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META-ANALYSIS

Acute liver failure: A systematic review and network meta-analysis of optimal type of stem cells in animal models

Jun-Feng Ma, Jian-Ping Gao, Zi-Wei Shao

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Abstract

BACKGROUND

The therapeutic effects of various stem cells in acute liver failure (ALF) have been demonstrated in preclinical studies. However, the specific type of stem cells with the highest therapeutic potential has not been determined.

AIM

To validate the efficacy of stem cells in ALF model and to identify the most promising stem cells.

METHODS

A search was conducted on the PubMed, Web of Science, Embase, Scopus, and Cochrane databases from inception to May 3, 2022, and updated on November 16, 2022 to identify relevant studies. Two independent reviewers performed the literature search, identification, screening, quality assessment, and data extraction.

RESULTS

A total of 89 animal studies were included in the analysis. The results of traditional meta-analysis showed that stem cell therapy could significantly reduce the serum levels of alanine aminotransferase [weighted mean difference (WMD) = -181.05 (-191.71, -170.39)], aspartate aminotransferase [WMD = -309.04 (-328.45, -289.63)], tumor necrosis factor-alpha [WMD = -8.75 (-9.93, -7.56)], and interleukin-6 [WMD = -10.43 (-12.11, -8.76)] in animal models of ALF. Further subgroup analysis and network meta-analysis showed that although mesenchymal stem cells are the current research hotspot, the effect of liver stem cells (LSCs) on improving liver function is significantly better than that of the other five types of stem cells. In addition, the ranking results showed that the possibility of LSCs improving liver function ranked first. This fully proves the great therapeutic potential of LSCs, which needs to be paid more attention in the future.

CONCLUSION

LSCs may have a higher therapeutic potential. Further high-quality animal

experiments are needed to explore the most effective stem cells for ALF.

Key Words: Acute liver failure; Stem cells; Animal model; Systematic review; Network meta-analysis

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Core Tip: Determination of stem cells with the highest therapeutic potential in acute liver failure is crucial for future animal and clinical research. In this pioneer study, we conducted traditional meta-analysis and network meta-analysis to explore the efficacy of stem cells from four aspects: Alanine aminotransferase, aspartate aminotransferase, tumor necrosis factor-alpha and interleukin-6. We found that although mesenchymal stem cells are the current research hotspot, liver stem cells may have higher therapeutic potential and should be the focus of future research. In addition, we point out the problems existing and the direction of improvement in the future through the quality evaluation.

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INTRODUCTION

Acute liver failure (ALF) is a clinical syndrome in which a large number of hepatocytes undergo necrosis resulting in severe liver function damage within a short period time. This condition occurs in the absence of chronic liver disease and is associated with severe complications, such as hepatic encephalopathy and even death, with a mortality rate of about 40%-62.2%[1-3]. The etiologies of ALF include heat pain poisoning, liver ischemia, viral hepatitis and autoimmune hepatitis, as well as prescription drugs, herbs and dietary supplements[1]. Currently, patients with ALF mainly receive medical supportive treatment due to the lack of specific therapy. Although liver transplantation is the only proven treatment, it is limited by the unavailability of donor organs, high medical costs, and the need for lifelong immunosuppressive drugs. Therefore, there is a need to develop newer and more effective therapeutic strategies.

Advances in clinical application of stem cells have provided a new perspective for ALF treatment[4]. Stem cells have many advantages, including self-renewal capacities, high proliferative activities, multi-directional differentiation, anti-inflammation, anti-apoptosis, and immunomodulatory activities. They can be obtained from various sources, such as the bone marrow, umbilical cord, adipose tissue, amniotic fluid and embryos among others[5,6]. After transplantation, naive stem cells or differentiated hepatocyte-like cells rapidly restore essential liver functions by replenishing functional hepatocytes and/or by stimulating endogenous liver regeneration processes through paracrine actions. Stem cell transplantation has been shown to increase the cumulative survival rate of ALF patients from 55.6% to 73.2%[7]. Stem cells improve damaged liver functions by increasing the activities of serum albumin, cholinesterase and prothrombin and reducing the levels of serum total bilirubin and alanine aminotransferase (ALT)[8].

Although the significance of stem cells in the treatment of ALF has been demonstrated in preclinical and clinical trials, there are still some controversies and obstacles that need to be resolved. For example, bone marrow mesenchymal stem cells (BMSCs) can be easily obtained without ethical problems and are considered the first-choice for autologous transplantation. However, their therapeutic effects are limited by easy aging and lack of vitality[9]. On the contrary, umbilical cord MSCs (UCMSCs) have become a new research hotspot because of their non-aging, low immunogenicity, and high proliferative activities [10]. Adipose MSCs (ADMSCs) can be prepared by minimally invasive methods, grow faster *in vitro*, and secrete abundant cytokines as well as growth factors related to immune regulation. Dysregulation of ADMSCs in clinical applications is significantly lower than that of embryonic stem cells (ESCs) or induced pluripotent stem cells (IPSCs)[11,12]. Therefore, ADMSCs are good candidates for ALF treatment. The non-invasive stem cell population, menstrual blood-derived stem cells (MenSCs), has been recently studied[13-15]. It has been postulated that MenSCs can express markers for both MSCs and ESCs, and can differentiate into the three germ layers. Fathi-Kazerooni *et al*[16] found that compared with BMSCs, MenSCs significantly reduced serum ALT and aspartate aminotransferase (AST) levels in ALF mice models. Therefore, the therapeutic potential of MenSCs cannot be ignored.

In conclusion, stem cells can improve liver failure. However, it is not clear which kind of stem cells has the best therapeutic effect. Solving this problem will improve the therapeutic effect and accelerate the clinical translation of stem cells. To date, few studies have directly compared the therapeutic effects

of different types of stem cells. In the absence of direct comparisons, systematic reviews and network meta-analyses can be used to perform direct and indirect comparisons between different interventions and estimate the ranking probabilities[17]. Therefore, we intend to compare the therapeutic potential of different types of stem cells through subgroup analysis of traditional meta-analysis and network meta-analysis. Our findings inform on optimal types of stem cells and provide a reference for animal experiments and clinical research.

MATERIALS AND METHODS

Inclusion and Exclusion Criteria

Patients and diseases (P): Animal models of ALF. Interventions (I): Stem cells. Control (C): (1) Positive control: Comparisons between different stem cells; and (2) Negative control: Blank, DMEM, PBS, saline. Outcomes (O): (1) Primary outcomes: ALT and AST levels; and (2) Secondary outcomes: Expression levels of genes or proteins of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Type of studies (S): Controlled studies.

Data sources and searches

Candidate studies were identified *via* searching PubMed, Web of Science, Embase, Scopus, *Reference Citation Analysis*, and Cochrane databases from their inceptions until February 3, 2022, and updated on November 16, 2022. Search terms were: "stem cell" OR "stem cells" OR "stromal cells" OR "stromal cell" OR "mesenchymal cell" OR "mesenchymal cells" OR "cell therapy" OR "cellular therapy" OR "progenitor cell" OR "progenitor cells" OR "cytotherapy" AND "liver failure" OR "hepatic failure". The retrieval processes for each database are shown in Supplementary Table 1.

Literature screening, data extraction, and quality assessment

Two independent reviewers performed the literature search, identification, screening, quality assessment and data extraction. Literature screening was based on the inclusion and exclusion criteria, and data extraction was based the pre-established information (including basic information, outcome indicators, and key elements of quality evaluation) shown in Supplementary Table 2. Based on SYRCLE's risk of bias tool for animal studies[18], 10 items in 6 aspects including selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases were used to evaluate the quality of included studies.

Statistical analysis

Traditional meta-analysis was performed using the Stata16.0 software. Odds ratios were used for count data, while weighted mean differences (WMD) rather than standardized mean differences (SMD) were used as effect indicators for measured data. In fact, when performing meta-analysis, WMD eliminates the influence of the absolute value on the results, so that the original weight and measure can truly reflect the experimental effect, and it is easy to understand when applied. However, SMD not only eliminates the influence of the absolute value, but also eliminates the influence of weights and measures on the results, which makes the results difficult to interpret and requires caution when interpreting the results. Therefore, it is a better practice to choose WMD in our study. Confidence intervals were also reported. Heterogeneity was analyzed by the χ^2 test while I^2 was used to determine the degree of heterogeneity. If the findings were not statistically different, the fixed effects model was used for meta-analysis. Conversely, the source of heterogeneity was further analyzed, and the random-effects model used for meta-analysis. Reliability of the traditional meta-analysis results were determined *via* sensitivity analyses.

Network meta-analysis was performed using the Winbugs1.4.3 software. The evidence network diagram of the comparisons for each treatment was drawn to show the current research status in the field. Then, a comparison-correction funnel chart was established to evaluate the possibility of publication bias and small sample effects in the included studies. The Bayesian network meta-analysis was performed using the Markov Chain Monte Carlo method, and ranking probability for each intervention calculated[19]. In addition, potential false positive factor (PSRF) was used to evaluate the convergence of the results. A PSRF value is between 1-1.05 indicated complete convergence, good model stability, and reliable analysis[20].

Traditional meta-analysis was performed by subgroups to explore the therapeutic effects of different types of stem cells. Only ADMSCs, amniotic fluid MSCs (AFMSCs), BMSCs, liver stem cells (LSCs), MenSCs, and UCMSCs were subjected to the network meta-analysis because studies using these six stem cells reported all the primary and secondary outcomes. In this case, the results of network meta-analysis based on different outcome indicators are comparable.

RESULTS

Literature search results

A total of 5436 articles were obtained from the preliminary search. After excluding repetitive articles and those that did not meet the inclusion criteria, 89 articles were finally included (Figure 1).

Basic information of the included studies

Among the 89 studies, there were 74 randomized controlled trials and 15 controlled studies. The animal species involved in the studies included Sprague-Dawley rats (22 studies), Wistar rats (6 studies), Albino rats (2 study), BALB/c mice (16 studies), C57BL/6 mice (29 studies), ICR mice (3 studies), NOD-SCID mice (2 studies), Swiss albino mice (1 study), dogs (1 study), pigs (6 studies), and F344-Fischer rats (1 study). In terms of gender, 66 studies included male animals, 10 studies included female animals while 13 studies did not report the gender of the animals. The animals weighed between 18 g-18 kg, were aged between 4 wk and 1.5 years, and the sample sizes were between 6-94. Modeling methods for ALF included partial hepatectomy (7 studies), carbon tetrachloride (25 studies), Concanavalin A (6 studies), D-galactosamine (D-GalN, 10 studies), D-GalN/lipopolysaccharide (27 studies), acetaminophen (9 studies), thioacetamide (4 studies), and ischemia-reperfusion injury (1 study). The types of stem cells that were assessed included ADMSCs (21 studies), UCMSCs (21 studies), AFMSCs (2 studies), BMSCs (39 studies), ESCs (1 study), IPSCs (2 studies), LSCs (2 studies), MenSCs (4 studies), and placental stem cells (PMSCs, 1 study). The transplantation routes for the stem cells included intraperitoneal injection (4 studies), liver parenchyma (3 studies), portal vein (6 studies), splenic vein (2 studies), tail vein (71 studies) and stent loading (3 studies). The transplantation timing for the stem cells was within 4 d after modeling, while the transplantation dose was between (1×10^5)-(1×10^8). The negative control groups were treated with normal saline (32 studies), PBS (35 studies), Blank (16 studies) and DMEM (6 studies). The basic information of the included studies is shown in Supplementary Table 2.

Risk of bias assessment results

Although there were 74 randomized controlled trials among the included studies, there were no reports on specific randomized grouping methods or whether covert grouping was implemented. Eighty-one studies reported that the baseline characteristics of experimental animals, such as age, sex and weight were balanced. Stem cell transplantation before modeling was performed in 4 studies. Seventy-eight studies reported randomized placement of animals during the experiments. Due to limited information provided by the included studies, it was difficult to determine whether animal breeders and/or researchers had been blinded. Only eight studies reported randomly selecting animals in measurement of the results. Thirty-one studies had blinded outcome evaluators. There was no study that reported on the loss or follow-up of animals. Although protocols were not available for all studies, all expected results were clearly reported. Findings from the risk of bias assessment are shown in Figure 2.

Meta-analysis results

Serum ALT levels: A total of 78 studies reported on serum ALT levels. Meta-analysis of the random effects model showed that ALT levels in the stem cell group were significantly suppressed, relative to those of the negative control group [WMD = -181.05 (-191.71, -170.39)]. Subgroup analysis revealed that the trend of improving liver functions was: IPSCs, LSCs, UCMSCs, ADMSCs, PMSCs, MenSCs, BMSCs. Notably, the treatment effects of AFMSCs and ESCs were not markedly different from those of the control group (Supplementary Figure 1). The evidence network diagram showed that BMSCs and ADMSCs are the current research hotspots (Figure 3A). Network meta-analysis revealed that the effects of LSCs in reducing ALT levels were significantly better than those of the other five stem cells (Table 1). The asymmetrical comparative correction funnel chart suggested that there was publication bias and small sample effects (Figure 4A). The ranking results showed that LSCs had the best effects in reducing ALT levels (Figure 5A).

Serum AST levels: Seventy-one studies reported serum AST levels. The meta-analysis of the random effects model showed that serum AST levels in the stem cell group were significantly lower than those of the negative control group [WMD = -309.04 (-328.45, -289.63)]. Subgroup analysis showed that the trend for effects of improving liver functions was: LSCs, IPSCs, UCMSCs, AFMSCs, ADMSCs, MenSCs, BMSCs (Supplementary Figure 2). The evidence network diagram showed that BMSCs and ADMSCs are the current research hotspots (Figure 3B). Network meta-analysis showed that the effects of LSCs in reducing AST levels were significantly better than those of the other five stem cells (Table 1). The asymmetrical comparative correction funnel chart revealed the possibility of publication bias and small sample effects (Figure 4B). The rank ranking results showed that LSCs have the best effects in reducing AST levels (Figure 5B).

Serum TNF-α levels: Twenty-six studies reported the expression of TNF-α at gene or protein levels. The meta-analysis of random effects model showed that TNF-α levels in the stem cell group were significantly suppressed, relative to those of the negative control group [WMD = -8.75 (-9.93, -7.56)]. Subgroup analysis showed that the reduction trend in TNF-α levels was UCMSCs, LSCs, ADMSCs,

Table 1 League table of network meta-analysis estimations

Interventions	Outcomes			
	ALT	AST	TNF-α	IL-6
ADMSCs *vs* AFMSCs	-564.66 (-2299.61, 1244.94)	333.03 (-3589.84, 4179.61)	-75.75 (-288.40, 135.39)	-83.75 (-240.12, 67.73)
ADMSCs *vs* BMSCs	-128.07 (-785.76, 520.77)	360.87 (-715.94, 1438.35)	-63.71 (-161.64, 37.04)	-72.42 (-155.32, 11.73)
ADMSCs *vs* LSCs	5933.08 (3909.44, 7997.17)[1]	9937.57 (6697.32, 13196.61)[1]	-55.42 (-215.17, 101.23)	64.61 (-91.38, 208.00)
ADMSCs *vs* MenSCs	-469.19 (-1740.82, 814.19)	45.27 (-2014.75, 2083.32)	-74.89 (-284.96, 141.77)	-86.05 (-240.23, 59.90)
ADMSCs *vs* Placebo	-667.52 (-1203.62, -120.99)[1]	-606.72 (-1506.56, 287.46)	-77.20 (-152.74, -1.88)[1]	-89.51 (-156.08, -28.79)[1]
ADMSCs *vs* UCMSCs	795.81 (46.64, 1560.45)[1]	1050.35 (-188.25, 2288.34)	37.30 (-72.01, 147.61)	-55.68 (-148.03, 34.58)
AFMSCs *vs* BMSCs	436.30 (-1349.95, 2166.93)	39.47 (-3802.42, 3952.36)	12.93 (-194.78, 220.48)	11.84 (-137.80, 164.77)
AFMSCs *vs* LSCs	6488.58 (3913.57, 9087.80)[1]	9604.11 (4643.34, 14441.26)[1]	18.65 (-224.35, 261.70)	148.58 (-49.07, 342.37)
AFMSCs *vs* MenSCs	82.07 (-1917.03, 2135.48)	-286.68 (-4498.58, 3913.27)	2.59 (-277.20, 281.02)	-2.79 (-200.21, 193.85)
AFMSCs *vs* Placebo	-114.65 (-1822.82, 1576.48)	-928.62 (-4714.44, 2892.00)	-1.11 (-201.89, 197.43)	-6.71 (-143.71, 133.84)
AFMSCs *vs* UCMSCs	1352.71 (-432.10, 3142.15)	724.02 (-3142.84, 4626.69)	114.19 (-99.91, 324.03)	28.52 (-122.68, 187.29)
BMSCs *vs* LSCs	6070.03 (4071.59, 8102.74)[1]	9576.33 (6336.42, 12727.97)[1]	7.85 (-144.90, 158.13)	136.42 (-15.99, 284.09)
BMSCs *vs* MenSCs	-341.05 (-1545.56, 858.89)	-322.93 (-2219.61, 1608.47)	-10.36 (-218.54, 200.58)	-13.77 (-163.34, 128.40)
BMSCs *vs* Placebo	-533.83 (-957.97, -124.80)[1]	-968.24 (-1654.55, -302.11)[1]	-13.45 (-79.67, 51.73)	-16.82 (-77.28, 33.66)
BMSCs *vs* UCMSCs	924.27 (248.03, 1604.46)[1]	693.88 (-410.09, 1801.40)	101.07 (-3.29, 205.02)	16.77 (-68.35, 99.68)
LSCs *vs* MenSCs	-6409.29 (-8678.76, -4138.55)[1]	-9871.16 (-13492.79, -6276.98)[1]	-19.14 (-258.98, 224.58)	-151.36 (-340.66, 43.49)
LSCs *vs* Placebo	-6600.26 (-8583.58, -4625.55)[1]	-10542.45 (-13603.53, -7407.19)[1]	-20.86 (-158.68, 119.97)	-154.33 (-290.19, -15.83)[1]
LSCs *vs* UCMSCs	-5138.64 (-7210.04, -3092.11)[1]	-8886.10 (-12104.64, -5633.36)[1]	93.80 (-66.86, 257.57)	-119.18 (-268.00, 37.35)
MenSCs *vs* Placebo	-204.31 (-1344.63, 944.68)	-639.98 (-2497.51, 1193.49)	-3.78 (-207.01, 193.79)	-3.14 (-138.85, 129.45)
MenSCs *vs* UCMSCs	1270.03 (-1.67, 2541.86)	1019.16 (-1023.95, 3022.83)	111.17 (-106.96, 327.48)	30.02 (-116.58, 179.83)
Placebo *vs* UCMSCs	1461.02 (918.17, 2019.69)[1]	1657.56 (793.12, 2525.40)[1]	114.68 (33.79, 197.01)[1]	33.87 (-27.82, 100.16)

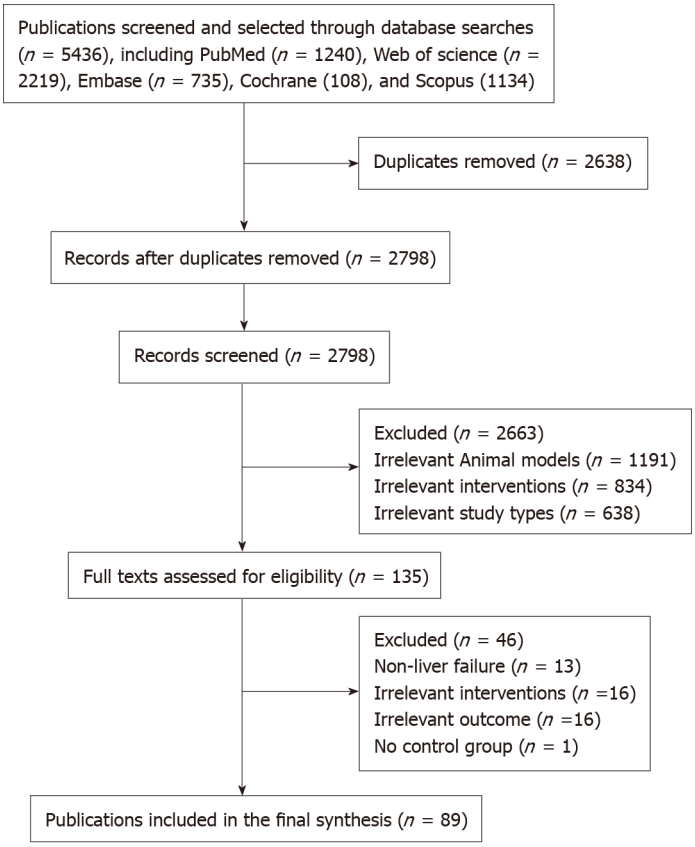
[1]Indicated statistical difference.

BMSCs: Bone marrow mesenchymal stem cells; LSCs: Liver stem cells; MenSCs: Menstrual blood-derived stem cells; AFMSCs: Amniotic fluid mesenchymal stem cells; ADMSCs: Adipose mesenchymal stem cells; UCMSCs: Umbilical cord mesenchymal stem cells; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.

MenSCs, BMSCs. The treatment effects of AFMSCs were not significantly different from those of the negative control group (Supplementary Figure 3). The evidence network diagram showed that BMSCs and ADMSCs are the current research hotspots (Figure 3C). Network meta-analysis showed that differences in the effects of all stem cells on reducing TNF-α levels were insignificant (Table 1). The asymmetrical comparative correction funnel chart suggested the existence of publication bias and small sample effects (Figure 4C). The rank ranking results showed that LSCs was most effective in reducing TNF-α levels (Figure 5C).

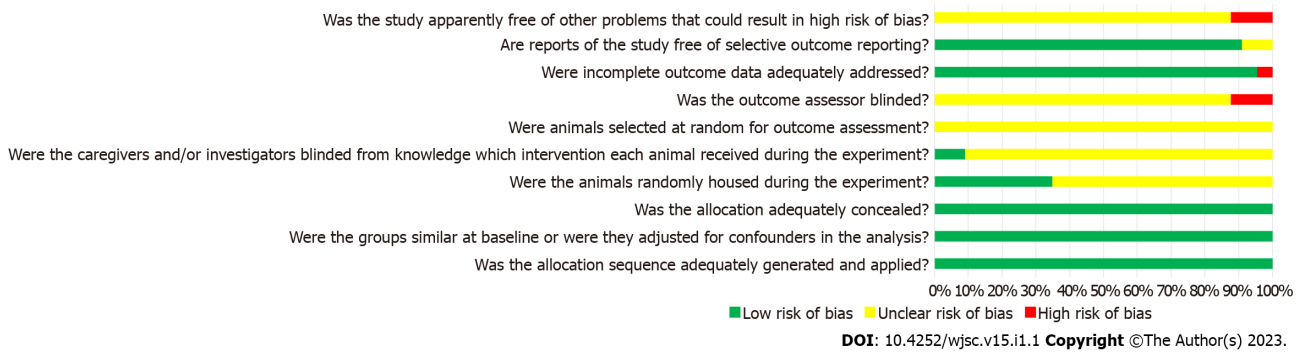
Serum IL-6 levels: A total of 20 studies reported the expression of IL-6 at gene or proteins levels. A meta-analysis of the random effects model showed that that IL-6 levels in the stem cell group were significantly lower than those of the negative control group [WMD = -10.43 (-12.11, -8.76)]. Subgroup analysis showed that the trend in reduction of IL-6 was LSCs, ADMSCs, UCMSCs, BMSCs, MenSCs. The treatment effects of AFMSCs were not markedly different from those of the negative control group (Supplementary Figure 4). The evidence network diagram showed that BMSCs and ADMSCs are the current research hotspots (Figure 3D). Network meta-analysis did not reveal significant differences in the effects of all stem cells in reducing IL-6 levels (Table 1). The asymmetrical comparative correction funnel chart showed the possibility of publication bias and small sample effects (Figure 4D). The rank ranking results revealed that LSCs have the best effects in reducing IL-6 levels (Figure 5D).

Reliability test of meta-analysis results: Sensitivity analysis was performed to test the reliability of the results from the traditional meta-analysis. The four outcome indicators showed that after one by one exclusion of certain studies, directions of the confidence intervals of the combined results of the



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Figure 1 Flow diagram for study selection. A total of 5436 articles were obtained from 5 databases. After removing repetitive articles and those that did not meet the inclusion criteria by reading the title, abstract, and full text, 89 articles were finally included.



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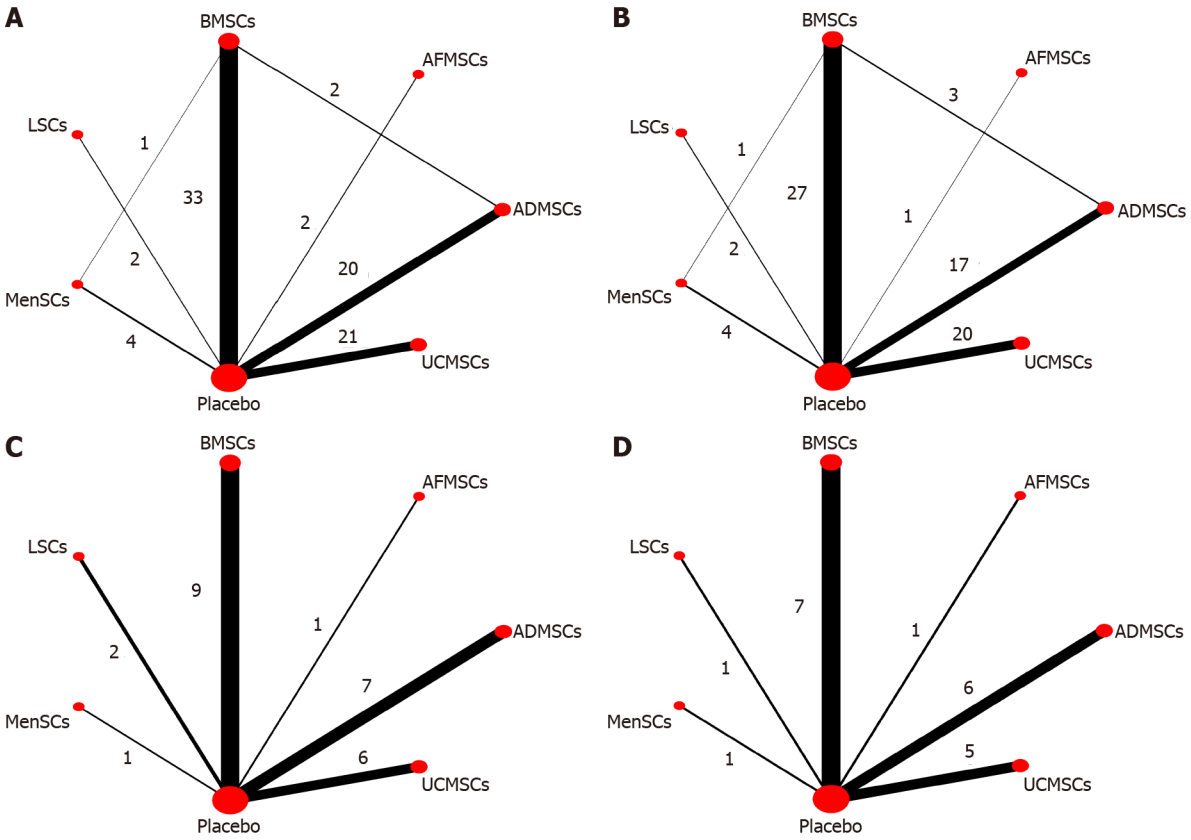
Figure 2 Risk of bias assessment results. 10 items in 6 aspects including selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases were used to evaluate the quality of included studies. The risk of bias of all studies about a certain item is expressed as a percentage.

remaining studies did not change, indicating that findings from the meta-analysis were robust and reliable (Figure 6).

Then, PSRF was used to evaluate the fitting effects of the model, after which the reliability of the network meta-analysis results was determined. The four outcome indicators showed that PSRF converged to 1 after a certain number of iterations, indicating that robustness and reliability of the network meta-analysis results were good (Figure 7).

DISCUSSION

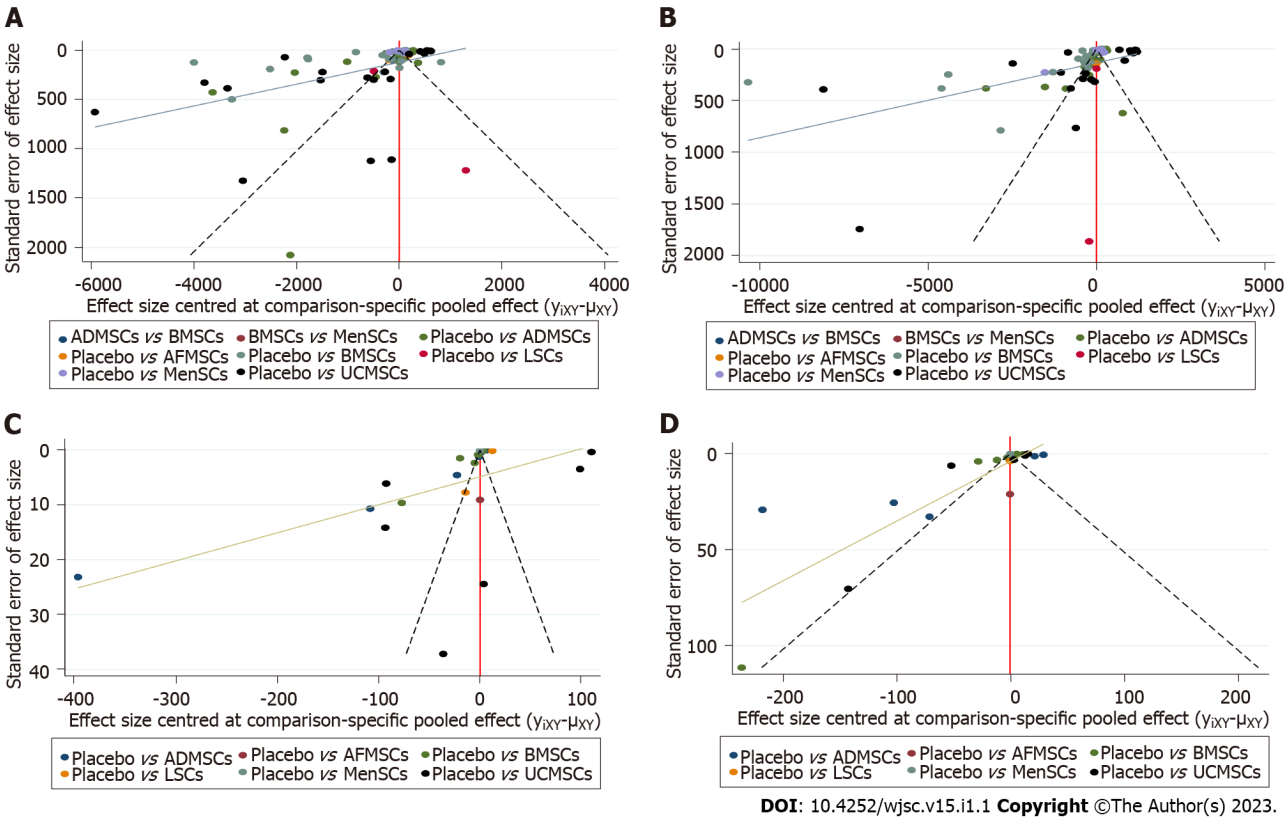
The mechanism for occurrence of ALF begins with hepatocyte necrosis[21], followed by damage to the membrane structure of hepatocytes and the release of AST and glutamic pyruvic transaminase (ALT)



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Figure 3 Evidence network diagram. Circle size represents sample size involved, thickness of the line segment represents the number of studies involving both interventions. A: Alanine aminotransferase; B: Aspartate aminotransferase; C: Tumor necrosis factor-α; D: Interleukin-6. BMSCs: Bone marrow mesenchymal stem cells; LSCs: Liver stem cells; MenSCs: Menstrual blood-derived stem cells; AFMSCs: Amniotic fluid mesenchymal stem cells; ADMSCs: Adipose mesenchymal stem cells; UCMSCs: Umbilical cord mesenchymal stem cells.

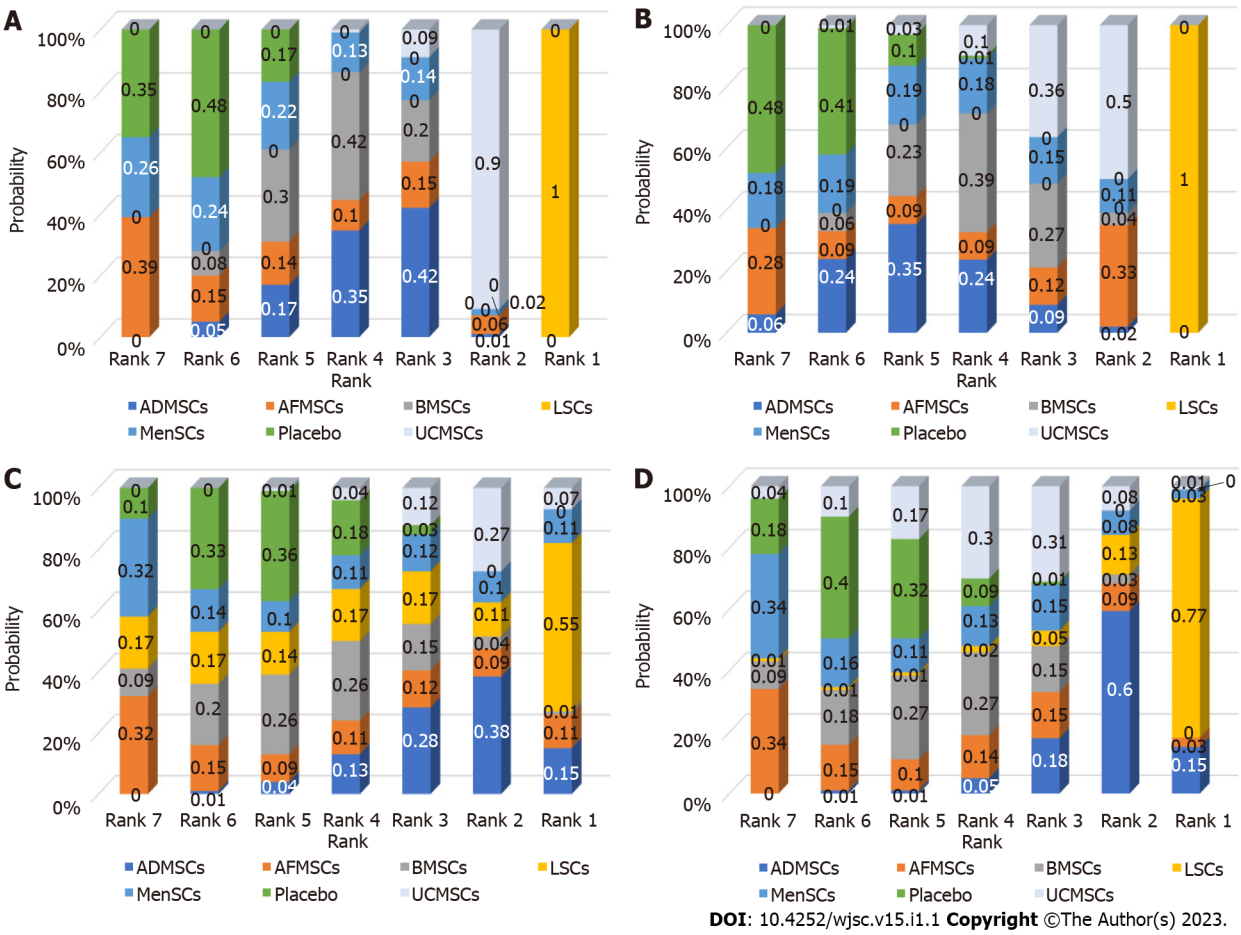
from hepatocytes into the bloodstream. Therefore, the serum levels of AST and ALT are the most reliable markers of liver injury[22]. Stem cells can protect the damaged liver by promoting hepatocyte regeneration and inhibiting hepatocyte apoptosis[23]. Consistent with findings from current animal studies, our meta-analysis showed that different types of stem cell transplantation can significantly suppress serum ALT and AST levels. This indicates the great therapeutic potential of stem cells in ALF. By confirming that stem cells can improve liver functions, there is a need to elucidate on their therapeutic potential to accelerate their clinical translation. About 10 different types of stem cells have been studied in ALF, however, a limited number of studies have compared the therapeutic effects of different types of stem cells. We found that only four studies compared the therapeutic potentials of different types of stem cells in ALF. Among them, three studies compared ADMSCs with BMSCs. They showed that the effects of the two kinds of stem cells on improving liver functions were comparable[24-26]. In another study, MenSCs were found to be significantly better than BMSCs in reducing ALT and AST levels[16]. Studies have reported that MSCs have the advantages of easy access, multipotential, anti-apoptosis, immunosuppression, and paracrine characteristics[27]. Therefore, MSCs are the most studied stem cells in the field of ALF. This is consistent with results shown in the evidence map of our network meta-analysis, that is, MSCs derived from the bone marrow, fat and umbilical cord are the current research focus. However, further subgroup analysis and network meta-analysis showed that they are not the best in improving liver functions. Besides, LSCs are more effective in reducing serum ALT and AST levels. LSCs are MSC-like cells in the liver. In addition to having the same surface markers as MSCs, such as CD29, CD73, CD44, CD105 and CD146, they also express specific hepatocyte markers (albumin, cytokeratin8, and cytokeratin-18) and embryonic markers (Nanog, Oct3/4, Sox2, Musashi, SSEA4, and Pax2)[28,29]. Studies have shown that LSCs improve liver functions by directly differentiating into hepatocytes and secreting cytokines that inhibit injury progression[29]. A possible reason why LSCs showed a better ability to repair liver functions is their greater ability to migrate to the damaged liver. For example, after intravenous transplantation, LSCs preferentially aggregate and play a role in the damaged liver, while MSCs are mainly trapped in the lungs and do not migrate to the damaged liver[29,30]. In addition, human LSCs do not express human leukocyte antigen II molecules and costimulatory molecules (CD40, CD80 and CD86), which reduces the severity of immune responses [31].



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Figure 4 The comparison-correction funnel plot. The dotted lines on both sides of the funnel plot represent 95% confidence intervals, and each point represents a study. If each point falls within the dotted line and is symmetrically distributed on both sides of the vertical line, there is no publication bias. On the contrary, it indicates the possibility of publication bias. A: Alanine aminotransferase; B: Aspartate aminotransferase; C: Tumor necrosis factor-α; D: Interleukin-6. BMSCs: Bone marrow mesenchymal stem cells; LSCs: Liver stem cells; MenSCs: Menstrual blood-derived stem cells; AFMSCs: Amniotic fluid mesenchymal stem cells; ADMSCs: Adipose mesenchymal stem cells; UCMSCs: Umbilical cord mesenchymal stem cells.

The most common causes of death in ALF patients is systemic complications with release of proinflammatory cytokines and impairment of associated molecular patterns of the necrotic hepatocytes, endothelial cells, and leukocytes[32,33]. Regardless of the cause, uncontrolled inflammation is crucial in ALF occurrence and development[34]. Reduction of pro-inflammatory cytokines and hepatocyte dysfunction is key in ALF treatment. Hepatocyte apoptosis is associated with excess activations of immune responses, usually mediated by TNF-α and IL-6[35]. TNF-α is a pro-inflammatory factor that regulates the expressions of pro-inflammatory genes, tissue factors and leukocyte antigen class I[36,37]. The TNF-α interacts with its receptor (TNF receptor I) to activate signal transduction pathways[38]. IL-6 is produced by Th2 cells, monocytes, and vascular endothelial cells, and is one of the core cytokines involved in liver injury development[39]. Excess secretion of IL-6 delays cell cycle progression and inhibits hepatocyte regeneration[40]. Therefore, plasma levels of TNF-α and IL-6 in ALF patients were significantly increased. Although previous studies have confirmed that MSCs from human cord blood can differentiate into hepatocyte-like cells *in vivo* and partially repair D-galactose/lipopolysaccharide-induced liver injury in mice models[41]. Menstrual fluid-derived stem cells showed the potential to restore liver functions in mice that had been subjected to 2/3 partial hepatectomy, and menstrual stem cell-derived hepatocyte-like cells were detected in the recipient liver [42]. However, transplanted stem cells are less likely to differentiate into functional hepatocytes and regenerate liver tissues within a short period of time. In early stages, stem cells protect against ALF *via* exerting their immunomodulatory and anti-inflammatory properties[43]. Consistent with the results of animal experiments, our meta-analysis found that stem cell transplantation significantly reduces TNF-α and IL-6 levels. In addition, consistent with ALT and AST levels, LSCs are the most potential stem cells for reducing serum TNF-α and IL-6 levels. The consistent results from four different outcome indicators prove that LSCs have the greatest therapeutic potential in ALF treatment. Our results are also in line with current research evidence, which has confirmed that serum IL-6 levels are positively correlated with ALT and AST levels. However, studies on LSCs are few, and the mechanisms by which they exert their repair effects have yet to be conclusively determined. There is also a lack of evidence for direct comparisons of LSCs with other stem cell types. Therefore, studies should elucidate on the roles of LSCs. In addition, due to differences in outcome reporting, AFMSCs, ESCs, and IPSCs were not analyzed in our network meta-analysis. This does not mean that other types of stem cells have limited therapeutic potential. On the contrary, studies should also focus on therapeutic effects of different types



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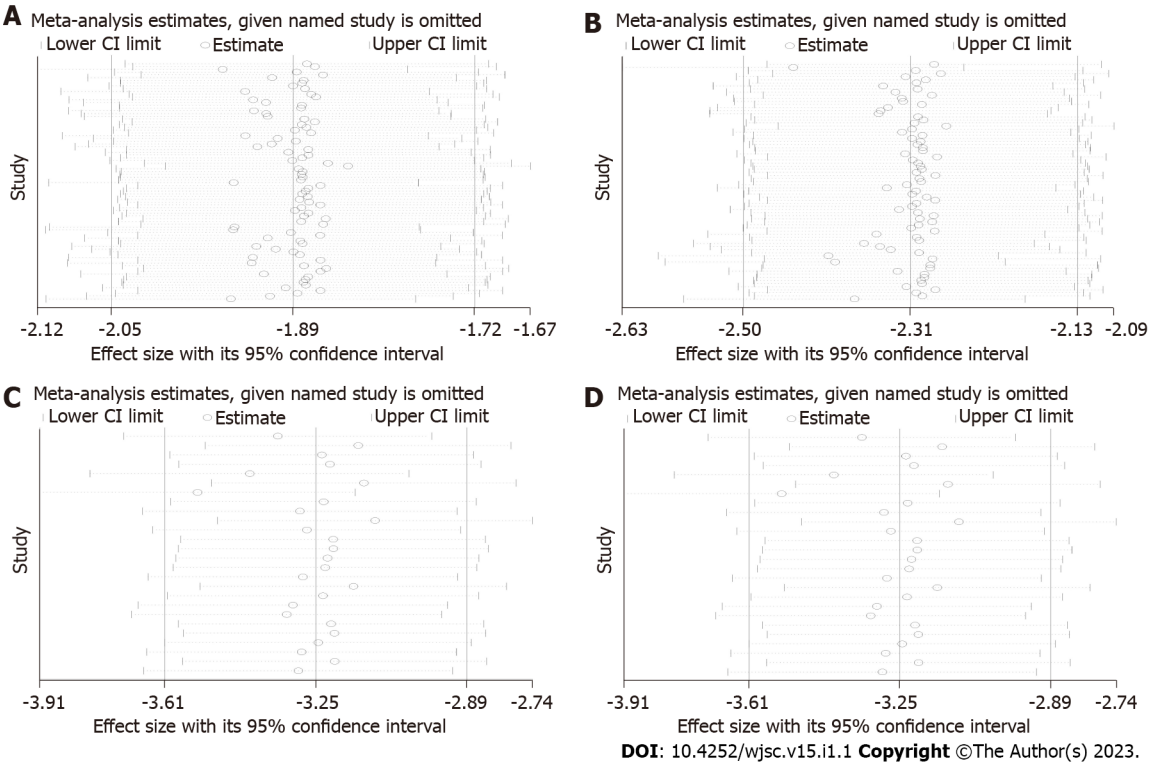
Figure 5 Ranking results. The higher the probability of being ranked first, the better the treatment effect of that intervention. A: Alanine aminotransferase; B: Aspartate aminotransferase; C: Tumor necrosis factor-α; D: Interleukin-6. BMSCs: Bone marrow mesenchymal stem cells; LSCs: Liver stem cells; MenSCs: Menstrual blood-derived stem cells; AFMSCs: Amniotic fluid mesenchymal stem cells; ADMSCs: Adipose mesenchymal stem cells; UCMSCs: Umbilical cord mesenchymal stem cells.

of stem cells, carefully compare their advantages and disadvantages, and construct stem cells with more potential through genetic modification and other means.

Evidence quality

Considerable heterogeneity among the included studies: The included studies involved 11 different animal species, 8 modeling methods, and there were wide variations in baseline characteristics of animals, such as body weight and age. In addition, there were differences in stem cell types, transplantation doses and routes, as well as in measurement and reporting of outcome indicators (such as protein levels, gene levels, *etc.*), resulting in greater heterogeneity among the included studies, which reduced the reliability of meta-analysis results. There is a need to standardize ALF animal models, experimental implementation and outcome measurements to improve the authenticity of experimental results and the reliability of systematic review results.

Insufficient internal authenticity of animal experiments: (1) Selection bias: Randomization and covert grouping of animals while ensuring the balance of their baseline characteristics is an important measure to avoid selection bias[18]. Although about 90% of the studies were randomized controlled trials and the baseline characteristics of the animals were balanced, no study reported the specific randomization method and whether they implemented concealed grouping, resulting in a certain selection bias; (2) Performance bias: Placement of animals, such as different light and temperature conditions, has an important impact on experimental results[18]. Although 87.64% of the studies randomized the placement of animals, no study had blinded animal breeders and researchers, resulting in a certain implementation bias; (3) Detection bias: Failure to blind the outcome assessors in animal experiments may lead to exaggeration of effects and produce false positive results[44,45]. Only 34.83% of the studies reported blinding the outcome assessors, and no study reported a specific blinding process. Therefore, studies should pay more attention to applications of blinding in experimental designs, and should provide more experimental details to improve the reporting quality; (4) Reporting bias: Selective reporting of results can lead to publication bias, which affects the reliability of experimental results[46].



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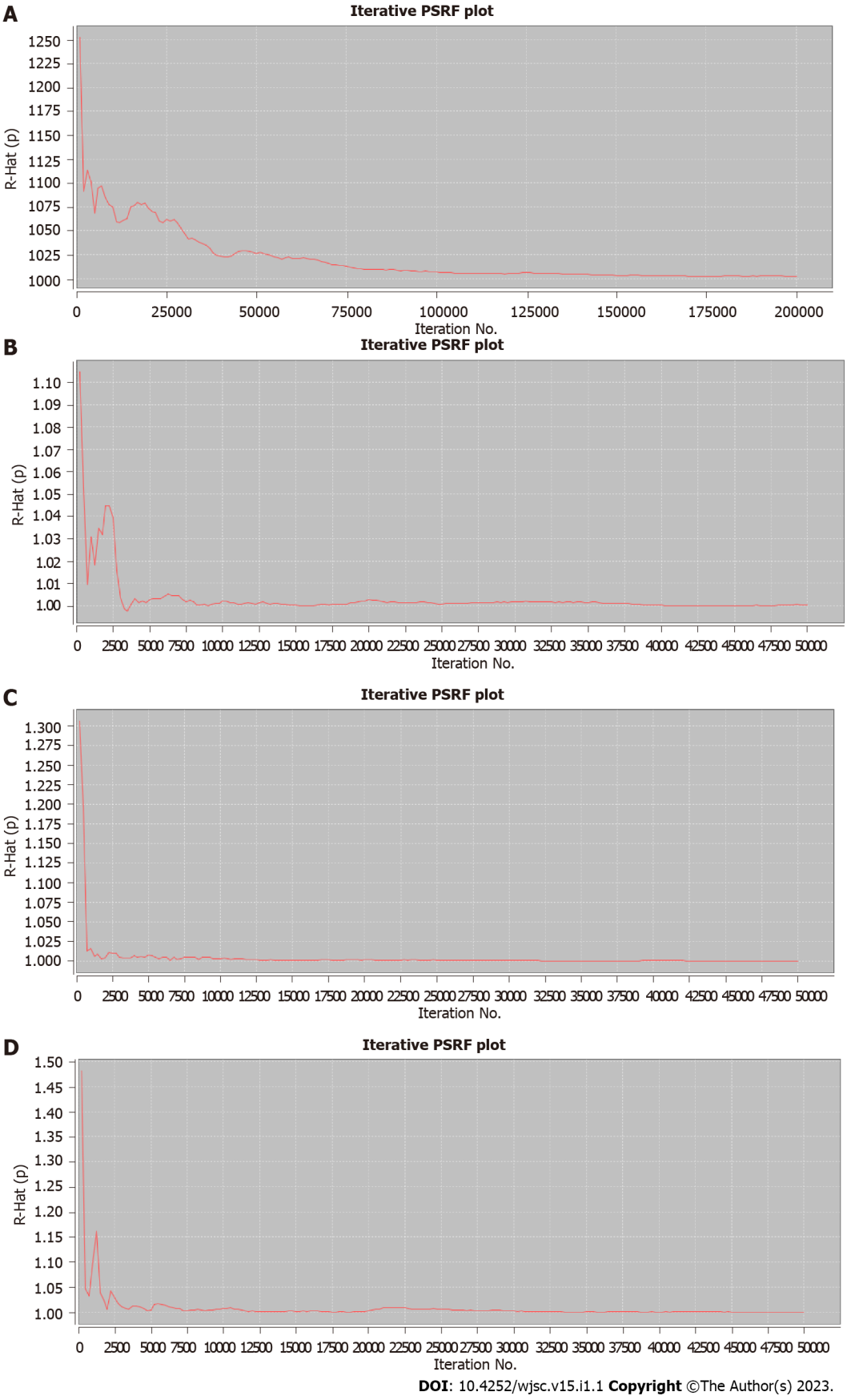
Figure 6 Results of sensitivity analysis. After excluding a certain study, the remaining studies were combined for analysis to see if there were any changes in the effect sizes. If the results of the remaining studies were basically the same as the previous total combined results (the range of the confidence interval did not change significantly), the results of the meta-analysis were considered to be reliable. Conversely, the results of the meta-analysis were considered unreliable. A: Alanine aminotransferase; B: Aspartate aminotransferase; C: Tumor necrosis factor-α; D: Interleukin-6. CI: Confidence interval.

Moreover, protocols were not available for all studies. Therefore, we suggest that animal experimental researchers prospectively register protocols to improve transparency; and (5) Publication bias: Negative results from animal experiments are difficult to publish[47]. If negative results are not included in systematic reviews, the effects of intervention will be overestimated. The corrected funnel chart revealed a certain publication bias in this field.

Limited external authenticity of included studies: External authenticity in animal experiments refers to reproducibility of results and feasibility of their translation to clinical research[48]. The lack of external authenticity in this study is reflected in the following aspects: (1) Although this study has preliminarily determined the best type of stem cells, the routes, dose, and timing of stem cell transplantation are also key factors for achieving desirable outcomes[49,50]. Until these experimental conditions are clearly explored, clinical translation of stem cells will remain immature; (2) In animal experiments, stem cells are often transplanted in early stages. However, clinical practice must be to recruit patients a few days or weeks after ALF; (3) Clinically, factors such as medical histories and physical conditions of patients may affect the efficacies of stem cells. For example, aging and diabetes can lead to impaired stem cell proliferation, decreased angiogenesis, and reduced wound healing. Animal experiments are difficult to simulate[51]; (4) The clinically used stem cells are all human stem cells, but in animal experiments, stem cells are obtained from various sources, which may lead to strong immune responses, affecting the authenticity of animal experimental results[50]; and (5) Stem cells have a potential risk of developing tumors because of their multi-directional differentiation ability, but no research has focused on the potential side effects of stem cells[49]. Therefore, studies should pay attention to effectiveness and safety of stem cell therapy.

Strengths and limitations

Strengths of this study: (1) As the first study in the ALF field, we systematically evaluated and analyzed the effects of different types of stem cells by subgroups, and pointed out the challenges and directions for improvement; (2) After testing the robustness and reliability of the results of traditional meta-analysis and network meta-analysis, we found that our results are very reliable; (3) In the absence of evidence for direct comparisons, the therapeutic potential of different stem cells was comprehensively compared through subgroup analyses of traditional meta-analyses and network meta-analyses; and (4) Based on the SYRCLE bias risk assessment tool, the internal authenticity of animal experiments was evaluated. At the same time, external authenticity was also evaluated.



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Figure 7 Results of potential false positive factor. After a certain number of iterations, the curve finally approaches 1, which indicates that the results of the network meta-analysis have good robustness and reliability. A: Alanine aminotransferase; B: Aspartate aminotransferase; C: Tumor necrosis factor-α; D: Interleukin-6. PSRF: Potential false positive factor.

Limitations of this study: (1) Due to the small number of studies and the large differences in treatment strategies of stem cells in different studies, subgroup analysis of transplantation doses and route of stem cell administration were not performed; (2) Searches were only performed in English databases, which may have led to a certain linguistic bias; and (3) Failure to search grey literature and conference abstracts may have led to a publication bias.

CONCLUSION

This analysis showed that stem cell therapy can significantly reduce ALT, AST, TNF-α and IL-6 levels in ALF animal models through a comprehensive analysis of 89 studies. Importantly, although MSCs are the current research hotspot, we found that LSCs have superior therapeutic effects in ALF. However, because of the low quality of evidence from internal and external authenticity of animal studies, future high-quality animal studies should aim at identifying the best stem cells for therapeutic purposes.

ARTICLE HIGHLIGHTS

Research background

The therapeutic effects of various stem cells in acute liver failure (ALF) have been demonstrated in preclinical studies, however, it has not been determined which stem cells have the best therapeutic implications.

Research motivation

The efficacy of stem cells in ALF has been demonstrated in preclinical and clinical trials. However, it remains unclear which stem cells have the most therapeutic potential. Addressing this issue is critical to improve the efficacy of stem cells and to accelerate the progress of basic and clinical research of stem cells.

Research objectives

To explore the best type of stem cells for ALF treatment and to promote the clinical translation of stem cell therapy.

Research methods

A systematic review and meta-analysis of stem cell therapy for ALF. A search was conducted on the PubMed, Web of Science, Embase, Scopus, and Cochrane databases. Two independent reviewers performed the literature search, identification, screening, quality assessment, and data extraction. The data was analyzed by STATA 16 and Winbugs1.4.3 software.

Research results

Stem cell therapy can significantly reduce serum levels of alanine aminotransferase, aspartate aminotransferase, tumor necrosis factor-alpha, and interleukin-6 in animals with ALF. Although mesenchymal stem cells are the current research focus, among the six types of stem cells included in the analysis, liver stem cells (LSCs) have the greatest therapeutic potential.

Research conclusions

LSCs have the best effects in treating ALF.

Research perspectives

In ALF treatment, stem cell therapy, especially LSCs should be paid more attention to. In addition, to improve on the quality of research, future animal studies should be carefully designed and reported.

FOOTNOTES

Author contributions: Ma JF undertook the design, guidance and writing of the paper; Gao JP and Shao ZW completed the literature retrieval, information extraction and data analysis.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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AIMS AND SCOPE

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REVIEW

Mesenchymal stem cells in ischemic tissue regeneration

Renata Szydlak

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Abstract

Diseases caused by ischemia are one of the leading causes of death in the world. Current therapies for treating acute myocardial infarction, ischemic stroke, and critical limb ischemia do not complete recovery. Regenerative therapies opens new therapeutic strategy in the treatment of ischemic disorders. Mesenchymal stem cells (MSCs) are the most promising option in the field of cell-based therapies, due to their secretory and immunomodulatory abilities, that contribute to ease inflammation and promote the regeneration of damaged tissues. This review presents the current knowledge of the mechanisms of action of MSCs and their therapeutic effects in the treatment of ischemic diseases, described on the basis of data from *in vitro* experiments and preclinical animal studies, and also summarize the effects of using these cells in clinical trial settings. Since the obtained therapeutic benefits are not always satisfactory, approaches aimed at enhancing the effect of MSCs in regenerative therapies are presented at the end.

Key Words: Ischemia; Mesenchymal stem cells; Regenerative medicine; Stem cell therapy; Clinical applications

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Core Tip: Mesenchymal stem cell (MSC) transplantation is an innovative therapy with positive therapeutic effects for many ischemic diseases. Ischemia of an area is defined as insufficient blood supply to specific tissues and various organs or individual parts of the body. The leading cause of tissue ischemia is the narrowing or blockage of the lumen of an artery, most often due to the formation of atherosclerotic plaques, thrombus, or spasms of a specific artery. Here, the potential therapeutic mechanisms of MSCs in ischemic diseases were discussed, along with examples of preclinical and clinical studies.

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INTRODUCTION

Ischemia of an area is defined as insufficient blood supply to specific tissues and various organs or individual parts of the body (*e.g.*, limbs). Reduced blood perfusion causes inadequate transport of oxygen and nutrients to tissue-resident cells, leading to ischemia and ultimately damaging tissues and organs. The leading cause of tissue ischemia is the narrowing or blockage of the lumen of an artery, most often due to the formation of atherosclerotic plaques, thrombus, or spasms of a specific artery[1]. Disruption of blood flow to particular organs or parts of the body may be chronic developing over several months or years or acute occurring suddenly (*e.g.*, during exercise) and usually takes a more rapid course often with a worse prognosis. Ischemia resulting from atherosclerotic lesions is most commonly found in the heart muscle, lower extremities, kidney, and brain[1-4]. Examples of ischemic diseases caused by the narrowing of the coronary and cerebral arteries that cause high morbidity and mortality in patients are myocardial infarction and ischemic stroke (Figure 1)[5]. Ischemia may entail several functional changes at the level of individual cells that build tissues, causing their dysfunction and death due to necrosis or apoptosis[6].

Standard treatments for ischemia include invasive and pharmacological control and treatments for the effects of ischemia in the damaged tissues. These methods emphasize improving the quality of and extending the patient's life, but they cannot fully reverse the effects of tissue ischemia in case of people suffering from congenital heart disease[7], ischemic heart failure[8], acute limb ischemia[9], critical limb ischemia[10], and ischemic stroke[11]. Even with the substantial progress of therapeutic strategies, these approaches do not provide the expected clinical benefits for all patients. Therefore, novel treatment pathways while replacing or supporting classic therapeutic approaches should continue to be investigated[12-14].

Currently, great hopes are placed on regenerative treatment, *i.e.*, therapies based on cellular preparations, including using various progenitor and stem cells (SCs) and their products[8,12,15]. Over the past decade, growing effort has been directed to the regenerative properties of SCs in relation to the biological treatment strategy of substitution of damaged cells in the tissue with new ones[16]. It is also believed that SCs may be involved in the neovascularization of ischemic tissues[17,18].

In 1970, Friedenstein *et al*[19] discovered an exceptional type of cell that has been extensively researched over the years for its potential use in regenerative medicine to treat ischemic damage[20]. These cells, called mesenchymal SCs (MSCs), reside in both young and adult donors [*e.g.*, umbilical cord (UC), Wharton's jelly (WJ) amniotic fluid, UC blood (UCB), placenta, adipose tissue (AT), bone marrow (BM), dental pulp (DP), and others], which has been a particular and exciting source of SCs for many years, mainly for autotransplantation and allotransplantation. Regardless of the tissue source, a cell that meets the criteria set out by the International Society for Cell Therapy (Table 1) may be qualified as MSCs[21].

They now account for the most popular SC population used in clinical trials worldwide (clinicaltrials.gov). MSCs derived from birth-related tissues have more promise due to better proliferative capacity compared with MSCs obtained from adult tissues. They are safe in terms of both sourcing and ethical aspects[22,23]. Due to several specific characteristics, these cells are essential candidates for regenerative therapies for ischemic tissues. Moreover, it is possible to use these cells to manufacture ready-to-use SC-based medicinal products. This review described the potential therapeutic mechanisms of MSCs in the context of ischemic disorder treatment. Exemplary clinical trials and procedures enhancing the therapeutic effect of MSCs were also discussed.

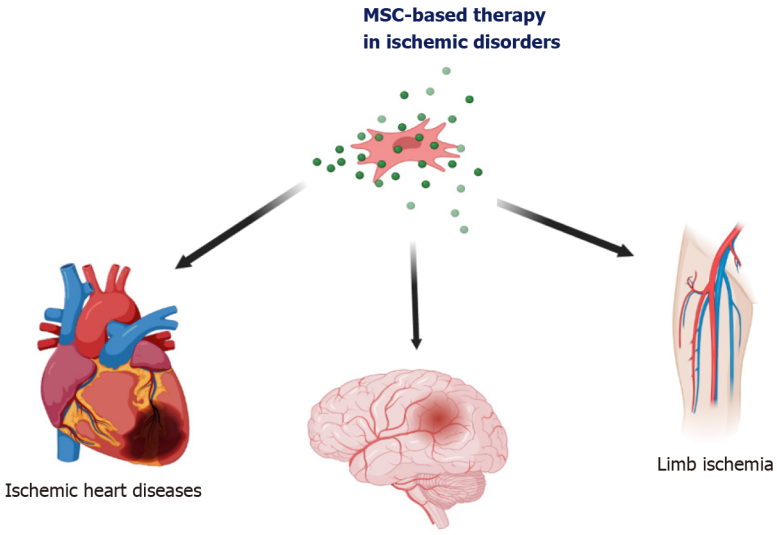
PATHOGENESIS OF ISCHEMIC DISEASES AND MSC-BASED THERAPY

The leading cause of ischemic disorders is the chronic inflammatory disease of the arteries, called "atherosclerosis". It can be caused by many risk factors, including aging, high blood pressure, diabetes, hypercholesterolemia, and smoking[24,25]. In the course of atherosclerosis, pathological damage and dysregulation of the endothelium lining the blood vessel wall, accumulation of lipids, smooth muscle cells, leukocytes, and foam cells are observed. Also, there is an aggregation of platelets in the lumen of the blood vessels, which leads to the formation of plaques narrowing the lumen of the vessels[26,27]. Moreover, in atherosclerotic lesions, increased expression of matrix metalloproteinases (MMP) and their participation in weakening the vascular wall through the degradation of the extracellular matrix (ECM) have been demonstrated[28].

Table 1 Minimal criteria for defining mesenchymal stem cells

Morphology, growth conditions	Spindle-shaped (fibroblast-like), adherent
Specific surface markers	CD73+, CD90+, CD105+, CD11b-, CD14-, CD19-, CD45-, CD79α-, MHC-II, HLA-DR-
Differentiation potential	Adipogenic, chondrogenic, osteogenic

HLA: Human leukocyte antigen; MHC: Major histocompatibility complex.



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Figure 1 Mesenchymal stem cell therapy in ischemic diseases. The mesenchymal stem cells can be use as biological drugs in the treatment of ischemic disorders of various organs, including brain ischemia in ischemic stroke, heart ischemia in acute myocardial infraction, Chronic ischemic cardiomyopathy, ischemic heart failure, and chronic lower extremity ischemia. MSC: Mesenchymal stem cell.

To compensate for the degradation of the ECM, smooth muscle cells migrate from the outer layers of the artery wall to the inner lining of the sheath to increase the collagen secretion rate[25]. It often causes undesirable remodeling as macrophages secrete cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 to induce smooth muscle cell apoptosis[25]. The unbalanced degradation rate of the ECM caused by increased collagen production results in the formation of atherosclerotic plaques with a thin fibrous collagen cap[29]. Injecting MSCs at this stage can modulate immune cell function, MMP activity, and the secretion of proinflammatory cytokines and restore collagen homeostasis[30]. In addition, due to hemodynamic changes and high shearing stresses in atherosclerotic plaques, ruptures and bleeding may occur, increasing platelet recruitment, coagulation processes, and thrombus formation[31]. Due to the secretion of proangiogenic factors and the ability to differentiate in the endothelium, MSCs can promote angiogenesis to restore blood flow to ischemic tissues for tissue regeneration and organ function restoration[32].

KEY PLAYERS IN ISCHEMIC TISSUE REGENERATION

Over the past decade, scientists and clinicians often discuss the regenerative properties of SCs in the context of biological treatment approaches. These strategies involve the replacement of damaged tissue cells with new SCs, including ischemic myocardium[16,33]. SCs are also considered cells that can participate in the neovascularization of ischemic tissues, which may also be associated with the improvement of the function of this organ[18,34,35].

One type of cell studied for years for their potential future use in regenerative medicine to treat myocardial damage or improve perfusion in limb muscle tissue is the mesenchymal/stromal SC (MSC) isolated from the BM, AT, birth-associated tissues, and other sites. These cells were found in both young and adult donors and have been an essential and exciting source of SCs, primarily for autotransplantation. They now account for the most often used SC population in clinical trials worldwide (clinicaltrial.gov)[18,36,37].

The history of studies on MSCs began in the 1970s when Friedenstein *et al*[19] observed colony-forming unit-fibroblasts (today known as MSCs). These cells constituted a fraction of adherent cells in the BM[19]. Moreover, their previous studies showed that subpopulations of BM cells could differentiate to other cell types, *i.e.*, osteoblasts[38]. These discoveries initiated an increase in interest in MSCs and the search for these cells in other tissues as well.

One of the primary and best-known sources of MSCs is BM[39,40]. Cells with similar morphology and biological characteristics can also be found in other tissues collected from adult donors, such as peripheral blood, AT, DP, and fetal tissues such as UCB and WJ[41].

BM-MSCs show morphological and phenotypic similarities to AT-MSCs and WJ-MSCs. These markers include CD29 +, CD44 +, CD73 +, CD90 +, CD105 +, CD166 +, human leukocyte antigen (HLA)-ABC +, CD11b-, CD14-, CD19a-, CD34-, CD45-, CD79-, and HLA-DR[42,43]. However, the source tissues from which MSCs are isolated differ in the content of these SCs. For example, the number of MSCs in the BM is lower than in AT[43,44]. The BM contains from 0.0017% to 0.02% of MSCs among all mononuclear cells, while in AT, these cells constitute 5.0% to 25.6% of all cells, which is the so-called stromal-vascular fraction obtained from this tissue[44-46]. The number of colony-forming unit-fibroblast colonies isolated from the same number of plated BM or AT cells is several times higher from AT than BM[42]. Also, WJ-MSCs cells have a higher frequency of colony-forming unit-fibroblasts than BM-MSCs cells[47]. Considering the differentiation potential of BM-MSCs, AT-MSCs, and UC-MSCs, these cells show a comparable capacity to differentiate into osteoblasts, chondrocytes, and adipocytes[42,48], confirming their mesodermal origin.

MSCs derived from various tissues can also be differentiated *in vitro* into phenotypically similar cells, including cardiomyocytes[49-53], vascular endothelial cells (ECs)[54,55], and nerve cells[56]. However, it has been shown that the effectiveness of such differentiation is variable and depends on the tissue origin[49,51,53,56]. Within one culture of MSCs, cells may be more or less predisposed to differentiate to a specific phenotype of the mature cell[57]. Despite the morphological and antigenic similarity between MSCs from different source tissues, the results of world studies showed that BM-MSCs may offer a different expression profile of many genes compared to placenta-derived MSCs[57], which suggests that MSCs obtained from BM, WJ, and AT may differ in terms of their molecular composition and their ability to differentiate.

The International Society for Cellular Therapy, to clarify the nomenclature and define the requirements of human MSCs and facilitate the comparison of test results between laboratories around the world, proposed three minimum criteria for characterizing human MSCs[21].

Due to numerous world studies showing the relatively wide potential of MSCs to differentiate into various types of tissues, these cells constituting a population obtained from mature tissues have for years interested scientists in the context of their potential use in tissue regeneration[58-60].

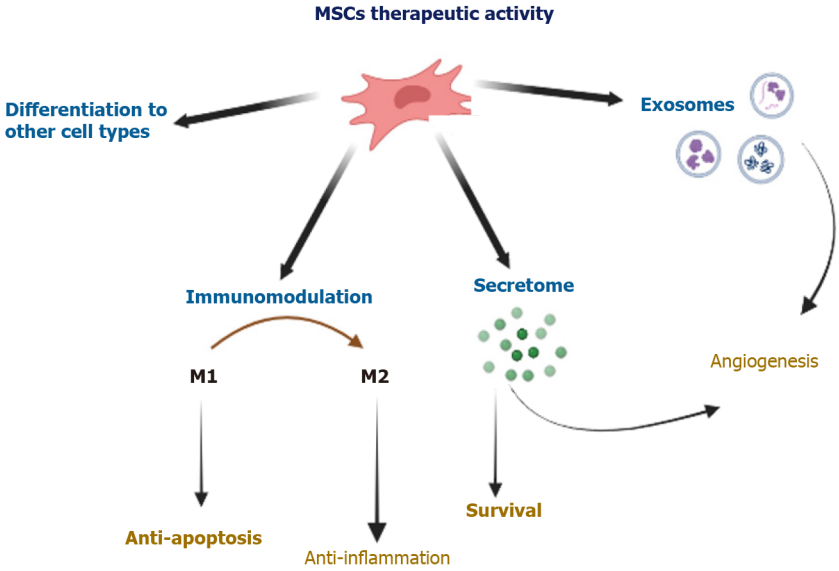
MSC MECHANISMS OF ISCHEMIC TISSUE REGENERATION

Multipotent MSCs show many features desirable from the point of view of their potential use in the regeneration of damaged tissues, not only in autologous but also in allogeneic transplants. Therefore, over the last decade, many studies have been undertaken to explain the mechanisms of action of these cells replaced by tissue damage. Figure 2 presents several essential mechanisms of action contributing to ischemic tissue regeneration.

Differentiation of MSCs

It was initially suggested that MSCs administrated into the area of tissue damage are able to differentiate into desired cell types, including muscle-building cardiac mesenchymal cells (CMCs), vascular smooth muscle cells, and ECs[59,61,62]. It was shown *in vivo* that MSCs injected to the heart muscle had a phenotype similar to differentiating cells, including CMCs and ECs[63-65]. Pochon *et al*[66] confirmed that WJ-MSCs differentiated into CMCs *in vitro*, expressing CMC markers and spontaneous throbbing, which might be evidence of their terminal maturation into specialized cells capable of playing their proper functions. Although MSCs show the potential to differentiate to CMCs *in vitro*, the fundamental issue is to restore damaged tissue *in vivo*. Effective delivery and retention must be emphasized here because if cells do not reach the target tissue, they cannot exert any therapeutic effect[60]. Even if studies have shown that MSCs can differentiate into various types of myocardial cells, an increasing number of studies show that this is not a main mechanism for their regenerative activity in the cardiovascular system.

Several articles now contain information on the neural differentiation of MSCs from various sources *in vitro*[67-69]. In the described studies, the prevailing view suggests that MSCs, derived from immature tissues due to their plastic properties, can differentiate more effectively into cells with neural phenotypes, showing the presence of typical linear markers[70]. Positive results of *in vitro* neural differentiation of MSCs were obtained in experiments using specific chemical compounds, growth factors, co-culturing with mature neurons, or the culture of three-dimensional aggregates[71,72]. Another study showed that neural differentiation of MSCs can be induced by a conditioned medium derived from



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Figure 2 Proposed therapeutic mechanisms of mesenchymal stem cells, including differentiation, immunomodulation, secretion of paracrine factors, and secretion of microvesicles. Mesenchymal stem cells (MSCs) can repair and/or rescue injured cells *via* differentiation into replacement cell types including endothelial cells, and cardiomyocytes. The immunomodulatory activity of MSCs includes the suppression of macrophage polarization to M1, though favors M2 polarization. MSCs can secrete a number of factors that stimulate the regeneration of damaged cells and tissues [*e.g.*, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), stromal cell-derived factor-1] and having a beneficial effect for neovascularization processes (*e.g.*, VEGF, IGF-1, HGF, fibroblast growth factor-2, platelet-derived growth factor-BB, placental growth factor), inhibiting the pathological remodeling of ischemic tissues [*e.g.*, matrix metalloproteinase 2 (MMP-2), MMP-9]. The exosomes secreted by MSCs contain cytokines and growth factors, signaling lipids, mRNAs, and regulatory microRNAs which can be responsible for the therapeutic effects. MSC: Mesenchymal stem cell.

cultures of olfactory ensheathing cells or Schwann cells[73]. Sotthibundhu *et al*[74] discovered that stimulation of autophagy MSCs could improve the efficiency of SC differentiation and the formation of neural-like cells.

Interaction of MSCs with other cells

MSCs can communicate with neighboring cells through direct cell-cell interactions, including gap junctions and tunneling nanotubes[75]. Moreover, MSCs are able to transport mitochondria through nanotubes and thus obtain cardiological protection by regaining the respiratory chain in myocytes[76]. To allow damaged tissue to regenerate, MSCs communicate with other cells in the damaged areas to recruit other types of SCs. For example, studies have demonstrated that CMCs can re-enter the cell cycle after supplementation with specific cytokines secreted by MSCs (*e.g.*, transforming growth factor-β)[77]. MSCs can also keep other cells to active migration to the area of tissue damage, as demonstrated by the trafficking of hematopoietic SCs to the damaged myocardium[78].

Immunomodulatory properties of MSCs

Despite the reported low rate of retention of MSCs in ischemic heart muscle after their injection, the results of many experiments showed improvement in the functional heart parameters, like inhibition of adverse tissue remodeling myocardium and left ventricle ejection fraction (LVEF)[33]. So, this begs the questions: Is the improvement in injured organ function following the administration of MSCs results only from the implantation and ability of MSCs to differentiate into specific cell types or is another mechanism also involved in this process?

No doubt, that in order to answer this question, other studies, which have focused on the immunomodulatory properties of MSCs, should be mentioned here[79-81]. The remarkable ability of MSCs to produce an enormous number of soluble factors, such as anti-inflammatory cytokines, enables them to modulate the immune system response[82,83]. For example, MSCs secrete the cytokines, IL-4 and IL-10, which inhibit the proliferation of T cells, the growth factor, hepatocyte growth factor, which inhibits the proliferation of CD4+ T cells, and transforming growth factor-β1, which with prostaglandin E2 inhibits the inflammation process[84-86]. Moreover, they encourage the maturation of monocytes towards anti-inflammatory macrophages type M2[79,87].

In addition, the most extraordinary attribute of MSCs is an immunological privilege. MSCs are known to be capable of avoiding and suppressing immune responses[88,89]. Most MSCs show the low expression of HLA class I and a lack of HLA class II markers. Due to this feature, they do not cause an immune conflict between the transplant recipient and the injected cells. Additionally, MSCs possess HLA-G, which is a key factor in the elimination of the fetus rejection by the maternal immune system

[90,91]. Because of the high expression of HLA-G, MSCs can modulate the tolerance of the immune system and it has a very beneficial effect on acceptance of the transplant.

Paracrine effects of MSCs

Another reported mechanism of therapeutic activity of MSCs can be attributed to the secretion of paracrine factors, including several cytokines, growth factors, and chemokines, that may regulate many regenerative processes at the MSCs implantation site[92]. Proangiogenic molecules produced by MSCs involve, among others, the protein fibroblast growth factor-2[92-94], platelet-derived growth factor[95], and an extremely proangiogenic vascular endothelial growth factor, supporting the proliferation of vascular smooth muscle cells and ECs as well as the migration and the new blood vessel structure formation[96].

On the other hand, molecules that promote ECM remodeling involve MMP1, MMP2, MMP9, a family of enzymes that degrade ECM structure, and TNF-α and the activator plasminogen that leads to ECM protein impairment[78,97]. A distinct category of molecules produced by MSCs are the factors responsible for MSCs survival, proliferation, and migration to the area of tissue injury, which involve fibroblast growth factor-2 supporting the proliferation of vascular smooth muscle cells and ECs, stromal cell-derived factor-1 reducing apoptosis and regulating cell migration, insulin-like growth factor-1 controlling cell differentiation and growth and inhibiting apoptosis, and a secreted frizzled-related protein-2 supporting CMC survival at the conditions of low oxygen availability *in vivo*[77,98].

Exosomes secretion

Extracellular vesicles are biological nanoparticle structures containing bioactive molecules, including protein, and nucleic acids. They can influence other cells and participate in intercellular communication over long distances[99]. Many studies on tissue regeneration mechanisms demonstrated that extracellular vesicles released by SCs can deliver bioactive molecules to target cells, which may influence the function of those cells, including the process of damaged tissue regeneration[100].

PRECLINICAL STUDIES: ANIMAL MODELS OF TISSUE ISCHEMIA

Most of the molecular and cellular mechanisms that affect the therapeutic potential of MSCs in the therapy of ischemic tissues were initially identified in animal models. The capacity of MSCs to survive in the recipient after administration and the ability to differentiate into Ecs and CMCs has been proven in acute myocardial infarction in a mini-swine model[65]. In this paper, Zhang *et al*[65] also confirmed the migratory activity of MSCs towards inflammation, inhibition of CMC apoptosis, stimulation of cardiac SCs, reduction of fibrosis, myocardium reverse remodeling, and enhancement of LVEF. It has been demonstrated that MSCs derived from UCB (UCB-MSCs) can reduce the acute myocardial infarction size by ≥ 50% and enhance LVEF[101]. The observed therapeutic effect may be due to the ability of MSCs to secrete bioactive factors. In turn, thanks to their immunosuppressive properties and the paracrine effect, MSCs can alleviate inflammation and ischemic heart disorders, contributing to the reduction of infarct size and improving LVEF through a paracrine effect[102].

The therapeutic efficacy of UC-MSCs and heart function improvement has been demonstrated[103]. Intravenous administration of MSCs has improved LVEF contractility, function, perfusion, and reverse remodeling[18]. The transplantation of MSCs in a rat model of acute myocarditis can reduce inflammation by decreasing the infiltration of an inflammatory cell, reducing CMC death, and remodeling adverse myocardium[104,105]. Based on the results of animal studies, hopes are high regarding enhancing many heart functions, such as reduction of scar tissue, myocardium reverse remodeling, increase in cardiac contractility, improvement of ejection fraction, and increase in heart perfusion. However, there is still a need for long-term observation of the effects of injecting MSCs to ensure the safety and efficacy of therapy.

In preclinical studies on transgenic animals of models of neurological diseases, significant functional improvement was observed after MSC cell transplantation[106]. The use of these cells may be related to their direct action, *i.e.*, replacement of damaged cells as a result of neural differentiation or to an indirect influence positively influencing the endogenous regenerative processes of the organism. In addition, preclinical studies on animals have confirmed the neuroprotective properties of MSC transplants, which may be linked to their production of numerous growth, anti-inflammatory, and anti-apoptotic factors important for neurons[107].

CLINICAL TRIALS: MSC AS MODERN THERAPEUTIC AGENTS IN ISCHEMIA

Clinical trials testing MSCs in regenerative therapy are growing (ClinicalTrials.gov). Regenerative therapies based on MSCs for several ischemic disorders are now carefully examined. The remedial effects obtained are promising and prove that the transplantation of MSCs may be beneficial in the

treatment of many diseases[108,109]. The concept of clinical application of MSCs may improve the health of patients suffering from many cardiovascular diseases[110]. So far, a few dozen studies have been registered in the international database of clinical trials to assess the safety and effectiveness of MSCs administration in the treatment of ischemic diseases.

The most popular source of therapeutic MSCs used in clinical practice is BM. The first MSC-based biological drug, AMI HeartiCellgram®, used in myocardial infarction therapy was also based on BM-MSCs. Lee *et al*[111] described the manufacturing procedure for this drug, and Kim *et al*[112] proved its effectiveness in a clinical experiment. The therapeutic benefits included the restoration of normal systolic heart function and the reduction of post-infarction scar tissue[113,114].

The pioneer pilot clinical study applying WJ-MSCs in treating ischemic disorders was performed by Musialek *et al*[115] in 2015. This group has shown the safety of injected MSCs as an off-the-shelf product, percutaneous allogeneic SC therapy in human acute myocardial infarction. Later observation proved no clinical adverse events in the treated tissue, except for a local rise in body temperature of 1 patient[115]. Currently, Musialek *et al*[115] are examining the safety and effectiveness of the "CardioCell" drug (WJ-MSC-based biological therapeutic) in a phase II/III randomized, placebo-controlled, double-blind clinical trial in several ischemic disorders [*i.e.*, acute myocardial infarction (EudraCT Number: 2016-004662-25), chronic ischemic heart failure (EudraCT Number: 2016-004683-19), and non-option critical limb ischemia (EudraCT Number: 2016-004684-40)][116,117].

A clinical trial with MSCs has shown the enhancement in heart muscle function in cases of heart failure. For example, Bartolucci *et al*[118] have shown that intravenous administration of UC-MSCs improved LVEF, functional status, and standard of living. Also, exosomes released by UC-MSCs can alleviate the effect of acute myocardial ischemic injury[119]. Scientists confirmed that the injection of UC-MSCs exosomes can greatly improve contractile heart function and minimize myocardial fibrosis. These bioactive bubbles protected heart cells from death and supported EC migration and angiogenesis. UC-MSCs have also been applied in a clinical study for the treatment of chronic coronary occlusion[17]. Also in this trial, the improvement the heart function and better left ventricular ejection fraction were reported[17].

So far, several clinical trials of ischemic stroke have shown that transplanting MSCs into patients with successful reperfusion therapy reduces the volume of lesions after stroke and promotes the regeneration of neurological function. This success is shown by improvements in human functional, behavioral, and sensorimotor assessments, such as the Barthel Index, Modified Rankin Scale, European Stroke Scale, Fugl-Meyer Scale, and National Institutes of Health Stroke Scale[120,121]. MSCs participate in the regeneration of ischemic tissues and organs with beneficial effects, as outlined above (Table 2). The therapeutic activity is presumed to include immunomodulation, cardioprotective effects, activation of endogenous repair processes, and tissue remodeling.

IMPROVING THE EFFECTIVENESS OF MSC-BASED THERAPY

MSCs can supply alternative therapy in the treatment of many disorders, but many studies have demonstrated that depending on the method of isolation, expansion, and delivery we can obtain cells with distinct functional features. The therapeutic benefits of MSC-based therapy involve paracrine activity, immunomodulation, and enhanced function of the damaged organ. However, not all patient responses to treatment are satisfactory; therefore this approach requires a deep understanding of the therapeutic actions of MSCs after injection into the recipient. The therapeutic efficacy of MSCs is affected by many factors, including the method of MSCs cultivation *in vitro*, the metabolic activity of the MSCs, the number of injected cells, the patient's genetic sensitivity, and the stage of the disease[136,137].

The selection of the appropriate source of therapeutic MSCs depending on the disease is crucial as more and more data show source-dependent variations in therapeutic activity such as levels of released trophic proteins or different differentiation capacities. There is much disagreement as to the therapeutic efficiency of MSCs derived from different tissues (fetal and adult sources). Therefore, extensive studies are desirable to obtain consistent data about remedial effects.

Another problem that is much debated is the type of transplant, *i.e.*, allogeneic or autologous. The results obtained from clinical trials showed no difference between the therapeutic effects of allogeneic and autologous MSCs in the treatment of ischemic cardiomyopathy[138]. The undoubted advantage of autologous transplants is the lack of burdening the cells with other diseases because in such cases the donors are healthy volunteers. Autologous SCs obtained from the patient do not have this privilege, which may limit the therapeutic effectiveness. In addition, it is an important issue to obtain the right dose of autologous cells. The autologous transplant requires the collection of appropriate tissue from the patient, isolation of SCs, and obtaining the necessary dose of therapeutic cells, which is a challenge as the disease and the patient's age may contribute to the reduction of the proliferative activity of MSCs [139,140].

To resolve these issues, scientists introduced allogeneic sources of SCs that can be used to produce ready-to-use biological products. The use of allogeneic SCs shortens the waiting time for a transplant. Allogeneic cells from young and healthy donors are used to produce biological drugs of quality that can

Table 2 Selected clinical trials of mesenchymal stem cell-based therapy in ischemic diseases

Disorder	Ref.	Trial number (acronym)	Type of transplant and stem cell source	Results
Myocardial infarction	Hare *et al*[36], 2009	NCT00114452	Allogenic BM-MSCs	No tumorogenicity, no arrythomogenicity
Myocardial infarction	Houtgraaf *et al* [37], 2012	NCT00442806 (APOLLO)	Autologous AT-MSCs	No adverse effects, decreased: Scar tissue; increased: Perfusion
Myocardial infarction	Gao *et al*[122], 2015	NCT01291329	Allogenic WJ-MSCs	Increased: Ejection fraction; decreased: Heart perfusion
Chronic ischemic cardiomyopathy	Hare *et al*[123], 2012	NCT01087996 (POSEIDON)	Allogenic *vs* autologous BM-MSCs	Increased: Ejection fraction; decreased: Scar tissue
Chronic ischemic cardiomyopathy	Karantalis *et al* [124], 2014	NCT00587990 (PROMETHEUS)	Autologous BM-MSCs	Local; increased: Contraction; decreased: Scar tissue
Chronic ischemic cardiomyopathy	Perin *et al*[125], 2014	NCT00426868 (PRECISE)	Autologous AT-MSCs	Increased: Left ventricular mass; increased: Contractility; increased: Perfusion
Chronic ischemic cardiomyopathy	Bartunek *et al* [126], 2013	NCT00810238 (C-CURE)	Preconditioned autologous BM-MSCs	Increased: Ejection fraction
Chronic ischemic cardiomyopathy	Bartolucci *et al* [118], 2017	NCT01739777 (RIMECARD)	Allogenic UC-MSCs	Increased: Ejection fraction
Ischemic heart failure	Teerlink *et al* [127], 2017	NCT01768702 (CHART-1)	Autologous BM-MSCs	Left ventricular reverse remodelling, increased: Left ventricular volume
Critical limb ischemia	Wijnand *et al* [128], 2018	NCT03042572 (SAIL)	Allogenic BM-MSCs	Safety, decreased: Pain rest
Critical limb ischemia	Norgren *et al* [129], 2018	NCT01732822 (EUCLID)	Allogeneic placental-derived MSCs	Increased: Amputation-free survival; decreased: Pain rest; increased: Tissue perfusion
Critical limb ischemia	Gupta *et al*[130], 2013	NCT00883870	Allogenic BM-MSCs	Decreased: Pain rest; increased: Ankle systolic pressure; increased: Ulcer healing
Ischemic stroke	Steinberg *et al* [131], 2016	NCT01287936	Modified BM-MSCs	Increased: Motor functions (ESS, NIHSS, Fugle-Meyer scales)
Ischemic stroke	Levy *et al*[132], 2019	NCT01297413	Allogenic BM-MSCs	Increased: Barthel index
Ischemic stroke	Savitz *et al*[133], 2019	NCT01273337 (RECOVER-Stroke)	Autologous BM-MSCs	Safety
Ischemic stroke	Laskowitz *et al* [134], 2018	NCT03004976	Allogenic UCB-MSCs	Safety and feasibility
Ischemic stroke	Jaillard *et al* [135], 2020	NCT00875654	Autologous BM-MSCs	Increased: Motor functions (NIHSS, Fugle-Meyer scales)

BM: Bone marrow; AT: Adipose tissue; WJ: Wharton's jelly; UC: Umbilical cord; MSCs: Mesenchymal stem cells; ESS: European Stroke Scale; NIHSS: National Institutes of Health Stroke Scale.

be stored frozen. The medicinal product prepared in this way, if necessary for clinical intervention, can be thawed at any time and administrated to the patient.

MSCs applied in the clinic as therapeutic agents must be carefully prepared, according to the good manufacturing practice and good clinical practice standards, with the established quality control system. The manufacturing process should be properly optimized in terms of therapy requirements to reach a sufficient remedial effect. MSCs applied in the clinic as therapeutic agents must be carefully prepared, according to the good manufacturing practice and good clinical practice standards, with the established quality control system. The manufacturing process should be properly optimized in terms of therapy requirements to reach a sufficient remedial effect. Here it should be emphasized that we can prepare therapeutic cells to treat different disorders, such as graft *vs* host disease, myocardial infarction, Crohn's disease, and others, however, the therapeutic benefits obtained may not be satisfactory. Therefore, there is still a need for a more sophisticated approach to obtaining highly effective biological medicines. The use of an appropriate approach to the production of therapeutic MSCs for the treatment of a particular disease should contribute to the achievement of satisfactory results.

Additionally, to adopt therapeutic MSCs for demanding conditions in the host body, preconditioning methods can be applied. This approach requires the presence of additional adverse factors (such as low oxygen availability or proinflammatory cytokines) during *in vitro* culture. Bernardo and Fibbe[141],

during the production of therapeutic MSCs, added proinflammatory cytokines to the culture medium, the concentration of which increased after acute myocardial infarction. In this study, an anti-inflammatory response of MSCs was observed within 24-48 h based on the analysis of the composition of the culture medium. The use of selected cytokines in MSCs cultures for the treatment of acute myocardial infarction enhanced anti-inflammatory secretory activity and therapeutic efficacy.

CONCLUSION

The remarkable ability of MSCs to regenerate damaged body parts to regain lost function is promising in many disorders, including ischemic diseases. Three properties of MSCs render them optimal for ischemic tissue repair and regeneration: (1) Immunomodulatory and immunoregulatory capacity beneficial to ameliorate abnormal immune responses; (2) Soluble and insoluble paracrine factor-generating potential; and (3) Endothelial differentiation.

While MSCs have several advantages, there are still many challenges to overcome. The unique immunomodulatory properties of MSCs are essential to their function, but the mechanisms of the immune regulation of MSCs have not been elucidated. Many factors can influence the therapeutic potential of MSCs, such as donor age, isolation and culture method, induction factors, oxygen concentrations, mechanical stimuli, and others. Hence, optimizing the culture conditions of MSCs may be an effective way to improve the therapeutic potential of MSCs for successful tissue repair.

FOOTNOTES

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The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

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REVIEW

Clinical trials using dental stem cells: 2022 update

Wen-Peng Song, Lu-Yuan Jin, Meng-Di Zhu, Hao Wang, Deng-Sheng Xia

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Abstract

For nearly 20 years, dental stem cells (DSCs) have been successfully isolated from mature/immature teeth and surrounding tissue, including dental pulp of permanent teeth and exfoliated deciduous teeth, periodontal ligaments, dental follicles, and gingival and apical papilla. They have several properties (such as self-renewal, multidirectional differentiation, and immunomodulation) and exhibit enormous potential for clinical applications. To date, many clinical articles and clinical trials using DSCs have reported the treatment of pulpitis, periapical lesions, periodontitis, cleft lip and palate, acute ischemic stroke, and so on, and DSC-based therapies obtained satisfactory effects in most clinical trials. In these studies, no adverse events were reported, which suggested the safety of DSC-based therapy. In this review, we outline the characteristics of DSCs and summarize clinical trials and their safety as DSC-based therapies. Meanwhile, we also present the current limitations and perspectives of DSC-based therapy (such as harvesting DSCs from inflamed tissue, applying DSC-conditioned medium/DSC-derived extracellular vesicles, and expanding-free strategies) to provide a theoretical basis for their clinical applications.

Key Words: Dental stem cells; Adult stem cells; Dental pulp; Tissue regeneration

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Core Tip: Since dental pulp stem cells were first isolated and identified in 2000, a variety of dental stem cells (DSCs) have been reported. DSCs have shown satisfactory clinical effects in the treatment of a variety of diseases and have great potential for clinical application. This paper will summarize DSC-based clinical trials and put forward the current limitations and perspectives to accelerate and extend the clinical application of DSCs.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are a population of unspecialized cells characterized by the properties of self-renewal and multidirectional differentiation[1,2]. Currently, MSCs are currently being explored for the treatment of many diseases, such as cardiovascular disease, neurodegenerative diseases, dental diseases, and metabolic diseases[1].

Dental SCs (DSCs) were reported to have similar features to MSCs[3]. Since dental pulp SCs (DPSCs) were first successfully isolated from the extracted third molar in 2000[4], multiple DSC types have been harvested from mature and immature teeth and their surrounding tissues, including periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), stem cells from exfoliated deciduous teeth (SHED), gingiva-derived mesenchymal SCs (GMSCs), and dental follicle progenitor cells (DFPCs)[5-7] (Figure 1). DSCs develop from the neural crest and express both stem cell markers and neural markers[8,9]. It was reported that DSCs have the potential for multipotent differentiation into osteogenic, chondrogenic, adipogenic, neurogenic, odontogenic, dentinogenic cells, and so on[10]. In addition to their self-renewal and differentiation properties, DSCs have also been reported to be involved in secretion, immunomodulation, and tumor processes[3,11]. Based on the characteristics of DSCs, many clinical articles and clinical trials have used DSCs in tissue regeneration and the treatment of various diseases, such as pulpitis, periapical lesions, and periodontitis[12].

In this study, the current status of clinical articles and clinical trials using DSCs in the treatment of various diseases and conditions are reviewed. In addition, current limitations and perspectives, including harvesting DSCs from inflamed tissue, applying DSC-conditioned medium (CM) and DSC-derived extracellular vesicles (EVs), and expanding-free strategies, are also discussed.

CHARACTERISTICS OF DSCS

Based on their various sources, DSCs are divided into DPSCs, SHED, PDLSCs, SCAP, GMSCs, and DFPSCs (Figure 1). DSCs are known to express not only mesenchymal and embryonic stem cell markers (such as CD44, STRO-1, and Nanog) but also neuronal markers because they originate from embryonic neural crests[8,9] (Table 1). However, they do not express CD34, CD45, or CD11b, which are defined as hematopoietic markers[7].

Similar to mesenchymal stem cells, DSCs showed the ability of self-renewal and multidirectional differentiation, such as osteogenic, chondrogenic, adipogenic, neurogenic, odontogenic, dentinogenic, cementogenic, and myogenic differentiation[13-16] (Table 1). In addition, even in the undifferentiated state, DSCs were able to secrete several angiogenic and neurotrophic factors, including vascular endothelial growth factor (VEGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glia-derived neurotrophic factor (GDNF), and β-nerve growth factor (β-NGF), to promote angiogenesis and tissue regeneration[17,18].

In addition, the immunomodulatory features of DSCs have also been the focus of a number of studies. First, it was reported that DSCs, like mesenchymal stem cells, faintly express the MHC class II antigen HLA-DR and maintain low immunogenicity[19-21]. Second, local tissue regeneration and inflammation could be influenced by the secretome of DSCs (including the production of inflammatory and anti-inflammatory cytokines and the regulation of immune cells), which is also regulated by the local inflammatory microenvironment[21-24]. Finally, the inflammatory microenvironment could impact the behaviors of DSCs, such as proliferation potential, migration, homing, and differentiation[22].

