression levels and sensitizing cells to differentiation stimuli^[382]. The distinct repression targets of PRCs and NuRD may explain why reprogramming efficiency is increased by overexpression of PRCs components^[383], but depletion of NuRD proteins^[384].

Finally, small molecule drugs targeting histone demethylases or DNA demethylases are also valuable tools for reprogramming since they can substitute for transcription factors^[385].

The information related to chromatin modifiers

in ESCs has facilitated the elucidation of their role in tumorigenesis. In addition to DNA hypo-methylation, reduced histone acetylation but enhanced histone methylation is an epigenetic feature characteristic of cancer cells^[386,387].

Polycomb HMT proteins are commonly upregulated in cancer^[388]. EZH2, the best studied PcG protein has been found to promote tumor growth by inhibiting pro-differentiation pathways and enhancing cell cycle progression^[389]. Furthermore HDMs, which reverse the action of HMTs, like LSD1, have also been implicated in oncogenesis^[390]. Increased HDAC activity usually characterizes cancer cells^[391]. Resetting normal acetylation levels through treatment with HDAC inhibitors (HDACi) has lowered tumorigenicity, suggesting HDACs as attractive targets for cancer epigenetic therapy^[392].

The role of chromatin regulators in CSCs has recently started to be under study with Polycomb Group (PcGs) proteins to be in the spotlight. EZH2 proved to be essential for the maintenance of breast and pancreatic CSCs^[393]. Intriguingly, EZH2 promotes NFκB signaling in ER-negative breast cancer cells^[243], that in turn has been shown to contribute to the generation of CSCs through a positive feedback loop involving IL6^[394]. In previous studies, silencing of EZH2 in glioblastoma CSCs significantly delayed intracranial tumor formation, demonstrating the necessity for EZH2 in CSC-driven tumorigenesis^[395], whereas treatment of prostate cancer cells with the PRC2 inhibitor DZnep (3-Deazaneplanocin A) inhibited CSC spheroid formation and decreased CSC frequency^[396]. Furthermore, EZH2 seems to contribute to the stemness phenotype of Ewing tumors by suppressing endothelial and neuroectodermal differentiation^[397]. Gupta *et al.*^[398] developed a model of phenotypic transitions to study stochasticity in regulating cell-state equilibrium in cancer cells and EZH2 was used as one of the main phenotypic markers for the CSC population.

Increasing number of studies implicate BMI-1, another PcG member, in cancer stemness. Its expression has been found elevated in many CSC populations such as in salivary adenoid cystic carcinoma^[399], prostate^[400], esophageal^[401], head and neck^[402], cervical^[403], colorectal^[404], laryngeal^[405] and ovarian^[406] CSCs. In addition it has been reported that depletion of Suz12 - a component of PRC2 complex - results in the blockade of mammospheres formation^[407] and increased apoptosis in colon CSCs^[408]. These findings highlight Suz12, as an essential regulator of CSCs.

Downregulation of the histone methyltransferase MLL1 reduces CSC self-renewal and tumorigenicity suggesting a role in CSCs^[409]. Additionally, HDMs like LSD1 are crucial for the biology of CSCs too. Wang *et al.*^[410] proved that inhibition of LSD1 inhibits the proliferation of pluripotent cancer cells but not that of normal somatic or non-pluripotent cancer cells. Except for histone methylation, histone acetylation is also essential for CSCs as a recent study showed that inhibition of HDACs limits the population of CSCs of

head and neck cancer^[411].

In conclusion, epigenetic regulators such as members of the Polycomb and Trithorax complexes, histone demethylases and histone deacetylases are essential for ESC pluripotency and at the same time can promote cancer stemness (Table 3). DNA methyltransferases are indispensable for both cell types and have been already reported as important targets for iPS generation and cancer chemotherapies (Table 3). Finally, DNA demethylases (TET 1, 2, 3) are critical targets for iPS generation, whereas their functions in CSCs await further clarification (Table 3).

MicroRNAs in ESCs and CSCs

The crucial role of microRNAs in mouse and human ESCs has been identified using Dicer and DGCR8 knockout mice. Dicer deletion resulted in embryonic lethality in mice^[412], while DGCR8-deficient mouse ESCs retained self-renewal capacity^[413].

Among the first families of microRNAs identified, miR-290-295 (miR-371 family, human homologous) and miR-302-367 clusters, which include the majority of miRNAs in mouse and human ESCs. Common characteristics of the two clusters is the promoter binding of the core pluripotency transcription factors (Oct4, Sox2 and Nanog)^[414] and the decrease of their expression during differentiation^[415]. Reintroduction of these miRNAs into Dicer1-knockout^[416] and DGCR8-knockout^[413] mice rescued proliferation and normal ESC self-renewal, respectively. It was found that these two clusters maintain the self-renewal by targeting retinoblastoma like 2 (Rb12), a repressor of DNA methyltransferases (Dnmt3a and Dnmt3b). The latter methylates CpG islands and epigenetically silences Oct4^[416,417]. In addition, these miRNAs were shown to regulate the G1/S transition of the ESC cell cycle, by repressing directly or indirectly the expression of the G1/S transition inhibitors (p21, Lats2, Rb1, Rb12 and Rb11)^[418,419].

Other miRNAs acting on the hESC cell cycle were studied by Qi *et al.*^[420], showed that miR-195 and miR-372 promote the transition of G2/M and G1/S, by suppressing the G2/M checkpoint kinase WEE1 and CDKN1A respectively^[420]. Furthermore, miR-92a and 92b were identified to target the *CDKN1C* gene and CDKN2B (known as p57), promoting in this way the G1/S transition^[421].

Beside their function in maintaining pluripotency, miRNAs play important role in the differentiation of ESCs. Tay *et al.*^[422,423] demonstrated that miR-134, miR-296 and miR-470 bind to the coding regions of Oct4, Sox2 and Nanog and suppress their expression and thus the self-renewal state. Similarly, miR-200c, miR-203 and miR-183 target Sox2 and Klf4^[267], while miR-145 was shown to repress human OCT4, Sox2 and KLF4 by binding to their 3'UTR, suggesting its role to regulate pluripotency^[424]. In another study, induction of differentiation caused the increase of miR-21 expression levels revealing its crucial role in stem cell

differentiation by targeting Nanog, Sox2 and Oct4^[425]. Moreover, the expression of miR-22 was also detected in high levels during ESC differentiation^[426]. Landgraf *et al.*^[427] demonstrated that miR-26a, miR-99b, miR-193, miR-199a-5p, and miR-218 are able to suppress the self-renewal of ESCs but the mechanism remains unclear. Another example of miRNAs that regulate the differentiation of ESCs is the miR-125 and miR-181 clusters^[428]. A recent study showed that miR-125 and miR-181 families suppress Cbx7, the primary Polycomb ortholog of the PRC1 complex, in undifferentiated ESCs. Their overexpression leads to the differentiation of ESCs via regulation of Cbx7^[428]. Furthermore, it was found that miR-34a, miR-100, and miR-137 are required for the differentiation of ESCs, and that they function in part by targeting Sirt1, Smarca5 and Jarid1b mRNAs^[429].

Let-7 is another miRNA family which has been widely involved in the establishment of the differentiated cell fate. Melton *et al.*^[430] identified that silencing of pluripotency and self-renewal of ESCs can be caused by the introduction of let-7 into DGCR8-knockout ESCs. In addition, let-7 binds to 3'UTR, inhibits expression of several stemness factors (c-Myc, Sall4n, Lin28) and induces ESCs differentiation^[430]. Interestingly, Lin28 forms a negative feedback loop with let-7, resulting from its blocking function during let-7 biogenesis at the Dicer processing step^[431,432]. Let-7 can also target the G1/S transition activators (cdc25a, cdk6, cyclinD1 and cyclinD2) that increase susceptibility of G1 phase cells to pro-differentiation signals and promote differentiation of ESCs^[431,432].

MiRNAs also play important roles in regulating CSC properties such as cell cycle exit and pluripotency, pro-survival and antistress mechanisms, EMT, migration and invasion, which contribute to tumor metastatic potential.

Because of the early discovery and better understanding of breast CSCs, miRNA studies are more advanced in this cancer type^[433]. Let-7 regulates the properties of CSCs and its overexpression results in the reduction of mammosphere and tumor formation, metastasis and cell proliferation. Moreover, let-7 diminishes the expression of HMAG2, c-Myc, and RAS and controls CSC properties^[434]. Another miRNA that is repressed in breast CSCs is miR-30. The inhibition of miR-30 induces metastasis and self-renewal of breast CSCs^[435] and the transfection of both let-7 and miR-30, causes a more complete reduction of mammospheres formation and CSC abilities in breast CSCs^[435].

Iliopoulos *et al.*^[436] showed the importance of an inflammatory positive feedback loop involving NF-κB, Lin28, let-7 and Il6 in the epigenetic maintenance of the cell transformation state following Src oncogenes activation. The same group using miRNA profiling defined a set of 22 miRNAs that are differentially expressed in normal vs CSCs that included the let-7 and the miR-200 families^[407]. Interestingly, Shimono *et al.*^[437] also identified that miR-200 cluster is down-regulated in breast CSCs and miR-200c reduces the

tumor formation. In addition, members of this family have the ability to regulate the breast CSC properties by repressing the BMI-1^[437] and a subunit of a polycomb repressor complex, SUZ12^[407]. Furthermore, several studies showed that miR-200 family and miR-205 modulate EMT, which is crucial for metastasis and tumor invasion. It was found that the expression of these miRNAs is decreased in cells undergoing TGF-beta induced EMT and their overexpression inhibits the EMT process. These miRNAs modulate the expression of EMT activators, ZEB1 and ZEB2, causing the inhibition of EMT program^[438]. In contrast, ZEB1 and ZEB2 activate EMT by forming a double negative feedback loop with the miR-200 family, resulting from their binding to promoter regions of miR-200 family members^[439]. As previously mentioned, miR-22 directly suppresses the expression of the TET family members (TET1-3), which are implicated in the demethylation of miR-200 promoter. In other words, miR-22 inhibits the activity of miR-200 cluster and promotes EMT and metastasis^[344]. Polytaichou *et al.*^[440] identified that miR-15/16, miR-103/107, miR-145, miR-335, and miR-128b inhibit breast CSC function and growth by directly inhibiting the expression of Suz12, Bmi1, Zeb1, Zeb2, and Klf4.

MiR-9/9*, miR-17 and miR-106b are the mostly represented miRNAs in the CSC population in glioblastoma. It was demonstrated that knocking down of miR-9 and miR-17 causes a reduction of neurosphere formation^[441]. Another study showed that miR-128 reduces glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo*^[442]. Furthermore, miR-128 overexpression modulates the properties of glioma^[443] and prostate CSCs^[400] by inhibiting BMI-1. Li *et al.*^[444] examined the role of miR-34a in brain tumor cells and human gliomas and they demonstrated that miR-34a causes cell cycle arrest, apoptosis or xenograft tumor repression, regulating the glioblastoma CSCs. In CD133⁺ CSCs of glioblastoma, miR-145 causes the inhibition of tumor formation as well as the reduction of CSC properties by targeting Sox2 and OCT4^[445]. miRNA expression profiles of glioblastoma stem cells and non-stem cells revealed that miR-451 is downregulated in the glioma CSCs. Transfection with the above miR leads to the inhibition of tumor growth of glioma CSCs and neurospheres formation^[446]. Another miRNA which is important for CSCs of brain tumors is miR-199b-5p. It was shown that miR-199b-5p blocks Notch signaling and inhibits the self-renewal capacity of medulloblastoma cells by targeting HES-1, a transcription factor of the Notch signaling pathway^[447].

Using prostate CSCs, Liu *et al.*^[448] performed miRNAs expression profiling and found that miR-34a, let-7b, miR-106a and miR-141 are downregulated whereas miR-301 and miR-452 are increased. They also showed that overexpression of miR-34a inhibits prostate CSC metastasis by targeting CD44, while knocking-down of miR-34a promotes tumor migration and development^[448]. MiR-320 was also investigated for

its role in prostate CSCs. Hsieh *et al.*^[449] illustrated that β -catenin is directly targeted by miR-320. Moreover, gene expression profile of miR-320-overexpressing prostate cancer cells revealed a diminished expression of both the genes involved in the Wnt/ β -catenin pathway and the markers of CSCs^[449].

As mentioned before, miR-34a does not play pivotal role only in glioma and prostate CSCs, but also in pancreatic and gastric CSCs^[450,451]. Overexpression of miR-34a in these two cancer types reduces sphere formation and tumor regeneration^[450,451]. Bu *et al.*^[452] reported that miR-34a inhibits colon CSC self-renewal and suppresses tumor formation. Particularly, miR-34a targets Notch-1 resulting in inhibition of Notch signaling which is frequently crucial in colorectal cancer^[452].

LncRNAs in ESCs and CSCs

A number of studies investigated the role of lncRNAs in self-renewal and differentiation of mouse and human ESCs. By performing genome-wide screening, Sheik Mohamed *et al.*^[453] identified four lncRNAs residing proximally to the genomic binding sites of Oct4 and Nanog. Two of them, the AK028326 and the AK141205 have been shown to be the direct targets of Oct4 and Nanog, while their overexpression or inhibition resulted in dramatic changes in the mRNA expression levels of the two core transcription factors, revealing their involvement in the regulatory network^[453]. Chakraborty *et al.*^[454] introduced a new technique combining knock-down and localization analysis of noncoding RNAs (c-KLAN) to study lncRNAs. By inhibiting Panct-1, a non-coding transcript, the amounts of Oct4 and Nanog mRNA were decreased, whereas the expression of differentiation markers increased (Gata6, Fgf5, and T-Brachyury)^[454]. To further examine the role of lncRNAs in ESCs, Wang *et al.*^[418] demonstrated the lncRNA-RoR function as a critical regulator of self-renewal and differentiation in hESCs. lncRNA-RoR prevents the suppression of Oct4, Sox2 and Nanog by miRNAs (miR-145) and forms a feedback loop with the core transcription factors and microRNAs^[418].

Concerning hESCs, the lncRNA_N1 lncRNA_N2 and lncRNA_N3 are important regulators of neuronal differentiation fate^[455].

LncRNAs have also been investigated in cancer. Their role is not well understood in CSCs, but recent studies have correlated some lncRNAs with CSC activity, based on their ability to promote metastasis^[103]. Gutschner *et al.*^[456] developed a metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) knockout model in human lung tumor cells, which confirmed the role of MALAT1 as a biomarker for lung cancer metastasis and revealed its ability to regulate genes associated with lung cancer metastasis^[456]. Furthermore, MALAT1 was implicated in cervical cancer by regulating gene expression (caspase-3, -8, Bax, Bcl-2, BclxL), while in a more recent study it was shown that MALAT1 promotes cell migration and proliferation of cervical

cancer cells^[457,458]. HOTAIR is another lncRNA which has been widely studied for its role in cancer. Gupta *et al.*^[459] demonstrated that HOTAIR is a strong metastasis and tumor invasiveness biomarker in primary breast tumors. This function is due to the recruitment of PRC2 that silences metastasis suppressor genes^[459]. Moreover, other studies suggest that HOTAIR is a potential biomarker for the existence of lymph node metastasis in hepatocellular carcinoma (HCCs)^[460]. As discussed earlier, lncRNA-RoR is an important ESC self-renewal regulator. Recently, it has been shown that its expression was negatively regulated by the NRF2 transcription factor in mammary stem cells^[461]. In particular, the inhibition of NRF2 led to an increase of both mammosphere formation in breast cancer cells and lncRNA-RoR levels supporting an involvement of lncRNA-RoR in tumorigenicity^[461].

To summarize, the above studies demonstrate an essential role of ncRNAs in stemness of ESCs and CSCs (Table 3). A better understanding of ncRNA biology will ultimately provide further insights into the molecular mechanisms of tumorigenesis and lead to the development of new therapeutic strategies against cancer.

DISCUSSION

In recent years there is a tremendous expansion of research focused on the biology of stem cells. As understanding of the differences and similarities of stem cells of various normal or abnormal developmental origins increases, so is the appreciation of their great complexity. In this review we have presented an overall comparison between embryonic and CSCs in terms of biomarkers (Tables 1 and 2), signaling pathways (Figure 1, Table 3), transcriptional and epigenetic regulators (Table 3).

Cell surface markers are very important for the characterization and separation of stem cells. Pluripotent (ES, iPS) cell markers are very useful for fast and effective enrichment when undifferentiated cells are required (positive selection). In addition, such markers are valuable in order to eliminate the pluripotent cells from their differentiated descendants (negative selection) before transplantation. Concerning cancer, a small proportion of cells that bears stem cell features - CSCs - are endowed with self-renewal and differentiation capacity and enhanced tumorigenicity. These cells are refractive to conventional therapies. In line with the above, promising therapies can be based on targeting CSC *via* their specific surface markers, as it has been shown in experimental models and clinical trials. An obvious disadvantage of such an approach is that while various markers are currently reported to mark or enrich for CSCs, no exclusive CSC markers are so far available to ensure high cell specificity of targeting. Table 2 shows combinations of markers that are used in order to identify CSCs from various cancer types. Among them, the major category are cell adhesion or

communication molecules CD44, Esa/EpCAM, CD166, CD129 and receptor-associated proteins such as Lgr5, c-met, c-kit. The enzymatic activity of ALDH can be a useful tool for separating CSCs, however a detailed identification of the active isoforms that are expressed in various cancer types is required^[462]. Most importantly, some ALDH isoforms can have functional roles in CSCs since they are directly involved in retinoic acid signaling and stemness. Another category of various CSC markers are the ABC transporters (Table 2) that actually render CSCs resistant to therapies. A recent promising method for CSC isolation is based on measurement of the intrinsic auto-fluorescence of epithelial CSCs due to the accumulation of riboflavin in cytoplasmic complexes containing ABCG2 transporters^[463]. Some CSC markers are also common with those of ESCs such as CD133, SSEA1, EpCAM and the pluripotency transcription factors Oct4, Sox2 and Nanog. Brescia *et al.*^[464] have demonstrated that silencing CD133 expression in human GBM neurospheres disrupts the self-renewal and tumorigenic properties of the neurosphere cells, indicating that CD133 could potentially be used as a therapeutic target in these tumors. Transcription factors regulating pluripotency could be particularly useful as biomarkers since they are not expressed in tissue stem cells. However, pluripotency factors are intimately cross-regulated and difficult to assess unless convenient assays are used. The importance of these common markers in tumor aggression and metastasis awaits further investigation.

Cell differentiation during embryonic development and cell transformation that leads to oncogenesis share common signaling pathways, suggesting that deregulation of embryonic signaling pathways in adult tissues might result in tumor progression by transforming adult stem and progenitor cells. Many signaling pathways are involved in the stemness of both pluripotent (ES/iPS) and CSCs (Figure 1, Table 2). Wnt, TGF/BMP and FGF are important regulators of ESC self-renewal and differentiation and are able to enhance the CSC population. On the other hand, Hedgehog and Notch pathways have great importance for CSCs and only marginal role in ESCs. Chemical inhibitors of the Wnt, Notch and Hh pathways have been proven valuable in combating CSC populations from diverse solid tumors^[79]. Agents that regulate Wnt signaling such as the CBP/ β -catenin antagonist ICG-001, that forces β -catenin to bind p300 instead of CBP, are tested in pre-clinical trials for the eradication of CSC^[464]. Gamma-secretase inhibitors are able to block the Notch signaling pathway resulting in inhibition of CSC proliferation and tumor regression^[79]. Cyclopamine and synthetic small molecules that antagonize Hh pathway are used alone or in combination with conventional antitumor agents for the inhibition of pancreatic and glioblastoma CSCs^[465].

Additional research on the regulation of normal stem cell response to external stimuli will offer alternative therapeutic means for cancer treatment.

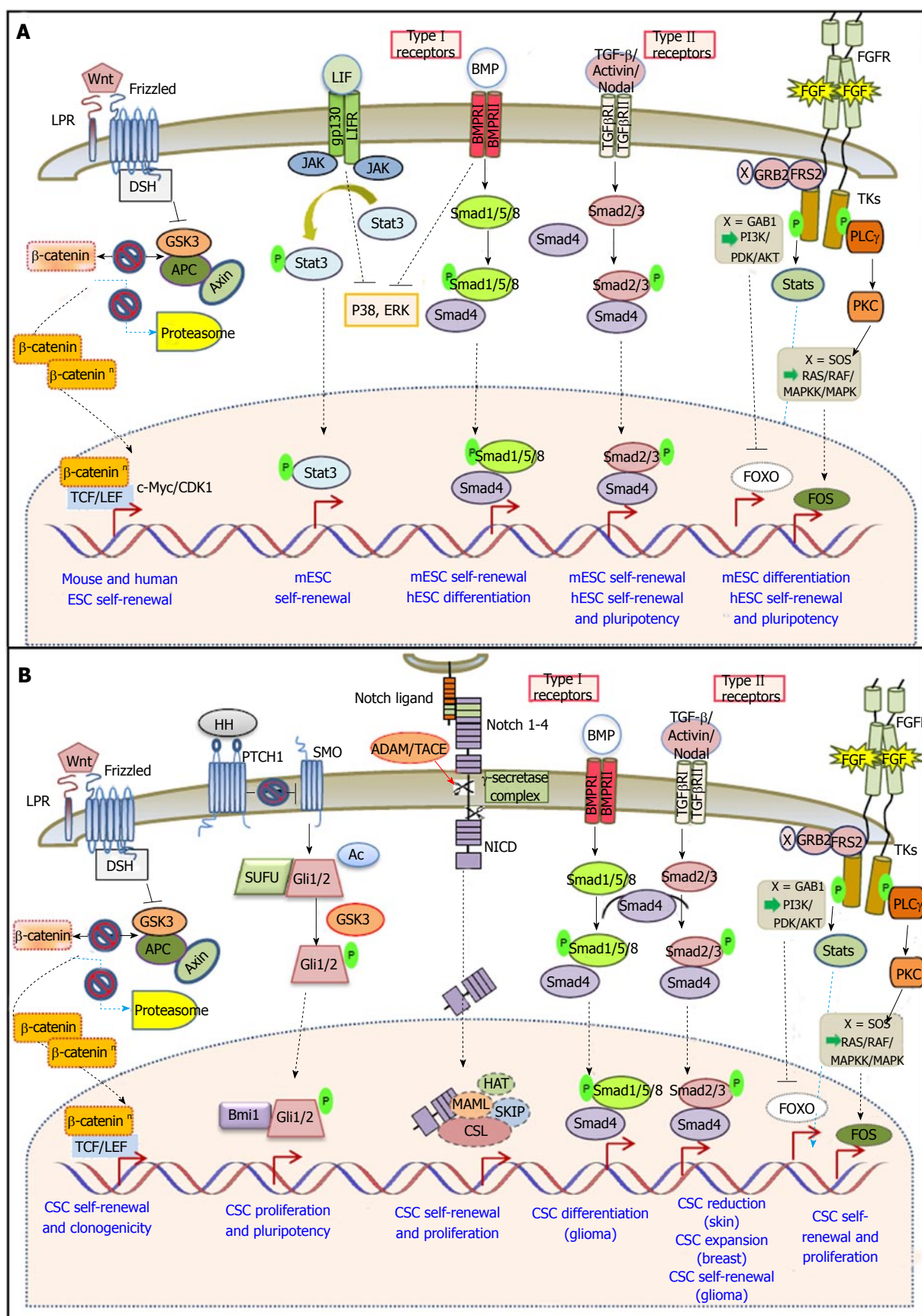


Figure 1 Core signaling pathways in embryonic stem cells and cancer stem cells. A: Mouse ESCs require concerted LIF/BMP signaling to sustain self-renewal and pluripotency, whereas hESC pluripotency depends on the activity of the TGF- β and the FGF pathway. Wnt signaling is important for pluripotency in both species; B: Wnt, Hedgehog, Notch and FGF signaling pathways are associated with CSC self-renewal, while BMP signaling is implicated in CSC differentiation. The TGF- β /Activin/Nodal pathway has a controversial role in CSCs depending on cancer type. ESCs: Embryonic stem cells; CSCs: Cancer stem cells; hESC: Human ESCs; APC: Adenomatous polyposis coli; Dsh: Dishevelled; FGF: Fibroblast growth factor; GSK3: Glycogen synthase kinase 3; JAK: Janus-family tyrosine kinase; LRP: Lipoprotein receptor-related protein; PKC: Protein kinase C; SUFU: Suppressor of fused homolog; TCF/LEF: Tcell factor/Lymphocyte enhancer binding factor.

Emerging evidence suggests that ESCs and CSCs may depend on common critical transcription factors (Table 3). In addition to the Myc oncogene that orchestrates a complex on its own (Myc-complex), CSCs also express members of the ESC core pluripotency complex such as Oct4, Sox2 and Nanog^[466]. Although Oct4, Sox2 and Nanog expression positively correlates with the development of CSCs, the role of Klf4 is highly context-dependent^[467]. While it promotes the self-renewal of various CSCs, it can inhibit EMT signaling pathway^[260,265] and attenuate lung and liver metastases^[468]. Pluripotency transcriptional factors may provide advantageous targets for the elimination of CSCs. For this purpose it would be important to characterize their regulatory circuits in CSCs to the same precision as it was previously attained in ESCs. This knowledge may provide novel approaches for combating cancer^[247,469].

In addition to transcription factors, critical regulators of gene expression in embryonic and CSCs are non-coding RNAs (ncRNAs). Interestingly, ncRNAs, which are important for ESC pluripotency such as lncRNA-RoR, miR-145, miR-200, miR-34a and let-7, can affect the generation of CSCs (Table 3). Accumulating evidence indicates that individual miRNAs and lncRNAs behave as tumor suppressors or oncogenes. Among them, the miR-200 family in combination with the TGF- β signaling are major determinants of the EMT transition that correlates tightly with the appearance of CSCs^[433,434]. Therefore, further investigation of their function in ESCs and CSCs may lead to clinical applications of non-coding RNAs in cancer diagnosis, treatment, and prognosis.

Epigenetic alterations are very important for the inheritance of the cellular phenotype properties. By governing chromatin state transitions and gene expression regulation, epigenetic processes have a crucial role in cell differentiation and tumorigenesis. Most importantly differentiated cancerous cells are driven into stemness *via* a profound epigenetic reprogramming that sustains the malignant phenotype^[470,471]. Although there is already accumulating information regarding epigenetic alterations and therapeutic use of epigenetic modifiers in cancer, less is known about their role in CSCs. Muñoz *et al.*^[471] recently reviewed the role of epigenetic effectors (such as DNMT, EzH2, BMI1 and MLL1) in leukemia and solid tumors and proposed that epigenetic targeting of CSCs may contribute to therapy. The advent of somatic cell reprogramming to pluripotency (iPS) offered a new way to test the effect of extreme epigenetic changes in tumor cells. Can cancer cells be reprogrammed to pluripotency and then induced to differentiate? Although not abundant, recent results in this area suggest a cancer context dependent response to the iPS process^[472]. In those cases, pluripotency was able to dominate over the cancerous phenotype leading to "normalization" although the neoplastic identity reappeared when cancer iPS cells were coaxed to differentiate to their lineage of origin^[472]. Thus reprogramming could offer a new way to track the history of tumor progression.

In conclusion, studies in the fields of stem cells, iPS and CSCs provide strong evidence of cross-complementing benefits. Insight of the tumorigenic properties of stem cells and their differentiated descendants is required before their use in cell replacement therapies^[473]. Understanding the biology and gene circuits shared by both normal stem cells and CSCs, is important for efficient cancer treatment. Cancer heterogeneity is contributed by both clonal and CSC components that are responsible for tumor aggressiveness, invasion and resistance to therapies. An emerging concept in cancer biology is that primary tumors may be composed of evolving and variable clones, each containing biologically distinct stem cells. As cell line models suffer from limited preservation of heterogeneity and newer models of patient derived xenografts are much more labor intensive and costly, alternative, *ex vivo* cancer models are important. As a result, use of the iPS approach may be useful. The hypothesis of normalizing cancer cells by epigenetic reprogramming has been recently under testing but its limits are not defined yet. Mixed initial results may reflect the heterogeneity of the experimental systems utilized, and various oncogenic and genome aberrations that may not be reversed by pluripotency. However, this is a new promising area that may generate diverse iPS-cancer clones from primary tumors. Thus even if the iPS process cannot "cure" cancer, it may provide, as with other human diseases, novel experimental models for studying cancer biology and drug discovery.

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Therapies targeting cancer stem cells: Current trends and future challenges

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Abstract

Traditional therapies against cancer, chemo- and radio-therapy, have multiple limitations that lead to treatment failure and cancer recurrence. These limitations are related to systemic and local toxicity, while treatment failure and cancer relapse are due to drug resistance and self-renewal, properties of a small population of tumor cells called cancer stem cells (CSCs). These cells are involved in cancer initiation, maintenance, metastasis and recurrence. Therefore, in order to develop efficient treatments that can induce a long-lasting clinical response preventing tumor relapse it is important to develop drugs that can specifically target and eliminate CSCs. Recent identification of surface markers and understanding of molecular feature associated with CSC phenotype helped with the design of effective treatments. In this review we discuss targeting surface biomarkers, signaling pathways that regulate CSCs self-renewal and differentiation, drug-efflux pumps involved in apoptosis resistance, microenvironmental signals that sustain CSCs growth, manipulation of miRNA expression, and induction of CSCs apoptosis and differentiation, with specific aim to hamper CSCs regeneration and cancer relapse. Some of these agents are under evaluation in preclinical and clinical studies, most of them for using in combination with traditional therapies. The combined therapy using conventional anticancer drugs with CSCs-targeting agents, may offer a promising strategy for management and eradication of different types of cancers.

Key words: Cancer stem cells; Targeted therapy;

Anticancer drugs; Biomarkers; Signaling pathways; Apoptosis

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Core tip: Cancer stem cells (CSCs) play important roles in tumor formation, metastasis and cancer relapse. In this article, we review the literature on the recent progress in developing anti-cancer stem cell strategies based on improved understanding of CSCs properties and molecular features. These novel therapeutic systems are designed with the aim of eradicating CSCs, by targeting surface specific markers and altering signaling pathways or mechanisms involved in CSCs maintenance and drug resistance, and also to disturb microenvironmental signals that sustain CSCs growth, with specific aim of impede CSCs regeneration and cancer relapse.

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INTRODUCTION

Tumor progression is explained by two models: The clonal (stochastic) evolution model and cancer stem cell model. The first one sustain that all transformed cell within a tumor have carcinogenic potential, with unlimited proliferation capacity, and the disease healing requires as therapy the elimination of all tumor cells^[1,2]. This hypothesis is supported by several studies demonstrating that a large number of cancer cells sustain tumor growth when are transplanted into histocompatible mice and the relevant results in this area depends on the xenotransplantation model used. Thereby, modifying xenotransplantation assay conditions can increase the detection of all tumorigenic cells^[3,4]. The second cancer evolution model sustain that tumors evolve from a small population of cells with self-renewal ability and high resistance to chemotherapy and radiotherapy. These cells were called CSCs or cancer initiating cells, due to their capacity to auto-regenerate, proliferate and induce tumor formation. Among their properties, resistance to standard oncology treatments is responsible for ineffectiveness of traditional cancer therapies and lead to tumor recurrence and metastasis^[5].

The CSCs model gained wide acceptance over the last years, based on continuous observations. Recent data suggests that CSCs might evolve from normal stem cells, progenitors or more differentiated cells

with whom they share many resemblance like: Self-renewing, differentiation to progenitor cells, expression of surface markers, common signaling pathways and a close association with microenvironment^[2].

There are two hypothesis regarding CSCs formation: (1) transformation of normal stem cells or progenitor cells into CSCs, process that occurs through multiple gene mutations as result of genetic and epigenetic instability^[6]; and (2) tumor cells progressively acquire stem cell properties through reversal of ontogeny based on oncogene-induced plasticity^[7].

Several studies suggest that epithelial-mesenchymal transition (EMT) process characterized by the repression of epithelial markers (*e.g.*, E-cadherin) and up-regulation of mesenchymal markers (*e.g.*, vimentin, fibronectin and N-cadherin) can also generates cells with stem-like properties^[8]. Mani *et al*^[9] using immortalized human mammary epithelial cells demonstrated that EMT induction results in the enrichment of cells with stem-like properties, an increased expression of stem-cell markers and an increased capacity of cells to form mammospheres. Gupta *et al*^[10] showed that E-cadherin inhibition and EMT induction, respectively leads to an increase number of CSCs in breast cancer cell populations, characterized by an increased resistance to chemotherapy.

CSCs were first described in acute myeloid leukemia, in which a population of CD34⁺CD38⁻ was noticed to possess stem cells capacities of proliferation, self-renewal and differentiation, and to reconstitute a heterogeneous cell population in nonobese diabetic/severe combined immunodeficiency mice^[11]. Later, CSCs were identified in various solid tumors including glioma^[12], as well as breast^[13], head and neck^[14], lung^[15], pancreatic^[16], liver^[17], stomach^[18], colon cancer^[19], *etc.*

Current review presents basic information about CSCs and discusses targeted therapeutic strategies developed for cancer eradication.

CSC: DEFINITION, CHARACTERISTICS, MARKERS

CSCs are auto-regenerating cells, able to proliferate and differentiate through symmetrical and asymmetrical cell divisions, with tumorigenic potential and specific surface markers useful for CSC identification and isolation. Additionally, several other properties like sphere forming capacity in serum-free medium or soft agar, dye exclusion ability based on over-expression of drug-efflux pumps (ATP binding cassette or multidrug resistance transporters), enzymatic activity of aldehyde dehydrogenase 1, are used to identify CSCs. However, the most important property of CSCs can be verified only by *in vivo* assay: Tumorigenicity in animal model, maintained even after serial transplantation.

The surface markers for CSCs vary according to

Table 1 Cell surface antigens present on cancer stem cells

Cancer type	Surface antigens	Ref.
Leukemia	CD34 ⁺ CD38 ⁺ CD47 ⁺ CCL-1 ⁺ CD96 ⁺ TIM3 ⁺ CD32 ⁺ CD25 ⁺	[11,20-24]
Brain	CD133 ⁺	[12]
Head and neck	CD44 ⁺ CD24 ⁺	[14]
Breast	ESA ⁺ CD44 ⁺ CD24 ⁺	[13,16]
Pancreatic	ESA ⁺ CD44 ⁺ CD24 ⁺	[13,40]
Lung	CD133 ⁺ CD44 ⁺	[15]
Liver	ESA ⁺ CD133 ⁺ CD90 ⁺ CD44 ⁺ CD24 ⁺	[17]
Gastric	CD44 ⁺	[18,38]
Colorectal	ESA ⁺ CD133 ⁺ CD166 ⁺ CD44 ⁺ CD24 ⁺	[19,26]

tumor type. Main surface markers for CSCs from solid tissue are CD133, CD44 and CD24. To these, several other more specific markers might be added, according to tumor tissue origin (Table 1). Thus, the phenotype epithelial specific antigen (ESA⁺) together with CD44⁺-CD24⁺ was described in pancreatic CSCs^[16], while ESA⁺CD44⁺CD24^{low} was the phenotype identified in breast CSCs^[13]. For liver CSCs the following combination ESA⁺CD133⁺CD90⁺CD44⁺CD24⁺ was proposed^[17]. For hematological malignancy CD34 and CD38 are main surface antigens^[11]. Subsequently novel markers have been found to be highly expressed on leukemia stem cells than normal hematopoietic stem cells. These include CD47^[20], C-type lectin-like molecule-1 (CLL-1)^[21], CD96^[22], TIM3^[23], CD32 and CD25^[24].

Although recent studies have contributed to a better understanding of CSCs surface molecules, the picture is not complete. It is often observed that CSCs do not express the same markers, or that normal cells also express these surface antigens. Therefore, it is not possible yet to certainly isolate CSCs, but only to identify a CSCs-enriched population. Consequently, identification of CSCs must be based on additional functional assays such as the ability to form spheres in serum-free medium and to initiate tumor growth after serial transplantation in immunocompromised animal models, based on their self-renewal capacity. However, these assays also have limitations due to microenvironment. Thus, the *in vitro* assay may not detect quiescent stem cells that are not capable to develop spheres due to lack of additional extrinsic signals needed for their activation. Moreover, in this type of assay there is a selection pressure imposed by specific culture conditions in presence of exogenous growth factors. Serial transplantation as well might have limitations, since considerable number of cells is required to induce tumor growth *in vivo*, due to insertion in a foreign microenvironment deficient in specific signals for survival and development^[25].

Therefore, to specifically address CSCs in further experiments, it is necessary to sort cells based on surface markers and subsequently to assess their functional abilities by *in vitro* and *in vivo* specific assays.

THERAPIES TARGETING CANCER STEM CELLS

Traditional therapies against cancer, chemo- and radiotherapy, have multiple limitations that result in treatment failure and cancer recurrence. These limitations are related to systemic and local toxicity because the agents are not selective enough and may affect also healthy tissue. An additional restriction is drug resistance due to CSCs specific properties like: Slow rate of division, high expression of drug-efflux pumps, high capacity for DNA repairing, and also to microenvironment characteristics: hypoxia and acidosis. Therefore, targeting CSCs became essential in treating cancer and preventing tumor relapse.

Recently, multiple strategies have been conceived with the specific aim of destroying CSCs and their niche. These include targeting specific surface markers, modulation of signaling pathways, adjustment of the microenvironment signals, inhibiting of drug-efflux pumps, manipulation of miRNA expression, induction of CSCs apoptosis and differentiation. A summary of these therapeutic strategies is presented in the Figure 1. At the moment, some of them are successfully used in clinic, mainly in combination with traditional therapies, and others are still under evaluation.

Targeting surface markers

The surface markers used for identification and isolation of CSCs are also important targets for therapy. Immunotherapy that involves antibodies targeting CSCs specific markers is often used as an adjunct to chemotherapy, radiotherapy and surgery. The most important CSCs associated markers together with strategies for targeting them are mentioned below.

CD133 (prominin-1) is a cell surface glycoprotein widely expressed on CSCs in solid tumors such as glioma^[12], lung^[15], liver^[17] and, colorectal cancer^[19,26]. Cancers with large CD133 subpopulation have a drug resistant phenotype and poor prognosis. For this reason, several strategies for anti-CD133 therapy have been generated. The polymeric nanoparticles loaded with paclitaxel (CD133NPs) targeting CD133 were tested on colorectal adenocarcinoma Caco-2 cells and proved to efficiently decrease the cell number and colonies formed. On the other hand, in xenograft model, the CD133NPs improved therapeutic efficacy compared to free paclitaxel treatment^[27]. Another anti-CD133 antibody constructed through the fusion with pseudomonas exotoxin 38, inhibited the progression of tumor growth after multiple intraperitoneal injections of drug over a period of 4-6 wk in xenografted mice with ovarian cancer. The elimination of CD133⁺ ovarian cancer cells resulted in long-term disease free tumor survivors^[28]. These studies suggest that anti-CD133 therapy might be associated with drug delivery, forming antibody-drug conjugates that enhance the effect on

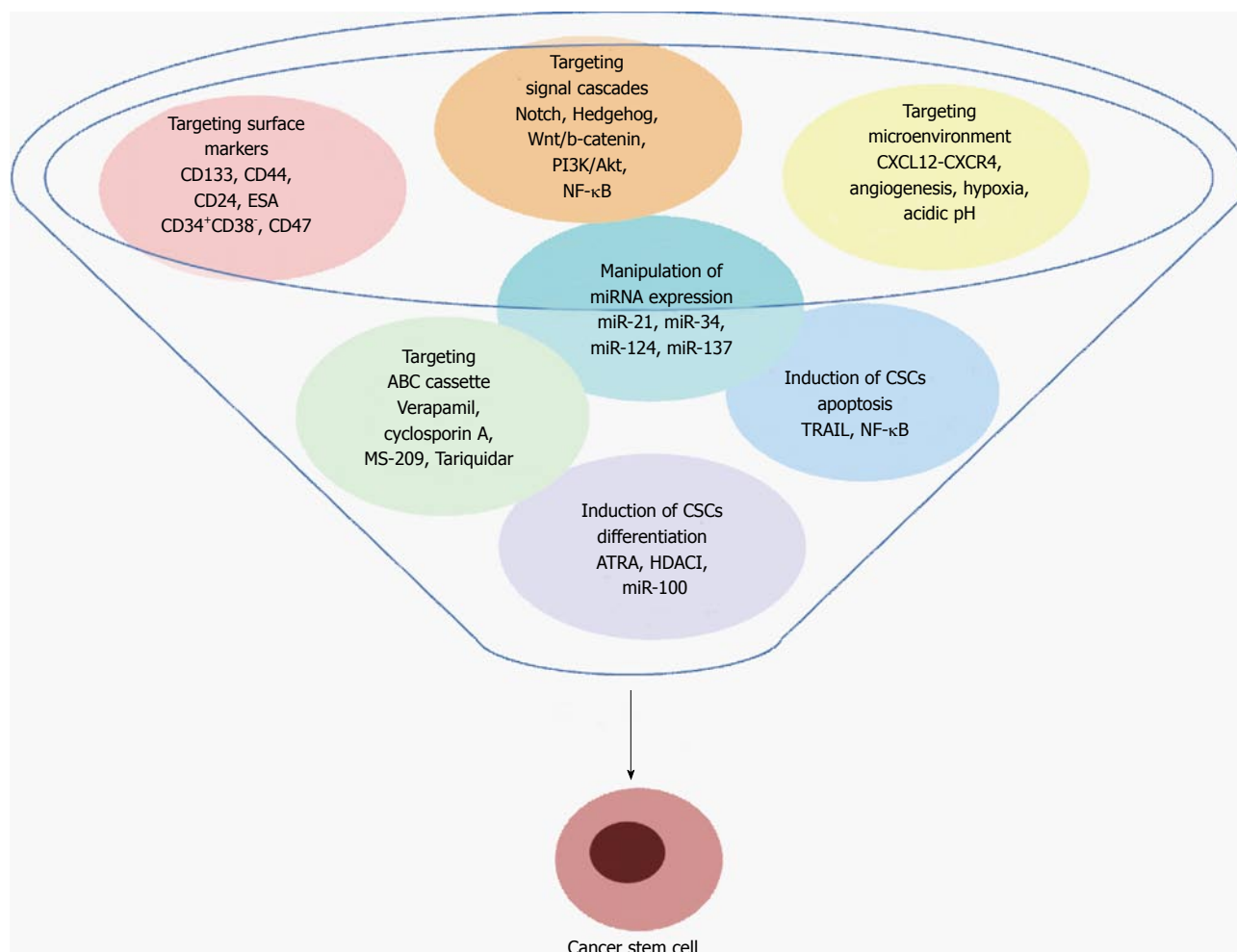


Figure 1 Therapies targeting cancer stem cells. Numerous therapies aiming to eradicate cancer stem cells have been developed during last year's. Here we highlighted most common seven approaches: targeting surface biomarkers, signaling pathways that regulate cancer stem cells (CSCs) self-renewal and differentiation, drug-efflux pumps involved in apoptosis resistance, microenvironmental signals that sustain CSCs growth, manipulation of miRNA expression, and induction of CSCs apoptosis and differentiation, with specific aim of hamper CSCs regeneration and cancer relapse. ATRA: All trans retinoic acid; HDACI: Histone deacetylase inhibitors; PI3K: Phosphatidylinositol 3-kinase; NF-κB: Nuclear factor kappa B; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand.