Based on the characteristics of DSCs, they have been widely studied in regenerative medicine and tissue engineering and have shown an amazing therapeutic effect on oral-facial, neurologic, corneal, cardiovascular, hepatic, diabetic, renal, muscular, tenogenic, dystrophic and autoimmune conditions in both animal and human models[21,25-27]. For example, the proliferation, paracrine effect, and multidirectional differentiation potential of DSCs support the application of DSCs in regenerative

Table 1 Characteristics of different types of dental stem cells

Cell types	Markers			Multidirectional differentiations
	Cell surface markers	Embryonic stem cell markers	Nerual markers	
DPSCs	CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146, STRO-1[7], CD81, CD49f[140], CD40, CD120a, CD261, CD262, CD264, CD266, CD121a, CD130, CD213a1, CD217, CDw210b[141]	OCT-4, Nanog[142], SSEA-1, SEEA-4[140], SOX-2[143]	βIII-tubulin, NFM, Nestin, CNPase[144], S100, CD271 [17]	Osteogenic, Odontogenic[145], Dentinogeni, Chondrogenic, Neurogenic, Myogenic, Adipogenic[13], Hepatogenic [146]
PDLSCs	CD13, CD29, CD44, CD49, CD73, CD90, CD105, CD146, CD166, CD271 [147], CD10[7], STRO-1[148]	SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, OCT-4, Nanog, SOX-2, REX1, and ALP[149]	Nestin, OCT-4, SSEA-4[9] CD271, SOX-10[147], SOX-2 [149]	Osteogenic, Cementogenic, Adipogenic, Chondrogenic, Neurogenic[13], Hepatogenic[149], Cardiac myogenic, Endothelial-like, Islet-like, Retinal ganglion-like[147]
SCAP	CD13, CD24, CD29, CD44, CD49, CD51, CD56, CD61, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, NOTCH-3[150], CD81, CD49f[151]	OCT-4, Nanog, SOX2 [143], CD49f[151]	βIII-tubulin, NFM, Nestin, CNPase[144], SOX-2[143], Vimentin, Survivin[150]	Osteogenic, Dentinogenic, Adipogenic [15], Neurogenic[13], Chondrogenic, Hepatogenic[150]
SHED	CD29, CD73, CD90, CD166[13], STRO-1, CD44[145], CD105[152], NOTCH-1, CD10, CD13, CD34, CD106, CD146, CD166, CD271[102]	OCT-4, Nanog, SSEA-3 [153], SSEA-4[152], NOTCH-1, OCT-4, SOX-2 [102]	βIII-tubulin, NFM, Nestin, CNPase, GAD, NeuN, GFAP [154], CD271, Vimentin, OCT-4, PAX-6, NSE, MAP-2, PSA-NCAM, TH[102]	Osteogenic, Odontogenic[145], Dentinogenic, Chondrogenic, Neurogenic, Myogenic, Adipogenic[13], Hepatogenic[155]
DFPCs	CD13, CD29, CD59, CD90[7], CD105, CD146[142], CD44, CD73, NOTCH-1, STRO-1[156]	OCT-4, Nanog[142], NOTCH-1, SOX-2[156]	OCT-4, SOX2[9], Nestin, SOX-2[156]	Osteogenic, Cementogenic, Odontogenic, Adipogenic, Chondrogenic[13], Hepatogenic[146]
GMSCs	CD13, CD29, CD44, CD73, CD90, CD105, CD146, STRO-1[16]	SSEA-4[16], OCT-4, Nanog[157]	Nestin, SOX10[16], βIII-tubulin, NFM, CNPase[144]	Osteogenic, Adipogenic, Chondrogenic, Neurogenic, Endothelial-like, Odontogenic[16], Myogenic[158]

ALP: Alkaline phosphatase; CD: Cluster of differentiation; CNPase: 2′,3′-cyclic nucleotide 3′-phosphodiesterase; GAD: Glutamic acid decarboxylase; GFAP: Glial fibrillary acidic protein; MAP-2: Microtubule associated protein 2; NeuN: Neuronal nuclei; NFM: Neurofilament medium chain; NGFR: Nerve growth factor receptor; NSE: Neuron-specific enolase; OCT: Octamer-binding transcription factor; PAX-6: Paired Box 6; PSA-NCAM: Polysialylated neural cell adhesion molecule; REX-1: RNA exonuclease 1 homolog; SOX: Sex determining region Y-box; SSEA: Stage-specific embryonic antigen; TH: Tyrosine hydroxylase; SHED: Stem cells from exfoliated deciduous teeth.

medicine (*e.g.*, dental pulp and bone tissue regeneration)[28,29]. The anti-inflammatory, immunomodulatory, and immunoevasive properties of DSCs also help in the treatment of plaque psoriasis[30] (Figure 1). DSC-based therapies have broad prospects for clinical application.

It is worth noting that the naming of mesenchymal stem cells and mesenchymal stromal cells remains controversial. Based on the position paper issued by The International Society for Cell & Gene Therapy (ISCT) Mesenchymal Stromal Cell (ISCT MSC) in 2005, mesenchymal stem cells are not equivalent or interchangeable with mesenchymal stromal cells[31]. Mesenchymal stem cells refer to progenitor cell populations with obvious self-renewal and differentiation functions, while mesenchymal stromal cells refer to large populations with significant secretion, immune regulation, and homing properties[32-34]. As we have just summarized, dental stem cells share some of the characteristics of both mesenchymal stem cells and mesenchymal stromal cells, and more consensus articles may be needed to further define the naming of dental stem cells.

DSC-BASED CLINICAL TRIALS FROM PUBLISHED ARTICLES

Pulpitis and pulp necrosis

Four studies were reported to treat pulp necrosis or irreversible pulpitis using autologous DPSCs or SHED, including a randomized controlled trial (RCT), two case series, and a case report[28,35-37] (Table 2). Xuan *et al*[28] applied SHED in the treatment of pulp necrosis caused by trauma and observed dental pulp tissue regeneration at 12 mo and 24 mo after transplantation. Meanwhile, the results also showed increased dental root length and decreased apical foramen width compared with traditional apexification treatment. Two case series reported by Nakashima *et al*[35,37] indicated that DPSCs transplanted with granulocyte colony-stimulating factor and gelatin sponges could increase pulp sensitivity and mineralization and recover the signal intensity (SI) of regenerated pulp tissue on MRI examination. Meza *et al*[36] transplanted DPSCs and leukocyte platelet-rich fibrin (L-PRF) harvested

Table 2 Dental stem cell-based clinical trials from published articles

Ref.	Registration ID	Conditions/diseases	Study design	Cell source	Administration route	Interventions		Follow-up period	Outcomes
						Test group	Control group		
Xuan *et al*[28], 2018	NCT01814436	Pulp necrosis	RCT	Autologous deciduous pulp	Implanted into injured teeth	SHED (n = 26)	Traditional apexification treatment (n = 10)	12 mo; 24 mo	Dental pulp tissue regeneration; no adverse events observed; the length of the root (↑); the width of the apical foramen (↓)
Nakashima *et al*[35], 2022	None	Irreversible pulpitis	Case series	Autologous dental pulp	Transplanted into the root canal	DPSCs + Gelatin sponge + G-CSF (n = 5)	None	1, 2, 4, 12, 24, 28, 32 wk	Pulp sensibility (↑); MRI examination showed similar SI between test teeth and untreated controls
Nakashima *et al*[37], 2017	None	Irreversible pulpitis	Case series	Autologous dental pulp	Transplanted into the root canal	DPSCs + Gelatin sponge + G-CSF (n = 2)	None	1, 4, 12, 24, and 48 wk	MRI examination showed similar SI between test teeth and untreated controls; mineralized tissue deposition (↑)
Meza *et al*[36], 2019	None	Irreversible pulpitis	A case report	Autologous inflamed dental pulp	Transplanted into the root canal	DPSCs + L-PRF (n = 1)	None	6 mo; 3 year	Delayed response to the cold test; positive response to electric pulp testing; dentin bridge formation
Shiehzadeh *et al*[38], 2014	None	Periapical lesions	Case series	Case 1 and case 3: Autologous apical papilla; case 2: Deciduous pulp	Case 1 and Case 3: Injected from root apex to cavity; case 2: Injected into the defect *via* a surgical approach	Case 1 and Case 3: SCAP + PEG-PLGA scaffold (n = 2); case 2: SHED + PEG-PLGA scaffold (n = 1)	None	Case 1: 30 d, 3 mo, 1 year; 2 year; case 2: 3, 6, 18 mo; case 3: 3, 6, 12, 24 mo	Developed mature apices; periapical tissue healing (↑)
Prasad *et al* [39], 2017	None	Periapical lesions	Case series	Allogeneic deciduous pulp	Transplanted into the root canal	SHED + Bioglass (n = 2)	None	7, 30, 90, 180, 365 d	Closure of open apex; periapical tissue healing; positive response to electric pulp testing and cold testing
Prasad *et al* [40], 2019	None	Periapical lesions	A case report	Allogeneic deciduous pulp	Transplanted into the root canal and periapical area	SHED + Bioglass (n = 1)	None	2 wk; 4, 12, 24 mo	Periapical tissue healing; positive response to electric pulp testing
Ferrarotti *et al* [41], 2018	NCT03386877	Periodontal intrabony defects	RCT	Autologous dental pulp	Implanted into bone defect sites consisted of MIST	Pulp micrografts + Collagen sponge (n = 15)	Collagen sponge (n = 14)	6 and 12 mo	PD (↓); CAL (↓); bone defect fill (↑); residual PD < 5 mm and CAL gain ≥ 4 mm (↑)
Sánchez *et al*	ISRCTN13093912	Periodontal intrabony	CCT	Autologous	Implanted into bone	PDLSCs + β-TCP (n = 9)	β-TCP (n = 10)	1, 3, 6, 9, 12 mo	CAL (-); PPD (-)

[42], 2020		defects		periodontal ligament	defect sites *via* surgical approach				
Feng *et al*[43], 2010	None	Periodontal intrabony defects	Case series	Autologous periodontal ligament	Implanted into bone defect sites *via* surgical approach	PDLPs + HA/TCP (n = 3)	None	3, 6, 12, 32, 42, and 72 mo	CAL (↓); PD (↓); GR (↑)
Chen *et al*[29], 2016	NCT01357785	Periodontal intrabony defects	RCT	Autologous periodontal ligament	Implanted into bone defect sites *via* surgical approach	PDLSCs sheets + DBBM (n = 20)	DBBM (n = 21)	2 wk; 3, 6, 12 mo	CAL (-); PD (-); GR (-)
Iwata *et al*[44], 2018	UMIN000005027	Periodontal intrabony defects	Case series	Autologous periodontal ligament	Implanted into bone defect sites *via* surgical approach	PDL-derived cell sheets + β-TCP (n = 10)	None	3, 6, 55 ± 19 mo	CAL (↓); PD (↓); bone height (↑)
Vandana *et al* [125], 2015	None	Periodontal intrabony defects	A case report	Autologous periodontal ligament	Implanted into bone defect sites *via* surgical approach	Periodontal ligament soft tissue + Gelatin sponge + Cementum scrapings (n = 1)	None	1 wk; 3, 6, 12 mo	CAL (↓); PD (↓); BMD (↑)
Aimetti *et al* [47], 2014	None	Periodontal intrabony defects	A case report	Autologous dental pulp	Implanted into bone defect sites *via* surgical approach	Pulp micrografts + Collagen sponge (n = 1)	None	6 mo; 1 year	PPD (↓); bone fill (↑)
Aimetti *et al* [46], 2018	None	Periodontal intrabony defects	Case series	Autologous dental pulp	Implanted into bone defect sites *via* surgical approach	Pulp micrografts + Collagen sponge (n = 11)	None	1 year	CAL (↓); PD (↓); bone fill (↑)
Aimetti *et al* [49], 2015	None	Periodontal intrabony defects	Case series	Autologous dental pulp	Implanted into bone defect sites *via* surgical approach	Pulp micrografts + Collagen sponge (n = 4)	None	6, 12 mo	PD (↓); CAL (↓); bone fill (↑)
Hernández-Monjaraz *et al* [48], 2018	ISRCTN12831118	Periodontal intrabony defects	A case report	Allogeneic dental pulp	Implanted into bone defect sites *via* surgical approach	DPSCs + Lyophilized collagen-polyvinylpyrrolidone sponge scaffold (n = 1)	None	3, 6 mo	PD (↓); TM (↓); bone fill (↑)
Barbier *et al* [57], 2018	EudraCT database 2014-001913-18	Post-extraction sockets	Split-mouth RCT	Autologous dental pulp	Implanted into postextraction sockets	Pulp micrografts + collagen matrix (n = 30)	Collagen matrix (n = 30)	6 mo	BMD (-); interdental septum height (-)
Cubuk *et al* [62], 2023	NCT04641533	Post-extraction sockets	Split-mouth RCT	Autologous dental pulp	Implanted into postextraction sockets	Pulp micrografts + L-PRF (n = 13)	L-PRF (n = 13)	7 d; 6 mo	PPD (-); CAL (-); vertical bone loss (-); relative bone density (-)
d'Aquino *et al* [58], 2009	None	Post-extraction sockets	Split-mouth CCT	Autologous dental pulp	Implanted into postextraction sockets	Dental pulp stem/progenitor cells + collagen sponge (n = 7)	Collagen sponge (n = 7)	7 d; 1, 2, 3, 12 mo	Rate of mineralization (↑); levels of cortical bone (↑); CAL (↓); BMP-2, VEGF (↑)
Tanikawa *et al* [63], 2020	NCT03766217	Cleft lip and palate	Historical control study	Autologous deciduous pulp	Placed into the alveolar defect *via* surgical approach	SHED + Hydroxyapatite-collagen sponge (n = 6)	rhBMP-2 + Hydroxyapatite-collagen sponge (Group I n = 8); Iliac crest bone graft (Group II n = 8)	6, 12 mo	Bone filling percentage (↑, compared with Group I at the 6-mo follow-up)

Manimaran *et al*[59], 2014	None	Mandibular osteoradionecrosis	A case report	Allogeneic dental pulp	Inserted into the defect after surgical curettage	DPSCs + PRP + TCP (n = 1)	None	2, 6 mo	Bone formation (↑)
Manimaran *et al*[60], 2016	None	Bone defect left by the resection of mandibular ameloblastoma	A case report	Autologous dental pulp	Packed inside the mesh and placed over the mandible after tumor resection	DPSCs + β-TCP + PRF + SVF (n = 1)	None	1, 10 mo; 1.5 years	Bone regeneration (↑); no recurrence of tumor
Brunelli *et al* [61], 2013	None	Sinus lifting	A case report	Autologous dental pulp	Implanted into sinus cavity	Pulp micrografts + Collagen sponge (n = 1)	None	4 mo	BMD (↑)
Koga *et al*[64], 2022	None	Erectile dysfunction	Case series	Allogeneic deciduous pulp	Injected into the penis	SHED-CM (n = 38)	None	After every injection	IIEF-5 score (↑)
Silva *et al*[65], 2022	NCT02728115	Huntington's disease with preexisting pulmonary nodule	A case report	Allogeneic deciduous pulp	Intravenous administrations	SHED (n = 1)	None	15, 30 d; 7, 24, 32 mo	Unified Huntington's disease rating scale (↓); not show long-term tropism or homing for the lung adenocarcinoma
Wang *et al*[93], 2010	None	Plaque psoriasis	A case report	Allogeneic gingival	Bolus injection	GMSCs (n = 1)	None	3 years	Psoriatic lesions fully cleared; no recurrence
Suda *et al*[67], 2022	NCT04608838; JapicCTI194570	Acute ischemic stroke	Study protocol	Allogeneic dental pulp	Intravenous administration	DPSCs	Placebo	Per 15 min (1-4 h); per 30 min (4-6 h); 12, 24 h; 2, 3, 8, 31, 91, 181, 366 d	No results
Nagpal *et al* [68], 2016	None	Chronic disability after stroke	Study protocol	Autologous dental pulp	Implanted into peri-infarct region *via* neurosurgical procedure	DPSCs	None	1, 6, 9, 12 mo	No results
Ye *et al*[69], 2020	ChiCTR2000031319: NCT04336254	COVID-19	Study protocol	Allogeneic dental pulp	Intravenous administration	DPSCs	Saline	2 h ± 30 min; 24 h ± 30 min; 90 d ± 3 d	No results

BMD: Bone mineral density; CAL: Clinical attachment level; CCT: Controlled clinical trials; COVID-19: Coronavirus disease 2019; DBBM: Deproteinized bovine bone mineral; GR: Gingival recession; G-CSF: Granulocyte colony stimulating factor; HA/TCP: Hydroxyapatite/tricalcium phosphate; IIEF: International index of erectile function; L-PRF: Leukocyte-platelet rich fibrin; MIST: Minimally invasive surgical technique; MRI: Magnetic resonance imaging; PD: Probing depth; PDL: Periodontal ligament; PDLPs: Periodontal ligament progenitor cells; PPD: Periodontal probing depth; PRF: Platelet rich fibrin; PRP: Platelet-rich plasma; PEG-PLGA: Poly (lactide-co glycolide)-polyethylene glycol; RCT: Random clinical trial; rh-BMP: Recombinant human bone morphogenetic protein; SI: Signal intensity; SVF: Stromal vascular fraction: TCP: Tricalcium phosphate; TM: Tooth mobility; VEGF: Vascular endothelial growth factor; SHED-CM: Stem cells from exfoliated deciduous teeth conditioned medium.

from autologous inflamed dental pulp and blood, respectively, to the root canal of irreversible pulpitis teeth and observed dentin bridge formation and a response to the cold test and electric pulp test.

Periapical lesions

In a case report and two case series, SCAP/SHED combined with a polyethylene glycol polylactic-polyglycolic acid (PEG-PLGA) scaffold and SHED combined with bioglass were used for the treatment of periapical lesions[38-40] (Table 2). Periapical tissue healing was found in the follow-up examinations

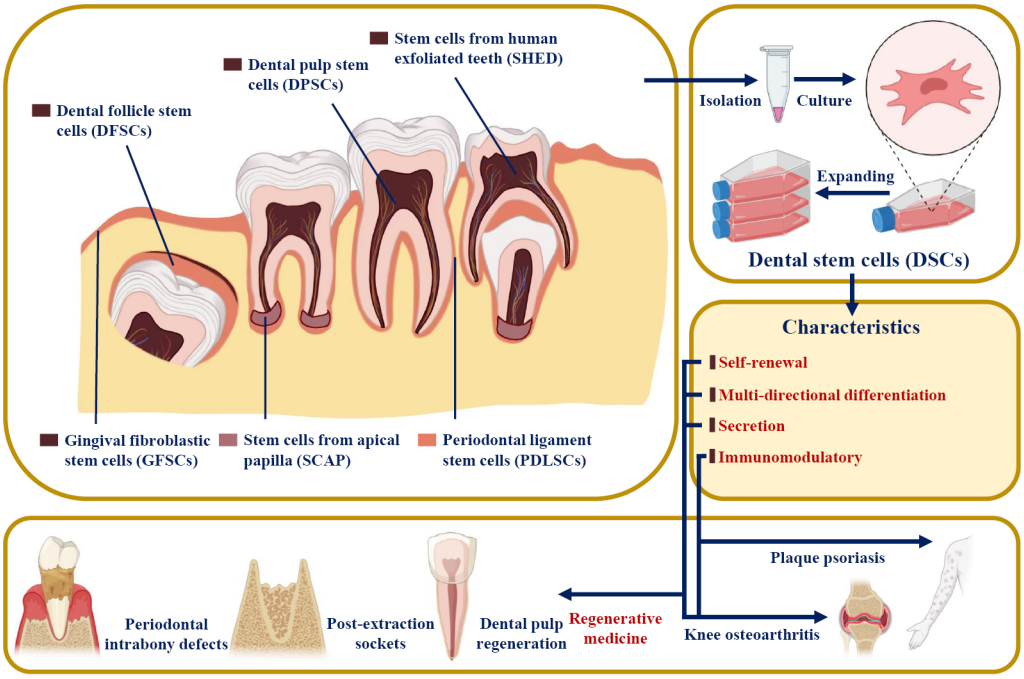


Figure 1 Tissue origin, harvest, characteristics, and clinical application potential of the various populations of dental stem cells. Dental pulp stem cells and stem cells from exfoliated deciduous teeth can be isolated from the inner dental pulp of permanent teeth and deciduous exfoliated teeth, respectively. Stem cells from apical papilla can be extracted from the apical papilla; periodontal ligament stem cells can be harvested from the periodontal ligament; and dietary fiber supplementation combinations can be derived from the dental follicle. Gingiva-derived mesenchymal stem cells can be extracted from gingiva. Citation: Sharpe PT. Dental mesenchymal stem cells. *Development* 2016; 143: 2273-2280[139]. Copyright ©The Authors 2016. Published by The Company of Biologists Ltd. The authors have obtained the permission for figure using from the Company of Biologists Ltd (Supplementary material).

of all three studies. It was reported a positive response in the test of dental pulp activity after SHED transplantation, suggesting the regeneration of pulp or pulp-like tissue, which does not occur in traditional root canal therapy[39,40].

Periodontal intrabony defects

There are two RCTs, a controlled clinical trial (CCT), three case series, and two case reports of DSC-based treatment for periodontal intrabony defects[29,41-46] (Table 2). The RCT of Ferrarotti *et al*[41] indicated that pulp micrografts applied with collagen sponges could significantly reduce PD and CAL and promote the regeneration of bone defects when compared with collagen sponges alone. Three case series and a case report using pulp micrografts/DPSCs and collagen sponges also reported similar results of periodontal benefits[46-49]. It was reported a novel approach using periodontal ligament soft tissue, gelatin sponges, and cementum scrapings, which reduced the CAL and PD of periodontitis teeth in their case report[45].

Although two case series demonstrated the periodontal benefits of PDLPs and PDL-derived cell sheets[43,44], significant differences in periodontal indices (including PD and CAL) were not observed between the test groups and control groups in the other two CCTs that applied PDLSC and PDLSC sheets[29,42]. Several factors might have contributed to the lack of significant differences in the outcomes, such as satisfactory scaffold material properties and small sample sizes. In these four studies, β-TCP, HA/TCP, and deproteinized bovine bone mineral (Bio-oss®) were applied as scaffold materials. Although some studies reported abilities to provide support for PDLSCs on osteogenic differentiation of these scaffolds*in vitro* and *in vivo*[50-53], only using these scaffolds also achieved great clinical benefits in the treatment of periodontitis[54-56]. The excellent performance of the scaffold may have overshadowed the contribution by PDLSCs. More clinical studies at multiple centers with different amounts and types of DSCs, more follow-up time points, and larger sample sizes are necessary, and the results of such studies would be meaningful.

Bone defects caused by other conditions

In addition to periodontal intrabony defects, DSCs were also used for the treatment of post-extraction sockets, mandibular osteoradionecrosis, bone defects after ameloblastoma resection, and sinus lifting [57-61] (Table 2). Two split-mouth RCTs reported by Barbier *et al*[57] and Cubuk *et al*[62] did not find significant differences in BD or interdental septum height between the pulp micrograft + scaffold (collagen matrix/L-PRF) group and the scaffold (collagen matrix/L-PRF) group after implantation into post-extraction sockets. However, in another split-mouth CCT designed for regenerating post-extraction

sockets, DPSCs combined with collagen sponges promoted the rate of mineralization, the levels of cortical bone, and the expression of bone morphogenetic protein-2 (BMP-2) and VEGF when compared with collagen sponge treatment alone[58]. Tanikawa *et al*[63] reported a historical control study comparing the effects of SHED, rhBMP, and iliac crest bone grafts in treating cleft lip and palate. The SHED group showed similar satisfactory performance in bone healing compared with iliac crest bone grafts and a higher bone filling percentage compared with the rhBMP group at the 6-mo follow-up[63].

Two case reports indicated that DPSCs combined with TCP could increase the bone regeneration of bone defects caused by osteoradionecrosis and ameloblastoma[59,60]. A case report by Brunelli *et al*[61] demonstrated that pulp micrografts + collagen sponges increased the BD in newly formed bone when applied for sinus lifting.

Other conditions

Koga *et al*[64] reported a case series that applied SHED conditioned medium (SHED-CM) to treat erectile dysfunction. In this study, the international index of erectile function (IIEF-5), which is clinically used to screen for erectile function and to assess treatment efficacy, was increased after SHED-CM injection into the corpus cavernosum of erectile dysfunction patients[64]. A case report indicated that SHED intravenous administrations could decrease the scale of unified Huntington's disease rating, which is designed to assess clinical performance and capacity in patients with Huntington's disease[65, 66]. Meanwhile, the patient with Huntington's disease also suffered from preexisting pulmonary nodules, and SHED injection did not result in long-term tropism or homing for the patient's lung adenocarcinoma[65]. In a case report by Wang *et al*[30], GMSCs were used to treat plaque psoriasis *via* bolus injection, and they observed fully cleared psoriatic lesions without recurrence.

Three clinical study protocols using DSCs have been published in recent years, including the treatment of acute ischemic stroke, chronic disability after stroke, and COVID-19[67-69].

DSC-BASED CLINICAL TRIALS FROM CLINICAL DATABASES

ClinicalTrials.gov (https://clinicaltrials.gov/) and the International Clinical Trials Registry Platform (ICTRP, https://trialsearch.who.int/) were screened for DSC-based clinical trials.

To date, there have been 21 clinical trials registered on ClinicalTrials.gov evaluating the use of DSCs in treating periodontitis (33.3%, 7/21), post-extraction sockets (4.8%, 1/21), edentulous alveolar ridge (4.8%, 1/21), cleft lip and palate (9.5%, 2/21), knee osteoarthritis (4.8%, 1/21), dental pulp necrosis (4.8%, 1/21), liver cirrhosis (4.8%, 1/21), type 1 diabetes (4.8%, 1/21), acute ischemic stroke (4.8%, 1/21), Huntington's disease (14.3%, 3/21), and COVID-19 (9.5%, 2/21) (Table 3). In addition to the 6 studies reported in ClinicalTrials.gov, 7 clinical trials were registered on the ICTRP using DSCs in the treatment of periodontitis (57.1%, 4/7), wrinkles (28.6%, 2/7), and hair loss (14.3%, 1/7) (Table 4). In all, 28 clinical trials were registered on these two platforms.

Several registered clinical trials applied two stages in one work. The most frequently appearing trial phases were phase 1 (42.9%, 12/28), followed by phase 2 (25%, 7/28), Phase 3 (7.1%, 2/28), and Phase 0 (3.6%, 1/28). There were 10 trials (35.7%) in which the phase design was not applied or not selected. One clinical trial reported the outcomes both on the registry platform and in a published article[63] (NCT01932164), and the published articles of seven trials stated the registered ID[29,42,62,63,67,69-71], while other trials did not publish any data.

Consistent with the literature, the proportion of clinical trials using DSCs to treat periodontitis was the highest. Eleven registered clinical trials researched the effect of DSCs on periodontitis (39.3%, 11/28). In these trials, various amounts, types, and injection times of DSCs and different application modes (such as DSCs, micrografts, cell sheet pellets, and cell sheet fragments) were applied. In addition, several scaffolds were used in combination with DSCs, including collagen sponges, deproteinized bovine bone minerals, β-TCP scaffolds, and hydroxyapatite-collagen scaffolds.

SAFETY ISSUES REGARDING DSC-BASED THERAPY

Although encouraging treatment effects on diseases have been achieved, the safety issues of stem cell-based therapy remain controversial, especially in long-term follow-up[72]. At present, the limitations of stem cell-based therapy are mainly focused on non-directional differentiation, accelerating tumor progression.

In addition, uncontrolled non-directional differentiation may have a great impact on the safety of stem cell transplantation. Breitbach *et al*[73] found that the encapsulated structures in the infarcted areas contained calcifications and/or ossifications in myocardial infarction mice after MSC injection. In another study, unselected bone marrow cells injected directly induced significant intramyocardial calcification in acutely infarcted myocardium[74].

Table 3 Dental stem cell-based clinical trials registered at clinicaltrials.gov

Ref.	Registration ID	Status	Diseases	Study design	Cell source	Administration route	Number of patients	Interventions		Follow-upperiod	Phase	Outcomes
								Test group	Control group			
-	NCT04983225	Recruiting	Periodontitis	Randomized; parallel assignment; double-blind (participant, investigator)	Dental pulp	Injecting into the periodontal defect site	36	DPSCs (1×10^6)/site; DPSCs (5×10^6)/site; DPSCs ($3\text{-}4 \times 10^7$)/three or four sites; DPSCs (1×10^7)/site; DPSCs (2×10^7)/two sites	Saline solution	90, 180, 360, 720 d	Phase 1	
-	NCT02523651	Unknown	Periodontitis	Randomized; parallel assignment; triple-blind (participant, investigator, outcomes Assessor)	Allogeneic dental pulp	Injecting into the periodontal defect site	40	DPSCs (1×10^6)	Saline solution	1 year	Phase 1/2	
-	NCT03386877	Completed	Periodontitis	Randomized; parallel assignment; triple-blind (participant, investigator, outcomes assessor)	Autologous dental pulp	Delivering into intrabony defect *via* minimally invasive surgical technique	29	Micrografts of DPSCs + Collagen sponge	Collagen sponge	6, 12 mo	Not applicable	
-	NCT01082822	Unknown	Periodontitis	Nonrandomized; parallel assignment; open label	Periodontal ligament	Implanted into bone defect sites *via* surgical approach	80	PDLSCs sheet fragment + DBBM (Bio-oss); PDLSCs sheet pellets + DBBM (Bio-oss); DBBM (Bio-oss)	Sham comparator	4, 12, 24 wk; 1 year	Phase 1/2	
-	NCT03638154	Completed	Periodontitis	Randomized; parallel assignment; double-blind (care provider, outcomes assessor)	Gingival	Implanted into bone defect sites *via* surgical approach	20	GFs + GMSCs + β-TCP	β-TCP	1, 3, 7, 14 d; 6 mo	Not applicable	
-	NCT03137979	Unknown	Periodontitis	Randomized; parallel assignment	Gingival	Implanted into bone defect sites *via* surgical approach	30	GMSCs + Collagen scaffolds; collagen scaffolds	Open flap debridement	1, 3, 6 mo	Phase1/2	
Chen *et al* [29], 2016	NCT01357785	Unknown	Periodontitis	Randomized; parallel assignment; open label	Autologous periodontal ligament		35		None	3-12 mo	Phase1	
Cubuk *et al* [62], 2023	NCT04641533	Completed	Post-extraction sockets	Split-mouth; randomized; crossover assignment; double-blind (investigator, outcomes assessor)	Dental pulp	Placing into the extraction socket	13	DPSCs + L-PRF	L-PRF	7 d; 6 mo	Not applicable	
-	NCT02731586	Unknown	Edentulous alveolar ridge	Single group assignment; open label	Allogeneic dental pulp	Introducing dental pulp-derived mesenchymal stem cells during placement of dental implants	10	Dental pulp-derived MSCs	None	3 mo	Early Phase 1	

Tanikawa *et al*[63], 2020; Pinheiro *et al* [70], 2019	NCT03766217	Completed	Cleft lip and palate	Randomized; parallel assignment; single-blind (outcomes assessor)	Autologous deciduous pulp	Placed into the alveolar defect *via* surgical approach	62	SHED + Hydroxyapatite-collagen sponge	Iliac crest autogenous bone graft	15 d; 3, 6, 12 mo	Phase3	
Tanikawa *et al*[63], 2020	NCT01932164	Completed; Has results	Cleft lip and palate	Single group assignment; open label	Autologous deciduous pulp	Maxillary alveolar graft by tissue engineering	5	SHED + Hydroxyapatite-collagen sponge	None	3, 6 mo	Not applicable	Percentage of bone filling at 6 mo postoperatively: 89.5%
-	NCT04130100	Unknown	Knee osteoarthritis	Randomized; parallel assignment; open label	Dental pulp	Intraarticular injection	60	Low dose of DPSCs; high dose of DPSCs	Sodium hyaluronate	12 mo	Early phase 1	
-	NCT01814436	Unknown	Dental pulp necrosis	Single group assignment;open label	Autologous deciduous pulp		80	Scaffold-free SHED-derived pellet	None	3-12 mo	Not applicable	
-	NCT03957655	Unknown	Liver cirrhosis	Randomized; parallel assignment; single-blind (outcomes assessor)	Autologous deciduous pulp	Peripheral vein infusion	40	SHED (1×10^6 cells/kg body weight)	Standard medication for viral hepatitis and cirrhosis	4, 8, 12, 16, 24 wk	Early phase 1	
-	NCT03912480	Unknown	Type 1 diabetes	Single group assignment; open label	Deciduous pulp	Intravenous drip	24	SHED (0.11 IU/kg body weight) + Insulin + oral hypoglycemic drugs	None	1, 2, 6 wk; 2, 3, 6, 9, 12 mo	Early phase 1	
Suda *et al* [67], 2022	NCT04608838	Completed	Acute ischemic stroke	Randomized;Parallel assignment;Quadruple-blind (Participant, Care Provider, Investigator, Outcomes Assessor);	Allogeneic dental pulp	Intravenously infusion	79	DPSCs (JTR-161, 1×10^8 cells); DPSCs (JTR-161, 3×10^8 cells)	Placebo	91, 366 d	Phase 1/2	
-	NCT02728115	Active, not recruiting		Nonrandomized; parallel assignment; open label	Allogeneic deciduous pulp	Intravenous administration	6	SHED (Cellavita HD, 1×10^6 cells); SHED (Cellavita HD, 2×10^6 cells)	None	1, 4 years	Phase 1	
-	NCT04219241	Active, not recruiting	Huntington's disease	Single group assignment; open label	Allogeneic deciduous pulp	Intravenous administration	35	SHED (Cellavita HD, 2×10^6 cells)	None	1, 2 years	Phase 2/3	
Wenceslau *et al*[71], 2022	NCT03252535	Completed	Huntington's disease	Randomized; parallel assignment; triple-blind (participant, investigator, outcomes assessor)	Allogeneic deciduous pulp	Intravenous administration	35	SHED (Cellavita HD, 1×10^6 cells); SHED (Cellavita HD, 2×10^6 cells)	Physiological solution without cells	Monthly for 14 mo	Phase 2	
Ye *et al*[69], 2020	NCT04336254	Recruiting	COVID-19	Randomized; parallel assignment; triple-blind (participant, investigator, outcomes assessor)	Allogeneic dental pulp	Intravenous injection	20	DPSCs (3×10^7 cells)	Saline	28 d	Phase 1/2	
-	NCT04302519	Unknown	COVID-19	Single group assignment; open label	Dental pulp	Intravenous injection	24	DPSCs (1×10^7 cells/kg body weight)	None	3, 7, 14, 28, 360 d	Early phase 1	

DBBM: Deproteinized bovine bone mineral; GFs: Gingival fibroblast; PRF: Platelet-rich fibrin; TCP: Tricalcium phosphate; DPSCs: Dental pulp stem cells; SHED: Stem cells from exfoliated deciduous teeth.

Similar to the regeneration of damaged tissue, tumors exert chemotactic effects on MSCs, affecting their recruitment to tumor sites[75-77]. Current studies have shown that MSCs have bidirectional, anti-cancer and pro-cancer, regulatory effects, which raises safety concerns for clinical application. On the one hand, MSCs are the major component of the tumor microenvironment and can be reprogrammed to the pro-tumorigenic phenotype by the tumor[78]. MSCs have been revealed to participate in the initiation, development, progression, and metastasis of multiple cancers[79]. The pro-cancer effect of stem cells may be achieved by secreting molecules that affect the phenotype of tumor cells, promoting tumor angiogenesis, cancer-associated fibroblast differentiation, cell-to-cell contact, or cell engulfment [76]. In recent studies, DPSCs and their conditioned medium were reported to promote the proliferation and carcinogenic properties of prostate cancer, oral cancer, breast cancer, and melanoma cells*in vitro*[80-82].

On the other hand, there is also evidence that MSCs can inhibit the growth of a variety of tumors, including breast cancer, Kaposi's sarcoma, hepatoma, glioma, and melanoma[76,83-85]. DPSCs and their conditioned medium also showed a suppressive effect on the development and migration of colorectal cancer cells through mitogen-activated protein kinase pathways[86]. In fact, there are few reports of primary pulp malignancies[87]. In a genome-wide RNA-seq study, phosphatase and tensin homolog (PTEN) expression in DPSCs was higher than that in BMSCs[88]. PTEN, a phosphatase, can metabolize phosphatidylinositol 3,4,5-triphosphate and directly oppose the activation of the oncogenic PI3K/AKT/mTOR signaling network[89]. At present, the regulatory effects of stem cells on cancer are still controversial, and the difference in results may be related to cell lines, cell doses, animal models, cancer types, treatment duration time, and other factors.

In conclusion, no adverse events were reported in the published clinical articles or clinical trials using DSCs, which suggested the safety of DSC-based therapy. However, based on current concerns about the safety of stem cell therapy, more *in vivo* studies on the safety of DSC-based therapies are of great significance.

CURRENT LIMITATIONS AND PERSPECTIVES

Harvesting DSCs from inflamed tissue

Most studies applied stem cells extracted from healthy dental tissue for treatment, but additional surgery (such as third molar extraction) might increase patient suffering. Harvesting stem cells from inflamed dental tissue could be an alternative method, although stem cell abilities might be affected[36,90,91].

Several studies have researched the different biological properties of DPSCs derived from normal and inflamed pulps (iDPSCs), and the results are still in dispute[92-98]. In some studies, DPSCs showed better self-renewal ability[92,93] and multidirectional differentiation capacities than iDPSCs[92], while in other studies, no significant difference was observed[94,95,98]. A study by Nie *et al*[97] indicated that DPSCs showed higher colony-forming, proliferative, and osteo/dentinogenesis abilities, while iDPSCs

Table 4 Dental stem cell-based clinical trials registered on the International Clinical Trials Registry Platform

Ref.	Registration ID	Status	Diseases	Study design	Cell source	Administration route	Number of patients	Interventions		Follow-up period	Phase	Outcomes
								Test group	Control group			
-	JPRN-UMIN000042791	Complete: Follow-up complete	Periodontitis	Randomized; parallel assignment; single-blind (participants)	Deciduous pulp	Gargle	30	Mouthwash containing SHED culture supernatant	Mouthwash without SHED culture supernatant	1 mo	Not applicable	
-	ChiCTR2100051466	Recruiting	Periodontitis	Randomized; parallel assignment; open label	Dental pulp	Bilateral multipoint injection on a single tooth	96	DPSCs (1×10^7 cells) for once; DPSCs (1×10^7 cells) for twice	Saline	90, 180, 360 d	Phage 0	
-	ChiCTR2100049178	Pending	Periodontitis	Randomized; parallel assignment; double-blind	Dental pulp	Local injection	36	DPSCs (1×10^6 cells) for single injection; DPSCs (5×10^6 cells) for single injection; DPSCs (1×10^7 cells) for single injection; DPSCs (1×10^7 cells) for single injection in 2 locations; DPSCs (1×10^7 cells) for single injection in 3-4 locations	None		Phage 1	
Sánchez *et al* [42], 2020	ISRCTN13093912	Completed	Periodontitis	Randomized; parallel assignment; single-blind (patients and examiners)	Dental pulp	Implanted into bone defect sites *via* surgical approach	20	DPSCs (1×10^7 cells) + hydroxyapatite-collagen scaffold	Hydroxyapatite-collagen scaffold	1, 2, 4, 12, 24, 36 wk; 12, 24, 36, 48, 60 mo	Not applicable	
-	JPRN-UMIN000045926	Complete: Follow-up complete	Wrinkles	Randomized; parallel assignment; single-blind (outcomes assessor)	Dental pulp		12	All-in-one gel containing immortalized DPSCs-CM solution and various beauty ingredients	No treatment	4 wk	Not applicable	
-	JPRN-UMIN000043528	Complete: Follow-up complete	Wrinkles	Randomized; parallel assignment; single-blind (outcomes assessor)	Dental pulp		12	All-in-one gel containing immortalized DPSC-CM solution and the latest peptide raw materials	No treatment	4 wk	Not applicable	
-	JPRN-UMIN000045897	Complete: Follow-up continuing	Hair loss	Nonrandomized; parallel assignment; open label	Deciduous pulp	Injection	22	SHED-CM; after SHED-CM injection, one dose of micrografts (Rigenera) followed by another SHED-CM injection; SHED-CM injection after one dose of micrografts (Rigenera)	None	6 mo	Not applicable	

DPSCs: Dental pulp stem cells; SHED-CM: Stem cells from exfoliated deciduous teeth conditioned medium.

demonstrated enhanced chondrogenesis, neurogenesis, angiogenesis, and adipogenesis capacities. Park *et al*[96] reported that iDPSCs appear to have higher osteogenic differentiation potential and lower neurogenic differentiation potential than DPSCs.

Differences in inflammation levels may explain the discrepancy in the biological properties of DPSCs and iDPSCs in various studies. Intense and rapid inflammatory stimulation irreversibly initiates pulp necrosis, while low insult levels of inflammation are able to cause reversible pulpitis and promote dentine regeneration[99]. DPSCs are a suitable source of stem cells for pulp nerve regeneration because of their neuronal differentiation potential. It was reported that acute inflammation with a high level of proinflammatory cytokines could reduce neural precursor cell (NPC) survival and inhibit the neuronal differentiation of NPCs, while chronic inflammation expressed a potentially neuroprotective phenotype and supported neuronal differentiation[100]. Meanwhile, age, sex, tooth position, and sample size are also confounding factors affecting the function of DPSCs, which should be considered in subsequent studies and clinical practice.

DSC-CM and DSC-EVs

The culture medium collected from cells in culture is known as CM. CM is applied as an alternative therapy for tissue regeneration, which is a less ethical issue because it uses cells indirectly. Koga *et al*[64] applied SHED-CM in the treatment of erectile dysfunction, which is the only record of its clinical use to the best of our knowledge.

DSC-CM contains a variety of cytokines associated with vascular and nerve tissue regeneration, such as VEGF, BDNF, β-NGF, GDNF and neurotrophin-3 (NT-3)[101,102]. To date, DSC-CM has been reported to have the potential to promote bone regeneration[103], periodontal regeneration[104], angiogenesis[105], pulp regeneration[106], and nerve protection/regeneration[105,107-109] with great possibilities for clinical application.

In addition, DSC-CM showed satisfactory anti-inflammatory and immunoregulatory effects. Several *in vivo* studies based on various animal models reported that intravenous injection or intranasal administration of SHED-CM improved liver fibrosis[110], acute liver failure[111], acute lung injury [112], Alzheimer's disease, temporomandibular joint osteoarthritis[113], Sjögren's syndrome[114], and rheumatoid arthritis[115] by exerting anti-inflammatory effects. Meanwhile, studies have also reported the effect of SHED-CM on promoting Treg cell differentiation[114] and M2-like macrophage induction [111,112], as well as inhibiting Th17 cell differentiation[114] and inflammatory macrophage activation [116].

In addition to DSC-CM, DSC-EVs harvested from cell-culture medium have also been deeply studied in recent years. Multiple studies have indicated the promotion effect of DSC-EVs on jawbone and calvarial bone regeneration[117,118], angiogenesis and cutaneous wound healing *in vivo*[119,120]. Li *et al* [121] also reported that DSC-EVs could alleviate cerebral ischemia-reperfusion by suppressing the inflammatory response, which is related to the inhibition of the HMGB1/TLR4/MyD88/NF-κB pathway.

The poor survival rate of implanted DSCs and host immunogenic reactions are the main drawbacks of applying DSCs directly. In some comparative studies, stem cell-derived CM showed similar and even better treatment effects on acute lung injury, Parkinsonism, and type 1 diabetes than the direct use of stem cells[112,122,123]. DSC-CM and its components (such as EVs) provide several key advantages over cell-based applications, including avoiding the risk of host immunogenic reactions, cost-effectiveness, long-term storage capacity, and simpler evaluation of safety and efficacy[104,124]. Accumulating evidence indicates the great potential of DSC-CM/DSC-EV-based treatment in clinical applications.

Expanding-free strategy

Despite encouraging results of differentiation and tissue regeneration, DSCs still require rigorous cell-expanding procedures to obtain a sufficient number of cells for treatment, which is costly with great technique sensitivity, often taking tens of days. The *ex vivo* expansion of stem cells often reduces their self-renewal and proliferation abilities[125]. Direct mechanical digestion or tissue transplantation are promising solutions to these limitations.

In recent years, using mechanical disaggregation of dental tissues instead of cell-expending procedures was successful for harvesting autologous pulp micrografts rich in progenitor cells[41,126]. In 2016, Monti *et al*[126] indicated that DSCs harvested by mechanical digestion (Rigenera® system, HBW, Turin, Italy) were fully comparable to stem cells obtained after enzymatic digestion. In this study, mechanical digestion-obtained DPSCs showed osteogenic, adipogenic, and chondrogenic differentiation abilities*in vitro* and were able to increase the regeneration of post-extraction sockets *in vivo* when applied with the collagen sponge[126].

Pulp micrografts harvested by mechanical digestion were also applied in the treatment of sinus lifting, post-extraction sockets, and periodontal intrabony defects[46,47,49,57,61,62]. One clinical trial using pulp micrografts was also designed for periodontitis management (NCT03386877), but the outcome was not reported. Different systems of mechanical disaggregation were applied in these studies, including BD Medimachine (BD Biosciences San Jose, CA, United States)[62], the Rigenera® system (HBW, Turin, Italy)[46,57,61], and the Medimachine System (Consul TS, Orbassano, Italy)[47, 49]. In brief, dental pulp is first collected from extracted teeth and then sent to the mechanical disaggregation system to obtain pulp micrografts. After filtration or without filtration, pulp micrografts are combined with the scaffold for transplantation.

In addition, Vandana *et al*[125] described a novel approach using stem cell assistance in the periodontal regeneration technique (SAI-PRT), which contained periodontal ligament soft tissue gelatin sponge scaffolds and cementum scrapings. In their research, SAI-PRT successfully bypassed*in vitro* culture and expanded PDLSCs, resulting in satisfactory defect filling of periodontal intrabony defects [125].

Embryonic stem cells, PSCs, and DSCs

Embryonic stem cells (ESCs) are pluripotent cells of great significance to developmental biology. They give rise to all types of germ layer cells in the embryo. The self-renewal ability and plasticity of ESCs make it possible to generate unlimited numbers of different types of cells *in vitro*[127]. Similar to embryonic cells, PSCs derived from different somatic cells also have the ability to immortalize and differentiate into the three germ layers[128]. The properties of these two cell types make them promising sources for stem cell-based therapy for various diseases and injuries. However, due to the limitations of ESCs and PSCs, adult stem cells (such as DSCs) still possess high application value.

First, ethical issues regarding the use of ESCs make their clinical application challenging[128]. Second, the preparation of autologous PSCs takes a long time (more than 3 mo) and has high medical cost, and the immune rejection issue of allotransplantation should be considered[129]. In addition, teratomas are germ cell tumors containing cells of two or three germ lines that always occur *via* uncontrollable stem cell proliferation and differentiation[130,131]. In experimental studies, stem cell transplants (especially ESC and PSC transplants) have been found to increase the risk of teratomas, raising safety concerns[131-133]. Previously, viral vector integration and contamination of animal-derived components also posed obstacles to the use of PSCs, but these problems have been addressed by innovative techniques, such as integration-free methods and xeno-free culture[134-136].

DSCs did not show unlimited proliferation potential and demonstrated poorer differentiation ability than PSCs and ESCs[137]. However, the advantages of DSCs over ESCs and PSCs, such as fewer ethical issues and lower teratoma risk[87,88,138], lower cost and shorter preparation period, harvesting from medical waste, and implementing therapeutic effects without gene editing, grant them greater potential for clinical applications in the future.

CONCLUSION

Many clinical articles and clinical trials of autologous and allogeneic DSCs have aimed to evaluate their therapeutic effects on various diseases, such as pulpitis, periapical lesions, periodontitis, cleft lip and palate and Huntington's disease. In most studies, satisfactory clinical treatment results were obtained, while clinical benefits of using DSCs were not found in some research. Although safety risks exist for stem cell-based therapies, safety issues have not been reported in the clinical applications of DSCs. In the future, in addition to continuing to study the efficacy and safety of DSC-based treatment, harvesting DSCs from inflammatory tissues, expanding-free strategies, and applying DSC-CM or DSC-EVs should be studied, as they have strong research value and application potential. Taken together, DSC-based therapy is a promising tool for the treatment of various diseases and can be further promoted.

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FOOTNOTES

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REVIEW

Immunomodulation: The next target of mesenchymal stem cell-derived exosomes in the context of ischemic stroke

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Abstract

Ischemic stroke (IS) is the most prevalent form of brain disease, characterized by high morbidity, disability, and mortality. However, there is still a lack of ideal prevention and treatment measures in clinical practice. Notably, the transplantation therapy of mesenchymal stem cells (MSCs) has been a hot research topic in stroke. Nevertheless, there are risks associated with this cell therapy, including tumor formation, coagulation dysfunction, and vascular occlusion. Also, a growing number of studies suggest that the therapeutic effect after transplantation of MSCs is mainly attributed to MSC-derived exosomes (MSC-Exos). And this cell-free mediated therapy appears to circumvent many risks and difficulties when compared to cell therapy, and it may be the most promising new strategy for treating stroke as stem cell replacement therapy. Studies suggest that suppressing inflammation *via* modulation of the immune response is an additional treatment option for IS. Intriguingly, MSC-Exos mediates the inflammatory immune response following IS by modulating the central nervous system, the peripheral immune system, and immunomodulatory molecules, thereby promoting neurofunctional recovery after stroke. Thus, this paper reviews the role, potential mechanisms, and therapeutic potential of MSC-Exos in post-IS inflammation in order to identify new research targets.

Key Words: Mesenchymal stem cells; Exosomes; Ischemic stroke; Immunomodulation; Inflammation; Exosome therapy

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Core Tip: Mesenchymal stem cell-derived exosomes (MSC-Exos) are an emerging strategy for treating ischemic stroke (IS) and have demonstrated certain achievements in animal studies. Here, we review and discuss the mechanisms of MSC-Exos in treating IS through immunomodulation, the current responses to the clinical limitations of MSC-Exos therapy, and the issues that need to be addressed in future MSC-Exos research.

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INTRODUCTION

Stroke is one of the leading causes of death and permanent disability on a global scale; ischemic stroke (IS) accounts for approximately 80% of stroke cases[1]. Currently, the mainstay of acute treatment for IS is limited to reperfusion by intravenous recombinant tissue fibrinolytic activator (tPA, thrombolysis) and rapid recanalization utilizing devices (thrombectomy)[2]. In clinical practice, however, the thrombolytic treatment conditions are strictly limited to presentation within 4.5 h of symptom onset[3]. Although the therapeutic window for thrombectomy has been extended to 24 h, there may be a risk of cerebral hemorrhage, occlusion after revascularization, and over-perfusion brain injury[4,5]. In recent years, other treatments that researchers have actively explored have also been prevented from being implemented on a large scale in clinical practice due to a variety of disadvantages. For instance, hypothermia treatment may reduce body metabolism while affecting neuronal death mechanisms, resulting in increased immunosuppression and susceptibility to infectious complications[6]. Prophylactic antibiotic treatment can decrease the incidence of infectious complications. However, antibiotic therapy is targeted, and broad-spectrum antibiotics can affect the body's normal flora if they are misused, which may increase organismal resistance[7]. By 2050, there will be more than 200 million stroke survivors and almost 300 million disability-adjusted life-years, 25 million new strokes, and 13 million deaths from stroke annually[1]. Therefore, there is a pressing need to discover effective treatments for IS that can be administered on a large clinical scale.

In acute stroke management, time is brain. The focus of stroke research should be on extending the time window for treatment. Examples include early measurement of immune biomarkers[8], improved efficiency of pre-hospital emergency transport[9], improved levels of care[10], and stem cell transplantation therapy[11]. Among these, stem cell transplantation therapy, which can extend the treatment window for IS to seven days, has become a hot research topic[11]. This also offers promising treatment options for patients outside the golden treatment period. MSCs are among the most hopeful candidates for stem cell therapy compared to other types due to their comprehensive source, ease of culture, pluripotent differentiation, immune tolerance, high survival rate, and strong paracrine effects [11-13]. It has previously been proved that nutrient factors and extracellular vesicles (EVs) secreted in situ by stem cells after transplantation enter the damaged brain and exert immunomodulatory, neuroprotective, angiogenic, and neural restructuring effects[13,14]. This phenomenon is known as the paracrine response (also called the "bystander" effect) and is the main mechanism by which stem cell's function. In comparison, exosomes are key effectors in the paracrine response of stem cells[14]. Mesenchymal stem cell-derived exosomes (MSC-Exos) therapy applied to stroke is superior to cell therapy in biodistribution, stability, safety, and development potential while ensuring therapeutic efficacy as an alternative therapy to stem cells.

In addition to the problem of a narrowing treatment window, the poor prognosis of IS is another pressing issue. Immunosuppression is the important cause of IS patients' poor prognosis and increased susceptibility. The inflammatory response underlies ischemic tissue damage. MSC-Exos, a highly promising treatment modality for brain injury, can effectively reduce neuroinflammatory reactions by modulating the immune system to promote recovery[15,16]. This paper reviews and discusses the immunomodulatory effects of MSC-Exos at the cellular and molecular levels following IS, as well as its application in therapy, in order to serve as a reference for future research and treatment.

IMMUNE RESPONSE AFTER IS

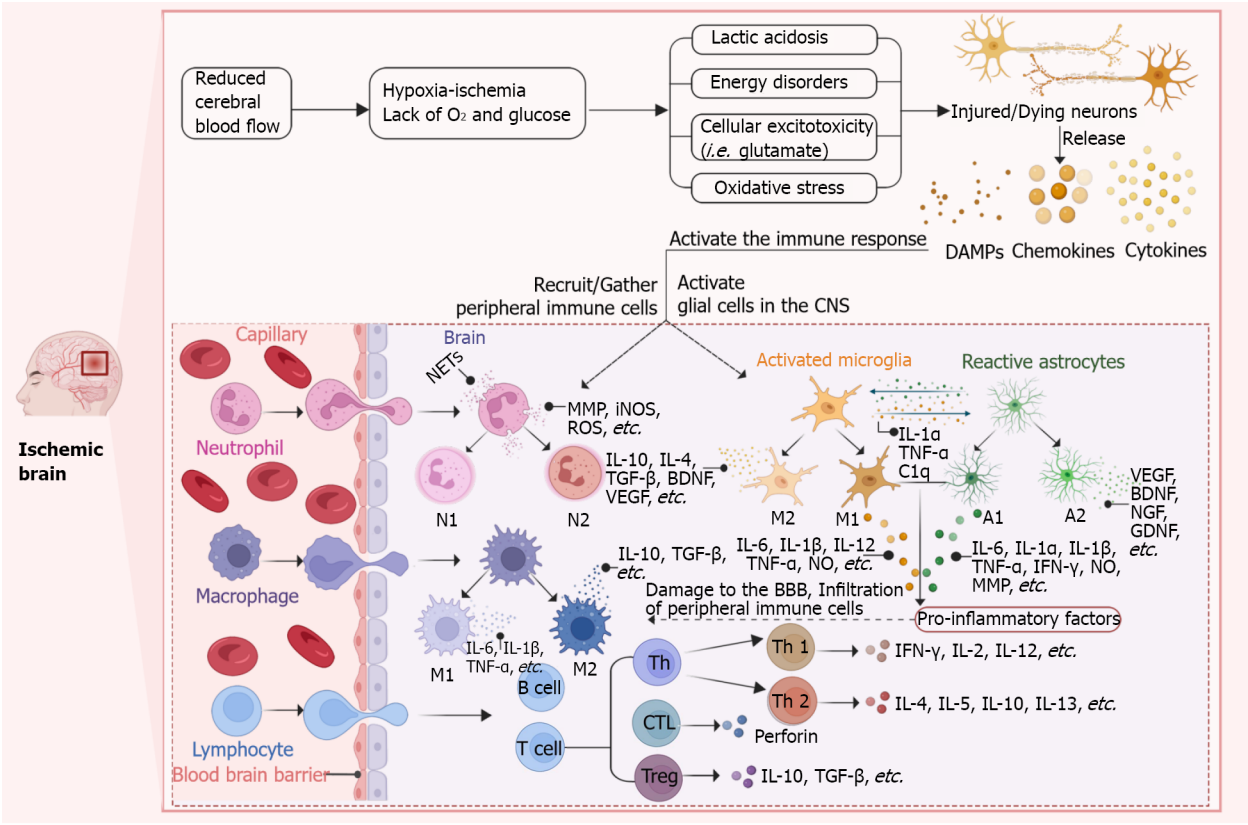
IS is caused by thrombosis or embolism, which could interrupt blood flow to the brain. After acute ischemic events, blood stagnation and altered hemodynamics restrict the availability of oxygen and glucose [oxygen-glucose deprivation (OGD). Then brain cell metabolism shifts from the oxidative phosphorylation to high levels of glycolysis, producing excess lactic acid[17,18]. Excessive accumulation

of lactic acid is able to trigger tissue acidosis, edema, blood-brain barrier (BBB) dysfunction, and extensive necrosis[18]. Firstly, once the Na^+/K^+ ATPase pump is affected, there will be an inward flow of Na^+ and an outward flow of K^+, which depolarizes the neuronal plasma membrane and promotes the release of excitatory neurotransmitters (including glutamate)[18-20]. Excess glutamate activates the N-methyl-D-aspartate receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor, thereby leading to cytotoxicity and cell death[19,21,22]. Next, an extracellular Ca^{2+} inward flow occurs after affecting the Ca^{2+} pump, which causes a dramatic rise in intracellular Ca^{2+}. Ca^{2+} overload activates calcium-dependent proteases, lipases, DNAases, kinase phosphatases, endonucleases, and other death signals, inducing ischemic core cell death[20,23]. Additionally, the Ca^{2+} influx activates nitric oxide synthase (iNOS), which subsequently generates oxygen radicals and peroxynitrite (ONOO-), causing oxidative stress in neural tissue[24]. Meanwhile, the depletion of ATP production and overproduction of reactive oxygen species (ROS) leads to mitochondrial dysfunction, further exacerbating oxidative stress [22,25]. In summary, OGD results in subsequent energy disturbances, lactic acidosis, cellular excitotoxicity, and oxidative stress, ultimately leading to brain cell damage or death. This is the initial step of ischemia-induced damage, which triggers the subsequent cascade responses. Injured/dying cells emit "danger signals" and thus activate the immune system (Figure 1).

Once the immune system is activated, immune cells enter the brain parenchyma sequentially. Microglia (MG), as the resident macrophages of the central nervous system (CNS), are the first to detect ischemia and rapidly activate in response[26,27]. MG recognizes "danger signals" [danger-associated molecular patterns (DAMPs)] released by dying and dead cells, primarily *via* the expressions of Toll-like receptors (TLR) and scavenger receptors. Then, the TLRs and scavenger receptors are activated, triggering a series of inflammatory events[28-30]. MG has been classified into two polarized phenotypes, including classical activation (pro-inflammatory, M1) and alternative activation (anti-inflammatory, M2). Anti-inflammatory cytokines [such as interleukin (IL)-4, IL-13, IL-10, and transforming growth factor (TGF)-β] activate the M2 phenotype. The M2 cells promote translocation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and proliferation-activated receptor gamma, and promote the secretion of anti-inflammatory IL-10, IL-4, TGF-β cytokines and growth factors (such as brain-derived neurotrophic factor and vascular endothelial growth factor) to suppress inflammation and enhance tissue repair[31,32]. In contrast, lipopolysaccharide (LPS) and interferon gamma (IFN-γ) activate the M1 phenotype. The M1 cells promote the transcriptional activation of nuclear factor-κB (NF-κB), a member of the signal transducer and activator of the transcription family (STAT), and promote the production of pro-inflammatory mediators like IL-12, tumor necrosis factor (TNF)-α, IL-6, IL-1β and NO, leading to the secondary brain damage[31,33,34]. Meanwhile, the chemokines and cytokines released by M1-MG and adhesion molecules highly expressed on endothelial cells can recruit peripheral blood leukocytes (including neutrophils, monocytes, and lymphocytes) to infiltrate the brain parenchyma, thereby mediating the adaptive immune response[28,30,31]. In the acute phase of brain injury, the M1 phenotype appears to predominate, whereas MG favors the M2 phenotype in the later stages. In addition, neurons can control MG activation by releasing "on" and "off" signals. MG is able to quickly recognize the "eat me" (CX3CL1) or "don't eat me" (*e.g.*, CD47-SIRPα and CD200-CD200R) signal on a neuron and engulf the live ischemic neurons[35]. In the same way like MG, macrophages can be polarized into two phenotypes, M1 and M2. The two are often described as MG/macrophages, because their roles in stroke are mostly similar[36]. However, in contrast, the main inflammatory factors produced by both are skewed. MGs secrete relatively high levels of ROS and TNF-α, while macrophages produce relatively high levels of IL-1β[37].

Astrocytes (Ast) are among the first brain cells to be activated after an ischemic event. Ast undergoes a dramatic transformation called "reactive astrocytosis" after ischemic injury, forming glial scarring[38]. Similar to MG, the harmful or beneficial effects of reactive Ast depend on the different phenotypes of Ast (neuronal toxicity phenotype A1 and neuroprotective phenotype A2)[38,39]. In addition to the activation of Ast by DAMPs, there is growing evidence regarding the importance of MG-Ast crosstalk for activating Ast. MG activation, followed by the release of IL-1α, TNF-α and complement component subunit 1q, induces the activation of A1-type reactive Ast[38,40]. A1-Ast secrete pro-inflammatory mediators, like IL-6, TNF-α, IL-1α, IL-1β, IFN-γ, NO, matrix metalloproteinases (MMP), superoxide and ONOO-, inducing neuron and oligodendrocyte death[38,41]. MG also induces the A2 phenotype of Ast and attenuates the inflammatory response. Li *et al*[42] have reported that Zinc finger E-box binding homeobox 1 (ZEB1) was highly expressed in MG of the ischemic hemisphere after experimentally induced strokes[42]. ZEB1 overexpression mediates the MG response primarily through a TGF-β1-dependent pathway and subsequently reduces CXCL1 production in Ast, thereby reducing neutrophil infiltration in the brain parenchyma. Likewise, Ast also can regulate the phenotype and function of MG through crosstalk between Ast and MG[43]. Thus, when the brain is disturbed, MG and Ast seem to respond as a unit.

Different from other immune cells, the number of lymphocytes infiltrating into the stroke brain is relatively small[27]. T lymphocytes can enter the brain hours after a stroke and are preferentially accumulated at the edge of the lesion[44]. The T cells infiltrating into the ischemic tissue mainly comprise $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T helper cells (Ths), and regulatory T cells (Tregs) [45]. Infiltrating MG/macrophages may stimulate the differentiation of activated $CD4^+$ T cells into Th1



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Figure 1 Schematic representation of the immune response after ischemic stroke. After ischemic stroke (IS), the blood-brain barrier (BBB) is disrupted and the central nervous system (CNS) and the peripheral immune system are able to interact with each other. The cerebral blood flow is significantly reduced immediately after IS, which limits the availability of glucose and oxygen. The initial ischemic event leads to energy disturbances, acidosis, cellular excitotoxicity and oxidative stress ultimately resulting in neuronal damage or death and subsequent activation of the immune response. Dying/dead neurons release "danger signals" such as danger associated molecular patterns (DAMPs), cytokines and chemokines to recruit and activate peripheral immune cells (neutrophils, macrophages and lymphocytes) and activate glial cells (microglia and astrocytes) in the central CNS. Activated glial cells release a range of cytokines and chemokines that also recruit peripheral immune cells into the brain parenchyma and further destroy the BBB. Activated cells polarize into different cell phenotypes or subtypes to secrete pro-inflammatory or anti-inflammatory factors that act to damage or protect. (Figure created with BioRender.com). DAMP: Danger associated molecular patterns; CNS: Central nervous system; NET: Neutrophil extracellular trap; MMP: Matrix metalloproteinases; iNOS: Influx activates nitric oxide synthase; ROS: Reactive oxygen species; IL: Interleukin; TGF-β: Transforming growth factor-β; BDNF: Brain-derived neurotrophic factor; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-α; NGF: Nerve growth factor; GDNF: Glial cell-derived neurotrophic factor; IFN-γ: Interferon-γ; BBB: Blood-brain barrier; CTL: Cytotoxic T lymphocyte; C1q: Component subunit 1q.

or Th2 cells[46]. Th1 cells are able to secrete pro-inflammatory factors like IFN-γ, IL-2, and IL-12 to exacerbate inflammation. In contrast, Th2 cells produce anti-inflammatory factors, such as IL-4, IL-5, IL-10, and IL-13, to suppress inflammation[47]. CTLs directly or indirectly kill neurons and aggravate brain damage through cell interactions and the release of perforin after antigen-dependent activation[48]. Tregs exert their protective effects mostly by inhibiting IL-1β and TNF-α through the expression of IL-10 [47,49]. The role of B lymphocytes in the immunology of stroke is not clear yet. Whereas, some studies have observed the local production of corresponding antibodies in the cerebrospinal fluid of stroke patients, indicating that B lymphocytes are indeed present in the ischemic brain and they may be involved in post-ischemic immunological events[50].

Neutrophils are the initial blood-derived immune cells to cross the BBB and invade ischemic tissues, and they can be detected as soon as 1 h after the event[51]. Neutrophils are activated and recruited to the injured brain parenchyma by inflammatory factors produced from some activated glial cells and dying neurons, and adhesion molecules expressed by endothelial cells (*e.g.*, intercellular adhesion molecule 1, P-selectin and E-selectin)[27,52,53]. Traditionally, the neutrophil aggregation has been considered detrimental to stroke. After infiltration into ischemic tissues, activated neutrophils produce inflammatory factors, such as MMP, iNOS, and ROS, and form neutrophil extracellular traps (NETs) to increase BBB permeability and exacerbate inflammation[54-56]. In addition, the accumulation of neutrophils can further block local blood flow, resulting in "no reflux" of the microcirculation[57]. Neutrophils also exhibit two kinds of phenotypes, comprising N1 (pro-inflammatory) and N2 (anti-inflammatory) phenotypes. Neutrophil's different phenotypes may shape other cellular effector functions and be cleared by phagocytosis of MG/macrophages[58,59].

In conclusion, the post-stroke ischemic environment induces immune cells to polarize into different phenotypes or type, acting either protectively or destructively. Hence, it is probably a promising mean to affect the immune cell heterogeneity and improve the post-stroke inflammatory environment.

EXOSOMES AS A REPLACEMENT THERAPY FOR MSCS ON IS

All above, it is clear that the immune responses following IS can influence the development of ischemic brain injury. Anti-inflammatory and immunomodulatory therapies have shown beneficial effects on several experimental stroke models[60]. Among them, MSCs transplantation is one of the most important therapeutic tools involved in regulating immunity and repairing ischemic tissues in clinical practice[61]. Initially, researchers have assumed that the primary mechanism of MSCs transplantation mainly involved in MSC's ability to differentiate into parenchymal cells to repair and replace injured tissues. However, many preclinical studies suggested that most MSCs were confined to the liver, spleen, and lungs, and only a few MSCs could reach the injury site, surviving and differentiating into neurons [62,63]. Interestingly, despite most transplanted MSCs stagnate in the organ, this does not prevent the therapeutic effect of MSCs transplantation. Thus, the distal therapeutic effect after transplantation of MSCs may be primarily attributable to the paracrine mechanism of MSCs[63]. MSC-Exos mainly mediate the paracrine secretion of MSCs. Exosomes are EVs with a single membrane structure of 30-150 nm in diameter, carrying proteins, lipids, nucleic acids (DNA, mRNA, miRNA, lncRNA, circRNA), and other substances[64]. When exosomes are circulating, the contents encapsulated within them can be delivered to target cells, mediating intercellular communication and regulating the function of the target cells[64,65]. This is essentially the role of the miRNAs contained by exosomes. MiRNAs are endogenous hairpin-loop structured non-coding RNAs, primarily binding to mRNA in specific ways to influence post-transcriptional events and regulate cellular behavior[66].

Furthermore, there are multiple advantages of transplanting exosomes rather than the entire "factory" (cell) into the body: (1) In terms of biodistribution, as nano-scale cellular secretions that could escape the phagocytosis of macrophages and readily cross the BBB to reach the brain parenchyma, they are considered to be natural therapeutic agents and innate drug delivery system for brain diseases[67]; (2) In terms of stability, exosomes have a stable bimolecular phospholipid structure that prevents the contents' biological activity from being broken down by extracellular hydrolytic enzymes[64]; (3) In terms of safety, compared to MSCs transplantation therapy, the cell-free therapy can avoid cell-mediated adverse effects, such as tumor formation, coagulation dysfunction, and infarction due to vascular occlusion[16]; and (4) In terms of development potential, exosomes can be enriched in large quantities within the culture medium (mass production) and easily retouched/retrofitted (controllable). Moreover, some studies comparing the therapeutic effects of MSC-Exos with MSCs in stroke rat models suggest that MSC-Exos treatment is indeed superior to treatment with MSCs themselves[68,69]. For above reasons, we believe that MSC-Exos is a crucial effector of MSCs to exert their immunomodulatory effects. Together with its unique advantages, MSC-Exos is expected to be a replacement therapy for MSCs in the treatment of stroke.

MSC-EXOS MODULATES IMMUNE RESPONSE AFTER IS

Recently, numerous studies have shown that MSC-Exos can promote recovery after stroke, *via* modulating the innate and adaptive immune responses activated after IS[70-72]. Firstly, MSC-Exos is able to regulate cell differentiation, activation, proliferation, and intercellular communication by delivering functional molecules to cells involved in immunity, for example, MG, Ast, macrophages, neutrophils, lymphocytes, dendritic cells (DCs), *etc.* (Table 1). There are three primary forms of action: (1) Through the signaling molecules on its surface as ligands binding to specific receptors on the target cell, the intracellular signaling pathways are regulated; (2) *via* fusing with the corresponding target cell membrane and releasing the contents into the recipient cell; and (3) by entering the target cell in the form of endocytosis and bringing the active factors into the cell[73]. Secondly, MSC-Exos can also mediate the immune response by down-regulating pro-inflammatory factors and/or up-regulating anti-inflammatory factors (Table 2).

MSC-Exos regulate the immune response through cells

MSC-Exos and CNS: MG is firstly activated after IS, as an immune sentinel of the CNS, exerting neuroprotective or neurotoxic effects[27,74]. A therapeutic strategy balancing the two polarization states of MG may become a future adjunctive stroke therapy. One study used protein blotting to analyze TLR-2, TLR-4 and TLR-6 levels in MG of ischemia/reperfusion (I/R) mice and found that the TLR/NF-κB pathway was activated in MG after an ischemic event, leading to the secretion of pro-inflammatory factors (IL-1β, TNF-α, IL-6, *etc.*) and that this signaling pathway was important in promoting M1 transformation and exacerbating the inflammatory response[75]. TLRs are pattern recognition receptors

Table 1 Mesenchymal stem cell-derived exosomes target cells to mediate immune responses on ischemic stroke

Origin	Targeted cells	Administration/cultivation routes	Pathways/factors involved	Function	Ref.
Human umbilical cord-derived mesenchymal stem cell exosome miR-26b-5p	Microglia	Tail vein injection and microglia co-culture	TLR signaling pathway	Balance microglia polarization	[75]
Bone marrow-derived mesenchymal stem cell exosome miR-182-5p	Microglia	Inject into the brain	TLR4/NF-κB		[81]
Mesenchymal stem cell exosome miR-223-3p	Microglia	Tail vein injection and BV-2 microglia co-culture	CysLT2R-mediated signaling pathway		[87,89]
Mesenchymal stem cell exosome miR-26a-5p	Microglia	Tail vein injection and BV-2 microglia co-culture	CDK6		[97]
Human umbilical cord-derived mesenchymal stem cell exosome miR-146a-5p	Microglia	Tail vein injection	IRAK1/TRAF6		[85]
Bone marrow-derived mesenchymal stem cell exosomes	Microglia	Tail vein injection	NLRP3 inflammasome		[99]
Bone marrow-derived mesenchymal stem cell exosome lncRNA H19	Microglia	BV-2 microglia co-culture	JAK/STAT		[100]
Adipose stem cell-derived exosome miR-30d-5p	Microglia	Tail vein injection and primary microglia co-culture	Autophagy		[91]
Mesenchymal stem cell exosome miR-542-3p	Neuroglia	Inject into paracele of mice	TLR signaling pathway	Mitigate OGD-induced glial cell damage	[71]
Mesenchymal stem cell exosomes	Astrocyte	Ventricular injection and astrocyte co-culture	Nrf2-NF-κB	Modulate astrocyte activation and ameliorate reactive astrogliosis	[104,105]
Bone marrow-derived mesenchymal stem cell exosome miR-138-5p	Astrocyte	Astrocyte co-culture	LCN2		[111]
Mesenchymal stem cell exosome miR-133b	Astrocyte	Tail vein injection	CTGF/RhoA		[118,119]
Human adipose-derived mesenchymal stem cell exosomes	Neutrophil	Neutrophil co-culture	IL-6	Increase neutrophil lifespan and enhance neutrophil phagocytosis	[122]
Wharton's jelly-derived mesenchymal stem cell exosomes	Neutrophil	Neutrophil co-culture	_		[123]
Adipose-derived mesenchymal stem cell-derived exosomes	Macrophage	Macrophage co-culture	MafB and Stat6	Balance macrophage polarization	[129]
Adipose-derived mesenchymal stem cell-derived exosomes	Macrophage	THP-1 cell co-culture	ROCK1/PTEN		[130]
Human adipose-derived mesenchymal stem cell exosomes	T-lymphocyte	T-lymphocyte co-culture	Markers	Inhibition of lymphocyte activation and proliferation	[135]
Bone marrow-derived mesenchymal stem cell-derived exosomes	B-lymphocyte	T-lymphocyte/B-lymphocyte co-culture	Specific mRNAs		[70]
Adipose-derived mesenchymal stem cell-derived exosomes	Dendritic cell	Dendritic cell co-culture	_		[142]

TLR: Toll-like receptors; NF-κB: Nuclear factor-κB; CysLT2R: Cysteinyl leukotriene receptor 2; LCN2: Lipid chainlipoprotein-2; JAK: Janus kinase; STAT: Signal transducer and activator of transcription; IRAK1: Interleukin-1 receptor-associated kinase 1; TRAF6: TNF receptor-associated factor 6; Nrf2: Nuclear factor erythroid 2-related factor 2; RhoA: Ras homolog gene family member A; CTGF: connective tissue growth factor; IL-6: Interleukin-6; ROCK1: Rho

associated coiled-coil containing protein kinase 1; PTEN: Phosphatase and tensin homolog; _: Refers to studies without detail the pathways

Table 2 Important immunological factors and their impact

Inflammatory mediators	Impacts	End of MSC-Exos transplantation/culture	Ref.
TNF-α	Pro-inflammatory	Decline	[71,75,81,85,91,100,104,111,130,140,141,143]
IL-1β	Pro-inflammatory	Decline	[81,85,100,104,111,140,141]
IL-6	Pro-inflammatory	Decline	[71,75,81,85,91,100,111,122,130,140-143]
iNOS	Pro-inflammatory	Decline	[81,91]
IFN-γ	Pro-inflammatory	Decline	[135]
IL-8	Pro-inflammatory	Decline	[130]
NLRP3	Pro-inflammatory	Decline	[99,145]
CysLT2R	Pro-inflammatory	Decline	[87,89]
CCL-2	Pro-inflammatory	Decline	[75]
MCP-1	Pro-inflammatory	Decline	[71,81]
IL-4	Anti-inflammatory	Raise	[91]
IL-10	Anti-inflammatory	Raise	[91,100,130,142,143]
TGF-β	Anti-inflammatory	Raise	[130,142]

TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IL-6: Interleukin-6; IL-8: Interleukin-8; iNOS: Influx activates nitric oxide synthase; IFN-γ: Interferon gamma; CysLT2R: Cysteinyl leukotriene receptor 2; CCL-2: C-C motif ligand 2; MCP-1: Monocyte chemotactic protein; IL-4: Interleukin-4; IL-10: Interleukin-10; TGF-β: Transforming growth factor-β; NLRP3: NLRP3 inflammasome; MSC-Exos: Mesenchymal stem cell-derived exosomes.

widely expressed on the surface of immune cells and play a key role in the immune response. NF-κB is a key regulator of the immune response and is intricately involved in MG/macrophage M1 and M2 phenotypic signaling[31,76,77]. Various miRNAs encapsulated in exosomes can regulate the expression of TLRs on MG surface, which act on NF-κB to influence MG polarization[29,30,78]. It has been reported that Cholesterol 25-hydroxylase (CH25H) is significantly increased during inflammation and contributes to the immune response by recruiting Iba-1-positive MG and activating TLR-3[79]. Meanwhile, an experiment used microarray to analyze the expression differences of miRNAs in ischemic brain tissue after exosome treatment, and found that miR-26b-5p expression increased significantly after exosome treatment and could target CH25H in MG to inactivate the TLR pathway to inhibit M1 polarization[75]. Besides, miR-542-3p prevents the expression of pro-inflammatory factors and the production of ROS by post-ischemic activated glial cells through inhibiting TLR[71]. miR-202-3p [80], miR-182-5p[81], MiR-181c[82], and miR-1906[83] also play a role in inhibiting M1 polarization through downregulation of TLR expression. Also, in a further explanation of the potential mechanism of miRNA-mediated TLR/NF-κB pathway, Liu *et al*[84] have documented that miR-216a-5p activates the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling cascade through inhibition of TLR4/NF-κB, enabling the M1 to M2 phenotypic shuttle[84]. Some data also suggest that miR-145-5p downregulates inflammatory responses by inhibiting the IL-1 receptor-associated kinase 1 (IRAK1)/TNF receptor-associated factor 6 (TRAF6) signaling pathway to reduce and increase the amount of M1-MG and M2-MG, respectively[85]. In contrast, overexpression of IRAK1 and TRAF6 is involved in the activation of TLR/NF-κB pathway and promotes the release of proinflammatory factors[78,85,86]. Hence, miR-145-5p may indirectly affect the TLR/NF-κB pathway by inhibiting the IRAK1/TRAF6 pathway. Exploring the crosstalk between TLR/NF-κB and other pathways may better interfere with MG polarization. As described above, exosome miRNAs acting on the TLR/NF-κB pathway and its upstream/downstream signaling pathways could influence MG phenotype as well as the expression of pro/anti-inflammatory factors to improve inflammation.

In the regulation of MG polarization, the TLR/NF-κB pathway has been studied the most. However, in stroke, MG polarization is complex and actually regulated by multiple factors. Thus, other pathways affecting the activation state of MG are discussed below. miR-223 is one of the most abundant miRNAs in MSCs and their exosomes. In vivo and *in vitro* experiments have revealed that miR-223-3p down-regulates the transcription and expression of Cysteinyl leukotriene receptor 2 (CysLT2R) to induce a conversion from deleterious M1 to beneficial M2 phenotype[87]. CysLTs secreted by dying/dead cells

are potent medium for inflammation. They are activated in various cell types during brain injury, further exacerbating the development of inflammation[88]. Zhao *et al*[89] conducted an in-depth study involving the miR-223-3p inhibiting CysLT2R expression *in vivo* and in vitro. They found that miR-223-3p reversed M1 polarization by apparently downregulating the expression of ERK1/2 downstream of CysLT2R, which led to a decrease in the secretion of pro-inflammatory factors and an increase in the secretion of anti-inflammatory and neurotrophic factors, thereby slowing down inflammatory damage [89]. In addition, miR-223-3p also effectively inhibits N-methyl-leukotriene C4/Leukotriene D4 to promote M1 to switch to M2[87,90]. Through targeting the autophagy-associated proteins Beclin-1 and Atg5, miR-30d-5p greatly inhibited autophagy-mediated polarization of MG towards M1 and reduced OGD-induced inflammatory responses[91]. Notably, autophagy may exert both beneficial and detrimental effects under IS conditions, depending on the degree of autophagy[92,93]. A moderate increase in MG autophagic activity can reduce MG activation and promote MG polarization towards the M2 phenotype, exerting a neuroprotective effect. Instead, excessive autophagy exacerbates cerebral ischemic injury. It has been demonstrated that regulation of autophagic flux and exosome biogenesis in MG plays a vital role in neuronal survival under conditions of cerebral ischemia[94]. Interestingly, the similar property was also reported in Ast[95,96]. As such, balancing the autophagic flux of immune cells after stroke may be a promising target for treating stroke. Cheng *et al*[97] have demonstrated that miR-26a-5p was downregulated and CDK6 was upregulated in MSCs-derived exosomes of middle cerebral artery occlusion (MCAO) and OGD model[97]. They then hypothesized that CDK6 might be a direct target of miR-26a-5p and further confirmed the correlation between exosome miR-26a-5p and CDK6 using a luciferase reporter gene assay. The data showed that miR-26a-5p inhibited MG apoptosis and attenuated I/R injury in mice by mediating CDK6 downregulation[97]. Besides, miR-424 can also reduce ischemic brain injury by targeting key activators of the G1/S transition in MG (including CDK6, CDC25A, and CCND1) to inhibit BV-2 MG activation[98]. CDK6 seems to be a good target of miRNAs in neuroprotection. As well as alleviating inflammation, MSC-Exos can also alleviate neuronal death by regulating MG polarization to downregulate inflammatory mediators relating to pyroptosis[99]. *In vitro* data suggest that the non-coding RNA H19 carried by MSC-Exos could attenuate M1 polarization and inflammatory responses by sponging miR-29b-3p and further inhibit neuronal apoptosis[100]. miR-29b-3p may prevent ischemic-hypoxic brain injury by activating the PI3K/Akt pathway *via* downregulating the protein phosphatase and tensin homolog (PTEN)[101]. Most studies have reported routes associated with miRNAs affecting M1 polarization, while studies acting on pathways associated with M2 polarization are still lacking and deserve further exploration.

Ast, the most abundant brain cells in the CNS, plays an essential role in neuroinflammation and neuroregeneration[40,41]. Following ischemic injury, Ast is activated by DAMPs and/or MGs and undergoes a transformation known as "reactive astrogliosis"[38]. Features include hypertrophy of the shape and overexpression of glial fibrillary acidic protein (GFAP)[102]. The activated Ast phenotype matches MG and is divided into pro-inflammatory A1 and anti-inflammatory A2. Notably, recent studies have shown that the inflammatory response mediated by Ast appears to last longer and induces more damage than MG[103]. This possibility further underlines the importance of targeted inhibition of Ast activation or induction of Ast phenotypic transformation in the treatment of IS. It has been demonstrated that Nrf2-related pathways are involved in the inflammatory response of Ast[104-106]. In one study, immunofluorescence experiments were performed after *in vivo* and *in vitro* administration of MSC-Exo, respectively, and protein blots showed that MSC-Exo reduced the expression of GFAP (Ast marker), C3 (A1 marker) and ki67 (cell proliferation marker) in LPS-stimulated cultured primary hippocampal Ast[104]. Meanwhile, the data show that MSC-Exo could reverse hippocampal Ast oxidation (*e.g.*, upregulation and nuclear translocation of Nrf2) and inflammation phenotypes (*e.g.*, NF-κB activation and translocation)[104]. These results suggest that MSC-Exo can inhibit inflammation-induced Ast activation by modulating the Nrf2-NF-κB signaling pathway. Nrf2 is a regulator of redox homeostasis and a target for the induction of inflammatory responses. In brain diseases with simultaneous inflammation and oxidative stress (*e.g.*, IS), the interaction between Nrf2 and NF-κB signaling pathway is the fundamental mechanism regulating these responses[107]. miR-146a-5p, one of the most abundant cargo miRNAs in human umbilical cord-derived MSC-Exos, dramatically decreased the expression of A1 markers [C3 and lipid chain lipoprotein-2 (LCN2)) by inhibiting the NF-κB signaling cascade, thereby reversing the neurotoxic phenotype of Ast[108]. Among them, LCN2 has been identified as a potent mediator of astrocyte neurotoxicity[109]. LCN2 secreted by reactive Ast can accelerate or propagate neuronal cell death and promote the activation of resting Ast and MG[109]. Moreover, a recent study identified high LCN2 expression in a mouse transient MCAO model and detected that IS patients with higher plasma LCN2 levels were more likely to develop a post-stroke infection[110]. Overexpression of miR-138-5p negatively regulates the LCN2 expression in Ast, thereby inhibiting inflammation and reducing ischemic nerve injury[111].

More importantly, the increase of reactive Ast results in further glial scarring. In the acute phase of IS, these physical barriers can limit the inflammation spread and infarct area to maintain CNS homeostasis. However, in the recovery phase of IS, their presence may impede the circulation and neurological tissue regeneration, affecting functional recovery in late stroke[41]. It has been demonstrated that in several previous cerebral ischemia and hypoxia models, transplantation of MSCs markedly reduced reactive Ast and further eliminated glial scarring around the lesion, promoting neuronal regeneration and

relieving inflammation[112,113]. Recently, the *in vitro* studies reported that MSCs improved brain function after transplantation mainly by reducing the number of hypertrophic Ast and GFAP overexpression through inhibition of p38 MAPK, JNK, and its downstream targets p53 and STAT1 activation by paracrine factors[114]. In addition, miR-124 attenuated Ast proliferation and migration by blocking the STAT3 pathway, thereby reducing the width of glial scarring and improving neurological function [115]. Meanwhile, miR-124 may also involve in the reprogramming neuronal progenitor cells by Ast through decreasing Notch1 expression and increasing Sox2 expression[115]. As Ast and neurons originate from the same precursor cells, Ast can be reprogrammed into neurons under some specific conditions, which can be achieved, for instance, by adjusting the expression of certain specific transcription factors (including Notch1, NeuroD1, Mash1, Ascl1, *etc.*) *in vivo*[115-117]. If Ast transdifferentiated neurons homed to the ischemic lesion and replaced lost neurons, this would help limit glial scar formation and neural connectivity after injury, contributing to neural remodeling. This may be used as an alternative therapy for neuronal replenishment after stroke in the future. Furthermore, exosomes can also mediate communication between MSCs and Ast to enhance neurological recovery after stroke. MSCs communicate with Ast and neurons by releasing miR-133b-containing exosomes and transferring miR-133b into neurons and Ast to promote neuroprotection regeneration[118]. Xin *et al*[119] further showed that miR-133b shared into Ast downregulated the expression of connective tissue growth factor, thereby reducing glial scar thickness in cerebral infarction[119]. All in all, Ast may be a potential target for the intervention in stroke. However, whether the responsiveness and function of Ast should be further reduced or enhanced may depend on the duration of the ischemic lesion, the location of the Ast, and the specific subtype of Ast.

MSC-Exos and peripheral immune system

Neutrophil: Neutrophils are the first peripheral immune cells to infiltrate into the brain parenchyma crossing the damaged BBB. Neutrophil infiltration after IS is now believed to be detrimental to stroke [27,54,56]. MSC-Exos can mitigate the harmful effects of neutrophils in several ways. Firstly, MSC-Exos can reduce neutrophil infiltration and inhibit neutrophil respiratory burst, thereby decreasing the expression of inflammatory mediators, including IL-1b, IL-6, and TNF-α, as well as suppressing the production of ROS in neutrophils[120]. Also, further studies have revealed that MSC-Exos may prevent the subsequent recruitment of monocytes/macrophages and lymphocytes after reducing neutrophil infiltration at the cerebral ischemia site[121]. Secondly, MSC-Exos inhibits neutrophil apoptosis and enhances neutrophil phagocytosis, then contributing to the clearance of cellular debris and eliminating inflammation and infection. This may result from the presence of IL-6 in MSC-Exos and its transfer into the neutrophil cytoplasm, which subsequently exerts an autocrine effect on neutrophils, thereby prolonging the survival of these cells and maintaining their effective function and viability to further improve the inflammatory response[122,123]. Thirdly, MSC-Exos inhibits the formation of terminal complement complexes on neutrophils *via* CD59, thus attenuating neutrophil activation and inhibiting the release of NETs and IL-17 from neutrophils[124]. Besides, human umbilical cord blood-derived MSC-derived EVs (exosomes) can also repair and enhance neutrophil mitochondrial function by transferring functional mitochondria, then reducing the formation of NETs[125]. Of greater importance, Soni *et al*[72] further investigated the differences in the regulation of neutrophil function by exosomes from different sources of MSCs. The results suggested that bone marrow-derived MSCs-derived exosomes (B-Exos) were more effective in prolonging the neutrophil lifespan. In contrast, adipose-derived MSCs (ADMSCs)-derived exosomes (A-Exos) were more prominent in increasing the phagocytic capacity of neutrophils and inhibiting the formation of NETs[72].

Macrophage: Activated macrophages are morphologically like MGs, which can be divided into neurotoxic M1 and neuroprotective M2 types[36]. Numerous experiments have demonstrated that MSC-Exos can effectively inhibit the effector function of M1 pro-inflammatory macrophages and/or promote the effector function of M2 anti-inflammatory macrophages, which contributes to alleviating the immune inflammatory response. It has been suggested that IFN regulatory factor (IRF) 5 could be reversibly induced by inflammatory stimuli in macrophages and IRF5 is associated with the plasticity of macrophage polarization (up- or down-regulation of M1 or M2 macrophage phenotypic marker expression)[126]. Overexpression of miR-22-3p promotes the polarization to macrophage M2, suppresses the inflammation, and attenuates I/R injury through downregulating IRF5, which is supported by increased expression of the M2 macrophage marker mannose receptor (CD206) and decreased expression of the M1 macrophage marker CD86[127]. B-Exos-derived miR-125a also exerts neuroprotective effects by targeting negative regulation of IRF5 to promote M2 phenotypic polarization [128]. Furthermore, A-Exos can promote M2 polarization by activating the M2 macrophage-specific transcription factors MafB and Stat6 to induce the expression of genes related to anti-inflammatory functions, supported by a mechanism that upregulates the expression of the M2 macrophage markers CD163, arginase-1 (Arg1) and CD206[129]. A-Exos also increased CD163, Arg1, CD206, TGF-β1, and IL-10 expression levels and decreased TNF-α, IL-6, and IL-8 expression levels by targeting the Rho associated coiled-coil containing protein kinase 1/PTEN pathway. The above results suggest that A-Exos may improve the inflammatory environment by promoting M2 polarization to increase the secretion of anti-inflammatory molecules and/or inhibiting M1 polarization from decreasing the

secretion of pro-inflammatory factors[130]. And similar to MG, miR-21, miR-146a, and miR-301a can also regulate macrophage polarization by inhibiting the TLR/NF-κB pathway[131-133].

Lymphocyte: First of all, T lymphocytes are at the heart of the adaptive immune system. Despite some subtypes of T lymphocytes exert a neuroprotective role in the early post-stroke phase, such as Tregs and Th2, on the whole, they have a negative impact on IS, as do neutrophils[45,134]. Accordingly, it appears to be a viable clinical treatment for stroke to modulate the differentiation, activation and function of various T lymphocyte subsets. In vitro studies indicated that A-Exos significantly inhibited the activation and proliferation of $CD4^+$ and $CD8^+$ T cells and reduced IFN-γ release, with directly immunosuppressive properties[135]. *In vivo* experiments showed that a dramatic reduction in the number of CTL was observed in a rat model of cerebral infarction injected intra-arterially with MSC-EV [136]. Another study has documented that MSC-Exos also promoted the Treg proliferation and induced the conversion of Th1 to Th2 by enhancing intracellular IL-10 and TGF-β secretion, thereby boosting its immunosuppressive capacity[137]. Soni *et al*[72] have further explored and found that MSC-Exos containing miR-146, miR-155, miR-21, and miR-29 may regulate the activation pathways of Th1 and Th2 [72]. And they showed that different sources of MSC-Exos all inhibited the proliferation of T lymphocytes. But in comparison, Wharton Jelly-derived exosomes and B-Exos had a better inhibitory capacity than A-Exos[72]. Additionally, DCs activate T cells through delivering co-stimulatory molecules, such as CD80 and CD86, to naive T cells[138]. However, MSC-Exos can reduce T-lymphocyte activity, increase IL-10 and TGF-β secretion, and reduce IL-6 secretion by inhibiting the maturation and differentiation of DCs[135]. Next, activation and isotype-transformed B-lymphocyte infiltration contribute to poor outcomes after IS. It has been previously reported that MSCs can reverse the unfavorable outcome by inhibiting B lymphocyte activation, proliferation, differentiation, and chemotactic response[139]. Recent studies have demonstrated that when co-cultured with lymphocytes derived from healthy human peripheral blood, B-Exos significantly inhibited lymphocyte proliferation and immunoglobulin M production, particularly exhibiting effects on B lymphocyte-specific mRNA expression[70].

MSC-Exos regulate the immune response through inflammatory mediators

In addition to immune cells, changes in inflammatory mediators, such as cytokines and chemokines, are also observed in ischemic area. Among them, TNF-α, IL-1β, and IL-6 are the more typical pro-inflammatory factors, and their expression is significantly upregulated after ischemic events. MSC-Exos containing lncRNA ZFAS1[140], lncRNAH19[100], miR21-3p[141], miR-146a-5p[85], miR-138-5p[111], and miR182-5p[81] was able to reduce immunosuppression by downregulating the secretion of TNF-α, IL-1β, and IL-6. Of these, lncRNA ZFAS1 and lncRNAH19 may be associated with the competitive binding of miR-15a-5p and miR-29b-3p[100,140]. MSC-Exos also down-regulate other pro-inflammatory factors such as IFN-γ, iNOS, and IL-8[81,91,130,135]. Apart from down-regulating pro-inflammatory factors, some MSC-Exos can up-regulate the expression of anti-inflammatory factors such as IL-10, IL-4, and TGF-β1[91,100,130,142,143]. Furthermore, some studies have shown that MSC-Exos could reduce the secretion of chemokines (*e.g.*, C-C motif ligand 2) and cellular chemotactic proteins (*e.g.*, monocyte chemotactic protein), thereby inhibiting the migration and aggregation of peripheral immune cells and alleviating the inflammatory response[71,75,81]. Inflammasomes are equally important inflammatory mediators in regulating the onset and progression of IS. NLRP3 inflammasome contains a caspase-1 precursor that cleaves to caspase-1 (Cl) upon stroke stimulation. C1 not only is a critical executioner of pyroptosis (cleave full-length GSDMD to release GSDMD N-terminus) but also can convert precursors of IL-1β and IL-18 into mature pro-inflammatory cytokines exacerbating inflammation[144]. Liu *et al*[99] found that NLRP3 inflammasome was downregulated in MCAO mice after MSC-Exos treatment, thereby reducing inflammation and pyroptosis. They also observed that MSC-Exos contributed to MG polarization towards the M2 phenotype by inhibiting NLRP3[99]. On the one hand, it may be due to the high plasticity of the MG phenotype, which can dynamically switch according to brain environmental variables[74]. On the other hand, it may be related to the amount of autophagy[91,99,145].

Collectively, MSC-Exos could improve the immune inflammatory response after IS *via* affecting the activation of MG/macrophages and Ast, reducing reactive astrocyte hyperplasia, decreasing excessive infiltration of neutrophils, balancing the functional status of T cell subsets, suppressing the proliferation of B cells, inhibiting DC maturation, and regulating the secretion of inflammatory mediators. Therefore, MSC-Exos exhibits immunomodulatory effects and may help to reduce neurological damage and promote neurological repair after IS.

FROM BENCH TO BEDSIDE: RESPONSES TO THE LIMITATIONS OF MSC-EXOS THERAPY FOR IS

To date, a growing number of studies have demonstrated the great potential of MSC-Exos in treating IS. However, lacking target ability of natural exosomes produced by MSCs has greatly limited their clinical

application[146]. Regarding exosome producing, the cell membrane invaginates to form endosomes, which in turn form multivesicular bodies (MVBs), and the MVBs finally fuse with the plasma membrane to release luminal vesicles (called exosomes) into the extracellular matrix (ECM)[147]. At present, basing on the production process of exosome, two strategies are proposed to improve the targeting ability of exosomes, comprising "cell engineering" (pre-isolation) and "exosome engineering" (post-isolation) [147] (Figure 2). For example, the cyclin (Arg-Gly-Asp-DTyr-Lys) peptide [c(RGDyK)] and the rabies virus glycoprotein (RVG) peptide have been used explicitly to target the brain. B-Exos loaded with cholesterol-modified miR-210 coupled to c(RGDyK) could bind to integrin αvβ3 on the BBB and deliver miR-210 to the site of cerebral infarction, thereby ameliorating post-stroke symptoms[148]. Additionally, c(RGDyK)-modified MSC-Exos loaded with curcumin (cRGD-Exo-cur) was used in a study, followed by tail vein injection to target the area of cerebral ischemic injury and enter neurons, MG and Ast, effectively inhibiting the inflammatory response and cellular apoptosis[146]. RVG fused with exosomes protein lysosome-associated membrane glycoprotein 2b could bind to acetylcholine receptors on the BBB and effectively deliver miR-124 to the infarct site, therefore promoting post-IS neurogenesis and reducing ischemic injury[149]. In another study, high-mobility group box 1 (HMGB1)-siRNA was loaded into RVG-modified exosomes (RVG-Exos) by electroporation and injected into an MCAO model *via* tail vein. The results showed that RVG-Exos loaded with HMGB1-siRNA was effective in reducing the level of brain apoptosis and infarct size and had the potential to target IS[150].

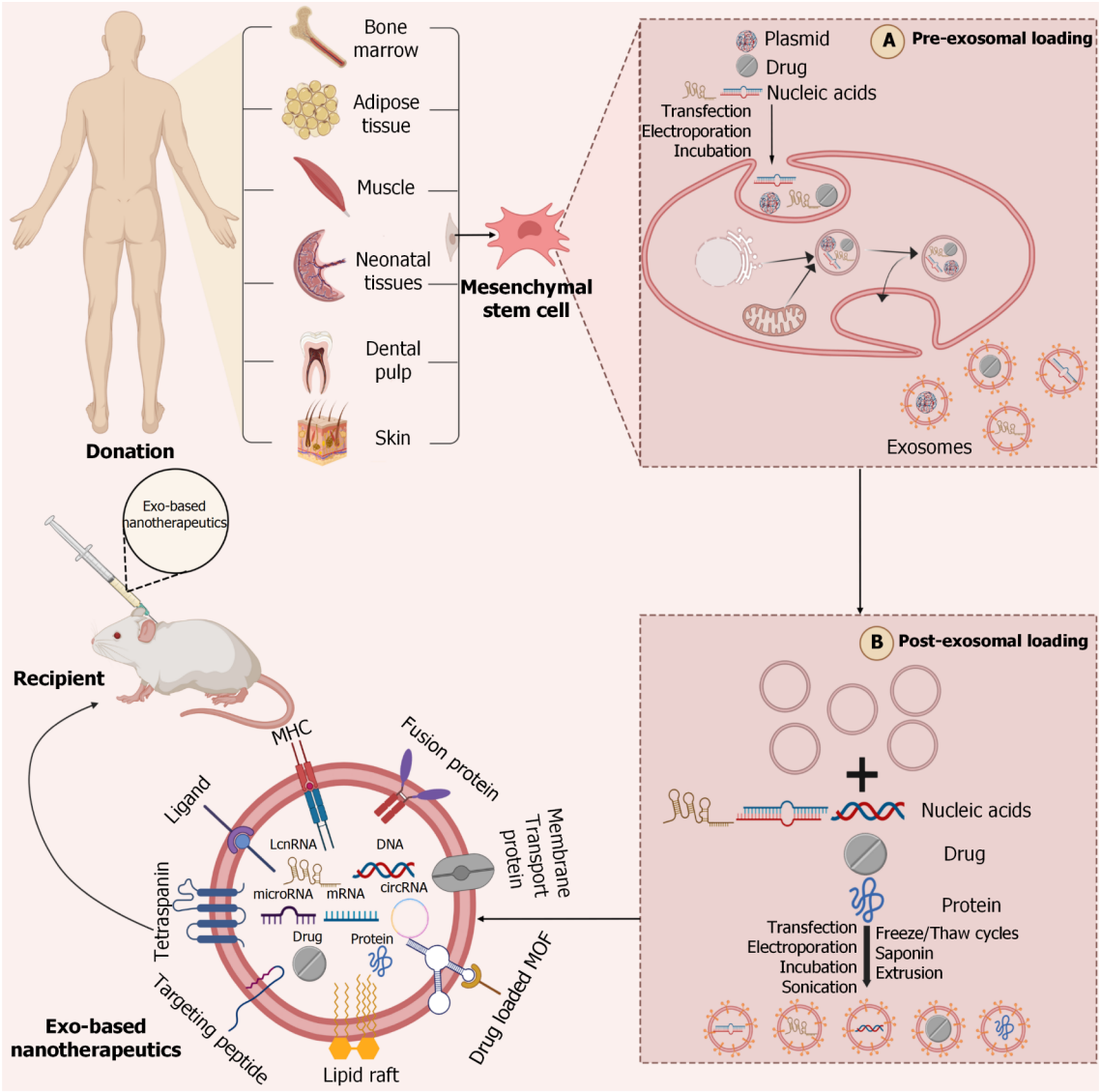
Next, the "low yield bottleneck" of MSC-Exos is also one of the main causes limiting its clinical application. Some researchers have illustrated that the pretreatment of MSCs appears to increase the yield of MSC-Exos. For instance, the three-dimensional porous scaffold structure increases the surface area for cell-ECM interaction, compared to the traditional two-dimensional culture of BMSCs. In addition, the three-dimensional culture more closely resembled the natural ECM conditions, providing a better environment for cell attachment and growth, thus substantially increasing the yield of MSC-Exos[151]. Some studies have indicated that cultures using microcarriers and hollow fiber bioreactor can provide cells a larger attachment area and further enhance the secretion of MSC-Exos[152,153]. Moreover, a recent study found that pretreatment of MSCs with small molecule modulators (N-methyl-dopamine and norepinephrine) tripled the production of exosomes without altering the intrinsic regenerative effects of MSC-Exos and the level of total exosomes protein expression[154].

To sum up, enhancing the targeting of exosomes by modifying them and improving the yield of exosomes by pretreating MSCs can both improve the therapeutic ability of exosomes. And further exploration of exosome improvement methods offers the possibility of transitioning from bench to bedside.

CONCLUSION

IS is a severe cerebrovascular disease that adversely affects patient's health and quality of life. A growing body of evidence suggests that the immune inflammatory response plays a critical role in pathogenesis of IS. It has emerged as a promising target for intervention in stroke therapy. After IS, the boundary between the CNS and peripheral immune system disappears due to the disruption of the BBB. Subsequently, the CNS and peripheral immune system can interact with each other, providing a unique opportunity to regulate the pathological process of IS and the repair process. At the same time, immunomodulation is one of the main mechanisms by which MSC and MSC-Exos exert their therapeutic effects on IS. MSC-Exos is expected to be an alternative therapy to MSC in treating stroke due to its parental cell-like capabilities and specific advantages. MSC-Exos exerts immunomodulatory effects mainly by affecting the inflammatory phenotype of glial cells in the CNS, inhibiting peripheral immune cell activation, proliferation, differentiation, and hyperinfilation, and regulating the secretion of immune-related molecules. Meanwhile, to complement or enhance the therapeutic suitability of exosomes, researchers are actively exploring novel methods to expand, modify or enhance their therapeutic capacity, such as modifying exosomes (to improve targeting) and pretreating MSC (to increase exosome yield).

Although the results of numerous preclinical studies have shown MSC-Exos to be one of the key breakthroughs in treating IS. However, the study of MSC-Exos in the treatment of IS is still in its infancy in clinical practice. Currently, there is only one study in the Clinical Trials Registry database to determine the effect of MSC-Exos administration on improving functional impairment after IS. This trial used the administration of miR-124-enriched isoform MSC-Exos to treat IS and entered into a phase I/II clinical trial (NCT03384433)[155]. There are many challenges to overcome to transfer MSC-Exos therapy further into the clinic: (1) Optimal duration of treatment and effective dose. Numerous studies have shown that post-IS inflammatory cells play a dual role (beneficial and detrimental) and the inhibition of the same pathway at the wrong time may exacerbate ischemic damage[27,36,38,45]. Therefore, during developing new therapeutic strategies for IS, we need to pay more attention to the duration of treatment. Interestingly, there are also cases where the timing of transplantation based on previous cellular therapies may affect the therapeutic outcome[156]. Thus, we need to further investigate the optimal timing of treatment with exosomes that may be influenced by parental cells. Most preclinical



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Figure 2 Strategies to enhance the targeting of mesenchymal stem cell-derived exosomes. Multiple strategies to be done before exosomes can be successfully translated into new technologies to improve the targeting ability of donor cells and therapeutic efficacy of chemical and biomolecular drugs. Current preclinical studies are focused on parental cells' modification (pre-isolation) (A); manipulation of exosome (post-isolation) (B). (Figure created with BioRender.com).

trials have currently chosen to administer a single dose of MSC-Exos in the acute phase of the stroke while showing beneficial effects. As such, the next step should investigate the effects of delayed-time dosing compared to acute phase dosing, in order to determine the optimal timing of treatment. However, determining the optimal timing of dosing may be difficult in practice, so we could further consider multiple repeat dosing; (2) A single research direction. Current experimental studies on the immunomodulatory aspects of MSC-Exos treatment with IS have focused on MG/macrophages, while other immune-related cells or factors remain poorly studied. Furthermore, the immune response following IS results from crosstalk within and between different cell types, which is complex and chronological. However, most experimental studies have usually explored a single mechanism of action mainly in a particular cell. There is no consensus on the exact molecular mechanism of MSC-Exos treatment of IS and further in-depth studies in multiple directions are urgently needed; (3) Lack of clinical trials; and (4) Stroke models combined with relevant clinical conditions. Current studies targeting MSC-Exos for treating IS have almost always been conducted in healthy animals. Therefore, when using stroke models, it should be as close as possible to achieve the clinical situation, like hypertension, diabetes, heart disease, atherosclerosis, secondary infections, *etc.*, as these diseases may affect the formation, treatment, and prognosis of stroke. To summarize, there are still many animal experiments and clinical trials to be finished before the fact that MSC-Exos can be applied as a routine treatment for stroke. However, based on the available evidence, we believe that MSC-Exos therapy is an emerging therapeutic strategy based on cellular therapy with great potential for future use in IS treatment, particularly in immunomodulation.

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MINIREVIEWS

Disease modeling of desmosome-related cardiomyopathy using induced pluripotent stem cell-derived cardiomyocytes

Shuichiro Higo

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Abstract

Cardiomyopathy is a pathological condition characterized by cardiac pump failure due to myocardial dysfunction and the major cause of advanced heart failure requiring heart transplantation. Although optimized medical therapies have been developed for heart failure during the last few decades, some patients with cardiomyopathy exhibit advanced heart failure and are refractory to medical therapies. Desmosome, which is a dynamic cell-to-cell junctional component, maintains the structural integrity of heart tissues. Genetic mutations in desmosomal genes cause arrhythmogenic cardiomyopathy (AC), a rare inheritable disease, and predispose patients to sudden cardiac death and heart failure. Recent advances in sequencing technologies have elucidated the genetic basis of cardiomyopathies and revealed that desmosome-related cardiomyopathy is concealed in broad cardiomyopathies. Among desmosomal genes, mutations in *PKP2* (which encodes PKP2) are most frequently identified in patients with AC. *PKP2* deficiency causes various pathological cardiac phenotypes. Human cardiomyocytes differentiated from patient-derived induced pluripotent stem cells (iPSCs) in combination with genome editing, which allows the precise arrangement of the targeted genome, are powerful experimental tools for studying disease. This review summarizes the current issues associated with practical medicine for advanced heart failure and the recent advances in disease modeling using iPSC-derived cardiomyocytes targeting desmosome-related cardiomyopathy caused by *PKP2* deficiency.

Key Words: Cardiomyopathy; Advanced heart failure; Induced pluripotent stem cell-derived cardiomyocytes; Desmosome; Genome editing; Gene therapy

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Core Tip: Prevention of advanced heart failure caused by cardiomyopathy is an urgent unmet need in the field of cardiovascular medicine. Desmosome, a cell-to-cell junctional component, maintains the structural integrity of heart tissues. Genetic mutations in desmosomal genes cause desmosome-related cardiomyopathy, an intractable disease refractory to standard medical therapies. This review introduces the recent advances in disease modeling of desmosome-related cardiomyopathy caused by *PKP2* mutations using induced pluripotent stem cell-derived cardiomyocytes.

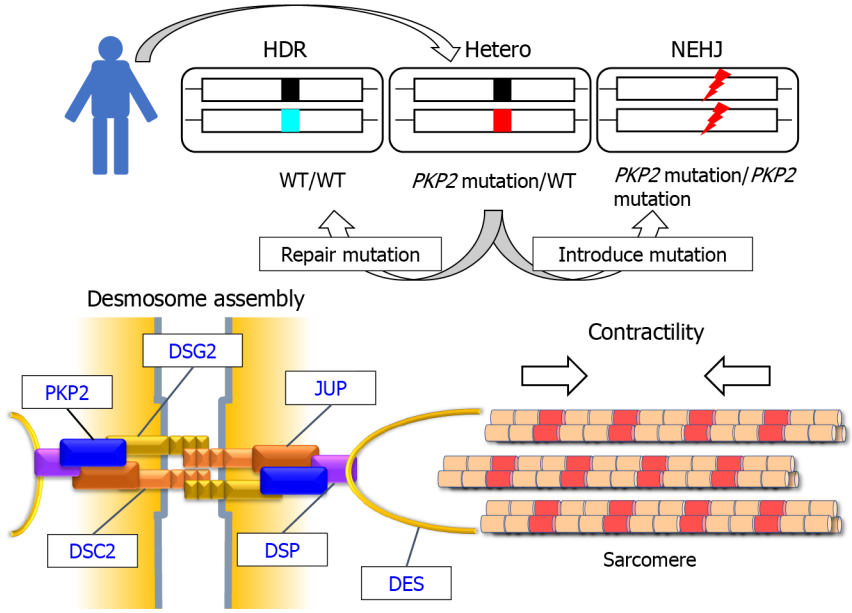
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INTRODUCTION

Heart failure is a clinical syndrome characterized by dyspnea, malaise, swelling, and/or decreased exercise capacity owing to impaired cardiac pumping function[1]. The established optimal medical therapies for heart failure have increased the survival rates of patients in the last few decades[2-4]. However, some patients are refractory to medical therapies and develop symptoms that are diagnosed as advanced heart failure. Currently, the therapeutic strategies available for these patients are heart transplantation and implantation of the ventricular assisting device[1,5]. Cardiomyopathy is a disease of cardiac pump failure due to myocardial dysfunction and is the major cause of advanced heart failure requiring heart transplantation[6-11]. Cardiomyopathies are differentially diagnosed mainly by using imaging modalities, including echocardiography, scintigraphy, computed tomography, magnetic resonance imaging, and cardiac catheterization. Based on the findings of these modalities, cardiomyopathies are classified into dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), or other rare cardiomyopathies, such as arrhythmogenic right ventricular cardiomyopathy (ARVC)[12]. Among 36,883 heart transplantation recipients registered in the International Society for Heart and Lung Transplantation Thoracic Organ Transplant Registry between 2010 and 2018, the major primary diagnoses were non-ischemic DCM (50.8%), ischemic cardiomyopathy (ICM) (32.4%) with coronary artery disease, RCM (3.5%), and HCM (3.4%)[13]. In Japan, cardiomyopathies [DCM (64%), end-stage HCM with left ventricular systolic dysfunction (12%), and ICM (9%)] account for more than three-quarters of underlying diseases among heart transplant recipients[14]. ARVC, a rare inherited disease, is characterized by the risk of life-threatening arrhythmias, myocardial dysfunction, and fibrofatty replacement of myocardial tissue, predisposing the patients to sudden cardiac death and heart failure[9,11]. The prevalence of ARVC among the registrants for heart transplantation is rare (0.3% and 1%-2% in the United Network for Organ Sharing registry[15] and Japan Organ Transplant Network[14], respectively).

DESMOSOME-RELATED CARDIOMYOPATHY IS CONCEALED IN ADVANCED HEART FAILURE

Recent clinical studies utilizing high-throughput sequencing technologies have elucidated the genetic basis of cardiomyopathies, identified various causative genetic variants, and revealed the correlation between genetic factors and clinical phenotypes or cardiac morphologies in patients with cardiomyopathies[16-20]. ARVC is an inherited disease caused by mutations in desmosomal genes (*PKP2*, *JUP*, *DSC2*, *DSG2*, and *DSP*) (Figure 1)[11,21,22]. These genes encode the structural components of the desmosome, a dynamic junction between cells that maintain the structural integrity of heart tissues[23,24]. The original disease phenotypes of ARVC are characterized by predominant right ventricular enlargement and contractile dysfunction. However, recent studies have reported left ventricular or biventricular involvement in patients with ARVC, resulting in the use of a broad phrase [arrhythmogenic cardiomyopathy (AC)][9,11]. Although the prevalence of AC in patients with advanced heart failure is rare, recent genetic analyses in large cohorts have demonstrated an increased incidence of desmosomal gene mutations in patients with DCM[18,25,26], which is the most frequent basal disease among heart transplantation registrants. Furthermore, homozygosity and compound or digenic heterozygosity of desmosomal genes are not rare, and patients with combined mutations exhibit a severe phenotype[27-30]. Recently, we identified *DSG2*-deficient cardiomyopathy caused by a rare homozygous stop-gain mutation in a patient initially diagnosed with idiopathic sporadic DCM[30]. *Dsg2* deficiency is associated with embryonic lethality in mice. Additionally, *Dsg2*-depleted embryonic stem cells do not proliferate[31]. However, a human male patient with a complete lack of *DSG2*



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Figure 1 Modeling impaired desmosome assembly and reduced contractility using isogenic induced pluripotent stem cell-derived cardiomyocytes with the precisely adjusted dose of *PKP2*. Heterozygous frameshift mutation in patient-derived induced pluripotent stem cells (iPSCs) was repaired through homology-directed repair. Homozygous frameshift mutations were introduced in *PKP2* through non-homologous end joining in patient-derived iPSCs. The generated isogenic iPSC-derived cardiomyocytes with the precisely adjusted expression of *PKP2* recapitulated impaired desmosome assembly and reduced contractility caused by *PKP2* deficiency. Desmosomal cadherin proteins (DSG2 and DSC2) form homo-dimers and hetero-dimers. PKP2 is a scaffold protein for desmosomal cadherins, JUP, and DSP. Desmosomes are linked to sarcomere structure *via* the intermediate filament protein DES that targets both desmosome and Z disc structure. HDR: Homology-directed repair; NEHJ: Non-homologous end joining; Hetero: Heterozygous mutation.

expression did not exhibit pathological phenotypes at birth but developed advanced heart failure during the teenage years. Immunohistochemical and transmission electron microscopy analyses of left ventricular heart tissues revealed that the loss of *DSG2* leads to aberrant deposition of desmosomal proteins and disruption of intercalated discs in cardiomyocytes. These findings suggest that desmosome-related cardiomyopathy is concealed in patients with advanced heart failure who are diagnosed with idiopathic DCM. As desmosome impairment is the most upstream molecular change in these patients, experimental studies must focus on elucidating the molecular mechanisms underlying the instability of cell-to-cell junctions to overcome advanced heart failure caused by desmosome-related cardiomyopathy. For disease modeling, patient-derived induced pluripotent stem cells (iPSCs) in combination with genome editing, which allows precise genomic modification of the targeted mutations, are powerful experimental tools to recapitulate pathological phenotypes based on the molecular factors of inherited cardiomyopathies[30,32-35].

PHENOTYPIC RECAPITULATION OF CARDIOMYOPATHY CAUSED BY PKP2 DEFICIENCY USING PATIENT-DERIVED IPSC-CMS

PKP2, which is encoded by *PKP2*, is a desmosomal protein localized to the outer dense plaque and functions as a scaffold for the other desmosome proteins DSG2, DSC2, JUP, and DSP[23,36] (Figure 1). Among the desmosomal genes, mutations in *PKP2* are most frequently identified in patients with AC [11,37-39], and have been extensively studied using patient-derived iPSC-CMs compared to other desmosomal genes (*DSG2*[30,40,41], *DSP*[42,43], and *DSC2*[44,45]). Various clinical phenotypes and pathological characteristics observed in patients with AC harboring *PKP2* mutations, downregulated desmosomal protein expression, upregulated lipogenesis, and increased apoptosis in heart tissues have been recapitulated using genetically engineered mouse models[11] and human cardiomyocytes differentiated from iPSCs[46-54] (Table 1). Most known mutations of *PKP2* are heterozygous and are missense, nonsense, and frameshift mutations. Studies on patient-derived iPSCs have identified that *PKP2* variants are heterozygous missense[48], heterozygous frameshift[46,47,49,50,54], homozygous frameshift[47,51], compound heterozygous, and frameshift[52] mutations. Disease-specific iPSCs are generated from fibroblasts[46-48,51], keratinocytes[49], adipose tissue-derived stromal cells[52], and peripheral blood mononuclear cells[54], whereas control iPSCs are generated from healthy subjects[46-49,51,52], human embryonic stem cells[50], or isogenic cells engineered from patient-derived iPSCs using genome editing[54]. Genome editing allows disease modeling by introducing heterozygous and

Table 1 Human disease model of *PKP2* deficiency using induced pluripotent stem cell-derived cardiomyocytes and experimental pathological phenotypes of arrhythmogenic cardiomyopathy

Genetic mutation	Origin of disease-specific iPSC	Experimental control	Desmosome proteins	Lipid accumulation	Apoptosis	Electrophysiology	Ultrastructure of desmosome	Contractility	Phenotypic rescue by gene replacement	Ref.
Heterozygous missense (c.1841T>C, p.L614P)	Dermal fibroblasts from a 30-yr-old male patient with AC	iPSCs from a 32-yr-old healthy male donor	Decreased JUP; No change in DSP, CDH2, and GJA1 (immunofluorescence staining at weeks 4-5)	Increased oil red O staining after exposure to adipogenic differentiation medium for 2 wk (oil red O staining)	NA	Ventricular-like action potential profile (single-cell patch-clamp recording (without control))	Increased cell width (TEM at weeks 4-5)	NA	NA	Ma *et al*[48]
Heterozygous frameshift (c.971_972ins, pA324fs335X); Heterozygous frameshift (c.148_151delACAG, p.T50SfsX110)	Dermal fibroblasts from a 30-yr-old male patient with AC	iPSCs from a healthy control	Decreased JUP and GJA1 (immunofluorescence staining)	Lipid droplet accumulation (TEM on day 40)	Increased apoptosis after serum starvation (TUNEL)	Prolonged field potential rise time (multielectrode array)	Widened and distorted desmosomes (TEM on day 40)	NA	NA	Caspi *et al* [46]
Homozygous frameshift (c.2484C>T leading to cryptic splicing); Heterozygous frameshift (c.2013delC, p.Lys672ArgfsX12)	Fibroblasts from a female patient with AC; Fibroblasts from a patient with AC	H9 human embryonic stem cell; iPSCs from cardiac fibroblasts of aborted fetus without a family history of AC	Nuclear translocation of JUP (immunofluorescence staining)	Increased lipogenesis after adipogenic stimulation for 4-5 wk (Nile red staining)	Increased apoptosis after adipogenic stimulation for 4-5 wk (TUNEL)	Slow intracellular calcium relaxation; Prolonged relaxation time (calcium imaging using Fura-2 acetoxymethyl on day 60)	NA	NA	NA	Kim *et al* [47]
Heterozygous frameshift (c.1760delT, p.V587Afsx655)	Dermal keratinocytes from a male patient with AC	iPSCs from dermal keratinocytes of a healthy control	Interrupted expression of DSP (immunofluorescence staining)	Lipid droplet accumulation after adipogenic stimulation for 4 wk (oil red O staining at months 3-4)	Genes associated with apoptosis remained unchanged (quantitative real-time PCR)	NA	NA	NA	NA	Dorn *et al* [49]
Homozygous frameshift (c.2484C>T leading to cryptic splicing)	Fibroblasts from a female patient with AC	iPSCs from a healthy control	Reduced JUP (immunofluorescence staining)	NA	NA	NA (decreased co-localization of NaV1.5 with PKP2)	NA	NA (increased pro-fibrotic gene expression after stretch)	NA	Martewicz *et al*[51]
Heterozygous frameshift (c.971_972InsT, p.A324fs335X)	A patient with AC	H9 human embryonic stem cells	Decreased membrane-localized JUP (immunofluorescence staining on day 34)	Increased lipid content (Nile red staining on day 34)	NA	Short action potential and slow spontaneous beat rate in engineered heart slices [optical mapping (relative to monolayer cardiomyocytes)]	NA	NA	NA	Blazeski *et al*[50]
Compound hetero-	Adipose tissue-	Gender-matched	Increased cytoplasmic and	No presence of	Not increased	Reduced sodium current	NA	NA	Restored	Khudiakov

zygous frameshift and missense (c.354delT, p.Y119MfsX23 and p.K859R)	derived mesenchymal multipotent stromal cells from a 14-yr-old female patient with AC	healthy donor	nuclear JUP levels (immunofluorescence staining on days 24-30)	lipid droplets (oil red O staining on day 24)	(PI staining at day 24-30)	density; Decreased action potential upstroke velocity (whole-cell patch-clamp and microelectrodes on days 24-30)			sodium current after lentiviral transduction of *PKP2*	*et al*[52]
Heterozygous and homozygous frameshift mutation (p.D109AfsX10, introduced mutation *via* genome editing)	Wild-type iPSC lines from two different donors with introduced heterozygous and homozygous frameshift mutations	Isogenic wild-type iPSCs	Decreased junctional localization of DSP and GJA1 (immunofluorescence staining); Impaired stability of junctional CDH2 (fluorescence recovery after photobleaching)	NA	NA	Prolonged action potential duration (optical voltage recording on day 30)	NA	Decreased systolic force (three-dimensional cardiac microtissues on day 40)	NA	Zhang *et al* [53]
Heterozygous frameshift mutation (c.1228dupG, p.D410fsX425)	Peripheral blood mononuclear cells from a female patient with AC	Isogenic iPSCs with corrected mutation (wild-type) and introduced homozygous frameshift mutations	Decreased area of desmosomes (DSG2, DSC2, and DSP) (immunofluorescence staining on day 14)	Lipid droplet accumulation in iPSC-CMs with homozygous frameshift mutations (TEM on day 28)	Increased apoptosis in iPSC-CMs with homozygous frameshift mutations (cleaved CASP3 expression on day 28)	Decreased propagation speed in iPSC-CMs with homozygous frameshift mutations (motion vector analysis on day 28)	Increased desmosome gap width (TEM on day 28)	Decreased contractility (contraction velocity and deformation distance evaluated using motion vector analysis on days 14 and 28)	Recovered contractility and desmosome assembly *via* AAV-mediated *PKP2* delivery	Inoue *et al* [54]

Gender of the patient or control donor is indicated if specified. Analytical methods along with time post-cardiomyocyte differentiation (if specified) are indicated. AAV: Adeno-associated virus; iPSC: Induced pluripotent stem cell; iPSC-CMs: Induced pluripotent stem cells-derived cardiomyocytes; PI: Propidium iodide; TEM: Transmission electron microscopy; NA: Not applicable; AC: Arrhythmogenic cardiomyopathy.

homozygous frameshift mutations in wild-type iPSC lines[53]. Decreased expression of desmosomal proteins, aberrant lipogenesis, and apoptosis of cardiomyocytes are observed in the heart tissues of patients with AC[9,55,56]. These pathological phenotypes are recapitulated in iPSC-CMs with *PKP2* mutations as determined using immunostaining[46-54], lipid staining[47-50], electron microscopy[46, 54], terminal transferase dUTP nick end labeling staining[46,47], and cleaved-CASP3 expression analysis [54]. Lethal arrhythmia is a hallmark of patients with AC. Arrhythmia phenotypes are recapitulated using iPSC-CMs with *PKP2* mutations as evidenced by the results of patch-clamp[48,52], multielectrode array[46], calcium imaging[47], and optical voltage recording[53]. In clinical settings, global or regional ventricular contractile dysfunction is defined as a major criterion for the diagnosis of ARVC in modified Task Force criteria[21] and Padua criteria[57]. However, the functional consequence in cardiomyocyte contractility caused by *PKP2* mutations has not been fully studied in human iPSC-CMs.

PKP2 DEFICIENCY AND CONTRACTILE DYSFUNCTION

We established iPSCs from a patient with AC harboring a heterozygous frameshift *PKP2* mutation

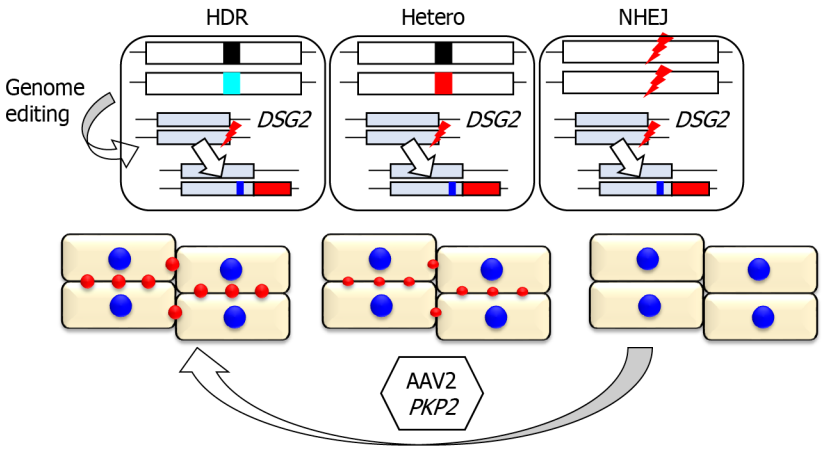
(c.1228dupG, p.D410fsX425) and generated an isogenic set of iPSC clones harboring three genotypes [heterozygous mutation (Hetero), homozygously corrected with homology-directed repair (HDR), and homozygously introduced frameshift mutations *via* non-homologous end joining (NHEJ)] using genome editing[54] (Figure 1). These isogenic sets of iPSCs comprise patient-derived Hetero-iPSCs, HDR-iPSCs with two-fold higher *PKP2* expression relative to Hetero-iPSCs, and NHEJ-iPSCs, which do not express *PKP2*, recapitulating both haploinsufficiency and complete loss of *PKP2*. After cardiomyocyte differentiation using the monolayer protocol with chemically defined medium[58], NHEJ-iPSC-CMs lacking *PKP2* expression exhibit lipid droplet accumulation, increased apoptosis, and decreased propagation rate (Table 1). However, patient-derived Hetero-iPSC-CMs with half-dose *PKP2* expression do not exhibit these pathological phenotypes, suggesting that the haploinsufficiency of *PKP2* is not sufficient to induce the above pathological phenotypes within 28 days after differentiation. In contrast, haploinsufficiency of *PKP2* decreased contractility, which was evaluated using motion vector analysis, within 14 days of differentiation. As the monolayer protocol confers strong contraction to iPSC-CMs on culture plates immediately after differentiation[58,59], continuous tensile overload may facilitate the contractile phenotype among isogenic iPSC-CMs. A recent study used isogenic iPSC-CMs in which heterozygous or homozygous frameshift mutation was introduced into wild-type iPSC-CMs[53]. The authors reported that *PKP2* deficiency decreased systolic force in three-dimensional cardiac microtissues. This further supported the functional relationship between *PKP2* deficiency and contractile dysfunction. An experimental study using cardiac tissue-specific *Pkp2* knockout mice demonstrated that the loss of *Pkp2* increased the distance between the cell periphery and DES, an intermediate filament protein in cardiomyocytes[60]. As DES connects Z-discs of sarcomeres to sarcolemmal costameres, desmosomes, and nuclear envelope[11,61], further experimental studies focusing on these cellular networks are required to elucidate the pathogenesis of desmosome-related cardiomyopathy.

DESMOSOME IMAGING USING THE ISOGENIC IPSC-CMS AND AAV-MEDIATED GENE REPLACEMENT

In the isogenic background, the haploinsufficiency of *PKP2* did not affect the localization or expression levels of desmosomal proteins in iPSC-CMs as evidenced by the results of immunostaining or western blotting analyses. However, the desmosome area represented by dot distribution on the cell periphery in Hetero-iPSC-CMs was significantly lower than that in HDR-iPSC-CMs[54], suggesting that desmosome assembly is impaired by *PKP2* haploinsufficiency. The impaired assembly of desmosomal proteins in human iPSC-CMs is supported by another study using isogenic iPSC-CMs. Fluorescence recovery after photobleaching experiments combined with lentivirus-mediated expression of fluorescent protein-tagged N-cadherin provided evidence that molecular stability of junctional N-cadherin is impaired by *PKP2* deficiency[53]. To trace the molecular behavior of endogenous proteins in cardiomyocytes, fluorescent tagging of the structural proteins through genome editing is a powerful tool[62,63]. However, fluorescent tagging of endogenous desmosomal genes might affect desmosome structures or cell-to-cell integrity in iPSCs or iPSC-CMs. We previously identified a patient with *DSG2*-deficient cardiomyopathy due to a rare homozygous stop-gain mutation and demonstrated that complete loss of *DSG2* in human iPSCs does not affect the differentiation or cellular morphology in iPSC-CMs[30]. These findings prompted us to use *DSG2* as the target of endogenous tagging by fluorescent protein to trace desmosome dynamics in live human iPSC-CMs. Genome editing targeting *DSG2* alleles was performed to establish the isogenic iPSC-CMs harboring identical two *DSG2* alleles comprising intact and knocked-in tdTomato alleles under the adjusted *PKP2* expression levels (Figure 2). The desmosome area (represented by desmoglein-2-tdTomato fusion protein) was significantly downregulated due to *PKP2* haploinsufficiency. Adeno-associated virus (AAV), a small, nonenveloped virus with a linear, single-stranded DNA, is widely used for gene therapy targeting human diseases, including heart failure[64,65]. AAV-mediated gene replacement of *PKP2* significantly restored the decreased contractility in Hetero-iPSC-CMs and NEHJ-iPSC-CMs, demonstrating the proof-of-concept for *PKP2* gene therapy in human cells. Furthermore, time-lapse imaging using NHEJ-iPSC-CMs captured the recovery of desmosomes, which gradually assembled at the cell periphery after AAV-mediated *PKP2* replacement (Figure 2). The established isogenic iPSCs harboring knocked-in tdTomato alleles allowed desmosome-imaging in living cells and provided distinct readouts for therapeutic development.

GENE REPLACEMENT THERAPY TARGETING HEART FAILURE

Several clinical trials using AAV-mediated gene replacement have been designed targeting cardiovascular disease[65,66]. A large-scale clinical trial was conducted as a randomized, multinational, double-blind, placebo-controlled phase 2 study targeting up to 250 patients with moderate-to-severe heart failure and reduced contractile function (CUPID2 trial)[67]. The study aimed to deliver



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Figure 2 Allele-specific fluorescent labeling of *DSG2* captures desmosome dynamics in isogenic induced pluripotent stem cell-derived cardiomyocytes. To establish a model for desmosome imaging, the tdTomato fluorescent reporter was knocked-in at the 3'-terminus of *DSG2* in the three established isogenic induced pluripotent stem cells (iPSCs) using genome editing. These isogenic iPSCs carried identical *DSG2* alleles comprising intact and knocked-in alleles distinguished by a synonymous single nucleotide variant (indicated as blue line). These iPSC-derived cardiomyocytes enable desmosome imaging and capturing desmosome recovery after adeno-associated virus-mediated replacement of *PKP2*. HDR: Homology-directed repair; NEHJ: Non-homologous end joining; AAV: Adeno-associated virus; Hetero: Heterozygous mutation.

sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) into heart tissues *via* intracoronary injection. SERCA2a regulates cardiomyocyte contraction and relaxation by transporting Ca^{2+} from the cytosol into the sarcoplasmic reticulum during diastole[68]. The deficiency of SERCA2a is associated with heart failure progression[69,70]. Although promising results were achieved in preceding preclinical and clinical studies[71-73], gene replacement of SERCA2a did not improve the clinical course of patients with heart failure[74]. The two clinical trials of gene therapy targeting patients with heart failure conducted in the same period (AGENT-HF[75] and SERCA-LVAD[76]) were terminated due to the neutral result of the CUPID2 trial and the lack of functional benefit. The amount of vector DNA in heart tissues obtained from patients who received gene therapy and subsequently underwent heart transplantation or mechanical circulatory support device implantation was low, suggesting that only a small proportion of cardiomyocytes expressed AAV-delivered SERCA2a in the myocardium. Although these clinical trials demonstrate the difficulty of gene delivery targeting human heart tissues, they provide the evidence for the safety of cardiac gene therapy and a basis for the design of future gene therapy trials. Recent genetic analysis clarified a large number of genetic mutations that cause cardiomyopathies with advanced heart failure in a loss-of-function manner and can be targeted by specific gene replacement therapy[77,78]. In desmosome-related cardiomyopathy, most of the identified mutations in *PKP2* are heterozygous[22,37,79,80]. However, in extremely rare cases, homozygous mutations of *PKP2* cause lethal infantile heart failure with left ventricular non-compaction or hypoplastic left heart syndrome[81-83]. No effective therapies are available for these patients who require a novel therapeutic approach for desmosome-related cardiomyopathy. Proof-of-concept studies for structural and functional recovery using both human iPSC-CM models and *in vivo* models are required for future clinical application.

CONCLUSION

Although human iPSC-CMs are immature and do not fully recapitulate *in vivo* heart tissues[59], tissue engineering approaches[84,85] will promote the maturation of iPSC-CMs and provide a useful tool in combination with genome editing. The isogenic iPSC-CMs that we established represent a human disease model that recapitulates reduced contractility and impaired desmosome assembly and provides a convenient cellular platform for therapeutic screening to examine upstream molecular targets of desmosome-related cardiomyopathy.

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FOOTNOTES

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MINIREVIEWS

Mesenchymal stem/stromal cells-derived exosomes for osteoporosis treatment

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Abstract

Osteoporosis is a systemic bone disease, which leads to decreased bone mass and an increased risk of fragility fractures. Currently, there are many anti-resorption drugs and osteosynthesis drugs, which are effective in the treatment of osteoporosis, but their usage is limited due to their contraindications and side effects. In regenerative medicine, the unique repair ability of mesenchymal stem cells (MSCs) has been favored by researchers. The exosomes secreted by MSCs have signal transduction and molecular delivery mechanisms, which may have therapeutic effects. In this review, we describe the regulatory effects of MSCs-derived exosomes on osteoclasts, osteoblasts, and bone immunity. We aim to summarize the preclinical studies of exosome therapy in osteoporosis. Furthermore, we speculate that exosome therapy can be a future direction to improve bone health.

Key Words: Mesenchymal stem cells; Exosome; Osteoporosis; Osteoblasts; Osteoclasts; Bone immunity

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Core Tip: Osteoporosis is one of the major diseases endangering bone health in the elderly. The existing treatment drugs have problems such as long-term administration and side effects; thus, it is fundamentally difficult to cure osteoporosis. Exosomes derived from mesenchymal stem cells (MSCs) are vesicles that deliver signals and molecules between cells and have shown substantial positive effects in pre-clinical trials. In this review, we summarize the latest progress of MSCs-derived exosomes in the regulation of bone metabolism.

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INTRODUCTION

Osteoporosis (OP) is a chronic systemic bone disease. It is characterized by decreased bone mass and destruction of bone microstructures, resulting in decreased bone mineral density (BMD) and ultimately an increased risk of fragility fractures[1]. The dynamic balance between bone formation and bone resorption is an important way to maintain normal bone metabolism. Osteoblasts promote bone formation by mineralizing the matrix, while osteoclasts degrade the bone matrix by secreting H+ and releasing cathepsin K, accelerating bone dissolution[2].

At present, the prevention and treatment of OP can be divided into adjuvant therapy and drug therapy. The therapeutic effects of drugs are mainly divided into two aspects: Inhibition of osteoclasts (inhibition of bone resorption) and stimulation of osteoblasts (promotion of bone formation). Among them, the drugs that inhibit bone resorption are denosumab, bisphosphonates and selective estrogen receptor modulators; and drugs that promote bone formation are teriparatide and abaloparatide. Romosozumab, a monoclonal antibody directed against sclerostin, has a dual regulatory effect, inhibiting bone resorption and promoting bone formation at the same time[3].

Although a variety of therapeutic drugs for OP have emerged, all the drugs mentioned above have side effects[4], and more novel and effective drugs and therapies are needed. There are new advances in the research on stem cells and their exosomes in tissue repair and treatment. Therefore, the new scheme of MSCs-derived exosomes for the treatment of OP has gradually become a therapeutic option[5].

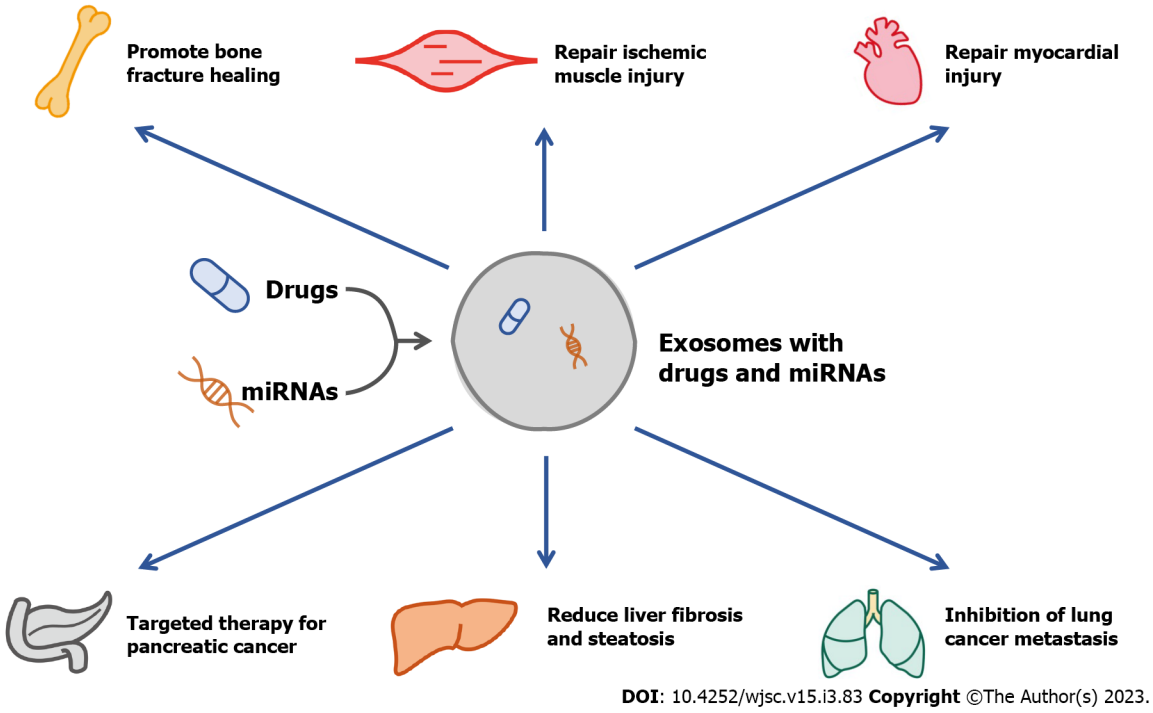
EXOSOMES AND MESENCHYMAL STEM CELLS

Exosomes are small vesicles secreted by cells with a diameter of 40 to 160 nm, which carry signals and molecules among cells. In addition, the exosomes secreted by the cells in diseased states contain specific microRNAs (miRNAs), which is helpful for the diagnosis of diseases[6]. As a carrier of signal transduction and drug delivery, exosomes also have good potential in the treatment of diseases, and their effectiveness has been confirmed by numerous animal studies[7-13] (Figure 1). In 1974, Friedenstein *et al*[14] first discovered that mesenchymal stem cells (MSCs) in bone marrow have the potential for osteogenic differentiation. Later, researchers continued to explore the function of MSCs and found that MSCs are pluripotent stem cells, which have the ability to differentiate into osteoblasts, adipocytes, chondrocytes, cardiac muscle cells and skeletal muscle cells[15]. Previous studies have found that therapy with MSCs can accelerate bone tissue repair and regeneration and maintain bone mass in OP[16,17].

MSCs-derived exosomes are likely to play a major role in mediating the therapeutic effect of MSCs [18,19]. A schematic diagram illustrating the mechanisms of MSCs-derived exosomes in the treatment of OP is shown in Figure 2. Therefore, MSCs-derived exosomes are expected to be a new biological agent in the future.

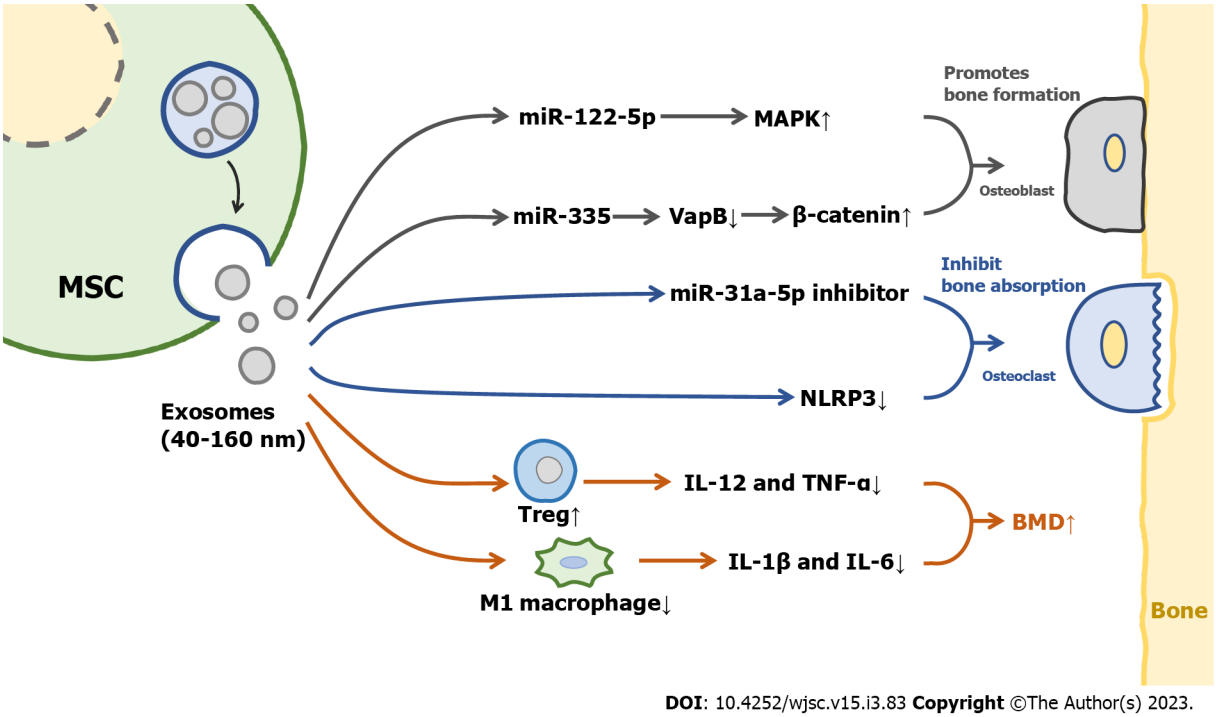
MSCS-DERIVED EXOSOMES IN THE TREATMENT OF OP

Exosomes have the characteristics of good targeting ability, high permeability and low toxicity. Therefore, exosomes therapy has become one of the hot spots in the study of OP treatment. Bone regeneration is one of the main modalities in the treatment of OP and bone fracture. MSCs-derived exosomes can promote the formation of new bone with vasculature, biomechanics, and histology[20]. MSCs-derived exosomes regulate bone metabolism and treat OP through signaling pathways, such as stimulating osteoblast differentiation from bone marrow stem cells, promoting osteoblast proliferation,



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Figure 1 Schematic diagram illustrating the therapeutic potential of exosomes in diseases of multiple organs. miRNAs: MicroRNAs.



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Figure 2 Schematic diagram illustrating the mechanisms of mesenchymal stem cells-derived exosomes in the treatment of osteoporosis. MSC: Mesenchymal stem cell; BMD: Bone mineral density.

inducing angiogenesis, and immunomodulation[21]. During treatment, MSCs-derived exosomes achieve the purpose of bone regeneration and treatment of OP by carrying and transporting proteins, miRNAs, and artificial synthetic drugs[22-24]. According to the pathogenesis of OP, exosomes are mainly involved in regulating the effects of osteoblasts, osteoclasts and bone immunity (Table 1).

Table 1 Summary of studies on mesenchymal stem cells-derived exosomes in osteoporosis treatment

Ref.	Exosome source	Cell type	Consequence
Zuo *et al*[24], 2019	BMSCs	BMSCs (after irradiation)	Alleviating radiation-induced bone loss by restoring Wnt/β-Catenin signaling pathway
Zhao *et al*[25], 2018	BMSCs	Osteoblasts (hFOB 1.19)	Promoting proliferation of osteoblasts *via* MAPK pathway
Liao *et al*[26], 2019	BMSCs	Osteoblasts	Promoting proliferation of osteoblasts by carrying miR-122-5p
Qi *et al*[27], 2016	hiPSC-MSCs	Osteoblasts	Promoting osteoblast proliferation, differentiation and bone formation
Hu *et al*[28], 2021	BMSCs	Osteoblasts	Promoting differentiation of osteoblasts by carrying miR-335
Zhang *et al*[31], 2021	AD-MSCs	Osteoclasts	Inhibiting NLRP3 inflammasome activation in osteoclasts
Nakao *et al*[32], 2021	GMSCs	Osteoclasts	Inhibiting RANKL and reducing osteoclast formation by carrying miR-1260b *via* Wnt5a/JNK signal pathway
Xu *et al*[33], 2018	BMSCs	Osteoclasts	Increasing the number of osteoclasts by carrying miR-31a-5p *via* RhoA pathway
Wei *et al*[40], 2019	BMSCs	Osteoblasts	Promoting osteoblast differentiation by inhibiting macrophage polarization and reducing the levels of inflammatory factors

BMSCs: Bone marrow mesenchymal stem cells; hiPSC-MSCs: Mesenchymal stem cells derived from human induced pluripotent stem cells; AD-MSCs: Adipose derived mesenchymal stem cells; GMSCs: Gingival tissue-derived mesenchymal stem cells; MAPK: Mitogen-activated protein kinase; NLRP3: NLR family pyrin domain containing 3; RANKL: Receptor activator of NF-κB ligand; Wnt5a: Wnt family member 5A; JNK: c-Jun N-terminal kinases.

EFFECTS OF MSCS-DERIVED EXOSOMES ON OSTEOBLASTS

Osteoblasts are derived from pluripotent MSCs, which are major functional cells in bone matrix synthesis, secretion, and mineralization. Exosomes can directly regulate the activity of osteoblasts and then affect OP. MSCs-derived exosomes promoted the cell cycle of hFOB 1.19 cells, a type of osteoblast, and activated their proliferative activity through the MAPK pathway[25]. It was also found that overexpression of miR-122-5p in MSCs-derived exosomes of bone marrow can increase the BMD of the femoral head through the MAPK pathway[26]. Co-culture of MSCs-derived exosomes with bone marrow MSCs from osteoporotic rats resulted in increased levels of osteogenesis-related proteins and mineral deposition; in addition, the use of MSCs-derived exosomes promoted bone regeneration in a rat model of calvarial defects[27]. One study showed that the infusion of bone marrow MSCs-derived exosomes carrying miR-335 into mice with fractures significantly accelerated fracture healing, as miR-335 in exosomes was able to inhibit VapB, activate the Wnt/β-catenin pathway, and promote osteoblast differentiation[28]. Notably, exosomal miRNAs derived from MSCs are critical in regulating the function of osteoblasts. These miRNAs participate in and regulate some key signaling pathways and alter protein expression in osteoblasts. The positive roles of exosomes in osteoblast proliferation and differentiation, and the excellent therapeutic performance of exosomes in bone repair and bone mass recovery, indicate the potential of MSCs-derived exosomes in the treatment of OP.

EFFECTS OF MSCS-DERIVED EXOSOMES ON OSTEOCLASTS

Osteoclasts originate from hematopoietic progenitor cells in the bone marrow, and play an osteolytic role by secreting H+ and cathepsin K[29,30]. Adipose MSCs-derived exosomes inhibited NLRP3 in the osteoclasts of diabetic OP rat models and increased BMD[31]. RANKL is a major factor in promoting the differentiation of preosteoclasts into osteoclasts, gingiva MSCs-derived exosomes can target Wnt5a in periodontal osteoclasts and inhibit the expression of RANKL, thus reducing the differentiation of osteoclasts[32]. Of course, as a carrier of miRNA, exosomes will also have an effect on osteoclasts by carrying miRNA. Bone marrow MSCs-derived exosomes delivered miR-31a-5p to osteoclasts, which increased osteoclast number and bone resorption. In addition, BMD significantly increased in a rat model treated with miR-31a-5p inhibitor[33]. These findings suggested that MSCs-derived exosomes can regulate osteoclast differentiation, increase bone density, and inhibit OP. Notably, bone marrow stromal cell-derived exosomes do not have any effect on osteoclasts at the surface of trabecular bone, and therefore do not play a role in preventing bone resorption[34]. There are few studies on the regulation of osteoclast differentiation by MSCs-derived exosomes. Therefore, we need to further explore the potential of MSCs-derived exosomes in the treatment of OP by regulating osteoclast differ-

entiation.

EFFECTS OF MSCS-DERIVED EXOSOMES ON BONE IMMUNITY

There is an intricate relationship between the immune system and the skeletal system. Activated immune cells release inflammatory mediators and cytokines that upset the balance of bone remodeling and activate osteoclasts, leading to bone loss and OP[35,36]. MSCs-derived exosomes can regulate the immune system[37]. After treatment with adipose MSCs-derived exosomes in mice with colitis, regulatory T cells returned to normal baseline level, and the levels of inflammatory factors such as interleukin (IL)-12 and tumor necrosis factor-α reduced[38]. Exosomes derived from adipose and bone marrow MSCs can significantly increase the level of type II collagen in articular cartilage, promote articular cartilage formation and accelerate the recovery of osteoarthritis in mice[39]. MSCs-derived exosomes promote the differentiation of bone marrow MSCs into osteoblasts by inhibiting the polarization of M1 macrophages and reducing the levels of inflammatory cytokines such as Il-1β and IL-6[40]. MSCs-derived exosomes can regulate immunity and inflammation in vivo. Unfortunately, little is known about their role in bone immunity. However, available findings suggest the potential of MSCs-derived exosomes in the treatment of OP by modulating bone immunity.

CONCLUSION

At present, drug treatment of OP still focuses on inhibiting bone resorption and promoting bone formation. Drug therapy has a good effect, but long-term use of drugs can cause serious side effects. In the field of bone regeneration, stem cell therapy has significant efficacy, and exosomes are one of the important carriers of cell information and factors. Therefore, MSCs-derived exosomes may be a promising biological agent in the treatment of OP. This review focuses on the mechanism of MSCs-derived exosomes in the treatment of OP, by delivering miRNA, regulating related targets, and regulating bone immunity. Therefore, the potential of MSCs-derived exosomes in the treatment of OP is anticipated, but further preclinical studies are needed to prove the safety and reliability of exosome therapy and provide strong evidence for the clinical conversion of exosome therapy.

FOOTNOTES

Author contributions: Shao J and Zhang WW were responsible for the concept design; Huo KL, Shao J and Zhang WW wrote the main manuscript text and prepared Figures 1 and 2; Yang TY provided technical support; all authors reviewed the manuscript.

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ORIGINAL ARTICLE

Basic Study

Mammalian Ste20-like kinase 1 inhibition as a cellular mediator of anoikis in mouse bone marrow mesenchymal stem cells

Tao Zhang, Qian Zhang, Wan-Cheng Yu

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Abstract

BACKGROUND

The low survival rate of mesenchymal stem cells (MSCs) caused by anoikis, a form of apoptosis, limits the therapeutic efficacy of MSCs. As a proapoptotic molecule, mammalian Ste20-like kinase 1 (Mst1) can increase the production of reactive oxygen species (ROS), thereby promoting anoikis. Recently, we found that Mst1 inhibition could protect mouse bone marrow MSCs (mBMSCs) from H_2O_2-induced cell apoptosis by inducing autophagy and reducing ROS production. However, the influence of Mst1 inhibition on anoikis in mBMSCs remains unclear.

AIM

To investigate the mechanisms by which Mst1 inhibition acts on anoikis in isolated mBMSCs.

METHODS

Poly-2-hydroxyethyl methacrylate-induced anoikis was used following the silencing of Mst1 expression by short hairpin RNA (shRNA) adenovirus transfection. Integrin (ITGs) were tested by flow cytometry. Autophagy and ITGα5β1 were inhibited using 3-methyladenine and small interfering RNA, respectively. The alterations in anoikis were measured by Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling and anoikis assays. The levels of the anoikis-related proteins ITGα5, ITGβ1, and phospho-focal adhesion kinase and the activation of caspase 3 and the autophagy-related proteins microtubules associated protein 1 light chain 3 II/I, Beclin1 and p62 were detected by Western blotting.

RESULTS

In isolated mBMSCs, Mst1 expression was upregulated, and Mst1 inhibition significantly reduced cell apoptosis, induced autophagy and decreased ROS levels. Mechanistically, we found that Mst1 inhibition could upregulate ITGα5

and ITGβ1 expression but not ITGα4, ITGαv, or ITGβ3 expression. Moreover, autophagy induced by upregulated ITGα5β1 expression following Mst1 inhibition played an essential role in the protective efficacy of Mst1 inhibition in averting anoikis.

CONCLUSION

Mst1 inhibition ameliorated autophagy formation, increased ITGα5β1 expression, and decreased the excessive production of ROS, thereby reducing cell apoptosis in isolated mBMSCs. Based on these results, Mst1 inhibition may provide a promising strategy to overcome anoikis of implanted MSCs.

Key Words: Mouse bone marrow mesenchymal stem cell; Mammalian sterile 20-like kinase 1; Anoikis; Integrin; Autophagy; Reactive oxygen species

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Core Tip: In isolated mouse bone marrow mesenchymal stem cell (mBMSCs), Mammalian sterile 20-like kinase 1 (Mst1) inhibition could ameliorate not only autophagy formation but also upregulate integrin (ITG) α5β1 expression (but not ITGα4, ITGαv, or ITGβ3). In addition, Mst1 inhibition-induced autophagy could scavenge the excessive production of ITGα5β1-triggered ROS. Therefore, Mst1 inhibition-based infusion may improve the survival of MSCs, thereby serving as an ideal candidate for clinical transplantation in pulmonary arterial hypertension.

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INTRODUCTION

Mesenchymal stem cell (MSC) therapy is characterized by anti-inflammatory, immunomodulatory, and regenerative properties, providing an attractive therapeutic approach for pulmonary arterial hypertension (PAH)[1]. Despite the therapeutic potential of MSCs for improving the outcomes of PAH patients[2,3], no more than 5% of cells survive after transplant[4]. Thus, the low survival rate of the grafted cells is widely perceived as the major hindrance for an MSC-based therapy for PAH.

Anoikis occurs when cells detach from the extracellular matrix and subsequently undergo apoptosis, and potentially acts as a major enabling factor for the apoptosis of transplanted cells[5,6]. Indeed, after isolation from the extracellular matrix (ECM) and injection into the circulatory system for transplantation, MSCs will undergo anoikis, also referred to as cell isolation-induced apoptosis, leading to a series of alterations in anoikis signalling pathways[3,7,8]. Anoikis can be induced by destruction of integrin (ITGs) signalling or deletion of ITGs genes[9]. After isolation, focal adhesion kinase (FAK), a key downstream target of ITGs, is recruited to focal adhesion sites, consequently activating cell survival signals, such as blocking caspase 3 expression[10]. However, it remains unknown whether ITGs signalling is involved in the process of anoikis in MSCs.

Autophagy is a dynamic process that maintains homeostasis by preventing the accumulation of excessive biomolecules and impaired cells and organelles. There is accumulating evidence of a link between autophagy and anoikis[11]. Previously, we demonstrated that mammalian Ste20-like kinase 1 (Mst1) inhibition could reduce H_2O_2-induced apoptosis of mBMSCs by inducing autophagy formation [12]. Mst1 is a serine/threonine kinase, known as a key mediator in cellular processes, including mediating the apoptosis[13]. However, the molecular mechanism by which Mst1 inhibition mediates autophagy and anoikis in isolated mBMSCs remains to be clarified.

In this study, we investigated the potential regulatory effect of Mst1 inhibition on ITGs signalling, autophagy and anoikis in isolated mBMSCs.

MATERIALS AND METHODS

Cell culture

The mBMSCs were obtained as previously described[12]. Cultured mBMSCs between passages 3 and 5 were selected for subsequent experiments.

Adenovirus infection

Adenovirus harbouring Mst1 short hair RNA (Ad-sh-Mst1) and the control vector for Mst1 shRNA (Ad-NC-Mst1) were purchased from WZ Biosciences (China). Vector details have been previously described [12]. The shRNA sequence targeting Mst1 in mice was GCCCTCACGTA GTCAAGTATT.

siRNA transfection

The small interfering RNAs (siRNAs) were obtained from GenePharma (China). The sense and antisense strand sequences of siRNA are as follows: Mouse siRNA-ITGα5, 5'-GCAGGGAGA-UGAAGAUCUACC' (sense) and 5'-UAGAUCUUCAUCUCCCUGCAG' (antisense); mouse siRNA-ITGβ 1, 5'-GGAGAACCACAGAAGUUUACA-3' (sense) and 5'-UAAACUUCUGUGGUUCUCCUG-3' (antisense); and siRNA-negative control (NC), 5'-UUCUCCGAACGUGUCACGUTT' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). Subsequently, 24 h after infection with Ad-sh-Mst1, mBMSCs at 75% confluence were transfected with ITGα5, ITGβ1 or NC siRNA (50 nM) using Lipofectamine RNAi MAX (13778500, Invitrogen) according to the manufacturer's instructions. The expression of ITGα5 or ITGβ1 was substantially blocked by the transfected siRNA.

Cell treatment

Petri dishes coated with polyhydroxyethyl methacrylate [Poly-HEMA, 529257, Sigma, United States of America (USA)] were used to prevent cells from adhering to the tissue culture plates. Briefly, poly-HEMA stock material was dissolved in 95% ethanol at a concentration of 12 mg/mL, and 1 mL of 12 mg/mL poly-HEMA was added to each well of a 6-well plate and then dried overnight on a clean bench. Cells were transfected as previously described. Cells (5×10^5) were coated with 12.5 mg/mL poly-HEMA in each well for a certain period of time.

To inhibit autophagy, cells were pretreated with 5 mmol/L 3-MA (189490, Selleck, USA) for 1 h and then cultured in poly-HEMA-precoated plates for a certain period of time.

Assay of intracellular ROS

As mentioned above, cellular ROS were assessed using the ROS probe 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), S0033, Beyotime Biotechnology, China)[12]. The mean fluorescence intensity was detected *via* flow cytometry.

Isolation-induced anoikis assay

Anoikis was analysed using an in situ Direct DNA Fragmentation Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL) Assay Kit [ab66108, Abcam, The United Kingdom of Great Britain and Northern Ireland (UK)]. After incubation in poly-HEMA-coated plates, the cells were collected and added to 70% ice ethanol for 30 min. Ethanol was then removed, and the cells were resuspended in washing buffer and then stained with a staining solution for 60 min. Prior to the addition of the PI/RNase A solution, the cells were washed twice with rinse buffer. Quantification analysis was performed by BectonDickinson Fluorescence Activating Cell Sorter (BD FACSDiva) software, [Ex/Em = 488/520 nm for fluoresceine isothiocyanate and 488/623 nm for propidium iodide)].

Anoikis was also detected by a CytoSelect™ 24-Well Anoikis Assay (XY-CBA-080, Cell Biolabs, USA) according to the manufacturer's instructions. Briefly, cells (1×10^6 cells/well) were cultured in each well of 24-well plate for 36 h before staining with ethidium homodimer (EthD-1) at 37°C for 1 h. The presence of red EthD-1 fluorescence in dead cells was observed by a fluorescence microscope, and cell viability was determined using a thiazolyl blue tetrazolium bromide (MTT) assay.

Flow cytometry

Cells were incubated in poly-HEMA-coated petri dishes for 36 h, centrifuged at 300 × *g* for 5 min and cultured in antibodies (ITGα4 [1/500 dilution, 553157, BD], ITGα5 [1/500 dilution, 557447, BD], ITGαv [1/300 dilution, 740946, BD], ITGβ1 [1/500 dilution, 561796, BD], ITGβ3 [1/100 dilution, 740677, BD]) for 1 h according to the operation manual.

Cell adhesion

After culture in poly-HEMA-coated petri dishes, the collected cells were resuspended in complete α-MEM and then plated in triplicate (5×10^4 cells/well) onto wells coated with fibronectin (10 g/mL), which was previously blocked with 1% BSA for 1 h. After 6 h, the cells were washed with phosphate belanced solution (PBS) and stained with crystal violet. Unbound dye was removed with PBS before adding a 10% acetic acid solution. The absorbance was read at 630 nm using a Multiskan MK3 microplate reader. The experiment was repeated three times. Cell adhesion was calculated according to the proportion of adhered cells in the control group.

Cytokine levels

The supernatants in each group were collected after culture in poly-HEMA-coated petri dishes for 36 h. The levels of anti-inflammatory cytokines were measured using a BD™ Cytometric Bead Array (CBA)

Mouse Th1/Th2/Th17 Cytokine Kit (561665, BD, USA) in accordance with the instruction manual. The levels of interleukin (IL)-4 (IL-4), IL-10, IL-17A and IL-6 in cell supernatants were measured using flow cytometry. Data analysis was performed as previously described[14].

Nude mouse tumorigenicity

All animal procedures were approved by the Animal Care and Use Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (IACUC protocol, Approval No. 2020-333). A total of 10 female nude mice (4 wk old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and raised in a specific pathogen-free environment. Mice were placed at a standard room temperature at a normal day-night cycle with free access to standard diet and water. Afterwards, 5.0 × 10^6 mBMSCs (n = 3), mBMSC/NC-Mst1 (n = 3), and mBMSC/sh-Mst1 (n = 4) were injected into the right flank near the hind legs of each nude mouse. The tumours were measured with a Vernier calliper every 4 d. Sixty days after cell inoculation, all mice were anaesthetized with ether, and tissues were collected.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed as previously reported[12]. mBMSCs were differentiated *via* 21-d exposure to osteogenic or adipogenic conditions, and total mRNA from mBMSCs subjected to these conditions and siRNA-transfected cells was isolated using TRIzol Reagent (15596026, Thermo Fisher Scientific, USA). The RNA was subsequently reverse transcribed into cDNA and amplified using the SYBR® Premix Ex Taq™ II kit (RR420, Takara, JPN) and d ABI 7500 real-time PCR system (Applied Biosystems). Each experiment was repeated three times. Data were normalized through the $2^{-\Delta\Delta CT}$ method using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The primer sequences are shown in Supporting Information Supplementary Table 1.

Western blot analysis

To determine protein expression, Western blot analysis was performed. After culture in poly-HEMA-coated plates, whole-cell protein extracts were prepared in radio-immunoprecipitation assay lysis buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% skimmed milk or Bovine serum albumin in Tris-Buffered Saline Tween-20 for 1 h and incubated overnight at 4 °C with the following primary antibodies (diluted by Western Primary Antibody Buffer, P0023A, Beyotime): Mst1 (1:1000, ab51134, Abcam), ITGα5 (1:1000, ab150361, Abcam), ITGβ1 (1:1000, ab179471, Abcam), phospho-FAK (Tyr397) [1:500, 3283S, Cell Signaling Technology (CST)], FAK (1:1,000, 3285S, CST), activated caspase 3 (1:1000, ab214430, Abcam), and caspase 3 (1:1000, ab18297, Abcam). GAPDH (1:1000, 5174S, CST) served as the loading control. Anti-rabbit IgG and HRP-linked antibodies (1:1000, 7074S, CST) were used. The relative protein expression levels were compared with GAPDH using ImageJ software.

Statistical analysis

All results are expressed as the mean ± SD. One-way Analysis of Variance was used for data analysis. $P < 0.05$ was considered statistically significant.

RESULTS

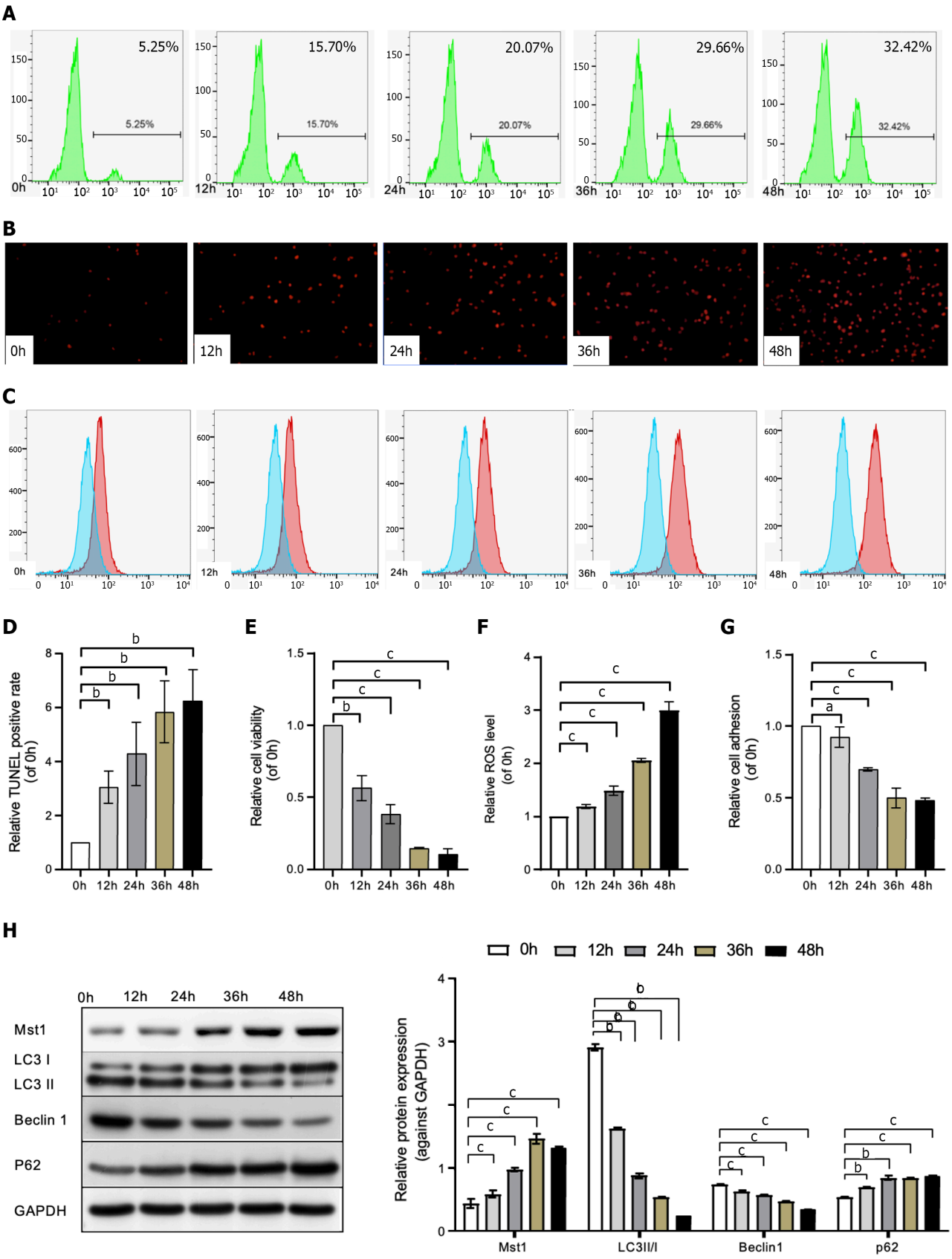
The loss of attachment to ECM increased the rate of aberrant cell apoptosis, ROS levels, and Mst1 expression and inhibited autophagy in mBMSCs

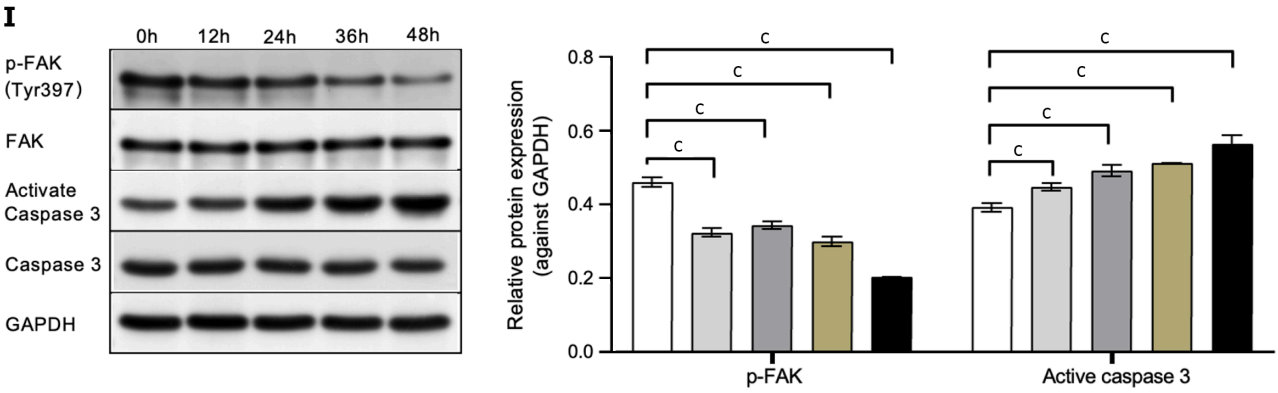
As the ability to reduce cell adhesivity to culture plates, Poly-HEMA was used to simulate an anchorage-independent culture condition. In present study, the sensitivity of mBMSCs to anoikis in Poly-HEMA-pre-coated condition were tested.

Using the TUNEL and Anoikis Assay Kit, the results showed an increased rate of mBMSC apoptosis in a time-dependent manner under poly-HEMA-induced isolated conditions (Figures 1A, B, D and E), suggesting that anoikis of mBMSCs could be induced in precoated poly-HEMA plates. In addition, the cell adhesion decreased at 24 h, 36 h, and 48 h compared with that at 0 h (Figure 1G).

Moreover, staining of intracellular ROS with the ROS probe DCFH-DA showed increased ROS levels at 24, 36, and 48 h compared with 0 h (Figures 1C and F), demonstrating the production of ROS in poly-HEMA-induced isolated mBMSCs.

To determine the alterations in Mst1 expression, autophagy and the FAK/Caspase 3 pathway in mBMSCs under isolated conditions, the protein level of Mst1, autophagy-related proteins (LC3 II/I, Beclin1, p62), p-FAK, and activated caspase 3 was detected by Western blot analysis. The data suggested that Mst1 was upregulated in isolated mBMSCs (Figure 1H). Moreover, the expression of p-FAK decreased, and the activation of caspase 3 increased in a time-dependent manner (Figure 1I). Similarly, LC3 II/I and Beclin1 expression was downregulated, and p62 expression was upregulated in a time-dependent manner (Figure 1H).


A
5.25%
15.70%
20.07%
29.66%
32.42%
0h
12h
24h
36h
48h
B
C
D
Relative TUNEL positive rate (of 0h)
E
Relative cell viability (of 0h)
F
Relative ROS level (of 0h)
G
Relative cell adhesion (of 0h)
H
Mst1
LC3 I
LC3 II
Beclin 1
P62
GAPDH
Relative protein expression (against GAPDH)
LC3II/I
Beclin1
p62



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Figure 1 Mouse bone marrow mesenchymal stem cells exhibit susceptibility to anoikis under detachment conditions. A: Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL)-positive cells (apoptotic cells) of mouse bone marrow mesenchymal stem cells (mBMSCs) cultured in precoated Poly-2-hydroxyethyl methacrylate conditions for 0 h, 12 h, 24 h, 36 h and 48 h using flow cytometry; B: Fluorescence staining images of dead cells; C: Reactive oxygen species (ROS) levels were measured using the ROS probe 2,7-Dichlorodihydrofluorescein diacetate by flow cytometry at 0 h, 12 h, 24 h, 36 h and 48 h; D: Quantitative analysis of the rate of TUNEL-positive cells; E: Quantification of live cells using a thiazolyl blue tetrazolium bromide assay; F: Quantitative analysis of the intracellular ROS level; G: Cell adhesion was expressed as fold changes between 0 h groups; H and I: Mammalian Ste20-like kinase 1 (Mst1), microtubules associated protein 1 light chain 3 II/I, p62, Beclin1, phospho-focal adhesion kinase, and activated caspase 3 expression levels at 0 h, 12 h, 24 h, 36 h and 48 h were evaluated by Western blot analysis; The expression of protein was expressed as the fold change relative to glyceraldehyde-3-phosphate dehydrogenase. Values are the mean ± SD of three independent experiments in each case. TUNEL: Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling; ROS: Reactive oxygen species; Mst1: Mammalian Ste20-like kinase 1; LC3-II/I: Microtubules associated protein 1 light chain 3 II/I; p-FAK: Phospho-focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. $^{c}P < 0.01$; $^{b}P < 0.05$; $^{a}P > 0.05$.

Mst1 inhibition upregulated ITGα5 and ITGβ1 expression in isolated mBMSCs

The mBMSCs were infected with adenovirus containing Mst1 shRNA. The effect of shRNA on inhibiting Mst1 expression were measured by qPCR and Western blot (Supplementary Figure 1).

Evidence has shown that ITGs, the heterodimeric cell surface adhesion receptors, mediates anoikis. In this study, the alterations of ITGs in isolated mBMSCs/sh-Mst1 were tested.

The expression profiles of ITGα5, ITGαv, ITGα4, ITGβ1, and ITGβ3 in poly-HEMA-treated mBMSCs were compared by flow cytometry. Compared with the control mBMSC levels, the poly-HEMA-treated isolated mBMSC levels of ITGα5, ITGαv, ITGα4, ITGβ1, and ITGβ3 were significantly decreased (Figure 2). Compared with isolated mBMSCs, isolated mBMSCs/sh-Mst1 show an upwards trend in ITGα5 and ITGβ1 expression. However, there was no difference of the expression profiles of ITGαv, ITG α4, and ITGβ3 between isolated mBMSCs and isolated mBMSCs/sh-Mst1 (Figure 2). This study suggested that the inhibition of Mst1 could reactivate the expression of ITGα5 and ITGβ1.

Suppression of Mst1 expression protected mBMSCs from anoikis by activating autophagy.

mBMSCs were cultured in precoated poly-HEMA plates for 36 h. A significant decrease in cell apoptosis was observed in mBMSCs/sh-Mst1 (Figures 3A, B, D and E). Similar to the above results, cell adhesion was ameliorated by silencing Mst1 expression (Figure 3G). These results indicated that Mst1 inhibition suppressed ECM-isolatedinduced anoikis in mBMSCs.

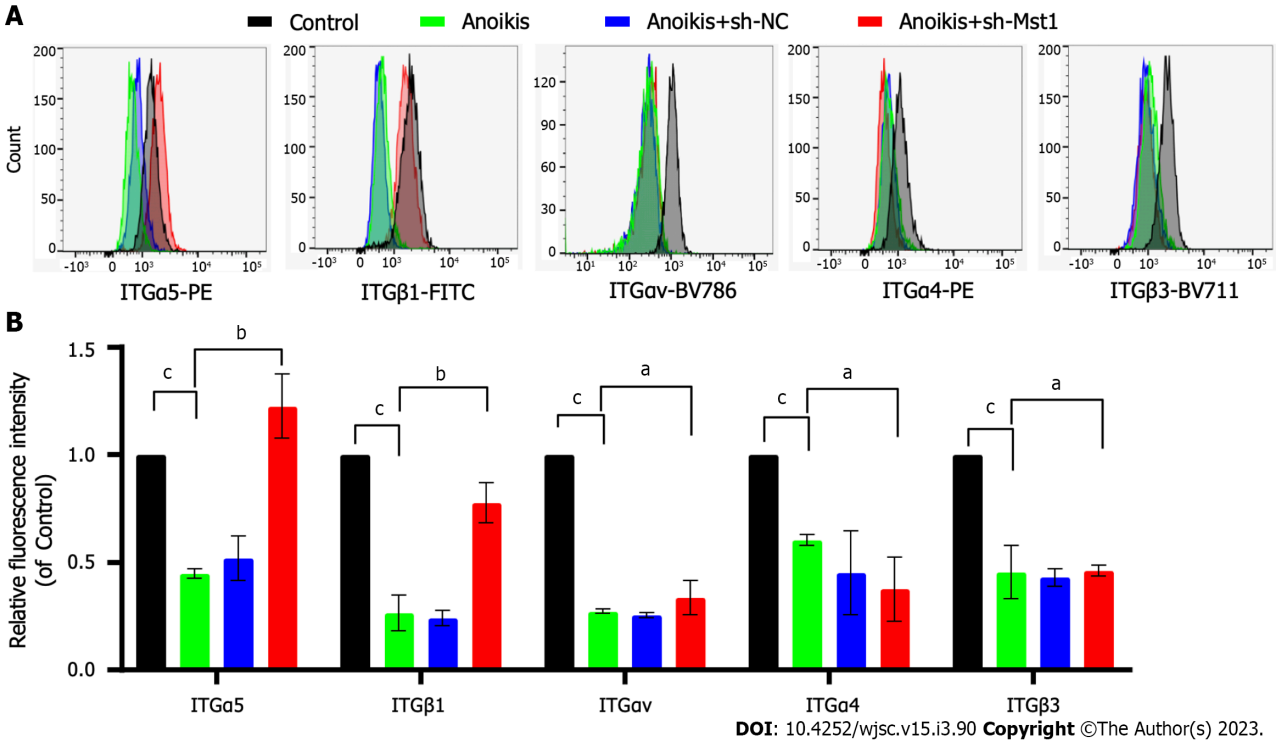
In addition, flow cytometric analysis confirmed decreased ROS levels in isolated mBMSCs/sh-Mst1 compared with those of isolated mBMSCs, whereas ROS levels were re-elevated by the autophagy inhibitor 3-MA (Figures 3C and F).

Western blot assay further suggested the above conception. FAK, has been recognised as the key mediator of cell–substrate adhesion. Western blot analysis results showed that mBMSC/sh-Mst1 exhibited robust FAK activation (Figure 3I). Similar to apoptosis, the activation of caspase can induce anoikis. Thus, we tested effect of Mst1 inhibition on the activation of caspase 3 by Western blotting. In Figure 3I, silencing Mst1 expression significantly inhibited caspase 3 activation in suspension-grown mBMSCs. This study indicated that silencing Mst1 expression could reactivate the FAK/Caspase3 pathway in anchorage-independent mBMSCs. However, 3-MA, an autophagy inhibitor, had no effect on the expression of ITGα5 and ITGβ1 or on cell adhesion (Figure 3I).

Consistent with the previous results, Mst1 inhibition reactivated autophagy in mBMSCs under isolated conditions, which can be demonstrated by the upregulated expression of LC3 II/I and Beclin1 and downregulated expression of p62 (Figure 3H). Furthermore, the number of mBMSCs/sh-Mst1 undergoing anoikis was increased after pretreatment with 3-MA (Figure 3H). In conclusion, the protective effect of Mst1 knockdown on anoikis in mBMSCs is associated with autophagy.

Inhibition of ITGα5β1 reversed the protective effects of Mst1 inhibition against anoikis in mBMSCs

To determine whether ITGα5 or ITGβ1 contributes to anoikis resistance in mBMSC/sh-Mst1 cells,



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Figure 2 Changes in integrin protein expression in detached mouse bone marrow mesenchymal stem cells/sh-Mammalian Ste20-like kinase 1. Cells were cultured in detached conditions for 36 h. A: Flow cytometry analysis investigating the expression of integrinα5 (ITGα5), ITGαv, ITGα4, ITGβ1 and ITGβ3; B: Data are expressed as the fold change compared to the control groups. Values are expressed as the mean ± SD, $n = 3$. ITG: Integrin. $^{c}P < 0.01$; $^{b}P < 0.05$; $^{a}P > 0.05$.

siRNA was used to knock down ITGα5 or ITGβ1 expression, respectively (Supplementary Figure 2).

In isolated mBMSC/sh-Mst1, cell apoptosis was increased, and cell adhesion was blocked by siRNA-mediated ablation of ITGα5 or ITGβ1 (Figures 4A, B, D, E and G). Similarly, p-FAK expression was downregulated and caspase3 activation was upregulated using ITGα5 or ITGβ1 siRNA (Figure 4I). In addition, LC3 II/I, Beclin1 and p62 expression was also reversed by ITGα5 or ITGβ1 siRNA (Figure 4H). In addition, the results in Figures 4C and F suggested that the ROS level was reduced by ITGα5 or ITGβ1 siRNA.

The properties and biological safety of mBMSCs/sh-Mst1

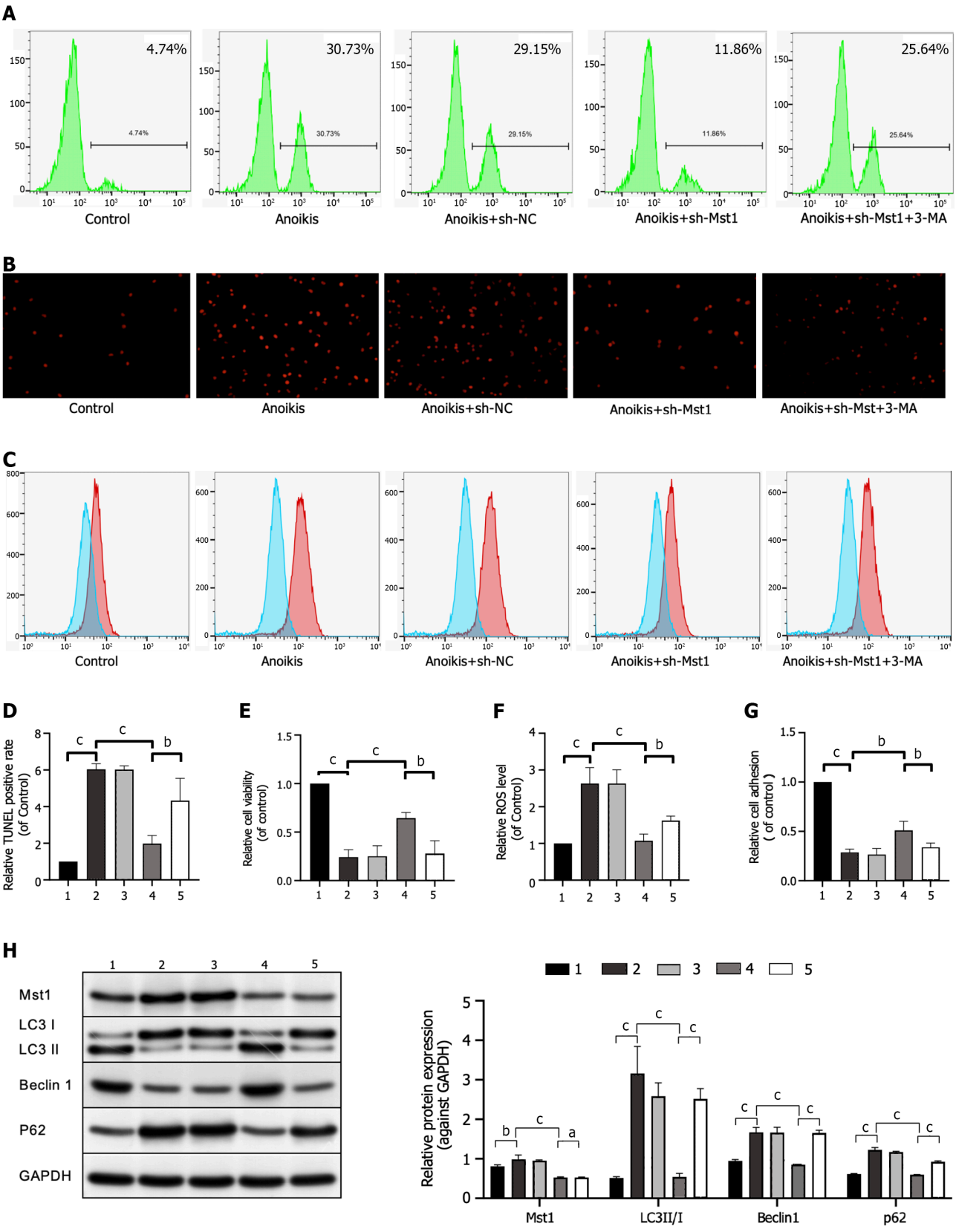
In isolated conditions, the levels of anti-inflammatory cytokines IL-4, IL-10 and IL-17A increased, while the level of pro-inflammatory cytokine IL-6 decreased in mBMSCs/sh-Mst1 compared with those of other mBMSCs (Figure 5A).

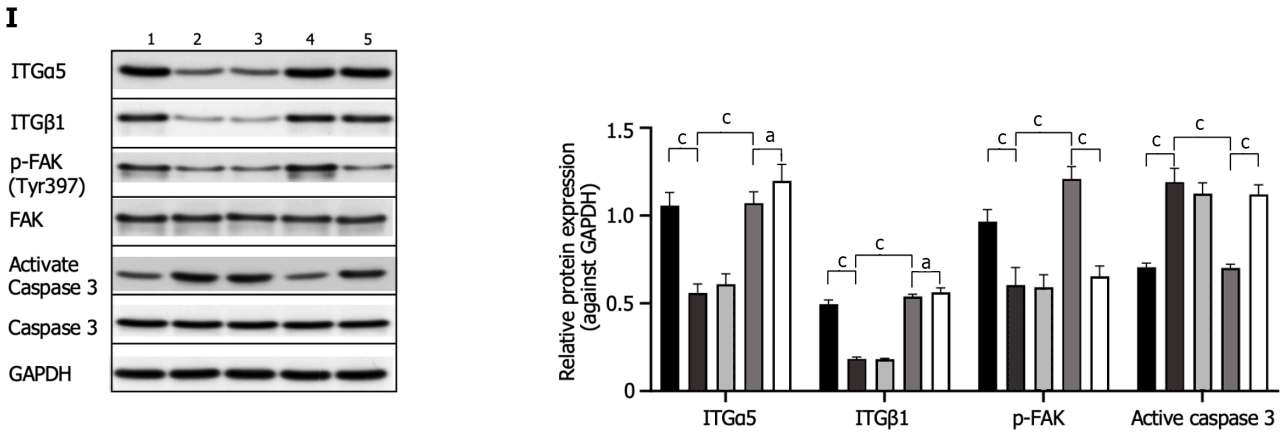
We assessed the effect of silencing Mst1 expression on the osteogenic differentiation of mBMSCs. In Figure 5B, Mst1 inhibition was correlated with increased osteogenic differentiation of mBMSCs. Subsequently, qPCR was performed to accurately determine the role of Mst1 inhibition on osteogenic differentiation in mBMSCs. As known as the markers of osteoblast differentiation, we tested the mRNA levels of runt-related transcription factor 2 (Runx2) and alkaline phosphatase (ALP). We found that the expression of Runx2 and ALP were both increased in mBMSC/sh-Mst1 (Figure 5B)[15].

There was no tumour-like mass in animals injected with mBMSC/sh-Mst1 after 60 d post-injection. After 60 d post-injection, we collected the subcutaneous tissue and the lung, liver, kidney and heart. There were no difference of the weights of the lung, liver, kidney and heart among each group (Figures 5C and Supplementary Table 2). It also showed that no stromal structures appeared in subcutaneous tissue of mBMSCs/sh-Mst1 groups (Figure 5C).

DISCUSSION

Convincing suggestion has confirmed that anoikis limits the therapeutic efficacy of MSC transplantation for tissue repair[16]. Herein, this study has proven that mBMSC/sh-Mst1 could survive after isolation from the ECM, and this response was mediated by the effect of Mst1 inhibition-induced autophagy on ITGα5β1-modulated production of ROS.


A
4.74%
30.73%
29.15%
11.86%
25.64%
Control
Anoikis
Anoikis+sh-NC
Anoikis+sh-Mst1
Anoikis+sh-Mst1+3-MA
B
Control
Anoikis
Anoikis+sh-NC
Anoikis+sh-Mst1
Anoikis+sh-Mst+3-MA
C
Control
Anoikis
Anoikis+sh-NC
Anoikis+sh-Mst1
Anoikis+sh-Mst1+3-MA
D
Relative TUNEL positive rate (of Control)
E
Relative cell viability (of control)
F
Relative ROS level (of Control)
G
Relative cell adhesion (of control)
H
Mst1
LC3 I
LC3 II
Beclin 1
P62
GAPDH
Relative protein expression (against GAPDH)
Mst1
LC3II/I
Beclin1
p62



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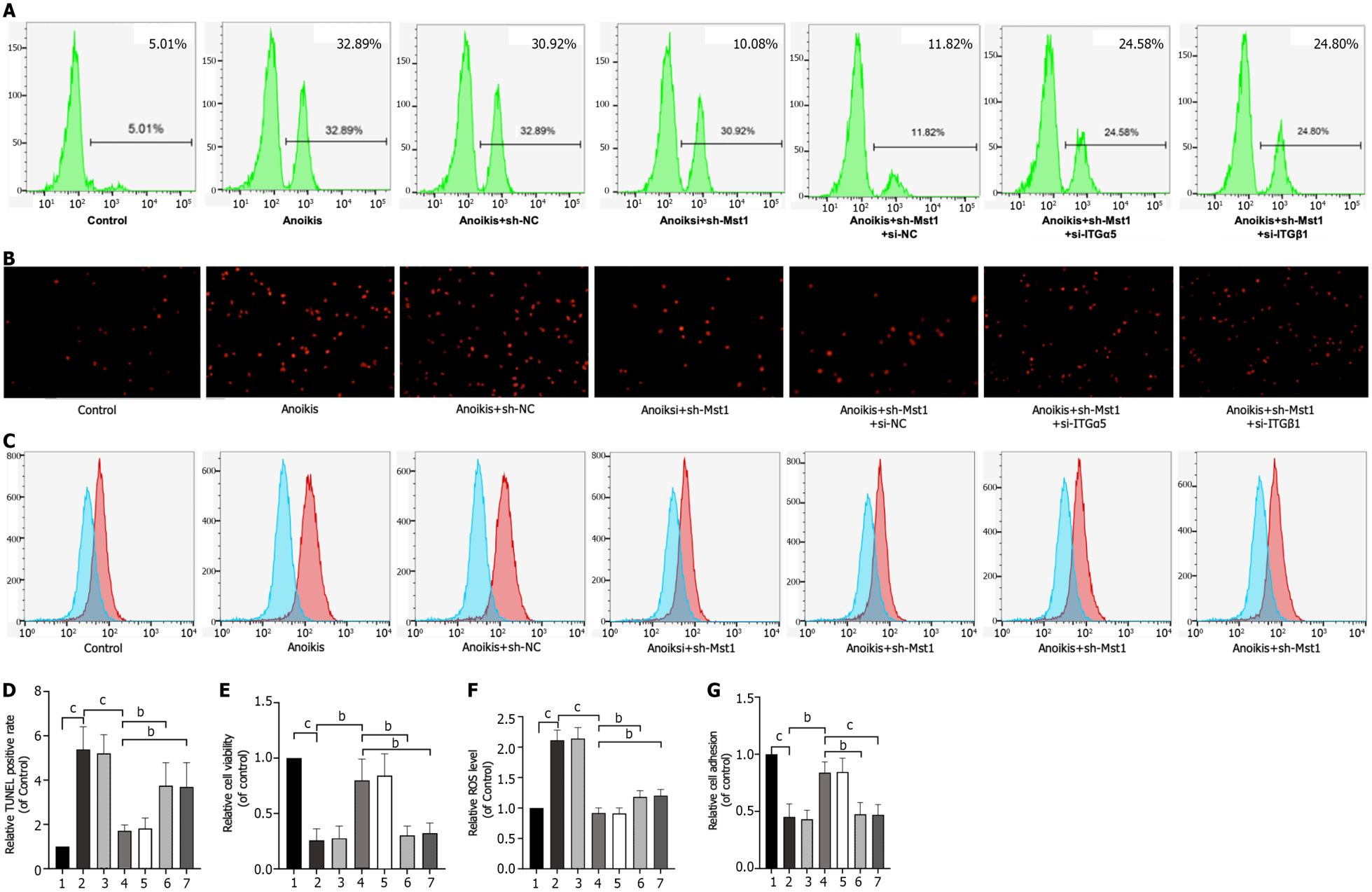
Figure 3 Mammalian Ste20-like kinase 1 inhibition-induced autophagy reduced cell apoptosis in detached mouse bone marrow mesenchymal stem cells. A: Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL)-positive cells analysed by flow cytometry; B: Representative images and quantification of fluorescence staining of dead cells; C: Reactive oxygen species (ROS) levels were measured using the ROS probe 2,7-Dichlorodihydrofluorescein diacetate by flow cytometry. D: Quantitative analysis of the rate of TUNEL-positive cells. E: Quantification of live cells using a thiazolyl blue tetrazolium bromide assay. F: Quantitative analysis of the intracellular ROS level. G: Cell adhesion was evaluated as the fold change compared back to the control groups; H and I: Mammalian Ste20-like kinase 1 (Mst1), microtubules associated protein 1 light chain 3 II/I, p62, Beclin1, phospho-focal adhesion kinase, and activated caspase 3 expression were evaluated by Western blot analysis. glyceraldehyde-3-phosphate dehydrogenase served as a control. Values are the mean ± SD, and experiments were completed in triplicate. 3-MA: 3-methyladenine; TUNEL: Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling; ROS: Reactive oxygen species; Mst1: Mammalian Ste20-like kinase 1; LC3-II/I: Microtubules associated protein 1 light chain 3 II/I; p-FAK: Phospho-focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. 1. Control group; 2. Anoikis group; 3. Anoikis + sh-NC group; 4. Anoikis + sh-Mst1 group; 5. Anoikis + sh-Mst1 + 3-MA group. $^{c}P < 0.01$; $^{b}P < 0.05$; $^{a}P > 0.05$.