CD133⁺ CSCs and eliminate them. Anti-CD133⁺ cell therapy was also tested on sarcoma CSCs, reducing proliferative capacity and resulting in decreased sarcoma tumor-initiating ability^[29]. Similar results were obtained for pancreatic and hepatic CSCs^[30,31].

However CD133 expression is not restricted to CSCs. Shmelkov *et al.*^[32] demonstrated that both CD133⁺ and CD133-metastatic colon cancer cells can initiate tumors. Also, Beier *et al.*^[33] demonstrated that CD133⁺ and CD133-glioblastoma cancer cells meet stem cell criteria, but they might reflect two biologically distinctive glioblastoma subtypes. The authors concluded that primary glioblastomas might develop either from different cells of origin or from related cell types that further acquired different molecular alterations.

CD44 is a transmembrane protein that mediates cell to cell adhesion and cell to extracellular matrix interactions, being a receptor for hyaluronic acid, selectin, collagen, osteopontin, fibronectin and laminin^[34]. CD44 is involved in cell proliferation, survival, migration, differentiation, apoptosis, self-renewal, niche preparation, epithelial-mesenchymal transition, and resistance

to apoptosis^[35,36]. It was found overexpressed in many tumor cells: breast^[13], bladder^[37], gastric^[38], prostate^[39], pancreas^[40], ovar^[41], colorectal^[26,42], hepatocellular^[43], head and neck^[44], acute myeloid leukemia (AML) CSCs^[45], etc. Targeting CD44 with monoclonal antibodies appears as a good strategy to eliminate CSCs. Treatment with anti-human CD44 monoclonal antibody can induce myeloid differentiation in patient-derived AML blasts, inhibits homing to the microenvironmental niche and alters stem cell fate^[46]. Marangoni *et al.*^[47] used an anti-human CD44 monoclonal antibody in combination with doxorubicin and cyclophosphamide to prevent relapse of aggressive breast cancer. Moreover, a mouse IgG1 anti-human CD44 receptor that inhibits CD44-STAT3 signaling pathways was used on human pancreatic cancer stem-like cells or MiaPaCa-2 cells and was found to decrease *in vitro* tumor sphere formation of CSCs, inhibiting pancreatic tumor growth, metastasis and tumor recurrence in xenografted nude mice^[48]. Similar results were obtained in colorectal, lung, bladder, larynx and breast cancers^[49,50].

CD47 is a transmembrane protein, receptor for

thrombospondin family members and for signal regulatory protein alpha (SIRP α)^[51]. It was found widely expressed on AML CSCs^[20] and almost all human solid tumor cells^[51,52]. Two anti-CD47 mAbs such as B6H12.2 and B6H12 were developed as strategy for cancer therapy. In a xenograft mouse model, B6H12.2 antibodies prevented the engraftment of human AML cancers stem cells and completely eradicated them^[20]. Administration of B6H12.2 prevented and inhibited growth of tumors derived from glioblastoma, ovarian, breast, colon, bladder cancer^[52], human non-Hodgkin lymphoma^[53], acute lymphoblastic leukemia^[54] and multiple myeloma CSCs^[55]. B6H12, a fully humanized anti-CD47, effectively inhibits aggressive leiomyosarcoma growth and metastasis in xenograft mice model^[56].

Other antibodies approved by FDA for the treatment of solid and haematological tumours, *e.g.*, rituximab (anti-CD20), cetuximab (anti-EGFR), trastuzumab (anti-HER2), bevacizumab (anti-VEGF-A), ipilimumab (anti-CTLA-4), pembrolizumab (anti-PD-1) are currently used in immunotherapy against tumor cells^[57].

Targeting signal cascades

One of the mechanisms by which CSCs manage to avoid or to survive cancer treatments seems to be represented by signals generated within the tumor microenvironment, due to dysregulation of signaling pathway networks^[58]. Like normal stem cells, CSCs use signaling pathways that are essential for self-renewal, proliferation and differentiation in order to preserve stem cell properties but the final result is carcinogenesis. Many studies have focused on signaling pathways dysregulation in CSCs attempting to find new strategy for cancer therapy; this line of research is promising mainly because many cancers present up- or down-regulation of the same signaling cascades. In this regard, CSCs can be identified by surface markers but also by the signals they send in tumor microenvironment^[59]. The major signaling pathways involved in the regulation of self-renewal and differentiation of normal and cancer stem cells are Notch, Hedgehog, Wnt/ β -catenin, NF- κ B, phosphatidylinositol 3-kinase (PI3K)/Akt, PTEN; sustained by aberrant activation of these pathways, CSCs have the capacity to initiate cancer and promote recurrence after the surgical removal of tumor^[60].

Notch signaling cascade is one major pathway involved in numerous critical cellular processes, including stem cell maintenance, progenitor cell proliferation and differentiation, and determination of cell fate during embryonic development^[61,62]. Notch signaling involvement in carcinogenesis and tumor progression seems to depend on tissue/cell-type. Thereby, Notch signaling was identified as oncogenic due to an increased activation in T-cell acute lymphoblastic leukemia, medulloblastoma, colorectal cancer, non-small cell lung carcinoma, hepatocellular carcinoma, melanoma, and breast cancer while in myeloid malignancies, head and neck squamous cell carcinoma, Notch displays tumor

suppressor functions^[63,64]. Notch signaling pathway comprises four Notch receptors (Notch 1, Notch 2, Notch 3 and Notch 4) and five ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1 and Jagged 2); depending on the cellular type, many studies have been focused on activation or inhibition of these proteins^[65,66]. *In vitro* and *in vivo* studies showed that human lung cancer lines presented an overexpression of Notch3; downregulation of Notch signaling, using a specific inhibitor was associated with decrease of tumor cell proliferation, induction of apoptosis, and inhibition of *in vivo* growth. Notch3 was also found to be overexpressed in resected human lung cancers and was associated with poor overall survival^[67,68]. Moreover, activation of Notch4 and Notch1 was noted breast CSCs, and inhibition of these receptors, especially Notch4, leads to decreased activity of breast CSCs *in vitro*, and reduced tumor development *in vivo*^[69]. In hepatocellular carcinoma, inhibition of Notch signaling pathway also reduced the invasion of tumor cells by downregulation of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor^[70]. In colorectal cancers, Notch1 signaling seems to have a dual role; it can increase the tumor progression but also counteracts β -catenin signaling whose activation is an important factor in human colorectal carcinogenesis^[71]. Many studies reported an overexpression of Notch signaling proteins in head and neck squamous cell carcinoma, sustaining a pro-oncogenic role of this signaling but recent exome sequencing analyses reported loss-of-function mutations in the *Notch1* gene in a significant proportion of patients with this malignancy^[72].

Notch signaling inhibitors tested in clinical trials include inhibitors of γ -secretase complex involved in Notch activation and antibodies against DLL-4 and Notch-1, -2 or -3 receptors^[43]. Results for phase 1 clinical trial using BMS-906024, one of these γ -secretase inhibitors, for patients with relapsed T-cell acute lymphoblastic leukemia showed at least 50% reduction in BM blasts in 8 of the 25 patients (32%)^[73].

Recently was reported the development of an antibody OMP-59R5 (tarextumab) which alone or together with chemotherapeutic agents can inhibit Notch2 and Notch3 function. The antitumor effect of OMP-59R5 was observed on xenograft tumors representing different types of epithelial cancers like breast, small-cell lung, ovarian, and pancreatic; and was associated with down-regulation of Notch target genes in tumor cells and with suppression of Notch3, HeyL, and Rgs5, expression in tumor stroma^[74].

Hedgehog is another pathway that plays an important role in stem cells maintenance and embryonic development, being involved in various cellular processes such as proliferation and differentiation; this cascade was also aberrant activated in several human tumors, including glioblastoma, breast cancer, pancreatic adenocarcinoma, multiple myeloma and chronic myeloid leukemia^[75,76]. Furthermore, Hedgehog signaling

contributes to development and maintenance of CSCs and acquisition of epithelial-to-mesenchymal transition, highlighting the involvement of Hedgehog cascade in cancer cell invasion, metastasis, chemotherapeutic resistance and tumor recurrence^[77]. Canonical Hedgehog signaling involves three ligands: Sonic (SHH), Indian (IHH), and Desert (DHH) with different expression depending on cell type; SHH is widely expressed, mostly during embryogenesis, IHH is found in hematopoietic cells, bone, and cartilage, while DHH is expressed in the peripheral nervous system and testes. Hedgehog signaling implies Hedgehog ligands binding to Patched receptor, Smoothened activation, and activation of transcriptional effectors that belong to the GLI family^[78,79]. Aberrant activation of Hedgehog signaling may be due to gene mutations, resulting in ligand-independent pathway activation, or by interaction with other molecular signaling pathways, such as Ras/Raf/MEK/Erk, PI3K/Akt/mTOR, and Notch. Thus, there are many studies sustaining that the combination therapies targeting more than one signaling pathway improves antitumor efficacy and survival in animal models^[80]. There are also several preclinical studies showing that in several cancers, Hedgehog signaling inhibition using specific inhibitors of Smoothened leads to the blocking of drug resistance, relapse, and metastasis. In basal cell carcinoma and medulloblastoma patients, Smoothened inhibitors such as vismodegib, BMS-833923, saridegib (IPI-926), sonidegib/erismodegib (LDE225), PF-04449913, LY2940680, LEQ 506, and TAK-441 were used as monotherapy with promising results^[81]. A recent study reported a significant negative association between GLI1 and GLI2 expression and overall survival, and also an increase of DHH plasma levels in acute myeloid leukemia patients. By *in vitro* and *in vivo* experiments the authors demonstrated that GLI1/2 inhibition induces apoptosis, and reduces proliferation, and colony formation in acute myeloid leukemia cells, and also increases the survival in murine models^[82]. Another study focused on biliary tract cancer showed that inhibition of Hedgehog and mTOR signaling pathways with rapamycin and vismodegib specific inhibitors results in decrease of Nanog and Oct-4 expressions and also in decrease of CSCs and ALDH-positive cells proliferation^[83]. In pancreatic cancer, the combination of focal radiation with Hedgehog inhibitors reduces lymph node metastasis, sustaining the involvement of this signaling pathway in carcinogenesis^[84].

Wnt/ β -catenin signaling pathway is one of the most deregulated pathways in many cancers, including leukemia, colon, breast and skin cancers. For instance, in human colon carcinoma, mutations in adenomatous polyposis coli (APC) result in aberrant activation of Wnt signaling and induce transformation of epithelial cells^[60]. There are also studies that support the involvement of Wnt signaling in self-renewal and maintenance of stem cells and CSCs, in several tissues like skin, intestine and

mammary gland^[85]. A minor subpopulation of breast tumor-initiating cells associated with drug resistance was identified in human breast cancer; *in vitro* and *in vivo* study showed that suppression of Wnt signaling inhibited sphere- and colony-formation of primary breast tumor cells and blocked tumor growth in murine models^[86].

Wnt/ β -catenin signaling pathway includes Wnt ligands, Frizzled receptors and a complex, composed of APC, Axin1, Glycogen synthase kinase 3- β , and CK1 (casein kinase 1) which stabilizes β -catenin^[87]. Wnt/ β -catenin inhibitors include small-molecule inhibitors (e.g., nonsteroidal anti-inflammatory drugs and natural compounds: aspirin, indomethacin, vitamins A and D derivatives) and biologic inhibitors (monoclonal antibodies, small interfering RNAs and recombinant proteins)^[2].

OMP-54F28 is an example of Wnt signaling inhibitor with interesting results in clinical trials, in several advanced solid tumors, such as ovarian, pancreatic and hepatocellular cancers^[88]. OMP-18R5 - an antibody targeting several Frizzled receptors - reduces tumor cell proliferation and tumor-initiating cell number in lung, breast, colon, and pancreatic tumors^[89]. *In vitro* study on lung CSCs showed that β -catenin nuclear transfer inhibitor PP significantly decreased colony formation and downregulated pluripotent stem cell signaling pathway, being a promising therapeutic approach in lung adenocarcinoma patients^[90]. The studies performed until now showed that Wnt inhibitors and modulators can eliminate drug-resistant CSCs and tumor-initiating cells but more studies are needed regarding the safety of this therapeutic approach, given the essential role of Wnt signaling in tissue homeostasis and repair^[91].

PI3K/Akt/mTOR signaling pathway is an important cascade of phosphorylation reactions, comprising several key molecules involved in carcinogenesis processes: PI3K - presents activating mutations in several human cancers; Akt - overexpression/activation of this protein kinase is associated with tumor metastasis and invasion; mTOR - protein kinase critical for cancer cell growth, cell proliferation, survival and angiogenesis^[59].

Many studies suggest the involvement of this signaling pathway in maintaining CSCs features. In breast cancer, inhibition of PI3K or Akt activity reduced generation and growth of CD44/CD24 mammospheres, leading to stem cell/mesenchymal phenotype loss and recovery of epithelial-like markers^[92]. In prostate cancer, PI3K/Akt/mTOR pathway deregulation is associated with CSCs quiescence and maintenance; moreover, prostate CSCs seem to present resistance to selective mTOR inhibitors^[93]. Another study showed that combination between PI3K/mTOR inhibitor (BEZ235) and radiotherapy increased radiosensitivity and apoptosis, and also reduced CSCs marker expressions in prostate cancer radioresistant cell lines^[94]. In endometrial tumors, PTEN/PI3K/Akt/mTOR pathway aberrant activation by miRNAs is a common event,

being involved in epithelial-mesenchymal transition and CSC maintenance^[95].

PTEN (phosphatase and tensin homolog) is a tumor suppressor and an inhibitor of PI3K and ERK activities, being one of the most inactivated tumor suppressor genes in cancers; many tumors present loss of PTEN function by mutations, deletions, transcriptional silencing, or protein instability affecting important cell processes such as survival, proliferation, energy metabolism and cellular architecture. Loss of PTEN activity was also linked to CSC development and proliferation in several cancers, including prostate, lung, intestinal, and pancreatic cancer^[95]. Moreover, PTEN loss and Akt activation lead to increase activity of β -catenin in hematopoietic stem cells while Notch signaling activation results in reduced PTEN expression in human T-cell acute lymphoblastic leukemia^[96].

Nuclear factor kappa B (NF- κ B) is a transcription factor constitutively activated in several tumors, being also associated with self-renewal and expansion of CSCs^[97]. NF- κ B interacts with many apoptosis-related proteins, including Bcl-xL, Bcl-2, survivin, cellular inhibitors of apoptosis (cIAPs), TRAF and cell cycle regulatory components, and NF- κ B aberrant expression was related to cancer development and progression, chemoresistance, chronic inflammation and autoimmune diseases^[60].

In hepatocellular carcinoma cell lines, one of the most activated signaling pathways is NF- κ B cascade; NF- κ B inhibition using SN50, suppressed tumor cell growth and the authors suggested that targeting NF- κ B signaling might specifically inhibit CSCs populations and it could be considered a new therapeutic strategy for hepatocellular carcinoma patients with poor prognosis^[97]. Pancreatic ductal adenocarcinoma characterized by a pronounced hypoxic tumor microenvironment presents epithelial-mesenchymal transition and stem-like features related to NF- κ B signaling activation. An *in vitro* study demonstrated that inhibition of NF- κ B with triptolide can reverse epithelial-mesenchymal transition and reduced migration, self-renewal activity, stem cell-related signaling^[98]. NF- κ B signaling is also considered an important therapeutic target in breast cancer; HER2-NF- κ B-HER2 pro-survival pathway seems to be activated in breast CSCs upon radiation therapy^[99].

Numerous studies have focused on identifying the molecular mechanisms and signaling pathways characteristic for CSCs, in solid and hematological malignancies. Notch, Hedgehog, Wnt/ β -catenin, NF- κ B, PI3K/Akt, and PTEN cascades present aberrant activation in cancers and they have been associated with high proliferative and metastatic capacity, self-renewal and differentiation of CSCs and also multi-drug resistance, being considered attractive targets for CSCs specific eradication. However, more studies are required to demonstrate the safety of these targeted therapies, considering the crucial role of these signaling pathways for normal stem cell maintenance^[97].

Targeting microenvironment

The tumor microenvironment provides necessary signals for CSCs maintenance, regulation of self-renewal and homeostatic processes such as angiogenesis, hypoxia and weakly acidic pH.

Interaction between CSCs and tumor stroma is ensured by CXCL12-CXCR4 axis. CXCL12 [also called stromal-derived factor-1 (SDF-1)] is a chemokine that binds its receptor CXCR4 and it is involved in migration, invasion and survival of normal and malignant cells^[100]. CXCR4 is highly expressed on several stem cells mainly haematopoietic, but also on endothelial, neural and embryonic stem cells, facilitating their response to gradients of CXCL12 produced in case of tissue damage and hypoxia. On the other hand, CXCL12 ligand has a constitutive expression on stromal cells in many tissues. It has an increased level in bone marrow, lymph nodes, lung and liver, and a low level in small intestine, skin skeletal muscle^[101].

Many studies have emphasized that under physiological conditions CXCL12-CXCR4 axis plays a crucial role in processes like normal development, tissue regeneration and repair. With respect to tumor growth, it was shown that CXCL12-CXCR4 axis ensures a close contact between cells and tumor stroma and consequently activates various signals related to cell growth, metastasis and chemoresistance^[102]. In human breast cancer CXCL12 generated by stromal fibroblasts exerts two types of effects on tumor growth: an endocrine effect by promoting angiogenesis *via* recruiting endothelial progenitor cells into the tumor mass, as well as a paracrine effect by acting directly on tumor cells through CXCR4^[103].

The important roles of CXCL12-CXCR4 axis in cancer led to an intense research for developing drugs that inhibit signaling through this axis. A series of inhibitors have been investigated, targeting either CXCR4 or CXCL12. Among CXCR4 inhibitors, AMD3100, also known as plerixafor, induces the rapid mobilization of hematopoietic stem and progenitor cells into the blood in mice and humans^[104], inhibits growth of intracranial glioblastoma and medulloblastoma xenografts by increasing apoptosis and decreasing the proliferation of tumor cells^[105], and can reduce the intraperitoneal dissemination of epithelial ovarian cancer^[106]. On the other hand, in a recent study it was shown that treatment with the AMD3100 diminished metastatic growth, but it didn't affect significantly the frequency of metastases or overall survival in a murine model of metastatic human non-small cell lung cancer^[107].

CTCE-9908 is another CXCR4 antagonist that proved to be effective in reducing both tumor growth and metastasis in xenograft mouse models of inflammatory breast cancer^[108] and also in an orthotopic model of esophageal carcinoma^[109]. A recent study highlighted the role of CTCE-9908 in decreasing the tumor invasivity and angiogenesis in prostate cancer^[110].

One of the most studied CXCL12 inhibitor is NOX-A12

that seems to suppress CXCL12-induced chemotaxis of chronic lymphocytic leukemia cells and to cause chemosensitization^[111]. In addition, Liu *et al.*^[112] observed that this compound had the potential to improve tumor response in glioblastoma multiforme animal model after irradiation.

CXCL12-CXCR4 axis can also promote angiogenesis. Liang *et al.*^[113] have shown that CXCR4 induces an increased expression of vascular-endothelial growth factor (VEGF) at both the mRNA and protein levels through the activation of PI3K/Akt pathway.

Tumor angiogenesis is another mechanism promoted by microenvironment that has been related with CSCs survival and tumor growth, since targeting VEGF can lead to normalization of the vasculature, decrease in tumor growth and disruption of the CSCs niche^[60,114]. Inhibitors against the VEGF/VEGFR-system are already in clinical use. Among the approved drugs that target VEGF/VEGFR-system are bevacizumab, anti-VEGF blocking antibody, and pazopanib, sorafenib and sunitinib, VEGFR-2 pathway inhibitors. Moreover, a series of compounds are tested in clinical trials or they are just in experimental phase^[115]. From those, fruquintinib (HMPL-013), a small molecule inhibitor very potent and highly selective against VEGFR family, is currently in phase II clinical studies^[115].

Another feature of the tumor microenvironment is hypoxia that is regulated by inducible transcription factors HIF-1 and HIF-2. Many studies correlated tumor hypoxia with tumor growth, cancer progression, metastasis, resistance to chemo- and radiotherapy. Therefore targeting HIF activity might represent an effective method of inhibit tumor metastasis and improve the outcome of chemo- and radiotherapy^[116,117].

In breast cancer, inhibition HIF activity in BrCa cells by using RNA interference or digoxin treatment prevent primary tumor growth and also reduce breast cancer dissemination in lungs by down-regulating the expression of angiopoietin-like 4 (ANGPTL4) and L1 cell adhesion molecule (L1CAM) 21860410. In a recent study, Gillespie *et al.*^[118] investigated the inhibition of HIF-1 α by using small interfering RNA in an orthotopic mouse model for glioblastoma. *In vivo* treatment reduced tumor growth and increased survival.

Another important therapeutic target can be represented by acidic extracellular pH, a major feature of tumor tissue that is the result of cancer cells increased metabolic activity and of the poor vascular perfusion of tumors^[119,120]. As it was shown in previous studies, tumor acidity can offer a selective advantage of cancer cells over the normal tissues and contribute to drug resistance^[119,121].

The acidity of tumor environment can be managed either by using delivery drugs that have specificity for acid environment, or can be reversed directly with systemic buffers or indirectly by using inhibitors for pH-regulatory pathways like carbonic anhydrase IX (CAIX).

Developing compounds that are tumoral acidic pH-

responsive is a dynamic area of research; liposomes, micelles, polymers and inorganic nanoparticles have been tested for anticancer therapy^[122]. An improved drug-delivery method is to encapsulate drugs into silica matrices like camptothecin and doxorubicin^[122,123].

Sodium bicarbonate is one of the systemic buffers that can be used to alkalinize the microenvironment. In intraductal tumors it have been shown that sodium bicarbonate can prevent or slow down the transition from in situ to invasive cancer^[124].

One approach, more captivating is to regulate cellular pH with drugs that inhibit CAIX, a hypoxia and HIF-1-inducible protein, overexpressed in many cancers. Data from phase III clinical trial suggest that CAIX score can be also used as statistically prognostic biomarker for survival in patients with high-risk nonmetastatic renal cell carcinoma (ccRCC)^[125].

Targeting the tumor microenvironment might affect communication with stroma and self-regulating process, the vasculature as the main nutrients supply, and essential features like oxygen level and acidity needed by CSCs, all these being fundamentals for CSCs maintenance. However these strategies are not specific enough and might affect also normal stem cells and their stroma. Consequently, a more particular approach is needed that addresses only tumor stroma.

Targeting ATP-binding cassette transporters

The ability to isolate and characterize CSCs from different tumors facilitated a more specific investigation of the therapeutic possibilities based on another distinctive feature of the CSCs, chemoresistance^[126]. CSCs identification by augmented efflux of Hoechst 33342 dye through ATP-binding cassette (ABC) transporters (defined as side population, SP cells) in flow cytometry analysis, proved to be very useful in isolating hematopoietic stem cells, and a variety of CSCs from solid tumors, including ovarian, breast, colon and hepatic cancers^[127-131]. Aberrant expression of ABC transporters is a major mechanism of chemoresistance in cancers cells, including cancer CSCs^[132] but may be generated by other mechanisms involved directly or indirectly in the process, from transcription to protein expression. All the mechanisms involved in ABC transporter modulation might be potential targets for overcoming chemoresistance in CSCs.

ABC transporter proteins are members of the ABC superfamily that have as most important physiologic role prevention of the accumulation of xenobiotics and toxic compounds in normal cells. These efflux pumps consist of a single or multiple sets of transmembrane domains and nucleotide binding domains. Nucleotide binding domains hydrolyze ATP giving power for the efflux while a variety of structurally unrelated substrates, including drugs, sugars, proteins, and metabolites are "pumped-out" through the transmembrane domains.

Among the numerous members of the ABC-transporters described to date, only few were well docu-

mented to be expressed in human CSCs: Multidrug resistance 1 (MDR1) or P-glycoprotein (Pgp)/ABCB1, multidrug resistance protein 1 (MRP1)/ABCC1, and breast cancer resistance protein (BCRP)/ABCG2/MXR/ABCP. These proteins differ in structure and in substrate specificity. MDR1 is a protein that confers cross-resistance to many antitumor agents, including anthracyclines, mitoxantrone, epipodophyllotoxins, and taxanes. ABCC1 is structurally similar to ABCB1 but shares only 15% amino acid sequence identity; it also generates resistance to anthracyclines, mitoxantrone, and epipodophyllotoxins but differs from MDR1 in the level of resistance to taxanes. BCRP also confers resistance to mitoxantrone, but anthracyclines resistance was found to depend on mutations at the codon 482^[133].

The new concept of targeting ABC transporters considers inhibitors as "CSC sensitizing agents". Clinical studies have attempted to overcome drug resistance by combination therapies in which a cytotoxic drug was given along with an ABC-transporter inhibitor. However, ABCB1 inhibitors have shown very limited effectiveness in clinical trials despite the debate on the fact that the clinical trials on the first generation of inhibitors have not been targeting CSCs but only the reduction of the tumors that express a particular drug transporter (usually ABCB1). If the stem cells are considered the principal culprits of drug resistance, the efficacy of ABC inhibitors would be better evaluated monitoring the relapse instead of tumor size reduction^[134]. However, ABC inhibitors would be most effective if are combined with an anticancer agent that specifically targets the stem cells.

Three generations of ABC transporter blockers were investigated until now and the fourth generation, based on natural compounds, is under development^[135].

First generation of inhibitor drugs were already in use for different conditions and also were able to block MDR1 (ABCB1), such as calcium channel inhibitors like verapamil, immuno-suppressants like cyclosporin A, anti-arrhythmics and neuroleptics like quinidine, reserpine, and yohimbine, and antiestrogens like tamoxifen and toremifene. The efficacy of these drugs was limited by their toxicity, which urged the development of drugs with less important side effects as first generation compounds.

Second generation of MDR1 modulators like R-verapamil, elacridar (GF120918), dofequidar (MS-209), valspodar (PSC833), biricodar (INCEL, VX-710), or timcodar (VX-853) were derived from first generation P-gp modulators. Although second generation modulators are less toxic than first generation modulators, they still induce important side effects due to non-selective inhibition of multiple cell ABC transporters and unpredictable pharmacokinetic interactions^[136].

Third generation modulators such as Zosuquidar (LY335979), oc144093 (ONT-093), laniquidar (R101933), and tariquidar (XR-9576), which are more selective inhibitors of ABCB1, ABCC1, ABCG2, are still in different phases of investigation as CSCs sensitizing

agents.

Although the expression of ABC transporters could render CSCs resistant to drugs, it is not the unique cause of resistance by ABC proteins, as multiple levels of regulation between transcription and protein expression might be susceptible to modifications. Future trends of drug development should consider such levels of regulation of ABC transporters that might be speculated in cancer therapy. One such direction should evaluate, for example, the potential of using miRNAs targeting specific RNA that results in its degradation. MiR-328, miR 519c, miR-520h, miR-487a, miR-181a are only few of the miRNAs reported to be involved in ABC transporter regulation.

Recently, was observed that the presence of side population (SP) cells in tumors is highly dependent on the driving genetic alterations of the tumor. For example, hepatic tumors driven by Myc, but not Akt and RAS, had a significant number of SP cells that show the same properties as chemoresistant tumor-initiating CSCs. These studies show that even the common mechanisms of chemoresistance may change under genomic alterations conditions that are very frequent in cancer. There are increasing evidence that small-molecule tyrosine kinase inhibitors (TKIs), such as AST1306, lapatinib, linsitinib, masitinib, motesanib, nilotinib, telatinib and WHI-P154, can inhibit ABC transporters, suggesting that these TKI might have also a direct effect on ABC proteins inhibitors^[137]. Effect of Akt, PI3K and mTOR inhibitors on ABC transporters trafficking and/or expression has been well documented^[138]. Particularly, LY294002, a derivative of quercetin, is able to inhibit PI3Ks, mTOR and ABCG2 and has been recently proposed as a new candidate to target efficiently ABCG2-expressing CSCs that depend on Akt signaling for survival. ABCG2 expression and the SP might be regulated by PTEN through the PI3K/Akt pathway, which was proposed as a potentially effective strategy for targeting CSCs^[139].

Using nanomedicines as delivery vehicles can enhance the therapeutic response in resistant tumors by bypassing efflux pumps or by increasing the concentration of drugs in CSCs at a given time point^[140].

Simultaneous targeting of genomic alterations that are responsible for different types of cancer and resistance phenotypes together with new strategies of delivery and retention of the drugs inside the CSCs, will allow the development of more selective therapy.

Manipulation of miRNA expression

MicroRNAs (miRs) are small non-coding RNAs (20 to 24 nucleotides in length) that negatively regulate post-transcription by binding to the 3'UTR of target messenger RNA, having a broad range of effects over self-renewal, differentiation and division of cells^[141]. MiRNA are involved not only in maintaining normal cell functions, but also might modify several signaling pathways that could transform stem cells into cancer stem cells. Aberrant expression of miRNA in cancer

stem cells was noted by many studies. MiRNAs differentially regulate the key properties of CSCs, including cell-cycle exit and differentiation, prosurvival and antistress mechanisms and EMT, migration and invasion, which increase tumor initiation and metastatic potential^[142]. They can act either as oncogenes or tumor suppressors.

Targeting oncogenic miRNAs can be achieved by antisense oligonucleotide inhibition. Studies have shown that miR-21 has been found to be frequently up-regulated in different CSCs^[143]. Thus, knockdown of miR-21 inhibits cell proliferation, migration and tumor growth in breast cancers^[144,145], ovarian^[146], and lung cancer^[147].

Several studies have exposed the potential of miRNA based therapeutics as a novel strategy towards cancer stem cells. MiR-34 is a target of tumor suppressor gene *p53* and is down-regulated in many cancers^[148]. Liu *et al.*^[149] showed that miR-34a, was underexpressed in CD44⁺ prostate cancer cells and increased expression of miR-34a in CD44⁺ prostate cancer cells inhibited clonogenic expansion, tumor regeneration, and metastasis. Moreover, Shi *et al.*^[150] demonstrated that transfection of synthetic miR-34a in CD44⁺ non-small cell lung cancer cell lines inhibited clonogenic expansion, and tumor regeneration *in vivo*. Consistently, forced expression of miR-124 and miR-137 in human derived glioblastoma stem cells leads to loss of their self-renewal and oncogenic capacity, leaving normal stem and precursor cells unharmed^[151].

Abnormal miRNA expressions lead to the initiation, development, and progression of cancer. Thus, miRNA based therapy that can correct abnormal transcripts at CSCs level show great potential for cancer cure.

Induction of CSCs apoptosis

Apoptosis is a critical mechanism that mediates death and survival through a complex signaling mechanism. Escape from this system is the precondition for cancer initiating cells. Usually, the apoptotic mechanisms are impaired during cancer development and progression.

Induction of apoptosis in CSC holds great promise for cancer therapy. Therefore, many compounds have been developed to target intrinsic and extrinsic apoptotic pathways. For example, activating of the death receptors [CD95 and trimeric human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)], part of extrinsic apoptotic pathway, leads to caspase-8 activation. Once activated, caspase-8 either directly cleaves and activates effector caspase-3 or, alternatively, processes Bid into the active fragment tBid, which translocates to mitochondrial membranes to initiate mitochondrial outer membrane permeabilization^[152]. Treatment with TRAIL in combination with various anticancer agents was reported to be effective in removing cancer stem cells. Thus, co-treatment with cisplatin was described to be very efficient in reducing triple negative breast cancer stem cells through inhibition of Wnt signaling

and increasing of apoptosis^[153]. Likewise, combined treatment with TRAIL and cytarabine or daunorubicin, has been shown to suppress the growth of acute myeloid progenitors^[154]. Furthermore, TRAIL in addition to Bortezomib, a proteasome inhibitor, was recently shown trigger apoptosis in glioblastoma stem cells^[155]. Moreover, mesenchymal stem cells (MSC) engineered to express TRAIL through transduction with a lentiviral vector, were used to activate apoptosis in of squamous and lung cancer stem cell population. Experiments were done by injecting TRAIL-secreting MSC subcutaneously into nude mice that hold tumors. Results showed that MSC migrate and localized near the cancer site and inhibited tumor growth through apoptosis induction^[156].

Another target in inducing CSCs death might be NF- κ B, a transcription factor linked to the control of apoptosis signaling. Mainly, NF- κ B inhibits apoptosis and promotes cell proliferation, inflammation, tumor promotion, angiogenesis and metastasis^[157-159]. In breast cancer, pharmacological inhibition of NF- κ B with small-molecules like parthenolide, pyrrolidinedithiocarbamate and its analog diethyldithiocarbamate preferentially target breast cancer stem cells. These results underline that NF- κ B activity is critical to maintain the survival of tumor-initiating cancer stem cells^[160]. Moreover, inhibition of NF- κ B by the proteasome inhibitor MG-132 together with the anticancer drug idarubicin induced apoptosis preferentially in the leukemic stem cells population but considerably less in normal hematopoietic stem cells^[161].

Targeting CSC population by reactivation of apoptosis programs has been shown in preclinical studies to offer the possibility to eradicate cancers. Future challenges should include the increasing of specificity and efficiency in targeting cancer stem cells and avoid toxicity of normal tissue stem cells.

Induction of CSCs differentiation

Traditional anti-cancer therapies successfully manage differentiated cancer cells but did not affect CSCs. This observation leads to another method to restrain cancer progression, induction of CSCs differentiation that became favored over self-renewal programs, diminishing CSC population. Many studies are currently in progress proposing various differentiation agents like: retinoic acid, histone deacetylase inhibitors, miRNAs, tyrosine-kinase and signaling pathways inhibitors.

Retinoic acid and its analogs (ATRA) are currently used to treat acute promyelocytic leukemia. Retinoic acid regulates several chromatin remodeling factors due to its interaction with retinoid receptors^[162]. Campos *et al.*^[163] demonstrated that ATRA induced differentiation of glioma CSCs, and showed that the anti-tumor effect is present both *in vitro* and *in vivo* experiments. Moreover, Ginestier *et al.*^[164] showed that modulation of retinoid signaling may promote self-renewal or induce differentiation of breast CSCs, and suggested that ATRA may be considered as targeted therapy for breast CSC

population.

Other agents that can affect cancer stem cell differentiation are histone deacetylase inhibitors (HDACI). To date, HDACI are used as differentiation therapy in several hematologic malignancies. Recently, this type of therapy was proposed for breast CSCs^[165] and head and neck CSCs^[166]. In the first study HDACI (abexinostat) induced CSC differentiation. Moreover, in the second study, HDACI (suberoylanilide hydroxamic acid and trichostatin A) altered the CSCs phenotype and also induced cell cycle arrest and apoptosis.

Petrelli *et al.*^[167] showed that miR-100 favors breast CSCs differentiation, converting a basal like phenotype into luminal. It induces the expression of a functional estrogen receptor and renders basal-like BrCSCs responsive to hormonal therapy. Vaz *et al.*^[168] suggest that pancreatic differentiation 2 (PD2) is a novel CSC maintenance protein, loss of which renders the CSCs more susceptible to drug-induced cell death. Knockdown of PD2 protein in CSCs decreased cell viability, enhanced apoptosis and diminish expression of CD133 and MDR2. Dong *et al.*^[169] demonstrated that long-term culture of glioblastoma cancer stem cells with imatinib mesylate, a tyrosine-kinase inhibitor, induced cell differentiation, confirmed by decreased expression of stem cell markers, CD133, Oct-3/4, nestin, and Bmi1, and increased expression of GFAP (astrocyte marker) and class III β -tubulin isotype (Tuj1, neuron marker), associated with reduced ability to form aggregates and colonies *in vitro* and tumorigenicity *in vivo*. Another strategy targeting signaling pathways in order to induce CSCs differentiation is directed against hyperactivated Akt/mTOR and the inhibited wild-type p53 pathways in glioma CSCs. Daniele *et al.*^[170] uses two inhibitors (FC85 and ISA27) that blocked proliferation and promoted the differentiation of GSCs by reducing Akt/mTOR signalling and reactivating p53 functionality.

Inducing CSC differentiation combined with CSC-targeted therapies and traditional therapies may bring real benefits in depleting cancer cells by increasing the efficacy.

CONCLUSION

In this review we have summarized recent advances in identification of CSCs molecular markers that might become targets for developing new therapies, aiming to hamper CSCs regeneration and cancer relapse. These novel therapeutic strategies are designed to target directly CSCs or to interrupt signals from the microenvironment that regulate important properties like self-renewal, differentiation and apoptosis resistance.

All these alternative therapies are very promising, but some of them not specific and might affect also healthy tissue since CSCs niche is similar and close to normal stem cell niche that can also be affected by compounds. There are also many ways by which CSCs could evade treatment. They reside in an area of low

oxygen and vascularity, preventing efficient delivery of the drugs.

Future challenges should include the increasing of specificity and efficiency in targeting CSCs, avoiding toxicity of normal tissue stem cells, and also new strategies of delivery and retention of the drugs inside the CSCs. These new therapies should increase the efficacy of existing drugs against aggressive cancers, and thus should prevent tumor relapse and enhance patient survival.

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REVIEW

- 1202 Dedifferentiated fat cells: A cell source for regenerative medicine
Jumabay M, Boström KI

ORIGINAL ARTICLE

Basic Study

- 1215 Polymeric vs hydroxyapatite-based scaffolds on dental pulp stem cell proliferation and differentiation
Khojasteh A, Motamedian SR, Rezai Rad M, Hasan Shahriari M, Nadjmi N

Contents

World Journal of Stem Cells
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Dedifferentiated fat cells: A cell source for regenerative medicine

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Abstract

The identification of an ideal cell source for tissue

regeneration remains a challenge in the stem cell field. The ability of progeny cells to differentiate into other cell types is important for the processes of tissue reconstruction and tissue engineering and has clinical, biochemical or molecular implications. The adaptation of stem cells from adipose tissue for use in regenerative medicine has created a new role for adipocytes. Mature adipocytes can easily be isolated from adipose cell suspensions and allowed to dedifferentiate into lipid-free multipotent cells, referred to as dedifferentiated fat (DFAT) cells. Compared to other adult stem cells, the DFAT cells have unique advantages in their abundance, ease of isolation and homogeneity. Under proper condition *in vitro* and *in vivo*, the DFAT cells have exhibited adipogenic, osteogenic, chondrogenic, cardiomyogenic, angiogenic, myogenic, and neurogenic potentials. In this review, we first discuss the phenomena of dedifferentiation and transdifferentiation of cells, and then dedifferentiation of adipocytes in particular. Understanding the dedifferentiation process itself may contribute to our knowledge of normal growth processes, as well as mechanisms of disease. Second, we highlight new developments in DFAT cell culture and summarize the current understanding of DFAT cell properties. The unique features of DFAT cells are promising for clinical applications such as tissue regeneration.