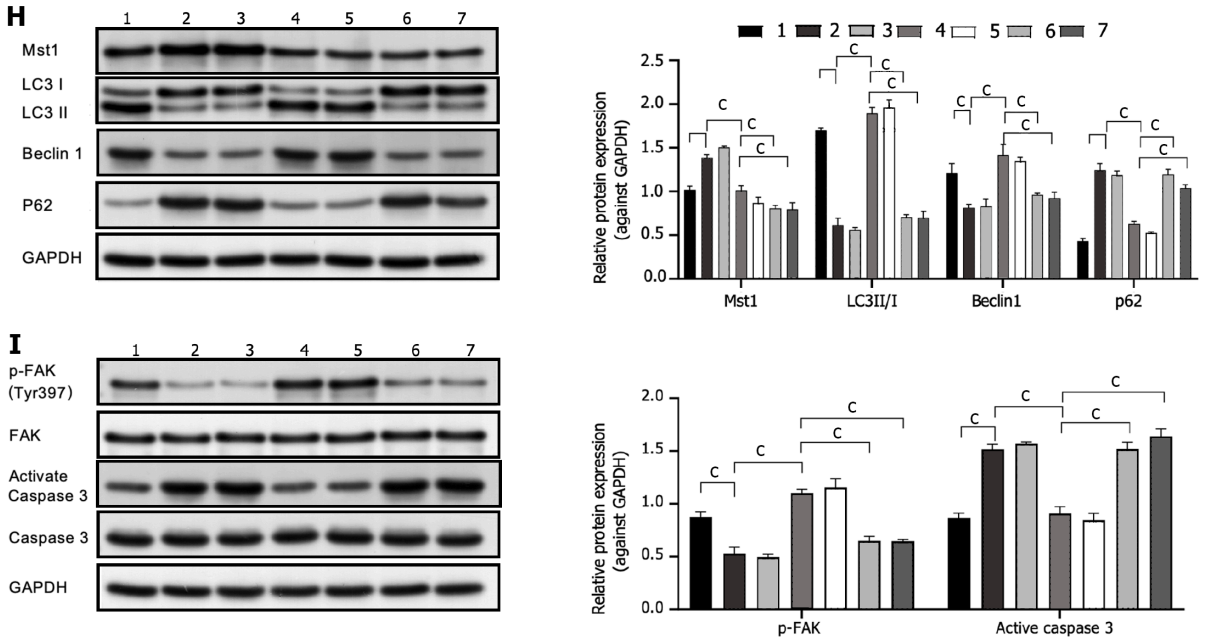
Corresponding alterations in cell-ECM isolation and autophagy also exist[17]. As a special type of apoptotic cell death, anoikis contributes to the loss of cell attachment to the ECM[18,19]. In the present study, we observed increased cell apoptosis and inhibited autophagy, as well as upregulated Mst1 expression in isolated mBMSCs. One hypothesis derived from a combination of previous studies is that Mst1 inhibition can not only overcome anoikis but also induce autophagy in isolated mBMSCs. In this study, we confirmed that mBMSCs averted anoikis by Mst1 inhibition-induced autophagy. Autophagy promotes cell survival or apoptosis in a stimulus-dependent manner. A series of experiments have elucidated the role of autophagy in promoting cell survival during anoikis[20]. Accordingly, our results established Mst1 inhibition-induced autophagy as a survival mechanism in isolated mBMSCs.

ITGs are transmembrane αβ heterodimers, with at least 18 well-known α and 8 β subunits. An increasing amount of experimental data has demonstrated that cells can overcome anoikis by changing ITGs expression[21]. In addition, ITGs-mediated cell adhesion to ECM is critical for maintaining appropriate cellular function and survival[22]. Therefore, the upregulation of ITGs allows cells to survive during anoikis[9,22,23]. This study has proved that the expression of ITGα5 and ITGβ1 were increased in cultured mBMSCs/sh-Mst1 under cell isolation conditions. Furthermore, upregulated ITGα 5 and ITGβ1 expression may be the underlying mechanism of anoikis resistance in mBMSCs/sh-Mst1. These results suggested the role of ITGα5β1 downstream of Mst1, as well as a collaboration between ITG α5 and ITGβ1, in anoikis-resistant mBMSCs/sh-Mst1.

ITGs relay signals from the ECM to initiate intracellular signalling through intracellular ROS production[24], by which p-FAK expression is mediated[25]. Moreover, a recent study confirmed that excessive or persistent increases in ROS levels might promote the process of anoikis[26]. However, high ROS levels may also promote the formation of autophagy, which could contribute to reducing ROS accumulation[27]. Despite the essential role of increased ROS levels in anoikis resistance reported in several studies[28], we still hypothesized the necessity of appropriate cellular regulation of ROS levels for anoikis inhibition. As a result, we speculated that a negative-feedback loop was formed among Mst1 inhibition-induced autophagy, Mst1 inhibition-triggered ITGα5β1 and ROS levels. Mst1 inhibition increased ITGα5β1 expression, thereby facilitating cell adhesion. In addition, Mst1 inhibition-induced autophagy reduced the level of ITGα5β1-triggered ROS in isolated mBMSCs, which contributed to the evasion of anoikis, elucidating why 3-MA did not affect the expression of ITGα5 or ITGβ1.

Mst1 has been known to play a key role in the signalling pathway that controls manifold cellular processes[29]. In the present study, silencing Mst1 expression was found to ameliorate the anti-inflammatory cytokine production, osteogenic differentiation capability and cell proliferation of mBMSCs, thereby making mBMSCs/sh-Mst1 an attractive target for anti-inflammatory, immunomodulatory, and regenerative therapies and potentially improving the curative efficacy of mBMSCs in PAH[1-3].


A
5.01%
32.89%
30.92%
10.08%
11.82%
24.58%
24.80%
Control
Anoikis
Anoikis+sh-NC
Anoiksi+sh-Mst1
Anoikis+sh-Mst1 +si-NC
Anoikis+sh-Mst1 +si-ITGα5
Anoikis+sh-Mst1 +si-ITGβ1
B
C
D
Relative TUNEL positive rate (of Control)
E
Relative cell viability (of control)
F
Relative ROS level (of Control)
G
Relative cell adhesion (of control)



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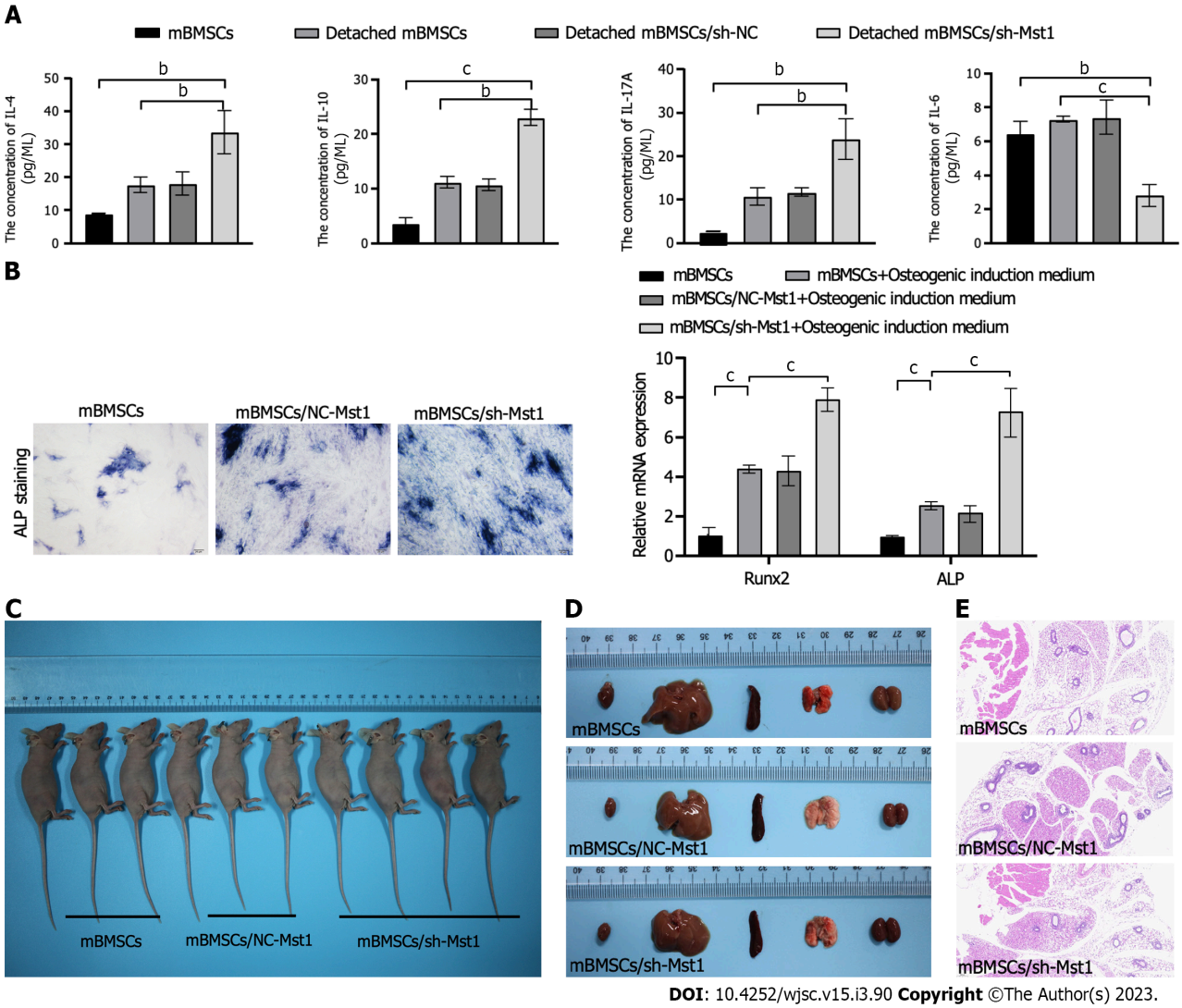
Figure 4 Upregulated integrinα5β1 expression triggered by Mammalian Ste20-like kinase 1 inhibition protected mouse bone marrow mesenchymal stem cells from anoikis. A: Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL)-positive cells analysed by flow cytometry; B: Representative images and quantification of fluorescence staining of dead cells; C: Reactive oxygen species (ROS) levels were measured by flow cytometry; D: Quantitative analysis of the rate of TUNEL-positive cells; E: Quantification of live cells using a thiazolyl blue tetrazolium bromide assay; F: Quantitative analysis of the intracellular ROS level; G: Cell adhesion was evaluated as the fold change compared back to the control groups; H and I: Western blotting was used to measure the expression levels of Mammalian Ste20-like kinase 1 (Mst1), microtubules associated protein 1 light chain 3 II/I, p62, Beclin1, phospho-focal adhesion kinase, and activated caspase 3. glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. Values are expressed as the mean ± SD. Measurements were performed in three replicates. 3-MA: 3-methyladenine; TUNEL: Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling; ROS: Reactive oxygen species; Mst1: Mammalian Ste20-like kinase 1; LC3-II/I: Microtubules associated protein 1 light chain 3 II/I; p-FAK: Phospho-focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. 1. Control group; 2. Anoikis group; 3. Anoikis + sh-NC group; 4. Anoikis + sh-Mst1 group; 5. Anoikis + sh-Mst1 + si-NC group; 6. Anoikis + sh-Mst1 + si-ITGα5; 7. Anoikis + sh-Mst1 + si-ITGβ1. $^{c}P < 0.01$; $^{b}P < 0.05$; $^{a}P > 0.05$.

Regardless of the extraordinary safety profile of MSC therapy verified in clinical trial data, several scholarly reviews have proposed that MSCs play a role in tumorigenesis and progression[18,30,31]. Therefore, the enhancement of the anti-anoikis ability of MSCs may promote tumorigenesis. However, in the present study, tumorigenic experiments in nude mice demonstrated the safety profile of mBMSC/sh-Mst1 administration.

CONCLUSION

In summary, the mechanism by which Mst1 inhibition acts on anoikis in mBMSCs was expounded in

this study. First, Mst1 inhibition was demonstrated to ameliorate not only autophagy formation but also ITGα5β1 expression. Second, Mst1 inhibition-induced autophagy could scavenge the excessive production of ITGα5β1-triggered ROS. Third, silencing Mst1 expression not only ameliorated the pluripotency of mBMSCs but also retained the safety profile of mBMSCs. Overall, Mst1 inhibition-based infusion may improve the therapeutic efficacy of MSCs, thereby serving as the ideal candidate for clinical transplant therapy in PAH.



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Figure 5 The properties and biological safety of mouse bone marrow mesenchymal stem cells/sh-Mammalian Ste20-like kinase 1. A: The levels of interleukin-4 (IL-4), IL-10, IL-17A and IL-6 were measured using a Cytometric Bead Array Cytokine Kit in detached mouse bone marrow mesenchymal stem cells (mBMSCs); B: Representative images of alkaline phosphatase (ALP) staining of mBMSCs after culture in osteogenesis induction medium for 21 d; C: Quantitative real-time polymerase chain reaction analysis of the relative mRNA expression levels of runt-related transcription factor 2 (Runx2) and ALP in mBMSCs cultured in adipogenesis induction medium for 21 d; D: Nude mice were subcutaneously implanted with mBMSCs (n = 3), mBMSCs/NC-Mst1 (n = 3), and mBMSCs/sh-Mst1 (n = 4). Representative images of the heart, liver, spleen, lung and kidney in each group 60 d postinjection; E. Paraffin-embedded subcutaneous sections derived from cell-injection sites were stained with haematoxylin and eosin. Data are the mean ± SD of three technical replicates. mBMSCs: Mouse bone marrow mesenchymal stem cells; Mst1: Mammalian Ste20-like kinase 1; IL: Interleukin; Runx2: Runt-related transcription factor 2; ALP: Alkaline phosphatase. $^{c}P < 0.01$; $^{b}P < 0.05$; $^{a}P > 0.05$.

ARTICLE HIGHLIGHTS

Research background

Anoikis plays a limiting role in the therapeutic efficacy of mesenchymal stem cells (MSCs). As a proapoptotic molecule, mammalian Ste20-like kinase 1 (Mst1) can increase the production of reactive oxygen species (ROS), thereby promoting anoikis. Recently, Mst1 inhibition was found to protect mouse bone marrow MSCs (mBMSCs) from H_2O_2-induced cell apoptosis by inducing autophagy and reducing ROS production. However, the influence of Mst1 inhibition on anoikis in mBMSCs remains unclear.

Research motivation

To investigate whether Mst1 inhibition could reduce anoikis in isolated mBMSCs.

Research objectives

To investigate the mechanisms by which Mst1 inhibition acts on anoikis in isolated mBMSCs.

Research methods

Poly-2-hydroxyethyl methacrylate-induced anoikis was used following Mst1 inhibition in mBMSCs. Integrin (ITGs) levels were tested by flow cytometry. Autophagy and ITGα5β1 were inhibited using 3-methyladenine and small interfering RNA, respectively. The alterations in anoikis were evaluated by Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling and anoikis assays. The levels of the anoikis-related proteins ITGα5, ITGβ1, and phospho-focal adhesion kinase, which activate caspase 3, and the autophagy-related proteins microtubules associated protein 1 light chain 3 II/I, Beclin1 and p62 were detected by Western blotting.

Research results

In isolated mBMSCs, Mst1 expression was upregulated, and Mst1 inhibition significantly reduced cell apoptosis, induced autophagy and decreased ROS levels. Mechanistically, we found that Mst1 inhibition upregulated ITGα5 and ITGβ1 expression but not ITGα4, ITGαv, or ITGβ3 expression. Moreover, ITGα5β1 upregulation and autophagy induction by Mst1 inhibition played an essential role in terms of the protective efficacy of Mst1 inhibition on averting anoikis.

Research conclusions

Mst1 inhibition ameliorated autophagy formation, increased ITGα5β1 expression, and decreased the excessive production of ROS, thereby reducing cell apoptosis in isolated mBMSCs. On this basis, Mst1 inhibition may provide a promising strategy to overcome the anoikis of transplanted MSCs.

Research perspectives

In isolated mBMSCs, Mst1 inhibition ameliorated not only autophagy formation but also ITGα5β1 expression (not ITGα4, ITGαv, or ITGβ3). Mst1 inhibition-induced autophagy scavenged excessive ITGα5β1-triggered ROS. Consequently, Mst1 inhibition-based infusion may improve the therapeutic efficacy of MSCs, thereby serving as an ideal candidate for clinical transplantation in pulmonary arterial hypertension.

FOOTNOTES

Author contributions: Yu WC contributed to the conception and design and manuscript writing; Zhang T contributed to conception and design, collection and assembly of data, data analysis and interpretation. Yu WC, Zhang T, Zhang Q performed the experiments; All authors participated in discussing, revising the manuscript, and approving the final manuscript; All authors have read and approve the final manuscript.

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OPINION REVIEW

Banking of perinatal mesenchymal stem/stromal cells for stem cell-based personalized medicine over lifetime: Matters arising

Cheng-Hai Li, Jing Zhao, Hong-Yan Zhang, Bin Wang

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Abstract

Mesenchymal stromal/stem cells (MSCs) are currently applied in regenerative medicine and tissue engineering. Numerous clinical studies have indicated that MSCs from different tissue sources can provide therapeutic benefits for patients. MSCs derived from either human adult or perinatal tissues have their own unique advantages in their medical practices. Usually, clinical studies are conducted by using of cultured MSCs after thawing or short-term cryopreserved-then-thawed MSCs prior to administration for the treatment of a wide range of diseases and medical disorders. Currently, cryogenically banking perinatal MSCs for potential personalized medicine for later use in lifetime has raised growing interest in China as well as in many other countries. Meanwhile, this has led to questions regarding the availability, stability, consistency, multipotency, and therapeutic efficiency of the potential perinatal MSC-derived therapeutic products after long-term cryostorage. This opinion review does not minimize any therapeutic benefit of perinatal MSCs in many diseases after short-term cryopreservation. This article mainly describes what is known about banking perinatal MSCs in China and, importantly, it is to recognize the limitation and uncertainty of the perinatal MSCs stored in cryobanks for stem cell medical treatments in whole life. This article also provides several recommendations for banking of perinatal MSCs for potentially future personalized medicine, albeit it is impossible to anticipate whether the donor will benefit from banked MSCs during her/his lifetime.

Key Words: Mesenchymal stromal/stem cells; Adult mesenchymal stromal/stem cells; Perinatal mesenchymal stromal/stem cells; Perinatal tissue; Stem cell bank; Personalized medicine

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Core Tip: This article reviews the current research related to the potential use of banked perinatal mesenchymal stromal/stem cells (MSCs) for future personalized medicine. This article also discusses the matters needing attention for cryopreservation of perinatal MSCs that are possible to be banked and stored over many years. Acknowledging the limitations and uncertainties of long-term cryopreservation of perinatal MSCs, several key recommendations need to be addressed in this article for future stem cell-based medical treatments.

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INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) possess their unique properties that have attracted great attention in regenerative medicine and tissue engineering. Understanding of the biological properties of MSCs has been continued over a long period of time. Approximately 50 years ago, Fridenshteĭn *et al*[1] found a minor subpopulation of transplanted bone marrow (BM) cells cultured in the diffusion chambers that can act as osteogenic stem cells to show the differentiation of these cells towards osteogenesis. MSCs were initially identified in animal BM in a series of studies as fibroblast-like cells that were plastic-adherent and formed discrete fibroblast colonies[1-3]. Such fibroblast colony-forming cells were also shown to display high proliferative capacity and osteogenic potential[3]. Caplan first coined the name "mesenchymal stem cells" in 1991 on the basis of their *in vitro* capacity to give rise to bone, cartilage, *etc*.[4], but, to date, the stem cell properties of these cells remain controversial due to the multipotent uncertainty of MSCs *in vivo*. The essential features of MSC population have been identified as the minimal criteria by the International Society for Cell & Gene Therapy (ISCT) based on the specific phenotypic markers, adherence to plastic, and the potential of tri-lineage differentiation (adipogenesis, chondrogenesis, and osteogenesis)[5]. Given the unknown self-renewal and differentiation properties *in vivo*, later, Caplan[6] proposed to change the name of MSCs to more accurately reflect their main immunomodulatory and trophic potential not for multipotency of MSCs[7]. In 2019, to further consolidate and clarify the nomenclature of MSCs unless rigorous evidence for stemness exists, the ISCT MSC committee offers a position on the functional definition of mesenchymal stem *vs* stromal cells[8].

MSCs are well known to be isolated from various adult tissues including BM, adipose (AD) tissue, skeletal muscle, dental pulp, and blood[9,10], as well as vascularized tissues[11,12]. Given the immunomodulatory functions, paracrine capacity, and tropic aspects of MSCs, as well as the lacking of human leukocyte antigen (HLA)-DR, a major histocompatibility complex class II molecule, the potential therapeutic properties of MSCs in clinical trials are being explored for MSC-based regenerative medicine. Typically, MSCs are expanded *in vitro*, tested, cryopreserved, and banked for later use in preclinical and clinical studies[13-15]. Biobanking of MSCs from perinatal/neonatal tissues such as umbilical cord tissue, placental tissue, as well as placenta-associated amniotic fluid (AF) and amniotic membrane (AM) for potentially personalized medicine in the future has become more popular over the last few years in China. These newborn tissues would be routinely thrown away after birth. Importantly, these neonatal tissues are an abundant and easily available source of MSCs at birth. In this rapidly growing field, usually, stem cell banking companies involved in the stem cell industry in close collaboration with hospitals in China take over neonatal tissue collection, and preparation and cryobanking of the perinatal MSCs proposed for personal or family use for future stem cell therapies when a person develops a disease. Banking of perinatal MSCs has attracted renewed attention not only in China and probably in many other countries as well. However, serious concerns have been also raised pertaining to the maintenance of functionality and stability of stem cells along with therapeutic potential of MSCs at the time of release years. It is hard to predict the true likelihood of perinatal MSC transplantation later in whole lifetime and, noticeably, there is great uncertainty regarding whether or not these MSCs can be used to treat certain diseases after decades. As such, this opinion review article highlights several key observations in regard to the limitation of perinatal MSCs stored in stem cell cryobanks for later personal or family use in the future, which should be widely considered in the settings of cryostorage to minimize the possible side effects of these MSCs for future stem cell therapies.

Importantly, this review provides several practical recommendations for banking of perinatal MSCs to better serve patients who might be desperately in need for potential personalized medicine for future stem cell-based treatments.

PERINATAL MSCS *VS* ADULT MSCS IN THEIR BIOLOGICAL AND THERAPEUTIC PROPERTIES

MSCs from adult and perinatal sources exhibit differentiated biological and therapeutic properties. The preclinical and clinical data reported in the literature are varied to this end. With a focus on whether the perinatal MSCs are worth biobanking, the pros and cons of perinatal *vs* adult MSCs need to be addressed in this section.

Compared to adult MSCs, the newborn stem cells obtained from perinatal tissues rarely carry any infectious diseases and reduce risk of exposure to environmental toxins. Perinatal MSCs have their own advantages such as easy availability, lacking stem cell variability, and comparably low risk of donor environmental effects, as well as immune privileged property. The diversity of MSC differentiation potential is also observed among MSCs from different source tissues. For example, Hou *et al*[16] conducted an analysis of single-cell RNA-seq using MSCs derived from various tissues. By comparison, umbilical cord-derived MSCs (UC-MSCs), one of the most explored perinatal MSC types, and BM-derived MSCs (BM-MSCs) exhibited the highest osteogenic potential, while AD-derived MSCs (AD-MSCs) and BM-MSCs had the highest potential of adipogenesis and chondrogenesis, respectively[16]. Meanwhile, UC-MSCs showed the highest immunosuppression as well as the highest stemness among all MSC samples in this study by Hou *et al*[16]. Although human UC-MSCs and BM-MSCs exhibited similar immunosuppressive properties, the differences in immunomodulatory effects of UC-MSCs and BM-MSCs were also suggested in a previous study[17]. In contrast, BM-MSCs expressed more indolamine 2,3-dioxygenase (IDO) in *in vitro* inflammatory environment, while UC-MSCs expressed more prostaglandin E2, interleukine-6, and programmed death-ligands 1 and 2. In addition, there were more T helper 17 cells inhibited and more regulatory T cells induced by UC-MSCs compared with BM-MSCs in co-culture[17]. In an experimental model of sepsis, BM-MSCs but not UC-MSCs were proposed to improve survival rate in septic mice due to the enhanced immunoregulatory properties through a variety of mechanisms[18]. In an *in vitro* study, UC-MSCs showed a higher angiogenic capacity in comparison with BM-MSCs and AT-MSCs[19]. Park *et al*[20] conducted an *in vivo* study to test the angiogenic potential of perinatal chorion-derived MSCs (C-MSCs), which revealed that C-MSCs significantly increased the capillary formation in the C-MSCs injected myocardium compared to UC-MSCs and BM-MSCs. Transplantation of UC-MSCs and BM-MSCs also showed similar therapeutic effects for type I diabetes in a mouse model as well as in treated patients with type I diabetes, respectively[21]. Compared to the mice with BM-MSCs treatment, the UC-MSCs treated mice had seemingly higher β-cell mass post-transplantation, although no significant difference was observed between the two treatment groups[21]. There was no difference in the therapeutic efficacy of BM-MSCs and UC-MSCs at one-year follow-up, albeit both types of MSCs decreased the levels of haemoglobin A1c and fasting and postprandial C-peptide[21].

Overall, the relevant preclinical and clinical studies to date by the use of adult and perinatal MSCs have yielded comparable results, which may contribute to a fuller understanding of their therapeutic potential in laboratory and clinical settings. The biological and therapeutic properties of both perinatal and adult MSCs are summarized in Table 1.

PERINATAL MSC BANKING FOR PERSONALIZED MEDICINE OVER LIFETIME: CONCERNS AND UNCERTAINTIES

Over the past 20 years of MSC translational research, clinical experiences have shown that MSCs are seemingly unmet medical needs[22-24]. Translation of stem cell potential into medical practices still confronts many challenges. Clearly, these challenges include the long- and short-term therapeutic safety and efficacy of transplanted MSCs, the sufficient capability of homing and engraftment, the long-term cultivation associated alteration of MSC therapeutic properties, loss of stem cell potency with culture time, heterogeneous functions, and consistency and stability of MSCs or MSC-based therapeutic products. While clinical studies have shown the therapeutic benefits of using MSCs in various human diseases, including cardiomyopathy, autoimmune diseases, diabetes and diabetic complications, bone and cartilage repair, and fibrosis[9], rigorous clinical evidence for MSC therapeutics is still actually lacking. The positive, negative, or mixed clinical results have also been frequently observed in MSC clinical studies. Currently, MSCs based medicine still remains unproven and experimental. Therefore, it is possible to consider the limitation and uncertainty of perinatal MSC biobanking for personalized medicine in the context of the future therapeutic potential. Several important issues illustrated by the use of adult MSCs but not well discussed in perinatal MSCs are exemplified in this section to extend the

Table 1 Perinatal *vs* adult mesenchymal stem/stromal cells in their biological and therapeutic properties

	Perinatal mesenchymal stem/stromal cells	Adult mesenchymal stem/stromal cells
Availability	Readily available	Readily available
Ethical concerns	Minimal crucial ethical concerns and possible religious barriers	Minimal ethical concerns and impossible religious barriers
Immune privileged characteristics	Inactively controversial for immune privileged characteristics	Actively controversial for immune privileged characteristics
Differentiation	Multipotent differentiation	Multipotent differentiation
Variability	Low possibility of stem cell variability and multipotent plasticity	Possibility of stem cell variability and multipotent plasticity
Heterogeneity	Diverse heterogeneities/low homogeneity	Diverse heterogeneities/low homogeneity
Age-induced senescence	Unconcerned about age-induced senescence *in vitro*	Concerned about age-induced senescence *in vitro*
Environmental effects	Low risk of donor environmental effects (such as virus, bacterium, chemical toxins)	Risk of donor environmental affects (such as virus, bacterium, chemical toxins)
Therapeutic safety	Comparatively safe	Comparatively safe
Therapeutic efficacy	Relatively low therapeutic efficacy	Relatively low therapeutic efficacy
Therapeutic mechanisms	Lacking of precise therapeutic mechanisms	Lacking of precise therapeutic mechanisms
Cost for banking	Expensive for banking	Expensive for banking
Stem cell nomenclature	Controversial stem cell nomenclature	Controversial stem cell nomenclature

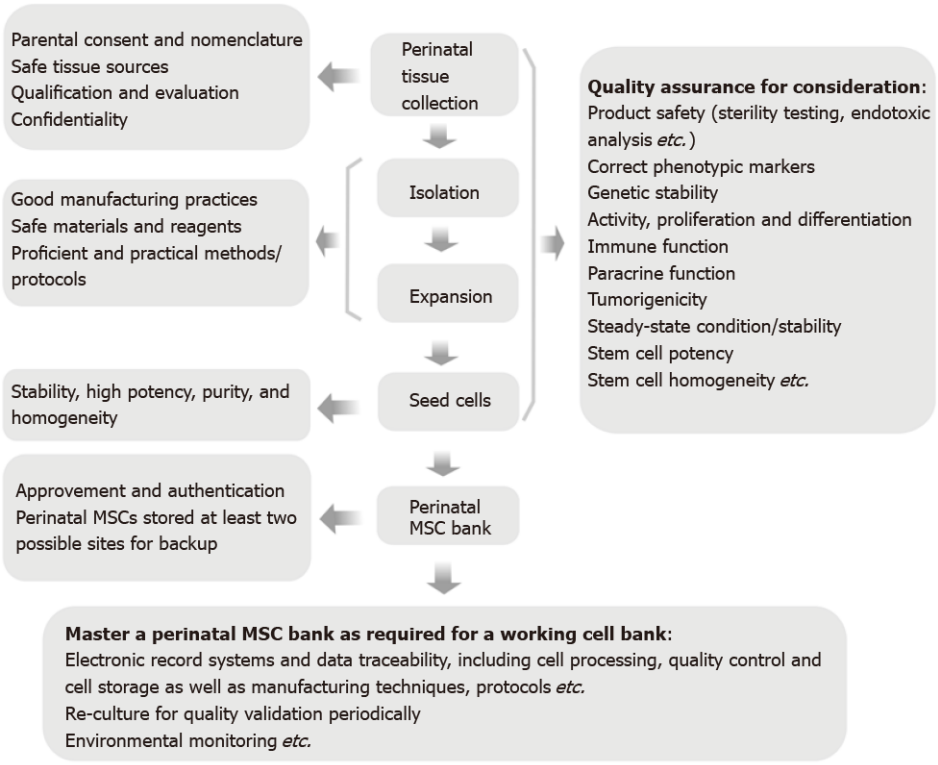
discussion regarding these relevant aspects of perinatal MSCs. The key issues have been identified to date, including the following.

Quality assurance before cryobanking for likely future personalized medicine

It is well known that functional properties and intrinsic multipotency of MSCs can be negatively affected by donor factors such as increased donor age, genetics, and health status. Banking of MSCs at their most potent state from perinatal tissues, the "best" cell source over one's lifetime, has been supposed for future use in need of regenerative therapies. Certainly, cryobanking of perinatal MSCs is a prerequisite in personalized medicine strategy; however, the great uncertainty remains concerning the final function of perinatal MSCs, the accessibility of MSCs (*e.g.*, a change in business circumstance), and therapeutic potential for the intractable diseases (*e.g.*, cancer) decades later. Due to no expiration date of the perinatal MSCs cryopreserved in biobanks, exploiting and developing new approaches to testing cellular variability and stability, functionality, and heterogeneity during the processing of preparation and banking of the end MSC products should be considered carefully for cell quality assessed over longer time periods. The quality assurance programs should be performed to ensure the quality of stem cell products during the whole banking process including the perinatal tissue collection, processing, testing, preparation, and storage, as well as additional analysis (Figure 1). It should be also considered that there should be minimal levels of differentiation of perinatal MSCs in the course of the overall culture period to preserve stem cells at their most multipotent state for future use. New quality control to meet the product standards is required to be developed during the process of banking of perinatal MSCs. Except for routine multiple experiments for cell quality assessment (*e.g.*, cell viability, proliferation, and differentiation potential), it is important to determine a panel of predicative *in vitro* tests for a system of quality assurance and these may be applied including but not limited to stem cell potency assay, spectral karyotyping, and genetic etiologies (Figure 1). Perinatal MSC potency assessment needs to be further validated for their therapeutic safety and effectiveness in the future. Specially, genetic etiologies associated with multi-factorial or monogenetic diseases may potentially influence stem cell safety. The wide analysis in identifying genetic/epigenetic etiologies is necessitated for therapeutic safety. For example, considering the importance of disease-associated individual single-nuleotide polymorphisms (SNPs), it is enabling to analyze and investigate whether stem cells carry disease-associated SNPs. Therefore, there is a critical need to further consider about whether it is worth biobanking of perinatal MSCs and whether it is to be stopped early if there is a family history of genetic conditions. Careful measuring and monitoring are extremely important before biobanking.

Reconsidering of pretreatment of perinatal MSCs before cryobanking

There are more and more *in vitro* and *in vivo* studies on pretreatment of MSCs, *e.g.*, pretreatment with cytokines or growth factors and hypoxia-priming, to improve their biological properties and therapeutic effectiveness. Acknowledging and understanding a beneficial role of pretreatment, the details given in this subsection describe the feature of pretreatment among MSCs from different source tissues including



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Figure 1 Operating model of banking perinatal mesenchymal stromal/stem cells. Perinatal mesenchymal stromal/stem cells banking process includes the perinatal tissue collection, isolation, stem cell expansion, testing, preparation, and storage. The quality assurance programs are performed during the whole banking process. MSCs: Mesenchymal stromal/stem cells.

adult and perinatal sources, specifically, with the BM appearing the most common. This would be conducive to a better understanding of the stem cell therapeutic advantages and matters needing attention before banking of perinatal MSCs.

Biological properties could be altered by pre-conditioning of MSCs during *in vitro* expansion. Cell culture variables should be documented and, conventionally, growth medium containing fetal bovine serum and supplemented with basic fibroblast growth factor (bFGF) is considered to be the "gold standard" medium for primary human MSC expansion[25]. To remove animal components, serum-free medium throughout the process of cell preparation is being developed for clinical applications and MSC cryobanking. Usually, serum-free medium contains various cytokines/growth factors. Medium supplementation with bFGF is shown to increase the proliferation capacity of BM-MSCs over multiple passages[26]. Expansion is further improved in UC-MSCs by addition of bFGF in growth medium and the highest cell yield is detected in UC-MSCs among MSC lines pre-treated with bFGF including BM-MSCs and AD-MSCs during the whole culture period[27]. Additionally, despite the increased expression of HLA-DR induced by bFGF in AD-MSCs, the addition of bFGF in culture media seems not to affect the expression levels of HLA-DR in UC-MSCs and BM-MSCs in the course of passages[27]. Furthermore, the percentage of HLA-DR positive cells increases after inflammatory priming of all these three types of MSCs expanded in the bFGF-supplemented media[27]. As regards MSC differentiation, on the one hand, pretreatment with bFGF enhances BM-MSC chondrogenesis during chondrogenic pellet culture, resulting in upregulation of collagen type X and matrix metalloproteinase 13, the hypertrophic markers, in pellet cultures after 5 wk of endochondral ossification[26]. On the other hand, the supplementation with bFGF in medium has been also shown to have a negative effect on chondrogenesis of BM-MSCs[28,29] as well as their adipogenesis and osteogenesis[29,30]. Studies in laboratories demonstrate a time-dependent contribution of bFGF signaling to the reduced osteogenic/adipogenic differentiation of BM-MSCs throughout the culture[30,31]. For instance, the activity of alkaline phosphatase, a marker of osteogenesis, is significantly reduced during osteogenic differentiation in the case of addition of bFGF after passage 4 compared to the control at the same passage of MSCs and, similarly, adipogenesis potential is also significantly decreased by time during differentiation[30].

Pretreatment of MSCs has been observed to enhance the biological properties of the frozen and thawed MSCs. For example, one study reports that there are more apoptotic cells in the post-thawed cryopreserved BM-MSCs than the live BM-MSCs in active culture[32]. These frozen-thawed BM-MSCs are susceptible to complement-mediated lysis[33] and T-cell mediated apoptosis[34]. Interestingly, frozen-thawed MSCs primed by interferon-gamma (IFN-γ) in the culture medium for 48 h prior to

cryopreservation may partly avoid the lysis by activated T cells[34]. The mechanism of MSC-mediated immunosuppression has been previously proposed as IDO expression in BM-MSCs[35] as well as UC-MSCs[36] induced by IFN-γ. However, while pre-licensing MSCs with IFN-γ have been seen to enhance their IDO expression *in vitro*[37,38], the pre-licensed MSCs by IFN-γ, compared to the unstimulated MSCs, have let to the loss of their effectiveness in rescuing retinal ganglion cells in a retinal ischemia/reperfusion injury mouse model[37]. Together, the results of pretreatment with cytokines including but not limited to bFGF and IFN-γ indicate the different effects on the biological properties of MSCs depending on their existing microenvironment (*e.g.*, inflammation) or the specific pathological contexts.

Apart from pretreatment with cytokines, other approaches can also be proposed to boost the therapeutic potential of MSCs, for example, microenvironmental hypoxia-primed MSCs[39]. One previous study shows that, compared to standard 21% O_2 tension, 3% O_2 can increase clonogenic potential, *in vitro* migration, and stemness of MSCs from the Wharton's jelly (WJ) of human umbilical cord (WJ-MSCs)[40]. Another previous study indicates that umbilical cord blood (UCB)-derived MSCs (UCB-MSCs) primed with hypoxia and calcium ions exhibit improved self-renewal, migratory, anti-inflammatory and immunomodulatory capacities, resulting in further improving therapeutic potential for graft *vs* host disease in an animal model[41].

As mentioned above, in theory, pretreatment of MSCs from adult or perinatal sources with cytokines can improve their biological and functional properties. However, previous *in vitro* and *in vivo* studies could not prove the therapeutic efficacy of the freshly cultured or post-thawed MSCs with cytokine pre-treatment. Microenvironment conditions (*e.g.*, hypoxia) have become a promising strategy prior to cryopreservation, which is possible for the enhancement of MSC-based therapeutic potential. *In vitro* and *in vivo* studies indicate that pretreatment strategy may benefit patients in a disease specific context. However, without knowing the context of a specific pathology, it is not possible to assess and predict the function of perinatal MSCs pretreated with cytokines for stem cell medical treatments after many years. Therefore, whether pretreatment of perinatal MSCs should become the standardization for biobanking for their future applications as stem cell-based treatments remains to be further investigated.

Pre-selection for biobanking to decrease the heterogeneity of perinatal MSCs

As known, there are diverse heterogeneities of MSCs including inter-donor/individual, inter-tissue/source, inter-cellular, and inter-colony heterogeneities involved in the manufacturing and biobanking of MSCs. MSC heterogeneity reflects the diversity of MSC-associated environments or niches. The present paper extends the relevant discussion regarding the pre-selection required prior to banking with the aim to control product consistency for a stem cell bank for maximizing the "homogeneity" of perinatal MSCs. As well known, MSCs including adult and perinatal MSCs derived different tissues differ in their functional characteristics. Wegmeyer *et al*[42] conducted one previous study to evaluate the source- and donor-dependent differential stem cell properties and found UC-MSCs, AM-derived MSCs (AM-MSCs), and BM-MSCs exhibited comparable differences between each other. Interestingly, UC-MSCs and AM-MSCs exhibited different growth characteristics and morphologies as well as high inter-donor variability of AM-MSCs but not UC-MSCs. In contrast, another previous report indicated that, compared to UCB-MSCs, placental tissue MSCs, and WJ-MSCs, umbilical cord lining-derived MSCs showed the highest proliferation and migration rates and prolonged survival in immunodeficient mice [43]. Concerning immunosuppressive properties, in contrast to placenta MSCs, both UC- and BM-MSCs significantly reduced the proliferation of activated CD4+ and CD8+ T cells[44]. Additionally, the heterogeneous proangiogenic properties of perinatal and adult MSCs were observed in one previous study, which showed that both BM-MSCs and placental chorionic villi MSCs had significant proangiogenic activities on endothelial cells in matrigel *in vitro* compared to AD-MSCs and UC-MSCs[45]. Importantly, compared to dental pulp MSCs and AD-MSCs, WJ-MSCs showed the strongest therapeutic efficacy in reducing fasting glucose levels in type II diabetic mice in one previous *in vivo* study[46].

As mentioned above, due to inconsistency of MSCs, different perinatal MSCs populations may have heterogeneous functional properties, consequently leading to different therapeutic efficiency. Therefore, a pre-selection may be required before stem cell banking to identify relatively homogeneous perinatal MSCs for potential later use in life. As known, pretreatment with cytokines before banking may be a suitable approach to support therapeutic potential of perinatal MSCs in the future as exemplified by IFN-γ pre-licensing. Peltzer *et al*[47] conducted a previous study to create an *in vitro* "tolerogenic" niche priming mimicking placental environment, which showed that primed perinatal MSCs (UC-, UCB-, AM-, and chorionic-MSCs) with IFN-γ could enhance their immunomodulatory potential in a dose- and donor-dependant manner. This study by Peltzer *et al*[47] may suggest that an approach to screen the large number of perinatal MSCs with cytokine priming will be beneficial for the consistency of banking MSCs. Sorting of perinatal MSCs may be another effective approach for pre-selection, for example, pre-selection of MSC subpopulation using surface markers to increase the purity of the expanded MSCs. A series of markers need to be considered in sorting of perinatal MSCs, including cell surface markers related to MSC clonogenicity, potency, differentiation, and immunomodulatory properties[48]. Practically, a few conventional approaches to increasing the consistency of perinatal MSCs may be also considered, such as the use of the single colony of MSCs and specific biomaterials (see below), and the handing of MSCs by the standardized protocols for manufacturing consistency.

Biomaterial scaffold approach to maintain the maximal biological properties of perinatal MSCs

MSCs within the body have their own physical microenvironments or niches to maintain their unique biological properties. In order to mimic a specific stem cell niche, various biomaterials such as alginate, chitosan, collagen hyaluronic acid, silica gel, silk fibroin, poly(lactic acid), and graphene-base materials are being explored for use as scaffolds/surfaces supporting the growth and differentiation of MSCs *in vitro* and *in vivo*. By comparing the conventional techniques with monolayer culture of MSCs, biomaterial scaffold approach such as cell surface modification and encapsulation technology for tissue engineering exhibits improved MSC survival and proliferation, increased differentiation potential, and maintenance of stem cell stemness[49,50]. Further studies indicate that biomaterials can enhance the paracrine function of MSCs[51]. While technical challenges for development of biomaterials remain the degradability, viscoelasticity/elasticity, architecture property, and compatibility, the non-toxic 3D porous biomaterials are already widely used for delivery of encapsulated UC-MSCs or BM-MSCs in pre-clinical studies[52,53] as well as in clinical trials[54,55]. Interestingly, cryopreservation of microencapsulated stem cells in alginate hydrogel has been reported to suppress ice formation that contributes to the effect of cryoprotection[56]. Importantly, there are no significant differences in cell viability and multi-lineage differentiation potential between the MSCs post cryopreservation either encapsulated or without encapsulation[56]. Similar studies have been also conducted with MSCs cryopreserved in degradable hyaluronic-acid based hydrogel[57]. In addition, intravenous injection of freeze-thawed mouse MSC encapsulates with microgel into recipient mice shows similar levels of cell survival as fresh non-cryopreserved MSC encapsulates[58]. As mentioned above, previous and current studies are instructive to note that using special biomaterial scaffold may be considered as a suitable new strategy for banking of MSCs from different source tissues including but not limited to perinatal tissues.

Dimethyl sulfoxide and dimethyl sulfoxide-free cryoprotectants

Dimethyl sulfoxide (DMSO), as a key cryopreservation agent, is most often used in the cryostorage to protect cells from mechanical and osmotic stress due to the formation of ice crystals[59]. DMSO is also observed to have significant influence on the viability, phenotype, and proliferation of MSCs, as well as cellular epigenome, and to induce changes in cellular processes[60,61]. As such, low concentrations of DMSO and DMSO with combinations of non-cytotoxic biocompatible agents/substances are being explored for MSC culture and cryopreservation in preclinical and clinical studies. For example, one previous study demonstrated that human BM-MSCs modified to express tumor necrosis factor-related apoptosis inducing ligand were cryopreserved in a low concentration (5%) of DMSO (accepted as nontoxic concentrations of below 10%) with 95% human serum albumin without affecting their biological properties[62]. However, a question may be raised as to whether DMSO at a very low concentration may not be sufficient to prevent freezing damage to MSCs.

Currently, there are different types of effective and non-toxic cryoprotective agents/compounds used as suitable replacements for DMSO, such as glycerol, hydroxyethyl starch, trehalose, and dextran[59,61]. One study reports that DMSO-free cryopreservation solutions composed of sugars, sugar alcohols, and small-molecule additives have been showed to retain MSC post-thaw viability, cell surface markers, and proliferation and differentiation potential[60]. The osmolyte-based freeze solutions also exhibit a more normal alignment of the actin cytoskeleton of MSCs compared to DMSO frozen cells[60]. A non-toxic cryoprotective agent, a combination of trehalose and glycerol, has been tested in another study for cryopreservation of AD-derived stem cells, and the cells cryopreserved with this cryoprotective agent presented high cell viability and proliferation and migration capacity after thawing[63]. Overall, while various studies are being devoted to possible improvement of DMSO as a cryoprotectant, some alternatives to DMSO are being evaluated as cryopretectants for cryopreservation of MSCs from different tissue sources including perinatal tissues. DMSO together with alternatives to DMSO have not been tested enough to advocate their use for biobanking of MSCs stored over many years. Therefore, the use of DMSO alone as a cryopreservation agent may not insure the final functional properties of MSCs for stem cell-based treatments after many years and the new freezing solutions need to be intensively investigated for long-term therapeutic stem cell cryopreservation.

Ethical and regulatory concerns

Banking stem cells using neonatal birth-associated tissues or other related sources began with the establishment of banking cord blood for hematopoietic cell transplantation in the early 1990s[64]. Given that perinatal MSCs have the positive characteristics of both embryonic stem cells and adult stem cells, the ethical issue involvement may be related to the use of embryonic stem cells. In particular, there are further ethical and regulatory issues that will challenge banking of perinatal MSCs. In China, currently, there are so many exaggerate advertisements directly to consumers on banking of life-saving perinatal stem cells to insure infants or family members against serious illnesses in the future. Parents are encouraged to make decision for the preservation of their children' stem cells in a private stem cell bank for the future health of their children. Indeed, the current applications of MSCs as stem cell medical treatments are sometimes also exaggerated by the social media. Parents or requestors need to pay thousands of dollars for the commercial banks of perinatal MSCs as well as an annual fee for the maintenance of stem cells. Therefore, physicians and clinical investigators in hospitals who have

financial conflicts of interest associated with stem cell banking should disclose any financial relationship. Currently, there is a lack of clarity in country-level regulations in China for the management of stem cell banking and the guidance document for quality control on the banking of MSCs. Accordingly, technical and ethical guidelines are imperative for the appropriate governance and restriction of banking perinatal MSCs to insure standardization for MSCs with good quality for future stem cell therapies. Technical and ethical guidelines should also be in line with the international standardized regimens such as guidelines from the International Society for Stem Cell Research and the International Stem Cell Banking Initiative. Lastly, the most established private banks of perinatal MSCs in China, previously engaged in cord blood storage, are paid by the parents and these banks store the source of stem cells for future use by the donor and, probably, the donor's relatives.

Correspondingly, an additional challenge is whether the source of MSCs with potential applications in the future can be shared by the donor-unrelated recipients. In this regard, many public banks that store the perinatal MSCs derived from the donated newborn tissues should be established free charge of anything for banking. In accordance, these banks are open for research and they store stem cells for all recipients in future personalized medicine including the donors, the donor's families, and those unrelated with donors when they might be desperately in need due to life-threatening diseases. Due to no data available for the use of public banks of perinatal MSCs in China, clinical applications of cord blood stem cells from the banks are illustrated for the perspective in the future. One representative example is that the public cord blood banks have released at least 30 times for the usage rate of previously cryopreserved cord blood stem cells as compared to the private banks worldwide[65]. Almost over the past 30 years, cord blood banks have been successfully achieved for safe storage and rapid availability for cord blood stem cell-based treatments[66]. Another example is that, in a cord blood bank (MEDIPOST Co., Ltd., Seongnam-si, Gyeonggi-do, Korea), cell populations were evaluated and, among 557 UCB units, 128 units cryopreserved for more than 10 years were used for transplantation [67]. Therefore, the useful value of public or private stem cell banks to society or the donors is important for the stem cell medicine potential in the future.

Reculturing post-thawed cryopreserved perinatal MSCs from stem cell banks at the release time

Usually, the freshly cultured perinatal MSCs are seemingly to be more potent than freshly thawed cells in therapeutic properties of MSCs. Some studies suggest that perinatal MSCs may lose their functionality with cryopreservation, which could in turn affect the efficacy. Whether the characteristics of thawed perinatal MSCs *vs* cultured MSCs would be changed needs to be further discussed here. One previous study showed that freshly cultured UC-MSCs were superior to cryopreserved and thawed UC-MSCs in regard to cell viability[68]. Another previous study demonstrated that osteogenic and chondrogenic capacities were slightly reduced in cryopreserved human UC-MSCs for one year, compared to non-cryopreserved UC-MSCs from the same donor[69]. In contrast, one previous study showed that short-term cryopreservation and subsequent thawing of UC-MSCs could not alter the specific MSC surface markers and the proliferation capacity[70]. There was no obvious difference in fibroblast-like morphology and cellular viabilities between freshly frozen thawed and culture-rescued UC-MSCs[71,72]. Frozen thawed and culture-rescued UC-MSCs also displayed similar osteogenesis, chondrogenesis, and adipogenesis[71]. Similarly, one previous study by Narakornsak *et al*[73] to compare the post-thawed human AF MSCs (AF-MSCs) after one-month cryopreservation to the non-frozen AF-MSCs showed no statistical differences between these cells in MSC surface markers, cell proliferation, chondrogenic differentiation, and immune privileged properties. Still, one previous study to analyze immunomodulatory activity *in vitro* indicated no significant differences in the suppression of activated T cells in the freshly thawed UC-MSCs *vs* the freshly cultured UC-MSCs[70]. Comparatively, there were different observations reported by assessing the potential in immunosuppression between freshly thawed and cultured UC-MSCs[71], as showed the freshly culture-rescued UC-MSCs to be more potent in immunosuppression than the frozen-thawed UC-MSCs.

In animal experiments, immunomodulatory activity in an adjuvant-induced arthritis rat model and angiogenic potential using a mouse model for hindlimb ischemia were observed and *in vivo* studies showed no significant difference in immunomodulatory and angiogenic potential of freshly thawed and freshly cultured UC-MSCs[70]. Treatment with the freshly thawed UC-MSCs improved the regeneration of rotator cuff tendon in an animal model after injection at 2 and 4 wk, respectively, which was comparable to the therapeutic potential for tendon regeneration by the use of the culture-rescued UC-MSCs[72]. In a ventilator-induced lung injury animal model, the fresh UC-MSCs as well as the fresh BM-MSCs were found to enhance injury resolution and repair, while the cryopreserved UC-MSCs were comparably effective[74]. As mentioned above, these experimental and preclinical data were provided to support short-term cryopreserved perinatal MSCs (*e.g.*, UC-MSCs) for stem cell-based applications in translational medicine. However, long-term cryopreserved perinatal MSCs need to be further explored to achieve preclinical and clinical safety and efficiency after intervention. Accordingly, it is advisable to continuously culture post-thawed perinatal MSCs for at least an additional passage at the release time for restoration of stem cell biological properties and, meanwhile, it is also required to conduct extra assessment of safety and therapeutic effectiveness.

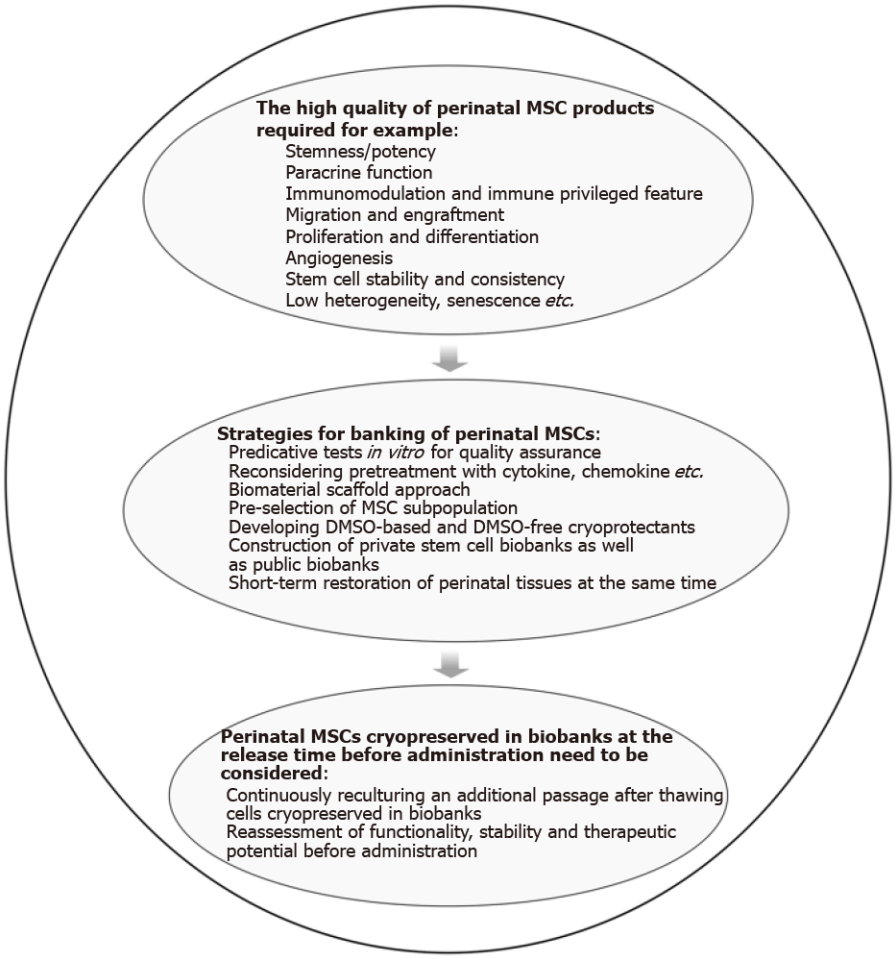
Short-term but not long-term cryopreservation of perinatal tissues for MSC preparation

Last but not least, a key issue has also been identified to date: Instead of banking perinatal MSCs, shot-term cryopreservation of perinatal tissues (*e.g.*, umbilical cord tissue) potentially for perinatal MSC storage for future stem cell-based treatments. Freezing of umbilical cords that yield post-thaw MSCs with high scalability is still controversial. One previous study reported that the frozen umbilical cord tissues for at least 2 wk were thawed and minced to generate a large number of MSCs by *in vitro* expansion[75]. UC-MSCs from cryopreserved and thawed umbilical cord tissues exhibited characteristics such as cellular phenotypes, immunosuppressive properties, and differentiation potentials similar to those of UC-MSCs from fresh tissues[75]. Another previous study reported that the fresh WJ cryopreserved for 30 d in liquid nitrogen can yield large numbers of post-thaw MSCs[76]. However, one previous study reported that although the cryopreserved umbilical cord tissue segments were thawed 5 years later to release MSCs after enzymatic digestion, those displayed poor recoveries and produced few viable UC-MSCs compared with fresh cord tissue samples[77]. Still, one previous study to assess the optimal isolation culture and cryopreservation methods to facilitate cord WJ-MSCs banking failed as no MSC cultures were obtained from the thawed cord WJ samples stored for 1 wk, 1 mo, or 6 mo in liquid nitrogen, respectively[78]. In addition, one study showed that cryopreservation of umbilical cord tissues for at least 1 mo in liquid nitrogen did not prevent the later collection of UC-MSCs by the explant method[79]. Currently, transplantation of UC-MSCs from fresh and short-term cryopreserved-then-thawed cord tissues is an issue of increasing interest. Similar to cord blood banks, perinatal umbilical cord tissue cryobanks are present in many other countries including United States, United Kingdom, Australia, and South Africa[80]. As a whole, given that cryopreservation cannot prevent the high cord quality, long-term restoration of perinatal umbilical cord tissue in banks may be unadvisable for potentially later use for post-thaw MSC preparation.

Collectively, when considering subsequent events, there are many limitations and uncertainties for stem cell medical treatments using perinatal MSCs banked over many years. Although most (if not all) aforementioned key issues, as summarized in Figure 2, are not specific to perinatal MSCs, such as cell quality insurance, heterogeneity, genetic etiologies, supplementation of scaffold, and use of cryoprotectants, these should be considered for the application of biobanking of perinatal MSCs. Research and development of different types of stem cell-based medication must be viewed with a forward-looking perspective. Importantly, when contemplating these issues including biological and technical concerns, this is a question as to whether any specific types of perinatal MSCs are worth biobanking. Without knowing the specific clinical applications, it is impossible to develop (*e.g.*, potency assay), and without a potency assay, it is not possible to assess the quality of perinatal MSCs stored by cryopreservation over many years. The question will always be raised as to what functions perinatal MSCs banked over many years can still actually play for the possible applications in their clinical practices. Realizing that short-term banking and restoration of perinatal MSCs is being routinely applied particularly in regenerative medicine and tissue engineering, long-term banking of perinatal MSCs as well as perinatal tissues for stem cell medical treatments within the lifetime should not be encouraged.

DISCUSSION

This opinion review focuses on several important points of view that are currently attracting people's attention. The review article does not diminish the clinical translational perspective of MSCs from either adult or neonatal tissues. Given the current situation of banking perinatal MSCs, the aim of this article is to contribute a balanced, comprehensive, and critical view in the settings of cryobanking of perinatal MSCs to probably maximize their potentially therapeutic activity in the future. As described above, there is no expiration date of cryostorage of the perinatal MSCs and it is not known about future timeline of stored perinatal MSCs for potential therapeutic applications. Therefore, the key issues have been identified in this article and, on the one hand, it is possible that the stored perinatal MSCs would be used for potential personalized medicine for a child when he/she develops a disease later on. On the other hand, it is too remote to assess the therapeutic benefit in the next decades. For example, by the time when a baby reaches the age of 50, no one knows whether these cells could be accessible and useful for personalized medicine at that time because there is a potential change for therapeutic benefit of perinatal MSCs. Although the short-term cryopreserved-then-thawed perinatal MSCs or the recultured MSCs are being widely used in pre-clinical and clinical studies, there are no data provided to support applications of long-term low-temperature storage of perinatal MSCs in banks, *e.g.*, cryopreservation of MSCs over a period of more than 10 years. Furthermore, it is not clear about how much the advanced techniques in science would be developed, for example, genome editing and precision medicine technologies to provide the best tools, probably, as opposed to MSC-based therapies looking forward 50 years. Science still drives the development of the advanced techniques and, eventually, the emerging advanced technologies are likely to influence the direction of therapeutic strategies in MSC-based translational medicine.



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Figure 2 Strategies and administrative requirements for high-quality stem cell banks. MSCs: Mesenchymal stromal/stem cells; DMSO: Dimethyl sulfoxide.

To date, banking perinatal stem cells and stem cell-derived newborn tissues have raised growing interest potentially for future stem cell applications. Due to less information available in scientifically understanding stem cell biology *in vivo* from the transplanted patients, such as stem cell proliferation, differentiation, immunomodulation, homing, and fate of MSCs, the true function and the precise mechanisms of the therapeutic benefit remain largely unclear. More importantly, given the unknown context of a specific pathology in the future, it should be noted as to whether perinatal MSCs are worth biobanking for a long period of time to achieve clinical efficiency. People believe that banking of perinatal MSCs may serve patients well one day in the foreseeable future, ideally for several years, when they may really need for the personalized medicine. But instead, the great uncertainties remain for potential use of the cryopreserved perinatal MSCs for stem cell-based treatments in the unforeseeable future. Acknowledging the limitations and uncertainties of banking perinatal MSCs for future potential personalized medicine, the following key recommendations should be addressed and the most of the recommendations proposed do not apply to the perinatal MSCs only.

For the sake of therapeutic safety and effectiveness in the future, a series of predicative tests *in vitro* for quality assurance as well as the additional analysis of genetic/epigenetic etiologies should be considered before cryobanking of perinatal MSCs. The novel strategic approaches, for example, biomaterial scaffold techniques, should be encouraged to use for maintaining the maximal biological and functional properties of perinatal MSCs in the routine banking practices.

Emerging practicable technologies would also be applied to yield a number of the desired stem cell types, *i.e.*, homogeneous and consistent perinatal MSCs, and, consequently, it is essential to develop novel biological technologies with a high yield of stem cells for cryobanking. The appropriate governance is required and banking of the perinatal MSC-based therapeutic products should comply with accreditation standards and the international standardized guidelines.

The public banks are to be built as a priority to better serve all recipients who might be desperately in need in the future and, therefore, the roles played by the public banks should not be underestimated. Lastly, instead of banking perinatal MSCs, short-term but not for long-term restoration of perinatal tissues, for instance umbilical cord tissue, may be suggestive of a possible approach of cell preparation

for stem cell-based medicine.

CONCLUSION

MSCs can provide therapeutic benefit given their unique biological characteristics, but, to date, there is still much to learn about stem cell science and medicine. MSCs derived from diverse tissues have their different functional properties and perinatal MSCs have their own advantages. Therapeutic properties of perinatal MSCs have been shown by multiple preclinical and clinical studies; however, banking of perinatal MSCs for personalized medicine in whole life remains to be unforeseeable. Therefore, it is extremely important for parents, physicians, and clinical investigators to be aware that there are limitations and uncertainties of banking perinatal MSCs for the future personalized medicine. Based on the above consideration, this opinion review article is conducted to address the concerns raised and provides several practical recommendations for banking perinatal MSCs for future potential personalized medicine. Accordingly, different strategic approaches can be employed in this rapidly growing field to improve this process, making perinatal MSCs available for future stem cell based therapies when needed to avoid banking poor-quality MSCs discarded as novel medical waste products in the future.

FOOTNOTES

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REVIEW

Obesity and cancer stem cells: Roles in cancer initiation, progression and therapy resistance

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Abstract

Obesity, the global pandemic since industrialization, is the number one lifestyle-related risk factor for premature death, which increases the incidence and mortality of various diseases and conditions, including cancer. In recent years, the theory of cancer stem cells (CSCs), which have the capacity for self-renewal, metastasis and treatment resistance, has been bolstered by increasing evidence. However, research on how obesity affects CSCs to facilitate cancer initiation, progression and therapy resistance is still in its infancy, although evidence has already begun to accumulate. Regarding the ever-increasing burden of obesity and obesity-related cancer, it is pertinent to summarize evidence about the effects of obesity on CSCs, as elucidating these effects will contribute to the improvement in the management of obesity-related cancers. In this review, we discuss the association between obesity and CSCs, with a particular focus on how obesity promotes cancer initiation, progression and therapy resistance through CSCs and the mechanisms underlying these effects. In addition, the prospect of preventing cancer and targeting the mechanisms linking obesity and CSCs to reduce cancer risk or to improve the survival of patients with cancer is considered.

Key Words: Obesity; High-fat diet; Cancer stem cells; Carcinogenesis; Metastasis

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Core Tip: Obesity increases the incidence and mortality of various cancers; however, research on how obesity affects cancer stem cells (CSCs) is still in its infancy. In this review, we discuss the association between obesity and CSCs, with a particular focus on how obesity promotes cancer initiation, progression and therapy resistance through CSCs and the mechanisms underlying these effects. In addition, the prospect of preventing cancer and targeting the mechanisms linking obesity and CSCs to reduce cancer risk or to improve the survival of patients with cancer is considered.

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INTRODUCTION

For millions of years, humans and their predecessors have evolved under the pressure of undernutrition, which selects a genotype that enables overeating, low energy expenditure, a high degree of calorie absorption and efficient energy storage in adipose tissue[1]. Therefore, with the development of the social economy in the past few decades, overnutrition and an increasingly sedentary lifestyle tip the balance from a few calories consumed but more expended to more calories consumed but little expended, leading to the pandemic of excess body weight, which is mainly measured by body mass index (BMI). Over the past four decades, the prevalence of overweight and obesity has nearly tripled globally. Between 1975 and 2016, the worldwide prevalence of obesity increased from less than 1% to 6%-8% among children, from 3% to more than 11% among men and from 6% to 15% among women[2]. Based on data from the Global Burden of Disease (GBD) 2015, overweight or obesity affects over 2.1 billion people, or nearly 30% of the global population[3]. Obesity was estimated to increase the economic burden by approximately 2 trillion United States dollars, or 2.8% of the global gross domestic product, and to lead to the loss of an estimated 5-20 years of life expectancy, representing one of the most serious unmet public health challenges of the 21st century[4-6].

Malignancy, a set of diseases caused by the interplay between genetic and environmental or behavioral factors, ranks as the third leading cause of premature death and disability attributable to excess body weight worldwide following cardiovascular disease and type 2 diabetes mellitus[7]. Recent studies have demonstrated that excess body weight is associated with higher risks of several types of cancer, including esophageal adenocarcinoma, multiple myeloma, and cancers of the gastric cardia, colon, rectum, biliary tract system, pancreas, breast, endometrium, ovary, and kidney[8]. In 2019, the estimated number of high BMI-related cancer cases accounted for 4.59% and 4.45% of all cancer-cause deaths and disability-adjusted life years, respectively[9]. Obesity can not only increase the risk of tumorigenesis but also promote the progression and metastasis of developed cancer and can affect the therapeutic efficacy and survival of patients with cancer[10].

Regarding the altered biological processes that occur in the context of obesity that contribute to cancer, the majority of studies have focused on common themes, including inflammation, hypoxia, angiogenesis and altered energy metabolism, which influence the proliferation and survival of cancer cells[10]. However, in recent years, emerging challenges in cancer management have promoted the proposal of many theories to explain the initiation and progression of cancer; one of them is the hypothesis of cancer stem cells (CSCs), which has been bolstered by an accumulating body of evidence [11]. CSCs, also referred to as treatment-refractory, tumor-initiating cells, constitute a small subpopulation of cancer cells within tumors capable of self-renewal, which can divide and differentiate into various tumor cell types (intratumoral heterogeneity). They can secrete antiapoptotic factors, undergo epithelial-to-mesenchymal transition (EMT), and display a higher performance of drug efflux pumps. Therefore, CSCs are preferentially aggressive and pose a high risk of therapy resistance and disease relapse[11]. With the rapidly increasing incidence of cancer attributable to obesity, a better understanding of the roles of obesity in CSC biology is of paramount significance. However, research in this area is in its infancy. In this review, we discuss the association between obesity and CSCs, with a particular focus on how obesity promotes cancer initiation, progression and therapy resistance through CSCs and the mechanisms underlying these effects. In addition, the prospect of prevention and targeting mechanisms linking obesity and CSCs to reduce cancer risk or to improve the survival of patients with cancer is considered.

OBESITY AND CSCS IN CANCER INITIATION

In the context of an increase in the global prevalence of obesity, large-scale epidemiological studies have demonstrated a compelling increased risk of tumorigenesis in individuals with obesity, and several landmark studies have summarized this evidence. To evaluate the strength and validity of the evidence for the association between adiposity and the risk of developing or dying from cancer, an umbrella review of the literature comprising 204 meta-analyses of large studies with limited heterogeneity or evidence of bias was published in 2017, which concluded that the associations for 11 cancers were supported by strong evidence, while others could be genuine, but substantial uncertainty remains[8]. In 2016, data from a meta-analysis reported by the International Agency for Research on Cancer supported relative risks of 1.5 to 1.8 in obesity for these tumor sites[12]. In GBD 2019, 13 cancer types were also found to be affected by a high BMI[9]. Although some inconsistencies in cancer types contributing to obesity were reported across these studies, a consistent and compelling association has been demonstrated in many cancer types, including esophageal adenocarcinoma, multiple myeloma, and cancers of the gastric cardia, colon, rectum, biliary tract system, pancreas, breast, endometrium, ovary, and kidney (Figure 1). In the majority of these cancers, the CSC theory has been established in tumorigenesis. For example, in the intestine, inactivation of the adenomatous polyposis coli (APC) gene can lead to the rapid and lethal generation of adenomas in intestinal stem cells (ISCs) but not in non-stem cells[13]. Breast cancer was found to originate from a rare population of mammary gland progenitor cells, the depletion of which significantly impaired tumor growth[14]. Therefore, to increase the risk of these cancers, obesity may disturb the normal biology of stem/progenitor cells residing in these tissues, which is conducive to their transformation.

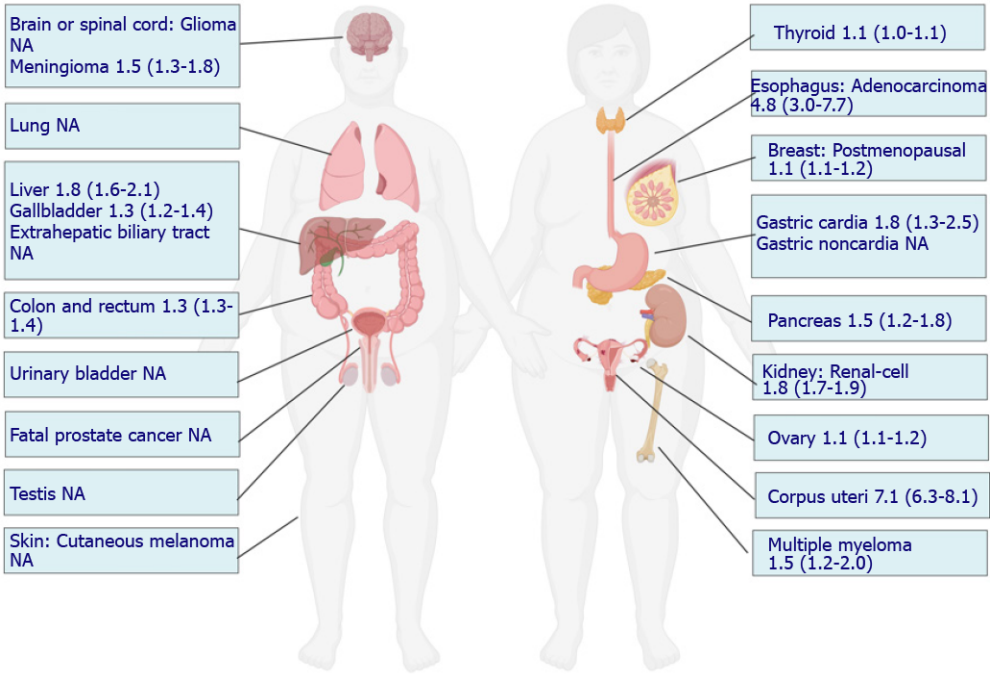
Except for compelling epidemiological evidence, no attempts have been made to investigate the biology of cancer-related adult stem cells in populations with obesity. Multiple animal models have been developed to recapitulate the effects of obesity or a pro-obesity diet on the initiation of cancer and have suggested that a high-fat diet (HFD) can promote tumorigenesis in the colorectum, prostate and liver[15-17]. However, the cellular origin of cancer was not defined in these studies. In recent years, research revealing the links between obesity or HFD and adult stem cells has increased (Table 1)[18-28]. Although discrepancies exist, the majority of these studies reported one or more of the following findings: Obesity or a HFD increases the depth or number of crypts in the intestine; non-stem cell progenitors in a HFD setting acquire stem cell attributes; the number and the capacity to form organoids of stem cells or progenitor cells are increased by a HFD; stem cells undergo autonomous changes in response to a HFD that poise them for niche-independent growth. Although studies exploring the initiation of carcinogenesis from these stem cells are very limited, the alterations in stem cells reported in these studies can predispose them to transformation. First, obesity or a HFD expands the pool of cells - both stem cells and progenitor cells - that can serve as the cellular origin of nascent cancers. Second, stem cells from mice on a HFD become functionally uncoupled from their niche in the organoid assay and *in vivo*, consistent with the hallmarks of cancer cells. Third, several studies have shown the possible links between these perturbations and tumorigenesis. For instance, when injected with azoxymethane, aberrant crypt foci (ACF), an early-appearing lesion of colon carcinogenesis, were increased in male mice fed a HFD[21]. In another study, more spontaneous intestinal low-grade adenomas and carcinomas were observed in HFD-fed mice than in standard diet-fed mice[19].

At present, elucidating how obesity and a pro-obesity diet contribute to the cellular origin of cancer in the intestine is the central focus of research, and data on stem cells in other tissues are very limited. Several reasons can explain such a tissue preference for studying the impact of obesity and HFD physiology on the initiation of cancer. First, robust epidemiological evidence has been accumulated for the increased risk of colon cancer in obese populations, and a better understanding of the altered biology of ISCs that occurs in the context of obesity will provide immeasurable public health benefits [10]. Second, the stem cell theory advanced most rapidly in ISCs, from which the histological architecture of the intestine has been well established[29]. Third, ISCs reside in the base of the intestinal crypt and directly interact with luminal nutrients, bacteria, and other intraepithelial and subepithelial cells, making the intestine an ideal system for studying the pathophysiological changes on a HFD[29]. Finally, the natural orifice of the intestine makes in situ manipulations for tumor induction or diagnostic tests easier than other *in vivo* cancer models[30]. Despite all of this, as consistent and compelling associations have been demonstrated between obesity and more than 10 cancer types, elucidating how stem cells in tissues other than the intestine are perturbed by obesity or HFD holds the same importance. Currently, the majority of obesity models are induced by HFD; however, other dietary patterns, such as a high-sugar diet and Western-styled diet, have also been shown to be obesogenic, and the effects of these dietary patterns on the initiation of cancer warrant further studies. Furthermore, the alterations in stem cell biology in tissues with increased cancer incidence warrant further investigation in obese human beings, not just in animals.

Table 1 Murine models of high-fat diet-induced obesity and cancer stem cells

Tissue	Findings	Ref.
Intestine	Increased crypt depth and villus height; increased number of Olmf4-positive ISCs; increased size of the enterospheres that developed from ISCs	[18]
Intestine	Increase in crypt depth; non-stem progenitor intestinal cells gain more stemness features and self-renewal; 50% increase in the number of Olfm4+ ISCs; 23% decrease in the number of Paneth cells; more likely to initiate mini-intestines; organoids had higher frequencies of Lgr5+ ISCs; ISCs by themselves had an increased capacity to initiate organoids	[19]
Intestine	Combined with Pten inactivation, obesity is insufficient to drive Lgr5+ ISC-derived tumorigenesis	[20]
Intestine	Increased aberrant crypt and crypt foci; increased proliferation of colonocytes per mouse	[21]
Intestine	Higher number of Lgr5+ stem cells per crypt	[22]
Intestine	Increased number of ISCs and progenitor cells; crypts are further likely to form mini-intestine organoids in a 3D culture	[23]
Intestine	Increased intestinal epithelial cell proliferation	[24]
Intestine	Reprograms Bmi1+ cells to function and persist as stem-like cells in mucosal homeostasis and tumor development	[25]
Intestine	Increased number of crypts; increased total numbers of ISCs and percentage of ISCs in S-phase; reduced numbers of Paneth and goblet cells	[26]
Lung	Increased number of AT2 cells; higher stem cell colony forming efficiency	[27]
Esophagus	Increased numbers of epithelial progenitors in Barrett's esophagus	[28]

ISC: Intestinal stem cell; Lgr5: Leucine-rich repeat-containing G protein-coupled receptor 5; AT2: Alveolar type-2.



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Figure 1 Relative risk of individual cancers at high body mass index. Data were obtained from a meta-analysis reported by the International Agency for Research on Cancer working group. The number represents the relative risk and its 95% confidence interval of the highest body mass index category *vs* normal body mass index. NA: Not available.

OBESITY AND CSCS IN CANCER PROGRESSION AND THERAPY RESISTANCE

In addition to promoting tumorigenesis, obesity might also promote the progression of established cancers, affect the efficacy of present forefront antitumor therapies and shorten the survival of patients with cancer. For instance, a meta-analysis including 86490 patients treated for clinically localized prostate cancer showed a moderate and consistent relationship between obesity and biochemical recurrence, and there was a 10% increase in biochemical recurrence per 5 kg/m^2 increase in BMI[31]. In the Carolina Breast Cancer Study phase 3, a high waist-to-hip ratio was found to be associated with a

high risk of metastasis[32]. Poor survival was also reported in overweight or obese patients with colorectal, endometrial and breast cancer[33-35]. In addition to the increased likelihood of recurrence, the poor prognosis of obese patients with cancer also results from the reduction in the efficacy of antitumor therapies[36]. Mechanistically, the link between obesity and increased recurrence, therapy resistance and poor survival is likely multifactorial, with some differences related to more advanced stages being attributed to reduced participation in routine screening or the systemic effects of obesity on drug pharmacokinetics and metabolism[37,38]. In addition to these explanations, emerging evidence has shown that the activation of stem cell programs in cancers can lead to progression, metastatic growth and therapy resistance[11].

The key roles of obesity in the activation of stem cell programs have attracted much attention in recent years; however, as in studies on the effects of obesity on cancer-initiating cells, the promotion effects of obesity on cancer through CSCs are also mainly limited to animal models, which are utilized to investigate how specific obesity-related factors induce the stemness of cancer cells. Knowledge about CSCs in obese patients with cancer is still not clear. For example, obesity increases inflammation in the tumor microenvironment (TME) through local and systemic adipokines, proinflammatory cytokines or hormones, which modulate the stemness of cancer cells[39]. In a mouse model of hepatocellular carcinoma, diet-induced obesity increased inflammatory signaling *via* STAT3, and this finding was associated with larger tumors with a cancer-stem-cell-like phenotype[40]. Prolonged culture of breast cancer cells, which developed from a fat-rich environment, with adipocytes increased the proportion of cells expressing stem-like markers *in vitro* and the abundance of cancer cells with metastatic potential *in vivo*[41]. Regarding the involvement of CSCs in therapy resistance, leptin was found to interfere with the efficacy of 5-fluorouracil (5-FU) in colon tumor stem cells by increasing cell viability and reducing 5-FU-induced DNA damage[42].

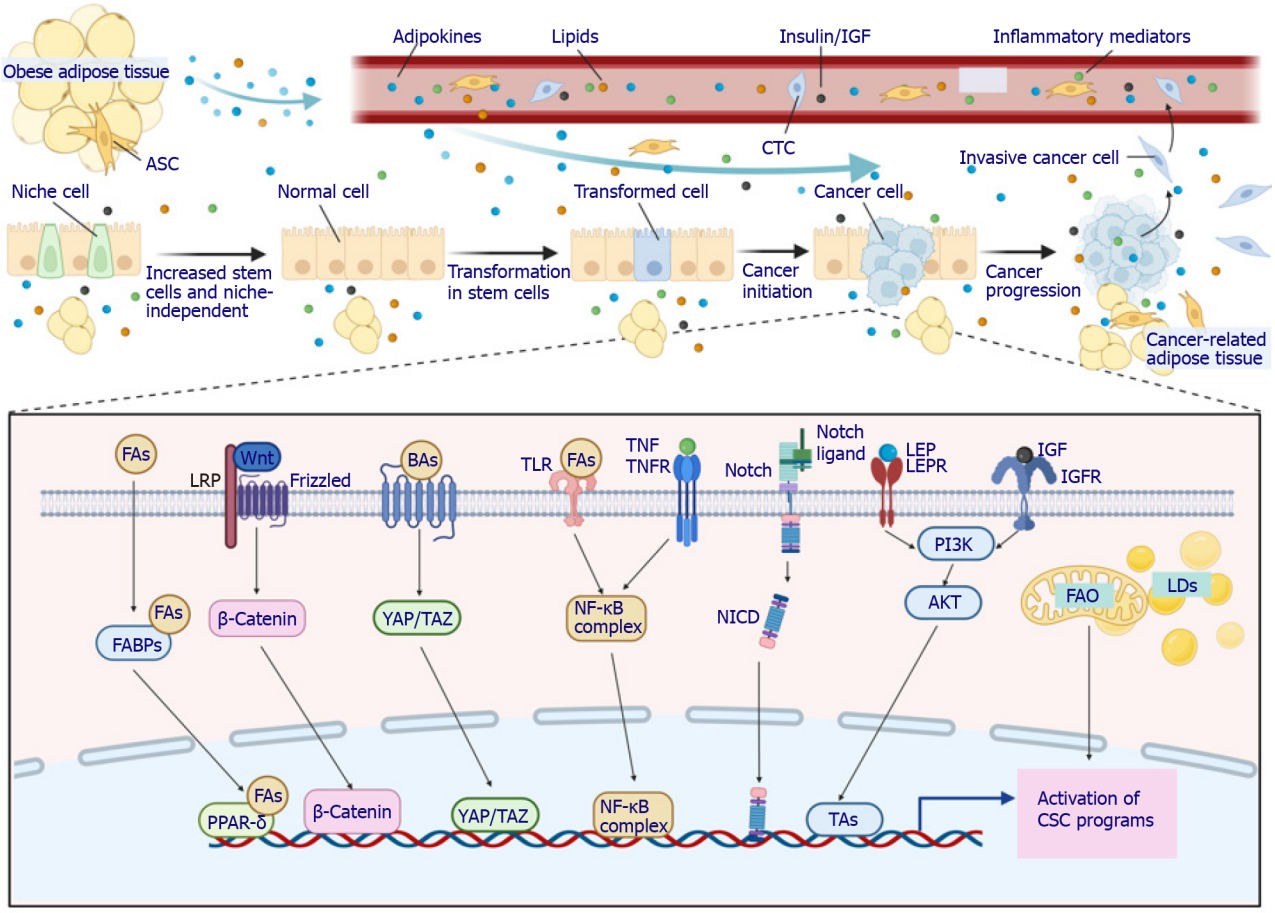
EMT is a reversible cellular process during which epithelial cells transiently acquire mesenchymal phenotypes, such as an elongated, fibroblast-like morphology as well as an increased capacity for migration and invasion[43]. It is now widely accepted that EMT has well-established roles in cancer metastasis[43]. In the majority of carcinomas, only CSCs exhibit aspects of EMT-program activation[44]. Various extracellular stimuli, including obesity-related factors, have been implicated in the induction of EMT programs. For instance, esophageal cancer cells cocultured with visceral adipose tissue taken from obese patients resulted in the induced expression of genes involved in EMT, which was also noted in tumor biopsies from obese patients[45]. Cytokines and growth factors released by adipose stem cells (ASCs) can induce EMT-like changes in various cancer cells[10]. The adipokine leptin has also been found to activate EMT programs to enhance the proliferation and metastasis of breast cancer cells[46]. Therefore, obesity can propel primary tumor cells toward EMT events, leading to malignant progression.

THE LINKS BETWEEN OBESITY AND CSCS

As discussed above, CSCs participate in every step of tumorigenesis promoted by obesity. Understanding the key links between obesity and CSCs, therefore, offers important potential to decrease the incidence and improve the outcomes of obese patients with cancer. Several main factors are considered to connect obesity and cancer: Components of pro-obesity diets, metabolic and hormonal alterations associated with obesity, dysfunctional adipose tissue in the TME, low-grade obesity-related inflammation, self-renewal and stemness pathways, and microbiome dysbiosis. Each of these factors is intimately linked to and cross-talks with each other. For example, fatty acids in pro-obesity diets accumulate in adipocytes, leading to expansion and dysfunction of adipose tissue, which is intimately linked to endocrine and paracrine dysregulation, such as increased circulating insulin, insulin-like growth factor-1 (IGF-1) and leptin. All of these alterations activate and maintain a prolonged low-grade inflammatory state, predisposing individuals with obesity to an increased cancer risk and poor outcomes[47-49]. Although these factors were mainly investigated in nonspecific conditions, their involvement in CSC biology is also beginning to accumulate evidence (Figure 2).

Components of pro-obesity diets

Although the mechanistic links are not completely understood, nutrient-sensing signaling activated by components of a pro-obesity diet has been shown to influence stem cell behavior and tumorigenesis. This was also indirectly suggested in a leptin-receptor-deficient (*db/db*) mouse model, which becomes obese on control diets and does not rely on a HFD. In *db/db* mice, the number of ISCs was reduced, while ISC function was not affected, highlighting that components of a pro-obesity diet can regulate stem cells independently of obesity[19]. As early as 1989, Blakeborough *et al*[50] reported that diets high in fat support a *Bacteroides*-dominated colonic microflora and increase the excretion of secondary bile acids to augment free radical production, which may overcome the antioxidant defense mechanisms of stem cells, causing DNA damage, tumorigenesis and proliferation of transformed stem cells. Since then, numerous studies have suggested for decades that a pro-obesity diet engages many diverse pathways in stem cells in various tissues that collectively contribute to tumorigenesis. For instance, a HFD increases



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Figure 2 Mechanisms linking obesity and cancer stem cells. In the obese microenvironment, the stem cell pool is increased and loses niche dependence, predisposing these stem cells to transformation. Several main factors are considered to connect obesity and cancer stem cells (CSCs): Components of pro-obesity diets, metabolic and hormonal alterations associated with obesity, dysfunctional adipose tissue in the tumor microenvironment, low-grade obesity-related inflammation, self-renewal and stemness pathways, and microbiome dysbiosis. These factors are intimately linked to and cross-talk with each other, synergistically leading to the activation of CSC programs through various signaling pathways. ASC: Adipose stem cell; BAs: Bile acids; CSC: Cancer stem cell; CTC: Circulating tumor cell; FAs: Fatty acids; FABPs: Fatty acid binding proteins; FAO: Fatty acid oxidation; IGF: Insulin-like factor; IGFR: Insulin-like receptor; LDs: Lipid droplets; LEP: Leptin; LEPR: Leptin receptor; LRP: Low-density lipoprotein receptor-related protein; NCID: Notch intracellular cytoplasmic domain; PI3K: Phosphatidylinositol 3-kinase; PPAR-δ: Peroxisome proliferator-activated receptor δ; TAZ: Transcriptional coactivator with PDZ-binding motif; TFs: Transcription factors; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TNFR: Tumor necrosis factor receptor; YAP: Yes-associated protein; NF-κB: Nuclear factor-kappaB.

leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) expression and promotes tumor growth in a xenograft model independent of obesity. Mechanistically, dietary fats stimulate vitamin A-bound serum retinol-binding protein 4 and retinoic acid 6, which are implicated in colon stem cell self-renewal, to activate the JAK-STAT3 pathway and boost Lgr5 expression and the tumorigenicity of ISCs [51].

The representative components of a pro-obesity diet, that is, fatty acids such as palmitic acid or oleic acid, were found to enhance the number and self-renewal potential of ISCs and to permit organoid body formation without supporting signaling from their niche cells in a well-designed study[19]. The molecular mechanism by which fatty acids deregulate ISCs to promote tumorigenesis was also delineated in this study. It was proposed that fatty acids can be transported to the nucleus by fatty acid-binding proteins or can be produced directly by lipid metabolism in the nucleus, where they increase the number and self-renewal of ISCs *via* peroxisome proliferator-activated receptor δ (PPAR-δ), a nuclear receptor that senses fatty acid derivatives, the synthetic activation of which mimics both the *in vivo* and *in vitro* impact of a HFD and fatty acid treatment[19]. In contrast, loss of PPAR-δ completely abrogated the effects of fatty acids on Lgr5+ ISC function with respect to organoid-initiating capacity [19]. The downstream signaling mediating the effects of PPAR-δ activation was attributed to WNT/β-catenin, as demonstrated by increased β-catenin staining and upregulation of its target genes (Jag1, Jag 2 and Bmp4) in ISCs and progenitors from HFD- and PPAR-δ agonist-treated mice[19]. Free fatty acids produced by obese fat lipolysis also serve as ligands for Toll-like receptor 4 (TLR4) on cancer cells to activate nuclear factor-kappaB (NF-κB), leading to an increase in CSCs[52]. Another critical element in a pro-obesity diet that is significantly elevated in some obese individuals, cholesterol, was also demonstrated to affect stem cell function, thus promoting tumorigenesis. In *Drosophila*, dietary

cholesterol modulated the differentiation of ISCs by stabilizing the Delta ligand and Notch extracellular domain and altering their trafficking in endosomal vesicles, while a low-sterol diet slowed the proliferation of enteroendocrine tumors initiated by Notch pathway disruption[53]. In a rodent animal model, evidence also showed that dietary cholesterol acts as a mitogen for ISCs, while disruption of cholesterol homeostasis dramatically enhances tumor formation in APC_{min} mice[54].

Although bile acids are not elements in a pro-obesity diet, their excretion is essential for the digestion and absorption of dietary fat and is increased when a HFD is consumed. Bile acids are endogenous agonists of the G protein-coupled bile acid receptor, the activation of which augments Yes-associated protein 1 (YAP1) signaling, leading to increased stem cell number and proliferation and enhanced organoid-forming capability of ISCs[55]. In addition, bile acids, such as tauro-β-muricholic acid and deoxycholic acid, can antagonize intestinal farnesoid X receptor (FXR), a master regulator of bile acid homeostasis. Antagonizing FXR in the intestinal lumen enhances the proliferation and DNA damage of stem cells, initiating the transformation of ISCs to a malignant phenotype and promoting adenoma-to-adenocarcinoma progression[56]. Conversely, selective activation of intestinal FXR by its agonist can restrict abnormal ISC growth and skew differentiation toward goblet cells, thus curtailing HFD-induced intestinal cancer progression[56].

Metabolic alterations

Aerobic glycolysis has long been viewed as the main metabolic characteristic of cancer cells. However, in recent years, CSCs have been found to be intimately dependent on lipid metabolism to maintain their self-renewal capability. Therefore, in addition to the abovementioned studies that investigated the regulation of CSC function by lipids as elements of a pro-obesity diet, numerous studies have explored the effects of lipids on CSCs at the cellular metabolism level. Metabolism of fatty acids and cholesterol, including *de novo* biosynthesis, storage and fatty acid oxidation (FAO), supports the stemness, proliferation and chemotherapy resistance of CSCs[57]. Metabolic analysis demonstrated that lipid synthesis, including *de novo* lipid biosynthesis, lipid desaturation, and cholesterol synthesis, displays high activity in CSCs, indicating that lipid synthesis plays critical roles in stemness maintenance[58]. Human breast cancer-derived data suggest that FAO promotes cancer cell stemness and chemoresistance. Blocking FAO resensitizes them to chemotherapy and inhibits CSCs in mouse breast tumors *in vivo*[59]. Furthermore, cytarabine-resistant acute myeloid leukemia cells, which are enriched in leukemic stem cells, exhibited increased FAO[60]. FAO is also responsible for the stemness and chemotherapy resistance in gastric cancer induced by mesenchymal stem cells (MSCs)[61]. To meet the critical functions of lipids in CSCs, lipid droplets, organelles that store neutral lipids, accumulate and are more abundant in CSCs in numerous types of cancer[57]. Although evidence connecting lipid metabolism and CSCs is increasingly accumulating, whether obesity can augment the lipid metabolic alteration in CSCs is not clear because studies on the metabolic adaptations of CSCs in obese environments are limited. However, the incidence of hyperlipidemia is higher in obese populations, and in obese individuals, CSCs more readily reside in a fat-rich TME, which may provide more lipids to CSCs. Therefore, theoretically, lipid metabolic alterations in obesity support the stemness of cancer cells, although further studies are warranted to validate such effects.

Another common metabolic alteration of obese patients is insulin resistance, leading to increased levels of circulating insulin and IGF-1, which contribute to the increased risk and mortality of several cancers in obese individuals. Mice with diet-induced obesity exhibited increased concentrations of plasma glucose, insulin, and IGF-1, which were significantly correlated with increased proliferation and self-renewal of ISCs, as well as decreased Paneth cell numbers[26]. In addition, insulin significantly increased the capacity of organoid formation *in vitro*[26]. Reports have suggested that the PI3K/AKT pathway is the major contributor to the abnormal renewal of ISCs endowed by insulin/IGF-1[62]. Therefore, insulin/IGF-1 signaling was suggested to mediate the effects of obesity on the function of stem cells, which is conducive to their transformation. Even insulin/IGF-1 levels in newborns are associated with the risk of future breast cancer, possibly resulting from an increased total number of stem cells[63]. Parallel to their function in normal stem cells, evidence suggests the roles of insulin/IGF-1 in cancer progenitor/stem cells from solid and hematopoietic malignancies. Insulin/IGF-1 and their receptors are overexpressed or overactivated in human thyroid, hepatic and breast CSCs and participate in the self-renewal, EMT and chemoresistance of cancer cells[64]. These emerging discoveries will undoubtedly promote renewed efforts aimed at targeting the insulin/IGF-1 system that contribute to CSC biology.

Hormonal alterations

Adipose tissue has long been viewed as an energy reservoir; however, this perspective has changed in recent years, as numerous bioactive adipokines, including more than 50 different metabolic and hormonal factors, cytokines and chemokines, were reported to be released by adipose tissue[65]. Two of the major adipose tissue-derived hormones are leptin and adiponectin, which have opposite effects. In contrast to lean adipose tissue, which mainly secretes the antimitogenic adipokine adiponectin in obesity, increased preadipocytes yield high levels of leptin, which has proangiogenic and promitogenic effects[66,67].

Leptin acts as a growth factor for many tissues, such as the mammary gland, lung, liver, and colonic epithelium[68]. The links between leptin and CSCs have been comprehensively studied in breast cancer [69]. In a diet-induced obese mouse model, mammary epithelial polarity was disrupted, which can contribute to overactivation of the PI3K/AKT pathway downstream of the paracrine effect of leptin expressed by neighboring adipocytes. Leptin expands the pool of stem/progenitor cells in the breast epithelium and causes mitotic spindle misalignment, which is an early step in tumor initiation[70]. The leptin receptor was found to be expressed on breast CSCs, and in orthotopically transplanted breast cancer, leptin can promote CSC enrichment[71,72]. Inactivation of the leptin receptor attenuated the expression of CSC transcription factors and reduced the self-renewal of cancer cells in tumor sphere assays[71]. Leptin-mediated cancer initiation, progression and therapy resistance through CSCs in other cancers have also been extensively investigated[69]. For instance, leptin was found to initiate the early transformation of colon cancer. ACF multiplicity, as early-appearing lesions of tumorigenesis, was increased by a HFD in *ob/ob* mice or in a genetic mouse (*db/db*) model with leptin receptor deletion[73, 74]. However, in *db/db* mice fed a control diet, the function of ISCs and the activity of PPAR-δ and Wnt/β-catenin signaling were not changed, indicating that obesity and elements in a pro-obesity diet may cause different alterations in the function of stem cells[19]. In addition, no leptin receptor was found on colonic stem cells, and leptin did not increase the pool of Lgr5+ stem cells, suggesting that leptin may be dispensable in the early stages of colon carcinogenesis[22]. Collectively, these findings indicate that although the crucial role of leptin in CSCs may be affected by the cellular origin of cancer, the potential of leptin pathways in cancer initiation and progression will lead to future areas of therapeutic management.

Although data are scarce, other adipokines with altered secretion in obese adipose tissue were also shown to affect the function of CSCs. For example, DeClercq *et al*[22] specifically investigated the effect of a HFD on colonic stem cell maintenance during cancer initiation and found that the number of stem cells and their proliferation capacity were significantly increased, while the incidence of apoptosis was decreased. The authors proposed that these effects are the result of decreased adiponectin signaling based on the findings that the reduction in stem cell number and increase in apoptosis were diminished in organoid cultures from obese mice treated with an adiponectin receptor agonist[22]. In addition, following a decrease in adiponectin signaling, obesity can increase tumorigenesis in the intestine[22]. Resistin, another adipokine, was highly associated with the transcription of genes related to CSCs in low malignant breast cancer cells and noncarcinogenic breast epithelial cells[75]. These adipokines with different effects on CSCs and their therapeutic translational potential need further research.

Dysfunctional adipose tissue in the TME

Despite the systemic effects of adipose tissue on CSCs through the secretion of circulating metabolic and hormonal factors, adipose tissue also constitutes an important part of the microenvironment of several cancers, and its dysfunction resulting from obesity is considered a critical determinant of cancer progression[76]. For example, cancer-associated adipose tissue obtained from obese patients with breast cancer was found to increase inflammatory breast cancer aggressiveness *via* the regulation of CSC markers[77]. Coculture of breast cancer cells with human-derived adipocytes increased the abundance of mammosphere-forming cells and stem-like cancer cells *in vitro* and increased tumor-initiating cells and metastasis in mouse models[41]. Mechanistic investigations demonstrated that immature adipocyte contact activates Src, thus promoting embryonic stem cell transcription factor upregulation, including Sox2, c-Myc, and Nanog, to mediate CSC expansion[41]. Moreover, Sox2-dependent induction of miR-302b further stimulated c-Myc and Sox2 expression and potentiated cytokine-induced CSC-like properties[41]. However, adipose tissue from different anatomical sites may have different effects on CSCs. For example, serial transplantation of pluripotent stem cells cultured in conditioned medium of breast cancer cells into mammary fat pads evoked the same features of breast cancer, while this result was perturbed following subcutaneous transplantation, indicating that mammary adipose tissue can synergize with secretory factors produced by cancer cells to transform normal cells into CSCs, while subcutaneous adipose tissue cannot[78]. Such performance differences may be caused by the various metabolic characteristics of adipose tissue from different anatomical sites determined by sex steroid hormones[79].

Among various adipose tissue cell types, ASCs are key players in adipose tissue. Under obese conditions, the biology of ASCs is dramatically altered, and ASCs can be recruited to sites of inflammation, including tumors[80]. ASCs are able to produce a large variety of circulating growth factors, cytokines and adipokines, which play important roles in CSC function. In addition to their systemic effects, ASCs represent an important cellular component in the TME. Therefore, in a breast cancer patient-derived xenograft model, cancers grown in the presence of ASCs had increased numbers of CD44+CD24− CSCs in the peripheral blood and had a higher tendency to form metastases[81]. This effect may be mediated by leptin, as the stable knockdown of leptin in obese ASCs led to a significant reduction in circulating CSCs[81]. In addition, ASCs reshape the TME and support the generation of CSCs, which are associated with radioresistance and chemotherapy resistance[82].