Key words: Adipocytes; Dedifferentiated fat cells; Adult stem cells; Pluripotent stem cells; Differentiation

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Core tip: Multipotent dedifferentiated fat (DFAT) cells provide evidence of plasticity in adipocytes. The newly established DFAT cells exhibit vigorous proliferation and multipotent abilities with advantages over other adult stem cells. Modified culture methods reduce the risk of contamination by cells from the stromal vascular fraction to a minimum. In *in vitro* and/or *in vivo* experiments have revealed adipogenic, osteogenic, chondrogenic,

myogenic, angiogenic and neurogenic potentials in DFAT cells. Moreover, the DFAT cells express embryonic stem cell markers and are similar to induced pluripotent stem cells in certain physiological aspects. Based on the abundance, ease of preparation, homogeneity, and multi-lineage potential, the DFAT cells are uniquely suited for regenerative medicine.

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INTRODUCTION

Adipose tissue is well known to house the largest energy reserve in the body. However, adipose tissue is more than just a simple storage depot. Adipocytes secrete hormones, growth factors and cytokines, such as leptin and tumor necrosis factor- α , as well as proteins related to immunological and vascular functions^[1-3]. Through this network of endocrine, paracrine, and autocrine signals, adipose tissue participate in energy homeostasis and is a global regulator of energy metabolism. Normal adipocyte function is also important for host defense and reproduction, and dysfunction may contribute to the development of pathological states such as insulin resistance^[4,5]. Interestingly, recent research indicates that mature adipocytes can be eliminated by dedifferentiation^[6-9]. With the advancement of tissue culture techniques it has been shown that mature adipocytes are able to dedifferentiate into progenitor cells, referred to as dedifferentiated fat (DFAT) cells. The DFAT cells are multipotent, and are able to redifferentiate into a variety of cell lineages^[10,11]. The DFAT cells may serve as an alternative source of adult multipotent cells, with significant potential for use in tissue engineering and regenerative medicine. In this review, we focus on the recent literature addressing dedifferentiation of mature adipocytes as well as the isolation, characterization, and multipotency of DFAT cells.

DEDIFFERENTIATION, TRANSDIFFERENTIATION, AND STEM CELLS

The ultimate goal of regenerative medicine is to restore structure and function of damaged tissues and organs. To successfully support new tissue development, three regenerative processes are essential: dedifferentiation, transdifferentiation, and reprogramming of cells. These are all involved in the transition of adipocytes to DFAT cells, which may be an appropriate model for enhancing our understanding of these phenomena.

Cellular dedifferentiation is considered a regression

of a cell from a highly specialized state to a simpler state that confers pluripotency, giving rise to undifferentiated progenitor cells. Transdifferentiation implies a process where one mature somatic cell transitions to another mature somatic cell. The induction of pluripotency in somatic cells is referred to as reprogramming. Stem cells are by definition able to self-renew, remain in an undifferentiated state, and differentiate along multiple cell lineages. Progenitor cells, on the other hand, exhibit less capacity for self-renewal and differentiate along one or a few lineages.

Dedifferentiation

Dedifferentiation is the basis of tissue regeneration. The first evidence of dedifferentiation during regeneration was found in plants^[12,13], where it is a common process during secondary growth and wound healing. However, in mammals, the capacity to regenerate subsequent to dedifferentiation is limited. For example, myotubes in newts are able to dedifferentiate and proliferate *in vivo*^[14], but this has not been shown for mouse myotubes. The *MYOD* and *MYOG* (myogenin) genes have been shown to be essential for myotube dedifferentiation. When mouse myotubes were treated with extracts from regenerating limbs of newts, *MYOD* and *MYOG* were downregulated, which allowed the myotubes to dedifferentiate and proliferate^[15]. Fortunately, recent studies have demonstrated that dedifferentiation can also occur in defined situations in many cell types in human tissue^[16,17].

Dedifferentiation and cell division are important intermediate processes in the process of switching phenotype, although they do not appear to be obligatory in all cases. Studies on the role of retinoblastoma protein (RB) and RB-like 2 showed that dedifferentiation of mature cardiomyocytes facilitated cardiomyocyte proliferation in cardiac hypertrophy^[18]. Furthermore, inhibition of the p38 mitogen-activated protein kinase induced mammalian cardiomyocytes to dedifferentiate, which may be essential for cardiomyocyte regeneration^[19,20]. However, other experimental data suggest that dedifferentiation may not be required for cardiomyocyte proliferation^[21]. It has also been observed that proliferation promoted by neuregulin, an essential extracellular ligand of the epidermal growth factor receptor during cardiomyocyte development, causes cardiomyocytes to reenter the cell cycle^[22,23]. Alternatively, dedifferentiation may cause cellular plasticity to emerge and allow rerouting of cells into different cell lineages. This could lead to intermediary transdifferentiation of the cell, and result in a progressive conversion into another terminally differentiated cell. Furthermore, dedifferentiation occurs during rare pathological events, which have been found in osteosarcoma, chondrosarcoma, and epithelial-myoeptithelial carcinoma in humans^[24-26]. A population-based study and a 20-year survey on soft tissue sarcoma also included cases of dedifferentiated liposarcoma^[27].

During normal cellular development, cellular dedif-

ferentiation has been shown to relate to the regenerating cells entering the cell cycle^[28]. Prolonged stress, injury, or activation of oncogenic pathways may trigger conversion of a “dedifferentiated” cell into a diseased cell, thereby opening the door for pathological changes. Hypoxia is believed to be the main factor driving the emergence of DFAT cells from adipocytes, as well as dedifferentiation of chondrocytes and smooth muscle cells^[29,30]. However, more studies are needed to fully evaluate the connection between hypoxia and dedifferentiation.

Specific injuries or manipulations stimulate cellular dedifferentiation^[31]. Cells lose their maturity and become susceptible to lineage modification. Dedifferentiation might occur, resulting in loss of cell function while the cells remain as undefined or resting cells as long as the insult persists. Thus, dedifferentiation would be a transition step prior to adopting a new identity, which would be distinguished by the reemergence of factors that redirect cell fate. Alternatively, dedifferentiation may be seen as an adaption to stressful stimuli, causing the cell to cease normal activity, thereby prohibiting progression toward cellular dysfunction, which in extreme cases could end in cell death. Thus, it is possible that specific changes in the intrinsic, environmental, or hormonal milieu might stimulate cells such as adipocytes to withdraw from the cell cycle, undergo dedifferentiation and acquire stem cell characteristics. Understanding the details of the dedifferentiation process would enhance our knowledge of normal regeneration.

Reprogramming

Mature cells acquire stem cell features by undergoing dedifferentiation prior to the acquisition of a new cell fate. The newly undifferentiated stem cells are primed to respond to specific cues and differentiate into a variety of cell lineages. Reprogramming can be attained through cell fusion with embryonic stem cells (ESC), somatic cell nuclear transfer, exposure to stem cell extracts, or induction of pluripotency by defined factors generating what are referred to as induced pluripotent stem cells (iPSCs). During the reprogramming, an erasure and remodeling of epigenetic marks occur including DNA methylation and modification of histone and chromatin structures^[32]. A major challenge in the field of iPSCs is to convert mature cells into pluripotent cells resembling ESC for use in transplantation therapies. The reprogramming of the somatic cells is induced by the transfer of pluripotent factors, many of which are oncogenes. It has also been suggested that iPSCs might be the product of dedifferentiation of somatic cells following oncogenic insult^[33,34]. To address the fear of tumorigenicity in iPSCs, a study recently showed that overexpression of core transcription factor genes or its activators support the maintenance of the cell type-specific transcriptional profile, thus inhibiting alterations in the expression of genes required for iPSC induction^[35]. The stable nature of gene signatures limits

dedifferentiation and promotes the cell type-specific transcriptional profiles.

Transdifferentiation

A normal, fully differentiated cell can either change its identity to a new cell type, referred to as transdifferentiation, or lose its functionality and revert to an immature state referred to as dedifferentiation. The transition to a new phenotype may occur directly or through dedifferentiation triggered by genetic factors or environmental cues. Intermediary transdifferentiation has been shown to occur when acinar cells undergo dedifferentiation into duct-like cells with exocrine as well as endocrine potentials^[36]. Another type of scenario is the conversion of phenotype without a detectable intermediate step, which is referred to as direct reprogramming. It is possible that dedifferentiated cells retrace normal development toward a different, sometimes closely related, cell lineage after reaching the progenitor state.

Different interventions, including naturally occurring events and experimental manipulations, might result in transdifferentiation of cells in unfamiliar compartments. Dedifferentiation is described as the entrance to such transdifferentiation. A recent study demonstrated that cultures of purified hepatic oval stem cells are capable of transdifferentiation into functional endocrine cells^[37]. It has also been reported that increased expression of CAAT/enhancer-binding protein (C/EBP) α and C/EBP β in differentiated islet β cells leads to reprogramming into macrophages without DNA methylation^[38]. Another example is fibroblasts that have transdifferentiated into cardiomyocytes after transfer of the transcription factors GATA4, MEF2C, and TBX5^[39].

The concept that terminally differentiated cells retain intrinsic plasticity increases the number of cell sources that could be used for tissue regeneration in cases of injury or disease. The generation of DFAT cells is an example of reemergence of plasticity in adipocytes, resulting in the ability to transdifferentiate into alternate cell types and to serve as a model for dedifferentiation and transdifferentiation.

DFAT cells and mesenchymal stem cells

The goal of tissue engineering is to repair and regenerate damaged organs with the help of stem cells, biomaterials, and cytokines. However, the limited availability of human stem cells that are able to differentiate along multiple lineages has hampered the progress and slowed the development of these treatments. While ESCs inherently exhibit nearly unlimited differentiation potential *in vitro* and *in vivo*, their use is constrained by scientific concerns regarding safety and efficacy, as well as by ethical, legal, and political concerns. An alternative approach is to use stem cells derived from adult tissues, which would circumvent most of these concerns.

Multipotent stem cells have been defined as a special kind of cells with a unique capacity to self-renew

indefinitely, which implies that the stem cells can be extensively expanded *ex vivo*. Human mesenchymal stem cells (MSCs) were initially derived from bone marrow, but have now been isolated from most types of tissue^[40], including the brain, dermis, periosteum, skeletal muscle, synovium, trabecular bone, vasculature, and adipose tissue, which is the most abundant and accessible source of adult stem cells^[41-43]. MSCs express cell surface markers like cluster of differentiation (CD)10, CD29, CD44, CD73, CD90, CD105, CD117 and STRO-1, but are negative for the hematopoietic lineage markers CD14, CD34, CD45 and HLA-DR. Identification of adipose-derived stem cells (ASCs) suggests that a pool of stem cells exists within the adult adipose tissue. The ASCs are derived from the adipose stroma vascular fraction (SVF), which includes all cells in adipose tissue except the white adipocytes. The ASCs are similar to MSCs in their expression of MSC markers, but lack expression of hematopoietic lineage markers and the endothelial markers CD31 and von Willebrand factor (vWF)^[43,44]. Studies have also identified a periendothelial pericyte-like subpopulation of ASCs, possibly due to the inclusion of vascular elements in the SVF. These cells express CD34, as well as mesenchymal, pericytic, and smooth muscle markers, including chondroitin sulfate proteoglycan (NG2), CD140a, and CD140b^[45], but are negative for CD31, CD45 and CD144. However CD34 and CD104b did not co-localize in these cells, suggesting that CD34+/CD31- cells in the adipose vasculature are not pericytes^[46].

The DFAT cells initially lack expression of CD34, CD31, CD146, CD45, and pericyte markers, distinguishing them from ASCs derived from the SVF^[6-9] (see below for further characterization of DFAT cells). Interestingly, lineage tracing in mice suggest that part of the stromal cells may be derived from adipocytes *in vivo*^[47], suggesting that ASCs and DFAT cells in part have the same precursor cells. The DFAT cells, however, constitute a more homogeneous cell population than the ASCs, further supporting a role of the mature adipocyte fraction as a source of stem cells^[48-50].

DFAT cells and iPSCs

Three major types of pluripotent stem cells have so far been identified, ESCs, iPSCs from reprogrammed adult somatic cells^[51], and multilineage-differentiating stress-enduring cells, referred to as Muse cells, isolated from mesenchymal human tissues^[52]. The Muse cells are considered MSCs, capable of forming cell clusters and expressing a set of genes associated with pluripotency.

All three cell types have factors that limit their use. Ethical concerns make the ESCs controversial, and heterologous transplantation of ESCs may produce immune rejection in the recipient. Transplantation of both ESCs and iPSCs run the risk of producing teratomas in the recipient because of their uncontrolled capacity of proliferation and differentiation. The paucity of Muse cells has so far been a limitation for their widespread

use. Other limitations to the use of iPSCs are derived from the fact that reprogramming genes have been introduced and remain with low efficiency expression in the host cells. One of the ways to overcome this problem is to achieve efficient transgene-free reprogramming using Sendai virus^[53]. Sendai virus remain in the cytoplasm and do not have the ability to integrate into the host genome. Most commonly, virus clearance is achieved by clonal propagation of primary colonies leading to isolation of sub-clones free of the viral genome. However, about 10% of the cells still have the virus after 10 passages^[54]. Although human is not the natural host for Sendai virus, and the virus is non-pathogenic to humans, the potential mucosal exposure to the virus remains a concern. Therefore, identification and development of new sources of pluripotent adult stem cells remain important.

DFAT cells have been shown to express ESC markers including the POU homeodomain protein Oct4, sex determining region Y-box 2 (SOX2), myelocytomatosis oncogene (*c-Myc*), and the homeobox protein Nanog, which are key factors in maintaining pluripotency^[55]. In addition, high alkaline phosphatase and telomerase activity further support similarities between DFAT cells and undifferentiated pluripotent stem cells. However, the expression of pluripotency markers decrease significantly in DFAT cells that have been cultured for longer than 2 wk. It is possible that the early expression of pluripotency markers in DFAT cells was missed in previous investigations where specific lineages were studied, causing the pluripotency in DFAT cells to be overlooked. Several investigators have also reported low levels of expression of pluripotency markers in human ASCs^[56-58], and other studies have revealed similar degrees of pluripotency in DFAT cells and iPSCs^[59,60].

The DFAT cells are unlike the iPSCs, in which simultaneous overexpression of the transcription factors Oct4, SOX2, *c-Myc*, and the Kruppel-like factor 4 leads to the generation of a pluripotent, ESC-like state in fibroblasts. After derivation from adipocytes, pluripotency emerges transiently of DFAT cells with expression of the same transcription factors as in iPSCs, as well as low levels of Nanog, stage-specific embryonic antigen (SSEA)-3, and CD105^[59]. The DFAT cells are able to differentiate into cells representative of the three germ layers, with no evidence of teratoma after injection of human DFAT cells in immunodeficient mice^[59]. It was recently shown that mature porcine adipocytes, in response to dedifferentiation, downregulate many genes important for lipid metabolism and upregulate genes involved in cell proliferation, cell morphology and regulation of cell differentiation^[53]. By this process, the dedifferentiated adipocytes achieved the appropriate DNA methylation status, underwent gene-reprogramming, and gained stem cell properties. Thus, the available data support that dedifferentiated adipocytes have the molecular signature of a reprogrammed cell similar to pluripotent stem cells.

ADIPOCYTE AND DFAT CELL ISOLATION, AND CHARACTERIZATION

Isolation and dedifferentiation of mature adipocytes

Large lipid accumulations make the white adipocytes naturally buoyant and therefore difficult to culture. This technical difficulty has in part made their *in vitro* characteristics inaccessible for study. Additionally, they have been considered to be in the terminal stages of differentiation and lacking proliferative activity, further decreasing the interest in their *in vitro* and *in vivo* behavior on a cellular level. It is more than five decades since Rodbell^[61] first succeeded in separating unilocular adipocytes from mature white fat tissue using collagenase treatment. Since then, most biochemical studies of adipocytes have made use of such dispersed cells. However, mature adipocytes do not attach to the bottom of tissue culture plates, but rather float to the surface of any given culture medium. To overcome this issue, Sugihara *et al.*^[9,62] proposed to use using ceiling culture as a way of culturing adipocytes. Most adipocytes lose their intracellular lipid and buoyancy with time *in vitro*. Ultimately, the cells have lost all lipids, appear fibroblastic, and proliferate to confluence. *In vivo*, tissue expanders were placed within the inguinal fat pad of rats^[63]. Expanded fat pads were then autotransplanted to a distant location. Histologic analysis demonstrated that the tissue-expanded fat pads had lost over half their original volume, and the adipocytes had become elongated, fibroblast-like cells. These changes were attributed to the mechanical forces of the expander but may represent adipocyte dedifferentiation. Interestingly, after these same "atrophied" fat pads were transplanted as autografts, they regained their previous volume, suggesting adipocyte redifferentiation. This supports the concept of an adipocyte equilibrium in which dedifferentiated adipocytes may withstand ischemic insult better than differentiated adipocytes.

In the last decade, several studies have explored the plasticity of mature adipocytes and introduced to them to the stem cell field. Adipocyte dedifferentiation is readily seen *in vitro*. Matsumoto *et al.*^[6] showed that adipocytes containing two nuclei were occasionally detected in adipocytes before they were placed in ceiling culture, but were frequently seen after 3 d of culture. Such binuclear adipocytes were always positive for BrdU in both nuclei, suggesting that the cells had entered S-phase and the nuclei had divided. The authors also performed time-lapse fluorescence microscopy, which revealed that fibroblast-like cells were indeed generated from lipid-filled adipocytes with single nuclei through asymmetric division^[6,64]. Another similar study demonstrated isolation of stem cells with characteristics of immature neural crest cells through asymmetric cell division in cultured human hair follicles *in vitro*^[65]. Asymmetric cell division permits a single mother cell to generate daughter cells that are distinct in size, shape, function and fate. The generation of two progeny with different

fates requires a highly regulated molecular program. In general, disruption of asymmetric cell division leads to the creation of two progeny that retain stem cell characteristics, but with reduced ability to achieve full differentiation^[66-69]. As far as we have observed, the emergence of DFAT cells from mature adipocytes occurs *via* two phenomena; mature adipocytes lose their lipid content and acquire a fibroblast-like shape, and asymmetric cell division of mature adipocytes into one lipid-filled adipocyte and another small daughter cells without lipid (Figure 1). Subsequently, the cells undergo dedifferentiation without the use of inducing agents, resulting in proliferative DFAT cells.

Recent studies exploring DFAT cells describe specific purification steps to ensure, to the extent possible, the purity of the initial preparation of mature adipocytes. Indeed, the purity of the primary adipocytes is essential in the preparation of DFAT cells, as to avoid the possibility of floating adipocytes "dragging" contaminating cells with them^[70]. Recently, we proposed a new method to prepare DFAT cells, using insert culture (Figure 2)^[59]. In this method, DFAT cells are generated from lipid-filled mature adipocytes isolated from small pieces of subcutaneous adipose tissue or human fresh lipoaspirate, washed repeatedly with phosphate-buffered saline until the washes are clear^[6,59,62]. Approximately 1 g of adipose tissue is minced and digested in 0.1% (w/v) collagenase solution (Collagenase type I) at 37 °C for 1 h with gentle agitation. After filtration through nylon filters (core size 100 µm) and centrifugation at 135 g for 3 min, the floating top layer of adipocytes is collected. The adipocytes are then washed repeatedly (usually three times) in Dulbecco's Modified Eagle Medium supplemented with 20% fetal bovine serum before further use. Adipocytes intended for DFAT cell generation are floated on top of medium in culture dishes or plastic tubes to let remaining non-adipocytes detach and sink to the bottom and be discarded after centrifugation. Adipocytes from the top creamy layer (30-50 µL) are subsequently transferred to 6-well plates fitted with 70 µm-filters and incubated for 5 d in culture medium. DFAT cells derived from the adipocytes sink through the filters and attach to the bottom of the dishes. The filters with remains of the adipocytes are removed after 5 d (Figure 2). This method of preparing DFAT cells does not include attachment of the adipocytes to plastic surfaces or ceiling culture, as previously described^[6-8,62,71]. In addition, this method allows the separation of the DFAT cells from the adipocytes as soon as they sink through the filter and attach to the bottom of the dish. This reduces the influence of adipocyte remnants on the surface of the medium on the characteristics of the nascent DFAT cells. We regularly collect up to 10000 DFAT cells during 5 d of collection. The inclusion of these additional steps not only enhances the purity of DFAT cells, but significantly increases the early expression of pluripotency markers

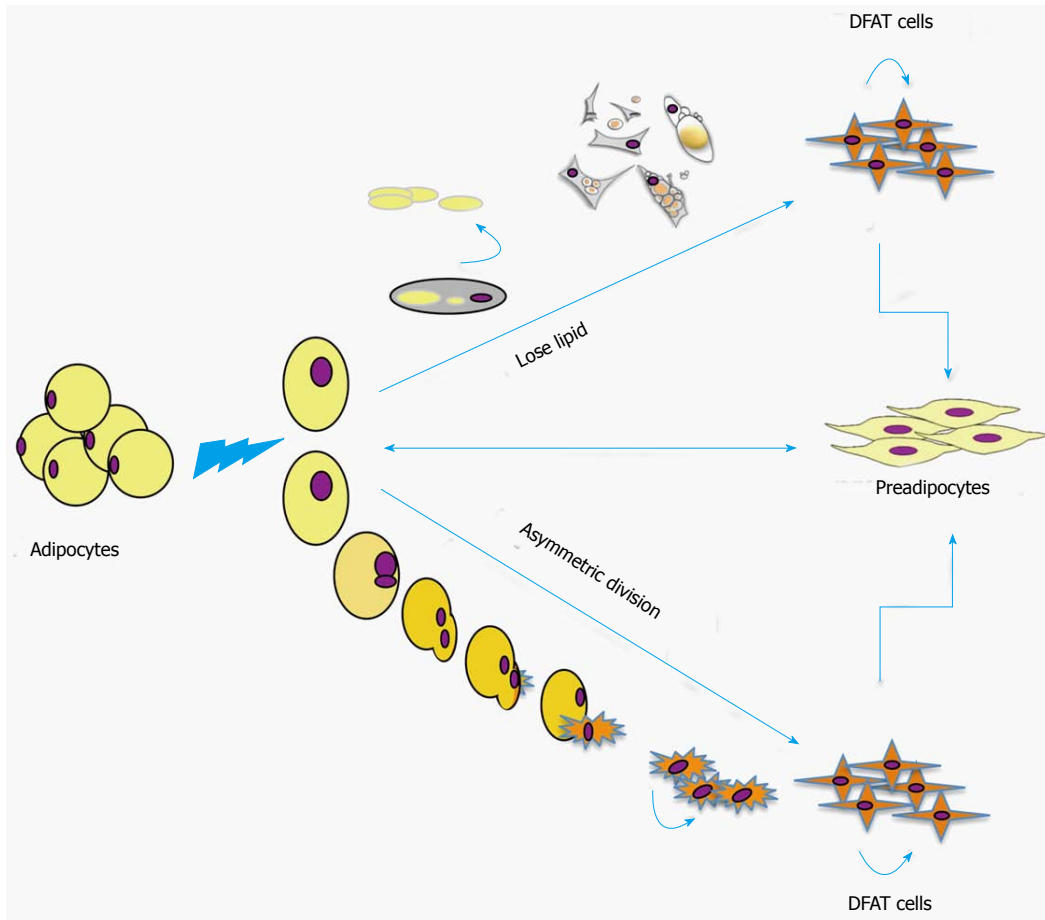


Figure 1 Dedifferentiation of adipocytes. Mature adipocytes lose their lipid content and acquire a fibroblast-like shape. Mature adipocytes also divide asymmetrically into one lipid-filled adipocyte and small daughter cells without lipid. The new lipid-free cells are referred to as DFAT cells. DFAT: Dedifferentiated fat.

previously described^[59].

Characterization of DFAT cells

Most studies on the DFAT cells have concluded that they are a largely homogeneous cell population with an immunophenotype similar to those of ASCs and other MSCs^[72,73]. Human ASCs are relatively heterogeneous and carry hematopoietic-associated markers such as CD11a, CD14, CD45, CD86 and HLA-DR, and low levels of the MSCs-associated markers CD13, CD29, CD34, CD44, CD63, CD73, CD90 and CD166^[73,74]. On the other hand, the DFAT cells are positive for CD13, CD29, CD44, CD90, CD105, CD9, CD166 and CD54, and negative for CD14, CD31, CD34, CD45, CD66b, CD106, CD117, CD133, CD146, CD271, CD309, HLA-DR and alpha-smooth muscle cell actin^[1,50,75]. Both ASCs and DFAT cells express HLA-A, -B and -C, which suggests that both cell types have allogeneic transplantation potential. In addition, we detected that 7.1% of the human DFAT cells expressed SSEA-3, and that most of the human DFAT cells expressed CD105, whereas mouse DFAT cells expressed Sca-1^[59]. This expression was maintained for multiple passages. However, the expression of stem cell markers in human DFAT cells varies with the donor's age, culture conditions, and the degree of differentiation.

DIFFERENTIATION POTENTIAL OF DFAT CELLS

DFAT cells have emerged as a potential cell source for regenerative medicine because of their transdifferentiation capability and similarity to ASCs and bone marrow MSCs^[75-77]. Multiple studies have demonstrated differentiation of DFAT cells into multiple lineages including adipogenic, osteogenic, chondrogenic, myogenic, angiogenic and neurogenic lineages (Figure 3). To monitor the fate of DFAT cells, investigators have used DFAT cells prepared from the adipose tissue of GFP-transgenic mice for transplantation into wild type mice^[64,78], or adipocyte protein 2-Cre^{+/+}; LacZ ROSA (R26R)^{+/+} double transgenic mice^[79]. Human DFAT cells used for injection of immunodeficient mice were traced by anti-human mitochondria staining^[59]. However, the potential need to maintain a specific cell identity once the DFAT cells have achieved a desired phenotype, or the methodology to do so, has not been assessed thus far.

Adipogenesis

Soft tissue reconstruction is an important aspect of tissue engineering, and adipose grafts are needed for minimally invasive injectable therapies in order to

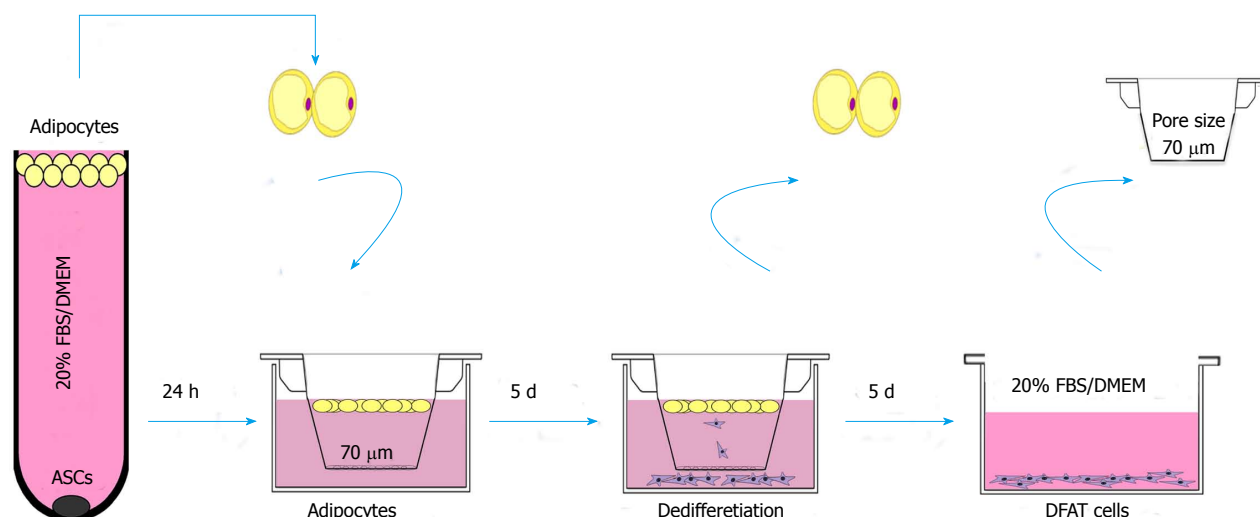


Figure 2 Schematic drawing of dedifferentiated fat cell preparation using preincubation and filters. Isolated adipocytes are incubated for 24 h in culture medium, before transfer to a new dish with filter, where the adipocytes remain for five days before the filter is removed. DFAT cells are allowed to sink through the filter to the bottom of the dish. The adipocytes and filter insert are then removed, and the new DFAT cells are cultured by regular methods. ASCs: Adipose-derived stem cells; DFAT: Dedifferentiated fat.

restore soft tissue volume. The most important features of adipose tissue as a cell source might be its relative expendability and the ease with which large quantities can be obtained with minimal risk. Correctly prepared adipocytes could therefore be a useful alternative for tissue augmentation, such as breast surgery with allogeneic material or tissue flap surgery.

Previous studies have confirmed that mature adipocytes easily redifferentiate into adipocytes^[6,7,11]. We demonstrated that mouse DFAT cells spontaneously underwent adipogenic differentiation without special treatment, whereas human DFAT cells required adipogenic induction^[51]. Another study showed that although DFAT cells expressed lower levels of lipoprotein lipase, leptin, and glucose transporter 4 compared to mature adipocytes, they still expressed important adipogenic markers such as peroxisome proliferator-activated receptor gamma (PPAR γ), C/EBP α , C/EBP β , C/EBP δ , and sterol regulatory element-binding protein-1c^[6,80]. In addition, the DFAT cells have been shown to have adipogenic capacity *in vivo*. Direct injection of DFAT cell into the subcutaneous portion over sternum of mice resulted in fat pad formation after 3 wk without the use of chemical induction^[7,81]. Furthermore, it was found that that PPAR γ and C/EBP α mRNA levels were higher in DFAT cells derived from intramuscular adipose tissue rather than visceral adipose tissue in pigs, suggesting a more active adipogenesis in intramuscular DFAT cells, as compared to visceral DFAT cells^[82]. It implies that DFAT cells from the same donor may differ in rates of redifferentiation and expression of molecular markers depending on the depot of origin.

Osteogenesis and chondrogenesis

DFAT cells can be derived from small amounts of subcutaneous adipose tissue regardless of the age of the donors, and may be useful in cell-based therapies for a

variety of diseases commonly affecting elderly subjects, including metabolic bone disorders and osteoporosis. An earlier study found that the transcription factors RUNX2 and SP7, secreted phosphoprotein 1, bone Gla protein, parathyroid hormone 1 receptor and SOX9 were expressed in DFAT cells, suggesting osteogenic and chondrogenic potentials^[6]. Osteogenic differentiation was stimulated by the addition of dexamethasone, β -glycerophosphate and L-ascorbic acid-2-phosphate to the culture medium. It was also stimulated by the addition of retinoic acid, an analogue of retinol that interacts with bone morphogenetic proteins (BMPs) to limit adipogenesis and promote osteogenesis^[83]. Chondrogenic induction, however, was facilitated by the addition of L-ascorbic acid-2-phosphate, proline, pyruvate, and transforming growth factor β 3. Appropriate mineralization of the cells was confirmed by alkaline phosphate, Alizarin Red S and von Kossa staining, whereas chondrocyte differentiation was confirmed by Alcian Blue staining. An experiment using implantation of DFAT cells in combination with collagen-based scaffolds further showed the ability of the cells to undergo osteochondrogenesis *in vivo*^[84]. Another study proposed DFAT cells as a cell source for periodontal regeneration, after the cells promoted osteogenic differentiation in co-cultures with periodontal ligament stem cells^[85]. Furthermore, the ability of human DFAT cells from the buccal fat pad to undergo osteoblastic differentiation appears to be higher than that of ASCs from the same fat depot^[86]. Thus, the DFAT cells may be attractive as a cell source for tissue engineering in bone disorders such as nonunion fractures and osteoporosis.

Myogenesis

Myocytes are generally divided into three categories: skeletal, cardiac and smooth muscle cells, which differ in their cellular characteristics and behaviors.

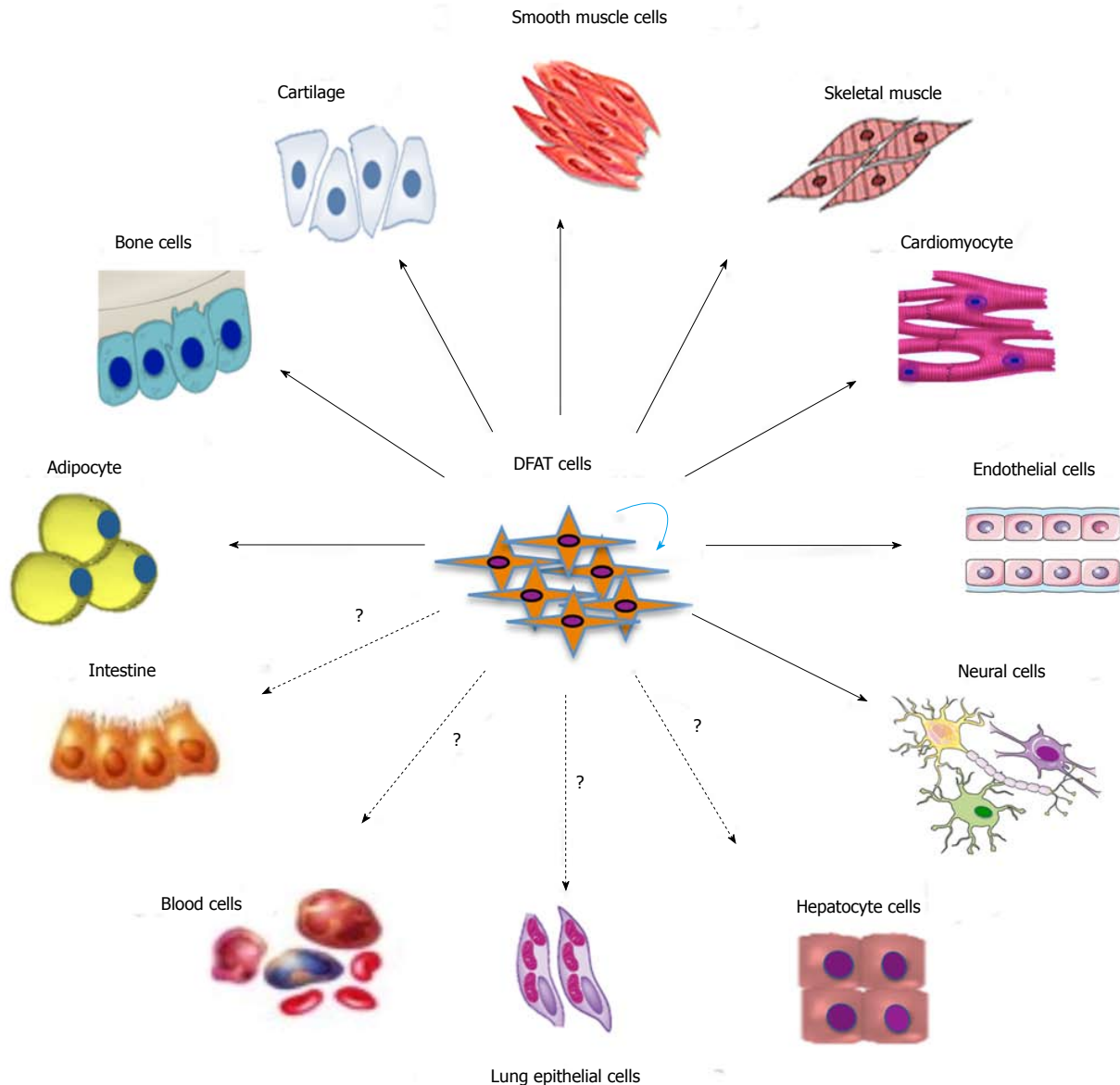


Figure 3 Schematic drawing of differentiation in dedifferentiated fat cells. The DFAT cells are able to differentiate into multiple lineages, including adipogenic, osteogenic, chondrogenic, myogenic, angiogenic and neurogenic lineages. Potential for differentiation into hepatocytes, lung cells, intestine cells and hematopoietic cells has not been reported. DFAT: Dedifferentiated fat.

Myogenesis is a multi-stage process resulting in the formation of muscular cells and tissue. It involves myoblast proliferation, secretion of fibronectin into the extracellular matrix, alignment of the myoblasts into multi-nucleated myotubes, and cell fusion^[87]. The two muscle-specific transcription factors Myf5 and MyoD are known to actively regulate these processes. Treatment with 5-azacytidine (Aza-C), a demethylating agent, led to induction of MyoD and Myogenin in DFAT cells and the formation of multinucleated cells with expression of myosin heavy chain, even though Myf5 was not expressed after induction^[88]. Another study found that DFAT cells underwent smooth muscle cell differentiation and contributed to the regeneration of bladder smooth muscle^[78]. It also was shown that DFAT cell transplantation promoted the recovery of the sphincter muscle and improved the urethral sphincter contractility

in a rat vaginal distension model^[89]. Our previous study demonstrated that DFAT cells were able to differentiate into cardiomyocytes *in vitro*, and injection of DFAT cells into ischemic rat hearts induced neovascularization and supported rehabilitation of the cardiac tissue^[64]. Although pluripotent stem cells have exhibited spontaneous cardiomyocyte differentiation, both *in vitro* and *in vivo*, none of the adult stem cell models studied thus far have spontaneously undergone cardiomyocyte differentiation *in vitro*, except for the DFAT cells^[8,90]. Other adult stem cell models have required either co-culture with isolated cardiomyocytes or treatment with methylation inhibitors and histone deacetylase inhibitors. However, mouse DFAT cells spontaneously differentiated into functional cardiomyocytes without specific induction or genetic treatment^[59]. Human DFAT cells, on the other hand, did not undergo spontaneous

cardiomyocyte differentiation, although they expressed cardiomyocyte markers after cardiogenic induction^[59]. Thus, cardiomyogenic differentiation differs between mice and human DFAT cells, a difference that could be explored to uncover pathways active in spontaneous cardiomyogenesis.

Angiogenesis

Blood vessels consist of an interior lining of endothelial cells surrounded by perivascular support provided by smooth muscle cells or pericytes. Human DFAT cells without induction have been shown to express low levels of endothelial cell or progenitor markers in culture such as the vWF, CD31 or CD34^[6,10,11], and to be stimulated to undergo endothelial differentiation by treatment with BMP4 and BMP9^[10]. They also differentiated into endothelial cells in established *in vitro* and *in vivo* models of angiogenesis^[91-93]. DFAT cells cultured in Matrigel® form tube-like structures that are stable for weeks and stain for endothelial markers, including CD31 and VE-cadherin^[10]. Some researchers have proposed the tube-like structures to be a result of the perivascular nature of DFAT cells, as suggested by the expression of the pericyte-related markers CD140b and NG2^[74]. Similarly, MSCs within adult mesenchymal tissues may differentiate into pericytes without induction by growth factors^[94,95]. In our experiments we initially did not detect pericyte markers in DFAT cells even though they appeared later during culture. Moreover, in Matrigel®, the human DFAT cells differentiated into cells that expressed either endothelial markers or α -smooth muscle actin, which suggests that the cells undergo differentiation into multiple cell types (unpublished observations). It is possible that interactions between endothelial cells and support cells, also derived from the DFAT cells, strengthen the tube structures and promote cell maturity. Thus, the DFAT cells are able to differentiate into endothelial cells *in vitro*, and participate in neovascularization *in vivo*.

Neurogenic and other lineages

Treatments of peripheral or central nerve injuries are still suboptimal despite significant advances in neuroscience and microsurgery. The standard treatment of autologous nerve grafting is unsatisfactory because of morbidity and loss of function at the donor site. Neural progenitor cells such as ESCs and neural stem cells derived from the adult central nervous system may provide new prospects although logistical, ethical, and immunological factors are likely to limit potential applications. However, recent studies have revealed differentiation of ASCs into early neural progenitors, which has generated interest in the field of regenerative medicine. Hsueh *et al.*^[96] observed that, when seeded on a chitosan-coated surface, human ASCs form spheres containing up to 19.5% nestin positive cells. Ahmadi *et al.*^[97] further reported that, after culture in serum-free medium, 51% nestin-positive cells could be generated from human ASCs. However, Zuk *et al.*^[43]

found that nestin was expressed in multiple cell types, including myogenic, endothelial, and hepatic cells, indicating that nestin expression alone is not suitable for the identification of neural cells, especially without functional analysis. Some studies suggest that adipose tissue stem cells are able to induce neurite outgrowth *in vitro*, as well as tissue-specific commitment to neural cell lineages and expression of the tropomyosin receptor kinase A neurotrophin receptor *in vivo*^[98,99]. Most recently, a study demonstrated that ASCs differentiated into astrocytes, oligodendrocytes, and functional neurons, which were able to generate tetrodotoxin-sensitive sodium currents^[100]. DFAT cells, prepared in parallel with ASCs, also had neurogenic potential and expressed neurotrophic factors such as brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. After transplantation, the DFAT cells supported functional recovery from spinal cord injury (SCI)-induced motor dysfunction in rats^[101]. Furthermore, the DFAT cells promoted remyelination and inhibited glial scar formation in SCI mice, possibly through cell-autonomous as well as cell-non-autonomous effects^[102]. It is possible that the neurotrophic factors secreted from the grafted DFAT cells contribute to the functional recovery. Further characterization of the capacity of DFAT cells to undergo neurogenesis, with particular focus on neurophysiologic and neurochemical signal transduction properties, is needed.

Even though hematopoietic lineage differentiation has not yet been reported in DFAT cells, studies comparing porcine-derived mature adipocytes and DFAT cells suggest that such potential exists^[60,103]. Upregulation of genes related to multiple lineages occurred during the dedifferentiation, including those related to hematopoietic cell differentiation.

Overall, DFAT cells appear to constitute an excellent source of cells for tissue engineering. However, the mechanisms of dedifferentiation, and whether it occurs under physiological or pathological conditions *in vivo*, need further exploration. In addition, culture conditions for the maintenance of stem cell characteristics, as well as induction of specific lineages, need development. DFAT cells can also be prepared to match the individual patient, thus allowing for replacement therapies using autologous transplantation. Although no human trial has ever been reported on DFAT cells, it should not deter us from anticipating their clinical application, with focus on tissue regeneration.

CONCLUSION

Multipotent DFAT cells provide evidence of plasticity in adipocytes. The recently characterized DFAT cell model exhibit robust proliferation and multipotency potential giving them an advantage over other adult stem cell models. Compared to bone marrow derived stem cells, white adipose tissue is mostly located subcutaneously and their abundance is generally guaranteed. Further-

more, access to mature adipocytes is obtained through less invasive methods such as liposuction, which have less physical and psychological effects on the donor. Compared to ASCs, DFAT cells comprise a more homogeneous population of cells. Modified culture methods reduce the risk of contamination by cells from the stromal vascular fraction to a minimum. *In vitro* and/or *in vivo* experiments have revealed adipogenic, osteogenic, chondrogenic, myogenic, angiogenic and neurogenic differentiation potentials in DFAT cells. Moreover, the DFAT cells express ESC markers and are similar to iPS cells in certain physiological aspects. Based on the abundance, ease of preparation, homogeneity, and multi-lineage potential, the DFAT cells are uniquely suited for regenerative medicine.

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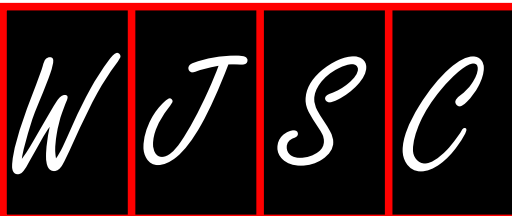
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Basic Study

Polymeric vs hydroxyapatite-based scaffolds on dental pulp stem cell proliferation and differentiation

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Abstract

AIM: To evaluate adhesion, proliferation and differentiation of human dental pulp stem cells (hDPSCs) on four commercially available scaffold biomaterials.

METHODS: hDPSCs were isolated from human dental pulp tissues of extracted wisdom teeth and established in stem cell growth medium. hDPSCs at passage 3-5 were seeded on four commercially available scaffold biomaterials, SureOss (Allograft), Cerabone (Xenograft), PLLA (Synthetic), and OSTEON II Collagen (Composite), for 7 and 14 d in osteogenic medium. Cell adhesion and morphology to the scaffolds were evaluated by scanning electron microscopy (SEM). Cell proliferation and differentiation into osteogenic lineage were evaluated using DNA counting and alkaline phosphatase (ALP) activity assay, respectively.

RESULTS: All scaffold biomaterials except SureOss (Allograft) supported hDPSC adhesion, proliferation and differentiation. hDPSCs seeded on PLLA (Synthetic) scaffold showed the highest cell proliferation and attachment as indicated with both SEM and DNA counting assay. Evaluating the osteogenic differentiation capability of hDPSCs on different scaffold biomaterials with ALP activity assay showed high level of ALP activity on cells cultured on PLLA (Synthetic) and OSTEON II

Collagen (Composite) scaffolds. SEM micrographs also showed that in the presence of Cerabone (Xenograft) and OSTEON II Collagen (Composite) scaffolds, the hDPSCs demonstrated the fibroblastic phenotype with several cytoplasmic extension, while the cells on PLLA scaffold showed the osteoblastic-like morphology, round-like shape.

CONCLUSION: PLLA scaffold supports adhesion, proliferation and osteogenic differentiation of hDPSCs. Hence, it may be useful in combination with hDPSCs for cell-based reconstructive therapy.

Key words: Human dental pulp stem cell; Stem cell; Tissue engineering; PLLA; Bone

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Core tip: Recently, the plasticity of postnatal stem cells from dental origin including human dental pulp stem cells (hDPSCs) has been suggested. Their osteogenic potential makes them valuable for craniofacial bone regeneration. hDPSCs can be easily isolated from dental medical wastes, extracted teeth, and expanded *ex vivo*. Combination of numerous postnatal stem cells and three-dimensional scaffold biomaterials has been used in bone tissue engineering. Selection of an ideal scaffold biomaterial is a challenging part of reconstructive surgeries. Current study aims to evaluate behavior of hDPSCs including adhesion, proliferation, morphology and differentiation on four different scaffold biomaterials. Our finding indicates that PLLA (Synthetic) scaffold supports adhesion, proliferation and osteogenic differentiation of hDPSCs. Therefore, it can be useful for the purpose of craniofacial tissue engineering.

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INTRODUCTION

Non-healing craniofacial defects occur frequently due to various factors such as trauma, infection, tumor, and congenital deformities^[1,2]. The treatment of large bone defects has been posed a challenge for reconstructive surgeons. The conventional treatment approach for treatment of craniofacial bone defects is harvesting and transplanting autogenous cancellous bone graft, particularly from ilia crest^[3]. However, the surgical procedures are invasive and it usually associated with donor site morbidities and limited availability^[4,5]. Another approach is utilization of allogeneic bone which is also limited by the risk of immunogenicity or disease transmission^[6].

The concept of harvesting adult stem cells (ASCs) in combination with appropriate three-dimensional (3D) scaffolds have been proposed as a promising alternative approach in reconstructive surgery^[7-12].

ASCs have been isolated from various tissues. In dental clinic teeth are often need to be extracted in order to avoid further complications including orthodontic treatments. Human dental pulp has been harvested from extracted teeth and shown to contain multilineage population of progenitor/stem cells, dental pulp stem cells (hDPSCs)^[13,14]. The differentiation capability of hDPSCs towards the osteoblast lineage and their accessibility and ease of *ex-vivo* expansion make them suitable cell source to be considered for repair of skeletal defects^[15,16].

Bone formation from hDPSCs is required a 3D structure provided by scaffolds. An ideal scaffold should provide an appropriate environment for cellular attachment, growth, and differentiation. A wide range of scaffold biomaterials have been developed for variety of applications in tissue engineering^[17]. Scaffolds can be categorized as following groups: (1) Allograft; (2) Xenograft; (3) Synthetic; and (4) Composite biomaterials. In order to select a suitable scaffold for craniofacial engineering, it is necessary to evaluate the cell-scaffold interactions *in vitro*. Current study is aimed to investigate hDPSCs behavior including cell adhesion, attachment, and differentiation on four commercially available scaffolds from given groups.

MATERIALS AND METHODS

hDPSC culture

hDPSC cultures are established from dental pulp tissues isolated from extracted teeth of healthy volunteer adults (aged 18-30) as previously described^[14]. Briefly, pulp tissues are gently separated from the crown and root and then digested in a solution of 0.075% collagenase type I (Sigma-Aldrich, St. Louis, Missouri, United States) for 1 h at 37 °C. hDPSCs are established by growing the primary cell suspension in stem cell growth medium containing DMEM-HG (Invitrogen, Grand Island, NY, United States), 10% FBS (Invitrogen, Grand Island, NY, United States) and 100 units/mL penicillin 100 mg/mL streptomycin (Invitrogen, Grand Island, NY, United States) in T-25 flasks overnight at 37°C and 5% CO₂, and non-adherent cells were removed by medium change. The remaining cells were cultured until they reached 80%-90% confluency. Cells were trypsinized using 0.05% Trypsin-EDTA (Invitrogen, Grand Island, NY, United States) and passaged at a ratio of 1:3 until the desired passages were reached. Passage 3-5 were used for subsequent experiments.

Scaffolds and cell seeding

Four commercially available bone-graft substitutes were studied. SureOss (HansGBR Biomaterial, Seoul, South Korea) is a freeze dried cortical bone allograft. It is a granular biomaterial with the particle size of 200-850

µm. Cerabone (Biotiss Biomaterial, Zossen, Germany) is a xenograft derived from the mineral phase of bovine bone. It is a granular biomaterial with the particle size of 0.5-1.0 mm. PLLA is a synthetic biomaterial. OSTEON II Collagen (Dentium, Gyeonggi, South Korea) is a composite biomaterials contains HA:β-TCP = 30:70 and natural type I collagen. It has particle size of 0.2-0.5 mm.

Approximately 100 mg of given biomaterial scaffolds were placed into 96-well plates. hDPSCs were seeded onto the scaffolds at a cell density of 10^4 cells/well in osteogenic medium (Invitrogen, Grand Island, NY, United States). The cells - scaffold constructs were maintained at 37 °C in a humidified 5% CO₂ atmosphere for 7 and 14 d.

Cell adhesion

hDPSCs adhesion on following scaffolds were assessed using scanning electron microscopy (SEM). Seven days after cell seeding, samples were fixed with 2.5% glutaraldehyde (MerckKGaA, Darmstadt, Germany) solution for 30 min. Specimens were then post-fixed with 1% Osmium tetroxide (Sigma-Aldrich, St. Louis, Missouri, United States). After cell fixation, the specimens were subsequently dehydrated by ascending grades of alcohol (25%, 50%, 75%, 96%, and 100% ethanol) for 15 min each step. The specimens were then allowed to dry in air. After complete drying, they were sputtered with gold and analyzed using a SEM imaging (Hitachi, Tokyo, Japan).

Cell proliferation

To determine the proliferation of hDPSCs on following scaffolds, DNA counting assay were performed using QIAamp® DNA Mini Kit, according to the manufacturer's description (Qiagen, Valencia, CA, United States). Briefly, cells at days 7 and 14 were harvested using 0.05% Trypsin-EDTA (Invitrogen, Grand Island, NY, United States). Then, cell pellets were collected by centrifugation and re-suspended in phosphate-buffered saline (PBS). DNA was collected with QIAam spin column. The amount of DNA (ng/mL) was measured using Nanodrop (Thermo Scientific, Waltham, MA, United States).

Cell differentiation

Osteogenic differentiation of hDPSCs on scaffold were evaluated at days 7 and 14 after cell seeding using alkaline phosphatase (ALP) activity assay. The cell-scaffold constructs were rinsed with PBS and homogenized in lysis buffer (pH 7.5, 10 mmol/L Tris-HCl, 1 mmol/L MgCl₂, and 0.05% Triton X-100). The resulting mixture was then centrifuged at 12000 rpm for 10 min at 4 °C. The cell lysate was mixed with *p* nitrophenol phosphate substrate solution (Sigma aldrich, St. Louis, Missouri, United States) and alkaline buffer solution (Sigma aldrich, St. Louis, Missouri, United States). After incubation at 37 °C for 15 min, the above mixture

was added to 0.5 N NaOH to stop the reaction and the absorbance at 405 nm was measured using ELIZA reader (BioTek, Winooski, VT, United States).

Statistical analysis

All quantitative data were expressed as mean ± SE. Analysis of variance and Post hoc tests was conducted for multiple comparisons. A *P* value less than 0.05 were considered to be significant.

RESULTS

Evaluation of hDPSC morphology and adhesion

The suitability of the 3D structure of scaffolds for cell seeding was assessed by observing cell morphology and adhesion using SEM imaging. SEM microphotographs after 7 d confirmed the adherence of hDPSCs in all biomaterial scaffolds (Figure 1-D) except SureOss (Allograft) (Figure 1A). Marked cell aggregation was observed on PLLA scaffold (Synthetic) (Figure 1D-F). hDPSCs were covered almost the entire PLLA scaffold surface. In contrast, fewer cells were aggregated on the surface of Cerabone (Xenograft) and OSTEON II Collagen (Composite) scaffolds (Figure 1B and C). The adherent hDPSCs on Cerabone (Xenograft) and OSTEON II Collagen (Composite) scaffolds demonstrated fibroblastic morphology with several cytoplasmic extension (Figure 1-F). The osteoblastic-like morphology, round-like shape, was observed on cells attached on PLLA (Synthetic) scaffold (Figure 1B, C, G and H).

Evaluation of hDPSC proliferation

The proliferation of hDPSCs on different scaffold biomaterials were monitored and quantified after 7 and 14 d culturing of the cells by means of DNA counting assay (Figure 2). DNA counting assay confirmed the SEM results. The amount of DNA was highest on PLLA group at both time points (Figure 2A and B).

Evaluation of hDPSC differentiation

The osteogenic differentiation capability of hDPSCs on different scaffold biomaterials was compared using ALP activity assay. The results are presented in Figure 3A and B. At day 14 both cells cultured on PLLA (Synthetic) and OSTEON II Collagen (Composite) scaffolds showed high level of ALP activity compared to cells on Cerabone (Xenograft). No significant difference observed between osteogenic activity of hDPSCs on OSTEN II Collagen and PLLA group (Figure 3B).

DISCUSSION

An ideal scaffold should interact with cells, support cell attachment and proliferation, and stimulate tissue regeneration. In current study, we investigated the hDPSC activity and growth behavior on four different 3D scaffold biomaterials. The scaffold biomaterials used

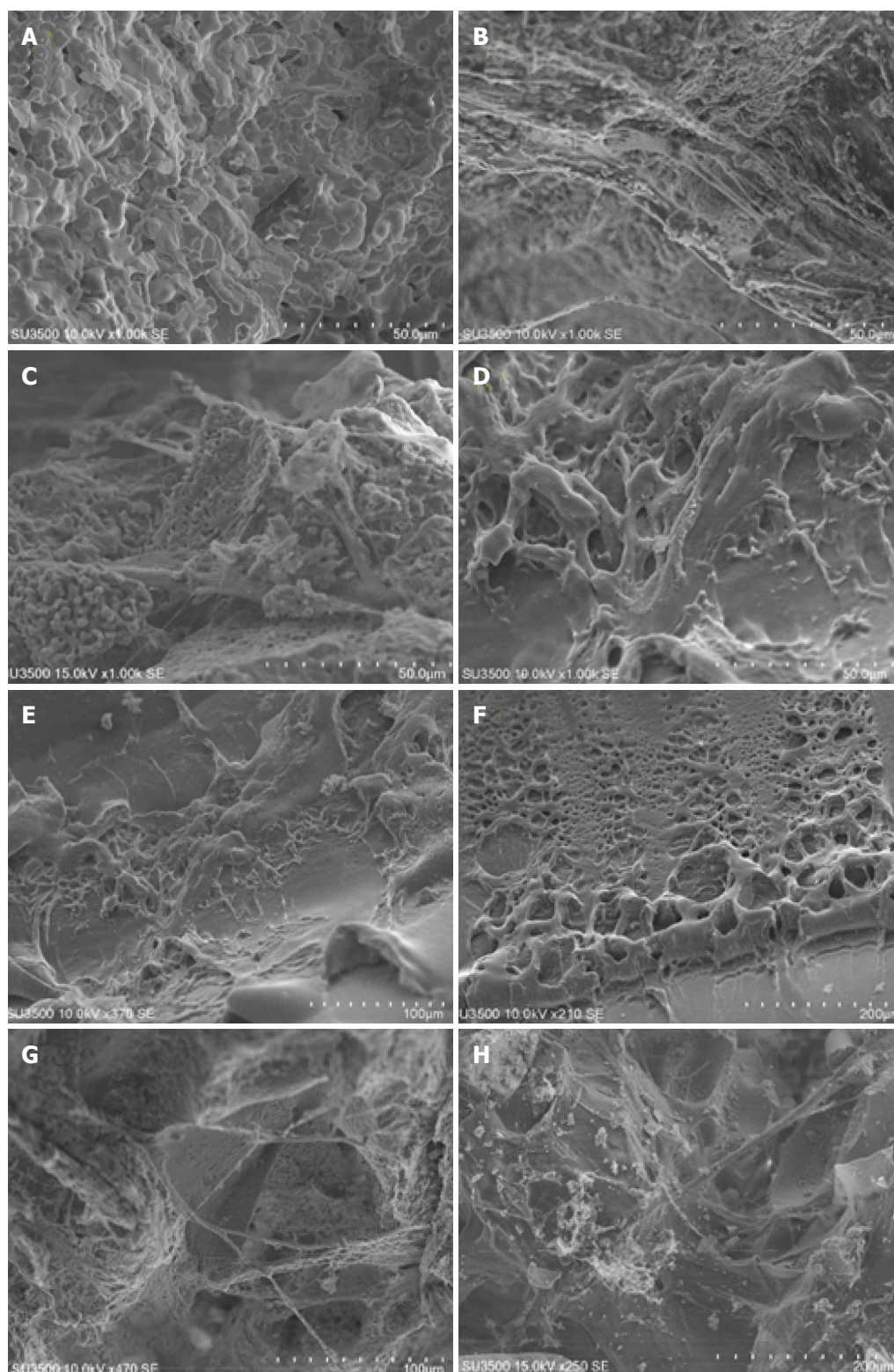


Figure 1 Scanning electron microscopy microphotographs of human dental pulp stem cells attachment and morphology on four different scaffolds. A: SureOss (Allograft); B: Cerabone (Xenograft); C: OSTEON II Collagen (Composite); D: PLLA (Synthetic); E and F: PLLA at lower magnification; G and H: Cerabone at lower magnification. Note that cells were covered almost the entire scaffold surface of PLLA (D-F). Also note that attached cells on PLLA surface showed fibroblastic morphology (D-F), whereas cells on Cerabone and OSTEON II Collagen demonstrated osteoblastic phenotype (B, C, G, H).

in this study are already commercially available for clinical applications. However, comparing their efficiency to provide structural support for hDPSCs is essential. hDPSCs has been selected for our observation, since

their clinical application seems feasible. Harvesting and isolating these progenitor population are relatively easy and a sufficient number of the cells can be provided in two weeks^[14,15]. In addition, since dental pulp stem

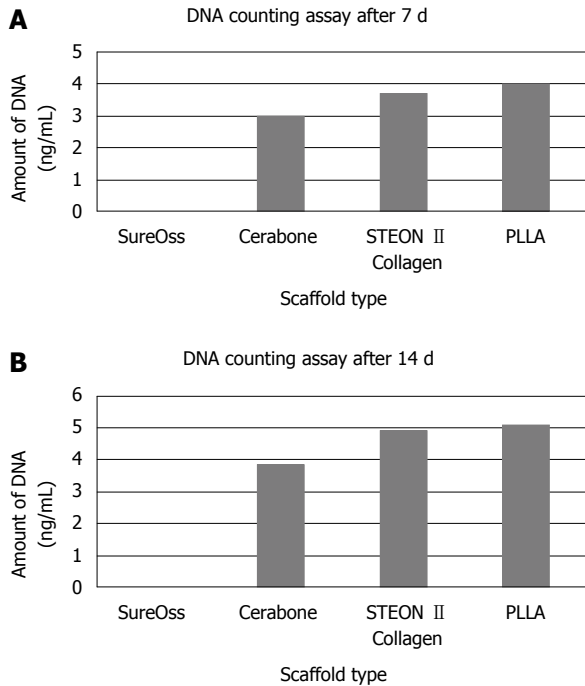


Figure 2 The quantitative analysis of human dental pulp stem cells proliferation by DNA counting assay after 7 (A) and 14 (B) d. Note that the highest amount of DNA was observed on PLLA group at both time points.

cells are derived from neural crest, different origin from mesoderm-derived bone marrow stem cells, they may be considered better candidate for repair of damages in neural crest-derived tissues including periodontium and craniofacial defects^[15,18].

We have shown that all scaffold biomaterials except SureOss (Allograft) support hDPSC adhesion, proliferation and differentiation. Among them, the highest cell proliferation was observed in the presence of PLLA, as it confirmed with both SEM and DNA counting assay. SEM showed almost all surface of PLLA scaffold was covered by adhering cells. PLLA is one of the few synthetic degradable polymers which approved by the Food and Drug Administration for clinical application^[19] and has been used extensively in reconstructive surgery in combination with mesenchymal stem cells (MSCs)^[20-22]. In our study, we have shown that MSCs derived from dental pulp tissues also are attached, proliferated, within PLLA scaffold.

In addition, SEM examination showed that cell morphology differs clearly between scaffolds. In the presence of Cerabone (Xenograft) and OSTEON II Collagen (Composite) scaffolds, the hDPSCs demonstrated the fibroblastic phenotype, which is the typical MSC morphology^[23]. However, the cells on PLLA (Synthetic) scaffold showed the round-like shape which is the typical osteoblastic phenotype^[24], indicating that PLLA scaffold not only can improve cell adhesion and growth, but also upregulate the osteoblastic phenotype.

Evaluating hDPSCs adhesion, proliferation and differentiation of HA/ β -TCP in composition with collagen, we have shown that despite the increase in ALP

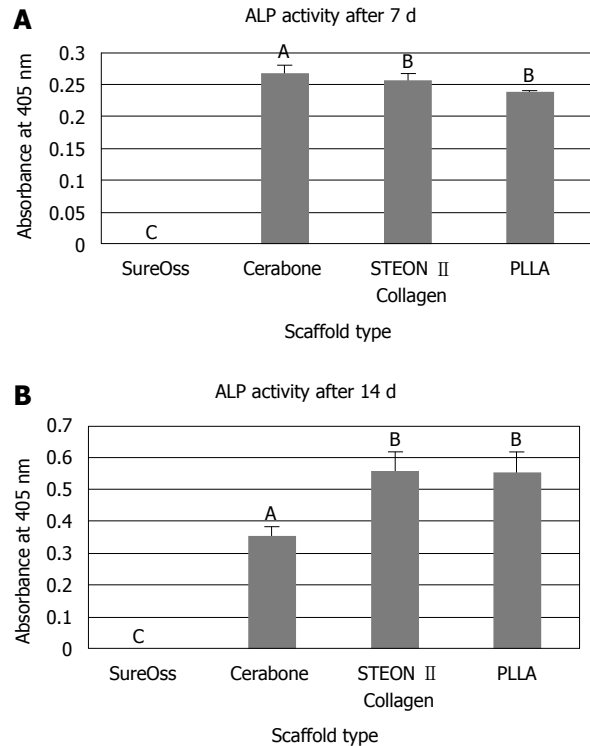


Figure 3 The assessment of osteogenic differentiation of human dental pulp stem cells by alkaline phosphatase activity assay after 7 (A) and 14 (B) d. ALP activity was highest on PLLA and OSTEON II Collagen groups at day 14. Note that no significant difference was observed between PLLA and OSTEON II Collagen. Also, note that different letters are indicated significant difference at $P \leq 0.05$. ALP: Alkaline phosphatase.

activity, the cellular attachment and proliferation was lower compared to PLLA scaffold. Phosphate Ceramics including HA and β -TCP have been described as an osteoinductive materials for many years^[25-27]. Despite their similarity to mineral component of human bone, their major drawbacks including their high resorption rate and low mechanical strength^[28] hampers their applications in clinics. The combination of these materials and collagen has been reported in various studies to reinforce their mechanical strength and decrease resorption rate^[29-31]. Moreover, several studies have been used both collagen and HA in combination with other scaffold biomaterial in order to mimic the natural environment of the bone^[32]. Akkouch *et al.*^[32], showed increased osteogenic capability of hDPSCs cultured on composite scaffold made of HA, collagen and poly (L-lactide-co- ϵ -caprolactone) (PLCL). However, there have been controversies among the literature regarding the beneficial effects of these phosphate ceramics on cellular attachment and proliferation. Study of Pereira-Junior *et al.*^[33], showed the slow growth of MSCs in the presence of HA granule. Similar study on MSCs seeded on β -TCP has shown the slow bone formation *in vivo*^[34]. In contrast, study of Kasten *et al.*^[35], showed that HA ceramics supported the cellular attachment and differentiation. Ling *et al.*^[36], compared the attachment, proliferation and osteogenic differentiation of DPSCs on composite scaffold containing HA, collagen and poly

(L-lactide) (PLA) with β -TCP. They demonstrated that although DPSCs had more mineralization on β -TCP, cell attachment and proliferation were higher in composite scaffold of HA, collagen and PLA.

Present study was designed to evaluate these bone substitute materials in response to MSCs derived from dental pulp. However, the result of this study needs to be confirmed *in vivo*, where the interaction of cell-scaffold with host environment is an essential factor.

Our findings indicate that PLLA (Synthetic) scaffold supports adhesion, proliferation and osteogenic differentiation of hDPSCs. Therefore, it can be useful for the purpose of craniofacial tissue engineering.

COMMENTS

Background

The treatment of large non-healing craniofacial defects has been posed a challenge for reconstructive surgeries. The conventional treatment approach for treatment of craniofacial bone defects is usually associated with donor site morbidities and limited availability. The concept of harvesting adult stem cells (ASCs) in combination with appropriate three-dimensional (3D) scaffolds have been proposed as a promising alternative approach in reconstructive surgery. Human dental pulp stem cells (hDPSCs) can be easily isolated from dental medical wastes, extracted teeth, and expanded *ex vivo*. Their osteogenic capability makes them appropriate source of ASCs. Bone formation from hDPSCs is required a 3D structure provided by scaffolds which should provide an appropriate environment for cellular attachment, growth, and differentiation.

Research frontiers

A wide range of scaffold biomaterials have been developed for variety of applications in tissue engineering. Scaffolds can be categorized as following groups: (1) Allograft; (2) Xenograft; (3) Synthetic; and (4) Composite biomaterials. In order to select a suitable scaffold for craniofacial engineering, it is necessary to evaluate the cell-scaffold interactions *in vitro*. Current study is aimed to investigate hDPSCs behavior including cell adhesion, attachment, and differentiation on four commercially available scaffolds from given groups.

Innovations and breakthroughs

The authors' findings indicate that PLLA (Synthetic) scaffold supports adhesion, proliferation and osteogenic differentiation of hDPSCs.

Application

DPSCs in combination with PLLA scaffold can be useful for the purpose of craniofacial tissue engineering.

Terminology

A scaffold is an artificial designed biomaterial which mimics extra cellular matrix and it is support cellular attachment, proliferation and differentiation.

Peer-review

The study is well designed and results are clear and support the conclusions.

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**REVIEW**

- 1222** High dose chemotherapy with stem cell support in the treatment of testicular cancer
Popovic L, Matovina-Brko G, Popovic M, Petrovic D, Cvetanovic A, Vukojevic J, Jovanovic D
- 1233** Towards *in vivo* amplification: Overcoming hurdles in the use of hematopoietic stem cells in transplantation and gene therapy
Nagreg MS, López-Vásquez L, Medin JA
- 1251** Multipotent pancreas progenitors: Inconclusive but pivotal topic
Jiang FX, Morahan G

ORIGINAL ARTICLE**Basic Study**

- 1262** Induction of CXC chemokines in human mesenchymal stem cells by stimulation with secreted frizzled-related proteins through non-canonical Wnt signaling
Bischoff DS, Zhu JH, Makhijani NS, Yamaguchi DT

Contents

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ABOUT COVER

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High dose chemotherapy with stem cell support in the treatment of testicular cancer

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Abstract

Testicular germ cell cancer (TGCC) is rare form of malignant disease that occurs mostly in young man between age 15 and 40. The worldwide incidence of TGCC is 1.5 per 100000 man with the highest rates in North Europe. After discovery of cisplatin cure rates of TGCC are very favorable between 90%-95% and unlike most solid tumors, cure rate for metastatic TGCC is around 80%. Metastatic TGCC is usually treated with 3-4 cycles of bleomycin, etoposide, cisplatin chemotherapy with or without retroperitoneal surgery and cure rates with this approach are between 41% in poor risk group and 92% in good risk group of patients. Cure rates are lower in relapsed and refractory patients and many of them will die from the disease if not cured with first line chemotherapy. High dose chemotherapy (HDCT) approach was used for the first time during the 1980s. Progress in hematology allowed the possibility to keep autologous haematopoietic stem cells alive *ex-vivo* at very low temperatures and use them to repopulate the bone marrow after sub-lethal dose of intensive myeloablative chemotherapy. Despite the fact that there is no positive randomized study to prove HDCT concept, cure rates in relapsed TGCC are higher after high dose therapy than in historical controls in studies with conventional treatment. Here we review clinical studies in HDCT for TGCC, possibilities of mobilising sufficient number of stem cells and future directions in the treatment of this disease.

Key words: High dose chemotherapy; Germ-cell cancer; Stem cell transplantation; Plerixafor

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Core tip: High dose chemotherapy with autologous haematopoietic stem cell transplantation is effective option in treating relapsed metastatic germ-cell cancer. We reviewed this topic in regard of clinical studies, optimal mobilising and conditioning regimens, with special review on plerixafor in this indication. We also analysed risk adapted approach in those patients and future directions in field.

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INTRODUCTION

Testicular germ cell cancer (TGCC) is a rare form of malignant disease that occurs mostly in young man between age 15 and 40. The worldwide incidence of TGCC is 1.5 per 100000 man with the highest rates in Northern Europe^[1]. One half of all TGCC are seminomas and other half are non-seminomas. The majority of TGCC arise from the gonads while around 5% arise from extragonadal sites in the body's mid-line: retroperitoneum, mediastinum or brain^[2]. After discovery of cisplatin TGCC cure rates have become very favorable ranging between 90%-95% and unlike most solid tumors, cure rate for metastatic TGCC is around 80%^[1,2]. Metastatic TGCC is usually treated with 3-4 cycles of bleomycin, etoposide, cisplatin (BEP) chemotherapy with or without retroperitoneal surgery and cure rates with this approach are between 41% in poor risk group and 92% in favourable risk group of patients^[3] (Table 1). Cure rates are lower in relapsed and refractory patients and many of them will die from the disease if not cured with first line chemotherapy^[3]. High dose chemotherapy (HDCT) approach was first used during the 1980s. Progress in hematology allowed the possibility to keep autologous haematopoietic stem cells alive *ex-vivo* in very low temperatures and using them to repopulate the bone marrow after sub-lethal dose of intensive myeloablative chemotherapy^[4].

RATIONALE FOR HIGH DOSE CHEMOTHERAPY

Resistance to chemotherapy is a major problem in the treatment of patients with malignant diseases. Large number of studies are directed towards finding and overcoming resistance mechanisms. One of the simplest and most logical way is to increase the dose of cytotoxic drugs^[5]. The evidence that higher doses

Table 1 Prognostic criteria for metastatic germ cell tumors^[3]

	Good prognosis group	Intermediate prognosis group	Poor prognosis group
Seminoma	90% of cases 5 yr PFS 82% 5 yr OS 86% All of the following criteria: Any primary site No non-pulmonary visceral metastases Normal AFP Any hCG Any LDH	10% of cases 5 yr PFS 67% 5 yr OS 72% Any of the following criteria: Any primary site Non-pulmonary visceral metastases Normal AFP Any hCG Any LDH	-
Non-seminoma	56% of cases 5 yr PFS 89% 5 yr OS 92% All of the following criteria: Testis/ retroperitoneal primary Non non-pulmonary visceral metastases AFP < 1000 ng/mL hCG < 5000 IU/L (1000 ng/mL) LDH < 1.5 × ULN	28% of cases 5 yr PFS 75% 5 yr OS 80% Testis/ retroperitoneal primary Non non-pulmonary visceral metastases AFP 1000-10000 ng/mL hCG 5000-50000 IU/L (1000 ng/mL) LDH 1.5-10 × ULN	28% of cases 5 yr PFS 75% 5 yr OS 80% Any of the following criteria: Mediastinal primary Non-pulmonary visceral metastases AFP > 10000 ng/mL hCG > 50000 IU/L (1000 ng/mL) LDH > 10 × ULN

AFP: Alpha-fetoprotein; hCG: Human chorionic gonadotrophin; LDH: Lactate dehydrogenase; PFS: Progression free survival; OS: Overall survival.

of cytotoxic drug kill more malignant cells has been well known for decades. Back in 1964, Skipper *et al*^[6] demonstrated that the curve showing the dose dependency of the treatments with cytotoxic drugs is very steep, indicating that even a small increase of the cytotoxic drug dose will kill more malignant cells. Also, this curve for cytotoxic drugs, unlike the curves from other drugs, has no plateau, which means that a constant increase of the dose of cytotoxic drugs leads to a steady increase in numbers of destroyed malignant cells^[6]. During the 1980s, Frei *et al*^[7] showed a dose-dependent killing of malignant cells in AKR and L1210 cell lines. Frei *et al*^[8] demonstrated the same on MCF7 breast cancer cells treated with alkylating agents BCNU, melphalan and nitrogen mustard. However, it was not possible to administer the 5 to 10-fold higher dose of chemotherapy *in vivo* due to the high toxicity and virtually lethal toxicity on bone marrow. Therefore, the researchers started to scrutinize bone marrow transplantation as a method for overcoming this high toxicity after chemotherapy^[9]. Afterwards, numerous studies of high dose chemotherapy and autologous stem cell transplantation in a large number of solid tumor cases were completed, however, this form of treatment

has remained standard practice only for TGCC^[10-13].

CLINICAL TRIALS WITH HDCT TGCC

As already mentioned, the majority of patients with metastatic TGCC are cured with standard chemotherapy: 3-4 cycles of BEP protocols^[3]. However, in patients with a poor prognosis, cure rate is below 50%. In these patients, and in patients with relapsed testicular cancer, unsatisfactory performance standard chemotherapy has directed researchers to search for new forms of treatment. The rationale of using high dose chemotherapy in chemo-sensitive cancer lead on investigators to start clinical trials with high dose chemotherapy and stem cell support^[3,14].

HDCT AS THE INITIAL TREATMENT IN PATIENTS WITH POOR PROGNOSIS

The initial studies of high dose chemotherapy for patients with poor prognosis in the first line setting were completed in the nineties. Motzer *et al.*^[15,16] from Memorial Sloan-Kettering Cancer Center in phase II studies demonstrated slightly better response to HDCT compared to a historical control with the standard dose chemotherapy (SDCT). In a study from 1993, 15 of 27 patients (56%) achieved a complete remission, 46% were free of disease, and 57% alive after a median of 31.2-mo follow-up^[15]. In another study by the same authors 30 patients were treated, 16 with etoposide, ifosfamide, cisplatin (VIP) chemotherapy, while 14 patients after VIP therapy, received HDCT, combination carboplatin, etoposide, cyclophosphamide (CEC). Patients selected for HDCT included those in whom tumour markers did not normalise after two cycles of chemotherapy. After a median follow-up of 30 mo, 15 (50%) patients remained progression-free. Patients treated with marker-dependent, early-intervention HDCT experienced longer survival^[16]. Bokemeyer *et al.*^[17] published in 1999 a match-paired multivariate analysis which compared the outcomes of patients with poor prognosis metastatic TGCC treated sequentially with standard VIP protocol and HDCT in a multicentric study including patients from German group studies and patients treated in two studies from Indiana University, with BEP or VIP conventional chemotherapy. High dose chemotherapy group included 147 patients, while 309 patients were in the SDCT group. Patients treated with HDCT had a longer progression free survival (PFS) 75% vs 59% ($P = 0.0056$) and a longer overall survival (OS) 82% vs 71% ($P = 0.0184$)^[17]. After that, Schmoll *et al.*^[18] from German Testicular Cancer Study Group (GTCSG) published a phase I/IIa study where they treated poor prognosis TGCC patients with a VIP-escalated protocol. After one cycle of standard VIP protocol, they applied dose escalated VIP with autologous stem cell transplantation, three to four cycles. Five-year PFS in this group of patients was 68%,

which is longer than the historical control with SDCT. After the advent of paclitaxel, and proven effectiveness of this drug in cisplatin-resistant TGCC, GTCSG announced the study of addition of a paclitaxel to dose-escalated VIP protocol^[19]. Addition of paclitaxel to high dose-VIP (HD-VIP) protocol resulted in higher response rate of 79%, and five-year PFS and OS of 64.1% and 75.2% respectively.

The only completed randomized phase III study is the one of Motzer *et al.*^[20]. This study included 219 untreated patients with metastatic TGCC intermediate and poor prognosis. One group of patients was treated with standard therapy, four cycles of BEP, while the experimental group received two cycles of BEP and afterwards two cycles high dose CEC (HD-CEC) protocol. Proportion of one year complete remission was not different in the two groups of patients (52% PEB + HD-CEC vs 48% BEP, $P = 0.53$). Benefit of a high dose chemotherapy, in this clinical trial, was observed only in those patients with unsatisfactory tumor markers decline. The study concludes that there is no benefit of adding a HDCT in this group of patients. Two other studies have started the third phase, but due to poor recruiting of patients they are not fully completed^[21-23]. The analysis of the included patients from high dose chemotherapy did not show the expected benefit in first-line treatment of metastatic TGCC with a poor prognosis. A review of studies of the first line is given in Table 2.

HDCT IN SECOND-LINE THERAPY

Therapeutic options of SDCT in patients with relapsed/refractory testicular cancer can achieve long-term remission of 25% of the cases with vinblastine, etoposide, ifosfamide (VeIP) protocol^[24], to about 65% of patients treated with paclitaxel, ifosfamide, cisplatin (TIP)^[25]. Considering the chemosensitivity of TGCC and relative modest results of conventional chemotherapy protocols, a large number of researchers have designed a variety of studies which applied HDCT with the support of haematopoietic autologous stem cell transplantation (Table 3).

Rodenhuis *et al.*^[26] have demonstrated a 54% PFS after a median follow-up of 37 mo in a phase II study on 35 patients. In this study they used two cycles of a HDCT after the induction with conventional chemotherapy. Similar design study was published in 2000^[27]. Two cycles of intensification were used and the results were almost the same as in the previous study. The same year, Motzer *et al.*^[28] demonstrated overall survival of 54% after a median of nearly three years of follow-up. They used the induction regimen with paclitaxel and ifosfamide, and three cycles of high dose protocol carboplatin/etoposide (TI-CE). A slightly worse result was achieved in the study by Rick *et al.*^[29] which included only one cycle of high dose protocol after the induction with three cycles of TIP. Three-year survival in this study was 30%. The explanation for the slightly

Table 2 Studies of first line high dose chemotherapy for poor prognosis patients

Ref.	Type of study	Number of patients	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Motzer <i>et al</i> ^[15]	Phase II, prospective	28	VAB-6 × 2 + HD-CE × 2	57	46	31
Motzer <i>et al</i> ^[16]	Phase II, prospective	30	VIP × 2 + HD-CEC × 2	48 (5 yr)	48 (5 yr)	60
Bokemeyer <i>et al</i> ^[17]	Comparative, retrospective	147 (HDCT) vs 309 (SDCT)	VIP × 2 + HD-VIP × 2 vs BEP/VIP × 4	82 vs 72 (2 yr) P = 0.0184	75 vs 59 (2 yr) P = 0.0056	21
Schmoll <i>et al</i> ^[18]	Phase I/II, prospective	221	VIP + HD-VIP × 3-4	73 (5 yr)	68 (5 yr)	48
Hartmann <i>et al</i> ^[19]	Phase I/II, prospective	52	VIP + T-HD-VIP	75 (5 yr)	64 (5 yr)	41
Motzer <i>et al</i> ^[20]	Phase III, prospective	108 (HDCT) vs 111 (SDCT)	BEP × 2 + HD-CEC × 2 vs BEP × 4	71 vs 72 (2 yr)	60 vs 57 (2 yr)	33
Daugaard <i>et al</i> ^[23]	Phase III, prospective	65 (HDCT) vs 66 (SDCT)	VIP + HD-VIP × 3 vs BEP × 4	86.1 vs 83 (2 yr)	66.1 vs 48 (1 yr)	NR
Necchi <i>et al</i> ^[22]	Phase II, prospective	43 (HDCT) vs 42 (SDCT)	BEP × 2 + HD-CpE + HD-Carbo vs BEP × 4	54.8 vs 55.8 (5 yr)	59.3 vs 62.8 (5 yr)	114

BEP: Bleomycin, etoposide, cisplatin; HD: High dose; HD-CE: High dose carboplatin, etoposide; HD-CEC: High dose carboplatin, etoposide, cyclophosphamide; HDCT: High dose chemotherapy; HD-VIP: High dose, etoposide, ifosfamide, cisplatin; NR: Not reported; OS: Overall survival; PFS: Progression free survival; SDCT: Standard-dose chemotherapy; VAB: Actinomycin D, vinblastine, cyclophosphamide, bleomycin, cisplatin.