Low-grade obesity-related inflammation

In recent decades, the contribution of inflammation to cancer initiation, progression and therapy resistance has regained enormous interest, and the association between inflammation and CSCs has also been explored extensively[83]. Obesity has long been considered a facilitator of mild, chronic, systemic inflammation. Along with the expansion of adipose tissue in obesity, hypoxia causes adipocyte stress and malfunction, recruiting different types of immune cells[84,85]. Both adipocytes and immune cells release numerous adipokines, cytokines, chemokines and hormones, which perpetuate the inflammatory state[84,85]. Therefore, it is reasonable to speculate that obesity can affect CSCs through low-grade inflammation. Although chronic inflammation is not induced consistently in obese mouse models, which may be related to the differences in feeding patterns and other combined interventions, current evidence indicates that inflammation might have a role in alterations in stem cells leading to tumorigenesis. Inflammatory mediators were found to be increased in the intestinal mucosa in mice with obesity or those fed a HFD, resulting from cytokine release by myofibroblasts and immune cells[86]. Local inflammation has been demonstrated to expand colon cell progenitors or stem cells and to induce their proliferation in the intestine. For example, HFD-induced obesity elevated both the colonic proliferative zone and stem cell zone in a pig model, and the proliferative zone was associated with an increase in the innate inflammatory markers TLR4, NF-κB, IL-6, and lipocalin-2[87]. In addition, activation of NF-κB, the central pathway downstream of the majority of proinflammatory cytokines, following local inflammation enhanced the reprogramming of non-stem enterocytes to acquire stem-cell-like properties, which expanded the pool of stem cells and generated tumor-initiating cells[88]. HFD-induced obesity promoted the phosphorylation of GSK3β and then increased the nuclear translocation of β-catenin, thereby activating the expression of WNT signaling target genes[89]. These effects were diminished by the deletion of tumor necrosis factor-alpha (TNF-α), indicating the role of inflammation induced by TNF-α in colon tumorigenesis associated with obesity[89]. Another inflammatory mediator, prostaglandin E2, was also found to be elevated by HFD in the circulation or in local tissues, leading to an increased number and division rate of stem cells[22].

Other proinflammatory mediators have also been demonstrated to facilitate CSC expansion. For example, IL-6 can induce malignant features in human ductal breast carcinoma stem/progenitor cells [90]. IL-8 treatment leads to breast cancer cells partially acquiring some stem-like cell attributes, thereby increasing their aggressiveness[91]. Chemokine (C-C motif) ligand 2, derived from cancer-associated fibroblasts, stimulates the stem cell-specific, sphere-forming phenotype in breast cancer cells and CSC self-renewal[92]. Although these studies were not carried out under obese conditions, these proinflammatory mediators are consistently elevated in obese individuals and are upregulated upon contact with cancer cells. The enrichment of CSCs may be partially attributed to these cytokines, as a few studies indeed found that proinflammatory cytokines, including IL-6, IL-8 and monocyte chemoattractant protein 1, are overexpressed in cancer-associated adipose tissue from obese patients and induce the stemness of cancer cells, while such effects were not found in nonobese patients[77]. Considering the importance of localized and systemic inflammation in the induction and maintenance of stemness in cancer cells and the definite association between inflammation and obesity, elucidating how these inflammatory pathways increase the risk of cancer incidence, progression and therapy resistance *via* CSCs holds great promise to decrease the burden of cancer in obesity.

Self-renewal and stemness pathways

Stem cells are proposed to reside in a distinctive microenvironment, that is, the stem cell niche, which induces and maintains the self-renewal and differentiation of stem cells. In the TME, niche signals also play critical roles in cancer cells acquiring more stemness. Although the pathways responsible for establishing a CSC phenotype are diverse and differ among cancer entities, developmental signaling pathways, including the Notch, WNT, Hedgehog and Hippo pathways, are commonly altered in CSCs and interact with each other and with other common oncogenic signaling pathways and have key regulatory functions that support the maintenance and survival of these cells, making them prime targets for anti-CSC therapy[82]. Obesity, HFD and abnormal adipocytes may engage in these self-renewal and stemness pathways directly or indirectly through increased local and systemic levels of many cytokines and adipokines. For example, in ISCs and progenitors from mice fed a HFD, the expression of Jag1 and Jag2, which are ligands for the Notch pathway and are normally expressed by neighboring niche cells, was increased by the activation of WNT/β-catenin, indicating that a HFD drives ISCs to niche independence[19]. Within the ISC niche, MSCs were expanded and secreted predominant levels of Wnt2b in the colon of HFD-fed mice, which promoted the growth of tumorigenic properties and accelerated the expression of CSC-related markers in colon organoids[93]. CSCs isolated from obese mice also exhibited enhanced Notch2 expression[94]. However, such direct evidence supporting the association between obesity and alterations in stemness pathways is scarce, and more studies are needed to test this model. Nonetheless, some emerging data demonstrate alterations in these stemness pathways in obesity-induced cancers. For instance, HFD consumption could upregulate the expression of β-catenin proteins in a mouse xenograft tumor model[95]. Notch signaling activity was increased in breast cancer cells following coculture with obesity-altered ASCs[96]. In addition, YAP, the major player in the Hippo pathway, dictates mitochondrial redox homeostasis to facilitate obesity-associated breast

cancer progression[97]. Although the contributions of CSCs were not examined in these studies, regarding the definitive effects of these signaling pathways on CSCs, it is reasonable to speculate that the upregulated activity of these pathways in obesity may promote the progression of cancer through CSCs and that targeting these pathways may be more promising in obesity-induced cancers.

Microbiome dysbiosis

The epithelial barrier surfaces of our body host a diverse microbial community, or microbiota, that is composed of a variety of microorganisms, such as bacteria, fungi, and viruses[98]. Substantial studies have reported that obesity or HFD markedly affects the composition of the commercial microbiota, especially in the intestine[48]. Obesity-induced perturbation of the gut microbiota has been demonstrated to influence stem cell phenotypes. For example, structural changes in the microbiota were associated with HFD-induced myeloid progenitor skewing of the differentiation capacity of hematopoietic stem cells[99]. The intestinal tract bacteria *Lactobacillus* induces the release of adiponectin by niche cells through NF-κB activation[100]. In pigs with HFD-induced obesity, the elevation of the proliferative zone and stem cell zone was associated with increased abundance of the gut bacterial phyla *Proteobacteria* and *Firmicutes*[87]. In addition to these initial data suggesting the association between obesity-related microbiome dysbiosis and the possible development of CSCs, evidence linking the microbiome in obesity to CSC function is lacking. Whether obesity-induced alterations in the composition of the host microbiota affect initiation, progression and therapy resistance and the underlying mechanisms need more investigation with the hope of providing more strategies for cancer prevention and treatment.

CLINICAL SIGNIFICANCE

If the above-discussed links between obesity and CSCs are founded on convincing evidence, an obvious question is whether targeting both can prevent or improve the outcomes of cancer. At present, targeting both obesity and CSCs has great challenges; however, progress is gradually being made. For example, bariatric surgery has been popularized globally, leading to more weight loss and longer maintenance than diet and lifestyle changes[101]. Intriguingly, in these patients with obesity who received bariatric surgery, a decrease in overall cancer diagnoses was observed[102,103]. However, surgical intervention does not guarantee the recovery of obese patients to a normal state and is typically a harmful method. Theoretically, obesity prevention represents the most promising and scientific solution, which requires joint efforts and cooperation from throughout the whole world[104]. However, under obesity pandemic conditions, exploring strategies to lower the incidence of obesity-related cancer represents the primary goal. Unfortunately, no experience has been gained. As low-grade inflammation plays a central role in linking obesity and cancer, anti-inflammatory therapy may be a promising direction, which has been validated in the prevention of colorectal cancer[105]. For obese patients with established malignancies, there is an urgent need to improve therapeutic efficacy and long-term survival. As mentioned above, various mechanisms have been proposed to link obesity and CSCs; thus, whether blocking these connections can prevent or delay the initiation and progression of cancer needs further study. Encouragingly, such strategies have been explored extensively, and some of them have already advanced into clinical use. For example, the clinical development of therapeutics targeting CSC-associated developmental signaling pathways has resulted in improved patient outcomes[82]. Some inflammatory factor-targeting therapies also show promising results in improving outcomes in patients with cancer[106]. Lifestyle interventions, such as reduced dietary intake and increased physical activity, were demonstrated to cause weight loss, leading to altered expression of inflammatory factors and maintenance of stem cell homeostasis[107,108]. Therefore, what awaits us next is to validate their efficacy in obese people with cancer.

CONCLUSION

As stated above, notwithstanding the clear and compelling link between obesity and CSCs, as well as an understanding of the mechanisms connecting them, this research area is still in its infancy. Most scientific research exploring the association of CSCs and obesity originates from mouse models or was inferred indirectly from the conclusions of different research fields. Therefore, there are still many major questions waiting for answers. For example, how can the profound differences in cancer incidence across different anatomical sites influenced by obesity be explained? Do the differences in the microenvironment across adult stem cell niches contribute to these variations? Regarding alterations in stem cell biology, how much overlap is there among animal models and obese patients? What role does the microbiome play in mediating the induction, maintenance and therapy response of CSCs? Does obesity differentially regulate normal and malignant stem cells, and how does it do so? How does obesity influence the crosstalk between CSCs and immunoediting? To what extent are stem cells conditioned in

obesity reversed when obesity is improved? Does obesity enhance the establishment of premetastatic niches? Can strategies aimed at targeting the mechanisms linking obesity and CSCs prevent the initiation and delay the progression of cancer?

Despite a wealth of unknowns, it is clear that obesity increases the stem cell pool and induces biological modulation in these cells, which predisposes these stem cells to transformation. Regarding the increased prevalence of obesity and its convincing association with cancer, programs are urgently needed to decrease the incidence of obesity. At this time, primary obesity prevention through public health policies, including dietary and lifestyle changes, represents a compelling approach toward a reduction in the incidence of obesity and its associated cancer. Identifying obese patients with increased cancer risk and developing appropriate management of obesity or applying cancer prevention methods such as anti-inflammatory agents in these populations represents another compelling approach toward a reduction in the burden of cancer in obesity. Several mechanisms have been proposed to explain the association between obesity and CSCs, and large amounts of agents targeting these pathways have been developed. Testing them in patients with obesity and comparing their efficacy with that in nonobese individuals are important components of future translational research and clinical trials.

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FOOTNOTES

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REVIEW

Clinical application prospects and transformation value of dental follicle stem cells in oral and neurological diseases

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Abstract

Since dental pulp stem cells (DPSCs) were first reported, six types of dental SCs (DSCs) have been isolated and identified. DSCs originating from the craniofacial neural crest exhibit dental-like tissue differentiation potential and neuro-ectodermal features. As a member of DSCs, dental follicle SCs (DFSCs) are the only cell type obtained at the early developing stage of the tooth prior to eruption. Dental follicle tissue has the distinct advantage of large tissue volume compared with other dental tissues, which is a prerequisite for obtaining a sufficient number of cells to meet the needs of clinical applications. Furthermore, DFSCs exhibit a significantly higher cell proliferation rate, higher colony-formation capacity, and more primitive and better anti-inflammatory effects than other DSCs. In this respect, DFSCs have the potential to be of great clinical significance and translational value in oral and neurological diseases, with natural advantages based on their origin. Lastly, cryopreservation preserves the biological properties of DFSCs and enables them to be used as off-shelf products for clinical applications. This review summarizes and comments on the properties, application potential, and clinical transformation value of DFSCs, thereby inspiring novel perspectives in the future treatment of oral and neurological diseases.

Key Words: Dental follicle stem cells; Oral disease; Neurological disease; Tissue engineering; Regeneration; Immunoregulation

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Core Tip: This review is intended to summarize and comment on the properties, application potentials, and clinical transformation value of dental follicle stem cells (DFSCs). Stem cells derived from dental SCs (DSCs) originating from the craniofacial neural crest exhibit dental-like tissue differentiation potentials and neuro-ectodermal features, making them a promising alternative for the treatment of oral and neurological diseases. Moreover, in contrast to other DSCs, DFSCs from the early-developing tissues exhibit a number of superior properties, including larger tissue volume, higher cell proliferation rate, more similar biological profiles to progenitor cells of origin, and better anti-inflammatory effects, *etc.* These advantages are part of the critical mechanism by which DFSCs exert therapeutic effects and are relevant for large scale scaling and industrial generation for clinical applications. Moreover, cryopreservation preserves the biological properties of DFSCs and enables them to be used as off-shelf products for clinical applications. Therefore, DFSCs could have great clinical prospects and translational value in oral and neurological diseases with natural advantages.

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INTRODUCTION

Stem cells are undifferentiated cells characterized by the ability of self-renewal, clonality, and differentiation into various types of cells[1]. With the development of cell biology and modern medicine, the application of stem cells has brought a new approach for restoring tissue defects and treatment of some refractory diseases[2,3]. To date, regenerative medicine has become an important branch of modern medical science and has played an increasingly important role in clinical treatment. Stem cells are a vital element of regenerative medicine, and different stem cell types have their own advantages and drawbacks[4]. With stem cell research deepening, it is crucial to explore the appropriate stem cells to solve clinical problems and obtain better clinical outcomes.

Stem cells for application in regenerative medicine are divided into the following two categories: Pluripotent and multipotent. Pluripotent stem cells include natural embryonic stem cells (ESCs) originating from the inner cell mass of the blastocyte and artificially induced pluripotent SCs (iPSCs)[5]. Multipotent stem cells refer to adult stem cells that exist in different tissues of the body, and their main function is general homeostasis and repair of injured tissues by differentiation[6]. Adult stem cells are also known as postnatal stem cells and mainly originate from either epithelial cells or mesenchymal cells [4].

Mesenchymal SCs (MSCs) are typical adult stem cells derived from mesenchymal tissues. In addition to the ability of self-renewal, high proliferation, and multidirectional differentiation, MSCs also present high immunomodulation and antiapoptotic capacity, to achieve the purpose of promoting tissue regeneration and disease treatment. Compared with ESCs, MSCs are multipotent but limited in terms of differentiation ability. However, the acquisition and clinical application of ESCs are also dramatically restricted to ethical, legal, safety, and source constraints[7]. Moreover, since iPSCs were first generated in 2006 by Takahashi *et al*[8] with four factors, a growing number of researchers have focused on the clinical application prospects and transformation value of iPSCs and their specialized differentiation cells. Nevertheless, iPSCs also present some worrying aspects as follows: (1) iPSCs have pluripotency, similar to ESCs, as well as the ability to cause possible teratomas while the specialized differentiated final product contains undifferentiated cells[9]; (2) there is possibility of tumor formation by integrated oncogenes, insertional mutagenesis, and disrupting tumor suppressor genes[9]; (3) epigenetic memories and genomic aberrations have been detected in reprogrammed cells[10]; and (4) human skin fibroblast-derived iPSCs have a 72% ultraviolet light-related damage, and human blood-derived iPSCs have a high prevalence of acquired BCL6 corepressor mutations (26.9% of lines)[11].

The first human trial using retinal pigment epithelium derived from iPSCs for the treatment of age-related macular degeneration was started in Japan in 2014 but was later suspended[12]. Recently, another study has reported a distinctive case of immature teratoma after the patient underwent autologous iPSC-derived cell therapy for diabetes. Two months after the cells had been injected into the deltoid muscle, a teratoma formed in the injection area[13]. Safety remains the most important criterion of a cellular product for clinical applications[9]; thus, more safety-related quality detections with iPSC-derived cell therapy should be performed. In contrast to ESCs, iPSCs, and iPSCs specialized differentiation cells, several studies have demonstrated that MSCs exhibit good safety profiles, making MSCs

the most widely used cell type for clinical applications at present[14-16]. To date, various MSCs have been discovered in different tissues, including bone marrow, umbilical cord, umbilical cord blood, placenta, amniotic fluid, hair follicle, adipose tissue, and dental tissues[17-26].

Since dental pulp stem cells (DPSCs) were first reported by Gronthos *et al*[27] in 2000, a growing interest has been observed toward the potential of dental stem cells (DSCs) for the treatment of oral and neurological diseases[28,29]. Six types of human DSCs have been isolated and identified at different stages of tooth development[30]. For instance, DPSCs, stem cells from apical papilla (SCAPs)[31], stem cells from human exfoliated deciduous teeth (SHEDs)[32], periodontal ligament stem cells (PDLSCs)[33], and gingival mesenchymal stem cells (GMSCs)[34] can be gained after tooth eruption, while dental follicle stem cells (DFSCs) are a special kind of DSCs, which can be obtained at the early developing stage of the tooth prior to eruption[35] (Figure 1).

The dental follicle is an ectomesenchyme-derived and loose connective tissue originating from the cranial neural crest. During the bud stage of tooth development, the dental follicle is formed and surrounds the dental papilla and enamel organ, which plays a critical role in tooth eruption *via* regulating bone resorption and formation[36]. In the late bell stage, the dental follicle gives rise to the supporting tissues of the tooth-periodontium, including cementum, alveolar bone, and periodontal ligament[37]. Compared with other dental tissues, dental follicle tissue has the obvious advantage of large tissue volume, which is the premise of obtaining a sufficient number of cells to meet the needs of clinical application. Meanwhile, the number of DFSCs obtained in the same passage is far greater than that of DPSCs, accordingly being more suitable for large-scale expansion and industrial generation[38]. Due to the origin of dental follicle tissue in the early stage of tooth development, it has been demonstrated that DFSCs have the following advantages over DPSCs. First, DFSCs exhibit a significantly higher cell proliferation rate and colony-formation capacity than DPSCs[38], which further suggests that DFSCs may be better able to meet the needs of clinical transformation in terms of quantity and quality. Second, DSCs originate from neural crest cells. DFSCs have more similar protein profiles with cranial neural crest cells (CNCCs) than DPSCs, and possess high potency in odontogenic differentiation *in vitro*[39], which demonstrates that DFSCs may have better transformation advantages for the treatment of neurological and oral diseases. Third, DFSCs have better inhibitory effects on the proliferation of proinflammatory lymphocytes and better promote the proliferation of anti-inflammatory Treg cells than SHEDs and DPSCs[40], which indicates that DFSCs have better immunoregulation capacity. Of note, the advantages in terms of quantity, quality, differentiation, and immunoregulatory properties, as mentioned above, are part of the critical mechanism by which MSCs exert therapeutic effects. In this regard, DFSCs appear to be the candidate cells with natural advantages for regenerative medicine compared with stem cells from other dental tissues. Indeed, as a kind of dental tissue-derived stem cells originating from the neural crest, DFSCs may be more advantageous in promoting oral tissue regeneration, including periodontium, dental pulp, and tooth regeneration, as well as in treating neurological injury and neurodegenerative diseases, such as spinal cord and brain injury, as well as Parkinson's disease (PD) and Alzheimer's disease.

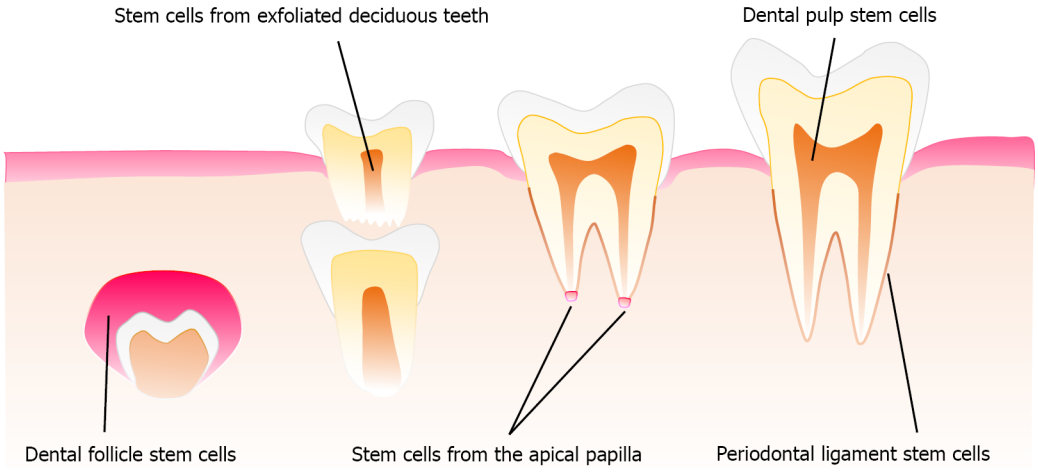
ORAL DISEASES AND FUNCTIONAL UNIT REGENERATION *VIA* DENTAL FOLLICLE STEM CELLS

Periodontitis and periodontal regeneration

The periodontium is a complex functional unit that plays a critical role in the oral cavity[41]. Periodontitis is a common and chronic inflammatory disease caused by plaque in the teeth[42]. As the disease progresses, gingival recession, loss of soft tissue attachment, and even intrabony defects can occur, ultimately resulting in premature tooth loss[43]. To date, while numerous conventional clinical treatments for periodontitis have been shown to control inflammation and aggressive progression, these strategies have not been able to achieve periodontal regeneration. Previous preclinical studies have demonstrated that the transplantation of stem cells presented the potential and provided new hope for periodontal regeneration[44,45].

The combination of DFSCs and hydroxyapatite scaffold forms a cementum-like matrix *in vivo* after transplantation into mice[35]. Scaffold plays a critical role in tissue engineering, providing support for transplanted stem cells and enhancing the therapeutic effects of tissue regeneration. The cell sheet technique prevents extracellular matrix degradation and provides a novel scaffold-free cell delivery strategy[46]. The extracellular matrix contains numerous growth factors and provides support to the cells without the need for additional scaffolds[47]. In addition, cell viability and function can be restored without the digestion operation step. A complex of dental follicle cell sheets forms periodontal tissue-like structures, including cementum-like structures and periodontal ligament with abundant blood vessels after transplantation into the subcutaneous areas of nude mice[48], demonstrating that DFSC sheets have the potential to achieve the goal of periodontal regeneration.

The tooth root development requires stimulation from Hertwig's epithelial root sheath (HERS), and DFSCs differentiate into cementoblasts during the epithelial-mesenchymal interaction process[49]. Accordingly, it has been demonstrated that the formation of cementum and periodontal ligament-like



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Figure 1 Main dental stem cells derived from different dental tissues. This image shows the six types of human dental stem cells isolated and identified at different stages of tooth development. Dental follicle stem cells are obtained at the early developing stage of the tooth prior to eruption.

tissue was enhanced at 5 wk after implantation into rat submentum when DFSCs had been pre-exposed to HERSCs[50], suggesting that establishing a microenvironment similar to tooth root development is important for periodontal regeneration. Moreover, appropriate microinflammation preconditioning is important for improving the regeneration and immunoregulation capacity of DFSCs and their secreted exosomes. Lipopolysaccharides (LPS) upregulated the expression of osteogenic and adhesion-related proteins in DFSC sheets, which showed good performance in canine periodontal regeneration[51]. Furthermore, LPS enhanced the paracrine activity and immunomodulatory effect of DFSCs, and LPS-preconditioned DFSC-derived small extracellular vesicles (sEV) were beneficial for repairing lost alveolar bone in rats[52]. A later study further clarified that LPS-preconditioned DFC-sEV inhibited intracellular reactive oxygen species (ROS) as an antioxidant; it reduced the NF-κB receptor activator ligand/osteoprotegerin ratio of PDLSCs by inhibiting ROS/Jun amino-terminal kinase (JNK) signaling under inflammatory conditions and promoted macrophages to polarize toward the M2 phenotype *via* ROS/extracellular signal-regulated kinase (ERK) signaling[53].

For clinical applications, autologous PDLSCs have been used in clinical studies (ClinicalTrials.gov Identifier: NCT01357785; www.isrctn.com Identifier: ISRCTN13093912), and the results showed no significant differences in the effect on intrabony lesions between PDLSCs groups and scaffold-only groups[54,55]. Although some previous studies have demonstrated that PDLSCs might be the first choice for periodontal regeneration[28,43], DFSCs can give rise to periodontal supporting tissues, including cementum, alveolar bone, and periodontal ligaments. Moreover, previous comparative studies have revealed that DFSCs exhibit a stronger capacity for the regeneration of cementum and periodontal attachment than PDLSCs[48,56]. Thus, DFSCs can be considered a better candidate cell source for periodontal regeneration.

Dental pulp necrosis and pulp regeneration

Dental pulp necrosis is an irreversible inflammatory dental disease that causes destruction and loss of the pulp tissue, resulting in the loss of teeth and even abscesses of the jaw[57]. Bacterial infections play a key role in the development of dental pulp necrosis. Bacterial invasion and colonization were observed in the pulp necrotic areas with caries exposure and symptomatic irreversible pulpitis. Additionally, bacterial penetration of blood vessels occurred, which may spread bacterial infections[58]. Before it leads to more serious consequences, endodontic treatment must be performed to remove the damaged pulp[59,60]. However, the tooth becomes more fragile and susceptible to caries, periapical infection, and fracture after endodontic treatment because of the loss of blood and nutrition supply[59].

Stem cell-based dental pulp regeneration has the objective of developing new methods to replace the conventional treatment of dental pulp necrosis. Scaffolds, stem cells, and growth factors have been used for dental pulp regeneration. Findings from animal studies have shown that DPSCs seeded in a collagen scaffold with dentin matrix protein 1 were able to induce the formation of dental pulp-like tissues in immunodeficient mice[61], and DPSCs pellets stimulated by bone morphogenetic protein 2 promoted the dentin formation onto the amputated pulp of dog teeth[62]. Later, autologous pulp stem cells and granulocyte colony-stimulating factor with a clinical-grade atelocollagen scaffold were transplanted into the dog pulpectomized teeth, promoting pulp/dentin regeneration[63]. A pilot clinical study using a similar strategy has also observed complete pulp regeneration in humans[64]. Moreover, a scaffold-free translation strategy has also been used for pulp regeneration. DPSC aggregates derived from the autologous canine tooth pulp induced the regeneration of three-dimensional pulp tissue equipped with

blood vessels and sensory nerves 12 mo after treatment[65]. Consequently, DPSCs are derived from the pulp tissue and it appears that DPSCs have the potential to promote dentin-pulp regeneration, while DFSCs have a similar capacity.

Previous studies have demonstrated that providing an inductive microenvironment with a suitable scaffold could achieve dentin and even dental pulp regeneration. Treated dentin matrix (TDM) is derived from animal or human dentin matrix treated with ethylenediamine tetraacetic acid, containing abundant collagen, noncollagenous proteins, and growth factors. Both rat and human TDM induce and support complete dentin regeneration, in addition to inducing transplanted DFSCs to differentiate into odontoblasts and express dentin sialoprotein and matrix protein 1[66,67]. The cell sheet technique promotes dental pulp regeneration. DFSC sheets were substituted for DFSCs and, in combination with TDM, were implanted subcutaneously into the dorsum of mice. New dentin pulp-like tissues were observed after eight weeks post-transplantation[68]. Moreover, native dental pulp extracellular matrix (NDPE) can be used to obtain prefabricated-shaped dental pulp. Dentin-pulp complex-like tissues and columnar odontoblasts-like layers arranged along the interface between newly formed predentin matrix could be found after DFSCs-NDPE-TDM transplantation to the jaw of miniature swine for 12 wk[69]. Therefore, DFSCs could also exhibit the capacity to regenerate the dentin-pulp tissues with suitable inductive scaffolds.

Tooth root and whole-tooth regeneration

The regeneration of whole teeth is a major objective and promise of oral regenerative medicine. To date, two main strategies have been used to achieve this goal. The first strategy is a combination of mesenchymal and epithelial cells to construct a bioengineered tooth germ, which is then transplanted into the alveolar socket. Parts of animals showed whole-tooth eruption around 3.5 mo after tooth-germ implantation[70]. However, several barriers should be addressed in future studies of bioengineered teeth, such as indiscriminate shape and smaller size than natural teeth[4,70-73]. The second strategy includes the direct reconstruction of the functional units, such as bio-tooth root with periodontal tissue-like structures and dentin-pulp-like tissues.

As with the combination of implants and crowns to replace missing teeth, researchers have explored implant-like scaffolds to reconstruct the root. As mentioned above, TDM has the potential to be a suitable scaffold for root reconstruction. Yang *et al*[68] used calcified human dentin to model an alveolar microenvironment and used DFSC sheets-TDM-DFSC sheets to reconstruct the pulp-root-periodontium structure. TDM could induce and support DFSC sheets to develop new dentin-pulp and cementum-periodontium-like tissues after subcutaneous transplantation into nude mice for 8 wk[68]. Moreover, with seed DFSCs, the complex of aligned PLGA/Gelatin electrospun sheet/TDM/NDPE generated tooth root-like tissues after 12 wk of transplantation in porcine jaws[69]. Shape-optimized TDM scaffolds with DFSCs were transplanted into the alveolar bone of swine, and ceramic crowns were installed. These bio-tooth roots not only regenerated histologically but also allowed masticatory functions and remained stable for 3 mo[74]. In nonhuman primates, a novel functional biological root complex was constructed based on DFSC sheets and *in vitro* three dimensional (3D) suspension culture. This complex was then transplanted into rhesus monkeys and gradually restored occlusal function and long-term masticatory function for 2 years during the evaluation period[75]. In this regard, DFSCs with suitable scaffolds have the potential to be suitable stem cells for whole-tooth reconstruction based on bio-booth root regeneration.

THERAPEUTIC POTENTIALS OF DENTAL FOLLICLE STEM CELLS IN NEUROLOGICAL DISEASES

Neural differentiation of dental follicle stem cells

As mentioned above, dental follicle is an ectomesenchyme tissue that originates from the cranial neural crest. DFSCs not only express the markers typical of MSCs [*e.g.*, cluster of differentiation (CD) 44, CD90, CD105] but also express neural cell markers (*e.g.*, Nestin, β-III tubulin, and CNpase)[76-78] and even embryonic stem cell markers (octamer-binding transcription factor 4 and sex-determining region Y-box 2)[79], indicating that DFSCs may retain some of the neural and embryonic features and can differentiate into neural-like cells. DFSCs display neural-like cell morphology with small neurite-like cell extrusions with a neuronal differentiation strategy[76]. Dental pulp comprises blood vessels, neural fibers, and connective tissue, and DPSCs exhibit extraordinary capacity to differentiate into neural-like cells and represent a potential source for neuronal regeneration therapies[80]. However, a comparative study has demonstrated that DFSCs possess more similar protein profiles to CNCCs than DPSCs[39]. Moreover, compared with DPSCs, the expression of CNpase, neurofilament protein, Nestin, and β-III tubulin of DFSCs was upregulated significantly after treatment in the same neural-induction condition [78]. From this perspective, DFSCs may be a better candidate cell type for neural differentiation and even for the treatment of neurological diseases based on pre-differentiation.

Therapeutic potentials of dental follicle stem cells in neurotrauma and neurodegenerative disorders

Spinal cord injury (SCI) is a severe neurological trauma that causes the impairment of sensory and motor functions[81]. The acute stage of injury is directly caused by trauma, including compression, contusion, and shear injury forces. Proinflammatory cells are then activated, releasing abundant inflammatory cytokines, which induce a cascade of secondary injury[82]. Many neurons, astrocytes, and other neural cells die in the injured area due to necrosis or apoptosis during the secondary injury[83]. Until now, current clinical strategies have not achieved satisfactory outcomes due to irreversible damage to neural cells. Stem cell-based therapies hold the promise of developing new approaches for the treatment of SCI. Several types of stem cells have been used for transplantation, such as neural SCs (NSCs), ES/iPS-derived NSCs, and MSCs[84]. However, the sources of NSCs are limited and also face ethical issues. ES/iPS-derived cells exhibit some of the aforementioned worrying aspects. Thus, MSCs may now be a better candidate cell type for SCI. Researchers have compared the therapeutic effects of MSCs from bone marrow and dental pulp. Transplantation of DPSCs promoted marked recovery of locomotor function in the hind limbs, while transplantation of bone marrow SCs (BMSCs) resulted in substantially less recovery of locomotor function in rats with complete SCI. The main mechanisms include inhibition of apoptosis in neurons, astrocytes, and oligodendrocytes; promotion of regeneration of disjunct axons; and differentiation into mature oligodendrocytes to replace lost cells[85]. Later, another comparative study explored the differences among DFSCs, SCAPs, and DPSCs for the treatment of SCI. Findings from an animal study demonstrated that all three types of DSCs, especially DFSCs, have the potential to promote functional recovery after SCI by reducing the inflammatory response, promoting neurite regeneration, reducing progressive hemorrhagic necrosis, and differentiation into mature neurons and oligodendrocytes but not astrocytes[77]. Moreover, scaffolds with DFSCs were also used to repair the spinal cord defect; for example, aligned poly-ε-caprolactone/poly-lactide-co-glycolic acid electrospun material allowed nerve fibers to pass through, and induced DFSCs to differentiate *in vivo*[86].

PD is a common and progressive neurodegenerative disorder characterized by tremors, rigidity, and bradykinesia[87,88]. The aggregates of ubiquitin and α-synuclein-positive protein, Lewy bodies, and the loss of dopaminergic neurons in the substantia nigra pars compacta are the main characteristics that define PD[89]. The incidence of the disease rises steeply with age, affecting approximately 1% of the population between the ages of 70 years and 79 years[90]. Although pharmacological approaches (such as amantadine and levodopa) and nonpharmacologic strategies (deep brain stimulation, exercise, and physical therapy) have been used in PD treatment[91,92], these therapeutic strategies only delay the progression of the disease and relieve the symptoms but do not achieve regeneration of dopaminergic neurons. There has been considerable excitement about the use of MSCs to treat neurodegenerative diseases *via* secretion of anti-inflammatory factors [*e.g.*, indoleamine (2,3)-dioxygenase (IDO), prostaglandin E2 (PGE2)], growth factors [*e.g.*, vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF)], and exosomes to achieve neuroinflammation attenuation and neural regeneration[93,94]. Various cell types, including BMSCs, adipose derived stem cells, umbilical cord mesenchymal stem cells (UCMSCs), and DSCs, and their neural-primed cells or accessory products (such as exosomes) have been used to treat PD[95-99]. The locomotive defect was recovered in PD rats after neural-primed SHEDs were transplanted and differentiated into neurons and dopaminergic neurons *in vivo*. The transplanted cells resided in the brains of rats and formed functional connections [98]. DFSCs have also been transplanted for the treatment of PD and survived in the transplanted regions of PD mice for more than 6 wk after surgery. DFSCs not only increased the number of dopaminergic neurons around them but also differentiated into tyrosine hydroxylase-positive cells, indicating that DFSCs may be a potential source in the exploration of possible therapeutic roles in PD [99]. In addition, other DSCs have been used to treat stroke, Alzheimer's disease, and a variety of peripheral nervous system diseases[100,101]. Based on the biological properties of DFSCs, they may also present promising therapeutic effects in the treatment of the aforementioned neurological diseases.

IMMUNOREGULATION OF DENTAL FOLLICLE STEM CELLS

Both in oral and neurological diseases, the immunomodulatory capacity is one of the most crucial functions of MSCs to facilitate the repair or regeneration of damaged tissues[51,77]. MSCs can regulate the proliferation, activation, maturation, and function of innate and adaptive immune cells *via* cell-to-cell direct contact, soluble cytokines, and exosomes[102]. As a kind of MSCs, DFSCs also present immunomodulatory characteristics. In acute lung injury models, DFSCs could suppress the production of proinflammatory cytokines, such as monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α; decrease the proportion of proinflammatory macrophage M1 phenotype; increase the level of anti-inflammatory cytokine IL-10 and the proportion of anti-inflammatory M2 phenotype both in bronchoalveolar lavage fluid *in vivo* and *in vitro* experiments[103]. Furthermore, DFSCs also exhibit great therapeutic potential in autoimmune diseases and chronic inflammatory disorders. DFSCs suppress the proliferation of T lymphocytes and lymphocyte apoptosis but increase the number of Tregs. DFSCs also reduce the secretion level of TNF-α but upregulate the level of IL-10 in peripheral blood mononuclear cells (PBMCs) of patients with rheumatoid arthritis[104].

Moreover, in inflamed mononuclear cell samples of patients with Crohn's disease, DFSCs also downregulate lymphocyte proliferation, CD4 + IL22BP T cell ratio, and the secretion of TNF-α and IL-6, but increase the frequency of Tregs and the level of IL-10[105]. From the case studies mentioned above, we can briefly summarize that DFSCs exert the common immune modulatory capacity *via* suppressing the proinflammatory immune cells [*e.g.*, T-helper (Th) 1, Th17, macrophage M1] and cytokines (*e.g.*, TNF-α, IL-1, IL-6) and increasing the number of anti-inflammatory immune cells (*e.g.*, Tregs, macrophage M2) and cytokines (*e.g.*, IL-4 and IL-10). Therefore, it is necessary to design experiments to examine the ratio or level changes in inflammatory cells and cytokines after co-culture of DFSCs with activated PBMCs, which can help us to screen the cells with superior immune properties and obtain better clinical outcomes.

CRYOPRESERVATION OF DENTAL FOLLICLE STEM CELLS

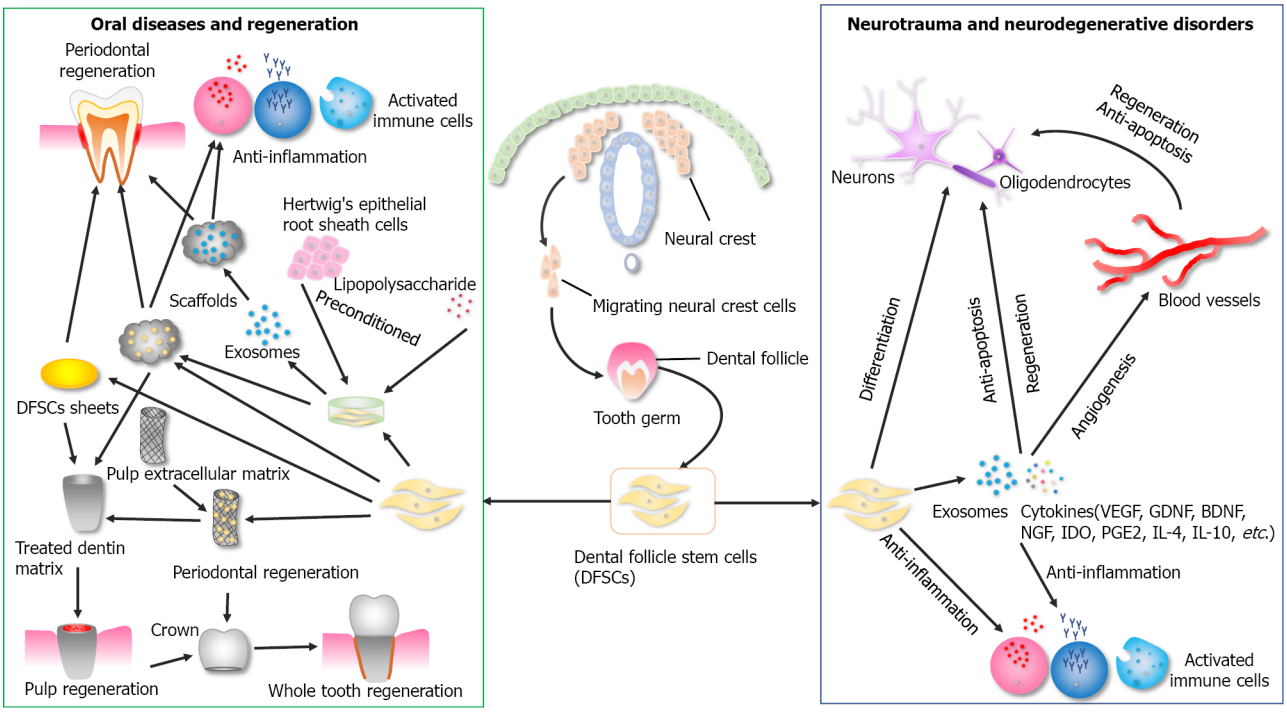
Stem cell-based therapies have been investigated for tissue engineering and treatment of many diseases for several decades, and cryopreservation can be used to effectively preserve stem cells. Cryopreservation is the process of gradually cooling cells or tissues to sub-zero temperatures, and finally preserving them in the gas phase liquid nitrogen (-150°C to -196°C) for an extended period. In this state, the biological activity of the cells is stopped, and their viability can be restored by careful thawing when needed[106]. Human DFSCs isolated from fresh and cryopreserved dental follicles show similar biological characteristics, such as proliferation ability, surface markers, and tri-linage differentiation capacities[107]. Moreover, the two types of DFSCs possess the same osteogenic differentiation potential and immunomodulatory properties for bone tissue engineering, resulting in the inhibition of adaptive immune response, which demonstrates that the stemness and immunomodulatory capacity of long-term-preserved dental follicle tissues can be restored[108]. Another study compared the biological characteristics between cryopreserved DFSCs and the cells from cryopreserved dental follicles. After 3 mo of cryopreservation, the cells from the cryopreserved dental follicles showed similar levels of stemness and apoptosis-related genes and exhibited similar osteogenic and adipogenic differentiation capabilities to cryopreserved DFSCs[109]. In this regard, both cryopreservation of DFSCs from fresh dental follicle tissues and direct cryopreservation of dental follicle tissues can preserve the biological properties of the cells. As a consequence, cryopreservation technology enables DFSCs to the off-the-shelf products for clinical applications.

Cryopreservation addresses the preservation of DFSCs. Another problem is that it is questionable whether allogeneic stem cells exhibit the same therapeutic effects as autologous stem cells. A previous study compared the therapeutic effects of allogeneic and autologous PDLSCs on periodontal tissue regeneration in a miniature pig model of periodontitis. Significant periodontal tissue regeneration was achieved in both transplanted groups without significant difference due to low immunogenicity and marked immunosuppression of T-cell antigen *via* PGE2[110]. Furthermore, allogeneic DSCs likely did not affect the therapeutic effects because of their inherent characteristics. However, more experiments should be performed to compare the outcomes of allogeneic and autologous DFSCs in different diseases.

In addition to cell cryopreservation, some biological materials can be cryopreserved. For instance, after being cryopreserved in liquid nitrogen with cryoprotectant for several months, the cryopreserved TDM exhibited superior mechanical properties, more dentin-related proteins, and a larger pore diameter than the fresh TDM. The cryopreserved TDM was also able to induce dental follicle cells to regenerate new dentin-pulp-like tissues[111], suggesting that the cryopreservation techniques address the preservation of biological materials and also enable them to be used as off-the-shelf scaffold for tissue engineering.

CONCLUSION

In this review, it was found that among MSCs, stem cells derived from dental tissue originating from the craniofacial neural crest exhibit dental-like tissue differentiation potential and neuro-ectodermal features, which makes them a promising alternative for the treatment of oral and neurological diseases (Figure 2). Moreover, in contrast to other DSCs, those from the early-developing tissues exhibit several superior properties, including larger tissue volume, higher cell proliferation rate and colony-formation capacity, more similar biological profiles to progenitor cells of origin, and better anti-inflammatory effects. These advantages are part of the critical mechanism by which MSCs exert therapeutic effects and are relevant for large-scale scaling and industrial generation for clinical applications. Cryopreservation preserves the biological properties of DFSCs and enables them to be used as off-shelf products for clinical applications. Therefore, DFSCs could have great clinical prospects and translational value in oral and neurological diseases with natural advantages.



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Figure 2 The origin of dental follicle stem cells and the main strategies/mechanisms of dental follicle stem cell-based therapies in oral and neurological diseases. Dental follicle stem cells (DFSCs) originating from the craniofacial neural crest exhibit dental-like tissue differentiation potential and neuro-ectodermal features. For the regeneration of periodontium and pulp, the main strategy is the combination of DFSCs or their accessory products (exosomes) with various scaffolds (such as treated dentin matrix, native dental pulp extracellular matrix, and hydroxyapatite). The cell sheet technique prevents the degradation of the extracellular matrix and makes it a natural scaffold. In addition, appropriate stimulation may enhance the therapeutic effect, such as preconditioning with Hertwig's epithelial root sheath cells or lipopolysaccharides. For the treatment of neurotrauma and neurodegenerative disorders, DFSCs enable tissue regeneration primarily by reducing inflammatory response and apoptosis, promoting angiogenesis, and differentiating into mature functional neurons and oligodendrocytes.

Currently, SHEDs, DPSCs, PDLSCs, and GMSCs have been used in clinics[54,65,112,113], and clinical trials of DFSCs are also forthcoming. For future clinical applications of DFSCs, several key points need further investigation. First, as one of the three essential elements in tissue engineering, the selection of stem cells is vital. Therefore, potency assessment and screening criteria should be established, including donor screening and culture system optimization. In addition to sterility, safety, activity, homogeneity, purity, and stability, the levels of released cytokines or markers associated with immunomodulation (*e.g.*, IL-4 and IL-10), dental tissue (*e.g.*, VEGF, dentin sialophosphoprotein), or neural regeneration (*e.g.*, Nestin, GDNF, and brain-derived neurotrophic factor, nerve growth factor) should be detected[17]. Furthermore, the potency evaluation system should contain the inhibition of proinflammatory immune cells (*e.g.*, Th1, Th17, and macrophage M1) and cytokines (*e.g.*, TNF-α, interferon-γ, IL-1, and IL-6), the promotion of anti-inflammatory immune cells (*e.g.*, Tregs, macrophage M2) and cytokines (*e.g.*, IL-4 and IL-10) after PBMCs co-culture with DFSCs *in vitro*, and the promotion of neuron and oligodendrocyte or multi-differentiation capacity that includes neural/osteogenic differentiation[79,103,105,114-120]. Then, *in vivo* experiments should be used to verify the correctness of the potency assessment system. Second, in the field of tissue engineering, appropriate scaffold materials have a synergetic effect on the promotion of regeneration with stem cells. Some of these materials include beta-tricalcium phosphate, collagen sponge, xenogeneic bone substitute, and TDM, some of which have been used in the clinic[54,55,74,113,121,122]. Without a doubt, the quest for more suitable scaffold materials remains a long-term process. Third, a wide range of bioactive factors and RNAs, including proteins, cytokines, proinflammatory components, extracts from biological materials, long non-coding RNAs, and microRNAs, have been used to enhance differentiation and immunomodulation capacities[53,123-127], and these strategies may further improve the therapeutic potential of DFSCs in clinical applications in the future. Collectively, with the development of materials and preconditioning strategies, and in combination with the natural superiority exhibited by DFSCs in terms of medicinal properties, DFSC-based therapeutics are a promising strategy for the future treatment of oral and neurological diseases.

FOOTNOTES

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REVIEW

Current status and prospects of basic research and clinical application of mesenchymal stem cells in acute respiratory distress syndrome

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Abstract

Acute respiratory distress syndrome (ARDS) is a common and clinically devastating disease that causes respiratory failure. Morbidity and mortality of patients in intensive care units are stubbornly high, and various complications severely affect the quality of life of survivors. The pathophysiology of ARDS includes increased alveolar–capillary membrane permeability, an influx of protein-rich pulmonary edema fluid, and surfactant dysfunction leading to severe hypoxemia. At present, the main treatment for ARDS is mechanical treatment combined with diuretics to reduce pulmonary edema, which primarily improves symptoms, but the prognosis of patients with ARDS is still very poor. Mesenchymal stem cells (MSCs) are stromal cells that possess the capacity to self-renew and also exhibit multilineage differentiation. MSCs can be isolated from a variety of tissues, such as the umbilical cord, endometrial polyps, menstrual blood, bone marrow, and adipose tissues. Studies have confirmed the critical healing and immunomodulatory properties of MSCs in the treatment of a variety of diseases. Recently, the potential of stem cells in treating ARDS has been explored *via* basic research and clinical trials. The efficacy of MSCs has been shown in a variety of *in vivo* models of ARDS, reducing bacterial pneumonia and ischemia-reperfusion

injury while promoting the repair of ventilator-induced lung injury. This article reviews the current basic research findings and clinical applications of MSCs in the treatment of ARDS in order to emphasize the clinical prospects of MSCs.

Key Words: Acute respiratory distress syndrome; Mesenchymal stem cells; Pulmonary edema; Inflammatory response; Tissue repair; Pulmonary fibrosis

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Core Tip: Acute respiratory disease syndrome (ARDS) is a common disease with high morbidity and mortality. ARDS is characterized by increased alveolar-capillary membrane permeability, influx of protein-rich pulmonary edema fluid, and surfactant dysfunction, resulting in severe hypoxemia. Mesenchymal stem cells (MSCs) have the self-renewal and multilineage differentiation properties, and their immunomodulatory abilities have been implicated in the treatment of disease. Herein, we discuss the pathophysiology of ARDS and recent research surrounding the clinical application of MSCs in the treatment of ARDS.

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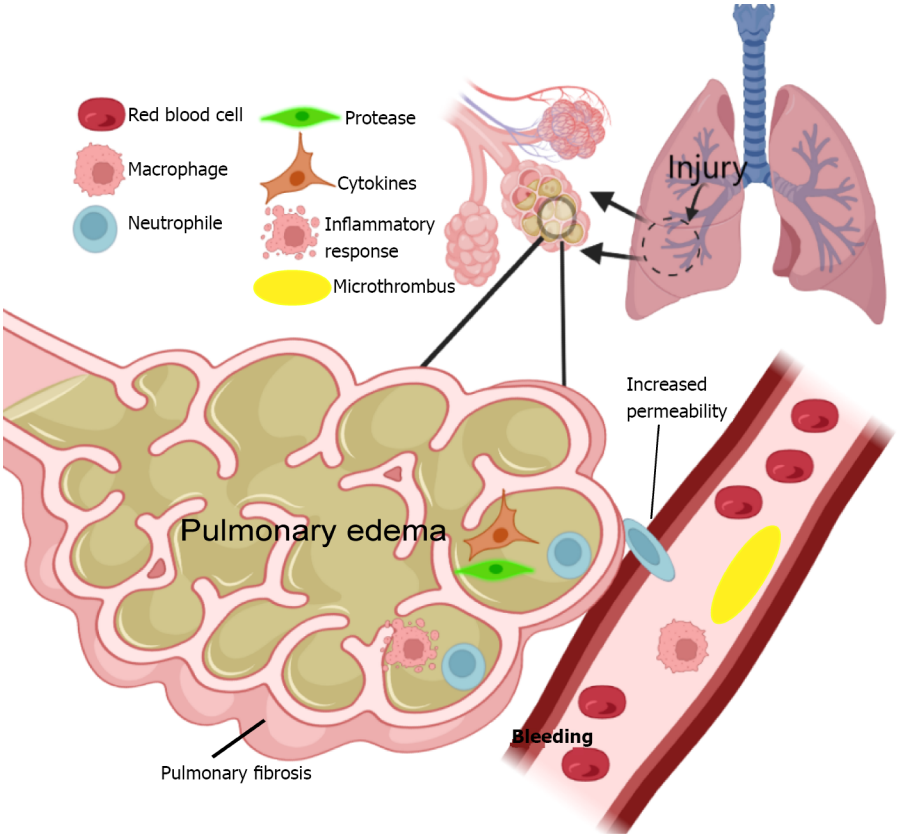
INTRODUCTION

Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is a clinicopathological condition characterized by increased lung fluid, decreased lung compliance, and severe hypoxemia[1,2]. ARDS was defined in 1994 by the American-European Consensus Conference[3]. After several decades of research and discussions, the current internationally recognized definition of ARDS is the Berlin definition which proposes three categories of ARDS based on the severity of hypoxemia: Mild [200 mmHg < arterial oxygen pressure (PaO_2)/fraction of inspired oxygen (FiO_2) < 300 mmHg], moderate (100 mmHg < PaO_2/FiO_2 < 200 mmHg), and severe (PaO_2/FiO_2 < 100 mmHg), along with explicit criteria related to the timing of the syndrome's onset, the origin of edema, and chest radiograph findings[4-6]. The pathogenesis of ARDS is characterized by an unregulated inflammatory cascade with increased pulmonary endothelial and epithelial permeability[7]. Endogenous chemicals and microbial products linked to cell injury are hypothesized to attach to receptors on epithelial cells and alveolar macrophages, triggering an immunological response. The unrestricted synthesis of reactive oxygen species, leukocyte proteases, chemokines, and inflammatory substances that results to gradual lung damage. The immune-mediated reaction is known as a "cytokine storm"[8,9]. The pathophysiological changes that occur during the development of ARDS are shown in Figure 1. Currently, the clinical treatment of ARDS is rather limited and is mainly based on organ function support, such as lung protective ventilation, liver and kidney function protection, gastrointestinal function protection, venous thrombosis prevention, and nutritional support [10]. Despite the profound understanding of the molecular mechanism of ARDS, improvement of pulmonary ventilation strategies, and strengthening of supportive care for critically ill patients, the prognosis of patients with ARDS is still unsatisfactory. Currently, the global mortality rate of ARDS exceeds 40%, whilst 6%–10% of patients with respiratory failure may develop ARDS in the emergency room[11]. The long-term sequelae of ARDS include long-term cognitive impairment, psychological disease, neuromuscular weakness, pulmonary dysfunction, and decline in quality of life because of long-term medical expenses[12]. Therefore, new and safer therapies are urgently needed for ARDS treatment.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) were first described by Friedenstein *et al*[13], as an adherent, fibroblast-like cell population in the bone marrow (BM) that could regenerate rudiments of bone *in vivo*[13,14]. After decades of research, it has been found that MSCs are present in a variety of tissues and organs and can also differentiate into a variety of cells to play related roles (Figure 2)[15,16]. However, the understanding of MSCs is still inadequate. MSCs were officially defined by the International Society of Cell Therapy in 2006 as follows: (1) MSCs must display plastic-adherent capacities; (2) A simultaneous



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Figure 1 Following acute lung injury, pulmonary vascular macrophages produce chemokines to increase vascular permeability, resulting in neutrophil aggregation. Neutrophils reach the pulmonary interstitium and alveolar space and release proteases, cytokines, and other harmful substances to destroy the alveolar microstructure, leading to cell dysfunction and eventually causing exudates and lung fluid to enter the alveolar space, resulting in the development of pulmonary fibrosis. Additionally, the disorder of pulmonary vascular coagulation and fibrinolysis caused by inflammation leads to pulmonary vascular microthrombosis, which is also one of the causes of pulmonary edema.

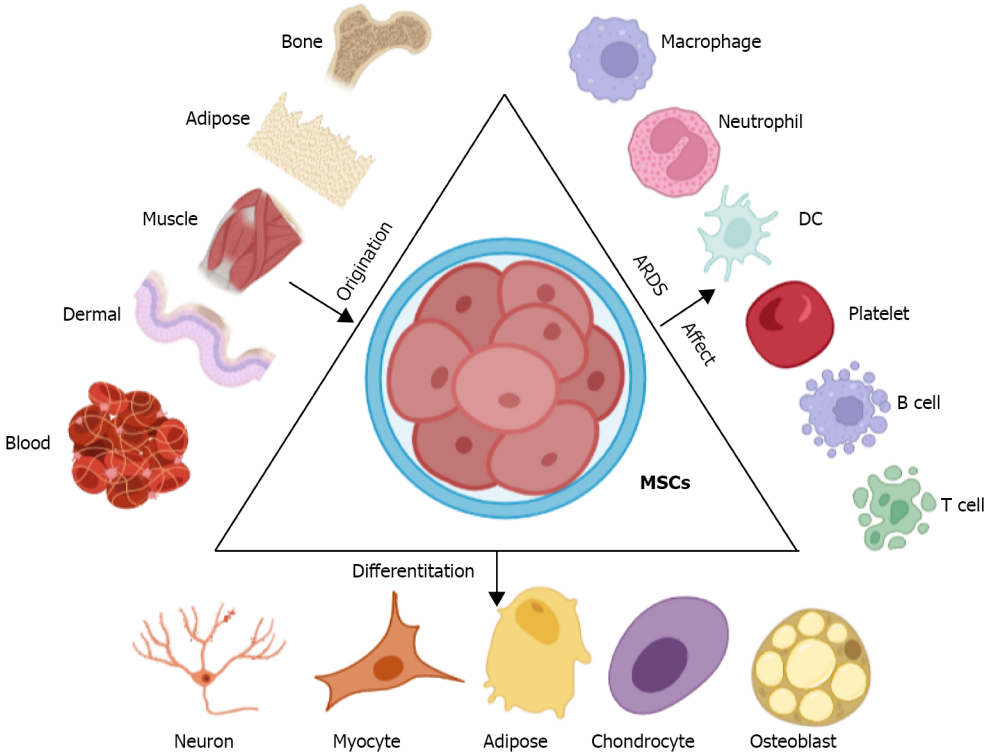
expression of stromal markers, an absence of hematopoietic or endothelial markers and human leukocyte antigen-DR surface molecules; and (3) An *in vitro* differentiation potential for osteoblasts, adipocytes, and chondroblasts[17,18]. The method of obtaining and culturing MSCs is simpler than other stem cells, and MSCs have broad application prospects in a variety of inflammatory-related diseases because of their unique immunomodulatory properties[19].

MSCs are considered a new approach for the treatment of ADRS[20]. The mechanism of MSCs in the treatment of ARDS is multifaceted, and the immunomodulatory effect of MSCs is a crucial aspect of it (Figure 3). At present, the collective view of the immune regulation ability of MSCs is based on the secretion of cytokines, such as tumor growth factor (TGF)-β and tumor necrosis factor-stimulated gene-6 [21]. By releasing a variety of cytokines and extracellular vesicles (EVs), MSCs play anti-inflammatory and anti-cell death roles and promote the generation of microcirculation, thereby promoting the clearance of bacteria and alveolar fluid, alleviating organ damage, thus alleviating ARDS-related symptoms[22-24].

CURRENT BASIC RESEARCH FOR THE USE OF MSCS IN ARDS TREATMENT

Immunoregulation ability of MSCs in ARDS

The hallmark of ARDS is a series of inflammatory responses. Uncontrollable inflammatory responses are known to cause catastrophic damage to various organs[25]. MSCs are pluripotent stem cells with immune properties that can secrete a variety of cytokines, such as anti-inflammatory factors, antiapoptotic factors, and antimicrobial peptides[26-28]. MSCs regulate immune activity *via* three different mechanisms: (1) Direct contact with tissue cells; (2) Production of a series of cytokines to regulate cell activities; and (3) Exerting immune effects by regulating the activity of T cells[29-31]. Currently, the MSC treatment of ARDS is mediated by controlling inflammatory responses. Therefore, the related mechanisms have become a hot research topic, and new research findings are constantly emerging, which introduce new views.



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Figure 2 Mesenchymal stem cells can be derived from a variety of cell types and can differentiate into different types of cells to play specific roles. In the pathological process of acute respiratory distress syndrome, mesenchymal stem cells can act on a variety of cells to play a protective role. MSC: Mesenchymal stem cell; DC: Dendritic cell.

MSCs regulate the immune activity of the dendritic cells: Conventional dendritic cells (cDCs) are unique antigen-presenting cells that bridge antigen immunity and innate immunity and can be activated by MSCs as regulatory DCs[32,33]. An existing study has shown that after lung injury induced by lipopolysaccharide, a large number of DCs accumulate in the lungs, which in turn aggravates lung inflammation and lung injury[34]. The underlying mechanism may involve the polarization of the T-helper cell (Th) 1 response and regulating neutrophil infiltration[35,36]. The aggregation of cDCs can lead to the activation of the Th1 pathway and aggravate the inflammatory response. At the same time, cDCs can also recruit neutrophils, prolong the life of neutrophils, upregulate innate immunity, and further intensify the inflammatory response[37,38]. MSCs also abolish the capacity of mDCs to migrate to chemokine (C–C motif) ligand 19, for DCs to display major histocompatibility complex class II peptide complexes recognized by specific antibodies, and for ovalbumin-pulsed DCs to support antigen-specific CD4+ T-cell proliferation[39].

Additionally, many studies have shown that MSC–EVs play key roles in the pathogenesis and progression of acute lung injury (ALI)/ARDS[40]. One of the underlying mechanisms may involve the potential impairment of antigen uptake, which may halt DC maturation[41]. MSC–EVs from the human BM may regulate the levels of maturation and activation markers (CD83, CD38, and CD80) and inflammatory cytokines [interleukin (IL)-6, IL-12p70, and TGF-β] *in vitro via* regulating the *CCR7* gene by carrying miR-21-5p[42]. Additionally, the emerging role of MSC–EVs in facilitating pulmonary epithelium repair, rescuing mitochondrial dysfunction, and restoring pulmonary vascular leakage has been shown[43,44]. Therefore, regulating the maturation of DC cells in the early stage of lung injury can effectively alleviate secondary lung injury[45] (Table 1).

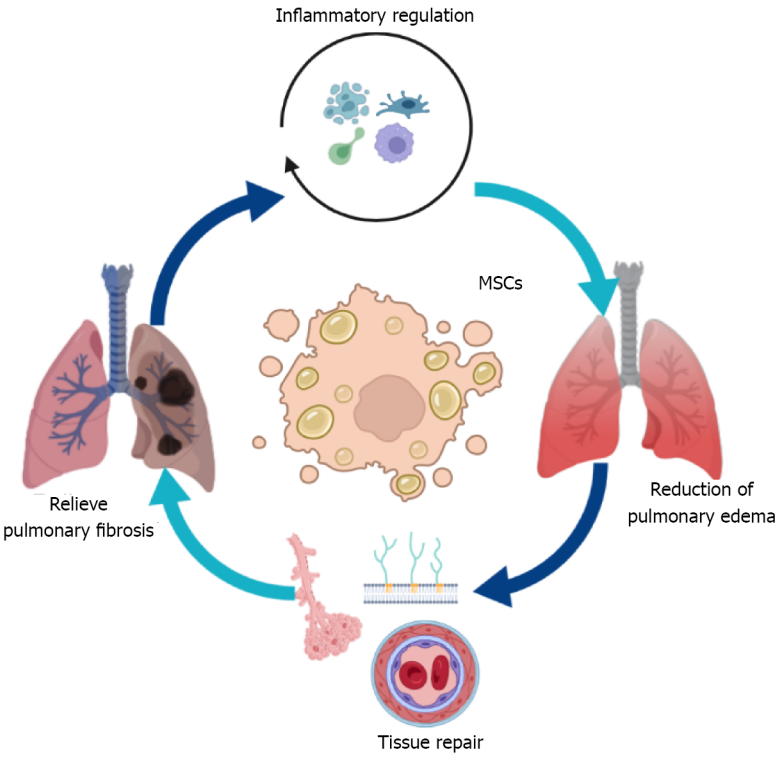
MSCs induce the activation of macrophages: Alveolar macrophages are guardians of the alveoli and airways, and interstitial macrophages are guardians of blood vessels and the lung interstitium[46]. After lung injury, tissue monocyte-derived macrophages accumulate and have increased viability in the lungs, and persist at the lesion for a long time after lung injury[47]. Macrophages respond in a variety of ways, including modulation of function (activation), the release of inflammatory chemical mediators that control immune cell recruitment, and the modulation of epithelial responses[48]. Macrophages can be divided into two phenotypes based on their functions, the M1 type in resting states and the M2 type in the activated state[49]. M1-type macrophages can secrete a variety of cytokines and participate in many processes including pro-inflammatory, pro-apoptosis, free radical formation, and matrix degradation pathways, while M2-type macrophages play a role in anti-inflammatory and anti-cell death

Table 1 Recent studies on the mechanism of mesenchymal stem cell action in acute respiratory distress syndrome

Ref.	Time	Animal/cell line	Interference	Pathway	Conclusion/main effect
Zhang *et al* [112]	2022	C-mice	Human dermal fibroblasts or MSCs were intravenously	CAP	MSC treatment significantly protects mice against bacterial pneumonia or LPS-induced lung injury *via* the CAP pathway. When the CAP was inhibited through vagotomy (VGX) and pharmacological and genetic ablation experiments, the anti-inflammatory effects of MSCs were markedly reduced in lung injury models
Kakabadze *et al* [113]	2022	Wistar rats	HPMSCs	-	HPMSCs have the ability to migrate and attach to damaged lung tissue, contributing to the resolution of pathology, restoration of function, and tissue repair in the alveolar space
Wang *et al*[114]	2022	C-mice	Human placental MSCs	Macrophage polarization pathway	Human PMSC treatment preferentially rescued resident M2 AMΦs over recruited M1 BMMΦs with overall M2 polarization to improve KP-related ARDS survival
Wang *et al*[115]	2022	SD rats	LRMSC/HMSC-C/HMSC-BM	-	Three kinds of LRMSC, HMSC-C and HMSC-BM are protective against LPS-induced lung injury, HMSC-C was more effective than LRMSC and HMSC-BM to treat LPS-induced lung injury
Zhang *et al* [116]	2022	C-mice	MSC derived microvesicles	KEGG pathway and GO function	MSV microvesicles treatment was involved in alleviated lung injury and promoting lung tissue repair by dysregulated miRNAs
Xu *et al*[60]	2022	BALB/c mice	Umbilical cord-derived MSCs	-	Transplantation of UC-MSCs transfected with SP-B could potentiate M2 macrophage polarization and further relieve LPS-stimulated lung injury
Xue *et al*[117]	2022	C-mice	Bone marrow-derived MSC	-	TGF-β1 from MSCs restored skewed Treg/Th17 levels induced by hypoxic- and LPS-stimulated conditions and reduced inflammation
He *et al*[118]	2022	Hnsclc cell line A549 (ATCC, CCL-185)	MSCs	CXCL12/CXCR4 signal axis	*In vivo* transplantation of MSCs significantly attenuated lung injury in ARDS, inhibited serum pro-inflammatory factors in mice, and down-regulated expression of apoptotic and focal factors in lung tissues
Zhang *et al* [119]	2022	C-mice	Mouse bone marrow-derived MSCs	Wnt/β-catenin transition signaling	MVs released from MSCs exerted protective effects on early fibrosis by suppressing EMT in LPS-induced ARDS
Meng *et al*[120]	2021	-	MSCs derived from normal mouse bone marrow	Akt/Mtor signaling	MTORC2 like mTORC1 as an important signaling of regulation of MSC-secreted HGF protective against LPS-induced lung endothelial dysfunction
Ishii *et al*[121]	2021	Adult male Fischer 344 rats	Adipose-derived MSCs	-	AD-MSCs enhanced the barrier function between lung epithelial cells, suggesting that both direct adhesion and indirect paracrine effects strengthened the barrier function of lung alveolar epithelium *in vitro*
Wang *et al*[122]	2021	C-mice	Bone MSCs	Vimentin-Rab7a pathway	MSCs can reach the damaged lung tissue through migration, reduce inflammatory responses and alleviate lung injury
Liu *et al*[123]	2021	SD rats	Bone marrow mesenchymal stem cell	Beclin-1	BMSC-derived exosomes were taken up by the alveolar macrophages and attenuated LPS-induced alveolar macrophage viability loss and apoptosis. Exosomes effectively improved the survival rate of ALI rats, which was associated with alleviating lung pathological changes pulmonary vascular permeability and attenuating inflammatory response

C-mice: C57BL/6 mice; CAP: Cholinergic anti-inflammatory pathway; HPMSCs: Human placental mesenchymal stem cells; PMSCs: Placental MSCs; AMΦs: Alveolar macrophage; BMMΦ: Bone marrow–recruited macrophage; Hnsclc: Human non-small cell lung cancer; KP: Klebsiella pneumonia; LRMSC: Lung resident MSC; HMSC-C: Human chorion-derived MSC; HMSC-BM: Human bone marrow derived MSC; KEGG: Kyoto encyclopedia of genes and genomes; GO: Gene ontology; SP-B: Surfactant protein B; AD-MSC: Adipose tissue-derived MSC; EMT: Epithelial–mesenchymal transition; SD: Sprague-Dawley; mTOR: Mammalian TOR; ARDS: Acute respiratory distress syndrome; ALI: Acute lung injury; TGF-β1: Tumor growth factor-β1.

processes during the inflammatory response, and promote angiogenesis and tissue repair[50-52]. The transformation of the M1/M2 phenotype helps subside the inflammatory response and alleviate tissue



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Figure 3 The main mechanism of mesenchymal stem cell therapy for acute respiratory distress syndrome. Mesenchymal stem cell can treat acute respiratory distress syndrome by regulating inflammatory response, reducing pulmonary edema, alleviating pulmonary fibrosis, and promoting tissue repair. MSC: Mesenchymal stem cell.

damage. In ARDS, this balance can effectively remove harmful substances and pro-inflammatory factors from the body and promote lung tissue repair. Conversely, the destruction of this balance aggravates the pathological development of ARDS. Studies have shown that MSCs can control the pathological development of ARDS by regulating the polarization of macrophages and effectively promoting the repair process in ARDS[53]. Basic studies have shown that MSC treatment reduces the expression of CD86 on macrophages in the ALI models, indicating that MSCs can inhibit the transformation of macrophages to the M1 phenotype[54]. Several mechanisms of MSCs have been described, such as: (1) MSCS can promote the phenotypic transformation of macrophages through paracrine secretion of soluble cytokines[55]; (2) MSCS promotes macrophage polarization through exosomes[51]; (3) Metabolic Reprogramming[56]; (4) MSCS regulate mitochondrial transfer[57]; and (5) Apoptotic and efferocytosis effects[58]. The involved signaling pathways are as follows: (1) The nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling pathway[59]; (2) The Notch signaling pathway; (3) The Janus kinase-signal transducer and activator of transcription signaling pathway[60]; and (4) The nuclear factor-kappa B signaling pathway[61].

MSCs regulate the T-cell balance: Imbalances between regulatory T cells (Tregs) and IL-17-producing Th17 are a sign of the development of inflammatory response in ARDS[62,63]. The main function of Th17 cells is to promote inflammation, release inflammatory factors, and play an important role in autoimmune diseases. Fortunately, they are precisely regulated by regulatory cells[64]. However, Treg cells can release anti-inflammatory factors (IL-4 and IL-10), control the inflammatory reaction process, and induce tissue damage repair[65,66]. Previous studies have shown that Tregs transferred into ALI animals can reduce the level of alveolar pro-inflammatory cytokines and inhibit neutrophil apoptosis and fibroblast recruitment[67,68]. A recent study also showed that a proportion of Th17 cells and Tregs > 0.79 was an independent predictor of 28-d mortality in patients with ARDS[62]. Therefore, maintaining the balance of Tregs and Th17 cells is crucial for patients with ARDS.

Existing studies indicated that, *in vitro*, MSCs repress the Th17 molecular program through the programmed cell death protein 1 pathway, prevent the differentiation of naive CD4+ T cells into Th17 cells, inhibit the production of inflammatory cytokines by Th17 cells, and induce Treg phenotype[69-71]. A study has shown that TGF-β1, as the main paracrine cytokine of MSCs, can significantly regulate the transformation of T cells into Tregs, disturb the Th17/Treg balance, and significantly contribute to the control of inflammatory response in ARDS[72]. It is also reported that MSCs can prevent the initial differentiation of CD4+ T cells into Th17 cells, inhibit the generation of inflammation, and induce the

generation of Tregs *in vitro*[73]. Several experiments have proved that controlling the level of Th17/Treg is the key to MSC-mediated control of the inflammatory response in ARDS. Therefore, it is extremely critical to find a method to regulate this balance, which may be the basis for a revolutionary breakthrough in the treatment of ARDS.

MSCs promote tissue repair

Lung epithelial cell and endothelial cell damage and the exudation of highly concentrated protein fluid are the basic pathological changes in ARDS. Therefore, the treatment of ARDS requires a combination of a lung-protective ventilation strategy and fluid manipulation[74]. The immediate effect of these strategies is that MSCs can directly participate in the reconstruction of lung injury by migrating to the site of lung injury, but this aspect has less impact on ARDS injury repair[75]. Evidence shows that MSCs can be directly transformed into type II alveolar epithelial cells to support the role of injured cells[74]. Meanwhile, cell-to-cell contact also provides a prerequisite for the control of inflammatory responses [76]. The formation and tissue damage of pulmonary edema is also related to the dysfunction of the pulmonary vascular system. The increased permeability of pulmonary capillaries leads to a series of serious consequences, such as the exudation of a variety of cells and cytokines and the formation of intravascular microthrombosis. Studies have shown that MSCs can also enhance the barrier system of the pulmonary vascular system, which is beneficial for promoting the repair of lung tissue[77]. Hepatocyte growth factor, angiopoietin-1, and keratinocyte growth factor secreted by MSCs can improve vascular endothelial barrier function[78-82]. Genetic engineering *in situ* has shown that MSCs could promote the potential of pulmonary angiogenesis[83]. Therefore, various results have shown that MSCs could inhibit pulmonary edema and also provide the basis for the regeneration of lung tissue.

Alleviation of pulmonary fibrosis

The cellular basis of the lung is composed of alveoli, various types of parenchymal cells, and BM-derived cells[84]. The interaction between various cells is crucial for maintaining the basic functions of the lungs[85]. Although lung protective ventilation strategies have been applied in clinical practice, ARDS survivors still have related health problems, and some patients develop fibroproliferative responses characterized by fibroblast accumulation and deposition of collagen and other extracellular matrix components in the lungs[86]. After ALI, vascular permeability increases, plasma exudates, and protein fluid aggregates, leading to pulmonary edema. Inflammatory factors from the coagulation/anticoagulation system and inflammatory system enter the lungs and damage the alveolar–capillary membrane barrier[87]. In the early inflammatory phase of ARDS, various immune cells continuously release a variety of harmful substances, including reactive oxygen species and nitrogen, as well as proteolytic enzymes such as elastase and matrix metalloproteinases, leading to lung endothelial and epithelial cell damage[86,88]. Persistent damage and failure to quickly repair this damage are the main factors that induce a pathological fibroproliferative response[89]. Late in the inflammatory response, massive and persistent accumulation of macrophages, fibroblasts, fibroblasts, and myofibroblasts in the alveolar space results in excessive deposition of fibronectin, collagen types I and III, and other components of the extracellular matrix[90,91]. The pro-fibrotic/anti-fibrotic balance is disrupted, and the fibrogenic effect increases dramatically, leading to irreversible pulmonary fibrosis. MSCs have shown gratifying advantages in anti-fibrotic effects. In preclinical models of lung fibrosis produced by bleomycin, silica, paraquat, and radiation, MSCs obviously show the ability to prolong life time[92-95]. However, MSC control of pulmonary fibrosis is also a double-edged sword. Studies have shown that MSCS can differentiate into ATII cells *in vitro*, inhibit the production of degradation enzymes, and thereby inhibit the secretion of pro-fibrotic factors by various immune cells[96]. There is also evidence that abnormally activated Wnt/β-catenin and TGF-β signaling pathways can induce the differentiation of pulmonary intrinsic MSCs into myofibroblasts and promote the development of pulmonary fibrosis [97].

CLINICAL EXPERIENCE USING MSCS FOR ARDS

To date, experience with the application of MSCs in patients is limited. The data of the available clinical evidence is summarized in Table 2. Wilson and his colleagues reported the results of the phase I stem cell research for ARDS treatment (START) in 2015[98]. Patients with moderate to severe ARDS received a single intravenous dose of low [1 × 10^6 MSCs/kg predicted body weight (PBW)], medium (5 × 10^6 MSCs/kg PBW), or high (1 × 10^7 MSCs/kg PBW) (n = 3/dose). All patients tolerated MSC infusion without prespecified infusion-related adverse events. High-dose MSCs improved daily SOFA scores compared with low-dose MSCs. Based on these promising results, the knowledge of the safety of administering MSCs to critically ill patients with ARDS is improving. A phase 2a clinical trial to evaluate the safety of BM-MSCs administered to patients with moderate to severe ARDS has also been conducted[99]. The primary outcome was safety, and secondary outcomes included respiratory, systemic, and serum biomarker endpoints. The study included 60 patients with ARDS, and intravenous Adipose-MSCs, 1 × 10^6/kg predicted body weight, *vs* the placebo was administered; however, there was

Table 2 Clinical study characteristics

Ref.	Cell type	Patient number	Outcome	Study design/evidence level	Publish time
Wilson *et al* [98]	MSC	9	No serious adverse events	Phase 1 clinical trial: A multicenter, open-label phase	2015
Matthay *et al* [99]	BM-MSCs	60	(1) No patients had any adverse events; (2) Mortality at 28 and 60 d was not significantly increased; and (3) ↑ Oxygenation index	Phase 2a safety trial: Prospective, double-blind, multicenter, randomized trial	2019
Yip *et al*[100]	UC-MSCs	9	(1) In-hospital mortality was 33.3% (3/9); (2) No serious prespecified cell infusion-associated or treatment-related adverse events; (3) ↓Circulating inflammatory biomarkers; (4) ↓Mesenchymal stem cell markers; and (5) ↑Immune cell markers	Phase I clinical trial: Prospective	2020
Lanzoni *et al* [107]	UC-MSCs	24	(1) No serious adverse events; (2) ↑Survival; and (3) ↓Inflammatory cytokines at day 6	Phase 1/2a clinical trial: A double-blind, randomized controlled trial	2021
Dilogo *et al* [108]	UC-MSCs	20	(1) ↑Survival; and (2) ↓Interleukin 6	Clinical trial: A multicentered, double-blind, randomized clinical trial	2021
Monsel *et al* [109]	UC-MSCs	45	(1) PaO_2/FiO_2 changes between D0 and D7 did not differ significantly; and (2) Clinical improvement	Clinical trial: A multicentered, double-blind, randomized clinical trial	2022
Grégoire *et al* [110]	BM-MSCs	8	(1) ↑Survival; (2) Clinical improvement; and (3) ↓Day-7 D-dimer value	A phase I/II Clinical Trial	2022
Kaffash Farkhad *et al* [111]	UC-MSCs	10	(1) ↑PaO_2/FiO_2; (2) ↓Serum CRP; (3) ↓IL-6, IFN-γ, TNF-α and IL-17 A; and (4) ↑TGF-β, IL-1B and IL-10	Phase 1 clinical trial: A single-center, open-label	2022

UC-MSC: Umbilical cord-derived mesenchymal stem cells; CDC: Cardiosphere-derived cells; BM-MCs: Bone marrow-derived mesenchymal stem cells; PaO_2/FiO_2: Arterial oxygen partial pressure/fractional inspired oxygen; CRP: C-reactive protein; CT: Computed tomography; ICU: Intensive care unit; ARDS: Acute respiratory distress syndrome; COVID-19: Coronavirus disease 2019; TNF-α: Tumor necrosis factor-alpha; IFN-γ: Interferon-gamma; TGF-β: Tumor growth factor-β; IL: Interleukin.

no difference in the outcome in patients treated with Adipose-MSCs *vs* the placebo. In another phase I study, nine consecutive patients were enrolled, between December 2017 and August 2019, the first three patients got low-dose human umbilical cord-derived MSCs, the following three patients received an intermediate dosage, and the last three patients received a high dose[100]. The results of the first phase of clinical trials demonstrated that a single dose of human umbilical cord-derived MSCs was safe and showed good results in all nine patients with ARDS. Swedish researchers tested the systemic administration of allogeneic BM-derived MSCs (2×10^6 cells/kg) in two patients with severe refractory ARDS, both of whom recovered from multiple organ failure and showed reduced markers of systemic and pulmonary inflammation[101]. In summary, clinical studies report that MSC administration is safe for patients with ARDS, with few adverse reactions. However, due to the relatively small number of patients in these studies, further research is needed to test the curative effect.

MSCS FOR CORONAVIRUS DISEASE 2019-INDUCED ARDS

Coronavirus disease 2019 (COVID-19) is an infectious disease responsible for the COVID-19 pandemic, caused by a novel coronavirus called severe acute respiratory syndrome-coronavirus 2[102,103]. COVID-19 has various respiratory and non-respiratory clinical manifestations, including mild or severe influenza-like syndrome, pneumonia, or respiratory failure, which may eventually lead to sepsis with multiple organ failure. The most common reason for being admitted to intensive care units is a respiratory failure caused by ARDS[104,105]. In a case series, Hashemian *et al*[106] found that multiple infusions of high-dose allogeneic prenatal MSCs are safe and can relieve the respiratory distress of severe patients with COVID-19 and inhibit the inflammatory response[106]. 24 participants were randomly assigned to either the umbilical cord-derived mesenchymal stem cell (UC-MSC) therapy or the control group in a double-blind, phase ½a randomized controlled trial. The UC-MSC treatment group got two intravenous infusions of $100 \pm 20 \times 10^6$ UC-MSCs, while the control group received two infusions of vehicle solution[107]. The primary endpoint was safety (adverse events) after 6 h; cardiac arrest or death within 24 h post-infusion) and secondary endpoints included patient survival at 31 d after the first infusion and time to recovery. Serious adverse events related to UC-MSC infusion were

not observed. Thus, UC-MSC is safe to inject into patients with COVID-19-induced ARDS. In subjects who received UC-MSC treatment, inflammatory cytokines decreased significantly on the sixth day. In a recent clinical research, 40 COVID-19 patients who were critically unwell got either saline or intravenous UC-MSCs[108]. The findings revealed that the survival rate of patients in the UC-MSCs group was 2.5 times greater than that of the control group. Among patients with complications, the UC-MSCs group had a fourfold greater survival rate than the control group. A multicenter, double-blind, randomized, placebo-controlled trial (STROMA–CoV-2) in France, with 45 enrolled patients, has also been conducted[109]. Patients were randomly assigned to receive three intravenous infusions of 1×10^6 UC-MSCs/kg or placebo (0.9% NaCl) over 5 d after recruitment. PaO_2/FiO_2 changes between D0 and D7 did not differ significantly between the UC-MSCs and placebo groups. Six (28.6%) of the 21 UC-MSCs patients and six (25%) of the 24 (25%) placebo patients had serious adverse events not related to UC-MSCs treatment. A phase I/II clinical study was also done in patients with severe COVID-19 to assess the safety and effectiveness of three intravenous infusions of BM-derived MSCs at 3-d intervals[110]. Eight intensive care unit patients requiring supplemental oxygen were treated with BM-MSCs. Survival was significantly higher in the MSC group at 28 and 60 d, but there was no significant difference in the number of invasive ventilation-free days, high flow nasal oxygenation-free days, oxygen support-free days, or intensive care unit-free days. MSC infusion was well tolerated, and no adverse effects associated with MSC infusion were reported. Furthermore, a single-center, open-label, phase 1 clinical trial enrolled 20 confirmed COVID-19 patients with mild-to-moderate degree ARDS, who were divided into two groups: The control and the intervention group (UC-MSCs)[111]. The patients received three intravenous infusions of UC-MSCs (1×10^6 cells/kg BW per injection) every other day. There were no adverse effects to cell infusion throughout the clinical study, oxygenation was greatly enhanced, anti-inflammatory factor levels were significantly increased, and pro-inflammatory factor levels were dramatically lowered. This intervention may reduce cytokine storms and restore respiratory function.

To summarize, MSCs from different tissues, such as BM, adipose, UC, and placental tissues, have entered the clinical trial stage. Some studies have used MSCs to treat COVID-19-induced ARDS.

PROSPECT

With the progress of scientific research, our understanding of the physiological and pathological processes of ARDS has gradually deepened, and the relevant treatment methods are also improving year by year. However, the final prognosis of patients has not improved much; therefore, it is particularly important to find a method to treat ARDS. MSCs have a variety of characteristics that are striking. At present, as a potential therapeutic method, MSCs have gradually entered the international arena of research and have been unanimously recognized by scientists worldwide. Their application has achieved some effect in improving the survival rate of patients with ARDS. However, because of various reasons, only a few clinical trials are conducted. Although the achievements of basic research are emerging endlessly, there is a theoretical basis for MSC use to enter clinical treatment, and the side effects of MSCs are not clear. Moreover, their clinical application involves ethical issues. As a cell therapy, its safety needs a lot of control experiments to be proven. This has hindered the successful application of MSCs. Fortunately, the basic experimental research on their mechanism of action is becoming more and more in-depth, and the application value of MSC therapy is also much clearer. Their successful application for the treatment of ARDS is expected to improve the quality of life of patients.

CONCLUSION

Due to the impasse that has been reached in the treatment of ARDS, MSC therapy has gained increasing attention. MSCs are known for their anti-inflammatory, differentiation, paracrine, and microvesicle transport abilities, which could perfectly target the pathological mechanisms of ARDS, providing a theoretical basis for treatment and precision treatment. Despite the current evaluation of MSC treatment of ARDS, further research is needed to observe the specific response to MSC treatment in the long term.

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FOOTNOTES

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REVIEW

Extracellular vesicles: Emerged as a promising strategy for regenerative medicine

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Abstract

Cell transplantation therapy has certain limitations including immune rejection and limited cell viability, which seriously hinder the transformation of stem cell-based tissue regeneration into clinical practice. Extracellular vesicles (EVs) not only possess the advantages of its derived cells, but also can avoid the risks of cell transplantation. EVs are intelligent and controllable biomaterials that can participate in a variety of physiological and pathological activities, tissue repair and regeneration by transmitting a variety of biological signals, showing great potential in cell-free tissue regeneration. In this review, we summarized the origins and characteristics of EVs, introduced the pivotal role of EVs in diverse tissues regeneration, discussed the underlying mechanisms, prospects, and challenges of EVs. We also pointed out the problems that need to be solved, application directions, and prospects of EVs in the future and shed new light on the novel cell-free strategy for using EVs in the field of regenerative medicine.

Key Words: Mesenchymal stem cells; Extracellular vesicles; Regenerative medicine; Cell-free strategy

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Core Tip: Extracellular vesicles (EVs) play a critical role in tissue repair and regeneration medicine *via* cell-to-cell communication. In this review, we elaborate and discuss both the recent research progress and the advancements in the therapeutic effects and limitations of EVs in tissue regeneration and engineering medicine. Moreover, we summarize the underlying molecular mechanisms related to EV repair effects and point out the problems that need to be solved, application directions, and prospects of EVs in the future.

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INTRODUCTION

Tissue and organ loss are challenging complications that are usually caused by severe diseases such as cancer and serious accidents and impose great burdens on patients' lives. Tissue regeneration and engineering medicine, which aims to repair lost cells and damaged organs caused by diseases or accidents, is achieving great progress sparked by numerous studies of stem cells, biomaterials and so on. Stem cells, especially mesenchymal stem cells (MSCs), play a critical role in regeneration medicine due to their strong self-renewal ability and diverse differentiation potential. However, there are several shortcomings of using MSCs in regeneration medicine, including cell source limitations, ethical controversies, low survival rates after cell transplantation, immune rejection, and risk of tumorigenesis after transplantation[1,2]. To solve the shortcomings of cell-based therapy, the paracrine action of cells has become a focus of research attention.

Recent research has focused on the secretions of cells and tissues. Extracellular vesicles (EVs) are heterogeneous lipid bilayer-surrounded vesicles secreted by various cell types, including immune cells, endothelial cells, epithelial cells, neuronal cells, cancerous cells, Schwann cells (SCs), and MSCs, that behave as crucial mediators of intercellular communication[3-6]. EVs participate in various types of physiological and pathological activities, including immune responses, homeostasis maintenance, inflammation, angiogenesis, and cancer progression, by transferring biological signals[7,8]. According to their size and origin, EVs can be classified in several ways. On the basis of their origin, EVs can be divided into three categories: Apoptotic bodies generated during cell apoptosis; microvesicles originating from budding cellular membranes; and exosomes derived from multivesicular bodies in fusion with the plasma membrane[4,9]. EVs also can be divided into small EVs, medium-sized EVs, and large EVs[3,10,11].

Increasing evidence indicates that EVs play a critical role in tissue repair and regeneration medicine *via* cell-to-cell communication. Moreover, several studies imply that the beneficial effects of MSCs on tissue regeneration may be attributed to their paracrine action by secreting EVs rather than MSC engraftment and proliferation[3,12,13]. Moreover, EVs themselves possess the ability to recruit endogenous cells and lead to their enrichment by releasing several chemokines, which may contribute to angiogenesis and tissue repair[14,15]. Therefore, EVs are an appropriate and hopeful new source for tissue repair and regeneration.

In this review, we elaborate and discuss both the recent research progress and the advancements in the therapeutic effects and limitations of EVs in tissue regeneration and engineering medicine (Figure 1). Moreover, we summarize the underlying molecular mechanisms related to EV repair effects (Table 1) and point out the problems that need to be solved, application directions (Table 2), and prospects of EVs in the future.

EVs

The discovery of EVs dates back to 1940, when a brand-new subcellular factor was identified in cell-free plasma by high-speed centrifugation. The subcellular fraction was identified and shown to consist of small vesicles by electron microscopy in the 1960s. The term "exosomes" was introduced when vesicles were isolated from cell supernatant in 1987, and these exosomes were found to be related to the removal of obsolete transmembrane proteins[10]. Currently, EVs are secreted by both normal cells and cancerous cells and act as a means of cell-to-cell communication. Signals are communicated through vesicle membrane proteins or by vesicle contents such as proteins, miRNAs, or long noncoding RNAs (lncRNAs)[16].

Table 1 The mechanism of extracellular vesicles in the regeneration medicine

	Source of EVs	Mechanism
Kidney	MSC-EVs	Inhibiting oxidation, apoptosis, and inflammation
	HLSC-EVs	Regulating angiogenesis, the cell cycle, regeneration, autophagy, proliferation[36-38,46]
	BMSC-EVs	
Liver disease	hiPSCs-EVs	Inhibiting hepatocyte apoptosis
	Hepatocyte-EVs	Supporting hepatocyte function
	MSC-EVs	Promoting angiogenesis
		Reducing inflammatory responses[48-53]
Cardiac muscle	ESCs-EVs	Vascularization
	iPSCs-EVs	Amelioration of apoptosis and hypertrophy
	MSCs-EVs	Promoting cell proliferation and migration[55-60]
	CDCs-EVs	
	BMSC-EVs	
Tendon	BMSC-EVs	Modulating macrophage phenotypes
	ADSC-EVs	Anti-inflammatory reaction
		Enhancing proliferation, migration, tenogenic differentiation of TSCs
		Regulating angiogenesis
		Modulating immune responses[62-68]
Wound healing	BMSC-EVs	Promoting re-epithelialization
	ADSC-EVs	Promoting collagen maturity and angiogenesis
	hENSC-EV	Enhancing cell proliferation and migration[69-74]
	HUVECs-EVs	
	iPSC-EVs	
Tooth and periodontal tissue	BMSC-EVs	Modulating the inflammatory immune response
	ADSC-EVs	Enhancing cell proliferation and migration
	DPSC-EVs	Promoting odontogenic differentiation
	DFC-EVs	Stem cell recruitment[76-83]
	PDLSC-EVs	
	HERS-EVs	
	SC-EVs	
Nerve	SKP-SC-EVs	Mediating axon regeneration
	BMSC-EVs	Regulating the phenotype of Schwann cells
	ADSC-EVs	Promoting angiogenesis
	GMSC-EVs	Regulating inflammatory reactions[84-94]
Bone	ADSC-EVs	Angiogenesis
	BMSC-EVs	Osteoblast proliferation
	SMSC-EVs	Intercellular communication
	PRP-EVs	Immune regulation[96-104]

EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells; HLSC: Human liver stem cells; hiPSCs: Human-induced pluripotent stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; CDCs: Cardiosphere-derived cells; hENSC: Human Endometrial stem cells; DPSC: Dental pulp stem cells; DFC: Dental follicle cell; PDLSC: Periodontal ligament stem cells; PRP: Platelet-rich plasma; SMSC: Synovium mesenchymal stem cells;

HERS: Hertwig's epithelial root sheath; BMSC: Bone marrow mesenchymal stem cell; ADSC: Adipose stem cells; GMSC: Gingiva-derived mesenchymal stem cell; SKP-SC: Skin-derived precursor Schwann cells; TSC: Trophoblast stem cells.

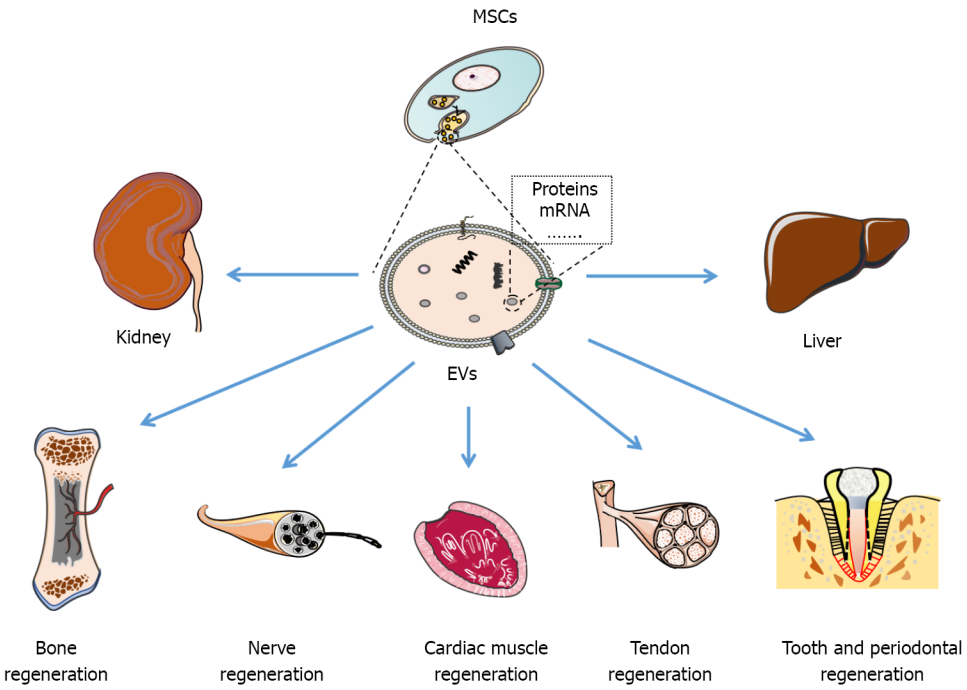
Table 2 Extracellular vesicles investigated in clinical studies

Research title	Interventions	Status
Treatment of Patients with Bone Tissue Defects Using Mesenchymal Stem Cells Enriched by Extracellular Vesicles	MSCs enriched by extracellular vesicles	Not yet recruiting
Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment for ARDS	Bone Marrow MSC Derived Extracellular	Not yet recruiting
Efficacy of Platelet- and Extracellular Vesicle-rich Plasma in Chronic Postsurgical Temporal Bone Inflammations	Plateletand extracellular vesicle-rich plasma	Completed
Use of Autologous Plasma Rich in Platelets and Extracellular Vesicles in the Surgical Treatment of Chronic Middle Ear Infections	Plateletand EVs-rich plasma	Recruiting
Extracellular Vesicle Infusion Treatment for COVID-19 Associated ARDS	ExoFlo	Completed
Safety of Mesenchymal Stem Cell Extracellular Vesicles (BMMSC-EVs) for the Treatment of Burn Wounds	Drug: AGLE-102 (BMMSC-EVs)	Not yet recruiting
Treatment of Non-ischemic Cardiomyopathies by Intravenous ExtracellularVesicles of CardiovascularProgenitor Cells	Extracellular vesicle-enriched secretome of cardiovascular progenitor cells differentiated from induced pluripotent stem cells	Not yet recruiting
Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment for Mild-to-Moderate COVID-19: A Phase II Clinical Trial	Drug: ExoFlo. Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles	Not yet recruiting
A Safety Study of IV Stem Cellderived Extracellular Vesicles (UNEX-42) in Preterm Neonates at High Risk for BPD	UNEX-42 is a preparation of extracellular vesicles that are secreted from human bone marrow-derived mesenchymal stem cells suspended in phosphate-buffered saline	Terminated
ExoFlo™ Infusion for Post-Acute COVID-19 and Chronic Post COVID-19 Syndrome	Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles	Not yet recruiting
Study of ExoFlo for the Treatment of Medically Refractory Ulcerative Colitis	ExoFlo. Intravenous administration of bone marrow mesenchymal stem cell derived extracellular vesicles	Recruiting
Study of ExoFlo for the Treatment of Medically Refractory Crohn's Disease	ExoFlo. Intravenous administration of bone marrow mesenchymal stem cell derived extracellular vesicles	Recruiting
Bone Marrow Mesenchymal Stem Cell Derived EVs for COVID-19 Moderate-to-Severe ARDS: A Phase III Clinical Trial	EXOFLO. Bone Marrow Mesenchymal Stem Cell Derived EVs	Recruiting
Pilot Study of Human Adipose Tissue Derived Exosomes Promoting Wound Healing	Adipose tissue derived exosomes	Not yet recruiting
Exosome Effect on Prevention of Hairloss	Placental Mesenchymal Stem Cells-derived Exosome	Recruiting
Expanded Access for Use of ExoFlo in Abdominal Solid Organ Transplant Patients	Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicles Infusion Treatment	Not yet recruiting
Safety of Injection of Placental Mesenchymal Stem Cell Derived Exosomes for Treatment of Resistant Perianal Fistula in Crohn's Patients	Placenal MSC derived exosomes	Active, not recruiting
Safety and Efficacy of Injection of Human Placenta Mesenchymal Stem Cells Derived Exosomes for Treatment of Complex Anal Fistula	Placenta MSCs derived exosomes	Recruiting
Safety and Tolerability Study of MSC Exosome Ointment	Exosome ointment	Completed
The Pilot Experimental Study of the Neuroprotective Effects of Exosomes in Extremely Low Birth Weight Infants	Exosomes derived from MSCs	Not yet recruiting

ARDS: Acute Respiratory Distress Syndrome; MSC: Mesenchymal stromal cells; COVID-19: Coronavirus disease 2019; EVs: Extracellular vesicles.