Table 3 High dose chemotherapy as second line treatment

Ref.	Type of study	Number of patients	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Rodenhuis <i>et al</i> ^[26]	Phase II, prospective	35	Conventional chemotherapy + HD-CTC × 2	NR	54	37
Bhatia <i>et al</i> ^[27]	Phase II, prospective	65	VeIP × 1-2 + HD-CE × 2	NR	57	39
Motzer <i>et al</i> ^[28]	Phase II, prospective	37	TI × 2 + HD-CE × 3	54	49	31
Rick <i>et al</i> ^[29]	Phase II, prospective	62	TIP × 3 + HD-CET × 1	30 (3 yr)	25 (2 yr)	36
Pico <i>et al</i> ^[30]	Phase III, prospective, randomized	135 (HDCT) vs 128 (SDCT)	VIP/VeIP × 3 + HD-CE × 1 vs VIP/VeIP × 4	53 vs 53 (3 yr)	42 vs 35 (3 yr)	45
Einhorn <i>et al</i> ^[31]	Retrospective	135	HD-CE × 2	NR	70	48
Lorch <i>et al</i> ^[32]	Phase II, prospective, randomized	111 (sequential HDCT) vs 105 (single HDCT)	VIP × 1 + HD-CE × 3 vs VIP × 3 + HD-CE × 1	47 vs 45 (5 yr) P = 0.057	49 vs 39 (5 yr) P = 0.057	90
Feldman <i>et al</i> ^[33]	Phase I/II, prospective	107	TI × 2 + HD-CE × 3	52 (5 yr)	48 (5 yr)	61
Lorch <i>et al</i> ^[34]	Comparative, retrospective	821 (HDCT) vs 773 (SDCT)		53.2 vs 40.8 (5 yr) P < 0.001	49.6 vs 27.8 (2 yr) P < 0.001	NR
Selle <i>et al</i> ^[36]	Phase II, prospective	45	Epi-Tax × 2 + HD Thio-Tax + HD-ICE × 2	66% (2 yr)	50% (2 yr)	26
Berger <i>et al</i> ^[37]	Comparative, retrospective	95 (HDCT) vs 48 (SDCT)	HDCT vs SDCT	P = 0.931	Median 8 vs 42 mo P < 0.001	NR
Nieto <i>et al</i> ^[64]	Phase II, prospective	42	BEC-GDMC + BEV + HD-ICE	65% (2 yr)	63% (2 yr)	NR

BEV: Bevacizumab; Epi-Tax: Epirubicine, paclitaxel; GDMC: Gemcitabine, docetaxel, melphalan, carboplatin; HD: High dose; HD-CE: High dose carboplatin, etoposide; HD-CET: High dose carboplatin, etoposide, thiotepa; HDCT: High dose chemotherapy; HD-CTC: High dose carboplatin, thiotepa, cyclophosphamide; HD-ICE: High dose ifosfamide, carboplatin, etoposide; NR: Not reported; OS: Overall survival; PFS: Progression free survival; SDCT: Standard-dose chemotherapy; Thio-Tax: Thiotepa, paclitaxel; TI: Paclitaxel, ifosfamide; TIP: Paclitaxel, ifosfamide, cisplatin; VeIP: Vinorelbine, ifosfamid, cisplatin; VIP: Etoposide, ifosfamide, cisplatin.

worse result in this study could be the application of only one cycle of high dose protocol.

The only prospective, randomized phase III study by Pico *et al*^[30] compared four cycles of conventional chemotherapy VeIP or VIP with three cycles of SDCT with the addition of one cycle of high dose carboplatin/etoposide (HD-CE) protocol. This study included a total of 263 patients. It did not demonstrate the superiority of the addition of one cycle of HDCT. Based on that study and study by Rick *et al*^[29], it was concluded that one cycle of HDCT was not sufficient to achieve better results in treatment compared to conventional chemotherapy, so further studies had two or even three cycles of HDCT.

The study which probably had the greatest impact on the practice of treating relapsed TGCC and utilization of HDCT was that by Einhorn *et al*^[31].

One hundred and eighty-four patients were retrospectively analysed, and 135 of 184 patients received two cycles of HD-CE protocol in the first relapse, while the other 49 were treated in second and subsequent relapses with the same protocol. After a median follow-up of four years, progression free survival in patients treated in the first relapse was 70%. Lorch *et al*^[32] compared one cycle of high dose therapy with three cycles of HD-CE. After long-term follow-up PFS was 49% vs 39% in favor of the sequential approach while overall survival did not differ between these two groups.

Table 4 High dose chemotherapy for third or subsequent lines, refractory/absolute refractory

Ref.	Type of study	Number of patients	Setting	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Vaena <i>et al</i> ^[38]	Retrospective	80	Second and subsequent lines, refractory	HD-CE × 2	40 (2 yr)	32 (2 yr)	24
Lotz <i>et al</i> ^[39]	Prospective	45	Second and subsequent lines, refractory/absolute refractory	Epi-Tax × 2 + HD Thio-Tax × 1 + HD-ICE × 2	23.5 (3 yr)	23.5 (3 yr)	36
Kondagunta <i>et al</i> ^[40]	Prospective	47	Second and third line, refractory/absolute refractory	TI × 2 + HD-CE × 3	NR	51	40
Einhorn <i>et al</i> ^[31]	Retrospective	49	Third or subsequent	HD-CE × 2	55	45	48
Lorch <i>et al</i> ^[41]	Retrospective	49	Third or subsequent, refractory	Various	17 (5 yr)	26 (5 yr)	48
Popovic <i>et al</i> ^[42]	Prospective	8	Fourth or fifth line, refractory	Epi-Tax × 2-3 + HD-CE × 1-2	Median 11 mo	NR	NR

Epi-Tax: Epirubicin, paclitaxel; HD: High dose; HD-CE: High dose carboplatin, etoposide; HDCT: High dose chemotherapy; HD-ICE: High dose ifosfamide, carboplatin, etoposide; NR: Not reported; OS: Overall survival; PFS: Progression free survival; SDCT: Standard-dose chemotherapy; Thio-Tax: Thiotepa, paclitaxel; TI: Paclitaxel, ifosfamide.

Feldman *et al*^[33] demonstrated in a prospective study of 107 patients a five-year PFS of 48% using the TI-CE protocol with three cycles of high dose chemotherapy.

A multicenter retrospective analysis of 1984 patients by Lorch *et al*^[34] compared the standard and high dose chemotherapy in patients with metastatic TGCC after progression on first-line chemotherapy. Patients were divided into five prognostic groups according to previously established criteria: Very low risk, low risk, intermediate risk, high and very high risk^[35]. Total of 1594 patients had all the data necessary for analysis, 773 of which received conventional chemotherapy, while 821 patients received HDCT. Two-year PFS and five-year OS was longer in the group with HDCT: 49.6% vs 27.8% (HR = 0.44; $P < 0.001$), 53.2% vs 40.8% (HR = 0.65; $P < 0.001$). This difference was seen in all prognostic groups except in low-risk group^[34].

Selle *et al*^[36] in the study TAXIF II demonstrated efficiency of a complex protocol which included several high dose cycles of paclitaxel, thiotepa, ifosfamide, carboplatin and etoposide, after induction with a combination of paclitaxel/epirubicin. The median PFS was 22 mo and OS was 32 mo. Two-year PFS was 50%, with Kaplan-Meier curve that showed a plateau at that value, and two-year OS of 66%^[36]. German Testicular Cancer Study Group retrospectively analyzed 143 patients and compared the HDCT ($n = 95$) with CDCT ($n = 48$). They showed a significantly longer median PFS 8 mo vs 42 mo ($P < 0.001$) with HDCT, but this difference was not seen when they analyzed overall survival^[37].

HDCT FOR REFRACTORY AND HEAVILY PRETREATED PATIENTS

Patients who progressed during standard cisplatin based chemotherapy have the worst prognosis. This group of patients also includes those who have not been cured after two lines cisplatin protocol. For this group of patients there have been several studies that, despite the very poor prognostic characteristics, showed some benefit from HDCT (Table 4).

Vaena *et al*^[38] retrospectively analyzed the results of HDCT in platinum-refractory patients. Two-year PFS was 32%, while two-year OS was 40%. Lotz *et al*^[39] applied a different concept in refractory patients. In TAXIF study they prospectively treated 45 patients with absolutely refractory metastatic TGCC. After mobilization therapy with paclitaxel/epirubicin, they gave two cycles of high dose ifosfamide, carboplatin, etoposide (ICE) protocol. Three-year PFS and OS were 23.5%^[39]. Kondagunta *et al*^[40] prospectively treated 47 refractory patients with high dose chemotherapy. After a median of 40 mo of observation the PFS was 51%. The Study by Einhorn *et al*^[31] treated 49 patients with HDCT in third and subsequent lines of chemotherapy. Time to disease progression and OS in these patients was 45% and 55% respectively. In patients with third and subsequent lines of therapy, Lorch *et al*^[41] reached five-year OS of 17% at five-year PFS 27%. In a pilot study, we have treated 8 heavily pretreated patients with HDCT. We used a modified TAXIF protocol^[39]. All patients had previously received four lines of different therapies. The median OS was 11 mo, with no long-term survival^[42,43].

HDCT FOR EXTRAGONADAL GCC

Extragenital GCC tumors occur most often in retroperitoneum and mediastinum and have worse prognosis compared to TGCC^[2]. Several studies with high dose chemotherapy administration has addressed this subgroup of patients (Table 5). Bokemeyer *et al*^[44] have treated patients with primary mediastinal germ cell tumors (PMNSGCT) initially with high dose chemotherapy. They included 28 patients and achieved 56% and 64% PFS and OS, respectively. Banna *et al*^[45] also used HDCT in the first line of treatment PMNSGCT and reached a three-year OS 41%. Rosti *et al*^[46] retrospectively analyzed 22 patients who had primary extragonadal non-seminomatous germ cell tumor (EGNSGCT) and received HDCT. Five-year survival in this group of patients was 75%. Hartmann *et al*^[47] and De Giorgi *et al*^[48] in two studies published in 1999 showed retrospective results of a treatment of

Table 5 High dose chemotherapy for extragonadal germ cell cancer

Ref.	Type of study	Number of patients	Setting	Protocol	OS (%)	PFS (%)	Median follow-up (mo)
Bokemeyer <i>et al</i> ^[44]	Phase I/II, prospective	28	PMNSGCT, first line	VIPx 1 + HD-VIP × 3	64 (5 yr)	56 (5 yr)	43
Banna <i>et al</i> ^[45]	Prospective	21	PMNSGCT, first line	BEP or VIP × 4 + HD-CEC × 1	41 (3 yr)	43 (5 yr)	52
Rosti <i>et al</i> ^[46]	Retrospective	22	EGCT, poor prognosis, first line	Various	75 (5 yr)	67 (5 yr)	50
Hartmann <i>et al</i> ^[47]	Retrospective	142	EGNSGCT, salvage	Various	12 (3 yr)	11 (3 yr)	45
De Giorgi <i>et al</i> ^[48]	Retrospective	59	EGNSGCT, salvage	Various	14 (PMNSGCT only)	14 (PMNSGCT only)	58

EGCT: Extragonadal germ cell tumor; EGNSGCT: Extragonadal non-seminomatous germ cell tumor; HD: High dose; HD-CEC: High dose carboplatin, etoposide, cyclophosphamide; HDCT: High dose chemotherapy; HD-ICE: High dose ifosfamide, carboplatin, etoposide; NR: Not reported; OS: Overall survival; PFS: Progression-free survival; PMNSGCT: Primary mediastinal non-seminomatous germ cell tumor.

Table 6 International Germ Cell Cancer Collaborative Group-2 prognostic criteria for relapsed germ cell cancer patients

Parameter	Score points			
	0	1	2	3
Primary site	Gonadal	Extragenadal	-	Mediastinal non-seminoma
Prior response	CR/PRm-	PRm+/SD	PD	-
PFI, mo	> 3	≥ 3	-	-
AFP salvage	Normal	≤ 1000	> 1000	-
HCG salvage	≤ 1000	> 1000	-	-
LBB	No	Yes	-	-
Score sum (0-10)				
Regroup into categories: (0) = 0; (1 or 2) = 1; (3 or 4) = 2; (5 or more) = 3				
Add histology points: Seminoma = -1; Non-seminoma or mixed = 1				
Final prognostic score: -1 = Very low risk; 0 = Low risk; 1 = Intermediate risk; 2 = High risk; 3 = Very high risk				

AFP: Alpha-fetoprotein; CR: Complete remission; HCG: Human chorionic gonadotrophin; LBB: Liver, brain, bone; PD: Progressive disease; PFI: Progression free interval; PRm-: Partial remission, markers negative; PRm+: Partial remission, markers positive; SD: Stable disease.

EGNSGCT after progression on first-line therapy. Results were rather modest with 12%-14% long term survival.

RISK ADAPTED APPROACH FOR USING HDCT

The first prognostic score related to the outcome of a HDCT was developed Beyer *et al*^[49]. They have analyzed a series of 310 patients treated with HDCT in four centers in Europe and the United States and by multivariate analysis of prognostic factors determined the data which had influenced the outcome. Progressive disease before HDCT, primary mediastinal localization, refractory or absolute refractory disease to cisplatin therapy and the value of human chorionic gonadotropin (HCG) over 1000 were independent factors for failure-free survival (FFS) after HDCT. These parameters separated patients into groups with good, intermediate and poor prognosis. Patients with good, intermediate

and poor prognosis had 51% FFS after HDCT, 27%, and 5% ($P < 0.001$) respectively. The International Prognostic Factors Study Group (Table 6) analyzed data of 1984 patients with TGCC, who have progressed after at least three cycles of cisplatin based chemotherapy. Patients' data were collected from 38 centers worldwide and 1594 patients had sufficient data for analysis. Patients were treated with SDCT or HDCT based on carboplatin. Factors that influenced the outcome were: Site of primary tumor, previous response to therapy, progression free survival on previously applied therapy, alpha-fetoprotein and HCG above 1000 and the presence of metastases in the liver, bone and/or bone^[35].

Based on these factors, patients were divided into five categories: Very low risk with a two-year PFS of 72%, low risk with PFS 51%, medium risk with 40%, high risk with 26% and very high risk with 6%. This is followed by the already mentioned retrospective analysis by Lorch *et al*^[34] which showed benefits in all prognostic categories, except in the low risk group. Given that the benefit was demonstrated even in the category of very low risk, there is a question in which prognostic groups, in patients with relapsed GCC, HDCT should be applied and which groups should receive conventional chemotherapy. Opinions differ greatly, and certain groups of authors believe that high dose chemotherapy should be applied in all patients with relapsed GCC, while some groups believe that patients with a low risk should be treated with the conventional chemotherapy in the second line, and HDCT should be applied in patients with medium and higher risk as well as in those with a low risk who relapse after second-line of conventional chemotherapy^[50]. Our position is closer to the second opinion.

STEM CELLS MOBILISATION AND OPTIMAL PROTOCOL OF CONDITIONING

Collection of sufficient numbers of hematopoietic stem cells is a key step in the further implementation of

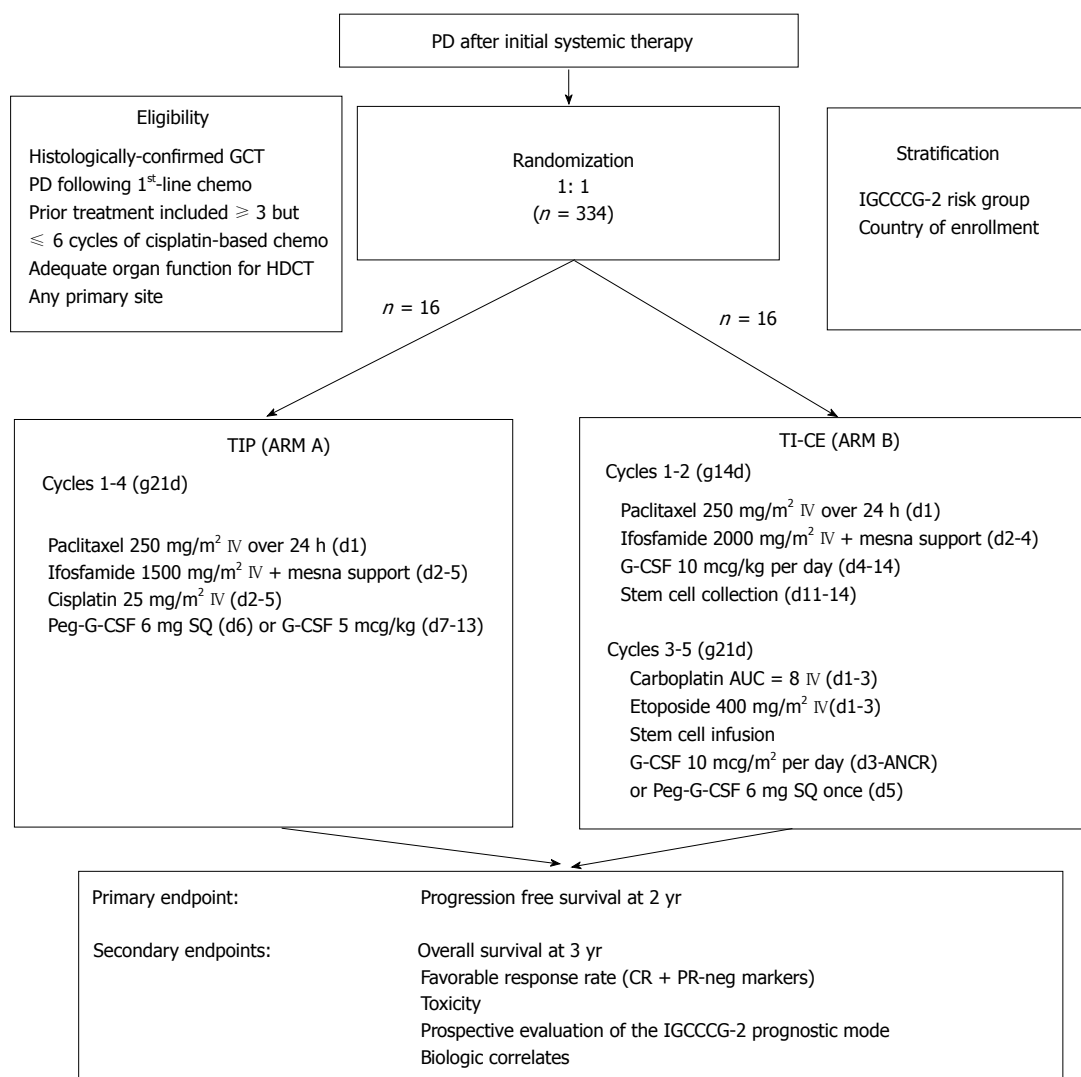


Figure 1 Randomized phase III trial of initial salvage chemotherapy for patients with germ cell tumors study (TIGER study) design^[62]. PD: Progressive disease; GCT: Germ cell tumors; HDCT: High dose chemotherapy; G-CSF: Granulocyte colony-stimulating factor.

a HDCT, and the possibility of treating patient with multiple cycles of HDCT. Combination of chemotherapy with granulocyte growth factor (G-CSF) is a standard for the mobilization of hematopoietic stem cells. However, in heavily pretreated patients, this method of mobilization is not enough to collect a sufficient number of stem cells. In our cohort of heavily pretreated patients median collected hematopoietic stem cells was 3.6×10^6 cells/kg of BW. Consequently, it was not possible for us to apply tandem transplantation in some patients^[43]. Some other authors as well conclude that the mobilization with chemotherapy + G-CSF was inadequate for obtaining a sufficient number of stem cells, especially in cases of highly pretreated patients^[4,51-53].

Plerixafor is the CXCR4 receptor antagonists which separate hematopoietic stem cells from bone marrow stroma and can lead to better mobilization of these cells into peripheral blood^[54]. After the positive outcome in poor-mobilisers with lymphoma and multiple myeloma increased enthusiasm for using plerixafor to mobilize hematopoietic stem cells in patients with TGCC. The four

smaller cohorts and three case studies^[52-60] showed the efficiency of plerixafor in heavily pretreated patients with TGCC. Worel *et al.*^[55] showed the efficiency of plerixafor in 33 patients with non-hematologic diseases, of which 11 were metastatic GCC. A total of 28 (85%) patients gathered a sufficient number of stem cells. Kobold *et al.*^[57] showed a series of 6 patients who had previously received chemotherapy for 3.5 lines metastatic GCC and were not able to mobilize a sufficient number of stem cells for transplantation. After the use of plerixafor, five of these six patients mobilized an adequate number of cells for a minimum one transplant. Kosmas *et al.*^[52], in a pilot study, showed stem cells mobilization in pretreated patients with GCC, in which 7 out of 10 patients could yield an adequate number of hematopoietic stem cells for transplantation. The remaining three, poor-mobilisers, have amassed an adequate number of stem cells after applying plerixafor. In all these publications, engraftment of stem cells obtained after the mobilization with plerixafor was adequate.

Despite attempts with different drugs that would

supplement carboplatin, such as thiotepa and ifosfamide, panelists of the third European consensus conference on the treatment of GCC, agreed that a combination of carboplatin and etoposide is a standard high dose protocol^[50].

FUTURE DIRECTIONS

Although HDCT is considered standard treatment option for relapsed GCC in most major cancer centers, there are still no level IA evidence for applying HDCT in the current recommendations for the treatment of GCC^[2,3,61]. The reason for this is the series of negative results of the randomized phase III studies by Motzer *et al.*^[20] in the first line, and Pico *et al.*^[30] in metastatic relapsed GCC, therefore TIGER (randomized phase III trial of initial salvage chemotherapy for patients with germ cell tumors) has been initiated^[62]. The study design is shown in Figure 1. The plan is to include 390 patients, a group of which will receive four cycles of TIP protocol, while the second group will receive TI-CE protocol with three cycles of a HDCT. The hypothesis is that the overall survival of patients should be 13% higher after the treatment with the HDCT.

The second concept is target therapy in addition to the treatment of relapsed GCC, for conventional, as well as for high dose chemotherapy. Vascular endothelial growth factor over-expression is an independent factor of poor prognosis for non-seminomatous germ cell tumor (NSGCT), especially for teratoma NSGCT which is the most refractory to chemotherapy^[4]. Voigt *et al.*^[63] showed a case of successful treatment of patients absolutely refractory to cisplatin with bevacizumab and HD-ICE protocol. At the last ASCO meeting, Nieto *et al.*^[64] presented a phase II study for the first and the second relapse of intermediate and high risk metastatic GCC. They combined bevacizumab with tandem HDCT. In the first cycle of HDCT they combined bevacizumab with gemcitabine, docetaxel, melphalan and carboplatin, while in the second cycle they combined bevacizumab with HD-ICE protocol. One-year and 2 year-OS were 72% and 65% respectively^[64]. In addition to these studies, there is an ongoing study TAXIF III, of the French group, with the addition of bevacizumab to HD-ICE protocol^[65]. Approximately 70% of embryonic GCT express CD30 receptor on cell surface^[66]. Brentuximab-vedotin is an anti-CD30 conjugated to a monoclonal antibody that has shown significant results in the treatment of Hodgkin's and peripheral T-cell lymphomas^[67]. The Italian group has started a phase II clinical study of efficiency brentuximab vedotin in refractory CD30-positive metastatic testicular cancer^[68].

CONCLUSION

Although there are no randomized phase III trials that support HDCT as an effective treatment option for patients with metastatic GCT, the majority of centres use this type of therapy in patients with intermediate

and high risk according International Germ Cell Cancer Collaborative Group-2 score^[35]. TIGER study might give a definitive answer whether HDCT should be a standard treatment for these patients, and a better understanding of tumor biology, detection of markers of resistance to cisplatin, as well as if adding target therapy such as bevacizumab should improve the treatment of GCT, especially in the group of patients with a poor prognosis.

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Towards *in vivo* amplification: Overcoming hurdles in the use of hematopoietic stem cells in transplantation and gene therapy

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Abstract

With the advent of safer and more efficient gene transfer methods, gene therapy has become a viable solution for many inherited and acquired disorders. Hematopoietic stem cells (HSCs) are a prime cell compartment for gene therapy aimed at correcting blood-based disorders, as well as those amenable to metabolic outcomes that can effect cross-correction. While some resounding clinical successes have recently been demonstrated, ample room remains to increase the therapeutic output from HSC-directed gene therapy. *In vivo* amplification of therapeutic cells is one avenue to achieve enhanced gene product delivery. To date, attempts have been made to provide HSCs with resistance to cytotoxic drugs, to include drug-inducible growth modules specific to HSCs, and to increase the engraftment potential of transduced HSCs. This review aims to summarize amplification strategies that have been developed and tested and to discuss their advantages along with barriers faced towards their clinical adaptation. In addition, next-generation strategies to circumvent current limitations of specific amplification schemas are discussed.

Key words: Gene therapy; Hematopoietic stem cells; *In vivo* selection; Chemical Inducer of Dimerization; Chemo-selection; Lentivirus

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Core tip: Though hematopoietic stem cell (HSC)-directed gene therapy is becoming a viable therapy for many disorders, optimization of clinical output needs improvement. One approach to circumvent lower efficiencies of gene transfer and/or engraftment is to apply *in vivo* amplification strategies. Here we review

various modules that have been developed and tested to mediate amplification of HSCs after gene transfer.

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INTRODUCTION

Hematopoietic stem cells (HSCs) are long-term, multipotent, self-renewing cells that reside in specialized bone marrow (BM) niches and are capable of generating and repopulating the entire spectrum of blood and lymphoid cells^[1,2]. Due to these unique properties, HSCs are targets for therapy for a number of hematological malignancies and many inherited blood disorders including β -thalassemia, sickle cell anemia, chronic granulomatous disease, and severe combined immunodeficiencies (SCID-X1 and ADA-SCID) among others^[3-8]. Additionally, HSC transplants have been used in attempt to correct other monogenic deficiencies, such as the mucopolysaccharidoses and Gaucher disease^[9-11].

There are still numerous drawbacks of allogeneic transplantation despite its clinical utility. Often, HSCs are collected from the patient's sibling, parents, or a matched donor. HLA-identical donors can be difficult to find and there are risks involved with the use of HLA-haploidentical or non-identical donors including rejection or poor engraftment of HSCs along with the occurrence of graft-versus-host disease (GVHD). Conditioning is also necessary for engraftment of HSCs, which can increase the risk of infections^[12-14]. As a consequence, HSC allo-transplantation is still considered a fairly risky intervention and is applied with caution in the clinic.

Gene therapy targeting patient-derived HSCs is a viable solution for some monogenic diseases^[15] (Figure 1A). Autologous transplantation has been well studied and detailed clinical protocols are available for this procedure^[3]. Additionally, autologous transplantation does not have a risk of GVHD associated with it and immune reconstitution after ablation occurs in a shorter period of time^[16,17]. Gene transfer into HSCs has been traditionally achieved by stable transduction of target cells using replication-incompetent retroviruses^[15]. There the expression of transgenes can be driven by constitutive or tissue-specific promoters, giving a range of control over the intended therapeutic intervention. Next-generation strategies are also being developed to correct original nucleotide mutations with the use of gene-editing technologies, such as TALENs and CRISPR-Cas9, though these remain to be optimized for clinical application^[18-20].

Over 2000 clinical gene therapy trials have been conducted to date^[4,15,21,22]. Most earlier trials employed

onco-retroviral vectors, which have shown to be clinically disadvantageous because of their tendency to integrate close to genes that are important for cell growth and proliferation, enhancing their expression and increasing the likelihood of developing leukemias^[4,15,23-25]. So far it appears that this genotoxicity and tendency towards insertional mutagenesis has been diminished with the introduction of HIV-1-derived, replication-incompetent, and self-inactivating lentiviral vectors (LVs), which do not show preferential integration near genes involved in cell growth and/or proliferation^[4,26-30].

There are other caveats to using HSCs as target cells for gene therapy that are a result of their unique biology. HSCs can be more difficult to transduce than some other cell types, partially owing to the difficulty of culturing them *ex vivo*. Longer-term culturing ensures that the cells will differentiate. Transduction also requires transient activation of the cell cycle, especially with onco-retroviral-based vectors since their downstream integration requires a breakdown of the nuclear membrane. As a consequence of *ex vivo* manipulation and cell-cycle activation, transduced HSCs often have lower engraftment potential and reduced longevity once engrafted. These additional limitations have also been partially addressed with the use of LVs, which need shorter transduction times and do not require target cells to be fully cycling^[31-35].

In spite of the progress made in HSC gene therapy with the implementation of recombinant LVs, there is ample room for additional improvements to increase therapeutic efficacy. Many active fields of research are geared towards optimizing gene therapy for HSCs. Efforts are under way to hone GMP-grade LV production to subsequently allow modulation of multiplicities of infection at a clinical level, whilst reducing the cost of gene therapy^[36,37]. Improvements are also being made to protocols for *ex vivo* handling and culture of HSCs with studies demonstrating enhanced transduction with shorter culture times and less activation, which have resulted from better understanding of the biology of HSCs and their BM microenvironments^[4,38,39]. In addition, in-depth studies of HSC biology have identified molecular targets for drugs that allow more efficient and safer mobilization of patient stem cells^[40-42]. The gene therapy field has also sought out methods to provide extrinsic selective pressure for transduced cells, though clear clinical utility of any system has yet to be demonstrated, especially in the context of reconstitutive HSC-directed gene therapy.

Reconstitution of deficient gene products in some inherited blood diseases leads to innate positive selective pressure *in vivo* for mature cells derived from transduced progenitor cells, especially when the gene product is necessary for the development or function of those cells. For example, reconstitution of the common gamma chain (γ /CD132) in SCID-X1 allows immune cells to develop normally, thus progeny cells are derived from successfully transduced HSCs almost exclusively^[17,43-45]. Selectivity for donor-derived late-stage

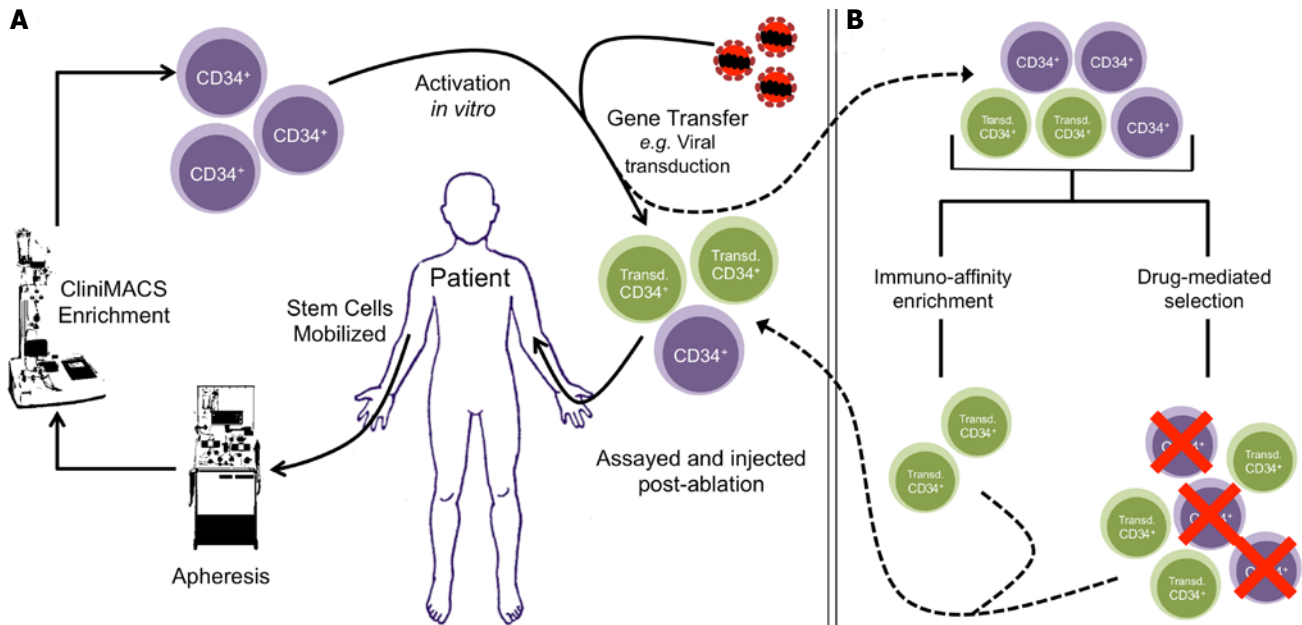


Figure 1 General outline of *ex vivo* hematopoietic stem cell gene therapy and pre-selection methods. A: CD34⁺ cells are enriched by CliniMACS after apheresis of peripheral blood of patients following mobilization. These cells are then briefly activated *ex vivo* and can be modified, commonly by viral transduction, to express a desired therapeutic protein. Cells are then assessed for quality control metrics and engrafted into patients following ablation; B: Pre-selection of transduced cells. Cells can be engineered to express an inert surface marker that can be used to immuno-enrich for the transduced population prior to engraftment. This strategy can increase the chances of hematopoietic reconstitution from the transduced population. Alternatively, cells can be given resistance to cytotoxic drugs. Pre-treatment of the cells *ex vivo* with drugs can kill off the non-transduced population. *Ex vivo* treatment allows the use of drugs that would normally not be efficacious in the bone marrow environment at a tolerable dose.

erythrocytes has also been observed in β -thalassemia patients that have received allo-transplantations; and a similar trend has been observed with gene therapy in mouse models of the disorder^[7,46,47]. HSC gene therapy may therefore become routine clinical practice some day for patients suffering from such hematological disorders that do not have matched donors, considering the innate advantage of such reconstituted cells.

There is also great potential of HSC-directed gene therapy for the treatment of non-hematological, monogenic enzyme deficiencies, such as those that lead to lysosomal storage disorders (LSDs) and other metabolic indications. In most cases, functional enzymes expressed after gene transfer into LSD patient cells have the potential to be secreted and subsequently taken up by other cells that do not have the transgene, a process termed metabolic cooperativity or "cross-correction". This occurrence has been demonstrated for a number of LSDs including Gaucher, Farber, and Fabry diseases^[48-51]. The current standard of care for many LSDs, enzyme replacement therapy (ERT), is actually a corollary of this phenomenon. HSC-directed gene therapy presents numerous putative advantages over conventional ERT, including sustained and continuous secretion of therapeutic enzyme by ubiquitously circulating cells, improvements in patient lifestyle by reducing the need for biweekly enzyme infusions, and overall cost savings. It is necessary to tailor HSC gene therapy for individual patients, however, which is incongruous with many current industrial business models, highlighting the necessity of shifting industrial focus from general to

personalized therapeutics.

Our laboratory is currently pursuing first-in-man HSC-based gene therapy for Fabry disease and is concomitantly demonstrating the utility of gene therapy for amelioration of Farber disease. However, in these cases, as with many such target disorders, expression of the functional gene product imparts no innate growth advantages to transduced cells. Vector-encoded transgenes alone or in tandem that allow extrinsic selective pressure to be applied *in vivo*, leading to an increased percentage of vector-transduced cells over background could therefore be highly beneficial in the context of HSC gene therapy for LSDs and many other monogenic deficiencies. Additionally, application of positive selective pressure could result in cell populations that have higher transgene expression, resulting in an increased therapeutic benefit.

In this review, we aim to summarize the various strategies that have been employed to date in attempts to increase vector-transduced HSC numbers, thereby increasing the efficacy of HSC-targeted gene therapy. In addition, we will discuss putative next-generation strategies aimed at addressing current shortcomings of applying selective positive pressure on transduced HSCs.

EX VIVO PRE-SELECTION STRATEGIES

Resistance to cytotoxic drugs

Selection of genetically modified cells is a compilation of laboratory techniques commonly applied to acquire

polyclonal cell lines after gene transfer. To achieve this, target cells are engineered to express proteins that confer resistance to drugs or proteins that allow selection by immune-affinity methods such as fluorescence- and magnetic-activated cell sorting (FACS and MACS, respectively)^[52,53] (Figure 1B). Ideally, proteins expressed for enrichment should have low or no endogenous expression in target cells, and should have no effect on the biology of the transduced cells or their progeny. Traditionally, xenogenic enzymes have been used to confer cells with resistance to pan-toxic drugs in this context. For example, neomycin and hygromycin phosphotransferases (NeoR and HygR) derived from bacteria are commonly used to provide protection against neomycin and hygromycin B, respectively^[54,55]. As such, first attempts at conferring resistance to cells for engraftment were made with these enzymes. However, the use of xenogenic enzymes in clinical protocols has been limited by their tendency to be highly immunogenic once such modified cells are engrafted^[56-58]. To address this, mutants of various endogenous enzymes have been used to confer resistance to other cytotoxic drugs. These enzymes are discussed in the section below in the context of *in vivo* selection. However, most drugs require prolonged *ex vivo* culture to effectively enrich for the gene-modified population. Prolonged *ex vivo* handling of HSCs reduces their usefulness post-selection due to a loss of “stemness” and engraftment potential^[31]. Thus, drug-mediated *ex vivo* pre-selection may not be ideal in current iterations for clinical purposes.

Cell-surface marking for immuno-enrichment

Transduced HSCs can also be enriched *ex vivo* with the use of cell-surface markers. Selectable cell-surface markers that have been studied for HSC marking and pre-selection include truncated forms of the human low-affinity nerve growth factor receptor (Δ LNGFR)^[59-62], the heat stable antigen (HSA/CD24)^[48,50,63,64], the human lymphocyte antigen T1 (CD5)^[65,66], and the human interleukin-2 receptor alpha chain (IL-2R α /huCD25)^[67]. In mouse allograft experiments, long-term engraftment of transduced and FACS-enriched BM cells along with hematopoietic cell marking has been demonstrated using CD24^[64] and CD5^[66] as selectable markers. However, it must be noted that those experiments did not include a control in which no pre-selection was applied prior to engraftment. This makes it difficult to unequivocally assess the contribution of pre-selection to the engraftment and repopulating ability of transduced cells.

Despite positive results in pre-clinical settings with the use of FACS for enrichment, it has more detrimental effects on cell survival, viability, and function than MACS, even though FACS can lead to higher purity^[68]. It is also difficult to physically and/or temporally achieve enrichment of large numbers of clinically-applicable cells by FACS. As such, MACS and analogous schemas are preferred for enrichment prior to engraftment in patients. Over 90% purity has been achieved with

MACS enrichment of Δ LNGFR-marked HSCs *in vitro*^[60] and similarly marked lymphocytes in clinical trials^[69-71]. In an allograft experiment with a mouse model of Fabry disease, BM mononuclear cells were transduced with a therapeutic vector capable of co-expressing α -galactosidase A and huCD25^[67]. Pre-selection of transduced cells by MACS led to a long-term increase of huCD25-marked peripheral blood mononuclear cells when compared to controls. Therapeutic benefit of pre-selection was demonstrated by a higher α -galactosidase A activity in plasma and most organs. Additionally, the utility of pre-selection in long-term HSC marking has been demonstrated in some cases by secondary transplant experiments^[64,67].

Since pre-selection strategies reduce the size of the transduced cell population^[60], however, their application to HSC gene therapy can be critically limited if there are difficulties in collecting large numbers of patient HSCs. *Ex vivo* expansion of HSCs is currently not a viable solution in order to compensate for reduced cell numbers since over-activation can have detrimental effects on their “stemness” and engraftment potential^[31]. In addition, pre-selection increases time of *ex vivo* manipulation, which increases costs and risks of contamination. It is therefore difficult to obtain a post-selection yield high enough to exert a therapeutic effect. Nevertheless, these studies demonstrate the benefit of enriching transduced cells *ex vivo* and that clinical translation may be augmented by higher yields of HSCs during their acquisition.

***IN VIVO* CHEMO-SELECTION STRATEGIES**

Various proteins have been shown to grant variable degrees of chemoprotection in the context of cancer therapy, such as ATP-binding cassette, sub-family B, member 1 (ABCB1), dihydrofolate reductase (DHFR), and *O*⁶-alkylguanine DNA alkyltransferase (MGMT). Overexpression of these proteins in HSCs has been pursued with the aim of protecting the hematopoietic compartment from the severe toxicity of many cytotoxic drugs used in cancer chemotherapy^[72].

Pan-resistance to chemotherapeutic agents using ABCB1

ABCB1 [also known as multidrug resistance protein 1 (MDR1); or P-glycoprotein 1 (P-gp1)] is a cell membrane transporter with broad specificity that pumps foreign compounds out of the cell and is also involved in lipid translocation^[73,74]. ABCB1 mediates chemoresistance in cancer cells in which its expression is upregulated^[75]. Overexpression of ABCB1 in murine BM was shown to confer protection to many chemotherapeutic agents such as vinblastin, doxorubicin, daunomycin, taxol, vincristine, etoposide, actinomycin D, colchicine, and paclitaxel^[76,77]. Early studies with mouse allografts showed *in vivo* selection of hematopoietic cells derived

from *ABCB1*-overexpressing HSCs, but it is unclear whether selection occurred at the stem cell level^[78,79]. Later studies demonstrated successful selection of human HSC-derived cells in the BM of murine xenograft models^[80,81]. In contrast to these outcomes, early autograft experiments in large animals and clinical trials demonstrated rather disappointing results. In a canine model, high toxicity was documented despite long-term *ABCB1*⁺-peripheral blood cell enrichment in the only surviving animal^[82]. In a study involving non-human primates, there was low initial *ABCB1* cell marking, drug-induced neutropenia, and no significant increase of neutrophil counts after drug treatment^[83]. In clinical trials, selection after drug treatment has been low and predominantly transient, albeit with little or no toxicity^[84-87]. The inefficacy of *ABCB1*-mediated selection may have been due to insufficient expression of the transgene in hematopoietic cells^[88]. Onco-retrovirally-mediated expression of *ABCB1* was found to be unstable due to cryptic splice sites within the cDNA^[89]. This issue was resolved by introducing a silent mutation that inactivates that splice site, which subsequently increased expression of onco-retrovirally-delivered *ABCB1*^[90,91]. Nevertheless, the robustness of this system must be reliably demonstrated in large animal models before it can be considered a feasible strategy to enrich HSCs after transplant for clinical gene therapy in patients.

Antifolate resistance using mutant DHFR

DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, a precursor required for the *de novo* synthesis of purines and some amino acids. Antifolate drugs such as methotrexate (MTX) and trimetrexate (TMTX) inhibit DHFR activity, thus blocking cell proliferation and promoting apoptosis in dividing cells. HSCs and myeloid progenitor cells, however, can employ nucleotide salvage mechanisms to escape antifolate toxicity^[92]. In order to overcome this, the nucleoside transport inhibitor nitrobenzylthioinosine 5'-monophosphate (NBTI/NBMPP-P) has been used in combination with MTX or TMTX^[92]. Transplanted HSCs have been engineered to over-express mutant forms of DHFR, such as DHFR^{L22Y}, that are resistant to antifolate agents^[93]. *In vivo* enrichment of transduced HSCs in murine allogeneic transplants has been demonstrated^[94,95]. However, translation of this method into large animal models has been rather discouraging. In a study in rhesus macaques that used a recombinant onco-retrovirus to deliver DHFR^{L22Y}, enrichment of cells derived from the transduced graft was only transient, indicating poor selection at the HSC level^[96]. To address this problem, enrichment of CD34⁺ progenitor cells in a xenograft transplant of human embryonic stem cells (hESCs) into mice has been demonstrated, though this enrichment was only modest and no clinically established methods to transplant hESCs exist^[97]. High toxicity and lethality has been documented in antifolate-mediated selection studies in dogs and rhesus macaques^[96,98]. Additionally, antifolate

toxicities are well documented when such compounds are indicated for treatment of cancer patients^[99-102]. These toxicities and the lack of positive evidence suggest that DHFR-mediated *in vivo* selection may not be useful for HSC gene therapy targeting monogenic diseases. Instead, it may be better suited to prevent graft rejection after HSC transplants, because antifolates would spare highly-proliferating T lymphocytes arising from transduced donor HSCs while eliminating alloreactive recipient T cells as shown recently *in vitro*^[103] and in a canine model^[104].

Selectivity using O⁶BG-resistant MGMT

MGMT repairs DNA damage by removing adducts from the O⁶ position of guanine, and thus confers resistance to the cytotoxic effects of alkylating agents such as dacarbazine, temozolomide (TMZ), procarbazine, and nitrosoureas such as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU)^[105,106]. MGMT is expressed at very low levels in the BM^[107,108]. MGMT overexpression was attempted in murine^[105,109,110] and human^[105] HSCs to confer BCNU resistance. A modest increase in resistance to BCNU was achieved in murine progenitors, both *in vitro* and *in vivo*^[105,109,110]. Human HSCs, however, had poor resistance *in vitro*^[111]. In order to sensitize the HSC compartment to alkylating drugs to achieve better *in vivo* selection, BCNU or TMZ have been co-administered with O⁶-benzylguanine (O⁶BG), a pseudosubstrate that irreversibly inactivates endogenous MGMT^[112]. O⁶BG-resistant mutants MGMT^{P140K} and MGMT^{G156A} have also been studied^[113]. The former is more commonly used in selection strategies, despite having a modest reduction in its DNA-repair activity compared to the wild-type enzyme^[113]. Indeed, MGMT^{P140K} has been shown to mediate selection of transduced HSCs in murine and canine allograft and autograft models^[114-117], and in murine xenograft models with human HSCs^[30,118,119]. However, high dose administration of BCNU and/or TMZ has been shown to cause toxicity^[120-122]. For example, selection experiments have shown up to 75% mortality in mice treated with TMZ^[120] or BCNU^[121], and 88% mortality in rhesus macaques treated with TMZ^[122].

Optimization of drug dosing by co-administering high doses of O⁶BG with low doses of BCNU or TMZ has partially ameliorated the cytotoxic effects of the alkylating drugs and allowed better engraftment of HSCs transduced at low MOIs^[121,123,124]. The improvements in survival are thought to be a result of lowering the threshold of MGMT expression required for resistance, which allows partial fulfillment of conditions expected in clinical trials. This dose-adjusted protocol has conferred successful chemoprotection of the hematopoietic compartment in a canine^[125] and a nonhuman primate model^[126], with no significant toxicity reported in the former. Despite survival of the macaques in the latter study, administration of chemoselective agents led to substantial peripheral blood cell depletion and enrichment of different blood lineages was highly variable^[126]. In the same study, use of a multi-

cistronic vector to co-express C46, which is an anti-HIV transgene, MGMT^{P140K}, and enhanced green fluorescent protein (eGFP) resulted in lower selective potential^[126]. From a translational point of view, the risk-to-benefit ratio is currently not in favor of implementation of such chemoselective strategies though further adjustments to drug regimens can be done.

In the context of gene therapy for murine models of β -thalassemia^[123], hemophilia A^[124], and hemophilia B^[127], amelioration of the disease phenotype has been enhanced with the use of bicistronic LVs encoding the therapeutic gene and an MGMT^{P140K}-based selection module. Additionally, increased expression of the therapeutic gene after drug selection in secondary^[123,124] and tertiary^[127] recipients of serial BMT demonstrated enrichment at the HSC level. MGMT-mediated enrichment of eGFP⁺ BM and peripheral blood cells has also been demonstrated in a murine model^[128]. Despite these promising results, there are still no reports of successful MGMT-mediated selection following gene therapy in large animals that we are aware of, wherein HSC selection may be less efficient because of their lower replication rates^[129]. However, autologous MGMT^{P140K}-transduced HSC transplants have been attempted in MGMT^{hi}, TMZ-resistant glioblastoma patients^[130]. Drug selection resulted in no significant extra-medullary toxicity and all three participants surpassed the median survival (12 mo) for glioblastoma patients with unmethylated MGMT-promoter status. Despite these promising results in the context of chemoprotection of the hematopoietic compartment during glioblastoma treatment^[130], it must be noted that gene-modified circulating blood cells were depleted from the patients with termination of treatment. As such, repeat administration of chemotherapy may be required for the use of this system for amplification of transduced HSCs for gene therapy.

Hypoxanthine-guanine phosphoribosyltransferase inactivation for 6-thioguanine resistance

While the strategies described above rely on the overexpression of a protein that confers chemoprotection to transduced cells, down-regulating endogenous enzymes necessary to activate cytotoxic drugs can achieve analogous outcomes. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is an enzyme involved in the purine nucleotide salvage pathway. HPRT can catalyze the addition of ribose 5-phosphate to the purine analog 6-thioguanine (6TG) to generate thioguanosine monophosphate (thio-GMP)^[131]. Thio-GMP is then converted into thiodeoxyguanosine triphosphate (thio-dGTP), which can be incorporated into DNA inducing futile mismatch repair and consequent apoptosis. It has been shown that BM cells of HPRT-deficient mice are resistant to 6TG treatment^[132]. Transplantation of HPRT-deficient BM into wild-type HPRT mice under 6TG selection resulted in good engraftment and long-term hematopoietic reconstitution in primary and secondary recipients^[133]. Furthermore, transduction of murine hematopoietic progenitor cells with a LV

encoding a short-hairpin RNA (shRNA) that targets HPRT can confer resistance to 6TG *in vitro*^[134]. The same knockdown strategy has shown effective enrichment in murine allograft models^[135] and human HSC xenograft models^[136]. This approach has some advantages over other drug selection methods described above. 6TG can be used for both pre-conditioning and chemoselection, and the shRNA sequence is very short, which makes it easier to include in a dual-gene vector. Also, ample information about 6TG dosage and toxicity is available because it has been used in the clinic for decades^[137]. A recent study suggests, however, that this method may be limited to enrichment of committed progenitor cells, which would decrease long-term efficacy with single-dosing regimens^[136]. Also, hereditary HPRT deficiency is the cause of Lesch-Nyhan syndrome, which has been associated with megaloblastic anemia^[138]. Therefore, it is necessary to carefully assess long-term consequences of HPRT deficiency in the hematopoietic lineage, especially in large animal models. Nevertheless, selective induction of an enzyme deficiency, as demonstrated by virally-induced HPRT knockdowns, may be a powerful method of introducing selective pressure following gene therapy.

ENGINEERED INDUCIBLE GROWTH AND SELECTION MODULES

Cytotoxic chemical inducers of dimerization

Discoveries relating to functional consequences of forcing proteins such as receptor tyrosine kinases into proximity with each other have allowed the use of protein engineering to confer specific biological characteristics to a subset of modified cells with exposure to various stimuli. For example, chemical inducers of dimerization (CIDs) are synthetic compounds that can be used to induce dimerization of proteins that are expressed as fusions to CID-binding domains (CBDs). With the use of CBDs, cell-fates can be made dependent upon the addition of CIDs (Figure 2A). One of the first examples of such a system utilized FK1012, a synthetic dimer of the immunosuppressant FK506 (Tacrolimus)^[139]. Proteins that can modulate cell biology, including proliferation^[140] and apoptosis^[141], have been engineered from growth factor receptors and the FK506 binding domain from FK506-binding protein (FKBP12). However, FK1012 retains the ability to bind endogenous FKBP12^[139]. This is undesirable from a clinical point of view because endogenous FKBP12 could sequester the drug, preventing its intended effect. In addition, FK1012 administration could affect the normal physiological role of FKBP12^[142-145]. As a result, the use of these systems has been limited, though thorough clinical evaluation of the drug has yet to be completed. However, amplification protocols can be envisioned wherein the underlying toxicities of FK1012, which are expected to be similar to FK506, are exploited. Future systems can be developed using other cytotoxic agents with their respective binding targets as CBDs fused to survival or growth signaling

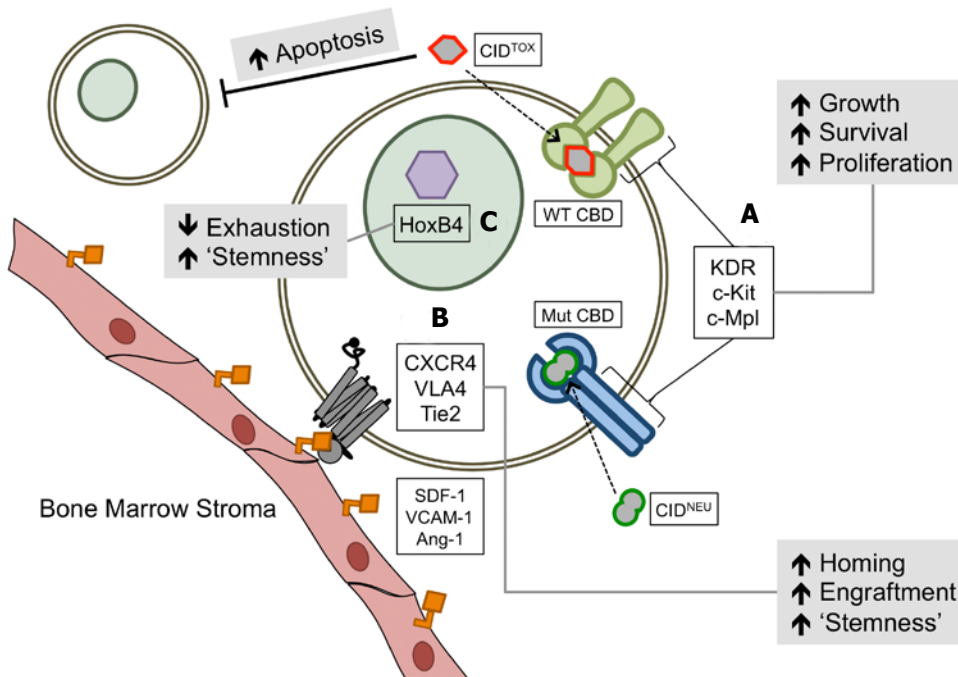


Figure 2 Summary of next-generation amplification modules. A: Fusion proteins comprised of chemical inducer of dimerizations (CBDs) such as FKBP12 (WT CBD) or F36V (Mut CBD) and receptors involved in hematopoietic stem cells (HSC) growth, proliferation, and survival. Activation of signaling by CIDs allows expansion of the transduced population. The use of cytotoxic CIDs (CID^{TOX}) can allow simultaneous depletion of the non-transduced population. FK1012 is a putative cytotoxic CID-binding domain. Examples of inert or neutral CIDs (CID^{NEU}) include AP20187 and AP1903; B: Controlled overexpression of HSC homing and adhesion molecules can increase the potential for therapeutic cells to survive and can promote long-term engraftment. Examples of such molecules include but are not limited to CXCR4, VLA4, and Tie2. Their corresponding ligands (SDF-1, VCAM-1, and Ang-1, respectively) are usually expressed on osteoblasts, osteoclasts, MSCs, and other cells that make up the bone marrow stroma; C: Downstream effectors of key signaling pathways involved in maintaining HSC phenotypes that are down-regulated during *ex vivo* handling of CD34⁺ cells can be reconstituted to prevent stem cell exhaustion and to increase long-term engraftment of transduced cells. HoxB4 is an example of a transcription factor that is activated in response to Wnt signaling and is key to maintenance of the stem phenotype of HSCs.

factors^[146]. Careful dosing can allow for simultaneous depletion of non-transduced cells and expansion of the transduced population (Figure 2A). Such studies have yet to be performed in clinically-relevant settings. Conversely, the application of such current systems remains risky as the continuous use of cytotoxic agents in general can have detrimental effects on patient quality of life. A potential solution is to aim for selective pressure to be applied to more mature cells, where dose reduction can be envisioned whilst clinical benefit is still achieved.

Neutral CIDs

Reverse-engineering of proteins and drugs that bind to them have led to numerous other CIDs and their respective CBDs. Progress in understanding the modularity of protein signaling has yielded numerous opportunities to generate CBD-signaling domain fusions. From a gene therapy perspective, ideal CIDs are those that would have little to no effect on any cells other than the transduced population. Ideal CBDs should also have no effect on the biology of transduced cells without the presence of a CID. As well, engineered polypeptides with CBDs should be derived from endogenous proteins where possible to minimize the potential immunogenicity of the fusions. In addition, unexpected effects of CID-induced dimerization of CBD fusion proteins should not occur in cells derived from transduced HSCs. "Bump and

hole" engineering of FK1012 and FKBP12 has yielded derivative CIDs such as AP20187 (B/B homodimerizer; Takara) and AP1903 (Rimiducid; Bellicum)^[147]. Such CIDs are chemically modified to prevent them from binding the original CBD. The derivative CBDs, such as FKBP12^{F36V} (F36V) for AP20187 and AP1903, have mutations that confer ability to bind the engineered CIDs^[147] (Figure 2A). These modifications allow such CID-CBD systems to be acceptable for use in gene therapy, as outlined above.

Amplification of HSCs using neutral CIDs

Multiple studies have been performed in cell, mouse, and canine models that utilize AP20187 and protein receptors fused to F36V^[148-154]. Our lab has previously shown the utility of a kinase insert domain receptor (KDR/CD309)-F36V fusion to control cell-fate in an AP20187 dependent manner, and we characterized the molecular mechanisms that are induced as a consequence of KDR dimerization in TF1 cells^[150]. The utility of this system has yet to be demonstrated in an animal model of HSC engraftment. The characterization of HSC cells and their signaling components have yielded other targets for F36V fusion and CID-mediated control of cell fate, such as c-Kit (SCF receptor)^[155], and c-Mpl (thrombopoietin receptor)^[149,152,156] (Figure 2A). It must be noted that these signaling components are not fully

unique to the HSC compartment and subsequent risk of unwanted proliferation can exist. That said, restricted expression of CBD-receptor fusions using HSC-specific promoters in gene transfer vehicles could reduce effects on non-target cells.

A long-term study in canines using an engineered thrombopoietin receptor, Mpl-F36V, has demonstrated the utility of intermittent use of AP20187 and AP1903 administration over the course of a number of years with no effect on the normal physiology of the dogs and no effect on the HSC compartment without drug administration^[152]. However, this study only involved long-term monitoring of two dogs and did not test the utility of such a system in a reconstitutive gene therapy context. Studies in healthy human volunteers (see below) may need to be conducted to unravel the effects of long-term administration of AP20187 in order to inform the FDA prior to administration in patients.

Unlike AP20187, AP1903 has gained more momentum with respect to its translation into the clinic. It induces dimerization through the same CBD as AP20187, extending the utility of studies that use F36V fusions^[150-156]. Most importantly, a phase I clinical trial in which a single infusion of AP1903 was administered to 28 healthy volunteers at a range of doses has already been conducted^[157]. No relevant adverse effects were observed at any of the doses. Furthermore, donor T cells that are genetically modified with a CID-inducible caspase-9 (iCasp9) suicide system have been administered to leukemia patients to enhance immune reconstitution in recipients of allogeneic HSC transplants^[158,159]. A single dose of AP1903 was sufficient to ameliorate the GVHD. T cell counts were reduced in as little as 30 min after drug administration followed by resolution of GVHD symptoms in 24 h. Further administration of AP1903 is being tested in patients receiving cell products that have been gene modified with iCasp9 (www.bellicum.com). The dosing for these patients has been informed by the pharmacokinetics observed in healthy volunteers^[157]. Long-term follow-up of patients from one study has been published with up to 4-year occurrence-free survival of patients receiving iCasp9-modified T cells and AP1903^[160]. However, it must be noted that repeated administration of the drug and associated safety profiles have not been published to the best of our knowledge. This is mainly because single-administration of the drug resolved symptoms in the studies conducted thus far and eliminated the need for further intervention^[158,159]. Additionally, no information about biodistribution and pharmacogenomics of AP1903 in humans is available - that we are aware of. As such, the drug is still considered experimental and there is no prior knowledge about its efficacy in the bone marrow niche other than that which has been observed in animal models^[152,161]. Further studies conducted in healthy volunteers to assess a full set of biological parameters can boost the clinical development of enrichment modules relevant to HSC-directed gene therapy.

Engineering alternative amplification modules

While FDA approval and consequential routine administration of existing CIDs in patients may be achievable in the future, there is value in stepping away from sole confinement to FKBP12-derived systems. Multiple other technologies exist that utilize either compounds known to be benign along with reverse engineered signaling domains or compounds and their respective binding domains derived from other species. For example, a recently demonstrated degron-system that uses an auxin-inducible domain derived from plants^[162] could be engineered to enhance Wnt signaling in HSCs by targeting axin degradation in an auxin analog-dependent manner^[163]. Mouse studies have shown no effect of this analog on normal physiology^[164]. In addition, reverse engineering of G-protein-coupled receptors (GPCRs) have led to the development of a variety of Designer Receptors Activated by Designer Drugs (DREADDs)^[165]. One DREADD has been designed to mediate chemotaxis of monocytes and neutrophils in a drug-dependent manner and highly promising results have been demonstrated *in vivo*^[166]. This DREADD is activated in response to Clozapine-N-oxide (CNO), a benign metabolite of the clinically-approved anti-psychotic agent, Clozapine^[167]. With our growing understanding of protein modularity, it is not difficult to envision the development of a similar DREADD that potentially activates HSC-specific survival and proliferative signals. Development of systems that utilize compounds with known pharmacokinetics and biodistribution that can be fast-tracked for FDA approval can provide a significant boost to *in vivo* enrichment strategies for HSC-directed gene therapy.

MODULES TO ENHANCE ENGRAFTMENT OF THE TRANSDUCED HSC SUB-POPULATION

HSCs are housed in specialized compartments in the BM. They use cell surface receptors and ligands to anchor themselves in their niche and to modulate signals for long-term survival and self-renewal^[4,168]. Roles of multiple signaling pathways have been elucidated; as well, cell surface proteins that can demarcate HSCs as single cells that can stably engraft and that are capable of reconstituting the entire hematopoietic system have been uncovered^[169]. It is therefore not difficult to envision novel clinical roles for these cell surface proteins. For example, plerixafor (Mozobil, Sanofi) is a drug that was developed to disrupt the interaction of C-X-C chemokine receptor type 4 (CXCR4, fusin) and C-X-C motif chemokine 12 (CXCL12, or SDF-1)^[170]. Greater numbers of CD34⁺ cells can be acquired with administration of plerixafor over the traditional mobilization strategy that uses granulocyte-colony stimulating factor^[171,172]. Recently, experiments have shown the potential of another drug, Bortezomib (Velcade, PS-341), in HSC mobilization *via* disruption of very late antigen 4 and

vascular cell adhesion molecules (VCAM-1) in mice^[173].

Enhancement of engraftment can also be envisioned using information resulting from such aforementioned studies. Specifically, interactions that can be disrupted for HSC mobilization can also be used to increase engraftment potential. To this effect, studies have shown stage-specific roles for CXCR4 in BM homing and survival of engrafted HSCs^[174,175] (Figure 2B). In addition, the benefit of virally-mediated CXCR4 transgene expression for HSC engraftment into a humanized mouse model has been shown with *ex vivo*-cultured human CD34⁺ cells^[176,177]. Concerns of long-term side effects of CXCR4 expression, such as diminished repopulating potential of the transduced cells and off-target expression in non-HSCs, still remain. For example, high CXCR4 expression is associated with worse prognosis in acute myeloid leukemia, amongst other cancers^[178,179]. Developments in transient cDNA introduction, conditional control of transcription, and/or robust, tissue-specific control of expression may minimize these concerns, though they remain to be tested. Should such technologies be developed in a clinically-relevant manner, multiple other cell surface targets for enhancing HSC homing, survival, and "stemness" could be utilized, such as Tie2, VLA4, and c-Kit^[168,180-182] (Figure 2B). Other methods relying on expression of downstream components of survival and self-renewal signaling such as homeobox protein Hox-B4 (HoxB4) can also be envisioned to be employed in a controlled manner^[183] (Figure 2C). Though introduction of transgenes may provide the ultimate solution to these challenges, other groups are focusing on development of small molecules and co-injection protocols as alternative methods; such as co-injection of BM-derived mesenchymal stem cells^[184-186].

LINEAGE-SPECIFIC ENRICHMENT

As discussed previously, it can be conceptually difficult to target therapeutic HSCs for *in vivo* amplification due to the unique microenvironment in the BM niche in which they reside and because of their inherently quiescent nature. Whilst it is generally agreed that the most clinical benefit can be obtained by targeting long-term precursor cells, it is not conclusively proven to be so. Examples of gene therapy that result in enrichment of cells derived from transduced HSCs, such as in SCID-X1 and β -thalassemia, provide proof-of-concept for the clinical utility of targeting a subset of the mature hematopoietic compartment for selection^[7,17,43-47]. Application of enrichment strategies in such a manner can overcome many of the hurdles of attempting to enrich the original HSC engraftment, such as bioavailability of the compounds used for enrichment and toxicities towards the core center of hematopoiesis. In addition, targeting mature cells, and more importantly, excluding the HSC compartment, reduces the chances of long-term complications in patients that have already gone through a risky intervention and reduces the likelihood of sporadic malignant disease arising from alterations

in "stem-like" progenitor cells. One conceptual disadvantage of such a targeted system is the lack of persistence of selection, since mature hematopoietic cells will be replaced over time. Enrichment protocols can be developed, however, that utilize either drug dosing that has little or no toxicity in normal cells, or compounds that specifically act on cells that arise from transduced HSCs. As such, continuous administration of the respective agent can provide for prolonged enrichment with minimal or no side effects.

IMPDH2 mutants for MMF resistance in T/B cell progeny

Inosine monophosphate dehydrogenase 2 (IMPDH2) is the rate-limiting enzyme involved in the *de novo* biosynthesis of guanosine monophosphate (GMP)^[187]. While most cells in the body have a salvage pathway, T cell activation and proliferation as well as B cell maturation are highly dependent upon this biosynthetic pathway^[188,189]. Mycophenolic acid (MPA) is a potent, non-competitive, reversible inhibitor of IMPDH2^[190-192]. Its prodrug, Mycophenolate Mofetil (MMF, Roche), is routinely used in the clinic as an immunosuppressant to control GVHD amongst other indications^[193]. Mutants of IMPDH2 that have diminished binding affinity for MPA have been described^[194,195]. The most potent amongst these is the combination of T333I and S351Y (IMPDH2^{IV}). The utility of this double mutant has been demonstrated in the context of donor T cell selection, both *in vitro* and in mouse models using primary human T cells^[196,197]. It should be noted that the total lymphocyte count in these experiments was dramatically lowered with MMF treatment, reducing the benefit of such a system with respect to T cell gene therapy^[197]. The dosage of drug used in that study, however, was considerably higher than that used to treat patients for GVHD. The effect of low-dose MMF treatment on engrafted cells expressing IMPDH2^{IV} has yet to be shown. In addition, it has been demonstrated that there is no biological effect of constitutive expression of this mutant enzyme on HSC differentiation^[194]. Therefore, use of such an enrichment strategy could exclude HSCs and all hematopoietic progeny other than T and B cells from being affected. That said, previous work has only described the use of IMPDH2^{IV} for application in T cell-related disorders, such as HIV treatment and prevention of GVHD^[194,197]. To our knowledge, *in vivo* use of this enrichment module in HSC gene therapy has not yet been demonstrated.

CID-dependent enrichment of gene modified progeny

Numerous examples exist of receptor-CBD fusions that can provide a proliferative advantage to subsets of mature cells. Most recently, an erythropoietin receptor (EpoR)-F36V fusion has been developed for use in facilitating AP20187-dependent erythropoiesis^[198]. The fusion is engineered with the minimal components of EpoR required for dimerization-induced signaling along with a myristoylation signal. The fusion is expressed under the control of an erythrocyte-specific promoter. The goal of that study was to design a system to

replace the necessity of recombinant Epo administration in anemic patients. The authors have successfully demonstrated CID-dependent erythropoiesis *in vivo* in a mouse model^[198]. Though this group has not shown the utility of their system in the context of HSC gene therapy, it can be postulated that it would be applicable for enhancement of treatment for disorders that affect erythropoiesis. It must be noted that such systems are hindered to date in their clinical translation due to their use of clinically-unavailable CIDs. Future studies utilizing clinically-available compounds and a variety of lineage specific growth signals are anticipated.

DISCUSSION

Safety of LV-mediated gene therapy

With the initial implementation of recombinant onco-retroviruses in gene therapy strategies, an emergent obstacle to be considered when genetically modifying long-term, stem-like cells is the potential for the development of malignancy^[4,15,23-25]. As discussed above, LVs greatly diminish the likelihood of integration near known oncogenes or tumor suppressors^[4,26-30]. However, multiple other mechanisms that can lead to gross cellular aberrations or changes in function of cells derived from transduced HSCs are still of concern. Though improbable, integrative modification of gene loci can lead to alternative splicing of putative oncogenes, or to the insertional inactivation of tumor suppressors^[199,200]. Additionally, a comprehensive understanding of oncogenesis has yet to be achieved in all its different forms, especially within the complex network of cells in the hematopoietic system. As such, continuation of long-term studies investigating the effects of transplanting patients with transduced HSCs is a necessity, especially with new knowledge being acquired regarding the functional importance of intergenic "junk" DNA.

One strategy to circumvent putative side effects of HSC gene therapy is to include suicide modules (or "cell-fate control" systems) in the transfer vector. Suicide modules refer to elements of therapeutic vectors that are capable of inducing specific cell-death of transgenically modified cells. This is especially important when considering the inclusion of amplification modules, which have not been thoroughly tested in patients. These systems can be designed to induce cell death by providing a surface target for antibodies, by inclusion of an inducible component that activates the apoptotic pathway, or by being able to activate a normally non-toxic prodrug. For example, there are multiple CID-based systems that bring together components of the apoptotic-signaling pathway^[158,159,201-203]. One of these, iCasp9, is currently being tested in patients receiving haploidentical donor T cell infusions amongst other indications, as discussed previously, though the CIDs being used have yet to acquire FDA approval^[158,159]. Additionally, such systems have been tested for their ability to eliminate autologous HSC engraftments in rhesus macaques^[204].

The ability to conditionally activate prodrugs has been a useful tool in molecular biology to induce killing of subsets of cells, though many of the enzymes used are derived from other species. For example, thymidine kinase (tk), derived from the herpes simplex virus (HSV-tk), can be used to render cells sensitive to the drug ganciclovir, a commonly employed laboratory technique^[205]. HSV-tk has been used to ameliorate GVHD in patients receiving allogeneic transplants and in anti-tumor suicide gene therapy^[71,206-209]. However, use of this system is limited by concerns of immunogenicity of non-human proteins that can cause elimination of otherwise useful cells^[56,57]. Additionally, ganciclovir and acyclovir are commonly prescribed for viral infections following engraftment, and use of HSV-tk can lead to unintended elimination of transduced cells^[210]. Our lab has developed a fusion protein comprised of the extracellular and transmembrane components of LNGFR (CD271) along with an engineered variant of human thymidylate kinase^[211]. This module combines the advantages of being able to overexpress a cell surface marker for tracking transduced HSCs and their progeny, since CD271 expression is absent in circulating blood cells, and the ability to activate azidothymidine to a toxic form.

Stem cell exhaustion and clonal selection

One of the principal advantages of *in vivo* selection is the potential for an increase in therapeutic benefit from an initially lower number of transduced repopulating cells. Yet, proliferative stress on few selected HSCs can occur, resulting in a gross negative, long-term impact on HSC proliferation and lineage differentiation. This is termed "stem cell exhaustion" and can eventually lead to BM failure in recipients. Such concerns have been studied in mice^[212] and dogs^[213] that underwent serial MGMT^{P140K}-expressing HSC transplantation under prolonged O⁶BG/BCNU treatment. Importantly, these studies revealed no apparent impairment in HSC repopulation, proliferation, or differentiation, suggesting that stem cell exhaustion may not be an issue, at least in the context of that mode of chemoselection. Further studies in long-lived, clinically-relevant models need to be conducted, however, especially in the context of HSC gene therapy, to demonstrate lack of long-term exhaustion within primary autologous recipients. *Ex vivo* selection may also be achievable without stem cell exhaustion with the co-expression of factors that maintain HSC "stemness", such as HoxB4^[183].

In vivo drug selection can also exacerbate clonal dominance, a phenomenon readily observed with the use of recombinant onco-retroviruses. Amplification strategies could augment the proliferative advantage of cells with proviral integration sites in or near proto-oncogenes, risking the development of hematopoietic malignancies. For example, analysis of tertiary MGMT-transduced BM recipients showed only 17 unique retroviral integration sites (RIS) following chemoselection^[212]. Most RIS in that study were in or near

genes involved in important cell regulatory processes, such as cell growth, cell development, and/or cell differentiation. However, this observation may be an artifact of the use of derivatives of murine stem cell retrovirus. In contrast, studies in canines and humanized mice that utilized recombinant LVs, and a mouse allograft study utilizing a foamy viral vector, showed no observable evidence of clonal dominance following chemoselection^[30,213,214]. Taken together, current data suggests that stem cell exhaustion and clonal dominance are unlikely to occur with amplification strategies, especially with the use of recombinant LVs. Nevertheless, a persistent concern remains when considering HSC gene therapy in patients. As such, an excellent strategy to address issues surrounding *in vivo* amplification of transduced HSCs is to apply enrichment to mature hematopoietic cells, as discussed previously. However, more studies need to be conducted to demonstrate clinical efficacy and safety of amplification of mature cells subsequent to HSC gene therapy.

Future considerations

Many of the enrichment strategies that have been suggested or tested for use in gene therapy applications are designed to be expressed in a constitutively “on” manner. While this may increase the potency of the given strategy, unexpected secondary effects on normal physiology may occur. Therefore, conditional expression cassettes for clinical use should be developed in order to minimize unwanted expression of amplification modules. This is paramount when considering the use of highly engineered or trans-species proteins, which can, over time, elicit immune responses against target cells. In addition, to avoid similar issues at a genomic level, use of innate promoters, such as that derived from the elongation factor 1- α locus (EF1 α), in contrast to virally-derived promoters, such as that derived from cytomegalovirus (CMV), should be considered for clinical application^[215].

Greater focus in the gene therapy field is placed upon the improvement of gene delivery methods and, in the context of HSC gene therapy, efforts are being made to increase the efficacy of various aspects of HSC acquisition, engraftment, and patient care. However, there is limited research to develop next-generation strategies for other aspects of HSC gene therapy that can improve clinical efficacy of this treatment modality. Clinically-feasible strategies need to be developed that allow for selection or enrichment of transduced, therapeutic HSCs after engraftment. In addition, strategies that use other target cell types, such as MSCs, should also be considered for tandem gene therapeutics, to increase the efficiency of correction mediated by HSC gene therapy. More specifically, enrichment strategies that utilize clinically-approved compounds with known pharmacokinetics and pharmacodynamics, such as MMF, need to be developed. Such systems have the potential to be employed in the clinic more quickly and can allow for

repeated administrations or continuous low dosing for long-term benefit. Dose adjustments can also be safely made to compensate for variability in patient pharmacogenomics. The development of modules that allow resistance to drugs used for the treatment of benign hematopoietic hyperplasias can encompass many of the aforementioned advantages.

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Multipotent pancreas progenitors: Inconclusive but pivotal topic

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Abstract

The establishment of multipotent pancreas progenitors (MPP) should have a significant impact not only on the ontology of the pancreas, but also for the translational research of glucose-responding endocrine β -cells. Deficiency of the latter may lead to the pandemic type 1 or type 2 diabetes mellitus, a metabolic disorder. An ideal treatment of which would potentially be the replacement of destroyed or failed β -cells, by restoring function of endogenous pancreatic endocrine cells or by transplantation of donor islets or *in vitro* generated insulin-secreting cells. Thus, considerable research efforts have been devoted to identify MPP candidates in the pre- and post-natal pancreas for the endogenous neogenesis or regeneration of endocrine insulin-secreting cells. In order to advance this inconclusive but critical field, we here review the emerging concepts, recent literature and newest developments of potential MPP and propose measures that would assist its forward progression.

Key words: Multipotent pancreas progenitors; Regeneration; Self-renewal; Clonogenesis; Differentiation

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Core tip: Diabetes mellitus is a pandemic health problem that currently affects approximately 400 million people worldwide and its incidence is increasing by 2%-3% per year. At present, insulin deficiency in diabetes is treated by exogenous insulin given as either multiple daily injections or continuous subcutaneous infusion (pump), which is associated with acute, potentially life-threatening metabolic disturbances as well as chronic, vascular complications with significant morbidity and mortality. The ultimate solution would therefore be regenerative therapies by which lost β -cells in disease processes could be restored/replaced by surrogate insulin-secreting cells including those derived from multipotent pancreas progenitors.

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INTRODUCTION

In contrast to what we would expect, modern humans encounter unprecedented health challenges, including the pandemic diabetes mellitus (DM), which is a major metabolic disorder worldwide with a progressively climbing incidence. This disease currently affects 387 million individuals, with one dying in every 7 s due to severe complications (<http://www.idf.org/diabetesatlas>). Among them, approximately 10% suffer from type 1 DM (T1DM), due to the absolute lack of glucose-responding β -cells destroyed by the patient's own immune system. Provided autodestruction of β -cells is under control, a permanent replacement approach may therefore be an ideal solution for T1DM, through regeneration *in situ* of endogenous β -cells, or by the replacement with donated glucose-responding islets, or of *in vitro* produced insulin-secreting β -cells from stem cell sources such as pluripotent stem cells (PSCs). The other 90% diabetic subjects are currently affected by type 2 DM (T2DM), resulting from the inability to react to insulin regulation by key metabolic tissues, the inability to regulate the generation of glucose from the liver and the dysfunction of endocrine β -cells^[1]. The latter is typically believed to be caused by the increase of β -cell death^[2]. Recently, accumulating evidence has suggested that β -cell dysfunction in T2DM is also caused by the dedifferentiation of glucose-regulating β -cells^[3,4]. Thus, the ability to restore function of failed endocrine cells would provide a novel redifferentiative treatment for T2DM. In order to develop regenerative medicine therapies to T1DM and T2DM, the interest to both stem cells and progenitors in the pancreas has recently been progressively increased.

In general, the concept of multipotent progenitors is exchangeable with that of stem cells. The latter are usually used to define undifferentiated primitive cells that have the capacity for self-renewal themselves as well as of differentiation into terminal functional cells. For example embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC, both together known as PSC) are able to produce all types of 210 cells that build up the body. Debatably, progenitors with multipotency are stem cells. Progenitors could be multipotent, oligopotent or unipotent based on their developmental potentials. Multipotent progenitors/stem cells are of fundamental importance to normal physiology and to regeneration during disease/injury repair. Multipotent pancreas progenitors (MPP) would be a rare subset present in developing and adult pancreas, and have a capacity for regeneration when required, even though

their location and origin have not yet been completely established and are still controversial. In this article, we will summarize knowledge on the candidate MPP along the natural route of endocrine pancreas development and in three functional components of the pancreas. We will also propose future research perspectives on the potential MPP.

CLASSICAL EMBRYOLOGY

The pancreas is an important digestive and endocrine organ originating from the endoderm of posterior foregut. The latter along the other two germ layers is derived from the inner cell mass and would develop into the pancreas primordia when a localized area of thickened columnar epithelia along the dorsal and ventral posterior foregut appears. The columnar endodermal layer evaginates into the neighboring mesenchymal tissues of mesoderm origin and gives rise to the dorsal and ventral buds of the pancreas, respectively. Whilst continuously proliferating and branching, two buds gradually fuse together due to the gut rotation movement. Subsequently, the primordial pancreas continues to expand, transform and, finally, differentiate into the mature organ. The mature pancreas is composed of the acinar compartment that secretes digestive enzymes and fluid, that are drained into the intestinal lumen by the pancreatic ducts and, the endocrine pancreas that secretes hormones (glucagon, insulin, somatostatin, ghrelin or pancreatic polypeptide) responsible for maintaining normal glucose metabolism.

The key developmental events in the human pancreas are different from what we know about from developing pancreas in mice^[5]. For instance, human dorsal bud is detectable at 26 d post conception (dpc), which is an equivalent age of embryonic day (E)9.5 in mouse embryos. The cellular developmental sequences in humans also differ from that in mice. Although they are not visible until 52 dpc, approximately 2 wk later than the equivalent stage at which they can be detected in mice, insulin-positive β -cells in humans appear precedent, unlike that in mice, to glucagon-positive α -cells at 8-10 wk of gestation^[6]. All islet cells are detectable at the end of the first trimester in humans^[6], but at later stages (E17.5) in mice^[7]. Finally, the dynamics of gene expression during embryonic development and in pathophysiological conditions also differ between the two species^[8]. Readers are referred to more reviews of human pancreas development elsewhere^[9-12].