Biogenesis and classification of EVs

EVs are classified by several traits, including their density, dimensions, and origin. Three subclasses of EVs have been reported recently. Although the sizes of these three main types of EVs may overlap, their biogenesis is different. Exosomes, whose dimensions range from 30-150 nm, are formed within the endosomal network, where endosomes target some protein/lipids for recycling or exocytosis. During early endosome transformation into late endosomes, proteins that are fated to be degraded or exported



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Figure 1 The repair and regeneration effects of extracellular vesicles in different tissue and organs. EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells.

are packaged into vesicles. Late endosomes, which contain small vesicles, fuse with the plasma membrane and finally lead to the secretion of small vesicles, named exosomes, into the extracellular space[16]. Microvesicles/Ectosomes originate from vesicles budding outward and fission of the plasma membrane directly, which is a dynamic interplay between redistribution and cytoskeletal protein contraction. The size of microvesicles/ectosomes is larger than that of exosomes, ranging from 50-2000 nm. Apoptotic bodies, ranging from 500-40000 nm, are formed during programmed cell death and contain organelles.

Isolation of EVs

Differential ultracentrifugation (UC), the gold standard EVs isolation approach, is the most reported and classical way to isolate EVs. Successive centrifugation was applied to eliminate large dead cells and cell debris. The supernatant acquired after successive centrifugation was used for another UC step at 100000 g to obtain the pellet related to EVs. The pellet was washed with PBS to obtain purified EVs[17]. UC has several advantages including low cost and low contamination risk with extra isolation reagents. UC is suitable for large volume preparation for its products is of high purity. However, UC is complicated, time-consuming and labor intensive. High speed centrifugation also may lead to potential mechanical damage. There are several isolation methods that can extract EVs in a more efficient way, including polymer-based precipitation[18], size exclusion chromatography (SEC)[19], ultrafiltration (UF)[20], flow field-flow fractionation[21], immunoaffinity capture[22], and microchip-based techniques [23]. Polymer-based precipitation is a commonly used strategy for EVs isolation. The principle of polymer-based precipitation is high hydrophilic water-excluding polymers can alternate the solubility of EVs. Highly hydrophilic polymers interact with water molecules surrounding the EVs to create a hydrophobic micro-environment, resulting in EVs precipitation[24]. Based on above principle, polyethylene glycol is well used in several popular commercial EVs isolation kits[25]. Polymer-based precipitation has high efficiency, but its products is easy to be contaminated by protein aggregates. SEC is according to the size of particle to realize the isolation. After adding to porous materials, substances eluted out in accordance with their particle size, with big particles eluted earlier[26]. Compared to UC, SEC is realized by the performance of passive gravity flow, which highly protect the structure and integrity of EVs[27]. UF uses filter membrane to isolate EVs from cell culture medium. Compared to traditional UC method, UF-based EVs isolation shortens time and presents relatively low requirements on experimental facilities. However, UF also has several shortages including the EVs production maybe limited due to clogging and membrane trapping[28,29]. Immunoaffinity capture is based on specific binding between EVs markers and immobilized antibodies such as Rab5, CD81, CD63, CD9, CD82, annexin, and Alix[30]. Immunoaffinity capture can harvest high-purity EVs with no chemical contamination. However, it is waste of antibodies and low yields[26]. The combination of the above methods is

also applied in the isolation of EVs. Moreover, there are several kits that are available for EV isolation, including ExoQuick and Total Exosome Isolation kits. However, the purity and quality of isolated EVs are extremely important because some soluble proteins or lipoproteins may be coisolated with EVs, leading to inaccurate experimental results. Therefore, isolation methods are of critical importance. Among all the methods, UC and UF followed by SEC are reported to be the most appropriate isolation methods because they can isolate high-purity and high-quality EVs[31,32].

Characteristics of EVs

EVs carry several specific surface markers, including proteins related to the cell membrane, annexin, flotillin and auxiliary proteins. Annexins and tetraspanins such as CD9, CD63 and CD81, which are often used for the identification of EVs, are located in the membrane of EVs. Moreover, EVs express ALIX, tumor susceptibility gene 101, VPS4 and heat shock proteins (HSP70 and HSP 90), which are associated with the biogenesis of EVs. EVs are encapsulated in a bilayer membrane, which can help in the safe transfer of their contents to secondary cells. Once released into the extracellular environment, EVs interact with recipient cells in three ways: (1) Endocytic uptake; (2) direct fusion with cells; and (3) adhesion to the cell surface and transmission of contents[4]. Among their contents, EVs are reported to contain large-scale genetic materials, such as mRNA, that play critical roles in cell-to-cell communication. miRNAs that are transferred by EVs also have an impact on biological functions, including cell proliferation, migration, and differentiation of recipient cells. EVs also carry several types of lipids that are related to EV structure, function and biogenesis[3]. In conclusion, EVs are heterogeneous and are composed of a bilayer membrane surrounding cargos that are indispensable for cell interactions.

REPAIR AND REGENERATION EFFECTS OF EVS

Kidney

Acute kidney injury (AKI) and chronic kidney disease (CKD) are two major causes of renal failure and exert great pressure on public health. AKI, the most common features of which are the rapid loss of renal tubular cells and a decline in renal function, usually leads to hospitalization[33]. As research has progressed, evidence has shown that MSC-EVs play a major role in treating AKI. EVs were first proven to be effective against AKI in 2009[34]. The intravenous administration of EVs not only alleviated or even reversed the detrimental effect on renal function caused by glycerol injection in an AKI model but also improved renal function and morphology by stimulating the proliferation of tubular epithelial cells (TECs)[35]. In a renal ischemia-reperfusion (I/R) injury AKI model, MSC-EVs accumulated in the renal tubules and facilitated with the recovery of kidney function through the Keap1-Nrf2 signaling pathway as well as the mitochondrial function of TECs[36]. Studies have also shown that EVs can reduce the presence of luminal cell debris, tubular hyaline casts and necrosis of tubular cells in an AKI model induced by toxins[37]. Moreover, EVs released by cells that were cultured under hypoxia could stimulate angiogenesis and help the formation of the peritubular microvasculature[38]. Furthermore, MSC-EVs attenuated mtDNA damage and inflammation after AKI through the mitochondrial transcription factor A (TFAM) pathway. MSC-EVs could attenuate renal lesion formation, mitochondrial damage, and inflammation in mice with AKI. TFAM overexpression (TFAM-OE) improved the rescue effect of MSC-EVs on mitochondrial damage and inflammation to some extent[39]. In general, MSC-EVs can relieve AKI not only by inhibiting oxidation, apoptosis, and inflammation, but also through regulating angiogenesis, cell cycle, autophagy, and cell proliferation[38]. There are multiple underlying mechanisms. Currently, it is believed that the repair effects of EVs on AKI are largely related to their transfer of genetic material and proteins[40].

CKD is a complex and long-term disease. The main trigger that causes CKD is diabetes. Hyperglycemia leads to glomerular and tubulointerstitial fibrosis, and the progression of fibrosis is the main reason for renal dysfunction[41]. Renal glomerulosclerosis and tubulointerstitial fibrosis are hallmarks of all types of CKD, including diabetic nephropathy (DN). Recent studies have shown that EVs are effective for the prevention of DN. For example, EVs can protect podocytes and TECs from apoptosis by secreting protective proteins, including transforming growth factor (TGF)-β1 and angiogenin[42]. Another study found that EVs derived from human liver stem cells (HLSCs) and MSCs could reduce or even revert the progression of profibrotic processes and finally ameliorate renal dysfunction and attenuate renal histopathological changes[43]. Moreover, studies have shown that EVs can ameliorate DN by inducing autophagy induction through the mTOR pathway. Rat bone marrow-derived EVs present nephroprotective and antifibrotic effects by upregulating autophagy through suppressing the mTOR pathway in a DN model[44]. Furthermore, HLSC-derived EVs could also prevent the development of CKD. HLSC-EVs not only present a regenerative and anti-inflammatory role but also downregulate profibrotic genes, including alpha smooth muscle actin, Col1a1 and TGF-β1, and modulate miRNAs that are related to the fibrotic pathway in the kidney of an aristolochic acid-induced CKD model[45]. In conclusion, MSC-EVs can eliminate the pathogenic damage of CKD by targeting renal fibrosis, reducing tubular atrophy and inflammation, and promoting angiogenesis to

facilitate with tissue regeneration[46]. EVs present a promising approach for renal repair and regeneration. Some evidence based on clinical trials has shown that EVs are safe and effective for CKD patients[47].

Liver disease

Liver dysfunction is classified into acute and chronic diseases, including hepatitis, alcoholic liver disease, fatty liver disease, cirrhosis, and hepatocellular carcinoma. Liver failure can manifest with several symptoms, such as jaundice, encephalopathy, cerebral edema, sepsis, and gastrointestinal bleeding, and its prognosis is relatively limited. Currently, liver transplantation is still the gold-standard therapy for liver diseases; however, it has many limitations, such as nonspecific treatment approaches, donor organ shortages and lifelong immunosuppressive therapy[48]. Although the liver possesses a great capacity for regeneration through the proliferation of mature liver cells, it can lose its function and lead to severe results when the injury progresses into a state of functional impairment, which may finally lead to liver failure or even death[49]. EVs can prevent further damage to injured liver cells. Studies have reported that EVs can reduce liver injury based on alanine aminotransferase and aspartate aminotransferase levels after CCl_4-induced liver impairment *in vivo*. Moreover, EVs promoted hepatocyte regeneration gene expression and $PCNA^+$ expression and induced quiescent hepatocytes to re-enter the cell cycle, ultimately assisting with hepatocyte proliferation. Another report showed that EVs derived from human-induced pluripotent stem cells (hiPSCs) could alleviate hepatic I/R injury by suppressing inflammatory responses, attenuating the oxidative stress response and inhibiting cell apoptosis[50]. Furthermore, Du *et al*[51] found that hiPSC-derived EVs could alleviate hepatic I/R injury by activating sphingosine kinase and the sphingosine-1-phosphate pathway in hepatocytes and ultimately promote cell proliferation[51]. EVs can not only be therapeutic but can also serve as diagnostic tools for liver disease and regeneration in the near future. A report outlined that hepatocyte-derived EVs played a key role in hepatocyte-to-hepatocyte communication and provided a new method for liver disease diagnosis, and progenitor cell-derived EVs offered a new opportunity for the treatment of liver diseases[52]. In conclusion, recent evidence supports that MSC-derived EVs inhibit hepatocyte apoptosis, support hepatocyte function, promote angiogenesis and hepatocyte proliferation, and reduce inflammatory responses by preventing immunocyte infiltration and inflammatory cytokine secretion [48]. In addition, animal model-based studies suggest that EVs may represent a novel and effective cell-free therapeutic agent as an alternative to cell-based therapies for patients with liver diseases[53].

Cardiac muscle regeneration

Myocardial repair and regeneration are important in the context of the increasing occurrence of heart failure and cardiac-related diseases. Key mechanisms related to cardiac repair and regeneration include survival and protection, inflammation reduction, angiogenesis, cardiomyogenesis and cell–cell communication. All these mechanisms work collectively and contribute to cardiac regeneration[54]. However, myocardial repair is slow and limited. Stem cell–based therapies have acted as an effective method for cardiac repair. Moreover, EVs, a key component of stem cell secretion, bring new hope to cell-free therapies for cardiac repair and regeneration. EVs of embryonic stem cells, iPSCs, MSCs, and cardiosphere-derived cells (CDCs) have been proven to be effective in cardiac repair[55]. Recent research has shown that EVs derived from murine iPSCs impart cytoprotective properties to cardiac cells *in vitro* and induce cardiac repair *in vivo* through vascularization, amelioration of apoptosis and hypertrophy and improvement in left ventricular function[56]. Moreover, iPSC-EVs contained numerous miRNAs (miR-17-92 cluster) and proteins [BMP-4, teratocarcinoma-derived growth factor 1 and vascular endothelial growth factor (VEGF)-C] that are related to cellular proliferation and differentiation, enhanced angiogenesis, and the prevention of apoptosis. Therefore, iPSC-EVs could induce angiogenesis, migration and anti-apoptosis of cardiac endothelial cells and finally induce superior infarct repair[56]. MSC-derived EVs also play a critical role in MSC-based therapy in cardiac diseases. Bian *et al*[57] found that intramyocardial injection of MSC-EVs could markedly enhance blood flow recovery in an acute myocardial infarction (MI) rat model. MSC-EVs could protect cardiac tissue from ischemic injury by promoting blood vessel formation. Receptors for growth factors, cytokines and signaling molecules are contained in EVs, among which Sonic hedgehog and platelet-derived growth factors have been proven to be effective for proangiogenic activities. The results of direct comparisons between MSCs and MSC-EVs have highlighted the beneficial effects of EVs. EVs also present a safer profile than their cells of origin because EV injection does not produce tumors[56]. Moreover, combinatorial treatment with both MSCs and their derived EVs exhibits advantages in MI treatment. Rat bone marrow mesenchymal stem cells (BMSCs) and their derived EVs improved cardiac function and reduced infarct size when compared to groups treated with BMSCs or EVs alone[57]. CDC exosomes were proven to enhance angiogenesis and promote cardiomyocyte survival and proliferation. Moreover, CDC exosomes improved cardiac function and imparted structural benefits after MI. miR-146a was the most highly enriched in CDC exosomes and mediated some of the therapeutic benefits of CDC exosomes[58]. EVs combined with biomaterials present better effects for the treatment of cardiac disease. Studies have shown that MSC-derived EVs incorporated into alginate hydrogels are a sustained delivery system that allows for better retention of EVs in the heart *in vivo* than EV single injection. EVs-Gel has a good effect on promoting angiogenesis, reducing the apoptosis and fibrosis of cardiac tissue,

enhancing scar thickness and improving cardiac function[59]. Another study showed that self-assembling peptide hydrogel-encapsulated exosomes could also promote cardiac repair due to the better retention and stability of EVs[60]. In general, EVs combined with biomaterials provide a novel approach for cell-free therapy and optimization of the therapeutic effects of EVs in MI.

Tendon regeneration

Tendons are soft tissues that connect muscles to bones. Tendon diseases and injuries have high morbidity owing to various sports and exercises. The effects of treatments for tendon diseases are limited due to the limited regenerative capacity of tendons. Current treatments for tendon injuries, including surgery and conservative treatments, always have side effects, including secondary injury and scar formation. Therefore, the ideal treatment is to promote tendon regeneration[61]. There are three important stages of tendon healing: Inflammation, proliferation, and remodeling. Suppression of the inflammatory response is critical for tendon repair[62,63]. Research has found that BMSC-EVs may help improving tendon healing by regulating macrophage phenotypes, creating anti-inflammatory environment, promoting apoptotic cell accumulation, and increasing the ratio of tendon resident stem/progenitor cells[64]. Moreover, EV-educated macrophages (EEMs), a kind of M2-like macrophage treated by EVs, present a more functional and regenerative ability to heal tendons. EVs isolated from MSCs were able to reduce the M1/M2 macrophage ratio and increase the number of endothelial cells 14 days after tendon injury. Injured tendons treated with exogenous EEMs presented improved mechanical properties, reduced inflammation and earlier angiogenesis[62]. Furthermore, Yu *et al*[29] found that BMSC-EVs could promote the proliferation, migration and tenogenic differentiation ability of tendon stem/progenitor cells (TSPCs). BMSC-derived EVs could activate the regenerative potential of endogenous TSPCs in tendon injury sites. EVs embedded in fibrin glue may allow the sustained release of EVs in injured regions and promote the regeneration of patellar tendon tissue in rats[15]. In addition to BMSCs, ADSC-EVs can also promote the proliferation and migration of tendon cells, reduce fatty infiltration, promote tendon-bone healing, improve the biomechanical properties of the tendon-bone junction and improve the mechanical strength of the repaired tendon[65,66]. The overexpression of H19 can enhance tendon regeneration potential. As an ideal drug carrier, EVs are a reliable delivery method to transmit lncRNAs. Reports have shown that engineered EVs with overload of H19 can regulate tendon regeneration by activating YAP through the H9-pp1-YAP axis[67]. In conclusion, MSC-EVs facilitate tendon regeneration by inhibiting apoptosis as well as enhancing the proliferation, migration and tenogenic differentiation of tendon stem cells and tenocytes. In addition, they can also regulate angiogenesis and modulate immune responses and extracellular matrix (ECM) remodeling of tendon tissue[68]. These results indicate that EVs have broad prospects in tendon repair and regeneration.

Skin and wound healing

EVs are currently widely used in wound healing and skin regeneration. EVs can participate in four stages of wound healing, namely, hemostasis, inflammatory response, cell proliferation and remodeling [69]. Both ADSC-EVs and BMSC-EVs exert benefits on cells related to skin wound healing, including fibroblasts, keratinocytes and endothelial cells, in different ways. BMSC-EVs mainly promote proliferation, whereas ADSC-EVs have a major effect on angiogenesis. BMSC-EVs and ADSC-EVs presented synergistic effects on wound healing[70]. Great progress has been made in the field of wound healing with the rapid development of the combination of EVs and biomaterials. EVs and glycerol hydrogels have synergistic effects on the proliferation of human skin fibroblasts. The full-thickness excisional wound model in mice showed that the fibrosis, vascularization, and epithelial thickness of wounds reached a maximum level after treatment with EV-loaded hydrogels. Moreover, research has found that EVs derived from human endometrial stem cells (hENSCs) contain several growth factors, including VEGF, basic fibroblast growth factor and TGF-1, which benefit angiogenesis. hENSC-EV-loaded chitosan hydrogel has positive impacts on wound healing by promoting angiogenesis and tissue granulation formation. hENSC-EV-loaded chitosan hydrogel could be an ideal scaffold for skin wound dressing and skin tissue regeneration[71]. EVs derived from HUVECs (HUVECs-EVs) could promote the proliferation and migration activities of keratinocytes and fibroblasts, which are two critical cells for skin regeneration. Gelatin methacryloyl (GelMA) hydrogel scaffolds combined with HUVEC-EVs could not only repair wound defects but could also achieve sustained release of EVs, which promoted re-epithelialization, collagen maturity and angiogenesis that ultimately contributed to wound healing[72]. In addition to wound healing and skin regeneration, EVs also play critical roles in skin aging and several skin diseases. For example, ADSC-EVs can relieve atopic dermatitis. ADSC-EVs can also promote cuticle hydration and ceramide synthesis and significantly reduce the secretion of inflammatory cytokines [interleukin (IL)-4, IL-5, IL-13, and IL-17] when applied *in vivo*[73]. Moreover, ADSCs could protect against oxidative stress by promoting the proliferation of human dermal fibroblasts (HDFs) and inhibiting reactive oxygen species and MMP production *via* the secretion of various cytokines. ADSC-EVs might be potential therapeutic tools for addressing the problem of photoaging [74]. Studies have also shown that EVs derived from human iPSCs could regulate the genotypic and phenotypic changes of HDFs induced by UV photoaging and natural aging. EVs derived from human iPSCs affected cellular responses related to skin aging, including the expression levels of MMP-1 and type I collagen and the proliferation and migratory ability of HDFs. iPSC-EVs can mediate intracellular

transportation and reconstruct the matrix in aging skin by enhancing the expression of structural proteins and regulating the expression of age-related proteins[75]. In general, EVs serve as efficient carriers of molecular cargos for wound healing and skin regenerative medicine.

Tooth and periodontal regeneration

EVs have been widely used in the repair of dental tissue, including dental pulp, dentin and periodontal tissue. Dental pulp stem cells (DPSCs)-EVs can bind to matrix proteins such as type I collagen and fibronectin, enabling them to be tethered to biomaterials. EVs are endocytosed by both DPSCs and human MSCs in a dose-dependent and saturable manner *via* the caveolar endocytic mechanism and trigger the P38 mitogen-activated protein kinase (MAPK) pathway. In addition, EVs can trigger the increased expression of genes required for odontogenic differentiation. In the generated pulp tissue, DPSC-EVs can also promote the expression of several growth-promoting factors, including TBG-β and BMP-2, which ultimately promote the repair of dental pulp tissue[76]. Moreover, DPSC-EVs can promote the proliferation and angiogenesis of vascular endothelial cells by activating the P38/MAPK pathway, which provides the possibility for the regeneration of vascular pulp tissue[77]. SC-EVs could promote DPSC proliferation and enhance neurite outgrowth, neuron migration, and vessel formation *in vitro*. SC-EVs facilitate dental pulp regeneration through endogenous stem cell recruitment *via* the SDF-1/CXCR4 axis without exogenous cell transplantation[78]. In addition to SC-EVs, EVs derived from Hertwig's epithelial root sheath cells could trigger regeneration of dental pulp-dentin-like tissue composed of hard (regenerative dentin-like tissue) and soft (blood vessel and neuron) tissue in an *in vivo* tooth root slice model by activating the Wnt pathway[79]. Periodontitis is the primary cause of tooth loss, but there is no effective treatment to repair inflammatory bone loss in periodontitis. In terms of periodontal tissue regeneration, studies have shown that EVs secreted by periodontal ligament stem cells (PDLSCs) are therapeutics for bone defects in periodontitis. EVs derived from healthy PDLSCs could rescue the osteogenesis capacity of endogenous stem cells under an inflammatory environment and promote the regeneration of alveolar bone by recovering the osteogenic differentiation ability of inflammatory PDLSCs through the inhinbitation of canonical Wnt signaling[80]. BMSC-EVs are also an ideal cell-free strategy for periodontal regeneration. BMSC-EVs could promote the regeneration of periodontal tissues through the OPG-RANKL-RANK signaling pathway to regulate the function of osteoclasts and affect macrophage polarization and TGF-β1 expression to modulate the inflammatory immune response, thereby inhibiting the development of periodontitis and immune damage in periodontal tissue[81]. Furthermore, the therapeutic effect of ADSC-EVs was the same as that of periodontal surgery in a rat periodontitis model[82]. Lipopolysaccharide (LPS)-induced dental follicle cell-derived EVs (L-D-EVs) could promote the proliferation of periodontal ligament cells. L-D-EV-loaded hydrogel applied in the treatment of periodontitis was beneficial to repairing lost alveolar bone in the early stage of treatment and maintaining the level of alveolar bone in the late stage of treatment in experimental periodontitis rats by decreasing the expression of the RANKL/OPG ratio *in vivo*[83]. EVs are presented as a novel cell-free therapeutic strategy for both dental pulp and periodontal regeneration.

Nerve regeneration

Neurological dysfunction usually causes great physical and psychological distress for patients. Autologous nerve transplantation is widely accepted as the gold standard for peripheral nerve repair, but its inherent defects greatly reduce its availability. Regeneration of peripheral nerves after injury remains a great challenge for researchers. EVs play a fundamental role in the physiological and pathological processes of the nervous system. There is growing evidence that EVs can play a neurotherapeutic role by mediating axon regeneration, activating SCs, promoting angiogenesis, and regulating inflammatory reactions. EVs from skin-derived precursor SCs (SKP-SC-EVs) could promote neurite outgrowth of sensory and motor neurons *via* the AKT/mTOR/p70S6K pathway *in vitro*[84,85]. SKP-SC-EV-incorporating silicone conduit nerve grafts could significantly accelerate the recovery of motor, sensory, and electrophysiological functions of rats; facilitate outgrowth and myelination of regenerated axons; and alleviate denervation-induced atrophy of target muscles, which raises the possibility of cell-free therapy in nerve regeneration[86]. Moreover, repair SCs (rSCs) could also release pro-regenerative EVs. Neuronal activity enhances the release of rSC-derived EVs and their transfer to neurons *via* the ATP-P2Y signaling pathway[87]. Mechanical stimuli could control the intercellular communication between neurons and SCs by changing the composition of miRNA in SC-EVs. MS-SC-EVs transferd miR-23b-3p from mechanically stimulated SCs to neurons, and lead the inhibition of neuronal Nrp1 expression, which was indicated the beneficial effect of MS-SC-EVs on axonal regeneration, and provided evidence for the role of miR-23b-3p-enriched EVs in peripheral nerve injury repair[88]. BMSC-EVs could promote the functional recovery of sciatic nerve injury and increase the expression of GAP-43, a marker of axon regeneration[89]. BMSC-EVs can also promote nerve regeneration by regulating the miRNA mediated genes which related to regeneration, such as vascular endothelial growth factor A and S100b[90]. In addition to BMSC-EVs, ADSC-EVs also benefited nerve regeneration. ADSC-EVs could increase neurite outgrowth *in vitro* and enhance regeneration after sciatic nerve injury *in vivo*[91]. Furthermore, ADSC-EVs contained mRNAs of neurotrophic factors, including NGR, brain-derived neurotrophic factor, ciliary neurotrophic factor and glial cell-derived neurotrophic factor. ADSC-EVs could deliver mRNAs as well as microRNAs that facilitate with neurotrophic factor

secretion and proliferation, support the SC repair phenotype, and provide a solid therapeutic evidence for nerve regeneration[92]. Gingiva-derived mesenchymal stem cell (GMSC)-derived EVs could obviously promote axonal regeneration and functional recovery of injured mouse sciatic nerves. GMSC-derived EVs promoted the expression of SC dedifferentiation/repair phenotype-related genes *in vitro*, particularly c-JUN, a key transcription factor that drives the activation of the repair phenotype of SCs during PNI and regeneration[93]. Revascularization treatment is a critical measure for nerve repair. A report revealed that EVs derived from hypoxic preconditioned HUVECs could facilitate MSC angiogenesis activity and the anti-inflammatory impacts of MSCs, which contribute to *in vivo* effective nerve tissue repair after rat spinal cord transection and provide inspiration for therapies based on stem cells and EVs[94]. In general, EVs are an effective therapeutic tool in nerve regeneration by mediating axon regeneration, regulating the phenotype of SCs, promoting angiogenesis, and regulating inflammatory reactions.

Bone regeneration

EVs play four potential roles in bone regeneration, namely, angiogenesis, osteoblast proliferation, intercellular communication, and immune regulation. EV-mediated intercellular communication between osteoblasts and osteoclasts may represent a novel mechanism of bone modeling and remodeling. EVs transmit signals between osteoblasts and osteoclasts to regulate bone remodeling. Osteoblasts release RANKL-containing EVs, which are transferred to precursor osteoclasts and promote osteoclast formation by stimulating RANKL-RANK signaling[95]. MSC-derived EVs (MSC-exos), with their inherent capacity to modulate cellular behavior, are emerging as a novel cell-free therapy for bone regeneration. Reports have revealed that EVs derived from osteoinductive BMSCs (BMSC-OL-EVs) contribute to bone regeneration *via* multicomponent exosomal miRNAs (let-7a-5p, let-7c-5p, miR-328a-5p and miR-31a-5p), which target Acvr2b/Acvr1 and regulate the competitive balance of Bmpr2/Acvr2b toward Bmpr-elicited Smad1/5/9 phosphorylation[96]. Moreover, lyophilized delivery of BMSC-OI-EVs on hierarchical mesoporous bioactive glass scaffolds showed the possibility of bone regeneration in a rat cranial defect model[96]. Umbilical MSC-derived exosomes (uMSCEXOs) also showed great promise in bone regeneration. uMSCEXOs encapsulated in hyaluronic acid hydrogel and combined with customized nanohydroxyapatite/poly-ε-caprolactone (nHP) scaffolds could repair cranial defects in rats by promoting the proliferation, migration, and angiogenic differentiation of endothelial progenitor cells *via* the miR-21/NOTCH1/DLL4 signaling axis[97]. Compared to EVs derived from uMSCs exposed to normoxia, EVs derived from uMSCs treated with hypoxia promoted angiogenesis, proliferation, and migration to a greater extent. Hypo-Exos facilitated with the recovery of bone fracture by exosomal miR-126 and the SPRED1/Ras/Erk signaling pathway. In addtion, hypoxia preconditioning promote the transferring of exosomal miR-126 through the activation of hypoxia-inducible factor-1α. Hypoxia preconditioning is an effective and promising approach for optimizing the therapeutic actions of MSC-derived exosomes for bone fracture healing[98]. Moreover, the comparison between adipose, bone marrow, and synovium-derived EVs (ADSC-EVs, BMSC-EVs, and SMSC-EVs, respectively) showed that ADSC-EVs, BMSC-EVs, and SMSC-EVs could facilitate the viability and migration of MSCs and possessed favorable capacities for chondrogenesis and osteogenesis. Among these three types of EVs, ADSC-EVs presented the best performance. The different effects between ADSC-EVs, BMSC-EVs, and SMSC-EVs could be attributed to the different factors associated with the focal adhesion, ECM-receptor interaction, actin cytoskeleton regulation, cAMP, and PI3K-Akt signaling pathways[99]. EVs constitutively expressing BMP2 could facilitate the effects of bone regeneration. BMP-engineered EVs potentiate the BMP2 signaling cascade, possibly due to an altered miRNA composition. EV functionality may be engineered by genetic modification of the parental MSCs to induce osteoinduction and bone regeneration[100]. EVs combined with biomaterials present a better effect on bone repair. Three-dimensional engineered scaffolds (PLAs) complexed with human gingival MSCs have therapeutic effects that can improve bone tissue regeneration[101]. EVs combined with tricalcium phosphate-modified scaffolds can promote osteogenic differentiation of cells and promote the recovery of cranial defects *in vivo* by activating the PI3K/Akt signaling pathway[102]. In addition to bone regeneration, MSC-EVs or platelet-rich plasma-derived EVs also have high therapeutic value for treating osteoarthritis by suppressing the inflammatory immune microenvironment. BMSC-derived exosomes can effectively promote cartilage repair and extracellular matrix synthesis and alleviate knee pain in OA rats[103]. In addition, the modification of EVs and the combination of EVs with biomaterials can enhance targeting effects and extend retention which contribute to an effectively treatment of OA [104].

PROSPECTS AND CHALLENGES OF EVS IN TRANSLATIONAL MEDICINE

Previous studies based on EVs have shed new light on the application of EVs in tissue regeneration, but there are some major problems that still need to be solved in the clinical translation of EVs. First, the storage of EVs is unstable. EVs can be stored at 80 °C for several months; however, the pH value of the storage solution and freeze-thaw cycles can affect EV activity. The transport and storage conditions of

EVs need to be further studied. Second, there is no effective method to isolate purified EVs in large quantities. At present, EVs are extracted mainly by UC, immunoadsorption, precipitation or microfluidic separation and are easily contaminated by proteins. Nowadays, there are several strategies to enhance the purity and quality of harvested EVs. From the perspective of stimulation of EVs secretion, many factors including protein regulations, thermal and oxidative stress, oxygen concentration, low pH (< 6.0), radiation, starvation can enhance the secretion of EVs from cells[105]. From the perspective of the methods of EVs isolation, how to simplify the isolation procedure and improve the EVs yield are the main two obstructions in the research field of EVs. Recent report reveals that the isolation procedure of EVs may be simplified with the improvement of newly EVs separation techniques including immunoaffinity, chromatography and polymer precipitation[106]. Moreover, with the development of commercial EVs isolation kits, EVs also can be extracted from limited sample in a short period of time. Artificial EVs is another way to realize large scale EVs generation. Artificial EVs generation technologies use the physical forces or chemicals (nitrogen cavitation[107], extrusion *via* porous membrane[108], sonication[109]) to break cells and release the cellular components. With reconstitution of the released lipids, proteins, and nucleic acids, artificial EVs can be generated in large quantity[105].

Moreover, improving the therapeutic efficiency of EVs is also a great challenge for applying EVs to clinical use. EV surface engineering can realize the EVs selective enrichment in specific cells and potentially tissues by introduction of targeting moieties[110]. To improve the therapeutic effects of EVs, a variety of methods (active drug loading, passive modification, electroporation, acoustic degradation, chemical transfection, *etc.*) can be used to modify EVs. Moreover, Cytochalasin B treatment and osmotic pressure can enhance the production of EVs with improved drug loading capacity[111]. Engineered EVs are a new strategy to enhance the expression of targeted proteins of EV-related RNA. These engineered EVs with specific targeted biomolecules can be used specifically for different therapeutic purposes, including as *in vivo* tracers or in targeted cell tracking, which could improve the efficacy of disease therapy and tissue regeneration.

CONCLUSION

In conclusion, EVs are intelligent and controllable biomaterials that can participate in a variety of physiological and pathological activities, tissue repair and regeneration by transmitting a variety of biological signals, showing great potential in cell-free tissue regeneration. Engineered EVs, which represent a clean, highly purified, and highly controllable means of achieving the sustained release of drugs, have broad prospects in future tissue regeneration engineering.

FOOTNOTES

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REVIEW

Human pluripotent stem cell-derived β cells: Truly immature islet β cells for type 1 diabetes therapy?

Helen Jiang, Fang-Xu Jiang

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Abstract

A century has passed since the Nobel Prize winning discovery of insulin, which still remains the mainstay treatment for type 1 diabetes mellitus (T1DM) to this day. True to the words of its discoverer Sir Frederick Banting, "insulin is not a cure for diabetes, it is a treatment", millions of people with T1DM are dependent on daily insulin medications for life. Clinical donor islet transplantation has proven that T1DM is curable, however due to profound shortages of donor islets, it is not a mainstream treatment option for T1DM. Human pluripotent stem cell derived insulin-secreting cells, pervasively known as stem cell-derived β cells (SC-β cells), are a promising alternative source and have the potential to become a T1DM treatment through cell replacement therapy. Here we briefly review how islet β cells develop and mature *in vivo* and several types of reported SC-β cells produced using different *ex vivo* protocols in the last decade. Although some markers of maturation were expressed and glucose stimulated insulin secretion was shown, the SC-β cells have not been directly compared to their *in vivo* counterparts, generally have limited glucose response, and are not yet fully matured. Due to the presence of extra-pancreatic insulin-expressing cells, and ethical and technological issues, further clarification of the true nature of these SC-β cells is required.

Key Words: Human pluripotent stem cells; Stem cell-derived β cells; Islet β cells; Type 1 diabetes mellitus; Cell replacement therapy

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Core Tip: Diabetes mellitus (DM) is a metabolic condition of absolute or relative deficiency in insulin. Since the discovery of insulin 100 years ago, there has been slow progress in the treatment of type 1 DM (T1DM) in clinical practice. In the scientific community however, there is much interest and progression in the research of human pluripotent stem cell derived insulin producing β-like cells, pervasively known as stem cell-derived β cells (SC-β cells). If they are determined to be genuine, scalable and functionally matured, SC-β cells have the potential to cure T1DM through cell replacement therapy.

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INTRODUCTION

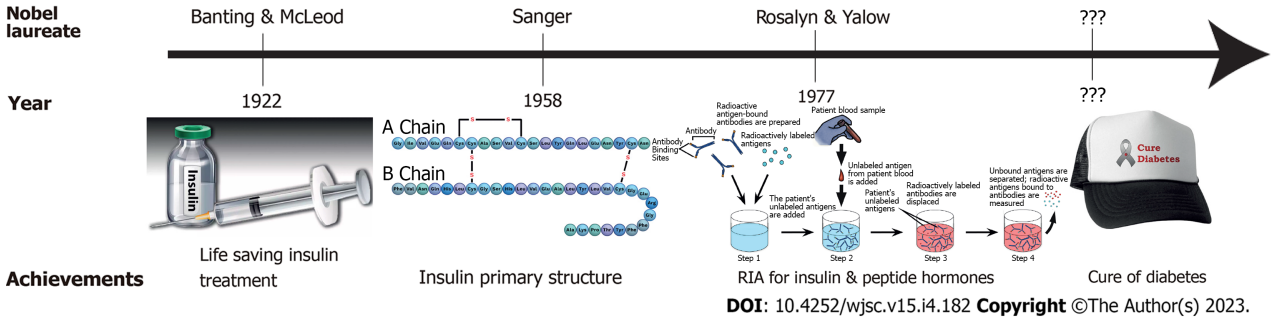
In this coronavirus disease 2019 pandemic era, there is a silent growing epidemic of significant public health burden with tremendous social and economic costs. This growing epidemic is not an infectious disease, but a chronic non-communicating metabolic disease - it is the epidemic of diabetes mellitus (DM). There was an estimated 537 million adults with DM globally in 2021[1], with the prevalence increasing each year due to the rising incidence of type 2 DM (T2DM) worldwide[2]. DM is a metabolic disorder characterised by a disruption in glucose homeostasis leading to hyperglycaemia, and broadly consists of 2 main types: T1DM and T2DM. T1DM is the absolute deficiency of insulin due to the autoimmune destruction of insulin-secreting β cells in the islets of Langerhans of the pancreas, and is usually first diagnosed in children and young adults. T2DM is the relative deficiency of insulin function due to insulin resistance in peripheral tissues, and sometimes with reduced insulin secretion due to dysfunctional or dedifferentiated β cells, usually occurring in adults.

Hyperglycaemia in T1DM and T2DM can cause various microvascular complications such as diabetic retinopathy and blindness, nephropathy and kidney failure requiring dialysis, as well as peripheral neuropathy and infected foot ulcers that lead to amputations. It can also cause macrovascular complications such as peripheral artery disease, coronary artery disease and stroke. These complications lead to significant morbidity and mortality, as well as substantial associated health and social costs[3,4].

INSULIN IS NOT A CURE FOR DIABETES

These were Sir Frederick Banting's words to the world during his Nobel Lecture for his 1923 Nobel Prize winning discovery of insulin. Subsequent discoveries on primary insulin sequences and radioimmune assay for insulin and other peptide hormones were also awarded the Nobel Prizes (Figure 1)[5]. A century later, unfortunately there is still no cure for DM, and life-long insulin replacement remains the mainstay of treatment for T1DM and controlling high blood sugar levels with antihyperglycaemic agents in most T2DM individuals. The continuous blood glucose monitoring and insulin pump known as artificial pancreas or bionic pancreas still presents the risk of developing complications, though reduced, because this and other current treatments cannot achieve physiological glucose homeostasis in patients[6,7]. These treatments themselves are also not without risks. Insulin as well as some oral anti-hyperglycaemics, such as sulfonylureas and glinides, are associated with the risk of hypoglycaemia which can lead to seizures, coma and even death[8,9]. Thus, there is a critical need for more effective and curative treatments to reduce the global burden of this disease.

The landmark proof-of-concept has demonstrated over the last 2 decades that clinical transplantation of donated human islets are able to restore β-cell function and achieve insulin independence immediately with improvement in glycaemic control and avoid the risk of hypoglycaemia episodes[10-13]. However, a large amount of approximately 340-750 million islet cells are required for successful transplantation in a patient of 68 kg weight[11,14]. Thus, the widespread application of donor islet transplantation is severely limited by the insufficient supply of human organ donor pancreases[15,16]. In addition to supply issues, another challenge to this treatment option is the prevention of transplant rejection, immune destruction and cell death of the transplanted islet cells[17]. To address the donor shortage issue, alternative scalable insulin-secreting tissues must be identified and developed. Due to their ability for theoretically infinite self-renewal and differentiation into all cell types in the body, human pluripotent stem cells (hPSCs) hold great promise for generating surrogate insulin-secreting cells *ex vivo*, pervasively known as stem cell-derived β cells (SC-β cells)[18-20] or SC-islets[21] in the literature. In order to help understand the true nature of these SC-β cells, we briefly introduce how islet β cells develop *in vivo*.



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Figure 1 Nobel prizes awarded for the endeavour towards curing diabetes. Insulin is not a cure for diabetes. Three Nobel prizes have been awarded in this endeavour[5].

IN VIVO DEVELOPMENT OF ISLET BETA CELLS

The pancreas is derived from the embryonic endoderm, one of the three germ layers, which is formed during gastrulation of embryogenesis. In addition to the pancreas, the definitive endoderm also gives rise to the liver, lung, thymus and other organs of the respiratory and digestive tracts[22]. The endoderm located in the foregut region gives rise to the dorsal and ventral buds of the pancreas which rotate to form one organ, then pancreatic epithelium is induced and expands, from which endocrine progenitors arise. The endocrine progenitors then differentiate into the β cells that secrete the hormone insulin, α cells that secrete the hormone glucagon, δ cells that secrete the hormone somatostatin, ε cells that secrete the hormone ghrelin, and PP cells that secrete the hormone pancreatic polypeptide. The pancreatic endocrine cells start to organize into clusters forming islets before birth, and the Islets of Langerhans become fully formed at around 2-3 wk after birth[22]. Human islets are made up of 40%-60% β cells and 30% α cells[22]. The adult pancreas is made up of exocrine cells that secrete digestive tract enzymes, duct cells that make up the ductal tree to transport digestive enzymes and islet cells that secrete hormones into the bloodstream for glucose homeostasis[23].

Mechanistically, the pancreatic islets are initiated by the transient expression of a high level of the transcription factor neurogenin-3 (NGN3)[24]. NGN3 is important in committing all pancreatic endocrine cell types, the deficiency of which leads to the absence of pancreatic endocrine cells[25,26]. The molecular mechanisms for the development of each pancreatic endocrine cells are not completely defined, however it is suggested that insulin-producing β cells are differentiated from the pancreatic progenitors that express transcription factor genes pancreatic and duodenal homeobox 1 (PDX1) and NK6 homeobox 1 (NKX6-1), and then turn on NGN3[27,28]. There are several β cell transcription factors, including PDX1, NKX6-1 and MAF BZIP transcription factor A (MAFA), which play a critical role in activating insulin transcription and regulating insulin secretion[29-31].

PDX1 is a homeodomain transcription factor homogenously expressed in the early pancreatic bud and its expression persists into mature β cells; the absence of PDX1 leads to agenesis of the pancreas [32]. NKX6-1 and the helix-loop-helix transcription factor Beta2/NeuroD determine islet cell differentiation during embryogenesis, and maintain specific islet cell hormone expression in adults[32]. Knockout of mouse *Nkx6-1* gene leads to a significant inhibition in the formation of β cells[33]. NeuroD is initially expressed in pancreatic epithelium during development, before being expressed in $NGN3^+$ endocrine progenitors, and finally exclusively expressed in β cells after birth. The absence of Beta2/NeuroD leads to reduced mouse endocrine cells, in particular β cells, increased apoptosis and arrestment in islet morphology[25,34]. Beta2/NeuroD is also a critical transcriptional activator of the insulin gene[35,36].

β-cell maturation including maturation of other clinically important cell types is a postnatal development process. For example maturation of mouse and human β cells takes place approximately 3 wk[37] and 26-44 wk after birth[38,39] respectively. The maturation process is controlled by transcription factors and exhibited in maturing at the gene, protein, subcellular, intercellular and metabolic levels.

ISLET BETA CELL MATURATION REGULATED BY TRANSCRIPTION FACTORS

Following β-cell specific NeuroD deletion, the mice developed glucose intolerance and the islets displayed features of foetal/neonatal β cells such as overexpression of glycolytic genes, lactate dehydrogenase (LDHA), Neuropeptide Y, and higher basal insulin secretion and oxygen consumption due to the reliance on oxidative metabolism of glucose[40-46]. That is, the glucose metabolic profile of mouse β cells without NeuroD was equivalent to immature β cells. The mutations of NeuroD cause maturity onset diabetes of the young[22,33]. NeuroD is also critical for maintaining a matured functional state of

islet β cells[40]. These data suggest that NeuroD regulates islet β-cell maturation, though its postnatal dynamic expression profile is not available. Thus, identification of Beta2/NeuroD activators may help mature hPSC-derived insulin-secreting cells *ex vivo*.

MafA is another transcription factor being demonstrated to regulate the maturation of islet cell organisation, β cell mass and β cell function from 3 wk of age in mice using the gene targeting strategy [47]. MafA expression reaches their adult levels at 3 mo in rats[48] coinciding with the obtaining of mature glucose stimulated insulin secretion (GSIS). Aguayo-Mazzucato *et al*[49] were the first to demonstrate that MAFA overexpression and the thyroid hormone triiodothyronine (T3) treatment are able to increase human foetal islet-like clusters, insulin secretion at 16.8 mmol/L glucose and proinsulin-to-insulin processing. Chromatin immunoprecipitation experiment showed binding of thyroid receptors to MafA promoter, thereby confirming that T3 directly regulates the expression of MafA[50]. The thyroid hormone receptor is also demonstrated to be expressed on human mature islets [51], though its postnatal development profile is unknown.

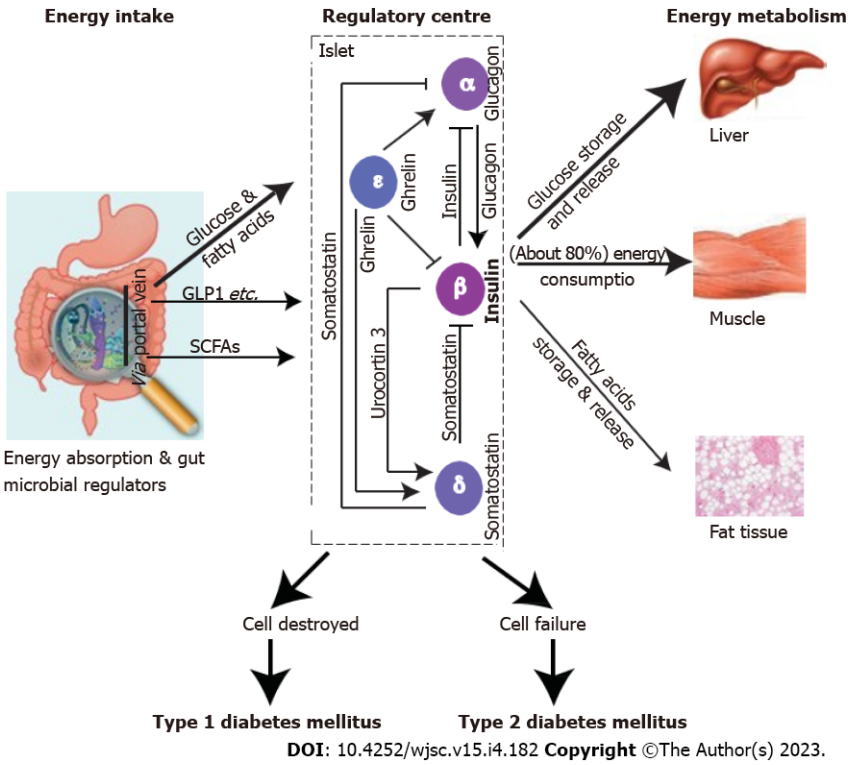
Furthermore, a recent study shows that the expression of the orphan nuclear transcription factor estrogen-related receptor gamma (ERRγ) is a hallmark of mature β cells[52]. ERRs consist of three paralogs in mammals, namely ERRα (NR3B1 or Esrrα), ERRβ (NR3B2 or Essrβ) and ERRγ (NR3B3 or Essrγ). ERRγ is progressively upregulated in mouse islets from 2 to 6 wk of age (5-fold higher in adults compared to neonatal β cells) and ERRγ transcriptional network promotes mitochondrial oxidative metabolism in mouse β cells, required for functional maturation of β cells and glucose homeostasis[52]. Mice with β cell-specific ERRγ deletion failed to develop a mature GSIS. With the developmentally deleted β cell-specific ERRγ knockout mouse islets, RNA sequencing (RNA-seq) revealed that the expression of 4189 genes were altered, with almost equal numbers of genes down- and up-regulated (2008 and 2182 genes respectively). Gene ontology analysis revealed that ERRγ-regulated genes are associated with processes critical for β cell function including ATP biosynthesis, cation transport, oxidative phosphorylation, electron transport and secretion[52]. However, data is not available on postnatal developmental expression of ERRs in human islet cells, which will have to be addressed in the near future. Identification of ERRγ activators may help mature hPSC-derived insulin-secreting cells *ex vivo*.

Expression of the *Sine Oculis* family of homeodomain transcription factors SIX2 and SIX3 increased with age in the human pancreatic islet β cells[53]. SIX2 and SIX3 are localised to the nucleus of adult human β cells but not detected in juvenile (under 9 years of age) β cells[53]. Using gain-of-function experiments in human β cell line, the EndoC-bH1 cells or primary juvenile human islets, evidence has demonstrated that expression of SIX2 or SIX3 were sufficient to enhance cardinal functions of human β cells[53]. Identification of SIX2 and SIX3 activators may therefore help generate matured hPSC-derived insulin-secreting cells *ex vivo*.

METABOLIC MATURATION OF ISLET BETA CELLS

Although rat islets acquired GSIS by postnatal day 21, a mature GSIS was only achieved by 3 mo[54], coinciding with the time when insulin dynamics reaches their adult levels[48]. The metabolic maturation is underscored by genes of important metabolic players in β cells such as glucose transporter 2, glucokinase, glucagon-like peptide-1 receptor and prehormone convertase 1 (encoded by *Pcsk1*) that were expressed from very low levels at postnatal day 2 to higher levels with increased age[48]. Similarly, the metabolic maturation is also underscored by genes transcribing for malate dehydrogenase, glycerol-3-phosphate dehydrogenase, glutamate oxaloacetate transaminase, pyruvate carboxylase and carnitine palmitoyl transferase 2 from much lower levels at neonatal postnatal day 2 to high levels at day 28[55]. In the same period, genes encoding proliferation regulators in β cell genes encoding platelet-derived growth factor receptor A, platelet-derived growth factor receptor B, platelet-derived growth factor B and fibroblast growth factor (FGF) receptor 1 are progressively downregulated. Mature β cells tend to have lower levels of LDHA and glycolytic genes, as it is downregulated by NeuroD from embryonic to adult islets, which then appears to possess the ability to correspond glucose metabolism with insulin secretion[56-58].

The molecular mechanism of mature β-cell secretion is well understood. The higher blood glucose levels stimulate its active transportation into the β cell cytoplasm, increasing the ATP/ADP ratio through glycolysis and oxidative metabolism and triggering the depolarisation of the β cell membrane and opening the voltage-gated Ca^{2+} channel. The AMP-activated protein kinase (AMPK) is a highly conserved sensor of intracellular adenosine nucleotide levels that is activated even with modest decreases in ATP production resulting in relative increases in AMP or ADP. In response, AMPK promotes catabolic pathways to generate more ATP, and inhibits anabolic pathways. The increase in cytoplasmic Ca^{2+} triggers the fusion of insulin granules with the plasma membrane for exocytosis of insulin contents. Coordinating with other islet cells, mature β cells produce insulin in adequate amounts and timeliness to maintain plasma glucose within a narrow concentration range[39,59]. Thus, β cell function is critical for mature GSIS. Destruction and failure of islet β cells will lead to T1DM and T2DM, respectively (Figure 2).



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Figure 2 Mature islets are a regulatory centre for glucose homeostasis. A simplified graphic representation of how mature pancreatic islets regulates glucose homeostasis. Skeletal muscle is the largest organ in the body (45%-55% body mass) and consumes about 80% insulin. GLP1: Glucagon-like peptide-1; SCFAs: Short-chain fatty acids.

MATURATION MARKERS OF ISLET BETA CELLS

To help with the characterization of whether hPSC-derived insulin-secreting cells *ex vivo* are matured, we briefly summarize maturation markers for *in vivo* islet β cells. Over the last decade, several potential markers for maturation of immature islet β cells were discovered. Blum *et al*[60] were the first to demonstrate that functional islet β cell maturation is marked by expression of the corticotropin-releasing factor family peptide urocortin 3 (UCN3), along with an increased glucose threshold.

Mature rat β cells expressed significantly higher levels of the gap junction connexion 36 gene (*Cx36*, also known as *Gjd2*) compared to neonatal immature counterparts, corresponding to a significantly higher membrane density of gap junctions and greater intercellular exchange of ethidium bromide[61]. Human mature islets predominantly express CX36 at mRNA and protein levels with β cell membrane harboring detectable levels of CX36 gap junction proteins[62]. Though the developmental profile of human islet CX36 is unknown, we speculate that the dynamic pattern of CX36 expression from human neonatal to mature β cells is similar to that in rats and CX36 is a potential maturation marker for matured hPSC-derived insulin-secreting cells *ex vivo*.

Our group recently showed that claudin 4 is the only tight junction molecule family member highly upregulated in the postnatal mouse islets and global deletion of this gene affects mature GSIS in a sex difference manner[63]. Thus, claudin 4 may also be a maturation marker for matured hPSC-derived insulin-secreting cells *ex vivo*.

THE DIFFERENTIATION OF SC-BETA CELLS *EX VIVO*

The advent of hPSC provided an important opportunity to overcome major challenges of clinical islet transplantation therapy through its accessibility, theoretically unlimited self-renewability and the boundless potential to generate an alternative source of donor insulin-secreting cells *ex vivo*[64,65]. The generated insulin-secreting cells can also be used for disease modelling and pharmaceutical drug testing to help establish therapeutics that improve cell function, survival and proliferation. Insulin-secreting cells differentiated from hPSCs that include human embryonic stem cells (hESC) and induced hPSCs (ihPSC) are ubiquitously termed as SC-β cells[18-20] in the literature. hESCs are generated from the inner cell mass of human blastocysts and have the infinite ability to proliferate as undifferentiated cells or differentiate into cells of all ectoderm, mesoderm or endoderm lineages[66,67].

Over recent years, there have been various protocols developed of *ex vivo* differentiation of SC-β cells [18-20] and SC-islets[18,21]. Thus far, hPSCs have been differentiated towards SC-β cells through a step-wise manner emulating *in vivo* pancreatic embryonic development[68-72]. The differentiation of hPSCs towards SC-β cells have been achieved with the application of growth factors, proteins or molecules to modulate signaling pathways to progress through each stage of pancreatic development, and is usually measured by expression of a couple of key transcription factors or C-peptide[22]. hPSCs (characterised by expression of Oct4) are first differentiated into definite endoderm cells expressing FOXA2 and SOX17 through application of a mix containing Wnt, activin A, inducer of definite endoderm, wortmannin, and sodium butyrate[22]. Then application of FGF10 and FGF7 differentiates the definite endoderm into gut tube endoderm expressing HNF1B and HNF4A[22]. The differentiation mixture containing retinoic acid, noggin KAAD-cyclopamine, FGF, and indolactum V leads to differentiation into pancreatic progenitors expressing PDX1 and HNF6, which further differentiates into endocrine progenitors (NKX6-1, NGN3, NKX2-2, PTF1A), and finally into β-cells (characterised by presence of C-peptide and insulin)[22]. We here summarize several representative protocols used to generate SC-β cells and SC-islets (Table 1).

Pagliuca *et al*[73]'s differentiation protocol was the first using specific and cocktail of inducing factors to differentiate hPSCs sequentially through 6 stages into SC-β cells (Table 1). At stages 5 and 6, there is however significant heterogeneity in the final population containing SC-β and SC-α cells, as well as SC-endocrine cells (resembling enterochromaffin cells) and non-endocrine cells (*e.g.*, exocrine cells such as pancreatic acinar, mesenchymal and ductal cells)[18]. Nevertheless, these cells appear to be stable, maintaining their identity as evidenced by their global transcriptional profiles during stage 6 cultures. At this stage, they also express the maturation marker SIX2 but several other β cell markers of maturity are not expressed such as UCN3, MAFA and SIX3[18].

The Velazco-Cruz *et al*[19]'s protocol was built upon and modified Pagliuca *et al*[73]'s protocol, and demonstrated that the SC-β cells had improved insulin secretion and greater gene expression of β cell markers compared to the cells generated with Pagliuca *et al*[73]'s protocol, but still much less than the average human islet (Table 1). Follow-up studies with the addition of differentiation factors or changes to the differentiation processes were unfortunately unsuccessful in producing more functional SC-β cells equal to human islet β cells[74-76].

In Balboa *et al*[20]'s protocol, the SC-islets had similar cytoarchitecture and functional insulin secretion pattern to islet β cells, though with immature glucose-induced mitochondrial respiration and instead retained pyruvate sensitivity - thus the SC-islets were not completely similar to functional adult islets (Table 1). Balboa *et al*[20]'s SC-β cells showed heterogeneous mature β cell marker expression, required further maturation *in vivo* after transplantation, showed upregulated expression of CHGB and MAFA after 6 mo, and did not express adult β cell factors RBP4 and SIX3[20].

Nevertheless, studies indicate that several current pancreatic progenitor differentiation protocols promote precocious endocrine commitment; ultimately resulting in the generation of non-functional polyhormonal cells[74]. The efficiency of differentiation decreases with each step, and at the final step there are very small amounts of SC-β cells that have a low insulin content, co-express insulin and glucagon, and usually respond poorly to glucose stimulation[22,70]. It was also found that these SC-β cells have little to no expression of maturation genes including MAFA and G6PC2[18,73,77]. Following transplantation, the amount of insulin secreted by SC-β cells rises[73,77,78] and the previously low or non-expressed genes of islet β cells such as MAFA, G6PC2, MNX1 and INS increases[79].

Cell purification steps will increase the safety of, and ability to upscale the manufacture of β cells. However, there are difficulties in including this step in large-scale manufacturing processes for production of reproducible PSC-derived cellular products with less variability in composition and function[80]. Several cell surface markers have been used to purify different developmental stages of PSC-derived cells[80]. Markers used include CD177 for anterior definitive endoderm cells[81], CD142, CD24 and glycoprotein 2 for pancreatic progenitors[82-85], CD49a for SC-β cells[18], and CD9 for negative selection of SC-β cells[86]. Monoclonal antibody against extracellular domain of claudin 4 might help enrich matured SC-β cells differentiated *ex vivo*.

Finally, a few maturation factors have proven useful in maturing SC-β cells *ex vivo*. For example, T3 enhanced the MAFA expression in the SC-β cells, and increased insulin content and insulin secretion at 16.8 mmol/L glucose[49]. Using an adenoviral ERRγ vector, overexpression of ERRγ increased glucose-stimulated C-peptide secretion in hPSC-derived insulin-secreting cells, thus may promote their functional maturation[52]. Therefore, identification of molecules that activate NEUROD, ERRγ, SIX2 and SIX3 will be important. Application of the activators individually or in combination may indeed promote functional maturation of genuine SC-β cells.

CURRENT *EX VIVO* SYSTEMS ARE DISTINCT FROM *IN VIVO* ISLET DEVELOPMENT NICHES

The current PSC differentiation protocols for insulin-secreting cells are mostly bulk cultures and consist of cocktails of inducing factors, which are generally based on accumulative knowledge generated from using the animal model systems. In these bulk cultures, there are cells types in the targeted lineage as

Table 1 A summary of several differentiation protocols for generating stem cell-derived β cells *ex vivo*

Differentiation protocol	Marker expression	Characterization	Ref.
The stem cell-derived β cell protocol is a 6-stage differentiation protocol using specific inducing factors for each stage (11 factors) to produce SC-islets from hPSCs, in a 3D suspension-based cell culture system (4-5 wk)	Pancreas progenitor marker: PDX1 (about 90% at stage 3). Endocrine markers: C-peptide, CHGA and NKX6-1. SC-β cell markers: INS, NKX6-1, ISL1, and others	Immunofluorescence. Ultrastructure. Insulin packaging into secretory granules. qRT-PCR. Static and dynamic GSIS. Functional test. Glucose-responsive Ca^{2+} flux, KCl depolarisation, ameliorate hyperglycaemia in diabetic mice. scRNA-seq. Purified SC-β cells with anti-CD49a	Pagliuca *et al*[73], 2014; Veres *et al* [18], 2019
Optimised 7 stage *in vitro* differentiation protocol of serial culture steps using factors such as vitamin C, ALK5 inhibitor, TGF-β receptor inhibitors, thyroid hormone (T3), R428 (AXL inhibitor), N-acetyl cysteine, Notch and BMP signalling inhibitors	Pancreas progenitor marker: PDX1 (about 90% at stage 3), NKX6-1. Endocrine markers: C-peptide, CHGA, NEUROD1, NKX2-2 and NKX6-1. β cell markers: INS, NKX6-1, ISL1, MAFB. Mature β cell markers: MAFA, ABCC8, IAPP, HOPX, NEFM, SIX2, G6PC2	Immunofluorescence. Transmission electron microscopy. qRT-PCR. Static and dynamic GSIS. Functional test in diabetic mice. Flow cytometry. Immunohistochemistry. Diabetes reversal within 40 d in mice. Metabolic analysis. Perifusion assay. Calcium imaging.	Rezania *et al*[78], 2014
Scalable 3D suspension culture system based on previous methods [101,102], with the addition of retinoic acid, cyclopamine (SHH inhibitor), Noggin (BMP inhibitor), then treatment with epidermal growth factor, KGF and Noggin (EKN). Followed by exposure to a cocktail of factors *e.g.*, TBP, ALK inhibitor, Noggin, TANK	Pancreas progenitor marker: PDX1 and NKX6-1 (90%). Endocrine markers: C-peptide, NEUROG3, NKX2-2 and NKX6-1. β cell markers: INS, NKX6-1, NKX2-2, PDX1. Mature β cell markers: MAFA, MAFB, PDX1, NKX6.1, NXK2.2, ISL1, PAX6, NEUROD1, and CHGA	Immunofluorescence. Transmission electron microscopy. qRT-PCR. Static and dynamic GSIS. Insulin biosynthesis and glucose metabolism, blood glucose reduction but not reversal of diabetes in mice. Flow cytometry. Western blot analysis. Statistical analysis	Russ *et al*[74], 2015
Six-stage differentiation strategy modulating TGF-β signaling by modulating Alk5i exposure, combined with controlling cell cluster size and use of enriched serum-free media culture	Pancreas progenitor marker: PDX1. Endocrine markers: CHGA (96%), C-peptide (73%), NKX6-1. SC-β cell markers: INS, CHGA, NKX2-2, PDX1, NKX6-1, MAFB, GCK, and GLUT1	Immunofluorescence. qRT-PCR. Static and dynamic GSIS. Flow cytometry. Light microscopy. Glucose responsive, first- and second-phase insulin release, improved glucose tolerance in mice. Western blot. Perifusion assay. Glucose tolerance test	Velazco-Cruz *et al* [19], 2019
Optimised differentiation protocol combining previous protocols. Changes made: Differentiation of hPSCs in adherent conditions until pancreatic progenitor stage. Then optimised with nicotinamide, epidermal growth factor, activin A and a ROCK inhibitor; a microwell aggregation step; and a final maturation step in suspension culture	Pancreas progenitor marker: PDX1. Endocrine markers: C-peptide. SC-β cell markers: INS. Mature β cell markers: INS, G6PC2, SIX2, GLIS3, RBP4, SIX3. HOPX, UCN3, IAPP, CPE and FXYD2 upregulated post engraftment. CHGB and MAFA upregulated 6 mo post-engraftment. B cell differentiation: SIX2, HOPX, ZBTB20. Insulin secretion genes: PCSK1, CPE, CHGB, ABCC8, FXYD2, GABRA2	Immunohistochemistry. Flow cytometry. Perifusion assay. Respirometry. Transmission electron microscopy. Electrophysiology. Exocytosis imaging. $[Ca^{2+}]_i$ imaging. $[cAMP]_m$ imaging. Metabolite tracing analysis. Ratiometric analysis. Transplantation study. scRNA-seq transcriptomic profiling. Glucose responsive biphasic insulin secretion. Glucose tolerance test	Balboa *et al*[20], 2022
Differentiation protocol using hCiPSC-islets by optimising pancreatic progenitor to β cells fate commitment by modulating signaling pathways and reconstructing islet spatial structure through 3D cell aggregates of posterior foregut-committed cells and combination of ISX9 and Wnt-C59 at stage 5	Pancreas progenitor marker: PDX1. Endocrine markers: C-peptide, CHGA and NKX6-1. β cell markers: PDX1, NKX6.1 and NKX2.2. Mature β cell markers: MAFA, UCN3	Immunofluorescence. qRT-PCR. Transmission electron microscopy. Static and dynamic GSIS. Glucose-stimulated calcium flux assay. Flow cytometry. scRNAseq. Glucose responsive biphasic insulin secretion, decrease HbA1c, restore endogenous C-peptide secretion. Glucose tolerance tests. Preclinical diabetic non-human primate transplantation study. Fasting blood glucose levels. Glycated HbA1c. scRNA-seq. Teratoma assay. Karyotype analysis. Calcium imaging. Cryo-electron microscopy. ELISA	Du *et al*[21], 2022

PDX1: Pancreatic and duodenal homeobox 1; SC-islets: Stem cell-derived islets; hPSC: Human pluripotent stem cells; C-peptide: Connecting peptide; CHGA: Chromogranin A; NKX6-1: NK6 homeobox 1; qRT-PCR: Quantitative real time-polymerase chain reaction; GSIS: Glucose-stimulated insulin secretion; SC-β cell: Stem cell-derived β cell; INS: Insulin gene; ISL1: ISL LIM homeobox 1; Ca^{2+}: Calcium; KCl: Potassium chloride; scRNA-seq: Single-cell RNA sequencing; anti-CD49a: CD49a (Integrin alpha 1) antibody; TGF-β receptor inhibitor/ALK5i: Transforming growth factor β-receptor I/activin receptor-like kinase 5 inhibitor; R428 (AXL inhibitor): A selective small-molecule inhibitor of AXL (bemcentinib, BGB324); BMP: Bone morphogenetic protein; NEUROD1: Neurogenic differentiation 1; NKX2-2: NK2 homeobox 2; MAFA: MAF BZIP transcription factor A; MAFB: MAF BZIP transcription factor B; ABCC8: ATP Binding Cassette Subfamily C Member 8; IAPP: Islet amyloid polypeptide; HOPX: HOP homeobox; NEFM: Neurofilament medium chain; SIX2: SIX homeobox 2; G6PC2: Glucose-6-phosphatase catalytic subunit 2; KGF: (FGF7) Keratinocyte growth factor; TBP: TATA-binding protein; TANK: TRAF family member associated NFKB activator; PAX6: Paired box 6; GCK: Glucokinase; GLUT1: Glucose transporter 1; ROCK inhibitor: Rho-kinase inhibitor; GLIS3: GLI-similar 3/zinc finger 3; RBP4: Retinol binding protein 4; SIX3: SIX homeobox 3; UCN3: Urocortin 3; CPE: Carboxypeptidase E; FXYD2: FXYD domain containing ion transport regulator 2; cAMP: Cyclic adenosine monophosphate; ZBTB20: Zinc finger and BTB domain containing 20; PCSK1: Proprotein convertase subtilisin/kexin type 1; GABRA2: Gamma-aminobutyric acid type A receptor subunit alpha-2; hCiPSC: Human chemically induced pluripotent stem cells; ISX9: Isoxazole-9; Wnt-C59: Nanomolar inhibitor of mammalian PORCN acyltransferase activity and blocks activation of all evaluated human Wnts; HbA1c: Hemoglobin A1c; ELISA: Enzyme-linked immunosorbent assay.

well as unwanted lineages. One or two *in vivo* biomarkers are selected based on *in vivo* islet lineage development to characterise targeted cells at different differentiated stages *ex vivo*. However, these *in vivo* biomarkers should not be extrapolated as biomarkers for the *ex vivo* differentiation conditions because of clear differences in spatiotemporal and microenvironment niches between the *in vivo* development and *ex vivo* differentiation (Figure 3). In other words, we do not yet fully understand the full regulatory program, or the molecular details of the 3D microenvironment niche for specific islet lineage development *in vivo* to guide the specific differentiation of hPSCs into insulin-secreting cells *ex vivo*.

EXTRAPANCREAS INSULIN-SECRETING CELLS

Perhaps the research community have also forgotten the fact that in our body, extra-pancreas insulin-secreting cells exist, which may complicate the efforts of generating genuine SC-β cells. Subverted to general knowledge, approximately a quarter of human foetal enteroendocrine K/L cells were recently shown to express high levels of insulin and other β cell genes including the transcription factor PDX1, by using samples of foetal and neonatal human small intestines derived from the endoderm during development[87]. Notably, the expression of UCN3 in the human foetal enteroendocrine K/L cells was higher than in foetal human pancreatic β cells[87]. These results were confirmed with single molecule fluorescence *in-situ* hybridisation of insulin mRNA combined with immunofluorescent antibody staining of the insulin protein[87]. Secondly, thymocytes that are derived from the foregut, adjacent to which gives rise to the pancreas, normally produce insulin to induce self-tolerance and protect the body from the autoimmune destruction of pancreatic insulin-secreting β cells[88]. Lastly, though the central nervous system is an ectoderm-derived organ, the neuronal progenitors derived from adult hippocampus and the olfactory bulb were demonstrated to undergo insulin biosynthesis[89]. Human *INS* mRNA expression is also detected in the hippocampus, amygdala and temporal lobe in addition to the olfactory bulb, cerebellar and pontine regions[90]. A historical account of the extrapancreas insulin-secreting cells is referred to in a recent review article[88]. These data suggest that it is possible that the current reported SC-β cells contain a varied percentage of non-pancreatic insulin-secreting cells. Future studies are required to increase the percentage of genuine insulin-secreting β-like cells in the *ex vivo* systems.

SC-BETA CELLS TRANSPLANTED INTO NON-HUMAN PRIMATES

To further test their functions, the chemically induced SC-islets were recently intraportally transplanted into immunosuppressed adult diabetic rhesus macaques[21]. Three months after the SC-islet transplantation, all four macaques reportedly had improvements in diabetic symptoms, glycaemic control, fasting blood sugar levels, hemoglobin A1c (HbA1c), and reduced exogenous insulin requirements[21]. However, after 5-6 mo, two of the macaques developed graft failure (the other two macaques died of immunosuppression-related complications)[21]. Autopsy conducted on the macaques found no evidence of teratoma or tumorigenesis, but levels of β cells had fallen. The authors concluded that the immunosuppression regimen used was not appropriate in preventing immune attack against the grafts[21]. Whether the short-term improvements in diabetic rhesus macaques are related to the immaturity of grafted SC-islets and/or the presence of non-pancreatic insulin-secreting cells needs to be determined in the future.

SC-BETA CELLS IN CLINICAL TRIALS AS A T1DM THERAPY

The first hPSC-derived, differentiated cell replacement T1DM therapy product named VX-880 was approved by the United States Food and Drug Administration for phase 1/2 clinical trials in March 2021. The VX-880 are SC-islets for T1DM patients with certain indications; that is, impaired hypoglycaemic awareness and severe hypoglycaemia[91]. The preliminary outcomes of the clinical trials were presented in June 2022 at the American Diabetes Association 82nd Scientific Sessions by Vertex, a United States Pharmaceutics company[91]. A half-dose of VX-880 in two patients was able to achieve glucose-responsive insulin secretion, significantly improve time-in-range (the amount of time that blood glucose level is measured to be within target blood sugar range 70-180 mg/dL or 3.9-10 mmol/L), reduce exogenous insulin requirements and improved HbA1c[91]. VX-880 was also well tolerated although with some largely mild or moderate adverse reactions[91]. For example, patient 1 showed blood glucose time-in-range change from 40.1% on 34.0 units per day of exogenous insulin at baseline to 99.9% and insulin independence at day 270 onwards. Patient 2 showed blood glucose time-in-range change from 35.9% on 25.9 units per day of exogenous insulin at baseline to 51.9% with a 30% reduction in exogenous insulin use at day 150[91]. Whereas these results are very promising, VX-880 requires a

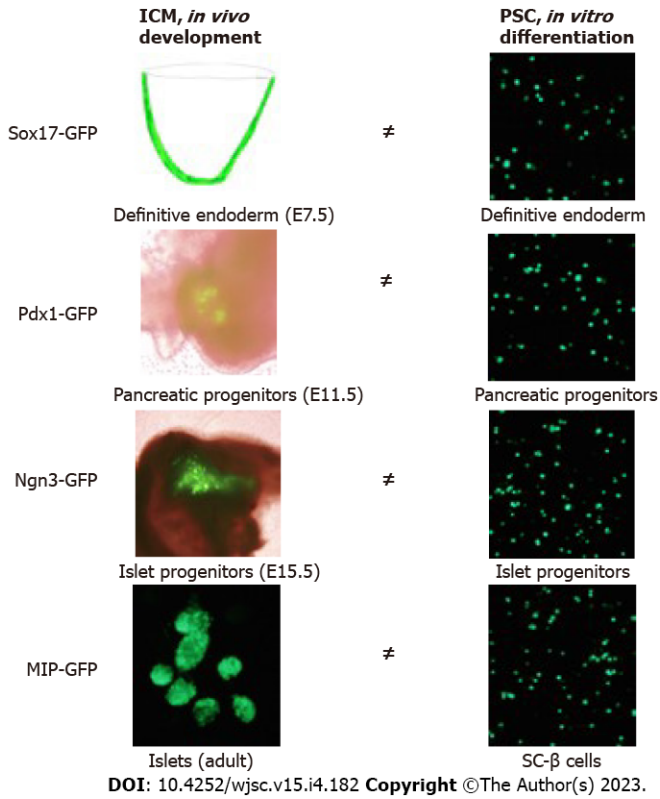


Figure 3 There are clear differences in spatiotemporal and microenvironment niches between the *in vivo* development and *ex vivo* differentiation of islet lineages. Details of lineage tracing mouse lines can be found in articles[103,104]. ICM: Inner cell mass; PSC: Pluripotent stem cell; Sox17: SRY-box transcription factor 17; GFP: Green fluorescent protein; PDX1: Pancreatic and duodenal homeobox 1, also known as insulin promoter factor 1; NGN3: Neurogenin-3; MIP: Mouse insulin 1 promoter; SC-β cells: Stem cell-derived β cells.

lengthy *in vivo* maturation period for blood sugar control (in patient 1) in contrast to donated islets retrieved from deceased persons, which achieved immediate insulin independence after transplantation into recipients[10-13]. The lengthy *in vivo* maturation period of grafted VX880 is a strong independent indicator that these SC-β cells are immature. It is also premature to claim the VX-880 SC-β cells are all genuine immature counterparts of islet β cells, as the duration and longevity of insulin independence was not yet available at the time of writing this article.

CONCLUSION

Immaturity of PSC-derived cells is a general obstacle, not only in the case of SC-β cells and SC-islets, but also other clinically important cell types[92]. Maturation biology is the final frontier in stem cell biology, of which our knowledge is still in its infancy. As summarised in Table 1, multiple hPSC differentiation protocols have been used in different laboratories. Consequently, off-target differentiation and aberrant differentiation from these protocols are more likely unavoidable, resulting in only a low frequency of genuine SC-β cells. Furthermore, the stage-specific differentiation factors selected may direct non-specific spatiotemporal differentiation, thus resulting in multiple cell types of the endodermal germ layer and even neuronal lineage origins. This may result in some differentiating cells along unwanted pathways and give rise to extrapancreas insulin-secreting cells. On the other hand, unwanted or off-target differentiated cellular products have accumulated in the bulk culture protocols and not been excluded for subsequent differentiation steps, which further increases the possibility of compromising the characterization through use of one or two developmental markers of *in vivo* cellular lineages. Finally, in addition to the above, there are still other challenges in this exciting field of research, such as ensuring SC-β cell survival post-transplantation given the highly vascularised islets are susceptible to ischaemic injury and loss of cell mass[93,94]. Developing methods that evade autoimmune attack in T1DM patients without the use of lifelong immunosuppression would be valuable[95].

Stage-specific *in vivo* pancreatic and islet lineage cell types would provide ideal positive controls for the *ex vivo* hPSC-derived insulin-secreting cells. Nevertheless, the human ethics issues and lack of human embryonic and foetal pancreatic tissues available prevent such reliable and precise comparison to be made between the islet lineage cells and the PSC-derived cells. However, future efforts should be made to resolve these issues. Similarly, it would be wise not to solely concentrate on undertaking

human β cell differentiation and maturation studies from hPSCs. Instead, investigating β cell differentiation and maturation from model animals will be invaluable and will facilitate the realisation of a curative stem cell therapy for people with T1DM.

In order to minimise confusion between the *ex vivo* differentiated insulin-secreting cells and islet β cells, our laboratory proposed a 4-criterial post-genomic concept for naming "β cells" a few years ago [96]. Recently, Kaestner *et al*[97] described many islet biologists/scientists much like the "Parable of the Blind Men and the Elephant" in terming "β cells". This appears to be the case in respect to claims made about SC-β cells without proper positive controls of corresponding *in vivo* islet lineage cells. Kaestner *et al*[97] further proposed six salient features of normal, fully functional mature β cells and made a recommendation to not name PSC-derived insulin-producing cells as "β cells", but conservatively as insulin-producing cells, insulin$^+$ cells or β-like cells, when there is no clear evidence that the six features of *in vivo* β cells are met.

The degree of single-cell RNA-seq (scRNA-seq) data similarity between the SC-β cells and donated islet β cells remains largely unclear. First, all scRNA-seq datasets of SC-β cells lacked a direct positive control from *in vivo* pancreatic and islet lineage cells. Second, most current scRNA-seq methods provide a high throughput but sacrifice full transcript coverage and sensitivity[98]. Third, as barcodes/inducers are introduced by the template switching of reverse transcriptase, strand invasion becomes problematic through systematic bias, namely biases from the introduction of artefacts. Fourth, loss of cDNA synthesis and bias in cDNA amplification leads to severe quantitative errors of these scRNA-seq methods[99]. Fifth, the current scRNA-seq methods suffer from impaired mRNA accounting. However, molecular spikes have significantly improved single cell mRNA accounting[100], adoption of the molecular spike method and further improvements may help address the above issues. As such, genuine SC-β cells will eventually become available as donor cells for establishing curative therapies for people suffering from T1DM in the not too distant future.

FOOTNOTES

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REVIEW

Mechanisms of analgesic effect of mesenchymal stem cells in osteoarthritis pain

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Abstract

Osteoarthritis (OA) is the most common musculoskeletal disease, and it is a major cause of pain, disability and health burden. Pain is the most common and bothersome presentation of OA, but its treatment is still suboptimal, due to the short-term action of employed analgesics and their poor adverse effect profile. Due to their regenerative and anti-inflammatory properties, mesenchymal stem cells (MSCs) have been extensively investigated as a potential therapy for OA, and numerous preclinical and clinical studies found a significant improvement in joint pathology and function, pain scores and/or quality of life after administration of MSCs. Only a limited number of studies, however, addressed pain control as the primary end-point or investigated the potential mechanisms of analgesia induced by MSCs. In this paper, we review the evidence reported in literature that support the analgesic action of MSCs in OA, and we summarize the potential mechanisms of these antinociceptive effects.

Key Words: Osteoarthritis; Pain; Inflammation; Mesenchymal stem cells; Regeneration; Analgesic mechanisms

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Core Tip: Osteoarthritis (OA) is the most common musculoskeletal disease, and it is a major cause of pain, disability and economic burden. Pain is the most common and bothersome presentation of OA, but its treatment is still suboptimal, which highlights the need for new analgesic agents for OA. Mesenchymal stem cells (MSCs) have been extensively investigated as a potential therapy for OA due to their regenerative and anti-inflammatory properties. The administration of MSCs resulted in significant improvement in joint pathology and function, pain scores and/or quality of life in numerous preclinical and clinical studies. Only a limited number of studies, however, addressed pain control as the primary end-point or investigated the potential mechanisms of analgesia induced by MSCs. So, this paper reviews literature for evidence of analgesic actions of MSCs in OA, and summarizes the potential mechanisms of these anti-nociceptive effects

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INTRODUCTION

Osteoarthritis (OA) is a disease of movable joints characterized by anatomic and/or physiologic derangements including cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function. It is initiated by micro- and macro-injury of the joint, which activates maladaptive repair responses producing abnormal tissue metabolism[1].

Clinical manifestations of OA include joint pain, tenderness, limitation of movement, coarse crepitus and, occasionally, effusion and mild local inflammation[2]. Diagnosis depends on the observation of signs and symptoms through clinical examination[3]. X-ray can be performed to help with differential diagnosis and in case of atypical features[4]. However, radiologic findings do not always complement the clinical presentation of pain[5].

Pathophysiology of OA is complex and is based on cartilage degeneration preceded by subchondral bone lesions. Historically, OA had been considered a disease of joint wear and tear, but recently, low-grade chronic inflammation has been found to play a key role in OA pathology. Both innate and adaptive central immunological mechanisms are involved in inflammation. Formation of ectopic bone and osteophytes are a characteristic pathologic features of OA[6]. Neuroinflammation and central sensitization mechanisms contribute to the development of chronic pain[7].

OA is the most common musculoskeletal disorder worldwide, and it poses a huge health and economic burden. It is considered a major cause of chronic pain, disability -due to diminished joint mobility and function-, and decreased quality of life[8,9]. Thus, finding effective and safe therapies for the treatment of OA is a significant clinical need.

Despite the recent progresses in understanding the pathophysiology of OA[7], the treatment of this condition is still suboptimal[10]. For low-grade OA, pain management and lifestyle changes are the only available therapeutic options, with total joint replacement considered the end-stage therapy. However, no effective therapeutic options are available that can stop the progress of the disease[10]. Analgesia may be achieved using nonsteroidal anti-inflammatory drugs (NSAIDs) (topical or systemic) as a first line, followed by paracetamol or tramadol. Less used medications include duloxetine and topical capsaicin. Intra-articular steroids are effective and recommended if other agents do not provide sufficient pain control, although their effect is short-termed[3]. Pharmacological therapies should be always accompanied by physical and psychosocial interventions, such as exercise, weight management and manual therapy. Patients whose joint symptoms are substantially impacting their quality of life and in which non-surgical management is ineffective or unsuitable should be referred for joint replacement surgery[3]. Table 1 summarizes the treatment guidelines for OA as recommended by the American College of Rheumatology and Arthritis Foundation[11].

PAIN IN OA

Pain is the major manifestation of OA and it significantly affects the function and quality of life of patients[12]. Both peripheral and central mechanisms contribute to OA pain. Peripherally, nociceptive signals may arise from the synovium, bone marrow, soft tissues and even cartilage in the advanced stages of the disease[13]. These inputs are modulated at the central level through mechanisms involving spinal and cortical sensitization, and the activity of discrete areas of the brain. Central sensitization may explain the poor correlation between pain severity in OA and the extent of cartilage damage, and the

Table 1 Recommended therapies for the management of osteoarthritis by the American College of Rheumatology and Arthritis Foundation

	Hand	Knee	Hip
Physical and psychosocial approaches			
Exercise[1]	√	√	√
Self-Efficacy and Self-Management Programs[1]	√	√	√
Weight loss[1]		√	√
Tai Chi[1]		√	√
Heat, therapeutic cooling[2]	√	√	√
Cognitive Behavioral Therapy[2]	√	√	√
Acupuncture[2]	√	√	√
Paraffin[2]	√		
Yoga[2]		√	
Pharmacological approach			
Oral NSAIDs[1]	√	√	√
Topical NSAIDs	√[2]	√[1]	
IA corticosteroids	√[2]	√[1]	√[1] (image-guided)
Acetaminophen[2]	√	√	√
Tramadol[2]	√	√	√
Duloxetine[2]	√	√	√
Chondroitin[2]	√		
Topical capsaicin[2]		√	

[1]Strongly recommended.
[2]Conditionally recommended.
Strongly and conditionally recommended approaches for the management of hand, knee, and hip osteoarthritis are shown. NSAIDs: Nonsteroidal anti-inflammatory drugs; IA: Intra-articular.

persistence of pain after joint replacement in some patients[14].

In the periphery, sensitization of afferent neurons is mediated by cytokines, chemokines and neuropeptides and is associated with low-grade inflammation and innate immunity[15], immune cell infiltration and activation, and damage-associated molecular patterns. These mechanisms involve early post-translational changes to receptor ion channels, followed by late transcription-dependent mechanisms, which produce changes to the chemical phenotype of the cell[16]. Animal models of OA demonstrated the role of nerve growth factor (NGF) in nociceptor sensitization after tissue injury, mainly through the tropomyosin receptor kinase A (TrkA) receptor. In addition, increased NGF levels were found in the synovial fluid of OA patients and were associated with pain[17,18]. Other molecules associated with the peripheral component of OA pain include the neuropeptide calcitonin gene-related peptide (CGRP)[19], interleukin (IL)-1β, IL-6 and tumor-necrosis factor α (TNF-α)[20].

Central sensitization occurs both at the spinal and supraspinal levels. In the spinal cord, acute pain is accompanied with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor activation by glutamate, followed by an early phase of sensitization, mediated by substance P (SP) acting on neurokinin 1 receptors, in which N-methyl-D-aspartate (NMDA) receptors are activated. The late phase sensitization witnesses gene induction with enhanced synthesis of prostaglandins and other local inflammatory mediators[14]. In models of OA, spinal cord exhibits enhanced transient receptor potential cation channel subfamily V member 1 (TRPV1) activity and increased levels of SP, CGRP, IL-1 α/β, IL-13, IL-17, TNF-α, L-selectin, tissue inhibitor of metallopeptidase inhibitor 1 and vascular endothelial growth factors[21]. In addition, activation of glial cells was found to contribute to spinal sensitization, indicating a strong neuropathic component of the OA associated pain[22]. Supraspinal sensitization occurs in several areas of the brain, including the rostral ventral medulla and the periaqueductal gray and it involves inflammatory mediators, such as prostaglandins, as well as serotoninergic and noradrenergic facilitation[23].

TREATMENT OF OA PAIN

Pain management in OA is still considered suboptimal. The use of paracetamol, once recommended as the first-line analgesic for OA pain, has now been reviewed after meta-analysis suggested that monotherapy with paracetamol may be ineffective[24] and that long-term treatment provides no more pain relief than placebo for most patients[11]. NSAIDs are effective on the short term to control OA pain and are thus strongly recommended. Their use, however, is limited by the adverse effect profile[11]. Intra-articular injections of steroids also showed short-term effectiveness (2-10 wk)[3], but they are limited in frequency of administration and may cause cartilage damage if used repeatedly[25]. The use of other analgesics, such as duloxetine, tramadol, and non-tramadol opioids is not supported by strong evidence of efficacy, as well as other supplements, such as hyaluronic acid, glucosamine, chondroitin, fish oil and vitamin D[11]. These factors highlight the need for the development of novel therapies that are both effective and safe on the long term.

MSCS AND THEIR THERAPEUTIC USES

Mesenchymal stem cells (MSCs) are multipotent adult stem cells likely derived from diverse embryonic lineages and isolated from different sources. They were discovered by Friedenstein in 1970[26]. The International Society for Cellular Therapy has proposed a set of standards to define MSCs: (1) Expression of a certain set of cluster of differentiation (CD) markers (CD105, CD73, and CD90); (2) Lack of expression of hematopoietic lineage CD markers (CD45, CD34, CD14 or CD11b, CD79α or CD19 and histocompatibility complex (HLA)-DR surface molecules); (3) Differentiation into osteoblasts (bone), adipocytes (fat) and chondroblasts (cartilage) *in vitro*; and (4) Plastic adherence and ability to form colony-forming unit fibroblasts[27]. MSCs from different sources showed differences in differentiation potential, immunophenotype, immunomodulatory activity, proteome, and transcriptome, producing their specific characteristics and features in their application.

MSCs are an attractive option and among the most frequently used stem cell types for clinical application and regenerative medicine due to numerous advantages, including self-renewal and differentiation, mostly due to their secreted trophic factors that mediate cell-to-cell communication. Moreover, immune rejection is an important concern with allogeneic cell-based therapy, but the lack of cell surface HLA class II molecules and T cell costimulatory molecules and their paracrine-mediated immunomodulatory activity, and secretion of immunomodulatory factors indicate that the MSCs have broad anti-inflammatory properties and active in tissue repair[28-31].

A large number of studies demonstrated the beneficial effects of MSC-based therapies to treat different pathologies, including neurological disorders, cardiac ischemia, diabetes, and bone and cartilage diseases[32,33], which indicate that MSCs holds great promise for cell therapies and tissue engineering.

Studies showed the ability of MSCs to migrate toward damaged tissues, which functionally influence the repairing of these tissues[34]. MSCs act to accelerate healing and reduce inflammation, which is essential to remove dead cells, and facilitate cell migration and proliferation in the injury site[35,36]. For instance, a study using a rat model of cardiomyopathy showed that MSC transplantation significantly improved cardiac function by induction of myogenesis and angiogenesis, resulting in decreased left ventricular end-diastolic pressure and increased left ventricular maximum[37]. Another study showed that basic fibroblast growth factor (bFGF) can promote the migration and survival of bone marrow MSCs *in vitro*, as the perfusion of the coronary vein with retrograde bFGF can enhance the graft transplantation of MSCs, promote the differentiation of MSCs to cardiomyocytes, and restore cardiac function[38]. Some studies used a laboratory-grown cell sheet patches of MSCs to repair large damaged areas instead of intravenous infusion of MSCs. Kim *et al*[39] transplanted adipose-derived stem cells (AD-MSCs) sheet to treat myocardial infarction in a rat model and showed that the stem cell sheet promoted cellular engraftment and upregulated growth factor and cytokine expression. An *in vivo* study of neonatal stroke rat model proved that intranasal delivery of MSC reduced ischemic brain damage and reduced white and gray matters loss[40]. The study attributed the healing in the injury site to an increase in cell proliferation after transplantation of MSCs.

In skeletal disorders, MSCs could be helpful in tissue repairing and regeneration through several mechanisms, including homing, angiogenesis, differentiation, and response to inflammatory condition [41]. Liu *et al*[42] used umbilical cord-MSCs (UC-MSCs) to treat rheumatoid arthritis in collagen-induced arthritis mice through suppression of T follicular helper cell differentiation partly *via* the production of indoleamine 2,3-dioxygenase. In addition, MSCs prevented arthritis progression by inhibiting both the number and function of follicular helper cell *in vivo*. Another study showed that transplantation of MSCs from allogeneic related donors treated severe progressive systemic sclerosis [43]. A third *in vitro* study treated bone marrow derived-MSCs (BM-MSCs) with all-trans retinoic acid, then co-cultured them with CD3/28-activated peripheral blood mononuclear cells derived from ankylosing spondylitis (AS) patients. The results showed that BM-MSCs treated with all-trans retinoic acid significantly decreased pathogenic cytokine, TNF-α, IL-17A and interferon-γ (IFN-γ) in AS[44].

MSCS IN OA

MSCs are the most studied stem cells for treating bone related diseases and the associated inflammation. The effects of treatment by MSCs on OA pathology and presentation was widely investigated *in vitro* and in different preclinical models of OA using different routes of administration of multiple types and amounts of MSCs. Numerous clinical trials also addressed the potential therapeutic role of MSCs in OA.

MSCs have the ability to differentiate into mesoderm-derived cells, including osteoblasts and chondrocytes. More importantly, MSCs are considered powerful immunomodulators and inflammation combatants rendering them suitable for many immunological and bone diseases where inflammation is a prominent component. OA is the most common form of arthritis and clinically variable in its severity. Patients with symptomatic form of OA suffer from intermittent attacks which are usually associated with joint pain. OA pathology encompasses the inflammation and degeneration of articular cartilage that defects its integrity and leads to subsequent changes in the subchondral bone. Many preclinical and clinical studies have corroborated the therapeutic potential of MSCs in alleviating the inflammation associated with OA and initiating the regeneration of the defective articular cartilage. A pilot study conducted by Song *et al*[45] reported positive outcomes of injecting autologous human adipose-derived MSCs (haMSCs) intra-articularly in patients with OA. Song *et al*[45] reported that a dosage of 5×10^7 haMSCs produced the desired improvement in the pain scale and restored the volume and the function of knee cartilage. Similarly, Lee *et al*[46] reported that single intra-articular injection of 1×10^8 AD-MSCs for patients with knee OA produced a significant reduction in the Western Ontario and McMaster Universities OA (WOMAC) index total score and the test related sub-scores for pain, stiffness and physical function at 6 mo post-injection. The range of articular motion remarkably enhanced after MSCs transplantation with no change in the joint space width of medial and lateral compartment and size of the cartilage damage[46]. On the other hand, Matas *et al*[47] found that intra-articular injection of two repeated doses of umbilical cord-derived MSCs (UC-MSCs) (20×10^6 *per* dose) at baseline and 6 mo was superior than using a single dose in lowering the WOMAC pain scores. Patients with knee OA who were recruited in Matas *et al* study experienced 86% reduction in pain and 89% reduction in disability with no progression in the chondral damage and intra-articular calcifications examined by magnetic resonance imaging at 12 mo[47]. Bastos *et al*[48] compared the effect of injecting autologous bone marrow-derived culture-expanded MSCs (hBM-MSCs) intra-articularly with or without the addition of platelet-rich plasma to intra-articular corticosteroid injections in patients with symptomatic knee OA. MSCs alone or in combination with platelet rich plasma were superior to corticosteroids in improving the Knee Injury and OA Outcome Score, increasing the range of motion and reducing the expression of IL-10, which is usually increased in OA knees, at 12 mo follow up[48].

The extracellular vesicles (EVs) derived from different MSCs have currently gained a wide interest due to their high therapeutic efficacy and safety profile. Li *et al*[49] injected UC-MSCs-EVs in the articular lumen of a rat model of OA created by the surgical transection of the anterior cruciate ligament (ACLT), and reported that the UC-MSCs derived EVs can deliver therapeutically effective miRNAs, including has-miR-122-5p, has-miR-148a-3p, has-miR-486-5p, has-miR-let-7a-5p, and has-miR-100-5p, which were able to promote the reprogramming of macrophages into anti-inflammatory M2-macrophages and increasing the level of inflammation inhibitory cytokine IL-10. Moreover, these miRNAs were effective in inducing the phosphoinositide-3-kinase-Akt signaling pathway, which is integral to prevent further degeneration of the knee cartilage and seize the progression of OA[49]. Duan *et al*[50] conducted an interesting study by isolating synovial MSCs and chondrocytes from the knee cartilage of patients getting total knee arthroplasty. After culturing these cells, they primed the MSCs with lipopolysaccharide (LPS) and isolated EVs from these preconditioned synovial MSCs. The EVs were injected in the right knees of mice model of OA created by surgical removal of anterior cruciate ligament and medial meniscus. It was found that the presence of Let-7b miRNA in the EVs isolated from LPS primed synovial MSCs were effective in reducing the cartilage damage, increasing the thickness of cartilage layer, and decreasing the level of the inflammatory and matrix lysis protein disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) while increasing the levels of aggrecan and type II collagen alpha 1 chain, which are essential for the interaction with hyaluronan and enhancing the load-bearing characteristics of the knee[50]. Likewise, Jin *et al*[51] reported that lncRNA MEG-3 existed in the exosomes derived from bone marrow MSCs (BM-MSCs) and prevents the apoptosis and senescence of chondrocytes through lowering the level of IL-1β and decreasing the inflammatory damage of knee cartilage, which helps in restoring the trabecular bone volume and density of the knee joint in OA rat model. Mao *et al*[52] observed that miR-92a-3p was significantly less in the chondrocytes isolated from the cartilage of OA patients who underwent total knee replacement. BM-MSCs derived exosomes can deliver miR-92a-3p following their injection in the knees of collagenase-induced OA mouse model, which enhances the differentiation potential of resident stem cells to chondrocytes and increases the synthesis of cartilage matrix. These protective and regenerative effects are mediated by the inhibition of Wnt Family Member 5A[52]. Huang *et al*[53] demonstrated that miR-206 was downregulated and E74-like factor 3 (Elf3) was upregulated in the femoral tissues of OA mouse model. The administration of BM-MSCs derived exosomes that have sufficient amount of miR-206 was effective in downregulating Elf3 and ameliorating the inflammation and apoptosis of resident osteoblasts besides increasing the expression of osteocalcin and bone morphogenetic protein 2 and

enhancing the deposition of calcium and the activity of alkaline phosphatase[53]. In the rat model of temporomandibular joint OA, Zhang *et al*[54] injected exosomes derived from MSCs. They reported a significant suppression of inflammation mediated by decreasing the level of IL-1β and increasing the levels of nitric oxide and matrix metalloproteinase 13 (MMP13), which promoted the synthesis of glycosaminoglycans required for matrix restoration and regeneration[54].

Genetic modification of MSCs is one of the most studied approaches to enhance their therapeutic and regenerative potential. It has been found that the transplantation of MSCs overexpressing platelet-derived growth factor (PDGF) or heme oxygenase-1 in the surgery-induced canine OA model can considerably suppress the destructive inflammation and increase the levels of aggrecan and collagen type 2 in chondrocytes. PDGF-MSCs were more effective in improving the limb function and reducing pain[55]. The accumulative evidence supported by the above mentioned studies highlights the importance of MSCs in mitigating OA inflammation and restoring the integrity and density of volume of articular cartilage and its associated matrix, which indicates that MSCs can be a possible new therapeutic tool for OA.