POTENTIAL MPP ALONG THE DEVELOPMENT OF ENDOCRINE PANCREAS

Remarkably, PSC can be guided to differentiate into definitive endoderm (DE)-like progenitors *ex vivo* by applying knowledge of *in vivo* developmental mechanisms (Figure 1). For example, this has been achieved

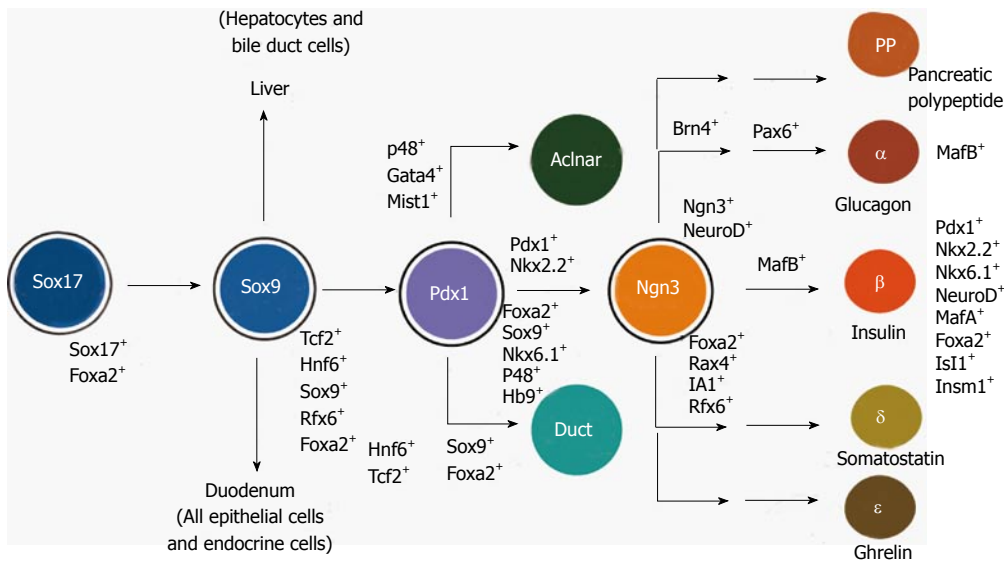


Figure 1 Multiple fate selections allow the development of the pancreas islet lineages. Committed from one of three germ layers (the ectoderm, mesoderm and endoderm) during gastrulation, the definitive endodermal cells (DE) are marked by the expression of Sox17 (the Sry-related HMG box transcription factor 17) and foxhead homeobox 2a (Foxa2). Along the anterior-posterior axis the DE is divided into foregut (giving rise to the lung, thyroid and esophagus), posterior foregut (PF), marked by the expression of the transcription factor hepatocyte nuclear factor 4a (Hnf4a) and hindgut (committing the intestine and colon). *In vitro*, retinoic acid would direct the DE cells to PF cells. Largely to the liver and duodenum, a fraction of the PF cells give rise to pancreatic progenitors (PP, marked by the expression of the transcription factor Pdx1). Mostly to the exocrine and ductal tissues, the PP commits to progenitors of the endocrine islet lineages [IP, marked by the expression of Neurog3, as well as neural differentiation 1 (NeuroD), insulinoma associated 1 (IA1), Islet 1 (Isl1), paired box factor 6 (Pax6) and Rfx6]. The IP then differentiates into at least five types of islet cells [α , β , δ (somatostatin), pancreatic polypeptide (PP) and ϵ (ghrelin)].

in culture by supplementing with a pharmacological level of activin A, a growth factor (GF) of the superfamily of the transforming GF β . Furthermore, human PSC-derived self-renewable DE-like cells have been reported to expand under the stimuli of four GFs^[13]. These DE-like endodermal progenitors have proliferated over 24 passages with an astonishing increase in cell numbers. To establish their reliability and utility in developmental biology, drug screening and regenerative medicine, PSC-derived DE-like cells should be biologically and transcriptomically compared to embryo-derived DE cells. Although further research is required, these endodermal progenitors may indeed function as pre-MPP in addition to possessing an incredible capacity for expansion.

Sox9-expressing MPP

Cystic epithelial colonies expressing E-cadherin first were generated by our group (Figure 2). After dissociation, fetal pancreatic cells produced cystic colonies containing β -cells in the presence of the basement membrane glycoprotein laminin 1, 1, 1 and a bone morphogenetic protein^[14,15]. Interestingly, spherical but not cystic colonies are generated in the presence of epidermal GF^[16], suggesting these GFs play different roles during development. We have not investigated whether all or only some fractions of cystoid epithelial cells stochastically commits to different lineages, though it seems possible that these cystic colonies originated from multipotent progenitors that are Sox9⁺ (see below).

Sox genes encode versatile regulators of stem/progenitor cell fate^[17], belonging to members of transcription factor family that contain the Sry (sex deter-

mining region Y) box-related high-mobility group. Sox9 for example critically regulates the development of multiple embryonic organs including the pancreas. This pivotal transcription factor is first detectable at E10.5 in the dorsal and ventral pancreases^[18] and at E13.5, Sox9-expressing progenitors (Figure 3) develop into the exocrine and endocrine lineages in the pancreas^[19]. However, by E16.5 the expression of Sox9 is progressively restricted to pancreatic ductal cells^[19]. Genetic tracing studies reveal that Sox9 is also expressed in organs of other posterior foregut origin such as the liver, the bile duct and the duodenum. For example, it is present in bile ductal cells adjacent to the portal vein from E16.5. Sox9 also is broadly detectable in the intestinal epithelia at E13.5 but confined to the crypt region from E18.5^[19]. Thus All Sox9-expressing cells in the posterior foregut region could be MPP. Supportive to these analyses, Sox9-expressing (Sox9⁺) multipotent progenitors purified from E11.5 Sox9-eGFP embryos generate expandable cystoid colonies that contain hormone-expressing cells in a laminin 1, 1, 1-enriched Matrigel-coated culture condition^[20].

Sox9⁺CD133⁺ ductal cells generates duct-like "ring/dense" colonies (1/5) in the culture of the Matrigel-containing methylcellulose-based semisolid medium. With the addition of the roof plate-specific spondin 1, a Wnt agonist, these ring/dense colony-forming cells can be serially dissociated and replated in the presence of Matrigel with an expansion of more than 100000-fold^[21]. In a laminin-containing hydrogel, the Sox9⁺CD133⁺ (cluster differentiation 133) cells are able to give rise to acinar/endocrine colonies^[21]. Further investigations

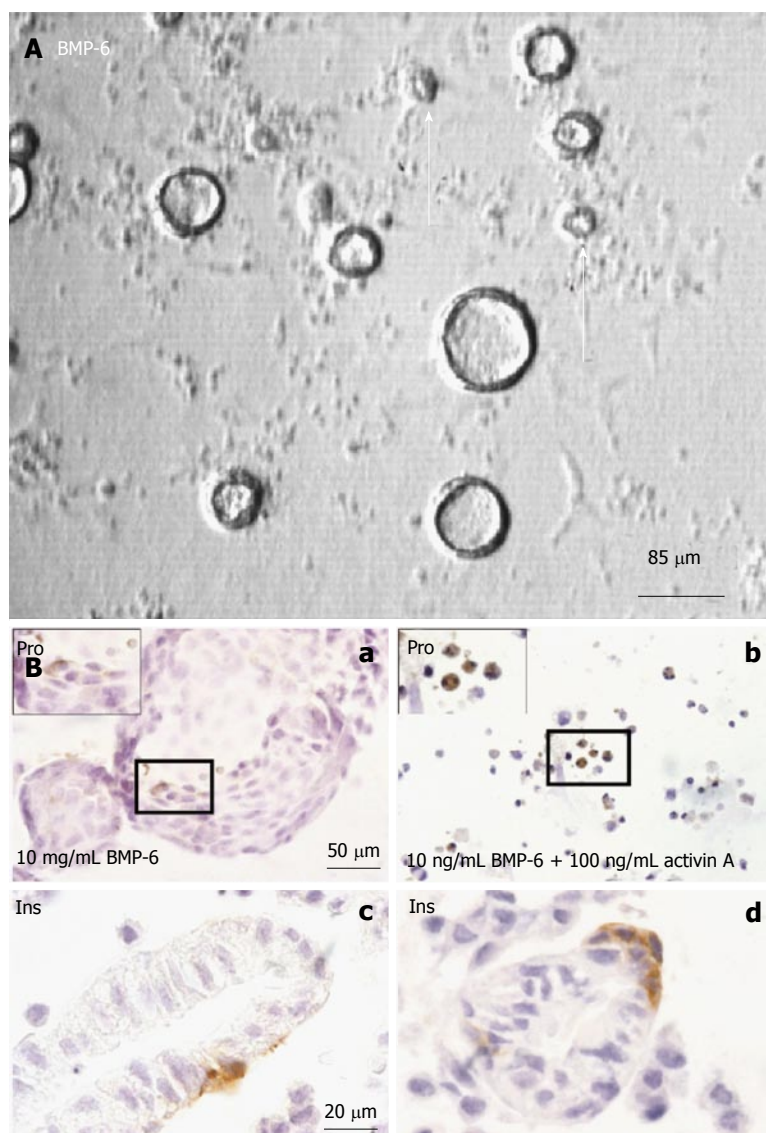


Figure 2 Cystic colony formation from dissociated fetal mouse pancreas cells. A: Phase contrast image showing that BMP-6 promotes colony formation. Open arrows indicate colonies $\leq 30 \mu\text{m}$; B: Immunocytochemical analyses: a: Proinsulin staining. Fixed colonies were stained with proinsulin antibody (brown); b: Activin A antagonizes colony formation; c, d: Insulin staining. Histological sections of harvested colonies were stained with anti-insulin antibody (brown). Adapted and modified from ref.^[14].

are needed to ascertain whether Sox9⁺CD133⁺ cells are identical to, or distinct from, the colony-forming Lgr5 [leucine-rich repeat-containing G-protein coupled receptor 5, also known as GPR49 or GPR67 (G-protein coupled receptor 49 or 67)]-expressing cells described below. However, Sox9-positive ductal and centroacinar cells are unable to produce β -cells in a few experiments for regeneration or trans differentiation, such as with cerulean-induced acute pancreatitis, an *in vitro* culture experiment, pancreatic duct ligation (PDL), partial pancreatectomy and a streptozotocin-induced diabetic model^[19,22]. Originated from the posterior foregut endoderm, a human bile duct progenitor population known as “biliary tree-derived cells”^[23] may have a MPP potential and give rise to islet lineages. However, the purified population, molecular profile and detailed developmental potential of these “tree-derived cells” require further investigations.

Pdx1-expressing MPP

A cluster of unique cells in the thickened DE epithelium along the dorsal and ventral surfaces of the posterior

foregut in E9.0-9.5 mouse embryos expresses the homeobox gene named Pdx1 (pancreas and duodenum transcription factor 1). The latter encodes a parahox homeobox-containing factor, critical for the establishment of primitive pancreas^[24] as well as the maintenance of functional β -cells^[25,26].

Studies have demonstrated that Pdx1-expressing (Pdx1⁺) progenitors are MPP because they give rise to acinar, ductal and islet tissues of the pancreas^[27]. Genetic lineage tracing studies revealed that these MPP reside in the termini of the tree-like branching ducts in the developing pancreas that also express the acinar transcription factor known as Ptf1a and the exocrine cell marker known as Cpa1^[28]. The Pdx1⁺ cells are capable of taking up the thymidine analogue bromodeoxyuridine (BrdU) and incorporating the latter into their genome during mitosis^[29], revealing that these cells are proliferative.

Using developmental biological knowledge, PSC have been manipulated to generate Pdx1⁺ cells that have been expanded for 16-fold co-cultured with pancreas-derived mesenchymal cells^[30]. To verify the proliferation

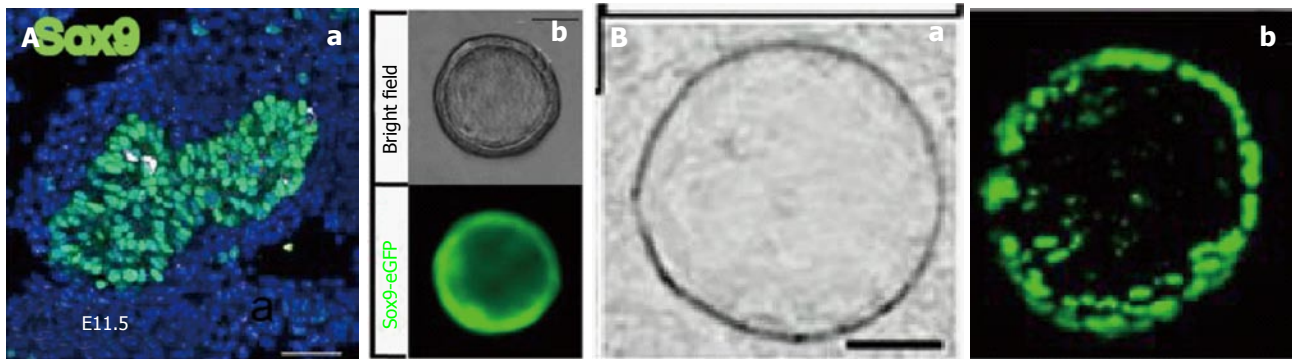


Figure 3 Cystic colonies generated from Sox9-tagged cells. A: Embryonic Sox9⁺ progenitors in the pancreas capable of generating cystic colonies; a: Sox9 is expressed in most ductal progenitors in E11.5 mouse pancreas; b: Cystic colonies are formed from purified Sox9-eGFP⁺ progenitors in E11.5 mouse pancreas; B: Purified Sox9⁺ cells in adult mouse pancreas capable of generating cystic colonies under a phase contrast (a) or a fluorescence microscope (b). Adapted and modified from [20,21].

of Pdx1⁺ cells, independent confirmation of this report will be required. We also need to ascertain whether these PSC-derived Pdx1⁺ cells share all or partial characteristics of embryonic pancreatic progenitors, because *Pdx1* is also expressed in extra-pancreatic tissues including other endoderm-derived non-pancreas organs [31].

In developing human pancreas, numerous PDX1⁺ progenitors are present between 8 and 21 wk of gestation [32,33]. These PDX1⁺ progenitors are frequently expressed SOX9 and highly proliferative [34], supporting the notion that pancreatic progenitors are committed from SOX9⁺ multipotent progenitors. The progressive increase of PDX1⁺ cells that co-express insulin or somatostatin is observed in this period of gestation [32]. Further research is required to investigate whether the PDX1⁺ progenitor pool is established by its self-renewal or by direct differentiation of the posterior foregut progenitors or both.

Strategies for proliferation, self-renewal and differentiation of pancreatic progenitors will be of importance in developmental biology and regenerative medicine. Interestingly, reserpine and tetrabenazine that inhibit the vesicular monoamine transporter-2 are shown to direct PSC-derived Pdx1⁺ cells to produce cells that express neurogenin 3 (*Neurog3*, also known as *Ngn3*) [35], a DNA-binding protein, belonging to the transcription factor family of the basic helix-loop-helix category. However, as a positive control, Pdx1⁺ progenitors purified from embryonic pancreas would have been tested with these two molecules to examine their capacity and efficiency to give rise to islet progenitors. Furthermore, caution needs to be exercised for the use of genetic lineage tracing in PSC differentiation *in vitro* because temporospatial cues are essential for the success of *in vivo* lineage tracing studies (see review by [36]). To enrich PSC-derived genuine pancreatic progenitors for further expansion and differentiation, identification of their specific markers would be highly valuable.

***Neurog3*-expressing pancreatic endocrine progenitors**

Approximately in E9.5 mouse embryos, within the

thickened posterior foregut DE epithelium there is a small cluster of cells that express a high level of the *Neurog3*, an endocrine determinant [27,37,38]. The cells that express *Neurog3* highly are the progenitors of endocrine pancreas as they develop into all islet lineage cells *in vivo*. Several studies verify the critical role of *Neurog3* in the ontogenesis of endocrine pancreas: Islet cells are not observed in *Neurog3* targeted mouse pancreas [37]; genetic tracing demonstrates that *Neurog3*⁺ progenitors differentiate into all five types of islet cells [27]; isolated adult *Neurog3*⁺ cells reappeared after PDL can, after inoculation into an embryonic pancreas *ex vivo*, give rise to five types of endocrine cells [38]. Nevertheless, a few *Neurog3*⁺ cells are observed to coexpress insulin in the dual fluorescence-tagged developing mouse pancreas [39]. Another laboratory reported that PDL allows the activation of *Neurog3* expression but the *Neurog3*-expressing cells are not able to complete the entire β -cell regeneration program [22]. Furthermore a recent study found that β -cell mass and insulin content are totally unchanged after PDL-induced injury [40], unresponsive to the conclusion of an active β -cell regeneration. The reason for these inconsistencies is unknown and future investigation is warranted to confirm or refute the conclusion.

Whereas the expression of mouse *Neurog3* mRNA in the developing pancreas plateaus approximately E15.5 [41] (approximately week 9 of gestation in humans), that of human *NEUROG3* is low prior to 9 wk, but increases sharply onward and remains very high until 17 wk [33]. Furthermore some cells coexpress both *NEUROG3* and insulin in the embryonic pancreas from 10 to 21 wk [32]. Although earlier studies showed that *Neurog3*⁺ cells could proliferate [42,43], clonal assays by "mosaic analysis with double marker" (MADM) have confirmed that *Neurog3*⁺ cells are quiescent and commit to only one cell type of the endocrine pancreas [44]. A recent study consistently demonstrated that the activation of *Neurog3* itself inhibits cell division by the activation of cyclin-dependent kinase inhibitor 1a [45]. The inconsistencies between the previous and recent reports require future research to reconcile. Again, it is formally

possible that PSC-derived Neurog3-expressing cells are not completely bona fide islet progenitors as Neurog3 is expressed in endoderm-derived non-pancreas organs.

Insulin⁺ multipotent progenitors

Embryonic insulin-expressing cells may be endocrine progenitors in the pancreas as they can give rise to other islet cell types in addition to β -cells at least during development^[46]. By employing fluorescence-tagged tracing strategies, multipotent progenitors that are insulin⁺ (arguably to be MPP) in the pancreatic tissues are believed to be originated from the Pdx1⁺ progenitors, instead from the ectoderm-derived neural crest. These MPP-like cells express several islet progenitor markers and are able to differentiate into all endocrine cells *in vivo*. The MPP express a low concentration of insulin and low copies or complete lack of glucose transporter-2, clearly different from functional mature β -cells^[47]. Nevertheless, studies have demonstrated that the expression of insulin gene is activated in Pdx1⁺ MPP, *via* Neurog3⁺ precursors to adult β -cells^[48,49], thus future investigation is needed to establish what stages at which insulin⁺ MPP are present: The embryonic, fetal and/or adult endocrine pancreas.

Human insulin⁺ multipotent progenitors have also been described with a similar developmental potential to the mouse ones. These cells, isolated from either mice or humans, could, after transplantation into diabetic mice, develop into functional cells to ameliorate hyperglycemia. Surprisingly, however, these insulin⁺ multipotent progenitors also gave rise to neural lineage cells *in vivo*^[47].

Ghrelin (ϵ)-expressing progenitors?

Ghrelin-expressing ϵ -cells are the fifth cell type in the endocrine pancreas and are first discovered in the stomach. Ghrelin is a polypeptide hormone composed of 28-amino acid residuals and known to negatively regulate insulin secretion from mouse, rat and human islets^[50]. Now it is well established that pancreatic ϵ -cells are detectable in mid-gestation in mice and humans and their number plateaus during the perinatal period^[51]. However, there are a substantial number of ϵ -cells in only human but not mouse and rat adult islets^[51], raising the possibility that ϵ -cells could participate in the regulation of glucose homeostasis.

Deletion of the *Arx* gene, encoding an α -cell transcription factor, drastically reduces the number of ϵ -cells^[52]. In contrast, knockout of *Nkx2.2*, *Pax4* or *Pax6* significantly increases the number of these cells at the expense of reducing other pancreatic endocrine cell types^[53,54]. Intriguingly, genetic studies of lineage tracing demonstrated that ϵ -cells give rise to α , PP and, to a lesser extent, β -cells in adult mouse pancreas^[55], suggesting that these cells have a unusual plasticity for trans differentiation towards, and may even be progenitors of, other islet cells. However, whether ϵ -cells would act as MPP of functional islets remains to be established.

Can islet β -cells behave like unipotent “progenitors”?

Functional duplication of insulin-secreting β -cells is first described by Dor *et al.*^[56] by using RIP (rat insulin promoter)-driving genetic lineage tracing. They revealed that mouse β -cells in the endocrine pancreas could be reproduced by RIP-expressing cells under the physiological condition or after partial pancreatectomy. This report however did not preclude the presence of and the role of MPP by assuming that all RIP-expressing cells are functional β -cells. Similarly, in a transgenic model using the RIP to govern diphtheria toxin expression resulted in 70%-80% β -cell apoptosis, disrupted architecture of endocrine pancreas and eventually in the development of diabetes. Withdrawing the expression of diphtheria resulted in a significant recovery of β -cell mass, islet architecture and of normoglycemia^[57]. Further analysis revealed that a subset of 20%-30% surviving “ β ”-cells played a major role in the β -cell regeneration and recovery of euglycemia^[57]. These studies suggest that islet β -cells are indeed facultative unipotent progenitor cells.

In the sophisticated double transgenic MADM (designated RIP-CreER; Rosa26^{GR}/Rosa26^{RG}) mouse pancreas, each RIP-expressing clone is made up of only 5 cells after one month of chase, slightly increasing to 8 cells by two months^[58]. The clusters that express RIP have been viewed as supportive evidence of regeneration of functional cells, but this should be treated with caution as discussed hereafter. A further study with a reductionist approach on the gene *Hnf4a* (hepatocyte nuclear factor 4a) suggested that the β -cell regeneration may be associated with the signaling cascade of the Ras/Erk pathway^[59] and even be modulated by cell cycle regulators such as cyclin D2^[60]. Collectively, it is critical to establish and identify the well-known RIP-expressing cells either in self-duplication or in dedifferentiation both *in situ* and *ex vivo*, as the latter may be a key MPP candidate for a T1DM replacement solution.

Furthermore, lineage tracing labeled with a thymidine analogue showed that β -cells are produced within an islet by rare self-renewable cells that have a long duplication-refractory time. Under stress conditions such as during pregnancy or after partial pancreatectomy, the number of self-renewable cells is dramatically elevated^[61]. However the molecular nature of these rare cells and the replication-refractory length have not been established. Future studies should characterize their molecular identity and reveal whether these self-renewable replicating cells are fully functional. Due to the ethical barrier and technical difficulties, similar studies are not possible to be undertaken to label human endocrine pancreas *in vivo*, but a similar research should be repeated with larger mammals.

The above described investigations of β -cell self-renewal as a regenerative mechanism have drawn considerable interest in last several years, due to its promise as a way to increase β -cell mass for the treatment of diabetes. However, these studies have not excluded

whether the insulin transcript (as controlled, for instance, by the RIP transgene) is only expressed in functional β -cells. There is increasing evidence suggesting that is not the case. First, the expression of insulin gene is detectable in the Pdx1^+ progenitors, dramatically increasing in Neurog3^+ precursors and peaking in mature islet β -cells^[48,49]. Second, the demonstration of insulin⁺ multipotent progenitors^[47] precludes insulin as an exclusive marker of functional β -cells. Third, insulin protein has been detectable in some mouse and human islet precursors as described above.

Taken together, the expression of insulin gene is clearly not exclusive for functional β -cells. It is formally possible β -cell populations in adults are maintained not only by the self-replication of functional glucose-regulating cells but also by the self-duplication and development of MPP. In order to demonstrate that MPP are indeed present in the adult endocrine pancreas, their clonogenesis must be established with isolated single cell MPP candidates *ex vivo*, with data generated from the intermediary stages of the clonogenesis, and with demonstrable ability to give rise to at least non- β endocrine cells *in vivo*.

MPP PRESENT IN THREE TYPES OF PANCREAS TISSUES

Accumulating evidence generated in *ex vivo* studies has suggested that MPP are present in three major tissues of the pancreas: The pancreatic ductal^[62-64], acinar and islet compartments^[65,66]. For instance, the application of flow cytometry has identified a potential MPP population in the mouse pancreas both in development and in adults^[67]. These candidates are characterized by the exhibition of the receptor c-met for hepatocyte GF, without the presence of hematopoietic lineage markers including CD45, c-Kit (stem cell factor receptor), Flk-1 and TER119. The purified population is able to give rise to several types of pancreatic cells *ex vivo* and generate pancreatic endocrine and exocrine cells *in situ* after transplantation^[67]. Nevertheless, the spatial localization and the molecular natures of these c-met positive cells are completely unknown and the clonogenesis has not been established at the single cell level.

MPP likely present in the ductal epithelium

Many studies suggest that MPP are present in the ductal epithelium. Bonner-Weir *et al.*^[68] first reported that human ductal epithelial cells in adult pancreas are able to differentiate into islet-like clusters containing insulin-secreting β -cells. Ramiya *et al.*^[63] showed that insulin-secreting islet-like clusters generated *in vitro* from mouse MPP are capable of ameliorating diabetes after being grafted under the kidney capsule. In cultures of human "pancreatic ductal cell aggregates" after isolation of the islets for transplantation, cells with fibroblast-like morphology appear known as pancreatic "mesenchymal stem cells (MSC)". These cells were

passed over 12 times and expressed an array of markers of bone marrow MSC such as CD13, CD29, CD44, CD49f (also known as α_6 integrin subunit), CD54, CD90 (also known as Thy1) and CD105. The pancreatic MSC are shown to be able to give rise to cross-germ layer cells such as endoderm-originated non-pancreas lineages^[69]. Utilizing culture protocols suitable for producing the neurospheres of ectoderm origin, ductal cells from mouse pancreas are shown to give rise to neurosphere-like clusters that subsequently give rise to a few types of endocrine cells, such as insulin-secreting β cells^[66]. Nevertheless, the exact nature of the special cells has not been established. A major limitation of the forementioned reports is the use of unenriched/unpurified cell preparations in addition to not demonstrating single-cell self-renewability.

Furthermore, numerous CK19^+ ductal cells and islet cells are detected after PDL^[38]. The lineage relationship between the ductal cells and islet cells has further been addressed using the genetic lineage tracing of the Cre-loxP system. Using the system, the Cre governs the promoter activity of carbonic anhydrase II gene, encoding a marker of adult ductal epithelia and controls the removal of the stop sequence (Rosa-loxP-stop-loxP-lacZ) in the reporter Rosa26 (R26R) mice. This resulted in Cre-driving β -galactosidase expression in ductal epithelia. After 28 d in normal or PDL pancreas, the activity of β -galactosidase is detectable in numerous ductal epithelia, localized acinar tissue and in up to 40% islet cells^[70,71]. These data have been viewed as further evidence that the ductal cells expressing carbonic anhydrase II are able to regenerate mouse endocrine cells. It remains unclear, nevertheless, how many cells that express carbonic anhydrase II have this regenerative capacity, how many potential MPP are present or whether a trans differentiation process also takes place.

Nevertheless, once an exon of the ductal epithelial marker gene *Tcf2* (T cell factor 2, also known as Hnf1b, hepatocyte nuclear factor 1b) is exchanged with the transgene containing Cre, the lineage tracing has demonstrated that the Tcf2^+ cells in the postnatal duct cannot develop into endocrine cells in both normal and PDL pancreas^[72]. However, a complicating factor in this report is that one allele of *Tcf2* was non-functional, leading to reducing by half the transcription of *Tcf2*. As heterozygous *Tcf2* mutant does not support pancreas development in mice and humans^[73,74], inactivation of one allele of *Tcf2* in the lineage tracing studies may have led to haploinsufficiency and affected the differentiation of potential MPP into functional islet cells.

Fbw7 (F-box and WD-40 domain protein 7), an ubiquitin ligase, is expressed in embryonic and adult ductal epithelial cells. Deletion of this gene stabilizes the heavily ubiquitinated *Neurog3*, and reprograms the ductal cells to α , β and somatostatin-producing δ -cells^[75]. This study suggests that pancreatic ductal cells are a latent MPP and Fbw7 is a critical cell-fate

regulator. Nevertheless, this report did not describe whether all or a small fraction of the ductal cells express Fbw7 and what the frequency of the reprogramming event is; both are critical to assess whether this might become a viable strategy to regenerate islet cells by suppressing the Fbw7 signaling. A fundamental assay on reprogrammed cells to ameliorate diabetes has also not been reported.

Interestingly, PDL robustly activates the Wnt signaling pathway and allows the regenerating ducts to express Lgr5^[76], a Wnt target which marks actively dividing stem cells such as those present in the intestine^[77]. Purified ductal Lgr5-expressing cells are also responsive to spondin 1 and form clonal 3D pancreatic organoids within the gel-forming Matrigel that generate ductal as well as endocrine lineages upon transplantation^[76].

MPP likely present in acinar tissue of the pancreas

In the clinic with experimental transplantation of donated islets from the cadaver's pancreas, the acinar cells are normally un-used. The discovery of a scientific and practical value for the acinar cells has hence drawn considerable attention. Cotransplantation with pancreatic cells from fetal mice under the kidney capsule in the immunocompromised mice, the acinar cells are reported to give rise to islet cells with undetectable β -cell division or cell fusion^[78]. The results imply that MPP or progenitor cells might be present in the acinar tissue of human adult pancreas. Moreover, the Cre-loxP lineage tracing analysis showed that acinar cells expressing amylase and elastase gave rise to insulin-producing cells in a rotating culture system^[79]. Nevertheless, a self-renewable assay at the single-cell level and its intermediate steps need to be demonstrated. Additionally, these studies have not excluded the possibility that some pancreatic acinar cells might have trans differentiated into surrogate insulin-expressing cells^[80,81]. This likelihood was supported by a previous study that showed that mouse acinar cells could be directly re-programmed *in vivo* to β -like cells by forced expression of three DNA-binding genes, namely, *Pdx1*, *Neurog3* as well as *MafA*^[82]. In addition, as the acinar cells from cadavers^[78] were not isolated by FACS, residual ductal or even islet cells present in the system may have compromised their conclusion. Supporting this view, a lineage tracing study *in vivo* did not provide evidence that the mouse acinar cells would give rise to insulin-secreting β -cells after 70%-80% pancreatectomy^[83]. Thus these inconsistencies remain to be addressed more carefully.

MPP likely present in the endocrine pancreas

A large body of evidence suggests that the islets of Langerhans harbor the MPP. The potential MPP are nestin⁺ and hormone⁻ present in both rat and human islets. These nestin⁺ cells are reported to expand in culture for approximately 8 mo and differentiate into cells that exhibit markers of liver (α -fetoprotein) and pancreas lineages (including amylase, CK19, PDX1, glucagon, insulin and neural-specific cell adhesion

molecule). These nestin⁺ MPP candidates may thus have been involved in the generation of new pancreatic islet cells^[65], potentially modulated by the incretin hormone GLP1, a processed product from the polypeptide pro-glucagon^[84]. It, however, remains unclear whether these nestin⁺ cells are either islet or duct-originated multipotent progenitors that transform into neurosphere-like structures as well as differentiated into β -like cells of the endocrine pancreas^[66], or the outgrown fibroblast-like cells that expand readily and differentiate *in vitro* into aggregates of non-typical hormone-expressing endocrine cells^[85]. Human endocrine cells transduced with a RIP-controlling transgene dedifferentiated into fibroblast-like cells that proliferate up to 16 population doublings without detectable insulin expression^[86]. Nevertheless, *in vivo* Nestin⁺ cells are mostly present in non-endodermal-derived compartments^[87,88]. Governed by their promoters, Pdx1- and RIP-expressing cells have not committed significantly *ex vivo* to the observed cells with fibroblast-like morphology^[89]. These discrepancies require clarifications in the future studies.

CONCLUSION

The presence of MPP remains to be conclusively established. Future development of the field needs to: establish essential criteria for MPP; screen and select cell surface antigens that can be used to generate antibodies for purification of candidate MPP and establish a simple, effective and robust *in vitro* assays and *in vivo* experimental protocols for the examination of the multipotency and lineage commitments of isolated MPP candidates.

The following minimal criteria have to be satisfied: (1) enriched or purified cell population by FACS or other technologies should form colonies at the single cell level; (2) single cells after purification should be studied *ex vivo* with their self-renewability; (3) colony cells would be able to differentiate *in vitro* into multiple functional cellular lineages; and (4) the clonogenic cells post transplanted into a recipient should be able to give rise *in vivo* to different terminally differentiated lineages. Thus, embryonic mouse Sox9⁺ multipotent progenitors and probably adult Sox9⁺ ductal cells satisfy some but not all criteria of MPP. Research into MPP would therefore be highly valuable for two reasons. First, MPP differentiation and trans differentiation of non- β -cell types in the pancreas may provide an important source for surrogate β -cells. Second, as there is a significant difference in regeneration capability of islets from mice, rats to humans, we should not extrapolate directly from rodent regenerative data to humans.

Future MPP research should apply integrated approaches, different from many previous *in situ* or *ex vivo* studies that did not target defined cell populations. Application of flow cytometry and cell surface markers would allow the separation of interested subsets for demonstrating *in vitro* the capacity of self-duplication, clonogenesis and differentiation. The latest technologies

such as single-cell RNA-seq, single-cell genomics and single-cell epigenomics^[90-92] should be applied to examine their developmental dynamics, differentiation pathways, gene interactions and genetic heterogeneity, and along with genetic studies to characterize their growth pattern, biological potential and lineage commitment *in vivo*. Lastly cross-sector, cross-institutional and global collaborations as well as the involvement of the biotechnological and drug companies will eventually deepen our understanding of MPP that assists the establishment of a platform towards a regenerative therapy for both T1DM and T2DM.

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Basic Study

Induction of CXC chemokines in human mesenchymal stem cells by stimulation with secreted frizzled-related proteins through non-canonical Wnt signaling

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Abstract

AIM: To investigate the effect of secreted frizzled-related proteins (sFRPs) on CXC chemokine expression in human mesenchymal stem cells (hMSCs).

METHODS: CXC chemokines such as CXCL5 and CXCL8 are induced in hMSCs during differentiation with osteogenic differentiation medium (OGM) and may be involved in angiogenic stimulation during bone repair. hMSCs were treated with conditioned medium (CM) from L-cells expressing non-canonical Wnt5a protein, or with control CM from wild type L-cells, or directly with sFRPs for up to 10 d in culture. mRNA expression levels of both CXCL5 and CXCL8 were quantitated by real-time reverse transcriptase-polymerase chain reaction and secreted protein levels of these proteins determined by ELISA. Dose- (0-500 ng/mL) and time-response curves were generated for treatment with sFRP1. Signal transduction pathways were explored by western blot analysis with pan- or phosphorylation-specific antibodies, through use of specific pathway inhibitors, and through use of siRNAs targeting specific frizzled receptors (Fzd)-2 and 5 or the

receptor tyrosine kinase-like orphan receptor-2 (RoR2) prior to treatment with sFRPs.

RESULTS: CM from L-cells expressing Wnt5a, a non-canonical Wnt, stimulated an increase in CXCL5 mRNA expression and protein secretion in comparison to control L-cell CM. sFRP1, which should inhibit both canonical and non-canonical Wnt signaling, surprisingly enhanced the expression of CXCL5 at 7 and 10 d. Dickkopf1, an inhibitor of canonical Wnt signaling prevented the sFRP-stimulated induction of CXCL5 and actually inhibited basal levels of CXCL5 expression at 7 but not at 10 d post treatment. In addition, all four sFRPs isoforms induced CXCL8 expression in a dose- and time-dependent manner with maximum expression at 7 d with treatment at 150 ng/mL. The largest increases in CXCL5 expression were seen from stimulation with sFRP1 or sFRP2. Analysis of mitogen-activated protein kinase signaling pathways in the presence of OGM showed sFRP1-induced phosphorylation of extracellular signal-regulated kinase (ERK) (p44/42) maximally at 5 min after sFRP1 addition, earlier than that found in OGM alone. Addition of a phospholipase C (PLC) inhibitor also prevented sFRP-stimulated increases in CXCL8 mRNA. siRNA technology targeting the Fzd-2 and 5 and the non-canonical Fzd co-receptor RoR2 also significantly decreased sFRP1/2-stimulated CXCL8 mRNA levels.

CONCLUSION: CXC chemokine expression in hMSCs is controlled in part by sFRPs signaling through non-canonical Wnt involving Fzd2/5 and the ERK and PLC pathways.

Key words: CXC chemokines; Mesenchymal stem cell; Osteogenesis; Differentiation; Wnt signaling pathway; Frizzled-related protein; Frizzled receptors

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Core tip: Chemokines have multiple functions during bone formation and fracture repair. The ELR⁺ chemokines classically have a role in blood vessel formation and were found to be stimulated by the non-canonical Wnt5a protein and also by soluble frizzled-related proteins (sFRPs) that are known inhibitors of both canonical and non-canonical Wnt signaling. This stimulation was mediated *via* the p44/42 extracellular signal-regulated kinase and phospholipase C pathways signaling through the non-canonical frizzled receptors 2 and 5. This is a newly identified role for the sFRPs in stimulation of ELR⁺ chemokines which may be involved in blood vessel formation during wound repair.

Bischoff DS, Zhu JH, Makhijani NS, Yamaguchi DT. Induction of CXC chemokines in human mesenchymal stem cells by stimulation with secreted frizzled-related proteins through non-canonical Wnt signaling. *World J Stem Cells* 2015; 7(11): 1262-1273 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i11/1262.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i11.1262>

INTRODUCTION

Bone fracture repair proceeds through a series of sequential steps including an inflammatory phase resulting in recruitment and differentiation of mesenchymal stem cells (MSCs) into osteoblasts, restoration of blood supply, subsequent soft (cartilaginous, in the case of endochondral repair) and hard (bone, in both endochondral and intramembranous) callus formation, and ultimately remodeling of the new woven bone into lamellar bone. During the initial inflammatory stage, neutrophils, macrophages, and lymphocytes migrate to the wound, fight infectious organisms, scavenge tissue debris, and begin the process of granulation tissue formation^[1]. Cytokines, chemokines, and growth factors released from these cells are necessary to initiate bone repair in the adult. The pro-inflammatory cytokine, tumor necrosis factor- α (TNF- α) is critical in both long bone fracture as well as intramembranous bone repair^[2,3]. TNF- α can highly induce members of the CXC chemokine family *via* NF- κ B signaling in osteoblasts^[4]. CXC chemokines can be grouped as to whether or not they contain a Glu-Leu-Arg (ELR) motif. ELR⁺ CXC chemokines, such as CXCL8 (IL-8), are present during the inflammatory phase to serve as chemoattractants for neutrophils^[5,6] and exhibit angiogenic activity^[7-9]. Chemokines without the ELR sequence are anti-angiogenic^[9].

Human MSCs (hMSCs) express CXCL8 mRNA^[10-12] and it has been reported that TNF- α can prime hMSCs to upregulate production of several CXC chemokines (highest upregulation with CXCL5 and CXCL8) and induce hMSC migration^[13]. In humans, CXCL8 is a ligand for both CXC receptor 1 (CXCR1) and CXCR2 whereas CXCL5 interacts solely with CXCR2. Angiogenesis in response to CXCL8 has only been associated with CXCR2 signaling^[14-16]. We previously demonstrated that CXCL8 expression can be stimulated with dexamethasone treatment during osteoblastic differentiation^[17] and by low extracellular pH^[18] in hMSCs. We also demonstrated that secreted CXC chemokines induced angiogenic tube formation of a human microvascular endothelial cell line (HMEC-1)^[17] consistent with *in vitro* angiogenesis.

The mouse CXC receptor (mCXCR) is functionally related to hCXCR2^[19]. Mice lacking the mCXCR (mCXCR2^{-/-}) have been described^[20] and some healing^[21] and bone^[22-24] defects have been reported. A second murine CXCR (mCXCR1) has also been identified; although, it has no discernable defect phenotype when inactivated (Jackson Laboratory Stock #005820). We have shown by DEXA and micro computerized tomography analysis that the mCXCR2^{-/-} mice (Jackson Laboratory Stock #002724) have an osteopenic phenotype with decreased trabecular bone volume, number, and thickness without any changes in bone formation and resorption indices^[25]. However, bone quality was affected as femurs had reduced stiffness and a lower ultimate load breaking point^[25]. There was

also a reduction in the blood vessel density in the newly repaired bone in a cranial defect model^[25]. During bone regeneration, ingrowth of blood vessels is required for endochondral bone formation^[1]. These results suggest a potential coupling of mMSC differentiation, bone formation, and angiogenesis in response to mCXCR signaling.

The Wnt family of secreted glycoproteins is involved in differentiation of an assortment of tissues^[26]. Wnts signal through specific seven transmembrane spanning G-protein coupled frizzled (Fzd) receptors *via* both canonical β -catenin signaling, and non-canonical Wnt/calcium and Wnt/planar cell polarity pathways^[27,28]. The highly conserved and redundant nature of the Wnt/Fzd system (19 Wnts and 10 Fzd in humans) only adds to the complexity of this system and confusion as to its role in osteogenesis.