EFFECTS OF MSCS ON OA PAIN

Despite the large number of preclinical studies investigating the effects of MSC administration on OA pathophysiology, only a small part of them included the effects on pain, and even less had pain control as the main outcome. This may be due to the difficulty of pain assessment in animal models of OA[56] and the complexity of the pain phenomenon[57]. In addition, reported results were contrasting, probably due to the variability in animal models and the methods of pain evaluation, as well as types of MSCs used and routes of their administration[56].

A number of *in vivo* studies, however, found an improvement in OA associated pain following treatment with MSCs. Transforming growth factor β1 (TGF-β1)-modified MSC-derived exosome was found to attenuate cartilage damage and pain behaviors in the ACLT model of OA by inhibiting angiogenesis, suppressing calcification of the cartilage zone and osteoclastogenesis[58]. Intra-articular injection of adipose derived MSCs (ADSCs) in rats diminished monoiodoacetate (MIA)-induced OA cartilage lesions by paracrine-based mechanisms and restored the OA associated mechanical allodynia and thermal hyperalgesia. In patients, ADSCs also reduced OA pain, as measured by the visual analog scale and WOMAC[59].

In a study by Zeng *et al*[60], bone marrow-derived MSCs (BMSCs) were enhanced by kartogenin (KGN) nanoparticles and administered to osteoarthritic rats. In addition to articular cartilage repair and enhanced chondrogenesis, KGN-enhanced BMSCs also ameliorated OA pain, as shown by increased weight bearing on the injured leg and decreased latency period after hot plate exposure. Administration of different concentrations of MSCs into OA rat knees improved both the histological damage and weight bearing distribution in the ACLT model[61].

Sakamoto *et al*[62] found that early intra-articular injection of adipose-derived MSCs resulted in significant suppression of inflammation and pain and prevented degenerative OA changes, but did not promote cartilage repair. In another study involving the MIA model of OA, STAT3 signaling pathway was suppressed by treatment of MSCs with STA21. Both intravenous (IV) and intra-articular (IA) administration of STA21-treated MSCs decreased expression of proinflammatory cytokines in the joint, which improved pain severity and cartilage damage[63].

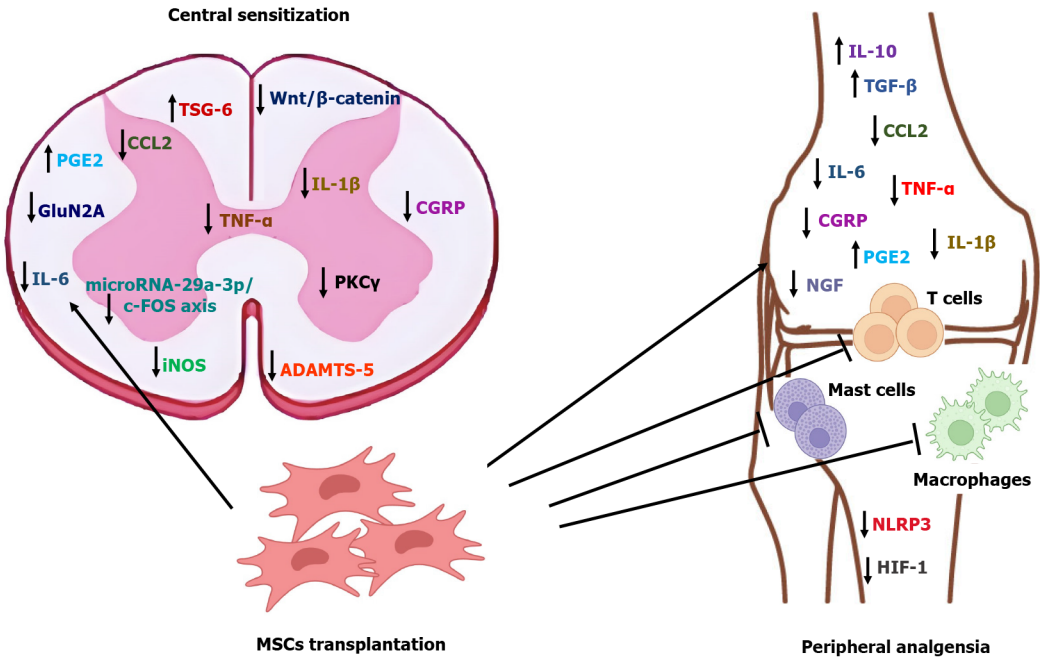
A trial of adult human bone marrow-derived mesenchymal stromal cells in the MIA model of OA caused significant pain reduction, along with articular damage repair. In patients, the same cells did not produce the significant improvement in pain scores[64].

Intra-articular injection of a large variety of MSCs in patients with knee OA was found to significantly improve joint pathology, disease symptoms, pain score and quality of life[65]. Human umbilical cord MSCs (hUC-MSCs) was found to improve pain scores and quality of life in OA patients. hUC-MSCs increase the expression of chondrocytes and activate anti-inflammatory mechanisms, preventing degradation of cartilage and bone[66]. A clinical trial of autologous bone marrow stem cells (BM-SC) concluded that a single IA injection of these stem cells significant reduced knee pain and improved quality of life[67]. Other clinical trials reviewed by Hwang *et al*[68] also resulted in improved joint function, reduction of pain severity and improved life satisfaction.

PROPOSED MECHANISMS OF ANALGESIA OF MSCS IN OA

Data from literature suggest that the analgesic action of MSCs in OA involves a peripheral component, originating in the joint tissues, and a central component related to central hypersensitization. This reflects the mechanisms of nociception reported in OA[69].

It can be speculated that a major antinociceptive mechanism of MSCs in OA is through inhibition of inflammation. MSCs secrete anti-inflammatory and growth factors that support their immunomodulatory, immunosuppressive and trophic capacities. These properties contribute to the regeneration of



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Figure 1 Graphical summary of proved and anticipated analgesic mechanisms of mesenchymal stem cells in osteoarthritis. Mesenchymal stem cell (MSCs) transplantation reverts central sensitization and induces peripheral analgesia due to their anti-inflammatory and immunomodulatory properties and by attenuation of specific pain pathways *via* exosomes-derived microRNAs and MSCs paracrine factors. TSG-6: TNF-stimulated gene 6 protein; CCL2: Chemokine (C-C motif) ligand 2; PGE2: Prostaglandin E2; GluN2A: N-methyl-D-aspartate receptor subunit 2A; IL-6: Interleukin 6; iNOS: Inducible nitric oxide synthase; TNF α: Tumor necrosis factor α; IL-1β: Interleukin-1β; CGRP: Calcitonin gene-related peptide; PKCγ: Protein kinase C γ; ADAMTS-5: A disintegrin and metalloproteinase with thrombospondin motifs 5; IL-10: Interleukin 10; TGF-β: Transforming growth factor β; NGF: Nerve growth factor; NLRP3: NLR family pyrin domain containing 3; HIF-1: Hypoxia inducible factor 1.

damaged cartilage and joint homeostasis, improving inflammatory and catabolic aspects of OA[70,71]. Initially, the bioactive substances secreted by MSCs change the inflammatory milieu in the joint from pro-inflammatory to anti-inflammatory, producing analgesia[65]. On the longer term, MSCs are inserted in the joint tissues and trigger the repair and regeneration of damaged tissues, including cartilage[72]. Numerous molecules have been reported to contribute to the antiinflammatory effects of MSCs in OA, including nitric oxide (NO), inducible nitric oxide synthase (iNOS)-27, IL-10, TGF-β, IL-6, IFN-γ, CCL2, hepatocyte growth factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and TNF-α[73-75]. Cyclooxygenase 2/prostaglandin E2 (PGE2) pathway plays a key role in the anti-inflammatory effect of MSCs in OA[76], where MSCs increase the levels of the antiinflammatory mediator PGE2[75]. Similar antiinflammatory results were also produced by MSC EVs[77]. Since these molecules and their related pathways are well established contributors to OA pain, their involvement in the analgesic mechanisms of MSCs in OA can be hypothesized.

Chemokine (C-C motif) ligand 2 (CCL2)/C-C chemokine receptor type 2 (CCR2) signaling is crucial in the development of knee OA pain. Neuronal CCL2 and CCR2 from dorsal root ganglion (DRG) mediate macrophage infiltration, while local CCL2/CCR2 signaling in the joint directly stimulates intra-articular CCR2 positive sensory nerves, producing knee hyperalgesia[78]. Interestingly, the analgesic effect of MSCs in different models of pain was associated with decreased levels of CCL2, produced by the downregulation of the NF-κB and c-Jun N-terminal kinase/mitogen activated protein kinase pathways[79,80], which suggests that a similar mechanism could be involved in the analgesic effect of MSC in OA.

Other molecules and pathways relevant to OA pain include: (1) NGF / TrkA; (2) CGRP; (3) IL-1β; (4) Pyrin domain-containing protein 3 (NLRP3) inflammasome; and (5) Wnt/β-Catenin[69]. NLRP3 inflammasome is a component of the innate immune system and is involved in the regulation of active IL-1β. NF-κB increases the expression of inactive NLRP3 and pro-IL-1 mRNA, followed by the assembly of the inflammasome, which results in the activation of caspase 1 and release of IL-1, IL-18, MMP13 and ADAMTS5. In OA, dysregulation of NLRP3 inflammasome in OA contributes to chronic pain[69]. MSCs were found to inhibit the NLRP3 inflammasome in macrophages[81], inflammatory cardiomyopathy[82] and inflammatory renal disease[83], which could indicate inhibition of NLRP3 inflammasome as a potential mechanism of MSCs induced analgesia in OA.

In OA, high levels of Wnt are associated with progressive joint damage[84] and hyperalgesia[69]. Since MSCs were found to inhibit the Wnt/β-Catenin pathway in a number of disorders[85,86], this pathway could be involved in MSC analgesic mechanism and should be investigated further. In the

study by Lee *et al*[63], administration of signal transducer and activator of transcription 3-inhibited MSCs reduced the levels of inflammatory mediators and chemokines in the OA joint, and this was associated with improvement in pain behavior and decreased TRPV1 expression in the dorsal root ganglion. This effect was more pronounced with IV administration, indicating a systemic immunomodulatory effect on inflammation.

The interaction of MSCs with immune cells, including macrophage, dendritic cells, T lymphocytes, and natural killer cells, also contributes to MSC antiinflammatory properties[87]. MSCs induce polarization of macrophages to an antiinflammatory M2 phenotype through: (1) Cellular interaction and paracrine factor-mediated mechanisms; and (2) exosome-mediated mechanisms. The first involves cytokines and hormones, and the latter depends on RNAs and other molecules[88]. Following an intra-articular injection of BM-MSCs in patients with knee OA, a decrease in synovial fluid levels of pro-inflammatory monocytes/macrophages and IL-12p40 was recorded[89].

Ai *et al*[90] investigated the effects of MSCs and derived EVs (MSC-EVs) on pain behaviors in the destabilization of the medial meniscus murine model of OA. It was found that treated OA mice did not display pain behaviors observed in untreated counterparts, and that did not result from reduced joint damage, but rather from a lack of knee-innervating sensory neuron hyperexcitability. MSC-EV treatment also prevented NGF-induced sensory neuron hyperexcitability. These results suggest that MSCs and MSC-EVs may reduce pain in OA by direct action on peripheral sensory neurons[90]. Another study[91] found that the intrathecal administration of umbilical cord blood MSCs (UCBMSCs) improved both pain behavior and inflammation in the MIA model of OA. This effect is regulated by LncRNA H19 and involves microRNA-29a-3p/ FOS axis. microRNA29a-3p, the target gene of LncRNA H19, and FOS mRNA were down-regulated after stem cell therapy, suggesting that microRNA-29a-3p and FOS might play a role in pain improvement. c-fos in spinal dorsal horn of rats was significantly down-regulated after UCBMSCs treatment, which may be the reason for pain improvement. Phosphorylation levels of NMDA receptor 1 (NR1), NR2B, protein kinase C γ (PKCγ), extracellular signal-regulated kinase in spinal dorsal horn of rats with OA pain decreased significantly after intervention of UCBMSCs, indicating that the central sensitization of rats with advanced OA pain decreased and the pain symptoms improved. Similar results were observed in astrocytes.

Intra-articular injection of human adipose tissue-derived MSCs was reported to improve pain behavior in a medial meniscal transection (MMT) rat model of OA[92]. These effects were attributed to the recruitment of endogenous cells through paracrine communication[93], and to a lesser extent, to direct engraftment coordinated with the local environment[94]. Paracrine factors excreted by MSCs help recruitment of stem and progenitor cells, repair of degraded tissue and, most importantly, counteracting inflammation. A similar result was reported after administration of bone marrow MSC (BMSC)-derived exosome in the MIA model of OA[95], which was found to inhibit CGRP and iNOS expression in the DRG, indicating relief of both inflammatory and neuropathic aspects of OA pain[95]. BMSC-derived exosome also attenuates the inhibitory effect of IL-1β on the upregulated inflammatory mediators.

In the meniscal transection (MNX) model of OA, different effects were reported on pain behavior and joint pathology with the use of early and late passage MSCs. Late passage MSCs attenuated established pain behavior, while early passage MSCs exacerbated it. Interestingly, none of them modified MNX-induced joint pathology, which suggests an analgesic mechanism not related to articular pathology[96]. SiMAG-labelled MSCs were detected within the synovial cavity at 29 d postinjection, indicating a peripheral site of analgesic action of the MSCs. The recorded decrease in serum TNFα indicated inhibition of systemic inflammation, which is the expected cause of pain relief[97]. Similarly, van Buul *et al*[56] reported that intra-articular injection of bone marrow mononuclear cells in the MIA model of OA significantly improved pain behavior – measured as weight bearing distribution - but did not affect cartilage damage, subchondral bone changes and synovial inflammation. Similar results were observed with the administration of MSCs and MSC-EVs in a mouse collagenase-induced OA model[98]. MSCs were also found to downregulate ADAMTS-5 expression, inhibit the expression of anti- CGRP and increase the expression of TNF-stimulated gene/protein-6 (TSG-6)[99]. These changes indicate the suppression of the central sensitization of pain.

Several pain pathways were found to be inhibited by MSCs in other types or models of pain. Small EVs from induced pluripotent stem cell-derived MSCs alleviated acute pain in tendinopathy by inhibition of mast cell degranulation and infiltration and the expression of proinflammatory cytokines and genes involved in the HIF-1 signaling pathway[100]. Human BMSCs relieved pain behavior in rodents by inhibition of neuronal hyperexcitability and primary afferent input, as well as suppression of GluN2A (N-methyl-D-aspartate receptor subunit 2A) tyrosine phosphorylation and PKC gamma (PKCg) immunoreactivity in the rostral ventromedial medulla[101]. In murine chronic constriction injury and spared nerve injury models, TGF-β1 was found to suppress spinal synaptic plasticity and DRG neuronal hyperexcitability *via* TGF-β receptor 1-mediated noncanonical signaling. This effect was mediated by paracrine mechanism by which BMSCs target C-X-C motif chemokine ligand 12-producing DRGs[102]. We thus anticipate that MSCs use in OA may exhibit their analgesic effects through the HIF-1, GluN2A tyrosine, PKCg and TGF-β signaling pathways. Figure 1 summarizes the established and proposed mechanisms of analgesia exerted by MSCs or MSC related EVs in OA.

CONCLUSION

Numerous *in vitro*, *in vivo* and clinical studies demonstrated the capability of MSCs in halting and/or reversing the progression of joint tissue damage in OA, as well as mitigating joint inflammation, improving pain sensation and enhancing overall patient quality of life. MSCs were able to produce an analgesic effect in models of OA through both peripheral mechanisms, mainly involving antiinflammatory processes, and on a central level, by preventing or reversing central sensitization. This evidence further reinforces the potential of MSCs as a safe and effective treatment for OA pain. Additional pathways which were observed in other types of pain may contribute to the analgesic mechanisms of MSCs in OA, and require further investigations.

FOOTNOTES

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REVIEW

Roles of cancer stem cells in gastrointestinal cancers

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Abstract

Cancer stem cells (CSCs) are the main cause of tumor growth, invasion, metastasis and recurrence. Recently, CSCs have been extensively studied to identify CSC-specific surface markers as well as signaling pathways that play key roles in CSCs self-renewal. The involvement of CSCs in the pathogenesis of gastrointestinal (GI) cancers also highlights these cells as a priority target for therapy. The diagnosis, prognosis and treatment of GI cancer have always been a focus of attention. Therefore, the potential application of CSCs in GI cancers is receiving increasing attention. This review summarizes the role of CSCs in GI cancers, focusing on esophageal cancer, gastric cancer, liver cancer, colorectal cancer, and pancreatic cancer. In addition, we propose CSCs as potential targets and therapeutic strategies for the effective treatment of GI cancers, which may provide better guidance for clinical treatment of GI cancers.

Key Words: Cancer stem cells; Gastrointestinal cancers; Promotion; Inhibition; Treatment

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Core Tip: This review summarizes the role of cancer stem cells (CSCs) in gastrointestinal (GI) cancers, focusing on esophageal cancer, gastric cancer, liver cancer, colorectal cancer, and pancreatic cancer. In addition, we propose CSCs as potential targets and therapeutic strategies for the effective treatment of GI cancers, which may provide better guidance for clinical treatment of GI cancers.

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INTRODUCTION

Cancer stem cells (CSCs) are small subgroups of undifferentiated cancer cells. CSCs possess an infinite self-renewal capability and a set of unique surface biomarkers[1]. Gastrointestinal (GI) cancer is the most common major malignancy, and includes esophageal cancer (EC), gastric cancer (GC), liver cancer (LC), colorectal cancer (CRC), pancreatic cancer and other related diseases[2]. The incidence of GI cancers is high. Recently, a growing number of studies have been conducted on the important role of CSCs in GI cancers. Research shows that CSCs are mainly involved in the growth, initiation, maintenance, survival, metastasis and recurrence of GI cancer. There are certain limitations in the methods of treatment for currently accepted GI cancers, often leading to treatment failure. This is due to CSCs resistance to chemotherapy and radiation therapy. In current treatment, CSCs cannot be erased, causing metastasis and recurrence of the tumor. Recently, studies have been conducted to clarify the signaling pathway which plays a critical role in the specific surface markers of CSCs and the self-renewal of CSCs. These cell surface markers as well as signaling pathways are potential targets for the treatment of GI cancer that provide the environment necessary for tumor growth[3-5]. Thus, certain therapies for CSCs will potentially help to eliminate the tumor.

This review aims to summarize the mechanism and treatment of CSCs in GI cancer, and to propose a potential target and therapeutic strategy for the treatment of GI cancer.

ROLES OF CSCS IN EC

EC, a common tumor of the digestive tract, causes a majority of cancer deaths[6]. EC includes esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). EAC may be associated with obesity and ESCC may be associated with drinking and narcotics. Approximately 300000 people die of EC worldwide every year. The incidence and mortality rates of EC vary from country to country[7]. Recently, the relationship between CSCs and the occurrence and development of EC has attracted more and more attention. Chen *et al*[8] discovered that esophageal CSCs activate matrix metalloproteinase 9 in EC cells and promotes cancer metastasis by the expression of placental growth factor. Moreover, CSCs expressing placental growth factor can promote[9] or suppress tumor angiogenesis by stimulating vascular endothelial growth factor[10]. Wang *et al*[11] found that by acting on ATG7-dependent β-catenin, OV6 CSCs can stably promote the progression of ESCC. In addition, studies have shown that exosomal O-GlcNAc transferase derived from esophageal CSCs can promote the suppression of cancer immunity by increasing programmed death-1 (PD-1) in CD8 T cells[12].

CSCs play a critical role in the treatment of EC. CSCs markers could help to identify the functions of CSCs in EC and can be used as targets for the treatment of EC. It was found that CD44, a CSC surface marker, can isolate and detect ESCC[13]. In addition, Lu *et al*[14] found CD133 and CXCR4 markers on the surface of ESCC, and the high expression of CD133-CXCR4 may become a marker for forecasting poor prognosis in patients with ESCC. Liu *et al*[15] found that Cripto-1 is a functional marker of CSC-like cells (CSLCs) and can predict the prognosis of patients with ESCC. In addition, studies have shown that MYH9 is a novel esophageal CSC marker and prognostic indicator, which promotes tumorigenesis through the PI3K/AKT/mTOR axis[16]. Studies have demonstrated that esophageal CSLCs can resist ferroptosis induced cell death through the active HSP27-GPX4 pathway. Hence, targeting HSP27 or GPX4 blockade is a promising therapeutic strategy to eradicate CSCs in ESCC[17]. Li *et al*[18] found that exosomal FMR1-AS1 can promote CSLCs homeostasis through TLR7-NFκB-Myc signaling in female EC. Song *et al*[19] analyzed clinical specimens and found that BCL-2 inhibitor AT101 could overcome drug resistance by targeting the CSC pathway and had good antitumor activity. In addition, Zarei *et al*[20] found that salinomycin could destabilize the low-pathway sensor TAZ in CSLCS and reduce the viability of esophageal CSLCs. Studies have shown that TRPV2 can maintain the growth of CSCs, and its specific inhibitor tranilast may be used as a targeted therapeutic agent for ESCC[21]. STAT3 and miR-181b activate each other *via* the CYLD pathway, thereby regulating the proliferation of esophageal

CSLCs[22]. Metformin can inhibit EC cell growth and sensitize EC cells to the cytotoxic effects of 5-FU by targeting components of CSCs and mTOR[23].

CSCs play an important role in the development, progress and future treatment of EC, but the associated research is inadequate, and the underlying mechanisms have not been fully explored. Therefore, more studies are needed to determine the mechanism and support the corresponding conclusions.

ROLES OF CSCS IN GC

GC is a malignant tumor derived from gastric mucosal epithelium. Most GCs are early adenocarcinomas with no apparent symptoms and are easily overlooked[24]. GC is the main cause of global cancer deaths. The treatment of GC involves surgery in combination with chemotherapy and radiotherapy, but the prognosis of terminal GC is still poor[25]. Over the past decade there is increasing evidence to show that CSCs have a significant role in GC development.

The concept that CSCs cause GC and may lead to invasion, metastasis, and treatment resistance has profound implications for anticancer therapy. Takaishi *et al*[26] implanted gastric CSCs (GCSCs) in the skin of immunodeficient mice, and a few weeks later observed that these GCSCs showed strong tumorigenic ability. Yang screened out GCSCs with serum-free medium, and found that these GCSCs had high tumorigenesis ability in nude mice, and high expression of GCSCs surface markers OCT4 and SOX2[27]. E-cadherin is a vital adhesion molecule, and its expression is closely related to the degree of cell adhesion[28]. Studies have found that the expression of E-cadherin in GCSCs is down-regulated, resulting in a decrease in the adhesion of tumor cells; thus, GCSCs are highly aggressive and easily metastasize to local lymph nodes or distant metastasis[29]. CSC Lgr5+ can promote the growth and proliferation potential of GC[30]. In addition, CSCs may maintain their viability through autophagy[31].

Identifying and targeting CSCs play a vital role in the treatment of GC. Chemotoxicity-induced exosomal lncFERO can regulate ferroptosis and stemness in GCSCs. Therefore, chemotherapy targeting the lncFERO/hnRNPA1/SCD1 axis could be an implicit strategy for CSCs-based GC therapy[32]. In addition, microRNA (miR)-375 can trigger ferroptosis by targeting SLC7A11, thereby attenuating the stemness of GCSCs[33]. CBX7 was found to positively regulate the stem-like characteristics of GC cells by inhibiting p16 and activating the AKT-NF-κB-miR-21 pathway[34]. Sezer *et al*[35] found that lymphatic metastasis-associated TBL1XR1 promotes the metastasis of gastric CSLCs by sensitizing ERK1/2-SOX2 signaling. Huang *et al*[36] found that SIRT1 inhibits both chemoresistance and cancer stemness in GC by launching a positive feedback loop of AMPK/FOXO3. The m6A methyltransferase METTL3 was found to promote oxaliplatin resistance in CD133+ GCSCs by improving PARP1 mRNA stability to increase base excision repair pathway activity[37]. *Celastrus orbiculatus* ethyl acetate extract can prevent GC growth by limiting the stemness of GCSCs by changing PDCD4 and EIF3H expression [38]. Methionine has been found to inhibit autophagy in GCSCs by promoting RAB37 methylation and phosphorylation. Therefore, supplementation with methionine γ-lyase can induce autophagy in GCSCs and inhibit tumor growth[36].

The role of CSCs in growth, metastasis, treatment and prognosis of GC is increasingly important. In the past few years, an increasing number of studies have been conducted on the mechanism of action of CSCs against GC and to offer promising targets and therapeutic tactics for GC treatment. Many treatments targeting CSCs have been developed, but there are limits to CSCs targeting therapy. It affects normal stem cells and causes tissue renewal problems. For example, Lgr5 is a marker associated with common stem cells in gastric tissue. mTORC1 can maintain self-renewal of the Lgr5 population, preventing cell differentiation and causing gastric tumorigenesis. However, the use of mTORC1 inhibitors may cause gastric glandular atrophy due to tissue malfunction, limiting its therapeutic use [39]. Therefore, more efforts are needed to treat GC based on CSCs.

ROLES OF CSCS IN LC

LC is the third most common cause of cancer associated deaths worldwide. This is due to its high recurrence rate, which after normal treatment, can reach 70%. Hepatocellular carcinoma (HCC) is the main pathology and causes approximately 80% of LC cases[40]. Liver CSCs (LCSCs) are now known to be responsible for HCC growth, metastasis and recurrence, in addition to failure of chemotherapy and radiation therapy[41]. These findings indicate that LC therapy kills most of the tumor cells, but cannot eliminate LCSCs and treatment can eventually fail as LCSCs survive and generate new tumors. Therefore, CSCs theory has provided new findings in the diagnosis, treatment and prevention of LC (Table 1).

LCSCs play a vital role in the development, progression, recurrence and drug tolerance of HCC. Studies have shown that LCSCs can accelerate tumor growth in primary cancer cells and metastasis of secondary tumors, causing the recurrence of HCC[42]. Studies have found that EpCAM-high HCC stem cells can promote tumor growth by upregulating CEACAM1 to weaken the capacity of natural killer

Table 1 Methods for targeting liver cancer stem cells in the treatment of liver cancer

Ref.	Genes/transcription factors/protein	Inducing way	Role
Park *et al*[43]	EpCAM-high HCC stem cells	Upregulating CEACAM1	Promoting the growth of LC
Deng *et al*[44]	Histone demethylase JMJD2D	Enhancing Sox9 expression	Promoting the growth of LC
Yang *et al*[45]	lncARSR	Targeting STAT3 signaling	Promoting LCSCs expansion
Chen *et al*[47]	4-PBA	Activating β-catenin signaling pathway	Promoting the growth of LC
Galizia *et al*[49]	CD133	-	Indicator of tumor recurrence
Haraguchi *et al*[51]	CD13	-	Causing drug resistance of LCSCs
Yang *et al*[52]	OV6	-	Causing drug resistance of LCSCs
Wei *et al*[53]	CD44	Regulating PES1	Promoting the growth of LC
Chen *et al*[55]	FOXM1	Reducing the expression of ALDH2	Inducing the apoptosis of LCSCs
Dou *et al*[56]	BC-02	Inhibiting CD13	Eradicate LCSCs
Zhou *et al*[57]	CD133-apt-Dox	Targeting CD133-expressing cells	Inhibiting the growth of LC
Feng *et al*[58]	MiR-124	-	Inhibiting LCSCs self-renewal
Jiang *et al*[59]	MiR-365	Regulating RAC1 pathway	Inhibiting the proliferation and invasion of HCC cells
Li *et al*[60]	MicroRNA-21	-	Inhibiting highly invade LCSCs
Si *et al*[61]	MiR-219	E-cadherin pathway	regulates the expansion of LCSCs
Dou *et al*[62]	MicroRNA-6838-5p	Down-regulating CBX4 expression and Inactivating ERK signaling	Inhibiting self-renewal and metastasis of Human LCSCs
Zhang *et al*[63]	MiR-589-5p	Inhibiting MAP3K8	Inhibiting the growth of LC
Wang *et al*[64]	HAND2-AS1	-	Promoting the growth of LC
Li *et al*[66]	Neuropilin1	The loss of neuropilin1	Inhibiting LCSCs
Wang *et al*[67]	ZBP-89	Inhibiting Notch1 signaling pathway	Regulating self-renewal of LCSCs

HCC: Hepatocellular carcinoma; LC: Liver cancer; LCSCs: Liver cancer stem cells.

cells to recognize and kill cancer cells[43]. Histone demethylase JMJD2D can enhance EpCAM and Sox9 expression to promote the self-renewal of liver CSLCs, promoting tumor growth[44]. Yang *et al*[45] found that lncARSR promoted HCC cell dedifferentiation and LCSCs expansion by targeting STAT3 signaling. Moreover, LCSCs release exosomes in a RAB27A-dependent manner which can lead to resistance to regorafenib in LC cells[46]. Chen *et al*[47] found that activating the β-catenin signaling pathway leads to upregulation of PPAR-α by 4-PBA, which in turn initiates LCSCs and promotes early HCC. Cao *et al*[48] found that RACK1 advances self-renewal and chemoresistance of CSCs in HCC by stabilizing nanoparticles.

The biomarkers of LCSCs are important targets in the treatment of LC. CD133 is one of the common surface markers of LCSCs, and studies have found that CD133 isolated from HCC cell lines has high proliferative and tumorigenic potential; thus, enhanced CD133 expression can also serve as a prognostic indicator for survival and tumor recurrence in patients with LC[49]. Yin *et al*[50] found that aldehyde dehydrogenase was expressed in LCSCs and was positively correlated with CD133 expression. CD13 is a marker of LCSCs, which has the function of dormancy and slow growth, and is the main reason for drug resistance of LCSCs[51]. The LCSCs marker OV6 was found to be chemoresistant, but this was reversed when lentivirus-delivered miRNAs targeting β-catenin were stably expressed. Therefore, targeting Wnt/β-catenin signaling could be a potential strategy to reverse the drug resistance properties of OV6 LCSCs[52]. Wei *et al*[53] found that CD44 regulated PES1 in LCSCs through miR-105-5p to promote tumor growth. Twist2 advances self-renewal of liver CSLCs by changing CD24[54].

LCSCs play a vital role in the treatment of LC. It was found that the transcription factor FOXM1 inhibits the dryness of LCSCs by reducing the expression of ALDH2, and inhibits proliferation, migration, invasion and tumorigenesis, while inducing apoptosis of LCSCs[55]. The CD13 inhibitor BC-02 can target CD13 and upregulate intracellular reactive oxygen species (ROS) and ROS-induced DNA damage to damage LCSCs. Therefore, BC-02 may be a potential therapeutic strategy to eradicate LCSCs and overcome chemoresistance in LC[56]. Studies have found that aptamer-based drug delivery agents

(CD133-apt-Dox) targeting CD133-expressing cells can impair the self-renewal ability of liver CSLCs and inhibit the growth of LC[57]. MiRNAs are important regulators of CSCs therapy in LC. Feng *et al*[58] found that forced expression of miR-124 can inhibit LCSCs self-renewal and tumorigenesis. It was shown that miR-365 regulated LCSCs through the RAC1 pathway and prevented the proliferation and invasion of HCC cells[59]. In addition, miR-21 downregulation can inhibit cell proliferation and highly invade LCSCs[60]. Si *et al*[61] found that miR-219 regulates the expansion of LCSCs through the E-cadherin pathway. Dou *et al*[62] showed that miR-6838-5p inhibited self-renewal and metastasis of LCSCs by down-regulating CBX4 expression and inhibiting ERK signaling. In addition, miR-589-5p inhibited MAP3K8 in HCC and inhibited CD90 CSCs[63]. It was shown that HAND2-AS1 can promote self-renewal of LCSCs and drive liver tumorigenesis, providing a potential new target for HCC treatment[64]. In addition, tumor-associated macrophages produce interleukin-6 and signal through STAT3 to improve the expansion of LCSCs[65]. Li *et al*[66] found that the loss of neuropilin 1 inhibits the LCSC population and blocks metastasis in HCC through epithelial-mesenchymal transition. Wang *et al*[67] found that ZBP-89 negatively regulated self-renewal of LCSCs by inhibiting the Notch1 signaling pathway.

Traditional HCC treatment mainly targets fast growing and differentiated HCC cells. However, a part of the emerging CSCs concept explains the failure of these therapies. The research progress of LCSCs has provided a new viewpoint on the possible adhibition in the clinical treatment of LCSCs. Detection of LCSCs is useful for predicting postoperative survival of patients. The development of treatment strategies for LCSCs may greatly improve the treatment of LC[68].

ROLES OF CSCS IN CRC

CRC is one of the most common cancers and the fourth most frequent cause of cancer death worldwide [69]. Despite the great progress in surgery and chemotherapy over the past decade, the five year survival rate of CRC patients is 50%-65%[70]. In the past few years, an increasing number of studies have shown that CSCs are closely associated with the occurrence and development of CRC, which provides promising directions for the diagnosis and treatment of CRC (Table 2).

CSCs are the major cause of drug resistance and disease recurrence in CRC. Studies have found that Lgr5 CSCs have a vital role in primary and metastatic colon cancer, and can promote tumor growth and metastasis[71]. In addition, most colorectal CSCs (CCSCs) express Lgr5 and form distant metastasis, which is a major factor driving CRC metastasis[72]. Recent studies have found that human CCSCs can give rise to vascular endothelial cells and constitute the vasculature in cancer tissue, which provides a new mechanism for tumor angiogenesis in cancer[73]. CD26 CCSCs can lead to CRC metastasis[74]. Razi *et al*[75] found that DCLK1 is a promising CCSC marker that changes tumor progression and invasion in a miR-137- and miR-15a-dependent manner. The JAK2/STAT3/CCND2 axis promotes the persistence and radioresistance of CRCs, which is the drug resistance mechanism for the continuous growth of CSCs after radiotherapy[76]. Liu *et al*[77] found that Sec62 promotes stemness and chemoresistance in human CRC by sensitizing the Wnt/β-catenin pathway. Studies have shown that CCSCs acquire chemoresistance by the upregulation of F-Box/WD repeat-containing protein 7 and the consequent degradation of c-Myc[78]. In addition, PD-L1 can maintain CSCs self-renewal by activating the HMGA1-dependent signaling pathway[79]. 5-FU improves the stemness of CRC through p53-mediated WNT/β-catenin pathway activation[80].

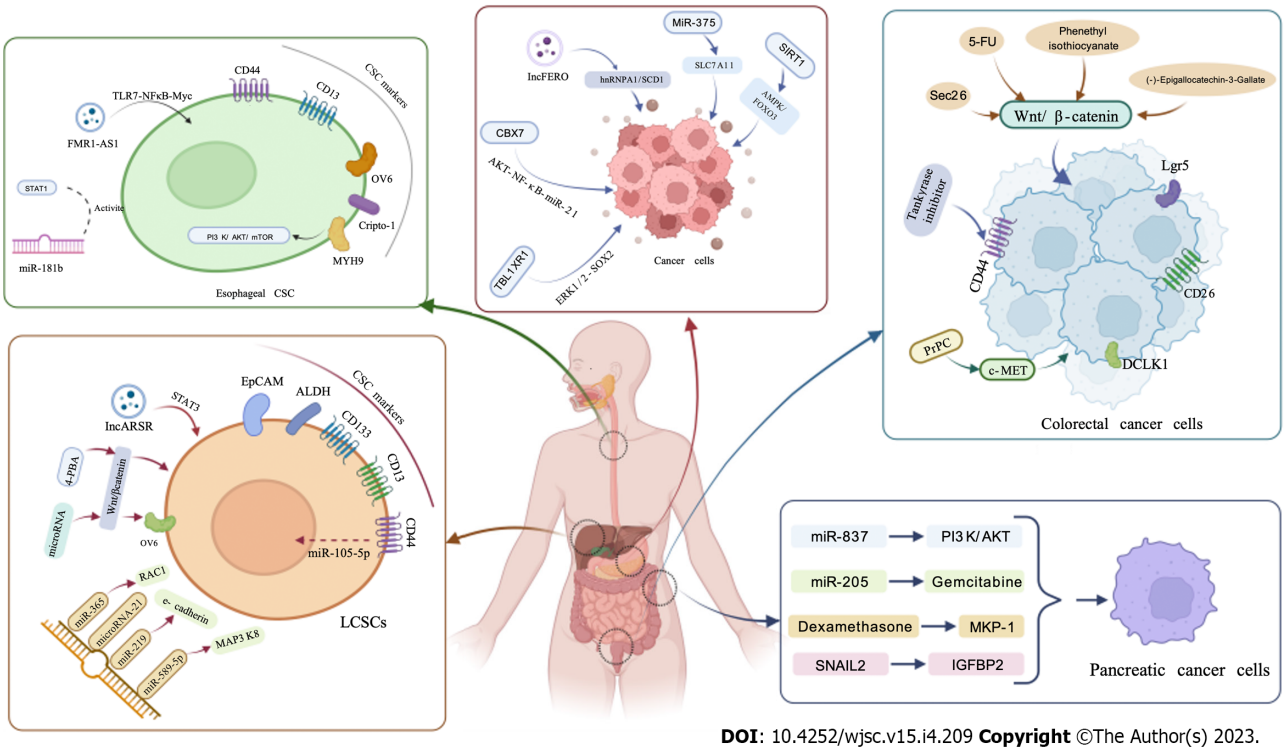
Whereas conventional therapies target proliferating and mature cancer cells, CSCs are mostly quiescent and poorly differentiated, so they could easily survive the chemotherapy attack. Therefore, novel therapies targeting CSCs are necessary. Chen *et al*[81] found that phenethyl isothiocyanate inhibits CCSCs by suppressing the Wnt/β-catenin pathway. In addition, (-)-Epigallocatechin-3-Gallate can also inhibit CCSCs by suppressing the Wnt/β-catenin pathway[82]. Studies have shown that AGR2 is a new stem cell marker that is changed by the canonical Wnt/β-catenin pathway in CCSCs and is important for stemness maintenance of CCSCs[83]. Jang *et al*[84] reported that tankyrase inhibitors downregulated c-KIT tyrosine kinase and prevented the growth of CD44-positive CCSCs. Liu *et al*[85] found that PTK6 interacts with JAK2 and phosphorylates to activate JAK2/STAT3 signaling, which can improve stemness and chemoresistance of CRC cells and reverse chemoresistance in CRC. Studies have shown that mithramycin A inhibits CRC growth by targeting CSCs[86]. Studies have also found that disruption of endolysosome RAB5/7 effectively eliminates CCSCs[87]. PrPC inhibits CSCs properties by interacting with c-MET in CRC cells[88].

According to the data accumulated during cancer research, CSCs have become a fundamental cause of cancer progression and resistance to treatment. Therefore, it is important to understand the biological, functional and clinical significance of CSCs in CRC tolerance in order to develop an effective treatment model for CRC patients. However, the practical clinical application of CSCs in CRC is still limited, and further studies and efforts are needed for clinical applications.

Table 2 Methods for targeting colorectal cancer stem cells in the treatment of colorectal cancer

Ref.	Genes/transcription factors/protein	Inducing way	Role
Fumagalli *et al* [72]	Lgr5	-	Promoting the growth of CRC
Cheung *et al*[74]	CD16	-	Promoting the growth of CRC
Razi *et al*[75]	DCLK1	MiR-137 and Mir-15a-dependent manner	Promoting the growth of CRC
Park *et al*[76]	JAK2/STAT3/CCND2 axis	-	Causing drug resistance of CCSCs
Liu *et al*[77]	Sec62	Activating the Wnt/β-catenin pathway	Causing drug resistance of CCSCs
Izumi *et al*[78]	F-Box/WD repeat-containing protein 7	-	Causing drug resistance of CCSCs
Wei *et al*[79]	PD-L1	Activating HMGA1-dependent Signaling pathway	Maintaining CSCs self-renewal
Cho *et al*[80]	5-FU	Activing p53-mediated WNT/β-catenin pathway	Promoting the stemness of CRC
Chen *et al*[81]	Phenethyl isothiocyanate	Suppressing Wnt/β-catenin pathway	Inhibiting CCSCs
Chen *et al*[82]	(-)-Epigallocatechin-3-Gallate	Suppressing Wnt/β-catenin pathway	Inhibiting CCSCs
Dahal *et al*[83]	AGR2	Regulating Wnt/β-catenin pathway	Regulating the stemness maintenance of CCSCs
Jang *et al*[84]	Tankyrase inhibitors	Downregulating c-KIT tyrosine kinase	Inhibiting the growth of CD44-positive CCSCs
Liu *et al*[85]	PTK6 interacts with JAK2	Activating JAK2/STAT3 signaling	Reversing chemoresistance in CRC
Quarni *et al*[86]	Mitramycin A	-	Inhibiting CCSCs
Lim *et al*[88]	PrPC	Interacting with c-MET	Inhibiting CCSCs

CCSC: Colorectal cancer stem cells; CRC: Colorectal cancer; CSCs: Cancer stem cells.



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Figure 1 The roles of cancer stem cells in gastrointestinal cancers. CSC: Cancer stem cells; LCSC: Liver cancer stem cells.

ROLES OF CSCS IN PANCREATIC CANCER

Pancreatic cancer is one of the most deadly human malignancies, the survival rate is 8% and the prognosis is the worst of all GI tumors[89-91]. It is now the fourth most common cause of cancer associated deaths worldwide. The tumorigenesis capacity of pancreatic cancer cells is different, and the proliferation and growth of pancreatic cancer are highly dependent on the presence of a limited subgroup of pancreatic cancer cells, called pancreatic CSCs (PCSCs)[92]. The concept of CSCs is recognized and some of the identified molecules and signaling pathways are associated with cancer diagnosis and treatment.

PCSCs contribute to the development and invasion of pancreatic cancer. It has been found that PCSC CD9 can promote the plasma membrane localization of glutamine transporter ASCT2, improving glutamine uptake in pancreatic cancer cells and promoting tumor growth[93]. Leng *et al*[94] found that SIRT1 coordinating with CRL4B can regulate PCSCs to promote tumorigenesis. In PCSCs, PAF1 interacts with DDX3 and PHF5A to regulate the expression of NANOG and other genes that regulate stemness. Therefore, knockdown of PAF1 reduced the development and progression ability of *in situ* pancreatic cancer in mice and its CSCs[95]. Bao *et al*[96] found that pancreatic CSLCs can promote tumor formation and rapid tumor growth by activating FoxQ1. Masuo *et al*[97] demonstrated that SNAIL2 can promote the tumorigenicity and chemotherapy resistance of PCSCs by regulating IGFBP2.

PCSCs have an important role in pancreatic cancer therapy. Studies have shown that PCSCs have longer telomeres and higher telomerase activity than tumor cells, which is associated with the expression of pluripotent genes (Nanog, Sox2, Oct3/4). Therefore, telomerase inhibition can lead to apoptosis of PCSCs, which is a suitable therapeutic approach against CSCs, especially in pancreatic cancer[98]. Yang *et al*[99] found that miR-873 could inhibit self-renewal and proliferation of PCSCs by blocking the PLEK2-dependent PI3K/AKT pathway. In addition, miR-205 can resensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine and act as a tumor suppressor miRNA[100]. JNK is required for PCSCs self-renewal and tumor initiation, as well as its survivin expression. Dexamethasone was found to induce the expression of MKP-1 through glucocorticoid receptor activation, thereby inactivating JNK and inhibiting tumor growth[101]. Urtasun *et al*[102] showed that simultaneous blockade of IGF-IR and EGFR/Her-2 using NVP-AEW541 and lapatinib could inhibit drug resistance in pancreatic cancer.

The role of CSCs in pancreatic cancer metastasis, recurrence and treatment has become very important. A large number of studies have allowed tumor stem cell markers to achieve metastasis, progression and resistance of pancreatic cancer cells, which provide potential targets and therapeutic directions for the treatment of pancreatic cancer. Nevertheless, there are still few practical clinical applications of PCSCs, and further studies are needed for clinical applications.

Recently, many studies have shown that CSCs are closely related to tumor recurrence, metastasis and drug resistance, especially in EC, GC, LC, CRC and pancreatic cancer (Figure 1). CSCs can be identified by a series of surface markers, including OV6, EpCAM, CD13, CD133 and CD44. Surface markers of CSCs are useful for cancer diagnosis and prognosis prediction. In addition, CSCs regulate tumor progression and therapeutic resistance through multiple mechanisms, including Notch, Wnt/β-catenin and other signaling pathways. There are many other elements that make CSC impressions, such as the tumor microenvironment and non-coding RNAs, including miRNAs and long non-coding RNAs.

CONCLUSION

Currently, we have developed a combination of chemotherapeutic agents and small molecule inhibitors to reduce CSCs and effectively treat GI cancer. As CSCs have many similar features to stem cells, the molecular signal pathway or mechanism that distinguishes the two cell subgroups is still unknown. Therefore, stem cell therapy is limited, and it is necessary to further study the biological difference between normal stem cells and LCSCs. Research on the therapy of GI cancer with regard to CSCs is still at the *in vitro* and animal experimental stage, and the precise molecular mechanism of CSCs in GI cancer requires further study. Therefore, more research is needed to promote the application of CSCs in clinical practice.

FOOTNOTES

Author contributions: Wu J and Dong P designed study and revised the manuscript. Xuan SH, Hua ML and Xiang Z analyzed data and performed manuscript drafting; He XL, Huang L and Jiang C searched the literature and collected data; Dong P and Wu J reviewed the results and made critical comments on the manuscript; all authors reviewed and approved the final version; Xuan SH and Hua ML contributed equally to this work; Wu J and Dong P contributed equally to this work.

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MINIREVIEWS

Harnessing and honing mesenchymal stem/stromal cells for the amelioration of graft-versus-host disease

Tang-Her Jaing, Tsung-Yen Chang, Chia-Chi Chiu

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Abstract

Allogeneic hematopoietic stem cell transplantation is a deterministic curative procedure for various hematologic disorders and congenital immunodeficiency. Despite its increased use, the mortality rate for patients undergoing this procedure remains high, mainly due to the perceived risk of exacerbating graft-versus-host disease (GVHD). However, even with immunosuppressive agents, some patients still develop GVHD. Advanced mesenchymal stem/stromal cell (MSC) strategies have been proposed to achieve better therapeutic outcomes, given their immunosuppressive potential. However, the efficacy and trial designs have varied among the studies, and some research findings appear contradictory due to the challenges in characterizing the *in vivo* effects of MSCs. This review aims to provide real insights into this clinical entity, emphasizing diagnostic, and therapeutic considerations and generating pathophysiology hypotheses to identify research avenues. The indications and timing for the clinical application of MSCs are still subject to debate.

Key Words: Mesenchymal stem/stromal cells; Graft-versus-host disease; Immunomodulatory; Adaptive immunity; Exosomes

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Core Tip: This article provides insights into the use of validated mesenchymal stem/stromal cells (MSCs) as a potential treatment strategy for graft-versus-host disease (GVHD) in hematopoietic stem cell transplantation (HSCT). Current prevention and treatment options involve immunosuppression, which can hinder immune recovery and limit the graft-versus-tumor effect. By using MSCs, clinicians can effectively treat GVHD, identify high-risk patients, and stratify patients based on disease severity. Therefore, MSCs can aid in promoting engraftment, ameliorating acute GVHD, and preventing chronic GVHD, making them an attractive option for HSCT.

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INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are multipotent cells with self□renewal abilities[1] that can be derived from different tissue sources. They attach to tissue culture dishes and express CD73, CD90, and CD105 but lack the expression of CD45, CD34, CD14, or CD11b, CD79α or CD19, and HLA-DR surface molecules. In vitro, MSCs can differentiate into osteoblasts, adipocytes, or chondroblasts[2,3]. MSCs can be effectively harvested without significant ethical concerns and have low immunogenicity. They have emerged as a promising cell source due to their regenerative and immunomodulatory potentials, limited ethical concerns, and low risk of tumor formation[4-6].

Malignancy relapse is a significant challenge in allogeneic hematopoietic stem cell transplantation (HSCT). Chronic graft-versus-host disease (GVHD) is associated with lower relapse rates, but the diagnosis, staging, and risk stratification of GVHD are challenging[7]. In this scoping review, we highlight recent evidence on different types of MSCs studied for GVHD, including bone marrow (BM), umbilical cord blood, placenta, adipose tissue, and others. MSCs have been found to inhibit immune cell proliferation and cytotoxic action, making them a potential treatment option for GVHD[8].

This review aims to provide a critical overview of the mechanisms by which MSC can treat GVHD, including immunomodulation, migration, homing, and clinical applications of MSC therapy. We searched peer-reviewed literature in PubMed and Embase to gather the latest information on this topic.

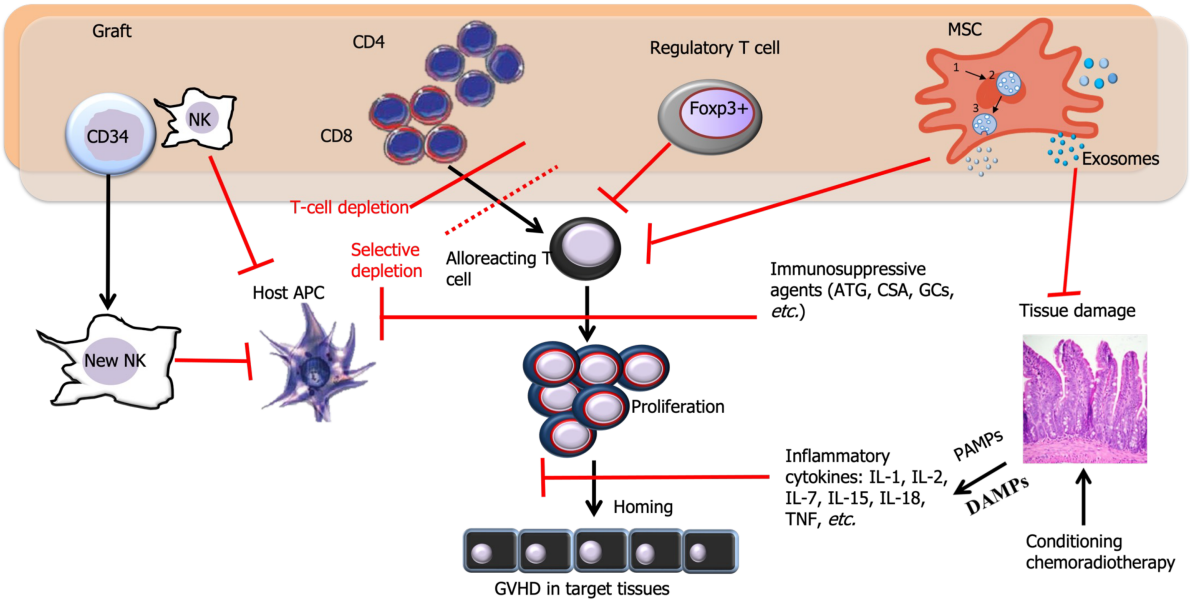
THERAPEUTIC STRATEGY OF GVHD

Immune pathways in GVHD

One of the most significant challenges in improving the prognosis for patients undergoing allogeneic HSCT is GVHD. This condition can be characterized as a rapid escalation in immune activation caused by massive target tissue apoptosis. The prevention of GVHD is primarily based on the use of calcineurin inhibitors and methotrexate, while the treatment of ongoing GVHD involves the use of corticosteroids. GVHD manifests as acute GVHD (aGVHD) in 53%-62.5% of the patients and chronic GVHD (cGVHD) in 20%-50.4% of patients[9,10], and the development of this complication may contribute to 6.3% of deaths following HSCT[9]. Although the administration of calcium inhibitors such as calcium sulphoaluminate can prevent the development of GVHD in some cases, about 19% of aGVHD II-IV cases are often resistant to all conventional therapy, resulting in a high mortality rate for these patients. Several potential second-line options have been proposed, including the use of MSCs. MSCs have attracted significant interest because they can actively undergo apoptosis by recipient cytotoxic cells [11]. Figure 1 illustrates the immune pathways involved in GVHD and the sites where therapy is used to block GVHD development.

Danger signals in aGVHD development

In a typical case of aGVHD, which occurs following a triptych course, symptoms begin with the prodromal phase caused by the underlying disease and conditioning regimens that secrete proinflammatory cytokines, mainly tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)[12]. Host conditioning facilitates donor cell grafting. Donor allograft T-cells are the primary effector cells for GVHD. However, tissue damage leads to the release of alarmins and the expression of pathogen-recognition receptors, triggering the next phase. This phase activates the innate immune system and, in turn, the adaptive immune system. Alarmins and exogenous pathogen-associated molecular patterns (PAMPs) elicit similar responses to relevant signals, and they belong to the group of damage-associated molecular patterns (DAMPs)[13]. DAMPs and PAMPs are potent stimulators for



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Figure 1 Immune pathways in graft-versus-host disease and sites where therapy is used to block graft-versus-host disease development (red bars). APC: Antigen-presenting cell; ATG: Antithymocyte globulin; CSA: Cyclosporine A; DAMPs; Damage-associated molecular patterns; Foxp3+: Forkhead box P3; GCs: Glucocorticosteroids; GVHD: Graft-versus-host disease; MSC: Mesenchymal stem cell; TNF: Tumor necrosis factor; NK: Natural killer cells; PAMPs: Pathogen-associated molecular patterns.

host and donor-derived antigen-presenting cells (APCs), which activate and enhance the responses of alloreactive donor T cells[14].

The immunosuppressive effects of MSCs are classified into soluble factor-mediated effects and cell-cell contact-mediated effects. MSCs suppress the proliferation and survival of activated T lymphocytes and reduce the release of inflammatory factors such as IL-2, TNF-α, IL-1β, and IFN-γ. By the same means, MSCs also reduce the number of Th1/Th2 and Th17 cells. Through cell-to-cell contacts, MSCs can stimulate the expression of transcription complexes related to Runt 1 (RUNX1), RUNX3, and CBFβ in Treg-specific regulatory regions to improve the stability of Foxp3[15]. MSCs have also been shown to be highly effective in inhibiting the cytotoxic effect, proliferation, and secretion of different cytokines of NK cells by directly contacting these cells and transforming their phenotype.

The effects of MSCs on B cells involve inhibiting their cell cycle progression by inducing G0/G1 cell cycle arrest and suppressing their proliferation. Additionally, the differentiation of B cells into IgM-, IgG-, and IgA-secreting cells is impaired by MSCs, thereby limiting their antibody production. Furthermore, MSCs can affect the chemotactic function of B cells[16].

GENETIC BASIS OF GVHD

Humanized mouse models

Most relevant models for studying human adaptive immune responses use immunocompromised mice whose immune system is reconstituted with human immune cells and immune system components. Lee *et al*[17] used a model of NSG mice reconstituted with human CD34 cells to evaluate the immunological safety of therapeutically compromising human MSCs. As major histocompatibility complex (MHC) molecules are the primary mediators of the allogeneic immune response, MHC expression levels are critical in the potential immunogenicity of cells. To investigate MSCs as a cellular therapy in GVHD, Tobin *et al*[18] treated NSG-PBMC humanized mice with human MSCs as a GVHD model. MSC treatment resulted in a reduction in liver and intestinal pathology and a significant increase in the survival of the GVHD NSG mouse.

In contrast to aGVHD, some MHC-mismatched animal models may mimic the features of cGVHD. However, due to the pathological resemblance between cGVHD and autoimmune diseases, there is a clear connection between the two entities, and the difference in cGVHD is primarily caused by the donor lymphoid graft[19]. These findings provide compelling evidence for the essential role of human leukocyte antigen (HLA) disparity in both aGVHD and cGVHD. The expression pattern of minor histocompatibility antigens (miHAs) determines the target organ involvement in aGVHD. The miHAs exhibit hierarchical immunodominance, which may contribute to the variability in GVHD variability [20].

Translation and clinical advances in GVHD

aGVHD: HLA mismatching is one of the most significant risk factors for aGVHD and cGVHD risk. HLA proteins are specifically encoded by MHC. In vitro studies have demonstrated that most T cells associated with GVHD are naïve T cells, whereas memory T cells mediate immunity against pathogens and the graft-versus-leukemia (GVL) effect[21]. Regardless of the graft source or conditioning intensity, the incidence of aGVHD is closely related to the number of HLA disparities. Although the impact of HLA disparity has been analyzed in the outcomes following allogeneic HSCT, relatively few studies have tried to correlate it with the incidence and severity of cGVHD. Some studies reported an association between HLA-A, -B, and -C disparity and aGVHD[22].

Although MHC antigens guarantee HLA matching, the donor and recipient may differ in various proteins presented in the form of HLA-peptide complexes to T cells that act as miHAs. The genomes include more than 10^7 polymorphic sequences outside HLA, and the role of miHAs is supported by genome-wide analysis of single-nucleotide polymorphisms[7]. The disparity in a single immunodominant miHA is insufficient to cause aGVHD, although T cells primed against a single miHA may induce tissue damage in a human skin explant model[23]. It is unknown whether the number of miHAs triggering a GVL response in a given transplant is significant or whether a small number of antigens play a dominant role[24].

cGVHD: In contrast, cGVHD has been considered an autoimmune disease based on its clinical features [25]. Some experimental studies have shown that T cells from animals with cGVHD are specific for a public determinant of MHC class II molecules and are therefore considered autoreactive. These autoreactive cells of cGVHD are often associated with an injured thymus and adverse selection.

Recent clinical data has highlighted a significant link between immune responses against ubiquitous miHAs and cGVHD. Since cGVHD usually occurs after allogeneic HSCT, aGVHD is its related risk factor. Unlike syngeneic GVHD, which results from deficient thymic selection[26], cGVHD typically arises after allogeneic HSCT and is characterized by chronic T-cell activation due to continuous exposure to miHAs. This chronic stimulation can cause target organ damage that resembles autoimmune features, where the target is miHAs for cGVHD and non-polymorphic autoantigens for autoimmune diseases. A study on female-to-male HSCT demonstrated a good correlation between the presence of antibodies to the Y-chromosome-encoded gene and cGVHD[27]. A study in female-to-male HSCT demonstrated a good correlation between the presence of antibodies to Y-chromosome-encoded genes and cGVHD, suggesting that miHAs may indeed be the targets. However, it is not yet clear whether the miHAs targeted in cGVHD are the same as those targeted in aGVHD. A murine study had shown that the type and selection of immunodominant miHAs can determine the target and character of GVHD damage[28].

Epitope spreading and the failure of appropriate regulatory mechanisms in aGVHD may result in donor T cells recognizing both non-polymorphic and miHA epitopes, perpetuating cGVHD. In contrast, T cells directed against miHAs with hematopoietic restriction may also mediate a GVL response in the absence of GVHD[29]. However, the relevant immunogenic targets for cGVHD remain speculative and confidential.

Potent immunomodulatory role of MSCs-derived exosomes in preventing GVHD

The safety and effectiveness questions regarding using MSCs remain unresolved, and conflicting effects have been noted due to the heterogeneity observed among MSCs. MSCs-derived exosomes (MSCs-Exo), a subgroup of extracellular vesicles released by MSCs, have shown therapeutic benefits for inflammatory diseases and cancers due to their ability to transport proteins and nucleic acids from donor cells to recipient cells of the same or different tissues, making it a suitable candidate for cell-free therapy. MSCs-Exo have been found to reduce inflammation and fibrosis in the skin, lungs, and liver, and inhibit Th17 cells while inducing Treg cells, making it a potential alternative method for the treatment of cGVHD. The activation of CD4+ T cells and their infiltration into the inflamed mouse lung were reduced in MSCs-Exo-treated mice[30]. MSCs-Exo, extracted from healthy donors' BM, suppress the expression of pro-inflammatory factors TNF-α and IL-1β but increase the level of anti-inflammatory factor TGF-β during *in vitro* culture[31].

Typically, MSCs-Exo are characterized by endosomes that bud inward and package into multivesicular bodies (MVBs). These MVBs fuse with the plasma membrane and deliver the exosomes into the intracellular space. However, exosomes can enrich several molecules as cargo, such as proteins/cytokines, DNA, RNA, and other nucleic acids. Exosomes, as secretory components of MSCs, transport cytokines, and growth factors of immunoregulation, such as transforming growth factor beta-1 (TGF-β 1), IL-6, IL-10, hepatocyte growth factor, signaling lipids, mRNAs, and regulatory miRNAs, which exert biological effects on recipient cells, such as cell-to-cell communication, tissue regeneration, metabolism, immune modulation, and homing of immune cells[32,33]. Diverse immune cells establish complex interactions with each other. MSCs-Exo might represent a novel cell-free therapy with unique competitive advantages over parent MSCs, such as no apparent risk of tumor formation or lower immunogenicity.

IMMUNE CELL LANDSCAPE OF GVHD

Antigen-presenting cells

Antigen-presenting cells (APCs) play a critical role in inducing aGVHD, with dendritic cells (DCs) being one of the most formidable cells in this regard[26]. Innate immunity activation during acute inflammation leads to DCs maturation and subsequent T cell priming, which is central to the potential antitumor benefits of aGVHD. Experimental data suggest that modulating perceptible DC subsets can influence aGVHD[34]. For instance, the absence of RelB signaling in host DCs or enhancing host CD8+ lymphoid DC subsets following HSCT significantly reduces aGVHD[35]. Other APCs, such as monocytes/macrophages, also play a crucial role in this phase. Some data suggest that the host B cells may reduce aGVHD in specific contexts. Although the precise mechanisms remain unclear when acting as APCs, MSCs from the donor, or host also reduce aGVHD.

Natural killer, γδ T, and natural killer T cells

Natural killer (NK) cells can directly kill tumor cells without specific immunization and also have a modulatory effect on aGVHD. In an allo-HSCT donor-to-F1 model, NK cells recognize the absence of donor class I on host APCs and eliminate them, resulting in a reduction of aGVHD reduction. Upon activation, NK cells may induce apoptosis of target cells through contact-dependent cytotoxicity primarily *via* perforin and granzyme[36]. Pro-apoptotic granzymes enter through perforin pores in the plasma membrane of target cells. Besides the cytotoxic activity, NK cell activation increases the secretion of various cytokines and chemokines, such as IFN-γ. However, the role of NK cells in GVHD remains controversial.

The infusion of donor γδ T cells may increase aGVHD, while the absence of host γδ T cells may reduce APC activation and aGVHD in an MHC-mismatched model. Conversely, in the absence of host γδ T cells, GVHD severity was not modified in an MHC-matched, miHA-disparate model of cGVHD. aGVHD could be more significant in patients with more considerable donor γδ T cells. The significance of γδ T cells in aGVHD and cGVHD is not fully understood and may reflect differences in immunobiology between the two or be solely a consequence of variation in the experimental models.

NKT cells, which are CD1d-reactive, are believed to play an immunoregulatory role in suppressing dysfunctional immune reactions, including GVHD[37]. The cumulative frequency of regulatory T cells (Tregs) is negatively correlated with GVHD development[38], and exogenous NKT cell infusion can reduce the degree of GVHD[39]. However, Treg populations have unstable Foxp3 expression, particularly those expanded in vitro. Because the expression Foxp3 is needed for the suppressive function, further research is necessary to determine if Foxp3 expression can be simplified, especially under pro-inflammatory conditions characteristic of the GVHD milieu[40].

T cells

The complex interactions between MSCs and T cells have been extensively studied, particularly *in vitro* culture techniques. MSCs may facilitate activated T cells in the phase G0/G1 cell cycle, yet apoptosis is not applicable[41-43]. MSCs may suppress or downregulate the proliferation of both naïve and memory T cells through cell-cell contact or mitogenic stimuli. This suppression is generally not MHC-restricted. MSCs can further decrease IFN-γ producing T cells and contribute to the T-cell skewing toward Th2 cells producing IL-4. cGVHD is a Th2 cell dominant disease process[12].

Regulatory T cells

MSCs activate *immune* responses that induce the expression of Tregs, which are a cluster of cells with a $CD4^+CD25^+$ $Foxp3^+$ phenotype that regulate the body's immune response. Tregs highly and constitutively express CTLA-4, which binds to CD80, and CD86 on DCs, leading to impaired DC maturation and blocking CD80/CD86 to CD28 on conventional T cells, thereby preventing costimulation, and T-cell activation. Lower Tregs and deficient Foxp3 expression have been associated with cGVHD in peripheral blood and mucosal biopsies. However, levels of Foxp3 mRNA in the CD25+ T cell compartment do not predict the development of cGVHD, demonstrating that the presence, or absence of Tregs must be considered in the context of their impact on aGVHD and cGVHD. An intriguing possibility is that the negative impact of calcineurin inhibitors on Tregs could exacerbate cGVHD as a consequence of the suppression of the alloreactive donor cytopathic and Tregs.

B cells

Host B cells attenuate aGVHD in an IL-10-dependent manner. Recent data provide a rationale for the pathogenic role of donor B cells in cGVHD[12], including a robust correlation between cGVHD and (1) The effects of antibodies against Y-chromosome-encoded miHA; (2) higher numbers of B cells with altered TLR9 responses; (2) levels of a B-cell-activating factor, which enhances survival and differentiation of activated B cells; and (4) in animal models, levels of autoantibodies. Besides, emerging data from the depletion of B cells with rituximab further supports the theory of the pathogenic action of B cells in cGVHD[44]. However, whether B cells are the effectors or inducers of cGVHD remains unknown.

SEARCH STRATEGY, STUDY SCREENING, AND SELECTION

We systematically searched the electronic bibliographic databases MEDLINE, EMBASE, and Google Scholar for studies published before November 2022 using the keywords: "graft-versus-host disease" OR "acute GVHD" OR "chronic GVHD" AND "mesenchymal stem cells" AND "mesenchymal stromal cells" AND "treatment response" AND "outcome." Publications were included if they met the following inclusion criteria: (1) Original research; (2) published in 2002 or later; and (3) specifically reporting on the use of MSCs in GVHD patients. Publications were excluded based on the following criteria: (1) Non-English literature; (2) small populations (n < 20 patients) or case studies; and (3) mixed population with non-GVHD patients. A meta-analysis was not performed for the limited number of published studies meeting the inclusion criteria. Pre-post design studies and case series were not included for lack of sustainability of the results. Additionally, reference lists of retrieved articles were cross-referenced for additional eligible articles.

RESULTS

This review provides an overview of clinical studies, animal models, and limited human patient trials regarding MSCs. MSCs have been widely studied and increasingly used in GVHD treatment since the first report of promising results by Le Blanc *et al*[45] However, the studies have reported varying outcomes, which could be contributed to differences in cell concentration and MSC infusion dose. While MSC infusion has shown quite promising results following GVHD prophylaxis failure, some clinicians still prefer using methylprednisolone and calcineurin inhibitors before cell therapy with MSCs.

In addition to suppressing inflammation, MSCs have other beneficial effects, including increased angiogenesis, reduced apoptosis, and modified extracellular matrix dynamics. These cells mediate immune system components like macrophages and neutrophils, improving tissue microenvironments. After the injury, MSCs can either promote or suppress the immune system to guide the whole-tissue regeneration process[1]. Clinical responses to MSC infusion assessed as early as one week after treatment may predict patients' overall survival, indicating the potential of MSCs in treating GVHD[45].

Although the paracrine effects of MSCs are known to mediate the modulation of the immune response, the mechanisms underlying this modulation are not yet fully understood. However, it has been found that under conditions of chronic hypoxia or co-stimulation with IFN-γ, MSCs express proteins that have the immunosuppressive capacity, such as IDO, HLA-G, PGE2, and FasL, which can modulate the immune response[46]. While other cytokines play a crucial role in immunosuppression, blocking highly expressed proteins can result in the setback of the human immunosuppressed state, leading to the growth, and proliferation of immune cells. Moreover, MSCs do not trigger the activation of immune cells as they lack CD40, CD80, CD86, and HLA-DR-stimulating molecules. Given that GVHD occurs following the infusion of immune cells donated by the same donors, suppressing the immune activity can improve the patient's prognosis. MSCs' expression of paracrine effects can regulate these donor immune cells through various mechanisms (Table 1)[47-62].

When MSCs are exposed to an insult, such as injury, or bacterial infection, MHC-II molecules facilitate the presentation of bacterial antigens, which induces further activation of T cells expressing IFN-γ. MHC-II is downregulated at high levels of IFN-γ, while B7-H1 is upregulated[45]. These presentation pathways are illustrated in Figure 2.

MSCs have been used to treat various conditions, including diabetes mellitus (DM), cardiovascular diseases, GVHD, and autoimmune diseases. Despite persistent questions, the immunomodulatory effects of MSCs make them a top choice for cell therapy. MSCs are early multipotent progenitors and non-hematopoietic cell populations that can be expanded ex-vivo to achieve large numbers necessary for *in vivo* use. Recently, adipose tissues, umbilical cord, placenta, and dental pulp have been recognized as multipotent sources of MSCs. MSCs can differentiate into a variety of cell types capable of osteogenic, chondrogenic, adipogenic, myogenic, and neurogenic differentiation. However, not all individual cells cultivated in tissue culture flasks result in the same degree of multipotency. Self-renewing progenitors can be identified in human BM, and it is currently unknown whether MSCs from other tissues exhibit this property. BM-MSCs are a critical source of multipotent stem cells and serve as a standard for comparing MSCs from different sources (Table 2)[49,63-85].

The term "mesenchymal stem cells" has been proposed as a more appropriate term than MSCs. These cells possess not only multipotency but also significant immunomodulatory and engraftment-promoting properties. They create a specialized microenvironment for HSCs by promoting the secretion of various inflammatory cytokines, chemokines, growth factors, extracellular matrix, and extracellular vesicles that are crucial for HSC differentiation, proliferation, and maintenance[86-88]. After *in vivo* biological application, MSCs secrete a range of cytokines and regulatory molecules with anti-inflammatory, wound healing, and regenerative effects, promoting the repair of endogenous tissues or tissue replacement. Beres *et al*[40] demonstrated that even in otherwise immunocompetent humans, allogeneic MSCs may graft, and differentiate through significant histocompatible barriers.

Table 1 Immunosuppressive effect exerted by mesenchymal stem/stromal cells from different sources on immune cells

MSC types	Mechanism of immunosuppressive effect	Ref.
BM-MSCs	Recipient-derived MSCs from patients with GVHD are analogous to MSCs from healthy volunteers	Copland *et al*[47]
	After MSC infusion, the ratio of Th1 cells to Th2 cells was reversed, with an increase in Th1 and a decrease in Th2 achieving a new balance	Zhou *et al*[48]
	BM-MSCs reduce the incidence and severity of GVHD by improving thymic function and induction of Tregs but not increase the risks of infections and tumor relapse	Zhao *et al*[49]; Selmani *et al*[50]
	HLA-G5 secreted by MSCs is critical to the suppressive functions of MSCs	Selmani *et al*[51]
MenSCs	MenSCs exhibit a higher capacity to migrate into the intestine and liver and not to their anti-inflammatory capacities	Luz-Crawford *et al*[52]
FL-MSCs	FL-MSCs demonstrates much longer-lasting immunomodulatory properties by inhibiting directly the proliferation and activation of CD4+ and CD8+ T cells	Yu *et al*[53]
UC-MSCs	UC-MSCs showed minimal expression of HLA-DR after activation and posed minimal risk of initiating an allogeneic immune	Kim *et al*[54]
	UC-MSCs alleviate SLE through upregulating Treg cells, which was partly dependent on HLA-G	Chen *et al*[55]
	UC-MSCs ameliorate GVHD and spare GVL effect *via* immunoregulations	Wu *et al*[56]
WJ-MSCs	WJ-MSCs exert immunosuppressive effects by cell-cell contact with activated T cells and in part through the soluble factor indoleamine 2,3-dioxygenase	He *et al*[57]
MC-, WJ- and BM-MSCs	The mixed populations of MSCs displayed all of the positive attributes of WJ-MSC and BM-MSC	Mennan *et al*[58]
AT-MSCs	The use of AT-MSC rather than BM-MSC could further preserve NK cell activity and favor GVL	Blanco *et al*[59]
hG-MSCs	hG-MSC treatment inhibited local inflammation of injured skin by suppressing inflammatory cells, reducing pro-inflammatory cytokine tumor necrosis factor-α, and increasing anti-inflammatory cytokine interleukin-10, which was promoted by hypoxia	Jiang *et al*[60]
CP-, BM- and AT-MSCs	CP-MSCs may have additional advantage over the other MSCs in terms of immunomodulation	Lee *et al*[61]
DP-MSCs	Immunomodulation and expression of trophic factors by dental MSCs increase their resistance to allogeneic NK cell lysis and their potential in vivo lifespan	Martinez *et al*[62]

AT: Adipose tissue; BM: Bone marrow; CP: Chorionic plate; DP: Dental pump; FL: Fetal liver; hG: Human gingiva; GVHD: Graft-versus-host disease; GVL: Graft-versus-leukemia; MenSC: Menstrual blood-derived mesenchymal stem cell; MSC: Mesenchymal stem cell; UC: Umbilical cord; WJ: Wharton jelly.

Similar to hematopoietic stem cells, MSCs have multi-organ specificity, and plasticity. In 2006, the International Society for Cellular Therapy officially defined MSCs as plastic practitioners under standard growing conditions, expressing CD73, and CD90 surface molecules while lacking CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR[2]. In addition, MSCs can differentiate into various mesodermal lineages including osteoblast, adipocyte, and chondroblast, to different degrees.

MSCs are capable of modulating both innate and adaptive immunity through the release of various soluble factors, including indoleamine 2,3-dioxygenase[11], IL-10, prostaglandin 2, nitric oxide, transforming growth factor-β, HLA-G5, and anti-inflammatory molecule TNF-α-induced gene/protein 6 [89]. These molecules are believed to play a key role in the immunomodulating effects of MSCs, which have been shown to be beneficial in certain immunopathological diseases, such as aGVHD, and type 1 DM. However, the precise mechanisms underlying this therapeutic potential are not yet fully understood. The literature suggests that the immunomodulating potential of MSCs involves interactions with both humoral and cellular components of the innate and adaptive immune systems. The literature refers to several fundamental cellular interactions. An integrated perspective on the utility of MSCs for GVHD has been strengthened by the recent findings that MSCs are induced to undergo necrosis/apoptosis by the recipient's cytotoxic cells and that this process is assumed to elicit MSC-induced immunosuppression[90]. This finding made it possible to reconcile the dilemma between the effectiveness of MSC and its apparent lack of engraftment and highlighted the crucial role of the patient in the promotion and administration of immunosuppression of MSCs. Recent research has shed light on the role of the patient in promoting and administering immunosuppression of MSCs, with evidence suggesting that MSCs are induced to undergo necrosis/apoptosis by the recipient's cytotoxic cells, leading to MSC-induced immunosuppression[90]. Table 2 provides an overview of recent studies on this topic, with 97 articles selected for full-text evaluation based on agreed-upon title and abstract criteria.

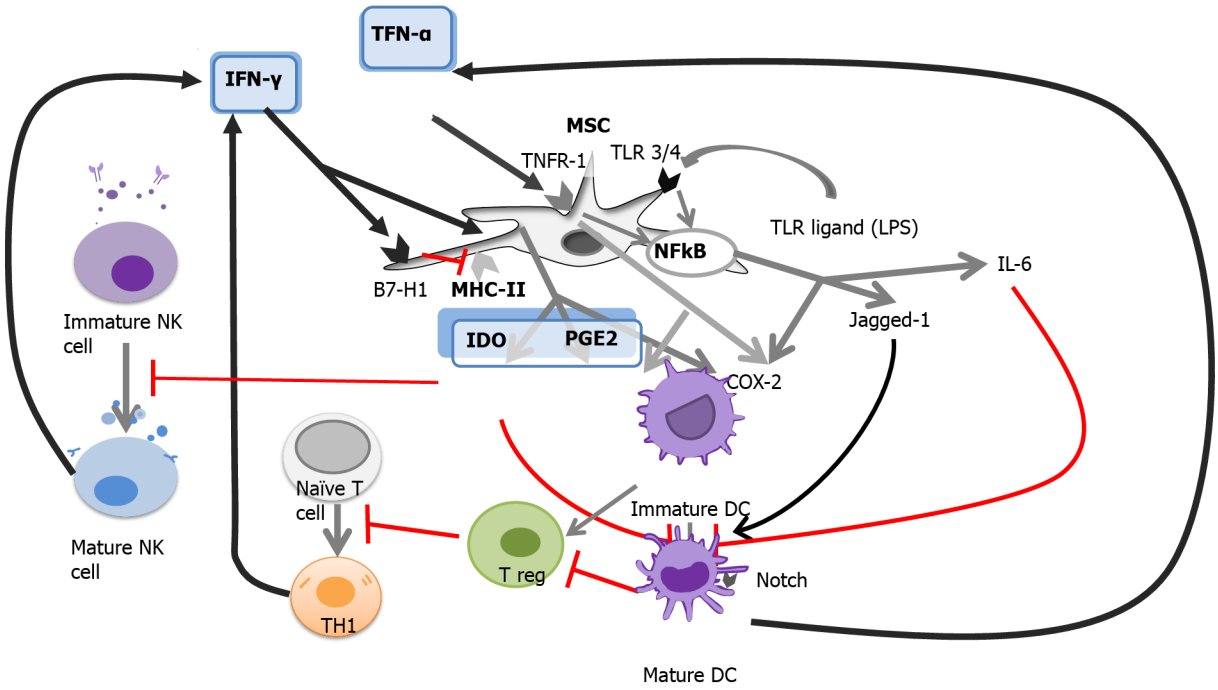
Innate immunity is primarily centered around the complementary system, with C3, and C5 being cleaved into anaphylatoxins C3a and C5a by convertases at the sites of inflammation. The labile C3

Table 2 Effects of different mesenchymal stem/stromal cells on refractory acute graft-versus-host disease

Study type	Patient No.	Indication	MSC type	Response criteria	Main findings	Ref.
Phase 2	55	Steroid-resistant, severe, aGVHD	BM	Glucksberg	CR: 30/55, better OS/TRM for complete responder	Le Blanc *et al* [63], 2008
Phase 2	31	Gr. II-IV aGVHD	BM	Glucksberg	CR: 77%; PR: 16%	Kebriaei *et al*[64], 2009
Pilot study	20	Co-transplantation with NMA mismatched HSCT	BM	Glucksberg	Decreased 1 yr GVHD death (10% *vs* 31%, $P = 0.04$). Better NRM & OS	Baron *et al*[65], 2010
Retrospective	37	Resistant Gr. III-IV aGVHD	BM	Glucksberg	CR: 65%, better TRM and OS	Ball *et al*[66], 2013
Multicenter trial	50	Resistant Gr. IV aGVHD	BM	Not mentioned	OR: 33%, CR: 17%, initial response and young age have better survival	Resnick *et al*[67], 2013
Prospective, single-arm, open-label	75	Severe refractory aGVHD	BM	IBMTR SI	OR on day +28: 61.3%, better OS for responder on day +100 (78.1% *vs* 31.0%; $P < 0.001$)	Kurtzberg *et al* [68], 2014
Phase 1	40	Resistant Gr. II-IV aGVHD	BM	Glucksberg	CR: 27.5%, OR: 67.5% on day +28; more CR in pediatric group	Introna *et al*[69], 2014
Phase 2	25	Refractory aGVHD	BM	Glucksberg	71% responded, CR 11/24, better OS for CR	Sánchez-Guijo *et al*[70], 2014
Prospective, nonrandomized	28 *vs* 19 without MSC	Refractory aGVHD	BM	Glucksberg	Decreased incidence and severity of cGVHD. Better OR and CR.	Zhao *et al*[49], 2015
Phase 2	48	Steroid-resistant aGVHD	BM	Glucksberg	CR: 25% on day 28, 50% lasting > 1 mo, with better OS	Te Boome *et al* [71], 2015
Compassionate use	58	Steroid-resistant aGVHD	BM	IBMTR SI	OR: 47%, but no improvement in OS	von Dalowski *et al*[72], 2016
Phase 2/3	25	Refractory Gr. III-IV aGVHD	BM	Glucksberg	Better OS for OR at 4-wk (CR: 6/25, PR: 9/25)	Muroi *et al*[73], 2016
Pilot study	33	Refractory aGVHD	BM	IBMTR SI	CR: 18/33, PR: 7/33, better OS in CR, no TRM in CR	Erbey *et al*[74], 2016
Compassionate use	26	Severe resistant aGVHD	BM	Not mentioned	OR: 77% on day +28 (CR: 5/26, PR: 15/26)	Kuçi *et al*[75], 2016
Phase 2 prospective RCT	62 *vs* 62 without MSC	cGVHD prophylaxis in haplo	Cord	NIH score	cGVHD: 27% (MSC) *vs* 49% in 2 yr ($P = 0.021$)	Gao *et al*[76], 2016
Phase 1/2	26	Steroid-refractory aGVHD	BM	Glucksberg	OR: 62% on day 28. Higher response rate in children. High NRM in adults	Salmenniemi *et al* [77], 2017
Pilot study	22	Refractory GVHD (Gr. 2-4 a or cGVHD)	BM or adipose tissue	Glucksberg/NIH score	CR: 45.8%, PR: 33.3%, better OS in CR/PR	Cetin *et al*[78], 2017
Retrospective	46	Refractory Gr. III/IV aGVHD	BM	Not mentioned	50% responded with better OS ($P = 0.0004$)	Dotoli *et al*[79], 2017
Phase 1/2	33	Steroid-refractory aGVHD	BM	Glucksberg	CR: 34%, PR: 50% on day 28. Better OS on day 90 and 1 yr ($P = 0.006$, 0.002)	Fernández-Maqueda *et al* [80], 2017
Phase 1/2	69	Refractory aGVHD	BM	Glucksberg	OR: 83% on day 28	Bader *et al*[81], 2018
Observational study	34 *vs* 34 without MSC	aGVHD	BM or adipose tissue	IBMTR SI	Better OS compared with historical control, $P = 0.0678$. MSC has no association with risk of infectious complication	Stoma *et al*[82], 2018
Retrospective	11 (study group 2)	Severe refractory aGVHD	Placenta derived decidual stromal cell	Glucksberg	73% 1 yr OS in study group 2 (albumin), 47% in group 1 (AB plasma), $P = 0.016$	Ringden *et al* [83], 2018
Retrospective	22	Severe refractory aGVHD	Cord	IBMTR SI	CR: 45.5%, PR: 13.6%	Bozkurt *et al*[84], 2019

Phase 3 RCT	151 *vs* 72 placebo	Severe refractory aGVHD	BM	IBMTR SI	Difference of durable CR (lasting > 28 d) not achieved (35% *vs* 30%, P = 0.42); Pediatric pts had better OR (64% *vs* 23%, P = 0.05)	Kebriaei *et al*[85], 2020

aGVHD: Acute graft-versus-host disease; BM: Bone marrow; cGVHD: Chronic graft-versus-host disease; CR: Complete remission; IBMTR SI: International Bone Marrow Transplant Registry severity index; MSC: Mesenchymal stem/stromal cells; OS: Overall survival; PR: Partial remission; RCT: Randomized controlled trial.



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Figure 2 The complex network of antigen presentation and immunomodulation. Mesenchymal stem/stromal cells (MSCs) exert immunomodulatory functions mainly *via* interactions with immune cells through cell-to-cell contacts and paracrine activity. The MSC secretome includes several cytokines, growth factors, and chemokines, and their immunomodulatory functions vary depending on the source of the MSCs, the target cells, and the microenvironment. COX-2: Cyclooxygenase-2; IDO: Indoleamine-pyrrole 2,3-dioxygenase; IFN: Interferon; IL: Interleukin; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2: Prostaglandin E2; TLR: Toll-like receptors; TNF-α: Tumor necrosis factor-α; DC: Dendritic cell; LPS: Lipopolysaccharide; NK: Natural killer cells.

convertases cleave C3 into C3a and C3b which can thereafter participate in forming distinct complexes and activate pathways for proliferation and protection against apoptosis through receptor binding. MSCs also secrete the factor H, which inhibits complement activation by limiting the activity of C3 and C5 convertases. In mice, MSCs promote pro-inflammatory repolarization and produce chemostatic cytokines, including IL-6, IL-8, GM-CSF, and macrophage inhibitory factors. IL-8, in particular, is a pro-inflammatory chemokine produced by multiple cell types that recruits leukocytes to sites of infection or tissue injury. Additionally, MSCs can inhibit mast cell degranulation and histamine release by binding allergens to allergen-specific IgE *via* FcRε on mast cells, providing a potential therapeutic benefit for allergic reactions[91].

The molecular interaction between NK cells and MSCs is complex and depends on the immune microenvironment and NK cell activation status. MSC can inhibit cytokine proliferation and production and interfere with NK cell cytotoxicity. They also inhibit monocyte maturation and differentiation into DCs, which are the primary type of APC and play a key role in T lymphocyte activation through antigen presentation. Monocytes and macrophages are important for tissue development, homeostasis, and injury repair. Activated MSCs produce chemokines that attract circulating monocytes to sites of inflammation and injury[92].

MSCs can regulate the adaptive immune system through multiple redundant pathways. They suppress the proliferation of T cells, IFNγ production, CD4 T cell differentiation, and CD8 T-cell cytotoxicity. Di Nicola *et al*[41] reported that MSCs can suppress T lymphocyte proliferation *in vitro* with autologous and allogeneic MSCs, including T lymphocytes cultured with DCs or lymphocytes in mixed lymphocyte reactions. MSCs can express and secrete programmed death-ligand 1 and 2, which suppress T-cell proliferation in the presence of MSCs, secrete IL-2, induce apoptosis, and promote the induction of an irreversible hyporeactive state[93]. In vivo studies suggested that MSCs may restore the balance

between T helper 1 and 2 cells in diseases associated with a shift to dominance of these T cell subpopulations[94]. *In vitro* models have shown that MSCs induce Tregs and maintain survival and suppressive phenotypes[95].

CONCLUSION

This article provides insights into the use of validated MSCs as a potential treatment strategy for GVHD in HSCT. Current prevention and treatment options involve immunosuppression, which can hinder immune recovery and limit the graft-versus-tumor effect. By using MSCs, clinicians can effectively treat GVHD, identify high-risk patients, and stratify patients based on disease severity. Therefore, MSCs can aid in promoting engraftment, ameliorating aGVHD, and preventing cGVHD, making them an attractive option for HSCT.

FOOTNOTES

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MINIREVIEWS

Role of brahma-related gene 1/brahma-associated factor subunits in neural stem/progenitor cells and related neural developmental disorders

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Abstract

Different fates of neural stem/progenitor cells (NSPCs) and their progeny are determined by the gene regulatory network, where a chromatin-remodeling complex affects synergy with other regulators. Here, we review recent research progress indicating that the BRG1/BRM-associated factor (BAF) complex plays an important role in NSPCs during neural development and neural developmental disorders. Several studies based on animal models have shown that mutations in the BAF complex may cause abnormal neural differentiation, which can also lead to various diseases in humans. We discussed BAF complex subunits and their main characteristics in NSPCs. With advances in studies of human pluripotent stem cells and the feasibility of driving their differentiation into NSPCs, we can now investigate the role of the BAF complex in regulating the balance between self-renewal and differentiation of NSPCs. Considering recent progress in these research areas, we suggest that three approaches should be used in investigations in the near future. Sequencing of whole human exome and genome-wide association studies suggest that mutations in the subunits of the BAF complex are related to neurodevelopmental disorders. More insight into the mechanism of BAF complex regulation in NSPCs during neural cell fate decisions and neurodevelopment may help in exploiting new methods for clinical applications.

Key Words: Neural stem/progenitor cell; BRG1/BRM-associated factor complex; Subunit; Proliferation; Differentiation; Neural developmental disorders

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Core Tip: There are several reviews in the literature contributed to the role of BRG1/BRM-associated factor (BAF) complex in neural cell specification and neural development diseases. We review recent progress indicating that BAF complex plays an important role in neural stem/progenitor cells (NSPCs) during neural development and neural developmental disorders. More progresses in the role of BAF complex subunits in balancing self-renewal and differentiation of NSPCs and neurodevelopment could finally be involved in highlighting new methods for clinical application.

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INTRODUCTION

Neural stem/progenitor cells (NSPCs) are self-renewing neural cells capable of differentiating into neurons, astrocytes and/or oligodendrocytes[1-3]. During development, NSPCs are critical for the establishment of the central nervous system (CNS)[4-6]. In the adult central neural system, neurogenesis plays a key role in fundamental processes, for example, memory, learning, the maintenance of normal tissue homeostasis, and the autonomous repair of pathological brain tissues[7,8]. NSPCs can be isolated from three canonical neurogenic niches in the spinal cord and brain, namely, the central canal (CC) in the spinal cord, the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle (LV) in the brain[9-13]. In addition, NSPCs have been identified in the developing cerebral cortex[14], olfactory epithelium[15] and outside the CC[16]. The fact that NSPCs can be isolated and propagated *in vitro* opens up new opportunities for medical research, and we hope they can be used to compensate for cell loss that features in several serious neurological disorders. Moreover, NSPCs can be created *in vitro* through induced differentiation from induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), expanding the pool of models for studying stem cells in health and illness[17,18]. For cell-based therapy approaches targeting the brain and spinal cord, NSPCs have emerged as focal points. However, the small amount of NSPCs present in this tissue has restricted the clinical uses of these cells. Although recent advancements in ESCs and iPSC research have meant that these cells can be novel sources of NSPCs, understanding NSC molecular regulation and NSPC applications, which are important for disease modeling and regenerative medicine, remain challenges[19-21].

The BRG1/BRM-associated factor (BAF) complex, which is a mammalian chromatin remodeler, regulates chromatin structure and transcription by providing subunit organization and nucleosome recognition in an ATP-dependent manner. Structurally, the BAF complex contains three modules, an ATPase, an actin-related protein (ARP) and a base module[22]. More than 30 major subunits of these modules can combine to form the complex[22-24].

By controlling chromatin structure, controlling the differentiation of NSPCs to produce different types of brain cells, and regulating transdifferentiation between cell types, BAF complexes perform crucial roles in the maintenance of a gene expression program. At various phases of brain formation, BAF complexes have specific roles and seem to be produced by the combinatorial assembly of their subunits. Moreover, intimate interactions between the BAF chromatin-remodeling complex and transcriptional machinery control the development of NSPCs[25]. With the combinatorial assembly of homologous component families, which enables their nonredundant functions, distinct BAF complex structures are made possible. ESCs, NSPCs, and postmitotic neurons all exhibit developmental stage-specific BAF assemblages during mammalian brain development. Neuronal BAF (nBAF) function is required for the development of mature postmitotic neuronal features along with long-term memory, and neural progenitor-special BAF (npBAF) structures are particularly important for modulating the rates and modalities of neural progenitor cell replication. Given the high prevalence of BAF subunit mutagenesis in neurological illnesses, it is obvious that BAF complexes perform a crucial role in controlling the rate of neuronal development, homeostasis, and plasticity. Understanding how BAF complex subunits influence BAF complex function and the roles played by different subunits will reveal disease pathogenesis and lead to novel treatments for related human disorders ultimately[26,27].

Here, we summarize recent progress in comprehending the role of the BAF complex in NSPCs. We focus on NSC-/NPC-specific BAF complex subunits and describe how they determine the balance between the self-renewal and differentiation of NSCs and are involved in several human neural developmental disorders. Supplementary Table 1 shows the abbreviations and their descriptions.

BAF COMPLEX SUBUNITS AND THEIR MAIN CHARACTERISTICS IN NSPCS

Among the major BAF complex subunits, there are 12 subunits expressed in NSPCs in the embryo cortex, olfactory bulb (OB) and spinal cord, adult brain and spinal cord, and human and mouse retina (detailed information and references are shown in Table 1).

BAF190A (BRG1/ SMARCA4)

Brg1 (Brahma-related gene 1) is necessary for NSPCs to remain in a state where they can react to gliogenic signals and to suppress neuronal commitment. Lack of Brg1 in NSPCs led to precocious neurogenesis, such that cells in the ventricular zone developed into postmitotic neurons before the start of gliogenesis in conditional brg1- mutant mice. As a result, these animals significantly lost the ability to differentiate astrocytes and oligodendrocytes. In addition, in vitro Brg1 deletion reduced growth factor-induced astrocyte development in gliogenic progenitors. Furthermore, it has been discovered that the levels of proteins associated with stem cell maintenance, such as Pax6, Sox1, and Musashi-1, are significantly lower in the ventricular zones of the mice with brg1-mutations[28]. The hippocampus experienced abnormal adult neurogenesis as a result of Brg1 deletion in nestin-expressing NSPCs. This abnormal adult neurogenesis initially decreased the number of adult NSPCs in the hippocampus, inhibited progenitor maintenance, and later decreased NSPC responsiveness to physiological stimulation. Brg1 deletion appears to hinder cell cycle progression mechanistically, which is partly because of increased p53 pathway activation and p21 expression. Defects in neurosphere formation brought on by Brg1 deletion were repaired by knocking down p53. These findings suggest that aNSPC and progenitor cell maintenance and responsiveness during neurogenesis are determined by epigenetic chromatin remodeling *via* a Brg1- and p53/p21-dependent mechanism[29].

Many transcription factors manipulating neuronogenesis have been ascertained in the adult and developing brain, and notably, the neurogenic transcriptional regulation factor Pax6, which can directly interact with the Brg1-carrying BAF complex in adult neural progenitor cells. Abolition of either Pax6 or Brg1 in the subependymal zone (SEZ) caused the offspring of adult NSPCs to switch to the SEZ ependymal cell lineage. In the interim, shifting neuroblasts changed to distinct glial cell lineages during or after reaching the OB. Several studies have revealed that there is a network with tripartite effector programing neuronal fate, which can be activated by Pax6-BAF and promote neurogenesis and the transformation from glia to neurons. According to the whole-genome analysis, the downstream regulatory factors of Pax6-BAF include Nfib, Sox11 and Pou3f4. While Brg1 is absent in SEZ and OB, the binding sites of Pax6 carried by Brg1 and most down-regulated genes will be down-regulated at the same time[30]. In the study of Jayaprakash *et al*[31], they find that Brg1 appears as a slender spherical structure so that it can provide a lager surface and form the further complex by binding to BAF57 and BAF60A. Furthermore, a neural Brg1 isoform interacts with the central neurodevelopmental transcriptional repressor REST/NRSF, suggesting that Brg1 can interact with transcription regulators and factors to conduct the BAF complex to specific sites[31].

BAF155 (SMARCC1) and BAF170 (SMARCC2)

Recent study found that conditional deletion of BAF155 can lead to the decrease of basal intermediate progenitors (bIPs), while the delamination of apical radial glial progenitors (RGs) can cause the increase of basal RGs (bRGs). During the progress, BAF155 has been proved necessary for the normal activity of PAX6 to regulate the gene expression. BAF155 can control the expression of the CDC42 effecting protein CEP4 in the Pax6-dependent way in order to regulate the stratification of progenitor. In addition, BAF155-dependent chromatin remodeling is also involved in regulating several human RG-specific genes, thereby acting on the generation of basal progenitors (BPs)[32].