The canonical pathway is characterized by Wnt binding to both Fzd and LRP5/LRP6 co-receptors resulting in activation of Disheveled (Dsh) which inhibits glycogen synthase kinase β (GSK3 β) phosphorylation. In the absence of Wnt binding, GSK3 β phosphorylation ultimately results in β -catenin degradation, preventing its nuclear translocation for activation of target genes. In murine models, evidence suggests that canonical Wnt/ β -catenin signaling is necessary for lineage commitment of pluripotent MSCs to osteochondroprogenitor cells, then to osteoprogenitor cells, and for differentiation to mature osteoblasts while suppressing both chondrogenesis and adipogenesis^[29,30]. However, in hMSC models, β -catenin and canonical Wnt3a can negatively regulate the differentiation of MSCs into the skeletal precursor cells that precede the appearance of the osteochondroprogenitor cells^[31-35]. Additionally, de Boer *et al.*^[36] reported a dose-response relationship in which lower levels of β -catenin stimulated hMSC proliferation while blocking adipogenesis; whereas, higher levels induced expression of alkaline phosphatase. The authors thus concluded that canonical Wnt/ β -catenin signaling could initiate osteogenic differentiation in the human system^[36].

Signal transduction through the non-canonical or β -catenin-independent Wnt pathways has also been shown to inhibit adipogenesis and chondrogenesis in MSC models and to stimulate osteogenesis^[33,37-40] mediated through activation of phospholipase C (PLC), and then through activation of the calcium-calmodulin kinase, nuclear factor of activated T-cells (NFAT), and protein kinase C (PKC) pathways or through the mitogen-activated protein kinase (MAPK) and RhoA pathways. Non-canonical Wnt signaling through traditional canonical Wnt ligands, Wnt3a and Wnt7b, and the non-canonical Wnt ligand, Wnt4, can also lead to osteogenic differentiation in both murine and human MSCs models through the activation of PKC and/or MAPK pathways^[33,41,42]. Levels of non-canonical Wnt5a are increased in the inflammatory environment during early fracture healing^[43] and non-canonical signaling (Wnt4 and Wnt5a) can affect the transition from

proliferative osteoprogenitors to mature osteogenic cells^[44,45]. However, as with canonical signaling, there are conflicting reports as to whether non-canonical Wnt5a can induce osteogenesis^[30,33].

Wnt antagonists which include secreted frizzled related proteins (sFRPs), that can inhibit both canonical and non-canonical Wnt signaling^[46], or the canonical Wnt/ β -catenin Dickkopf (Dkk) inhibitors may also contribute to osteoblast differentiation and mineralization^[30,38,47]. sFRP1 knock-out mice exhibit increased trabecular bone mass due to reduced osteoblast and osteocyte apoptosis^[48] suggesting that Wnt signaling is involved in bone formation. Additionally, long bone fracture healing is enhanced in sFRP knockout mice through canonical Wnt signaling as a consequence of MSCs directed to differentiate into osteoblasts rather than towards cartilage^[49]. However, high sFRP1 levels expressed early in fracture repair in this model would suggest that both canonical and non-canonical Wnt signaling are inhibited in early callus formation. Wnt5a/5b expression was decreased in the sFRP1 knockouts; although, contrary to conventional thought, canonical Wnt7a and Wnt1 were elevated^[49]. There have been other reports of sFRPs enhancing rather than inhibiting Wnt activity^[50] through mechanisms which may involve: (1) sFRP-Wnt binding to each other and facilitating transport and binding of Wnts to Fzd receptors on distant cells; or (2) binding to both Wnt molecules and Fzd receptors simultaneously to activate downstream Fzd signaling^[51]. Many repair processes are stimulated by sFRP2-Wnt interactions including the enhancement of vascular density during granulation tissue formation^[52]; inhibition of cardiomyocyte apoptosis during cardiac repair^[53]; establishment of MSC-endothelial and smooth muscle contacts to stabilize new blood vessel formation^[54]; and stimulation of angiogenesis by sFRP1 (FzIA) independent of VEGF, bFGF2, or angiopoietin1^[55].

A key observation made by several laboratories is that canonical Wnt/ β -catenin signaling may be important in osteoblastogenesis through the cooperation of Wnt signaling with other known osteogenic factors such as BMP-2 and BMP-4^[47,56]. Thus taken together, canonical Wnt/ β -catenin signaling appears to be involved in determining a specific tissue fate of MSCs. However, further effects of Wnt signaling (both canonical and non-canonical) on osteogenic differentiation is dependent on several factors including: The species from which the MSCs are derived, the specific Wnt (and Fzd receptors) expressed, the stage of osteogenic differentiation, the amount of β -catenin available to translocate to the cell nucleus, and other biologically active molecules (*e.g.*, growth factors) present in the MSC's microenvironment^[30,57]. These other factors could include the ELR⁺ CXC chemokines which are also elevated in the inflammatory phase of healing and which have been shown to be stimulated by non-canonical Wnt5a^[58,59]. In this article, we report the observation that sFRP treatment of hMSCs leads to an increased expression of ELR⁺ CXCL5 and CXCL8 which may serve

Table 1 siRNA oligonucleotide sequences

Gene	Qiagen product name	Qiagen catalog No.	Human target sequence
siFZD2	Hs_FZD2_5	SI02757433	CACGGTCTACATGATCAAATA
siFZD5	Hs_FZD5_5	SI02757650	TAAGGTTGGCGTTGTAATGAA
siROR2	Hs_ROR2_6	SI00287525	CTGGTGCTTTACGCAGAATAA
siScrambled	Ctrl_Control_1	SI03650325	AATTCTCCGAACGTGTACAGT

Table 2 Reverse transcription-polymerase chain reaction primer sequences

Gene	Human primer sequence
<i>hCXCL5</i>	5'GCTGGTCCTGCCGCTGCTGTG3' 5'GTTTCTCTGTTTCCACCGTC3'
<i>hCXCL8</i>	5'GCCTTCCTGATTCTGCAGC3' 5'TCCAGACAGAGCTCTCTCC3'
<i>18S Ribosomal RNA</i>	5'CGGGTCATAAGCTTGCGTT3' 5'CCGCAGGTTACCTACGG3'
<i>FZD2</i>	5'CCTCAAGGTGCCATCCTATCTCAG3' 5'GTGTAGCAGCCGACAGAAAAATG3'
<i>FZD5</i>	5'CCTACCAACAAGCAGGTGTCC3' 5'GGACAGGTTCTTCTCGAAA3'
<i>ROR2</i>	5'TCCTTCTGCCACTTCGTGTTCC3' 5'TGCTTGCCGTTCTCTGTAATCC3'

to attract MSCs to the wound or to couple angiogenesis to osteogenesis in the early phase of bone repair.

MATERIALS AND METHODS

Cell culture

hMSCs, growth supplements, and basal medium were purchased from LonzaWalkersville, Inc. (Walkersville, MD). hMSCs from several donors were used: 19 years old male (Lonza Lot #6F4393; race unknown); 20 years old Caucasian male (Lot #0000351482); 27 years old Black male (Lot #0000318006). Cells were grown in complete medium (HMSCGM) at 37 °C under 95% air/5% CO₂ atmosphere and subcultured once a week at 60%-70% confluence.

For osteoblastic differentiation, hMSCs were treated every 3-4 d with osteogenic medium (OGM) consisting of complete growth medium with 50 mmol/L ascorbic acid-2-phosphate, 10 mmol/L β-glycerophosphate, and 10⁻⁷ mol/L dexamethasone (Sigma-Aldrich, St. Louis, MO).

Cells (passage 2-7) were plated at 5000-10000 cell/cm² in HMSCGM and allowed to adhere for 4 h prior to exposure to OGM ($n \geq 3$ for all experiments). Differentiation toward the osteoblastic lineage was monitored by detection of mRNA levels of the reporter gene alkaline phosphatase or by Alizarin Red staining for calcium at 28 d as previously described^[17]. Qiagen RNeasy Miniprep columns (Qiagen, Inc., Valencia, CA) were used to isolate RNA at the specified time-points. In some experiments exogenous sFRPs (varying concentration from 0-500 ng/mL; PeproTech Inc., Rocky Hill, NJ), Dkk1 (50 ng/mL, PeproTech), and L-cell or Wnt5a-conditioned medium (CM) (1:1 mixture with

HMSCGM) were added as needed. The PLC signal transduction inhibitor (U73122) and control (U73343) were used at 10 μmol/L (Calbiochem, San Diego, CA). Effects of siRNA inhibition of receptors were determined by transfection of hMSCs with siRNA (150 ng/mL) using the HiPerFect transfection reagent (Qiagen) followed by treatment of the cells with sFRPs for 48 h before gene expression analysis. siRNA were purchased from Qiagen and the nucleotide sequences indicated in Table 1. A scrambled oligonucleotide siRNA was used as a negative control.

Quantitative reverse transcriptase-polymerase chain reaction

Relative mRNA levels of various genes were determined by real-time RT-PCR using the Opticon Continuous Fluorescence System (Bio-Rad Laboratories, Inc., Hercules, CA) and the SYBR Green RT-PCR kit (Qiagen). Primers used for RT-PCR are indicated in Table 2.

PCR reactions were performed in triplicate. Reactions consisted of reverse transcription at 50 °C (30 min), inactivation at 95 °C (15 min); followed by 50 cycles of denaturing at 94 °C (15 s), annealing at 60 °C (30 s), and extension at 72 °C (30 s). Gene expression changes were calculated and normalized to 18S ribosomal levels and the reference time point using the 2^{-ΔΔC(T)} method^[60].

ELISA analysis

Secreted CXCL5 protein levels were determined with the human CXCL5/ENA-78 DuoSet (R and D Systems, Minneapolis, MN) after concentration of supernatants with microcon centrifugal filters (EMD Millipore Inc, Billerica, MA). Culture supernatant samples were compared to CXCL5 standard curves and were run in duplicate.

Western blot analysis

Cells were plated in 35 mm dishes and treated with OGM medium (7 d). sFRP1 (150 ng/mL) was added and cell lysates isolated at indicated time points in PhosphoSafe Extraction Reagent (EMD Chemicals, Gibbstown, NJ). Proteins were separated (SDS-PAGE), transferred to polyvinylidenedifluoride membrane, and probed with ERK-specific pan or phospho-antibodies (Cell Signaling Technology, Danvers, MA). Immunoreactive proteins were detected using the ECL kit (GE Healthcare Bio-sciences, Piscataway, NJ) and levels quantitated using AlphaView Software (ProteinSimple, San Jose,

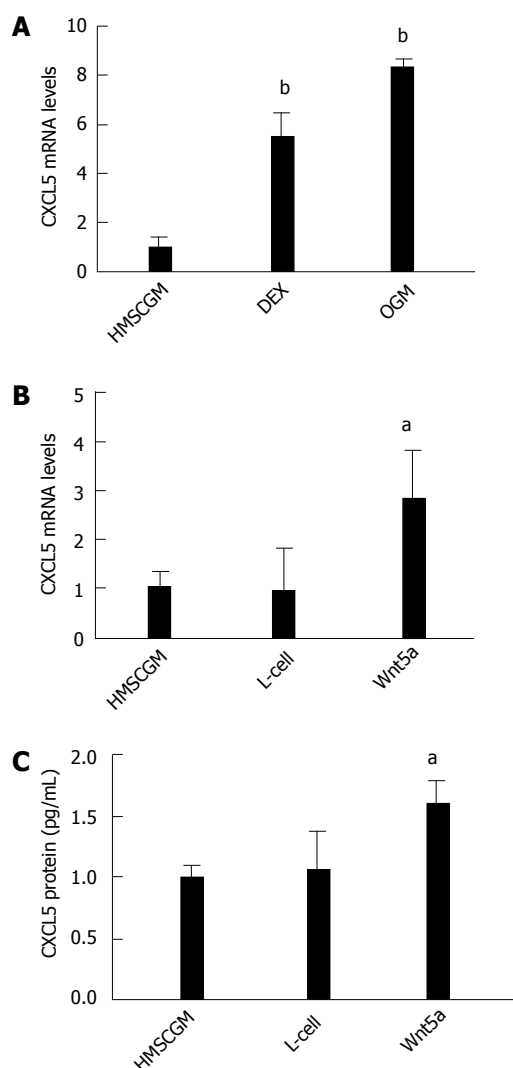


Figure 1 CXCL5 chemokine induction in human mesenchymal stem cells treated with osteogenic medium or non-canonical Wnt5a. A: CXCL5 mRNA levels are induced at 7 d in complete osteogenic medium or medium containing 0.1 $\mu\text{mol/L}$ dexamethasone. Conditioned medium containing non-canonical Wnt5a induces (B) mRNA expression and (C) protein secretion. All values are mean $\pm$ SD. ^a $P < 0.05$; ^b $P < 0.01$ vs HMSCGM-treated group. HMSCGM: Human mesenchymal stem cell growth medium; DEX: Dexamethasone; OGM: Osteogenic medium; L-cell: Conditioned medium from L-cells; Wnt5a: Conditioned medium from L-cells expressing Wnt5a.

CA).

Statistical analysis

Data values are reported as mean $\pm$ SD. Statistical analysis (1-way ANOVA with the Bonferroni method for multiple comparisons between pairs or non-parametric Mann-Whitney *t* test) was performed using GraphPad Prism software. Differences from negative controls were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

We had previously demonstrated that mRNA and protein for CXCL8 (IL-8) and CXCL1 (GRO α)^[17] were

induced in hMSCs exposed to osteogenic differentiation medium (OGM) containing ascorbate-2-phosphate, β -glycerophosphate, and dexamethasone. To see if another angiogenic CXC chemokine, CXCL5 (ENA-78), was also induced by osteogenic differentiation, RNA from hMSCs treated with OGM was analyzed for CXCL5 expression levels. OGM treatment for 7 d stimulated CXCL5 mRNA levels approximately 8-fold ($P < 0.01$; Figure 1A). Dexamethasone alone (0.1 $\mu\text{mol/L}$) in the presence of proliferating medium (HMSCGM) increased CXCL5 mRNA by 5.5-fold ($P < 0.01$).

Non-canonical Wnt signaling has also been associated with osteogenic differentiation of hMSCs^[32,61]. Since osteogenic differentiation using OGM or dexamethasone alone resulted in CXCL5 mRNA expression, we then explored if treatment of hMSC cells with non-canonical Wnt5a protein was able to stimulate the expression of CXCL5. hMSC cells were treated for 7 d with CM from L-cells overexpressing Wnt5a protein or control L-cell CM. CM containing Wnt5a induced the expression of CXCL5 mRNA 3-fold ($P < 0.05$) compared to the lack of stimulation of CXCL5 in both non-osteogenic medium (HMSCGM medium) or control L-cell medium (Figure 1B). CXCL5 protein secretion was also increased 1.5-fold ($P < 0.05$) above controls (Figure 1C).

We next sought to inhibit all Wnt signaling, both canonical and non-canonical Wnt signaling, using sFRPs. Surprisingly and unexpectedly, sFRP1 increased CXCL5 mRNA levels 3-fold ($P < 0.01$) at 7 d and approximately 8-fold ($P < 0.01$) at 10 d of culture in HMSCGM medium (Figure 2A). To see if canonical Wnt signaling inhibition was responsible for the unexpected stimulation of CXCL5, hMSCs were treated with Dkk1 which binds to the low density lipoprotein receptor related protein 6 (LRP6) to inhibit canonical signaling. Unlike sFRP-1, Dkk1 addition did not induce an increase in CXCL5 levels at 7 or 10 d and in fact significantly inhibited basal mRNA expression levels more than 50% ($P < 0.01$) at 7 d (Figure 2A). To see if the effect of sFRP1 on CXCL5 was unique amongst the other sFRP family members, sFRPs 2, 3, or 4 was each added separately to the medium (150 ng/mL) for 7 d and levels of CXCL5 protein secreted into the medium determined. All four sFRPs added independently significantly stimulated CXCL5 protein secretion 3-4-fold ($P < 0.01$) over unstimulated vehicle control (Figure 2B).

We next tested if CXCL8 mRNA levels are also stimulated by sFRP1 treatment. sFRP1 treatment increased CXCL8 mRNA levels in a dose-dependent manner (Figure 2C). Maximum stimulation of CXCL8 mRNA expression (approximately 5-fold) was observed at a concentration of 150 ng/mL sFRP1 ($P < 0.05$). A time-course study of CXCL8 mRNA expression stimulated by sFRP1 (150 ng/mL) showed maximal expression levels ($P < 0.05$) between 5 and 7 d of culture (Figure 2D).

In an effort to explore the mechanism of the sFRP1-stimulated increase in both CXCL5 and CXCL8 expres-

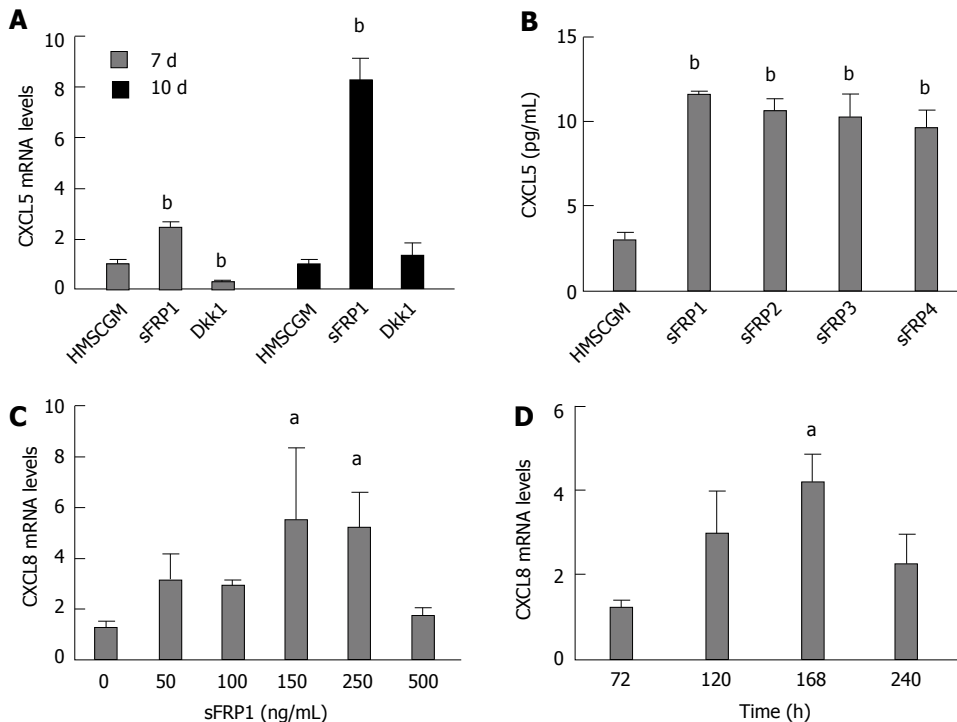


Figure 2 Secreted frizzled-related protein-stimulated expression of CXCL5 and CXCL8 in human mesenchymal stem cells. A: sFRP1, an inhibitor of canonical and non-canonical Wnt signaling, induces expression of CXCL5 mRNA. Dkk1, an inhibitor of canonical Wnt signaling does not induce CXCL5; B: All four of the sFRPs stimulated CXCL5 protein secretion as determined by ELISA analysis of cell supernatants. sFRP1 induces expression of CXCL8 mRNA in a dose- (C) and time- (D) dependent manner with maximum expression at 150 ng/mL and 7 d post-treatment. All values are mean $\pm$ SD. $^aP < 0.05$; $^bP < 0.01$ vs HMSCGM-treated group (A, B), vs untreated (C), or 3-d CXCL8 mRNA levels (D). HMSCGM: Human mesenchymal stem cell growth medium; sFRP: Secreted frizzled-related protein; Dkk1: Dickkopf-related protein 1.

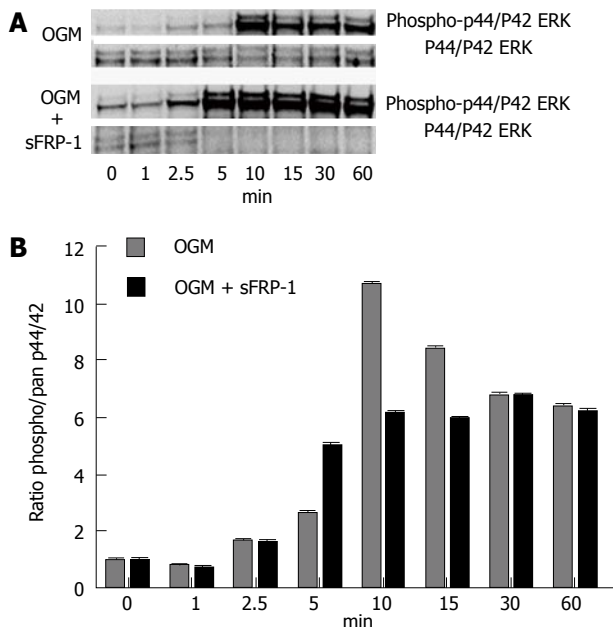


Figure 3 Phosphorylated state of mitogen-activated protein kinase p44/42 extracellular-signal-regulated kinases in response to secreted frizzled-related protein 1 stimulation. Western Blot time-course analysis of pan and phosphorylated states of the MAPK p44/42 ERK proteins (A); The ratio of phospho/pan p44/42 ERK reaches a maximum at 5 min in the presence of OGM and sFRP1 (B); whereas, in OGM alone the maximal ratio level is not reached until 10 min post stimulation (representative experiment). sFRP1: Secreted frizzled-related protein 1; MAPK: Mitogen-activated protein kinase; ERK: Extracellular-signal-regulated kinases; OGM: Osteogenic medium.

ssion, we characterized the phosphorylated state of the extracellular signal-regulated kinase (ERK) p44/42, a member of the MAPK pathway, since it has been previously reported that CXC ligand expression can be increased *via* MAPK activation^[62-66]. p44/42 was shown to be phosphorylated maximally at 5 min in the presence of OGM and sFRP1, whereas maximal p44/42 phosphorylation occurred at 10 min in OGM alone (Figure 3).

To see if other G-protein coupled signaling mechanisms could be involved in sFRP stimulation of CXCL8 mRNA expression, the PLC inhibitor, U73122, was added (10 μ mol/L) to the HMSCGM medium and CXCL8 mRNA levels determined after 3 d of sFRP treatment. Both sFRP1 and sFRP2 in the presence of the inactive isomer, U73343, enhanced CXCL8 mRNA by approximately 3 to 5 fold ($P < 0.05$). However, U73122 prevented the increase in both sFRP1- and sFRP2-stimulated CXCL8 mRNA levels returning them back to HMSCGM control levels (Figure 4).

Since Fzd receptors are G-protein coupled seven transmembrane receptors, it was further investigated if the sFRP stimulation of CXCL8 could be through interactions with specific frizzled receptors. Fzd2 and Fzd5 have been associated with non-canonical Wnt signaling^[67,68]; whereas, Fzd7 is associated with canonical signaling^[69]. sFRP1, 2 and 3 all stimulated CXCL8 mRNA levels in the presence of a scrambled

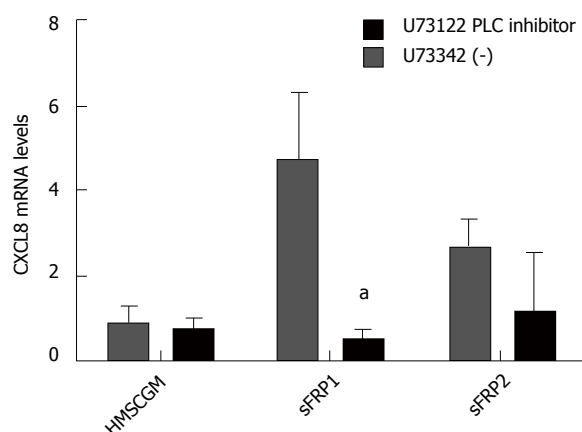


Figure 4 Modulation of secreted frizzled-related protein-stimulated induction of CXCL8 mRNA. sFRP1/2-stimulated CXCL8 mRNA expression is suppressed in the presence of the PLC inhibitor U73122 suggesting that signaling is *via* the Wnt/calcium non-canonical pathway (values are mean $\pm$ SD). ^a $P < 0.05$ vs HMSCGM-treated levels. sFRP1/2: Secreted frizzled-related protein 1/2; PLC: Phospholipase C.

siControl siRNA (7 to 10-fold for sFRP1 and sFRP2; 3-fold for sFRP3). In the presence of siRNA to Fzd2 and 5, the sFRP-stimulation was almost entirely inhibited back to baseline levels ($P < 0.05$; Figure 5A and B). siRNA to Fzl7 did not have any effect on sFRP1 or sFRP2 induction of CXCL8 mRNA (data not shown). The receptor tyrosine kinase-like orphan receptor-2 (RoR2) has been shown to be either a stand-alone receptor or co-receptor with Fzd in non-canonical Wnt signaling^[70,71]. siRNA directed against RoR2 inhibited sFRP1-stimulated CXCL8 mRNA expression by approximately 65% but did not have an effect on sFRP2-stimulated CXCL8 mRNA expression (Figure 5C).

DISCUSSION

sFRPs have been traditionally thought to act as Wnt signaling antagonists by binding to Wnt molecules and preventing them from binding to Fzd receptors thus inhibiting signal initiation^[72]. Interaction of Wnts with Fzd receptors has been hypothesized to occur *via* interaction with the extracellular cysteine-rich domains (CRDs) found in Fzd receptors. sFRPs also contain N-terminal CRDs, but without the transmembrane domain characteristic of Fzd receptors^[73], and it has been demonstrated that sFRP interaction with Wnts occurs through binding within this CRD^[74,75]. Additionally, Bafico *et al.*^[75] found that at least one sFRP could also bind to a selected Fzd (e.g., Fzd6) and hypothesized that heteromeric complex of Fzd and sFRPs would render Fzd receptors nonfunctional.

However, there has been emerging evidence that sFRPs may not only function as Wnt antagonists or anti-morphogens but also serve as molecules that promote differentiation of specific tissues. It has been suggested that sFRPs may aid in Wnt protein distribution and signaling within tissues. Wnt proteins interact with heparin sulfate proteoglycans thereby limiting diffusion

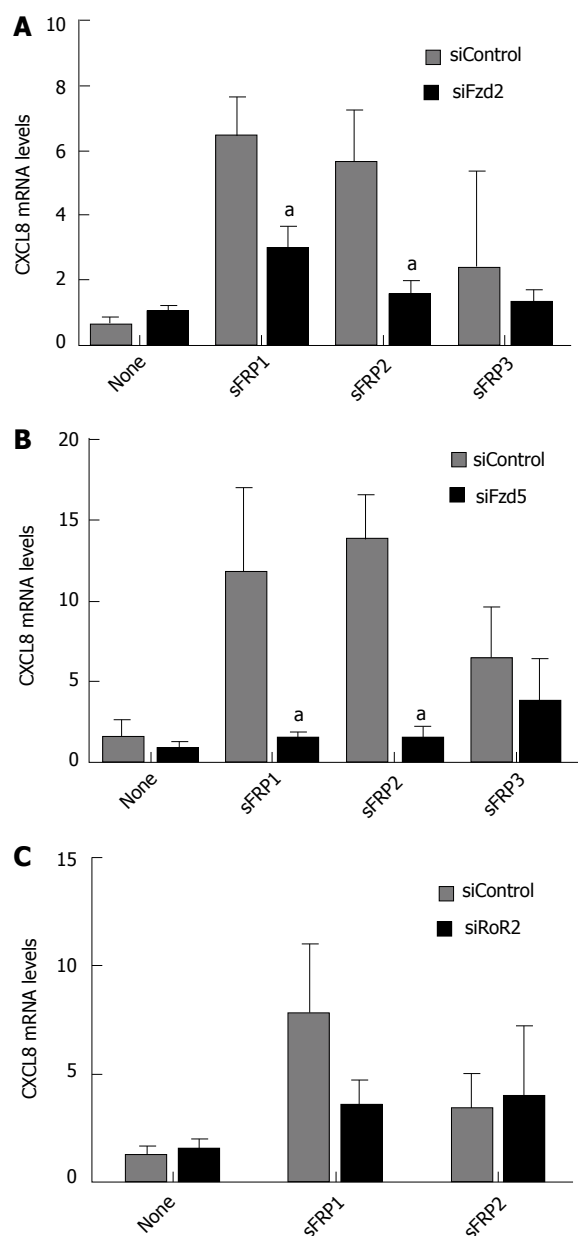


Figure 5 Effect of siRNA directed to frizzled receptors or receptor tyrosine kinase-like orphan receptor-2 on secreted frizzled-related protein-stimulated CXCL8 mRNA levels. sFRP-stimulated CXCL8 mRNA expression is inhibited in the presence of siRNAs directed toward non-canonical (A) Fzd2 and (B) Fzd5 or (C) the non-canonical Frizzled co-receptor RoR2. All values are mean $\pm$ SD. ^a $P < 0.05$ vs untreated siControl levels for each graph. sFRP1-3: Secreted frizzled-related protein 1-3; siFzd2/5: siRNA to the non-canonical frizzled receptor 2/5; RoR2: Receptor tyrosine kinase-like orphan receptor-2; siRoR2: siRNA to RoR2; siControl: Scrambled control siRNA.

of the Wnt proteins within tissues. sFRPs may compete with this binding, enabling the Wnt-sFRP "transport" complexes that are formed to diffuse along a more extended gradient allowing for longer range Wnt signaling within the tissue^[76] thereby aiding in tissue generation or differentiation. In one mouse model system, MSCs engineered to overexpress protein kinase B (also known as Akt) produce high levels of sFRP2 which can effectively limit cardiac muscle infarct size through the inhibition of cardiomyocyte apoptosis^[53].

The increased sFRP2 led to increased levels of nuclear β -catenin thus enhancing canonical Wnt signaling and increased transcription of anti-apoptotic genes such as Birc1b and to a lesser extent Bcl2.

Others have reported links between non-canonical Wnt signaling and sFRP-mediated differentiation processes. Chung *et al.*^[77] reported that sFRP3 could increase osteoblast differentiation in the mouse pre-osteoblastic cell line, MC3T3-E1, by increasing alkaline phosphatase, osteocalcin, and promoting mineralization of MC3T3-E1 cultures. Endostatin, which promotes degradation of β -catenin independent of GSK3 β , did not abrogate sFRP3-stimulated osteogenic differentiation suggesting that non-canonical Wnt signaling may be involved in the sFRP3 effect. Esteve *et al.*^[78] also reported that chick sFRP1 enhanced retinal differentiation by increasing the generation of retinal ganglion and photoreceptor cells independent of cell proliferation. This group noted that canonical Wnt- β -catenin signaling was not involved in this process; although, they did find that phosphorylation of GSK3 β down-regulated its activity while promoting retinal cell differentiation. The authors were unclear if non-canonical Wnt signaling was involved in the sFRP1 findings at that time. In a subsequent communication, this group reported that chick sFRP1 binds to Fzd2 to stimulate axonal outgrowth from retinal neurons^[79]. Furthermore, the action of sFRP1 on the retinal ganglion cells was dependent on cAMP and cGMP in a pertussis toxin-sensitive manner suggesting that sFRP1 acted as an agonist for Fzd2 non-canonical Wnt signaling.

Several reports have shown that sFRPs were involved in stimulating angiogenesis through canonical Wnt signaling but independent of VEGF signaling^[54,55,80]. In studies utilizing MRL/MpJ mice, which have enhanced regenerative capacity, it was found that MRL/MpJ bone marrow MSCs showed decreased expression of cyclin D1, Sox2, and Axin2, which are target genes of canonical Wnt signaling. Concomitantly, sFRP2 and sFRP4 expression was found to be significantly up-regulated^[52] in these cells. It was also reported that sFRP2 overexpression in mouse MSCs that were then injected into the cardiac peri-infarct area reduced infarct size and improved cardiac function similar to that seen when MRL/MpJ MSCs were injected. Of note, vascularization of granulation tissue was also enhanced by sFRP2 overexpression. This was also reported in another MRL/MpJ MSC engraftment wound healing model^[52] whereby sFRP2 overexpression in mouse MSCs increased levels of several angiogenic factors including FGF2 receptor, PDGF receptor beta, VEGF, and angiopoietins among others. While the authors concluded that the increased sFRP2 inhibited canonical Wnt signaling which may be related to the increased angiogenesis, non-canonical Wnt signaling was not examined. Furthermore, the expression of ELR⁺ CXC chemokines that are also angiogenic was not assessed.

There are a number of steps that occur in new blood vessel formation, several of which have been linked to sFRP1 signaling, including endothelial cell

(EC) spreading, proliferation and migration, vascular channel formation, and blood vessel stabilization. sFRP1 has been shown to enhance angiogenesis in a chick chorioallantoic membrane model of angiogenesis and to increase blood vessel density in a tumor implantation model^[55]. EC spreading was hypothesized to be a result of an interaction of sFRP1 with Fzd4 and Fzd7 thereby blocking Fzd activity and has also been shown to be independent of canonical Wnt- β -catenin signaling; although, this process still involving GSK3 β upstream of Rac 1 signaling^[80]. sFRP1 has also been shown to stimulate EC migration and chemotaxis *in vitro*, increase EC branching in capillary structures when cultured on Matrigel, and inhibit EC apoptosis^[55]. EC and vascular smooth muscle cell proliferation are also inhibited as evidenced by slower entry into S-phase as well as decreased expression of the cell cycle components cyclin D1 and cdk4^[81]. This latter inhibition of vascular cell proliferation appeared to be dependent on inhibition of canonical Wnt- β -catenin signaling but not MAPK signaling through ERK1/2. Vessel maturation and stabilization of EC channels by pericytes or MSCs were also enhanced by sFRP1 stimulated cell-cell interactions between MSCs and ECs or smooth muscle cells in a GSK3 β -dependent manner. Interestingly, sFRP1 increased α -smooth muscle actin expression in MSCs suggesting differentiation of MSCs to pericytes which are involved with blood vessel stabilization. Furthermore, the localization of β -catenin at cell-cell junctions rather than intranuclear locations could further support a non-canonical Wnt signaling mechanism. Others have also reported that sFRP2 can also stimulate EC migration and tube formation as well as inhibit hypoxia-induced EC apoptosis through a non-canonical Wnt-calcium pathway involving an increase in NFATc3 nuclear translocation^[82,83].

The mechanism of how sFRPs stimulate angiogenesis is currently unknown. sFRP1, which did not induce expression of the known angiogenic factors VEGF or FGF2, did increase expression of PDGF-BB which is involved in postnatal blood vessel maturation^[84]. Expression of other angiogenic factors such as the ELR⁺ CXC chemokines could potentially be the result of sFRP1 actions. Indeed we are the first to report here that sFRPs are able to induce the ELR⁺ CXC chemokines CXCL5 and CXCL8 in hMSCs. Rauner *et al.*^[85] has shown that human bone marrow MSCs stimulated with pro-inflammatory factors (lipopolysaccharide or TNF- α) resulted in Wnt5a and Ror2 increases in mRNA and protein. The expression of the ELR⁺ CXC chemokines, CXCL1, CXCL2, and CXCL5, was also increased with Wnt5a treatment of these hMSCs. Additionally the CC chemokines, CCL2, CCL5, CCL7, and CCL19 were also upregulated, although, to a lesser extent than the CXC chemokines CXCL1 and CXCL5. Albers *et al.*^[59] also reported that Fzd9 knockout mice demonstrated an osteopenic phenotype caused by decreased bone formation which was unrelated to canonical Wnt signaling^[59]. The presumed non-canonical Wnt regulation

of bone mass in Fzd9-deficient mice was also shown to have significantly decreased CXCL5 expression. In these studies, treatment of wild type osteoblasts with Wnt5a showed a 12-fold increase in CXCL5 mRNA. In a fracture repair model in Fzd9-deficient mice, protein expression of CXCL5 and CCL2 in the healing callus was diminished in comparison to wild-type, and overall new bone in the Fzd9 knockout mice was reduced^[86]. Most recently, Zhao *et al.*^[87] reported that in human dental pulp cells, non-canonical Wnt5a significantly induced CXCL8, CCL2, and CCL5 mRNA and protein expression, as well as increasing CXCL1 mRNA expression. CXCL5 expression was not tested in response to Wnt5a stimulation in this model.

The signaling mechanism(s) responsible for sFRPs induction of ELR⁺ CXC chemokine expression are unknown. Our results suggest that ELR⁺ CXC chemokine stimulation by sFRPs in human bone marrow-derived MSCs is *via* non-canonical Wnt signaling. MAPK, specifically though ERK1/2, and PLC pathways appear to play a role in sFRP stimulation of the ELR⁺ CXC chemokines. PLC signaling can be upstream of MAPK/ERK^[88] and perhaps the non-canonical Wnt-calcium pathway is involved. Our findings that inhibition of sFRP-induced ELR⁺ CXC chemokine expression by PLC as well as demonstration of ERK phosphorylation upon sFRP stimulation of MSCs are consistent with potential sFRP signaling thru serpentine G protein-coupled receptors such as the Fzd receptors. Furthermore, our data demonstrating prevention of sFRP1-stimulated CXCL8 mRNA induction with siRNA-directed inhibition of the non-canonical Fzd2 and Fzd5 are also consistent with a role of sFRP-Fzd receptor interaction in ELR⁺ CXC chemokine genesis. How RoR2 either acting as a co-receptor with non-canonical Fzd receptors or independently fits in to the regulation of ELR⁺ chemokine expression is currently unknown. Since these angiogenic chemokines are expressed during the inflammatory phase of wound healing, these chemokines could contribute to several aspects of bone repair including attraction of additional MSCs to the site for differentiation or attraction of endothelial cells for generation of vascularized granulation tissue and stimulation of angiogenesis as we had previously demonstrated^[17]. Thus, in addition to regulation of Wnt signaling as inhibitory substances, our study adds to a growing body of knowledge on the stimulatory functions of sFRPs. Specifically, a novel function of sFRPs in stimulating angiogenic chemokines can be envisioned that may aid in wound and bone repair.

COMMENTS

Background

Mesenchymal stem cells (MSCs) have the capability to differentiate into several cell types including adipocytes, chondrocytes, and osteoblasts and thus have high potential as treatment for repairing bone defects. This process requires the interaction of various growth factors, chemokines, and signaling pathways resulting in the necessary inflammatory, angiogenic, and osteogenic stages of bone repair.

Research frontiers

Although much attention has been placed on the role of the major angiogenesis proteins (vascular endothelial growth factor and fibroblast growth factors) and the Wnt system in bone repair, not much research has been conducted on the role of the ELR⁺ chemokines in this process. These chemokines also have important functions in inflammation and blood vessel formation and have been shown to be stimulated by non-canonical Wnt signaling and during osteogenic differentiation of MSCs.

Innovations and breakthroughs

In this report, the authors demonstrate that treatment of human MSC (hMSC) with the soluble frizzled-related proteins (sFRPs), which should inhibit both canonical and non-canonical Wnt signaling, actually stimulates the expression of the angiogenic CXC ELR⁺ chemokines CXCL5 and CXCL8. CXC ELR⁺ chemokine stimulation was mediated through the non-canonical frizzled receptors 2 (Fzd2) and Fzd5 Wnt receptors and the RoR2 co-receptor. This adds to the data suggesting non-canonical Wnt control of several bone formation processes through expression of the ELR⁺ chemokines and identifies a potential new role for the sFRPs in coupling ELR⁺ chemokine angiogenesis to bone repair.

Applications

Many recent reports have focused on the use of native or genetically engineered MSCs as a treatment to speed up or enhance the quality and mineralization of bone in wound and bone defect models. The ability of the sFRPs to stimulate ELR⁺ CXC chemokines detailed in this study may suggest another avenue for manipulation of bone formation pathways and may eventually lead to a therapeutic treatment to hasten bone healing and return bone strength back to pre-injury levels.

Terminology

MSCs: Multipotent stromal cells that can be differentiated into several cell types including cartilage (chondrocytes), bone (osteoblasts), fat (adipocytes) and muscle (myocytes); ELR⁺ CXC chemokines: Family of small cytokines secreted by cells and containing a Cys-X-Cys domain. CXC chemokines can be further divided into those with or without a Glu-Leu-Arg (ELR⁺) motif. ELR⁺ CXC chemokines are angiogenic. ELR⁻ CXC chemokines are angiostatic. Wnt signaling: family of signaling molecules (Wnts) and Fzds that are important in many developmental pathways including cell fate, proliferation, and differentiation; sFRPs: Family of proteins that inhibit Wnt signaling by acting as soluble, decoy receptors preventing Wnt binding to Fzds.

Peer-review

The paper found that CXC chemokine expression in hMSC is controlled in part by sFRPs signalling through non-canonical Wnt involving Fzd2/5 and the ERK and PLC pathways. The results are interesting.

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