The study of Narayanan *et al*[33] in 2015 revealed a molecular mechanism of controlling the global chromatin state and transcriptional program during development, which is mediated by the BAF complex. They found that BAF155 and BAF170 knockout can cause elimination of the entire BAF complex with all kinds of BAF subunits in cortical development, with the overall increase of the abundance of the repressive marks (H3K27me2/3), decrease of active chromatin mark H3K9Ac, and down-regulation of gene expression. Both BAF155 and BAF170 subunits are expressed in early cortical progenitors (E10.5-E14.5), and BAF170 is replaced by BAF155 in late cortical progenitors[34]. Specifically, between E12.5 and E14.5, apical progenitors express BAF170 and BAF155. They exhibit comparable expression patterns at the height of upper layer neurogenesis (E15.5), with BAF170 being missing from VZ progenitors but expressed in the cortical plate but expressed in the cortical plate[34]. While BAF170 overexpression resulted in the acquisition of a feature similar to that acquired by the Pax6-loss brain, with depletion of overlying layer neurons, BAF170 loss in Emx1t cortical progenitors exerted an effect opposite to that of Pax6 elimination and caused an aberrant growth of IPs and a bias toward the acquisition of an overlying layer identity[34]. By the recruitment of the transcriptional suppressor REST to Pax6 targets, the BAF170-containing npBAF complex indirectly suppresses neurogenesis in its early stages[34,35]. Interestingly, the transcription of BAF170 and Brm subunits, which replace BAF155 and Brg1 subunits in the esBAF, respectively, begins at the same time when ES cells commit to developing into neural progenitors[33,36]. According to a recent functional invest-

Table 1 BRG1/BRM-associated factor complex subunits in neural stem/progenitor cells

BAF complex subunits (aliases)	NSPC types	Function	Interaction signalings/factors	Related neural developmental diseases	Ref.
BAF45A (PHF10)	oNSC, E10.5-E11.5 cortical NPCs, E10.5-E16.5 spinal cord VZ	NSPC proliferation	BRG1	-	Bachmann *et al*[15], 2016; MacDonald *et al*[89], 2022; Lessard *et al*[41], 2007
BAF45D (DPF2)	SGZ, SVZ, spinal cord central canal GFAP + radial glial cells	PAX6 expression, contributes to NSPC induction	PAX6, BRG1	CSS	Liu *et al*[42], 2017; Wang *et al*[44], 2019; Chen *et al*[43], 2022; Vasileiou *et al*[69], 2018; Knapp *et al*[70], 2019; Milone *et al*[71], 2020
BAF47 (SMARCB1)	oNSC	-	BRG1	CSS, kleefstra syndrome, Nicolaides-Baraitser syndrome	Bachmann *et al*[15], 2016; Tsurusaki *et al*[72], 2012; Santen *et al*[74], 2013; Wieczorek *et al*[75], 2013; Kleefstra *et al*[88], 2012; Gossai *et al*[73], 2015
BAF53A (ACTL6A)	oNSC, NPCS in the neural tubes of E11.5 embryos	BAF53A is essential for NPC proliferation	miR-9 and miR-124	-	Bachmann *et al*[15], 2016; Lessard *et al*[41], 2007; Yoo *et al*[45], 2009
BAF55A (SS18)	NPCs in VZ	SS18 is required for NSC self-renewal	BRG1	-	Staahl *et al*[48], 2013
BAF57 (SMARCE1)	Adult human OBNSCs	NSC proliferation	Neuregulin-1	CSS	Wieczorek *et al*[75], 2013; Kosho *et al*[67], 2014; Marei *et al*[52], 2012; Zarate *et al*[76], 2016; Pirotte *et al*[63], 2010
BAF60A (SMARCD1)	Oligodendrocyte precursors	-	BRG1, BAF155 and BAF170	CSS, Nicolaides-Baraitser syndrome	(Yu *et al*[54], 2013; Hsiao *et al*[53], 2003; Machol *et al*[78], 2019; Nixon *et al*[77], 2019
BAF60C (SMARCD3)	NPCs in human retinas as well as mouse retina, cortex and spinal cord	Keeps the progenitors in a proliferative state -	Notch signaling	-	Lamba *et al*[55], 2008
BAF155 (SMARCC1)	oNSC, adult human OBNSCs	Proliferation and maintenance of ONSCs forebrain development	BRG1, PAX6	ASD	Lessard *et al*[41], 2007; Marei *et al*[52], 2012; Narayanan *et al*[33], 2015; Neale *et al*[84], 2012
BAF170 (SMARCC2)	oNSC, in postnatal DG, in RGL progenitors	oNSC proliferation, affects NSC proliferation, differentiation, forebrain development	BRG1	CSS, ASD, Nicolaides-Baraitser syndrome	Lessard *et al*[41], 2007; Machol *et al*[78], 2019; Narayanan *et al*[33], 2015; Neale *et al*[84], 2012; Tuoc *et al*[38], 2017
BAF190A (BRG1/SMARCA4)	Mouse VZ, SGZ, SEZ, NSCs/NPCs, NCC	NSC maintenance and neuronal differentiation	PAX6, BAF45D	CSS, ASD	Tsurusaki *et al*[72], 2012; De Rubeis *et al*[85], 2014; Matsumoto *et al*[28], 2006; Petrik *et al*[29], 2015; Ninkovic *et al*[30], 2013
BAF250A (ARID1A)	Developing cortex	Regulates NSPC proliferation and differentiation during cortical development	BRG1	CSS	Tsurusaki *et al*[72], 2012; Liu *et al*[40], 2021

BRG1: Brahma related gene 1; BRM: Brahma; BAF: BRG1/BRM-associated factor; NSPCs: Neural stem/progenitor cells; oNSCs: Olfactory neural stem cells; CSS: Coffin-siris syndrome; VZ: Ventricular zone; OBNSCs: Olfactory bulb-derived neural stem cells; ASD: Autism spectrum disorder; KSS: Kleefstra syndrome spectrum; DG: Dentate gyrus; RGL: Radial glial-like; SEZ: Subependymal zone; NCC: Neural crest cells.

igation, Pax6 is directly associated to the BAF complex in aNSCs that contained the Brg1, and its neurogenic activity was abolished in the lack of the Brg1[30]. BAF170 and BAF45 also carry domains with putative DNA-binding capacities[37]. In the neonatal DG, the BAF170 member of the complex is found in radial glial-like (RGL) cells and cell types implicated in following stages of adult neurogenesis together with mature astrocytes. The pool of RGL cells in the DG is reduced by conditional knockout of BAF170 during late cortical neurogenesis and also adult brain neurogenesis, which encourages terminal astrocyte differentiation. A moderate loss in spatial learning was produced by the induced abolition of BAF170 in the DG throughout adult brain neurogenesis, with the reversal test showing the greatest impact on spatial learning. These findings indicate a specialized role for adult neurogenesis in the DG

regulating adaptive behavior and show the engagement of BAF170-reliant chromatin modification in neurogenesis and cognition of hippocampus[38].

BAF250A (ARID1A)

The core ATPase, Brg1 or Brm, BAF250A or BAF250B, a homologous dimer of BAF155, or a heterologous dimer of BAF170 and BAF155 are just a few examples of the different subunits that npBAF can contain[27]. Mammal BAF is significantly different from its yeast analogue, losing some members while obtaining new core members, such as BAF45a/b/c/d, BAF57, BAF250A/B, SS18/CREST, beta-actin, BRD7, and BRD9. Notably, BAF250A/B are the most commonly mutated subunits of BAF complex in related human neurological diseases[36]. Loss of BAF250A or BAF250B in mouse models leads to early embryonic lethality[39], indicating that, despite their homology, both genes are essential for early development.

Recent genome sequencing and many clinical studies have revealed that mutations of BAF250A is closely related to microcephaly and mental retardation. Liu *et al*[40] generated a *baf250a* conditional knockout mouse line and found that cortical thickness was reduced in the developing cortex. Radial glial cell proliferation is inhibited by loss of BAF250A function, which also increases the rate of cell damage during late cortical neurogenesis and causes abnormal expression of genes involved in proliferation and differentiation. Therefore, for the purpose of better understanding the pathogenic mechanisms and creating new treatments for neurodevelopmental disorders brought on by its mutations, BAF250A may be one of the gene possibilities that is worthwhile being researched[40].

BAF45A and BAF45D (DPF2)

BAF45 family proteins, including BAF45A, BAF45B, BAF45C and BAF45D, are subunits of the BAF complex[41]. BAF45A, an npBAF, is required for the self-renewal and proliferative features of neural cells[41]. BAF45A is identified in olfactory neural stem cells (oNSCs), E10.5-E11.5 cortical NPCs, and E10.5-E16.5 spinal cord ventricular zone NSCs[15,41]. BAF45D is expressed in the SGZ of the LV, SGZ of the DG, and the CC of the adult spinal cord. Co-expression of BAF45D and glial fibrillary acidic protein (GFAP), a marker protein of radial glial cell-like cell was found in the SGZ, SVZ, and CC of the adult spinal cord. According to the results of quantitative examination, BAF45D is selectively expressed in the adult neurogenic regions of neurons in CNS. What's more, BAF45D is necessary for the induction of PAX6, a determinant for neuroectoderm that regulates adult neural stem/progenitor cell self-renewal and specific neural fate, during neuroectodermal differentiation of H9 cells[42].

Recently, BAF45D has been shown to regulate spinal cord neural stem/progenitor cell (SCNSC/SCNPC) fate mediated through the SMAD-PAX6 axis. SCNSCs exhibit increased DNA-BAF45D compared to that in human ESCs[43]. It has been reported that postmitotic neurons express BAF45B/C protein but lack the paralogous BAF45A/D, which confers neuronal properties[25-27,41]. The protein expression of BAF45D in adult rat NSPCs and neurons but not astrocytes may imply a role for BAF45D in the differentiation of NSPCs[44].

BAF53A (ACTL6A)

The BAF53 subunit is encoded by the BAF53A (ACTL6A) or BAF53B (ACTL6B) gene, with the latter exclusively expressed in differentiated neurons[41].

BAF53A is specific to the npBAF complex and can be replaced by BAF53B in the nBAF complex in neurons[26,45]. miR-9 and miR-124 control the transition of postmitotic neurons from the BAF complex's BAF53A to BAF53B subunit[46]. These miRNAs' expression is derepressed in neurons during cell development, which encourages the replacement of BAF53B in the nBAF complex by suppressing the expression of BAF53A. Increased progenitor proliferation can result from BAF53A's continued expression[46]. These miRNAs have an interacting location in the 3′UTR of BAF53A, and their expression is constrained in progenitors by REST and its corepressors[47]. As a result of decreased chromatin accessibility at particular neural transcription factor-binding sites brought on by BAF53A loss, cell cycle-related genes are repressed, preventing the advancement of the cell cycle and cell differentiation[48].

BAF57 (SMARCE1)

BAF57, subunit in the BAF complex core, is highly conserved in the BAF complexes of vertebrates[49]. The DNA-binding capabilities of its primary structural characteristic, a high-mobility group domain, imply that BAF57 may perform topological functions when the BAF complex moves in or out a nucleosome. BAF57 specifically interacts with a variety of proteins which aren't part of the BAF complex. BAF57 demonstrates particular functionalities as a result of these interactions. For example, in the embryo, it interacts with the transcriptional cosuppressor Co-REST to enable the neuronal genes inactivation in nonneuronal cells throughout development[50]. Interestingly, global gene expression profiling results indicated that BAF57A was specifically upregulated in human NSPCs of OB (OBNSPCs) but not in human embryonic NSPCs (hENSC)[51]. In cultured NSCs, BAF57 interacts with the overexpressed intracellular domain of neuregulin-1 and leads to a decrease in the NSC proliferation rate[52].

BAF60A (SMARCD1)

The BAF60A/B subunits define an ESC-specific assembly of BAF, named esBAF[27]. The BAF subunits BAF60A binds to BRG1, the primary factor that drives the BAF complex to particular genomic locations, and interacts with transcription regulators and factors[31]. Nuclear receptors and the BRG1 complex may have an additional important and direct relationship that is necessary for recruitment of promoter and subsequent chromatin modification[53]. Low levels of BAF60A and BAF45B/D expression are found in oligodendrocyte precursors, and these expression levels are upregulated upon differentiation [54]. However, their specific roles in NSPCs are still unclear.

BAF60C (SMARCD3)

During development, another crucial BAF complex component called BAF60C also has neuronal progenitor-specific actions. BAF60C is expressed in neural progenitors in the mouse and human retina, as well as the human cortex and spinal cord[55]. BAF60C expression decreases during neural differentiation, and through its interaction with the Notch pathway, its overexpression keeps progenitors in a proliferative condition. Finally, Muller glia that resume the cell cycle following neurotoxic damage express BAF60C once more[55].

BAF55A (SS18)

BAF55A, also called SS18, is a member of the npBAF complex. In NSCs, SS18 knockdown results in cell cycle exit and self-renewal failure[48]. Moreover, SS18 is adaptively activated to respond to ethanol exposure, protecting fetal NSCs against complete loss of miR-9-2[49].

BAF47

BAF47, a ubiquitous BAF complex subunit, is expressed in oNSCS[15]. However, the exact function of BAF47 remains largely unknown.

BAF COMPLEX FUNCTIONS IN BALANCING SELF-RENEWAL AND DIFFERENTIATION OF NSPCS

The number of neural stem cells expands through symmetrical division, and through asymmetrical divisions, they maintain the capability to self-renew and produce other differentiated cells[56]. Normal neural development requires the maintenance of the dynamic balance between the proliferative cells and differentiated cells of neural stem cells, which controls the number of stem cells and neurons and protects the body from diseases such as glioblastoma[57]. Furthermore, neural stem cells respond to several molecular signals during neurogenesis, especially those mediated by the Wnt and Notch signaling pathways[58]. For instance, Notch signaling is a significant mechanism regulating the balance between the quiescence and differentiation of neural stem cells[59]. By suppressing the Notch pathway, the homeostasis of neural stem cells can be altered, promoting neural differentiation[60].

Several BAF family members, which act as components or modulators of the Notch and Wnt/β-catenin signaling pathways, have been shown to exert either positive or negative effects on the differentiation of neural stem cells[55,61]. On the one hand, neural differentiation can be promoted by BAF155 and BAF170, which act on regulators of Wnt/β-catenin signaling-related genes, including H3K27me3 and H3K4me2[15,62]. On the other hand, BAF45a and BAF53a contribute to the proliferation and self-renewal of neural stem cells[41]. The repression of BAF53a during neural differentiation is realized through its 3'-UTR and the cooperative influence of miR-9 and miR-124[45]. Brm and BAF57 interact with the neuregulin-1 (Nrg-1) intracellular domain, reduce proliferation and promote differentiation of neural stem cells in vitro, possibly by reversing the direction of signaling in a pathway[63]. Furthermore, it has been shown that the overexpression of BAF60c maintains the proliferation of neural progenitors by interacting with the Notch signaling pathway[55]. The BAF complex may also make direct contact with the neurogenic transcription factors that are critical for neural stem cell specification or differentiation. Notably, the subunit composition of BAF complexes undergoes a profound switching during differentiation; for instance, BAF40c is replaced by BAF40a, the function of which is activated by Pax6, Tbr1, and Tbr2[64]. Moreover, BAF155 in the primate brain has been shown to be involved in the normal activity of Pax6, thereby regulating the specification of progenitors during cortex development [32].

Overall, BAF complexes function as both agonists and inhibitors of neural differentiation. Notably, certain similarities and differences have been revealed through the approaches used in the aforementioned studies. A study by Narayanan *et al*[32] was carried out by generating cortex-specific BAF155-knockout mice, while in another study, Nguyen *et al*[62] adopted a transgenic mouse model in which both BAF155 and BAF170 were knocked out, eliminating the entire BAF complex during late cortical neurogenesis. Their double-knockout model offered a novel and practical method for examining the function of complete BAF complexes during cerebral development[65]. Furthermore, a study by Bi-Lin *et al*[66] was conducted after the neural crest-specific knockout of BAF155 and BAF170 in $Wnt1^{Cre/+}$ and

Pax3$^{Cre/+}$ mice, and the results demonstrated that the BAF complex modulated the gene expression network and pathways, including the Notch signaling pathway, critical for neural crest development.

NSPC-RELATED BAF COMPLEX SUBUNITS IN NEURAL DEVELOPMENTAL DISEASES

Given the importance of BAF complexes in creating and sustaining a chromatin state that keeps proper transcriptional output during NSPC proliferation, differentiation and responses to exogenous or endogenous stimuli, it is clear that several neural developmental disorders have been linked to NSPC-related BAF complex subunits (Table 1 and Figure 1).

Coffin-Siris syndrome

Coffin-Siris syndrome (CSS) is a unusual congenital neural developmental disorder characterized clinically by intellectual disability (ID), progressive facial thickening, hirsutism, recurrent infections, difficult feeding, and restricted growth of the distal fifth phalanx and nails[67,68]. Genes encoding NSPC-related BAF complex subunits have been found to have mutations recently thanks to extensive human exome sequencing and genome-wide association research, including BAF45D[69-71], BAF47[72-74], BAF57[67,75,76], BAF60A[77], BAF170[78], BAF190A/BRG1[72], and BAF250A[72]. Different brain midline abnormalities, similar to those seen in people with CSS, were shown to be caused specifically by BAF47, BAF57, and BAF250B mutations in mice with a heterozygous neural system-specific SMARCB1 defect and a partial dysfunctional mutation in a BAF core member gene, indicating significant clinical suggestions for BAF complex-related ID/neurodevelopmental diseases[79].

Within a cohort of 15 unrelated individuals on the CSS pathway disorders registry, who were carriers of a SMARCA4 variant, they showed differences in the severity and number of learning disabilities and health problems. Two of them with novel nonsense variants appeared to acquire a non-organ/system affected phenotype with minor learning/behavioral differences[80]. A recent report described a Chinese woman presenting with on the CSS pathway disorders registry, who were carriers of a SMARCA4 variant, they showed differences in the severity and number of learning disabilities and health problems. Two of them with novel nonsense variants appeared to acquire a non-organ/system affected phenotype with minor learning/behavioral differences and endocrine dysfunction. Whole-exome sequencing was used and led to the identification of a heterozygous missense variant in the SMARCC2 gene of the proband[81].

Nicolaides-Baraitser syndrome

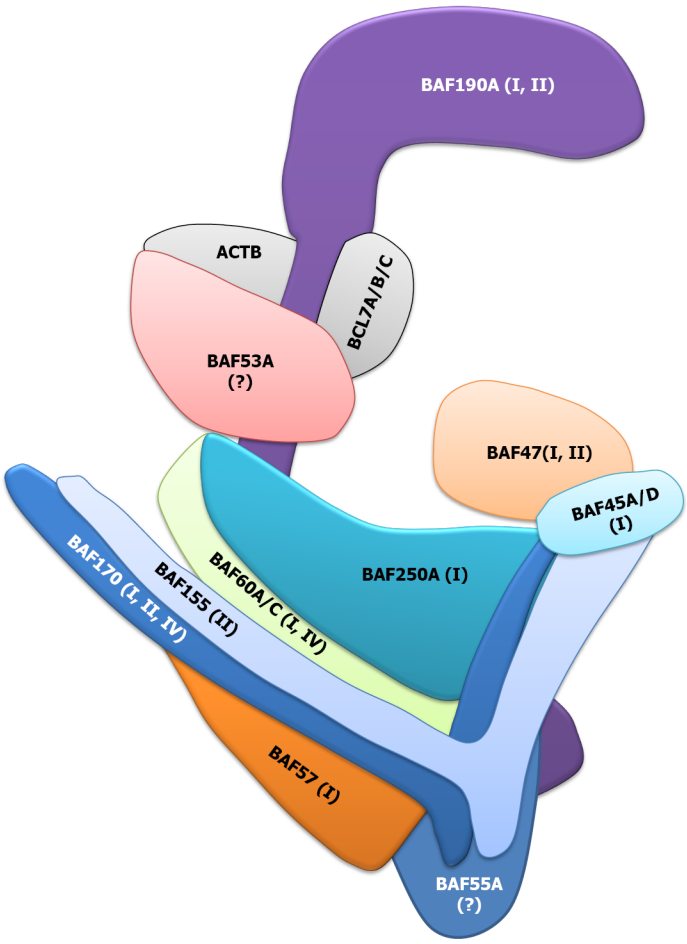
The clinical features of patients with nicolaides-Baraitser syndrome (NCBRS) caused by mutations in the SMARCA2 gene, remarkably resemble those of CSS patients[82]. The characteristics of hands and feet are where NCBRS and CSS most clearly diverge. While typical CSS patients have hypoplasia or agenesis of the fifth fingernail with or without engagement of the nail phalanges, typical NCBRS patients have pronounced distal phalanges and interphalangeal joints[75]. Five individuals with mutations in BAF60A exhibited developmental delay, small hands and feet, hypotonia, ID, and difficult feeding. According to TRIO exome sequencing, these mutations developed in four of the five patients[77]. A missense mutation (p. Arg366Cys) of BAF47 also has been reported to be associated with NCBRS[75]. Furthermore, variants in BAF170 have also been found in patients presenting with overlapping ID syndromes related to other BAF subunits, such as those identified in CSS and NCBRS[78].

Autism spectrum disorder

Autism is a neurodevelopmental disease which is caused by both environmental and genetic factors, presented as speech problems, limited and repetitive behaviors and social skills barrier[83]. Recent exome-sequencing studies on individuals with autism have found mutations in the BAF155, BAF170[84] and BAF190A[85] genes. But at least seventy percent of the cases, the fundamental genetic cause is still unknown.

Kleefstra syndrome spectrum

Kleefstra syndrome spectrum (KSS) is a recognizable syndrome characterized by ID and induced by a hybrid mutation in the euchromatin histone methyltransferase 1 (EHMT1) gene[86,87]. According to the research of Kleefstra *et al*[88], KSS could result from mutations in any complex protein including MLL3, MBD5, NR1I3, BAF47, or EHMT1. The EHMT1 mutation is not present in every patient with the primary symptoms of Kleefstra syndrome. In fact, de novo mutations in four genes that code for epigenetic regulators, including BAF47 (SMARCB1), were found in four of the nine individuals who did not have EHMT1 mutations[88]. And their research using a Drosophila model showed that MLL3, MBD5, NR1I3, BAF47, and EHMT1 directly interact with one another[88].



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Figure 1 BRG1/BRM-associated factor complex subunits in neural stem/progenitor cells and the related neural developmental diseases. The BRG1/BRM-associated factor (BAF) complex subunits in neural stem/progenitor cells are shown (in colors), including BAF45A/D, BAF47, BAF53, BAF55A, BAF57, BF60A/C, BAF155, BAF170, BAF190A, and BAF250A. The other two types of the BAF complex subunits, β-actin and BCL7/A/B/C, are shown in grey. Neural developmental disease types are listed as, I: Coffin-Siris syndrome; II: Autism spectrum disorder; III: Kleefstra syndrome; IV: Nicolaides-Baraitser syndrome; "?": Unknown. BAF: BRG1/BRM-associated factor.

CONCLUSION

Considering recent research progress, we suggest that three aspects of BAF complexes should be investigated in the near future. First, BAF complexes are crucial for development of neural system and differentiation of neural cells, including the specification of neural fate and functionality[4]. BAF complex subunits play key roles in the manipulation of gene expression, and the distinct ontogenetic stage-specific BAF complex functions in neural stem/progenitors and postmitotic neuronal cells that are derived from the combinatorial organization of these subunits[26]. Sequencing of human whole-exome and genome-wide association studies have recently demonstrated the link between mutants of BAF complex subunits and neurodevelopmental illnesses like CSS, NCBS, KSS and ASD[89-92]. Focus must be placed on the roles played by BAF complex organization during neural development as well as the ways in which mutations in well-known BAF complex subunits contribute to certain neurodevelopmental illnesses[93]. Secondly, both loss- and gain-of-function techniques to evaluating neural network development at the molecular, single-cell, and network activity levels should be used in order to comprehend how BAF complex gene mutations might cause the phenotypic convergence of CSS, NCBS, KSS, and ASD. For example, ASD and KSS genes focus primarily on the level of neural network communication and provide insight into the pathophysiology of phenotypic consistency disorders[79, 94]. Thirdly, recent investigations have shown that BAF complexes are frequently involved in human cerebral development abnormalities, providing fresh mechanisms and corresponding paths for therapeutic intervention, all of which should be looked into. In recent years, small compounds that target the BAF complex subunits have become viable therapeutic agents. Researchers in the field have been motivated to recognize and take use of the whole spectrum of therapeutic options as a result of the creation and evaluation of novel drugs that target a variety of BAF complex subunits[95].

FOOTNOTES

Author contributions: Ke NY and Zhao TY collected the data and wrote the manuscript; Wang WR and Qian YT collected the data; Liu C conceived the manuscript, collected the data, and wrote the manuscript.

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Bone marrow mesenchymal stem cell-derived exosomal microRNAs target PI3K/Akt signaling pathway to promote the activation of fibroblasts

Fang-Qi Li, Wen-Bo Chen, Zhi-Wen Luo, Yi-Sheng Chen, Ya-Ying Sun, Xiao-Ping Su, Jun-Ming Sun, Shi-Yi Chen

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Abstract

BACKGROUND

Fibroblast plays a major role in tendon-bone healing. Exosomes derived from bone marrow mesenchymal stem cells (BMSCs) can activate fibroblasts and promote tendon-bone healing *via* the contained microRNAs (miRNAs). However, the underlying mechanism is not comprehensively understood. Herein, this study aimed to identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets, and to verify their effects as well as mechanisms on fibroblasts.

AIM

To identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets and verify their effects as well as mechanisms on fibroblasts.

METHODS

BMSC-derived exosomal miRNAs data (GSE71241, GSE153752, and GSE85341) were downloaded from the Gene Expression Omnibus (GEO) database. The candidate miRNAs were obtained by the intersection of three data sets. TargetScan was used to predict potential target genes for the candidate miRNAs. Functional and pathway analyses were conducted using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively, by processing data with the Metascape. Highly interconnected genes in the

protein-protein interaction (PPI) network were analyzed using Cytoscape software. Bromodeoxyuridine, wound healing assay, collagen contraction assay and the expression of COL I and α-smooth muscle actin positive were applied to investigate the cell proliferation, migration and collagen synthesis. Quantitative real-time reverse transcription polymerase chain reaction was applied to determine the cell fibroblastic, tenogenic, and chondrogenic potential.

RESULTS

Bioinformatics analyses found two BMSC-derived exosomal miRNAs, has-miR-144-3p and has-miR-23b-3p, were overlapped in three GSE datasets. PPI network analysis and functional enrichment analyses in the GO and KEGG databases indicated that both miRNAs regulated the PI3K/Akt signaling pathway by targeting phosphatase and tensin homolog (PTEN). *In vitro* experiments confirmed that miR-144-3p and miR-23b-3p stimulated proliferation, migration and collagen synthesis of NIH3T3 fibroblasts. Interfering with PTEN affected the phosphorylation of Akt and thus activated fibroblasts. Inhibition of PTEN also promoted the fibroblastic, tenogenic, and chondrogenic potential of NIH3T3 fibroblasts.

CONCLUSION

BMSC-derived exosomes promote fibroblast activation possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing.

Key Words: Exosome; MicroRNA; Fibroblast; Mesenchymal stem cell; Tendon-bone healing

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Core Tip: Exosomes derived from bone marrow mesenchymal stem cells (BMSCs) can activate fibroblasts and promote tendon-bone healing *via* the contained microRNAs (miRNAs). Supported by bioinformatics tools, this study identified two BMSC-derived exosomal miRNAs, has-miR-144-3p and has-miR-23b-3p, were overlapped in three GSE datasets. Bioinformatic analysis revealed that both miRNAs regulated the PI3K/Akt signaling pathway by targeting phosphatase and tensin homolog (PTEN). Experiments *in vitro* confirmed that interfering with PTEN can affect the phosphorylation of Akt and thus the activation of fibroblasts. These results suggested a potential mechanism by which BMSC-derived exosomes promote tendon-bone healing.

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DOI: https://dx.doi.org/10.4252/wjsc.v15.i4.248

INTRODUCTION

Tendon-bone insertion (TBI) injury, such as rotator cuff tears and anterior cruciate ligament injuries, is one of the common injuries in daily life and sports. Thus, how to promote tendon-bone healing becomes an important issue in research and clinical practice. The normal TBI has a transitional structure consisting of four gradated layers including bone, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon[1,2]. This complex structure can disperse force from bone to tendon, preventing stress concentration[3]. However, current therapeutic strategies cannot restore this structure, hence raising the risk of re-injury.

Fibroblasts play a key role in tendon-bone healing. In the early stages of TBI injury, α-smooth muscle actin positive (α-SMA+) fibroblasts help form Sharpey-like fibers to withstand strength at TBI and participate in early ligament remodeling by producing collagen and restoring in situ tension[4,5]. Mesenchymal stem cells (MSCs) are a kind of stem cells with strong proliferation capacity and multilineage differentiation potential, which can differentiate into myoblasts, osteoblasts, adipocytes and chondroblast[6]. MSCs can be extracted from bone, adipose tissue, blood, and amniotic membrane [7]. They have multiple functions including immunomodulatory, anti-inflammatory, anti-apoptotic, and angiogenesis promotion, making them ideal candidate cells for tissue engineering research[8]. Recent evidence indicates that conditioned medium, primarily containing exosomes of MSCs, can stimulate the activation of fibroblasts, thereby promoting tendon-bone healing[9-12]. However, the underlying mechanism is not comprehensively understood.

Bioinformatics has played an important role in exploring disease mechanisms by allowing parallel processing of large volumes of high-throughput sequencing data. To identify key bone marrow MSC (BMSC)-derived exosome microRNAs (miRNAs), three Gene Expression Omnibus (GEO) datasets were interrogated using bioinformatics tools in this study. TargetScan, a software designed to predict miRNA binding sites, is often used to analyze target genes of miRNAs[13]. Metascape, a powerful gene functional annotation analysis tool, enables pathway enrichment and biological process (BP) annotation [14]. STRING database allows for assessing the functional associations among target genes[15]. Cytoscape software enables visualization of miRNA-target gene pairs.

Therefore, using the above database tools, this study aimed to identify overlapped BMSC-derived exosomal miRNAs in three GSE datasets and verify their effects as well as mechanisms on fibroblasts, so as to facilitate further studies to verify the role of exosomal miRNAs and their potential in promoting tendon-bone healing.

MATERIALS AND METHODS

The workflow of this study was described (Figure 1).

Data collection

Three BMSC-derived exosomal miRNA expression microarray datasets (GSE71241, GSE153752, and GSE85341) were retrieved from the GEO repository on 20 April 2022. (https://www.ncbi.nlm.nih.gov/geo). GSE71241[16], GSE153752[17], and GSE85341[18] included nine, four and one BMSC-derived exosome samples respectively. The above datasets were produced independently using the GPL18743, GPL16791, and GPL22300 platforms, respectively.

Venn diagram analysis and prediction of target genes

After obtaining the top 100 BMSC-derived exosomal miRNAs in each dataset, Venn diagram analysis was used to identify overlapped miRNAs in the three datasets through online tool Venny (version 2.1) (https://bioinfogp.cnb.csic.es/tools/venny). The overlapped miRNAs were considered as candidate miRNAs. Potential target genes for the candidate miRNAs were predicted by TargetScan, an experimentally validated database of miRNA-target interactions[13].

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses

Functional and pathway analyses of the predicted target genes were conducted using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively, by processing data with the Metascape[14].

Construction of target gene-protein-protein interaction and miRNA-gene networks

Target gene data was uploaded to the STRING database[15] to assess the functional associations among the target genes of candidate miRNAs. Highly interconnected (hub) genes in the protein-protein interaction (PPI) network were analyzed using Cytoscape software (version 3.9.1). After candidate exosomal miRNAs and their hub target genes were identified, Cytoscape was used to visualize the resulting miRNA-gene network.

Cell culture and treatment

The NIH3T3 fibroblast (Shanghai Institutes for Biological Sciences) was used as a model *in vitro* to further determine the effect of SF1670 [a specific phosphatase and tensin homolog (PTEN) inhibitor] on fibroblasts[19]. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When the cells reached 80% confluence, they were digested with 0.25% trypsin and sub-cultured 1:3. To determine the effect of SF1670, NIH3T3 cells were starved for 24 h with DMEM containing 0.5% FBS and then treated with or without SF1670 2 μM for the indicated time. NIH3T3 fibroblast was seeded and cultured into six-well plate.

Western blot assay

Protein was extracted and analyzed using an established method[20]. Anti-phosphorylated Akt (p-Akt) (CST, United States, 1:1000) and anti-Akt (CST, United States, 1:1000) were used as primary antibodies. Each group contains 3 protein samples for calculation.

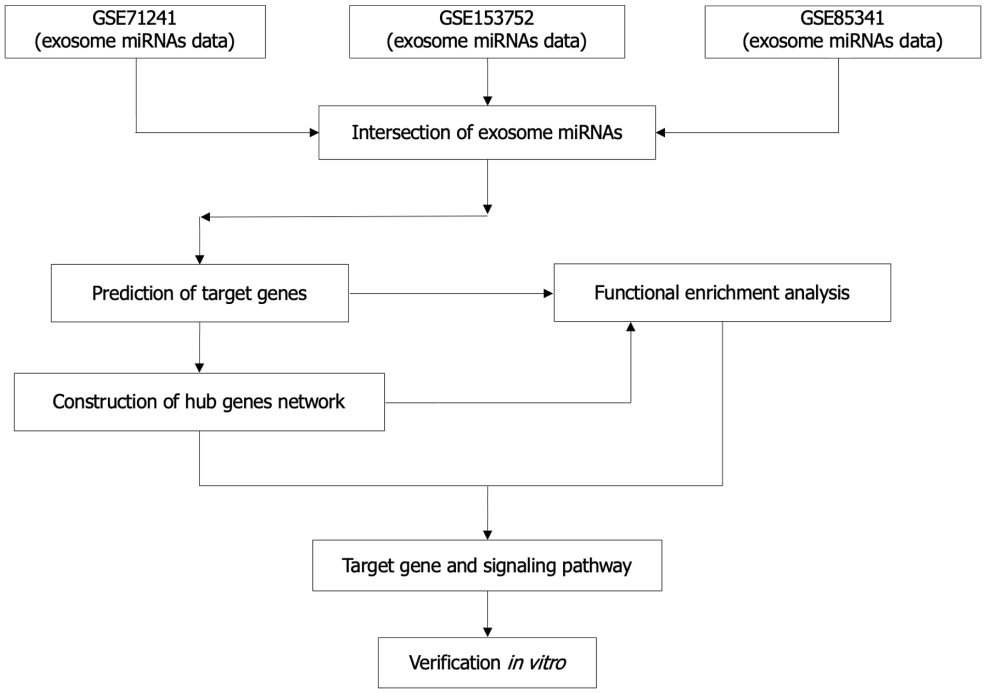
Quantitative real-time reverse transcription polymerase chain reaction

RNA was extracted and analyzed using the previous method[21]. Total RNA was obtained by the Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by Nanodrop. RNA was then reversely transcribed by the PrimeScript RT reagent kit (Takara Bio). Specific primers used in the experiment are from PrimerBank (Table 1). The operation was performed on the ABI7900 Real-Time PCR System (Applied

Table 1 Primers used in the experiment

Genes	Primers (5' - 3')
PTEN	F: TCAGCCGTTACCTGTGTGTG
	R: TCCTTGTCATTATCTGCACGC
α-SMA	F: GACAATGGCTCTGGGCTCTGTAA
	R: CTGTGCTTCGTCACCCACGTA
Vimentin	F: GACGCCATCAACACCGAGTT
	R: CTTTGTCGTTGGTTAGCTGGT
Tenomodulin	F: CCATGCTGGATGAGAGAGGTT
	R: CCGTCCTCCTTGGTAGCAGT
Col I	F: CCCGGGTTTCAGAGACAACTTC
	R: TCCACATGCTTTATTCCAGCAATC
Sox 9	F: GAGCCGGATCTGAAGAGGGA
	R: GCTTGACGTGTGGCTTGTTC
Col II	F: GGGAATGTCCTCTGCGATGAC
	R: GAAGGGGATCTCGGGGTTG
GAPDH	F: ACTCCACTCACGGCAAATTC
	R: TCTCCATGGTGGTGAAGACA

PTEN: Phosphatase and tensin homolog; α-SMA: α-smooth muscle actin positive.



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Figure 1 The flowchart of this study. miRNAs: MicroRNAs.

Biosystems). The expression of mRNAs relative to the expression of GAPDH was calculated and normalized to the control group.

Luciferase reporter assay

Validation of miR-144 and miR-23b binding to 3′-UTR was performed using Dual-Glo Luciferase assay

system (Promega) as recommended by the manufacturer. The relative luciferase activities were determined by calculating the ratio of firefly luciferase activities over Renilla luciferase activities. All experiments were repeated three times in triplicate. Two constructs of pmirGLO luciferase reporter plasmid were generated: MUT-PTEN (with mutation of part of miRNA binding site sequence) and WT-PTEN [containing the wild-type (WT) miRNA binding site sequence]. 500 ng of the pmirGLO luciferase reporter plasmid and appropriate miRNA plasmid were co-transfected with lipofectamine 3000 (Invitrogen) into NIH3T3 cells. 48 h after transfection, the luciferase expression was determined using the Dual-Glo Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol. The pRL-TK vector (Promega) containing Renilla luciferase was also co-transfected for normalization in all relevant experiments.

Bromodeoxyuridine assays

The Bromodeoxyuridine (BrdU) incorporation assay was performed using Cell Proliferation ELISA kits (1647229; Roche Applied Science, Mannheim, Germany). Briefly, NIH3T3 cells were plated at 5000 cells/well in 96-well culture plates in complete media. After attaining 70% confluence, cells were treated with media containing 10% FBS. BrdU solution (10 μM) was added after intervention. The cells were dried and fixed, and cellular DNA was denatured with FixDenat solution (Roche Applied Science) for 30 min at room temperature. A peroxidase-conjugated mouse anti-BrdU monoclonal antibody (Roche Applied Science) was added to the culture plates and cells were incubated for 90 min at room temperature. Tetramethyl-benzidine substrate was added and the plates were incubated for 15 min at room temperature. The absorbance of samples was measured using a microplate reader at 450-620 nm.

Wound healing assay

Cells at logarithmic growth stage were taken and placed in a six-well plate with a cell density of 5×10^5/well. Three multiple wells were set for each group. When the cells were adherent to the wall in a single layer, a pipetting tip was used to vertically scratch the six-well plate to avoid tilting. The suspension cells were cleaned and removed with PBS and cultured in an incubator with 5% CO_2 at 37 °C. Photographs were taken at 0 and 24 h under the microscope. The wound healing area was measured using ImageJ[22].

Collagen gel contraction assay

Cell contraction was determined using a kit from Cell Biolabs (San Diego, CA). Briefly, cells were seeded in collagen solution according to the manufacturer's instructions and release from the plates with a sterile spatula. Gel images were taken after release and the surface area of each gel was measured using ImageJ[22].

Immunofluorescence

NIH3T3 cells were cultured in a 24-well plate. The cells were fixed with 4% paraformaldehyde and incubated with 0.5% Triton X-100 (Sigma) and then blocked with goat serum (Biyuntian Company, China) for 1 h. The cells were then incubated overnight at 4 °C with the primary antibody. The secondary antibody was applied, and the cells were incubated for 1 h in the dark. Finally, the nuclei were counterstained with DAPI for 15 min. The stained cells were photographed using a fluorescence microscope.

Statistical analysis

All experiments were performed at least three times. Data were analyzed with GraphPad Prism 7.0 (GraphPad Software, La Jolla, United States) and were presented by mean ± SD. Significance was typically analyzed by student's *t*-test, one-way ANOVA followed by post hoc LSD test, and two-way ANOVA followed by multiple *t*-tests. $P < 0.05$ was regarded as significant.

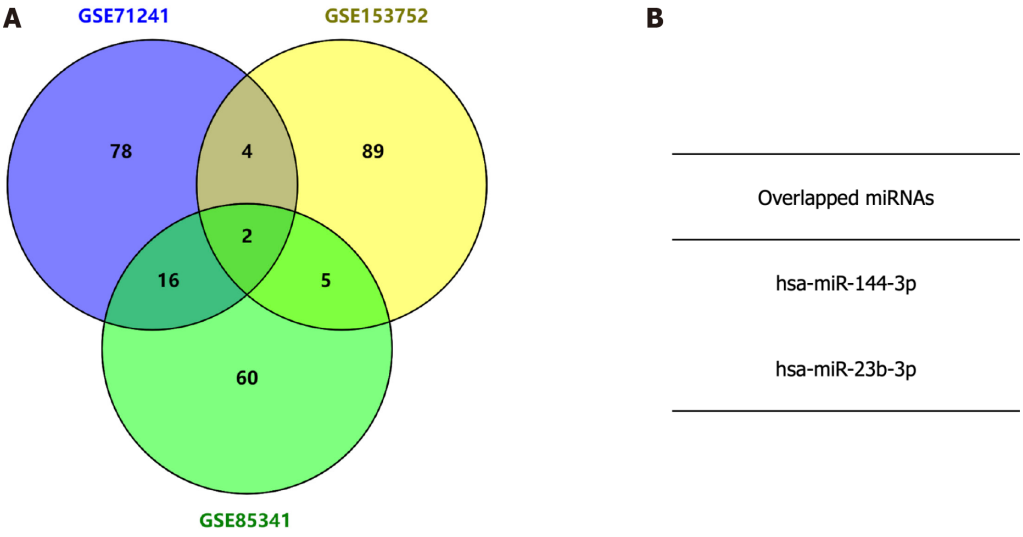
RESULTS

Identification of candidate exosomal miRNAs and target genes

Three independent BMSC-derived exosomal miRNA expression microarray datasets (GSE71241, GSE153752, and GSE85341)[16-18] were downloaded from the GEO database. Subsequently, top 100 BMSC-derived exosomal miRNAs in each dataset were obtained. The Venn diagram analysis demonstrated two miRNAs in the intersection of the three datasets (Figure 2). The target gene interactions of two candidate miRNAs, hsa-miR-144-3p and hsa-miR-23b-3p, were assessed by the TargetScan[13]. In total, 1048 and 105 potential target genes were identified for miR-144-3p and miR-23b-3p, respectively.

Functional enrichment analysis of miRNAs' target genes

GO functional annotation analysis revealed that the most enriched terms for the target genes of miR-



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Figure 2 Identification of candidate microRNAs by intersecting three exosome microRNA expression microarray datasets. A: Venn diagram analysis showing the top 100 exosome microRNAs (miRNAs) in the three Gene Expression Omnibus (GEO) datasets; B: Identification of two miRNAs overlapping between the three GEO datasets. miRNAs: MicroRNAs.

144-3p were 'head development' and 'regulation of kinase activity' in the BP, 'postsynapse' in the cellular component (CC), and 'transcription factor binding' and 'kinase activity' in the molecular function (MF) (Figures 3A-D). On the other hand, the target genes of miR-23b-3p were mostly enriched in 'phospholipid metabolic process' in the BP, 'nuclear speck' and 'centrosome' in the CC, and 'chromatin binding' in the MF (Figures 4A-D). KEGG pathway analysis revealed that most of target genes of miR-144-3p enriched in 'pathways in cancer' and 'PI3K-AKT signaling pathway' (Figure 3E); most of target genes of miR-23b-3p enriched in 'pathways in cancer' (Figure 4E).

Construction of target gene-PPI and miRNA-hub gene networks

A PPI network was constructed through the STRING database[15]. Interactions with an overall score of more than 0.4 were considered significant. Further processing was carried out using Cytoscape software. Then, the cytoHubba plugin was used to determine the top 10 hub genes based on the Degree algorithm. The top 10 predicted hub genes of miR-144-3p and miR-23b-3p were presented (Figures 5A and B). GO and KEGG analysis revealed that these hub genes were mostly enriched in 'regulation of MAPK cascade', 'regulation of phosphatidylinositol 3-kinase signaling' and 'negative regulation of cell differentiation' in the BP (Figure 5C, Table 2), 'fibrillar center' and 'focal adhesion' in the CC (Figure 5D, Table 3), and 'protein kinase activity' and 'transcription factor binding' in the MF (Figure 5E, Table 4). Most of these genes were mainly enriched in PI3K-Akt signaling pathway (Figure 5F, Table 5). Interactional analysis showed that PTEN and sonic hedgehog (SHH) are potentially co-regulated by miR-144-3p and miR-23b-3p (Figure 5G).

PTEN is verified as the targets of miR-144-3p and miR-23b-3p

The phosphatase and tensin homolog deleted on chromosome ten (PTEN), was predicted as the hub gene of miR-144-3p and miR-23b-3p (Figures 6A and B). To further validate the putative binding sites, WT and mutated 3'-UTR of PTEN were cloned into the pmirGLO vector. The transcripts of the target gene were then evaluated by dual luciferase reporter assays. Compared with the control group, luciferase reporter assays showed that miR-144-3p mimics and miR-23b-3p mimics significantly decreased the luciferase activity of WT-PTEN but did not show a significant effect on the luciferase activity of MUT-PTEN (Figures 6C and D). In addition, miR-144-3p mimic significantly down-regulated the mRNA levels of PTEN, while miR-144-3p inhibitor up-regulated the mRNA levels of PTEN (Figure 6E). Similarly, miR-23b-3p mimic down-regulated the mRNA level of PTEN, while miR-23b-3p inhibitor up-regulated the mRNA level of PTEN (Figure 6F). These results indicated that miR-144-3p and miR-23b-3p repressed the expression of PTEN by specifically binding with and subsequently inducing the degradation of mRNA.

miR-144-3p and miR-23b-3p regulate the proliferation, migration, and collagen synthesis of NIH3T3 fibroblasts

To evaluate the effect of the identified miRNAs on fibroblast biology and activation, analysis of proliferation and migration was then performed 24 h after transfection of miR-144-3p and miR-23b-3p respectively. Compared with the control group, proliferation and migration capacity of NIH3T3 cells in

Table 2 Top 10 clusters with Gene Ontology Biological Processes analysis of hub-gene targets

Term	Description	LogP	Log(q-value)	Count
GO:0048732	Gland development	-12.279	-7.927	9
GO:0048608	Reproductive structure development	-10.269	-6.495	8
GO:0061458	Reproductive system development	-10.245	-6.495	8
GO:0030900	Forebrain development	-8.922	-5.472	7
GO:0043408	Regulation of MAPK cascade	-8.603	-5.240	8
GO:0045596	Negative regulation of cell differentiation	-8.549	-5.238	8
GO:0022612	Gland morphogenesis	-8.482	-5.208	5
GO:0014066	Regulation of phosphatidylinositol 3-kinase signaling	-8.316	-5.077	5
GO:0060322	Head development	-8.138	-4.931	8
GO:0060284	Regulation of cell development	-8.045	-4.868	7

Table 3 Top 10 clusters with Gene Ontology Cellular Components analysis of hub-gene targets

Term	Description	LogP	Log(q-value)	Count
GO:0045121	Membrane raft	-4.441	-2.311	4
GO:0098857	Membrane microdomain	-4.436	-2.310	4
GO:0001650	Fibrillar center	-4.076	-2.035	3
GO:0009898	Cytoplasmic side of plasma membrane	-3.849	-1.860	3
GO:0098562	Cytoplasmic side of membrane	-3.638	-1.688	3
GO:0005770	Late endosome	-3.205	-1.308	3
GO:0005788	Endoplasmic reticulum lumen	-3.103	-1.227	3
GO:0031983	Vesicle lumen	-3.041	-1.174	3
GO:0005925	Focal adhesion	-2.726	-0.896	3
GO:0030055	Cell-substrate junction	-2.699	-0.873	3

Table 4 Top 10 clusters with Gene Ontology Molecular Functions analysis of hub-gene targets

Term	Description	LogP	Log(q-value)	Count
GO:0002020	Protease binding	-5.941	-3.326	4
GO:0004712	Protein serine/threonine /tyrosine kinase activity	-5.299	-2.910	5
GO:0004672	Protein kinase activity	-4.802	-2.546	5
GO:0003682	Chromatin binding	-4.726	-2.507	5
GO:0008134	Transcription factor binding	-4.694	-2.487	5
GO:0031490	Chromatin DNA binding	-4.457	-2.319	3
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	-4.435	-2.310	5
GO:0019904	Protein domain specific binding	-4.395	-2.280	5
GO:0061629	RNA polymerase II-specific DNA-binding transcription factor binding	-4.331	-2.228	4
GO:0016301	Kinase activity	-4.267	-2.193	5

transfection groups were significantly increased (Figures 7A and B). Furthermore, overexpression of miR-144-3p or miR-23b-3p upregulated the expression of Col I and a-SMA in NIH3T3 cells (Figure 7C). These *in vitro* transfection data indicated that increment in intracellular miR-144-3p or miR-23b-3p stimulated NIH3T3 fibroblast proliferation and migration, and promoted fibroblast-myofibroblast differentiation and collagen synthesis.

Table 5 Top 10 clusters with Kyoto Encyclopedia of Genes and Genomes Pathway analysis of hub-gene targets

Term	Description	Log*P*	Log(*q*-value)	Count
hsa04510	Focal adhesion	-10.807	-6.755	7
hsa05200	Pathways in cancer	-9.488	-5.834	8
hsa04151	PI3K-Akt signaling pathway	-9.088	-5.538	7
hsa01521	EGFR tyrosine kinase inhibitor resistance	-9.046	-5.538	5
hsa05215	Prostate cancer	-8.593	-5.240	5
hsa05224	Breast cancer	-7.682	-4.608	5
hsa05165	Human papillomavirus infection	-7.560	-4.511	6
hsa05213	Endometrial cancer	-7.435	-4.444	4
hsa05225	Hepatocellular carcinoma	-7.392	-4.437	5
hsa05230	Central carbon metabolism in cancer	-7.102	-4.181	4

EGFR: Epidermal growth factor receptor.

Inhibition of PTEN promote cell proliferation, migration, collagen synthesis and change the gene expression of fibroblastic-, tenogenic-, and chondrogenic-related factors in NIH3T3 cells

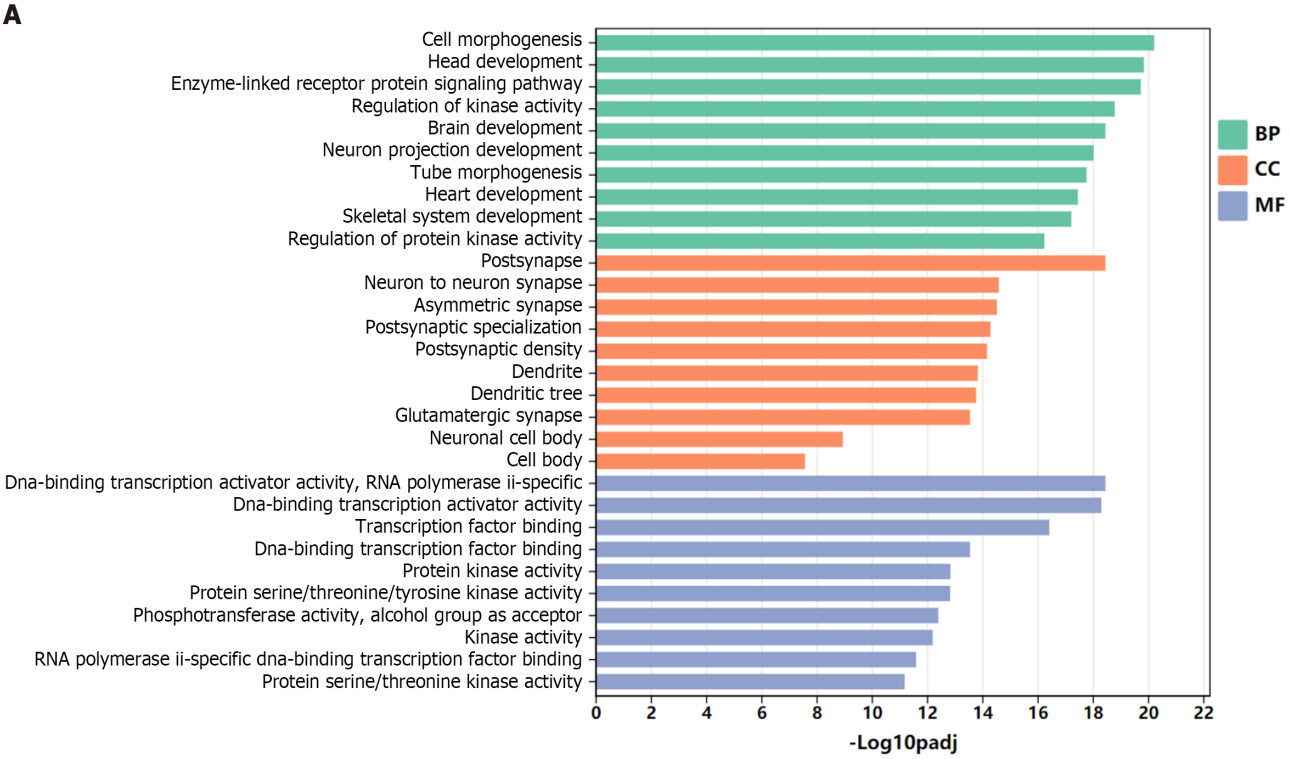
To explore whether BMSC-derived exosome promoted tendon bone healing by directly targeting PTEN in fibroblasts, SF1670, a specific PTEN inhibitor that binds to the active site of PTEN and increases cellular PtdIns(3,4,5)P3 levels and phosphorylation of Akt *in vitro*[19], was used to examine the effect of PTEN inhibition on NIH3T3 cells. The results showed that SF1670 significantly increased the phosphorylation level of Akt compared with DMSO group (Figure 8A). Moreover, BrdU and wound healing assays showed that SF1670 significantly increased proliferation (Figure 8B) and migration (Figure 8C) of NIH3T3 cells. Collagen contraction assays indicated that SF1670 significantly enhanced the contractility of NIH3T3 cells (Figure 8D). Finally, the properties necessary for tendon-bone healing was examined by quantitative real-time reverse transcription polymerase chain reaction. The results showed that the mRNA level of fibroblast-related genes (α-SMA and vimentin), tenogenic-related genes (TNMD and Col I), and chondrogenic-related genes (SOX-9 and Col II) were significantly up-regulated by SF1670 (Figure 9).

DISCUSSION

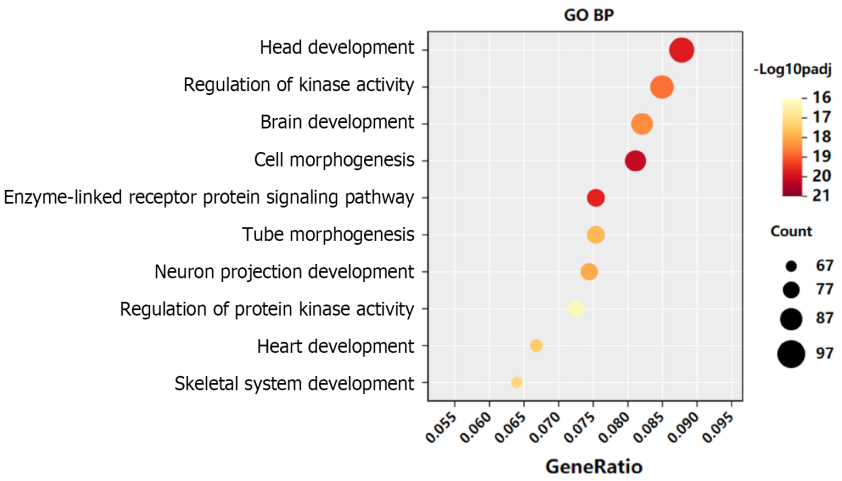
Tendon-bone healing has always been an important challenge in orthopedics and sports medicine research and clinical practice. Fibroblasts play a key role in early ligament remodeling and a large number of studies have verified the effective role of MSC-derived exosomes in tendon-bone healing[9-12]. However, because the status of MSCs may be influenced by their generations or donors, the expression of exosomal miRNAs may be more or less different. This presents difficulties in identifying the roles associated with MSC-derived exosomal miRNAs. On ground of this, three GEO datasets were interrogated through bioinformatics tools to determine key BMSC-derived exosomal miRNAs. Two candidate miRNAs (miRNA-144 and miRNA-23b) were obtained by intersecting the top 100 exosomal miRNAs in each dataset (Figure 2). Their target genes and hub genes were obtained subsequently. GO annotation and KEGG pathway enrichment analysis suggested that these genes were involved in multiple cellular processes (*e.g.*, regulation of kinase activity, transcription factor binding, and phospholipid metabolic process) and signaling cascades (*e.g.*, focal adhesion, and PI3K-Akt pathways). A miRNA-target gene interaction network indicated that PTEN was co-regulated by miRNA-144 and miRNA-23b (Figure 5G).

PTEN functions primarily *via* dephosphorylation of PIP3 to PIP2, resulting in negative regulation of the PI3K/Akt activity[23]. The PTEN/PI3K/Akt nexus participates in various physiological and pathological conditions, and plays an important role in regulating cell growth, apoptosis, metabolism and other processes[24]. Being the direct target of PTEN, PI3K/Akt is also involved in activation of fibroblasts[25,26]. In this study, the effect of PTEN on fibroblasts was also demonstrated. The results showed that inhibition of PTEN increased the level of Akt phosphorylation and promoted the proliferation and migration of fibroblasts (Figure 8), which suggest that PTEN plays an important role in the activation of fibroblasts.

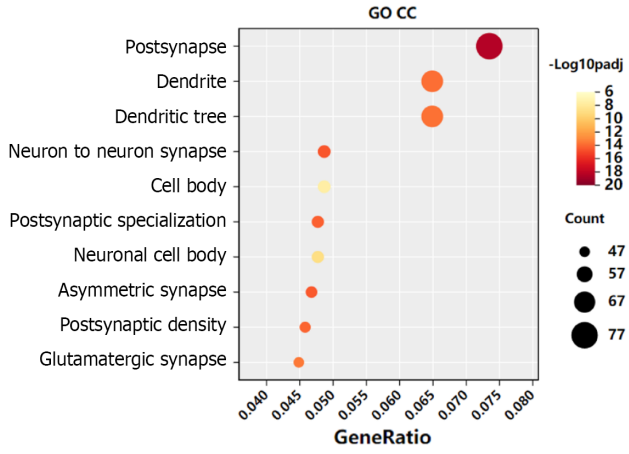
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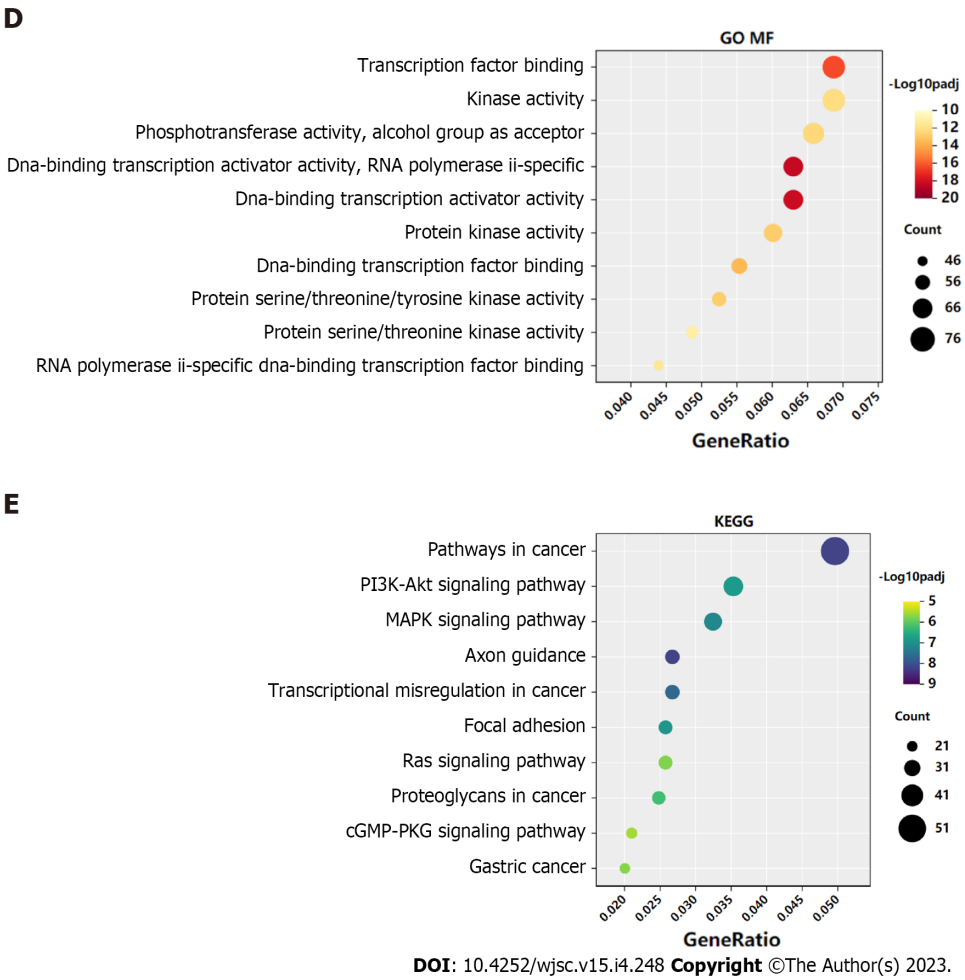

Cell morphogenesis
Head development
Enzyme-linked receptor protein signaling pathway
Regulation of kinase activity
Brain development
Neuron projection development
Tube morphogenesis
Heart development
Skeletal system development
Regulation of protein kinase activity
Postsynapse
Neuron to neuron synapse
Asymmetric synapse
Postsynaptic specialization
Postsynaptic density
Dendrite
Dendritic tree
Glutamatergic synapse
Neuronal cell body
Cell body
Dna-binding transcription activator activity, RNA polymerase ii-specific
Dna-binding transcription activator activity
Transcription factor binding
Dna-binding transcription factor binding
Protein kinase activity
Protein serine/threonine/tyrosine kinase activity
Phosphotransferase activity, alcohol group as acceptor
Kinase activity
RNA polymerase ii-specific dna-binding transcription factor binding
Protein serine/threonine kinase activity
0 2 4 6 8 10 12 14 16 18 20 22
-Log10padj
BP
CC
MF

B


GO BP
Head development
Regulation of kinase activity
Brain development
Cell morphogenesis
Enzyme-linked receptor protein signaling pathway
Tube morphogenesis
Neuron projection development
Regulation of protein kinase activity
Heart development
Skeletal system development
0.055 0.060 0.065 0.070 0.075 0.080 0.085 0.090 0.095
GeneRatio
-Log10padj
16 17 18 19 20 21
Count
67 77 87 97

C


GO CC
Postsynapse
Dendrite
Dendritic tree
Neuron to neuron synapse
Cell body
Postsynaptic specialization
Neuronal cell body
Asymmetric synapse
Postsynaptic density
Glutamatergic synapse
0.040 0.045 0.050 0.055 0.060 0.065 0.070 0.075 0.080
GeneRatio
-Log10padj
6 8 10 12 14 16 18 20
Count
47 57 67 77



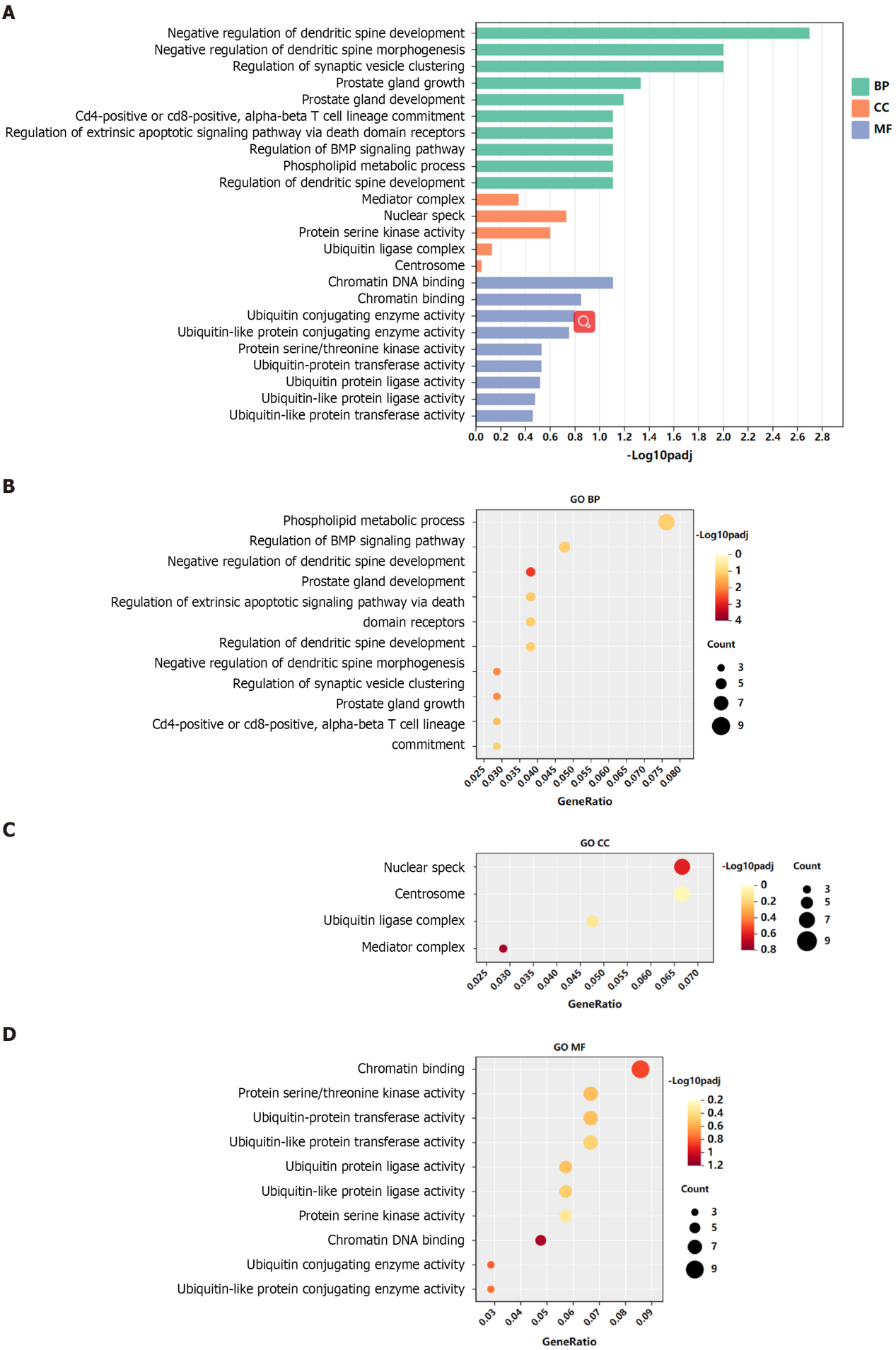
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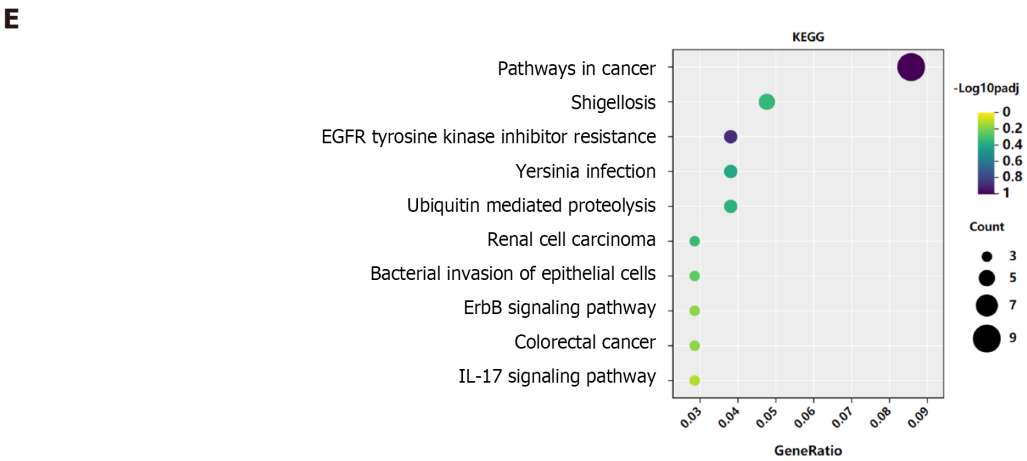
Figure 3 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of genes targeted by hsa-miR-144-3p. A-D: Top 10 Gene Ontology biological process, cellular component, and molecular function terms enriched in target genes of hsa-miR-144-3p; E: Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched in target genes of hsa-miR-144-3p. BP: Biological process; CC: Cellular component; MF: Molecular function; GO: Gene Ontology; KEEG: Kyoto Encyclopedia of Genes and Genomes.

Normal TBI has a transitional structure consisting of four graduated layers including bone tissue, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon tissue[27], which means that fibrogenesis, tenogenesis and chondrogenesis are key characteristics of tendon-bone healing. As the markers of fibrogenesis, α-SMA and vimentin are expressed in activated fibroblasts[28]. Similarly, tenomodulin (TNMD) and collagen I, collagen II and Sox-9 are well-acknowledged indicators for representing the activation of tenogenic and chondrogenic process[29,30]. By inhibiting PTEN, the results showed increased expression of fibroblastic, tenogenic and chondrogenic markers in fibroblasts (Figure 9), implying that PTEN and PI3K/Akt pathway may be targets for promoting tendon-bone healing.

In addition, this study found that SHH was also regulated by both miRNA-144 and miRNA-23b (Figure 5G). HH signaling pathway is a highly conserved pathway involved in embryonic development, tissue homeostasis and stem cell maintenance[31]. HH signaling is also involved in regulating proliferation of MSCs in adult tissues and plays critical roles in promoting tendon-bone healing[32-34]. SHH, one of three HH family members in mammals, acts in the early stages of development to regulate patterning and growth[35], and plays a crucial role in bone healing[36]. These evidences suggested that MSC-derived exosomal miRNAs may promote tendon-bone healing in more than one way.

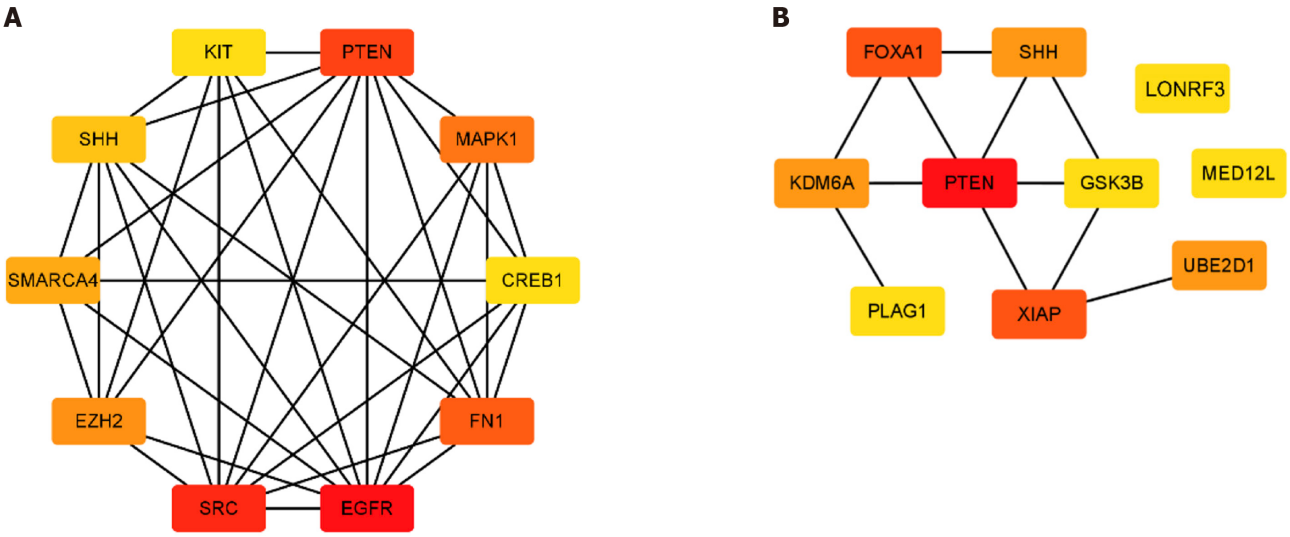
The outcomes of the current study were guaranteed by several factors. In this study, three BMSC-derived exosomal miRNA expression microarray datasets were searched from the GEO repository to avoid batch differences. Moreover, due to the costs, technical challenges, and lack of biomarkers suitable for specific exosomes, it is difficult to isolate large number of pure and specific exosomes from mixtures of different vesicle types in a large volume of solution[37]. Therefore, this study identified the key pathway of exosomal miRNA through comprehensive analysis, providing a theoretical basis for developing methods to stably regulate the activation of fibroblasts and thus promote tendon-bone healing.

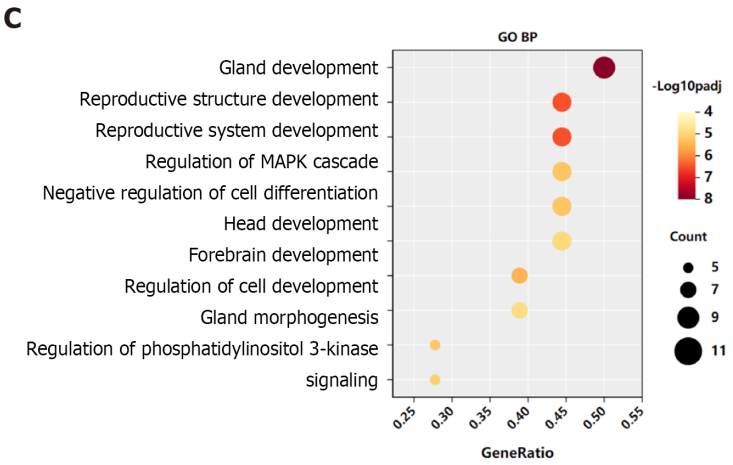

A
Negative regulation of dendritic spine development
Negative regulation of dendritic spine morphogenesis
Regulation of synaptic vesicle clustering
Prostate gland growth
Prostate gland development
Cd4-positive or cd8-positive, alpha-beta T cell lineage commitment
Regulation of extrinsic apoptotic signaling pathway via death domain receptors
Regulation of BMP signaling pathway
Phospholipid metabolic process
Regulation of dendritic spine development
Mediator complex
Nuclear speck
Protein serine kinase activity
Ubiquitin ligase complex
Centrosome
Chromatin DNA binding
Chromatin binding
Ubiquitin conjugating enzyme activity
Ubiquitin-like protein conjugating enzyme activity
Protein serine/threonine kinase activity
Ubiquitin-protein transferase activity
Ubiquitin protein ligase activity
Ubiquitin-like protein ligase activity
Ubiquitin-like protein transferase activity
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8
-Log10padj
BP
CC
MF
B
GO BP
Phospholipid metabolic process
Regulation of BMP signaling pathway
Negative regulation of dendritic spine development
Prostate gland development
Regulation of extrinsic apoptotic signaling pathway via death
domain receptors
Regulation of dendritic spine development
Negative regulation of dendritic spine morphogenesis
Regulation of synaptic vesicle clustering
Prostate gland growth
Cd4-positive or cd8-positive, alpha-beta T cell lineage
commitment
0.025 0.030 0.035 0.040 0.045 0.050 0.055 0.060 0.065 0.070 0.075 0.080
GeneRatio
-Log10padj
0 1 2 3 4
Count
3 5 7 9
C
GO CC
Nuclear speck
Centrosome
Ubiquitin ligase complex
Mediator complex
0.025 0.030 0.035 0.040 0.045 0.050 0.055 0.060 0.065 0.070
GeneRatio
-Log10padj
0 0.2 0.4 0.6 0.8
Count
3 5 7 9
D
GO MF
Chromatin binding
Protein serine/threonine kinase activity
Ubiquitin-protein transferase activity
Ubiquitin-like protein transferase activity
Ubiquitin protein ligase activity
Ubiquitin-like protein ligase activity
Protein serine kinase activity
Chromatin DNA binding
Ubiquitin conjugating enzyme activity
Ubiquitin-like protein conjugating enzyme activity
0.03 0.04 0.05 0.06 0.07 0.08 0.09
GeneRatio
-Log10padj
0.2 0.4 0.6 0.8 1 1.2
Count
3 5 7 9

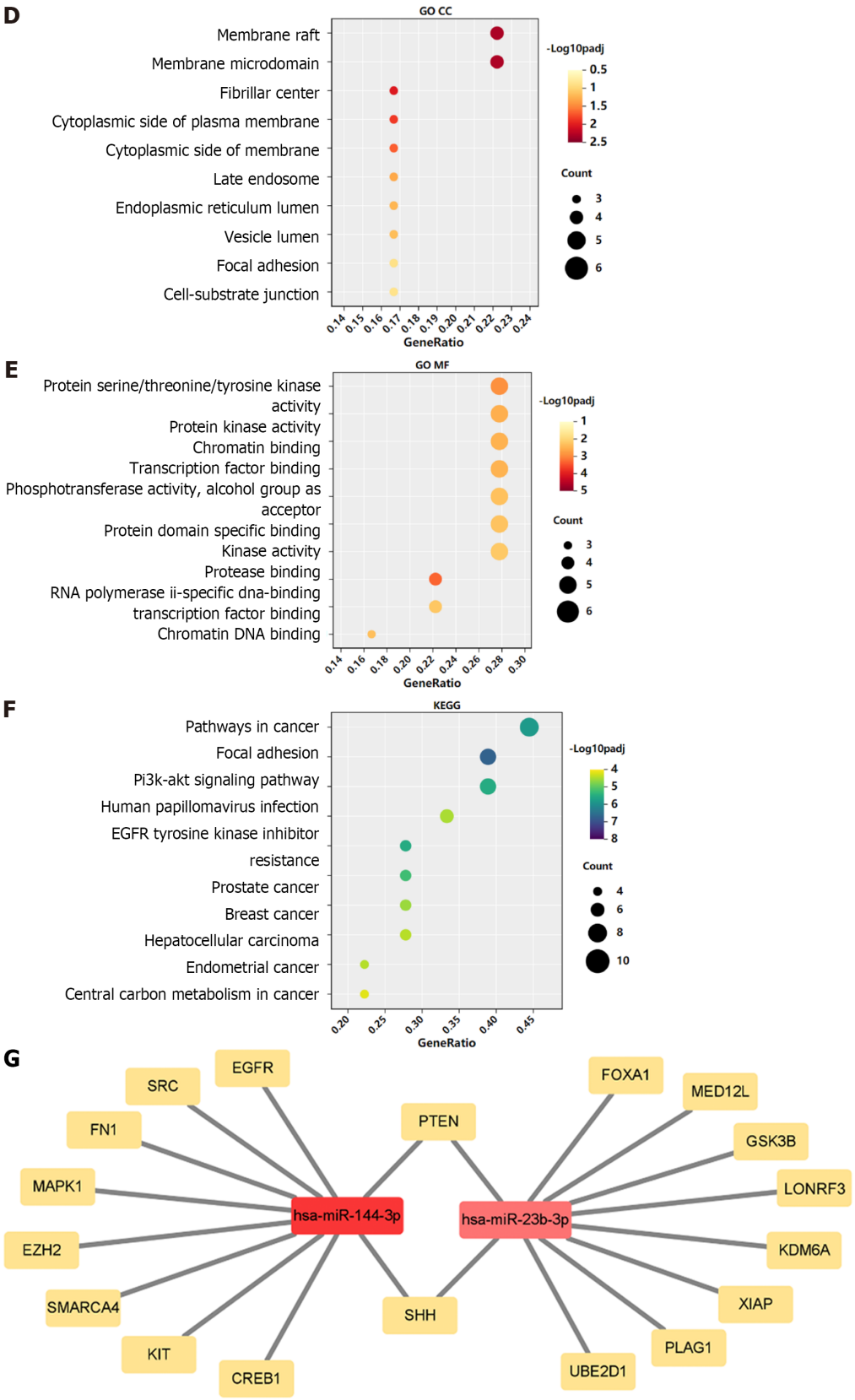


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Figure 4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of genes targeted by hsa-miR-23b-3p. A-D: Top 10 Gene Ontology biological process, cellular component, and molecular function terms enriched in target genes of hsa-miR-23b-3p; E: Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched in target genes of hsa-miR-23b-3p. BP: Biological process; CC: Cellular component; MF: Molecular function; GO: Gene Ontology; KEEG: Kyoto Encyclopedia of Genes and Genomes.

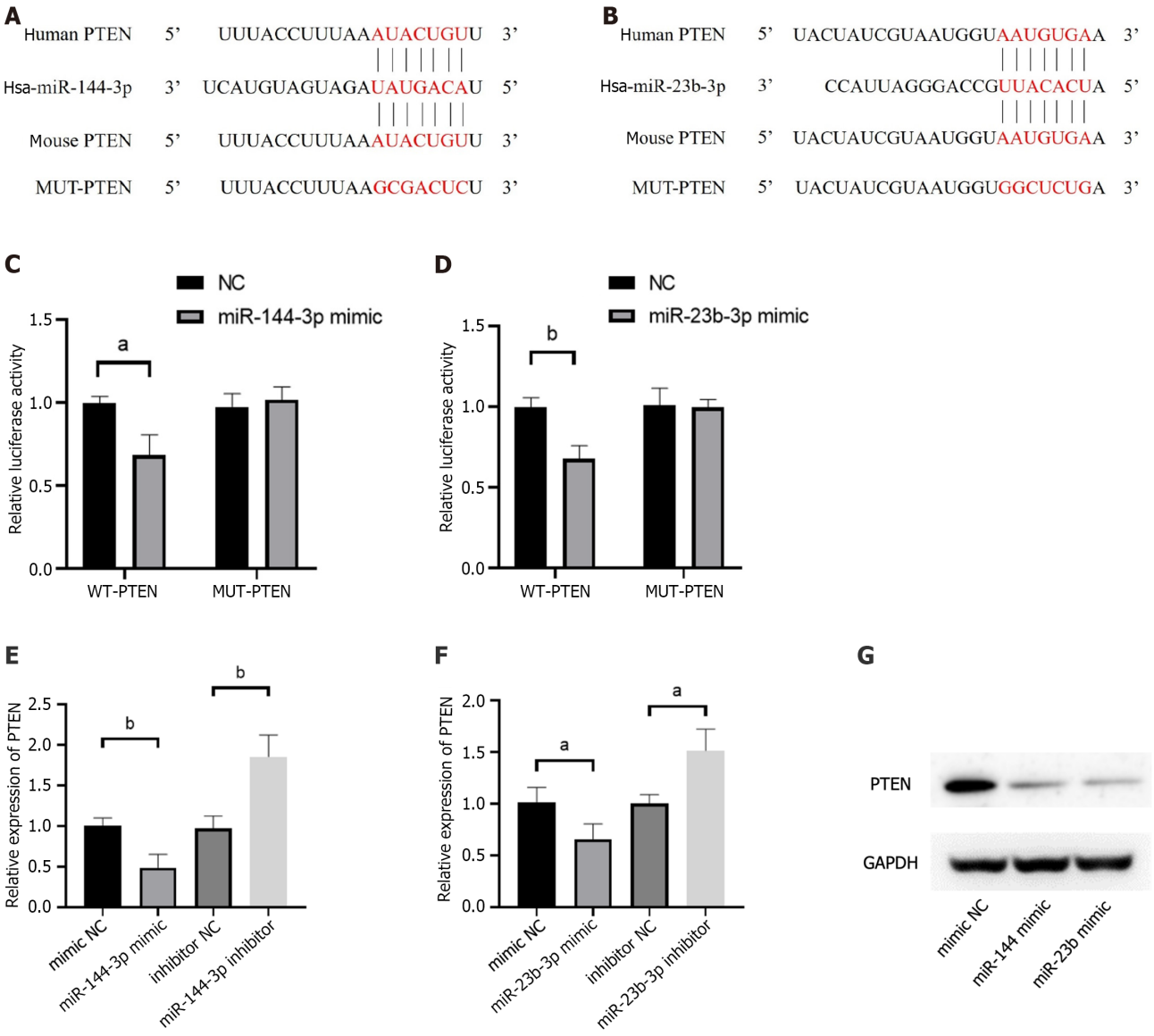






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Figure 5 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of hub-gene targets. A: Top 10 hub-gene targets for hsa-miR-144-3p; B: Top 10 hub-gene targets for hsa-miR-23b-3p; C-E: Top 10 Gene Ontology biological process, cellular component, and molecular function terms enriched in the top 20 hub-gene targets; F: Top 10 Kyoto Encyclopedia of Genes and Genomes pathways enriched in the 20 hub-gene targets; G: Interaction network of the two microRNAs and their hub-gene targets.



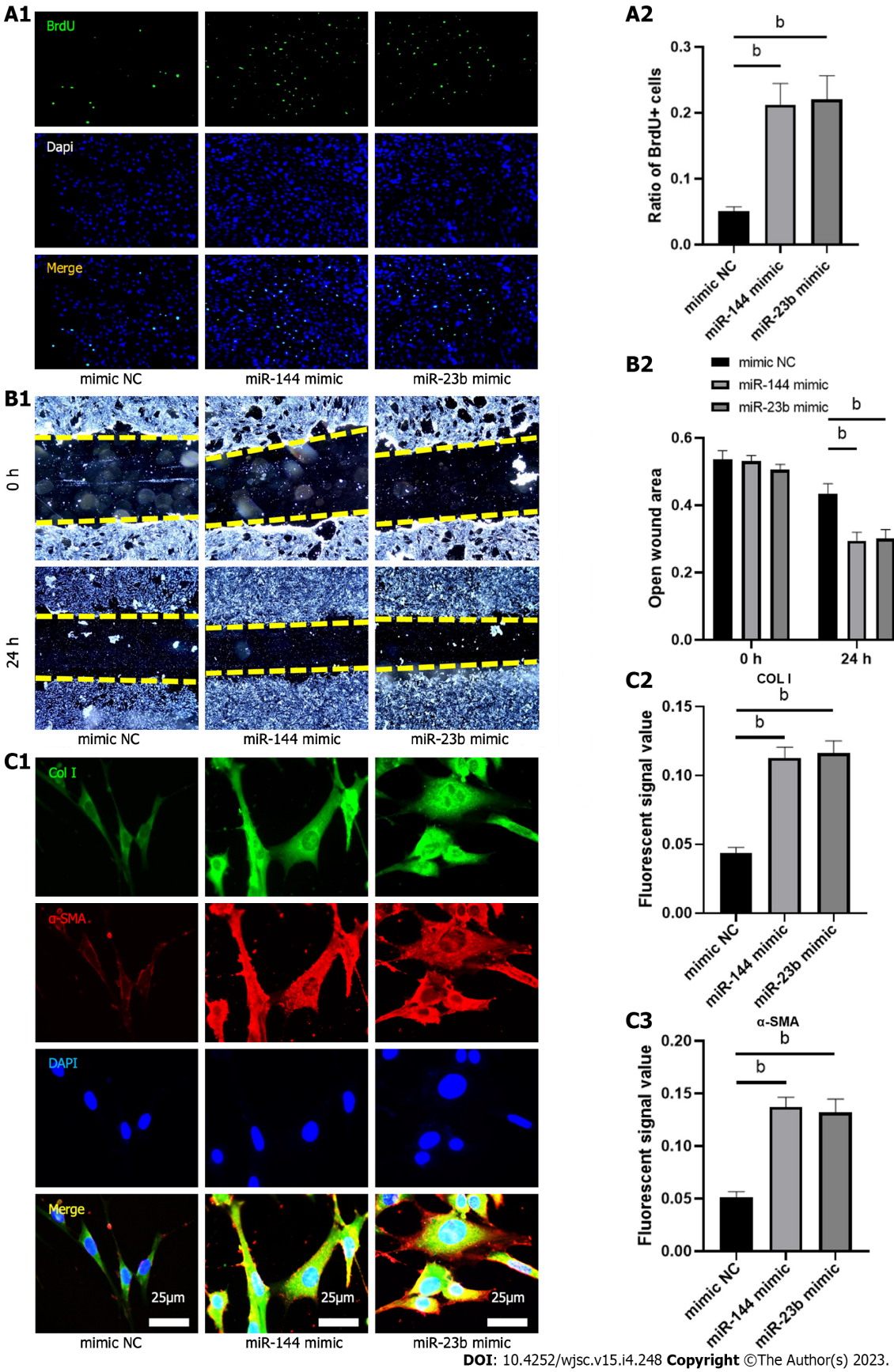
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Figure 6 Phosphatase and tensin homolog is verified as the targets of miR-144-3p and miR-23b-3p. A: Human and mouse sequence of phosphatase and tensin homolog (PTEN) for miR-144-3p binding site; B: Human and mouse sequence of PTEN for miR-23b-3p binding site; C: The relative luciferase activity was tested after co-transfection of WT/MUT pmirGLO-PTEN-3'UTR and miR-144-3p mimics or their control groups in NIH3T3 cells; D: The relative luciferase activity was tested after co-transfection of WT/MUT pmirGLO-PTEN-3'UTR and miR-23b-3p mimics or their control groups in NIH3T3 cells; E: The mRNA levels of PTEN in NIH3T3 cells transfected with miR-144-3p mimics or miR-144-3p inhibitors; F: The mRNA levels of PTEN in NIH3T3 cells transfected with miR-23b-3p mimics or miR-23b-3p inhibitors; G: Western blot showing the PTEN expression levels after transfected with miR-144-3p mimics and miR-23b-3p mimics. n = 3 per groups. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.

However, the outcomes should also be interpreted with caution. The biological functions of exosomal miRNAs are diverse, but this study only took the overlapped target gene, *i.e.*, PTEN, for analysis. Thus, further exploration is needed to pinpoint the specific functions of each miRNA. In addition, this study only used murine NIH3T3 cells *in vitro*. Experiments *in vivo* is needed to verify the reconstruction of the natural gradient structure of TBI.

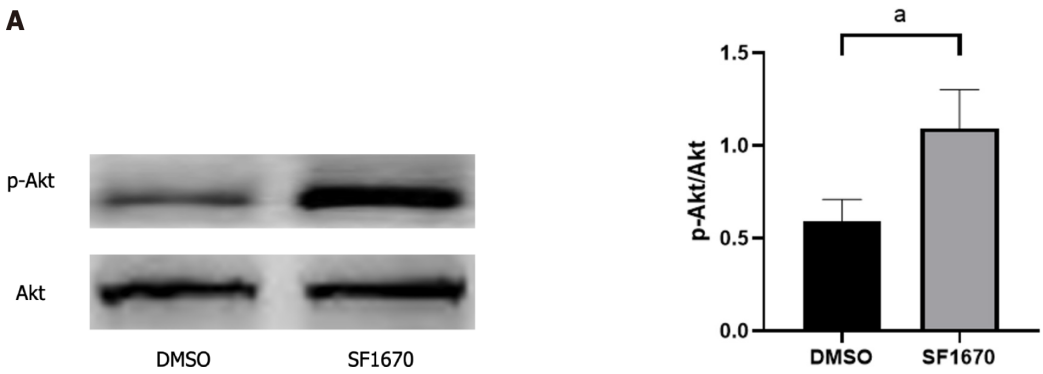
CONCLUSION

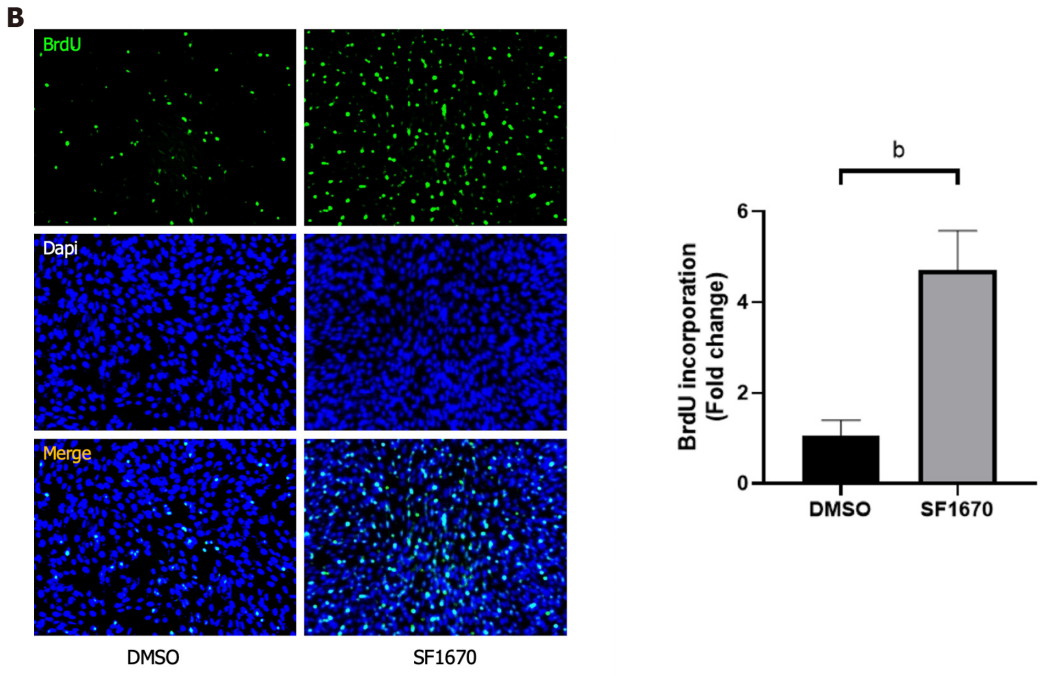
BMSC-derived exosomes promote fibroblast activation possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing.

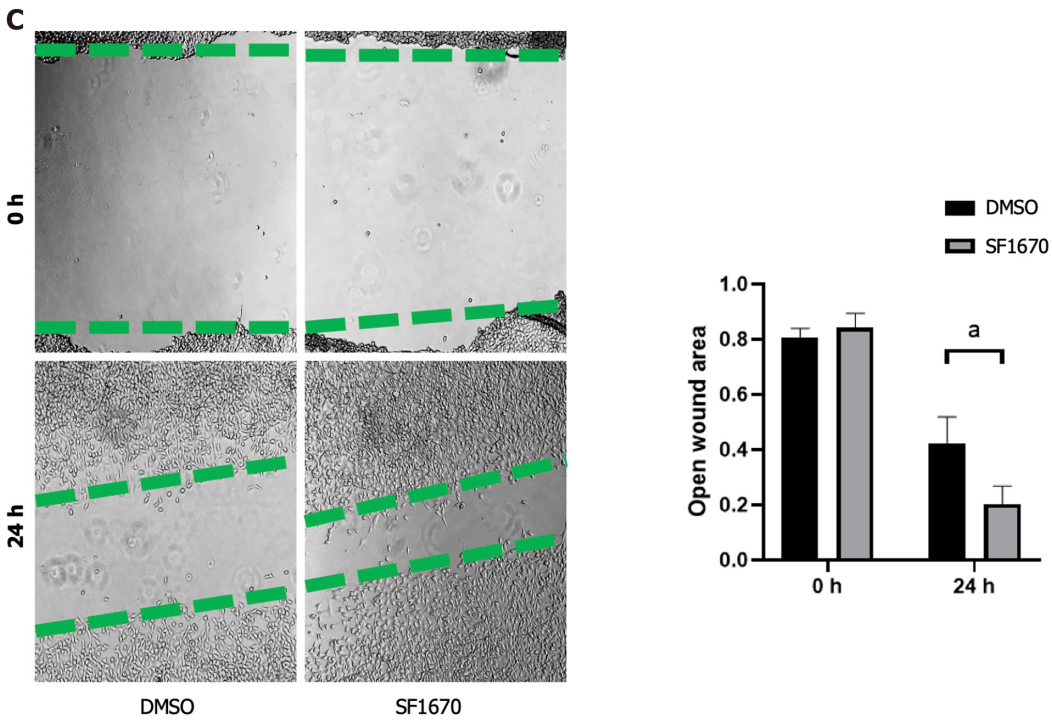


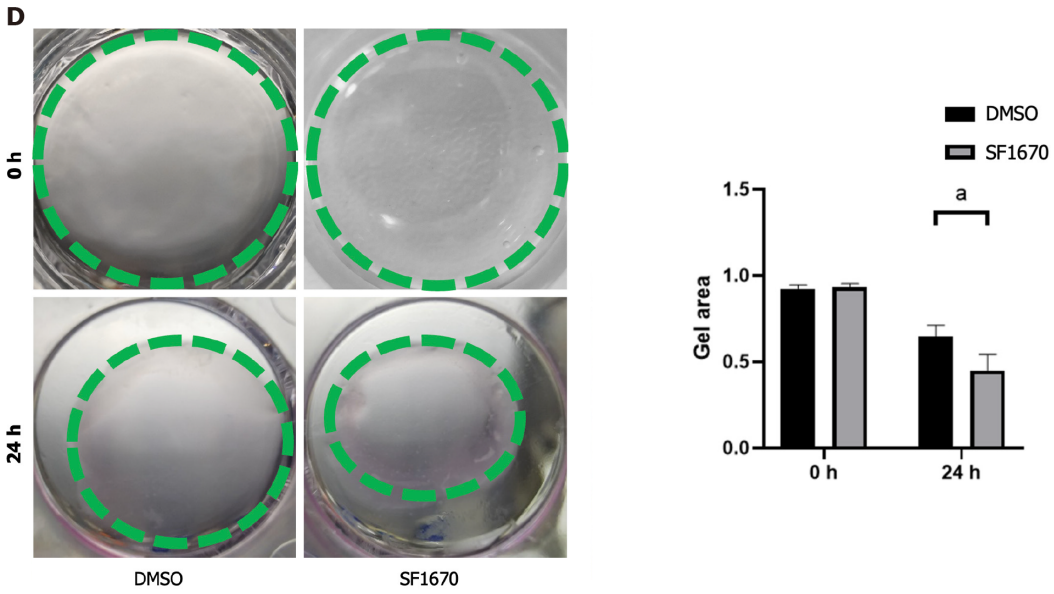
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Figure 7 MiR-144-3p and miR-23b-3p regulate the proliferation, migration and collagen synthesis of NIH3T3 fibroblasts. A: Bromodeoxyuridine assay was performed to measure the proliferation ability; B: Wound healing assay was performed to measure the migratory capability; C: Immunofluorescence was performed to determine the effects of miR-144-3p and miR-23b-3p on COL I and α-smooth muscle actin expression. n = 3 per groups. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$. α-SMA: α-smooth muscle actin.


A
p-Akt
Akt
DMSO
SF1670
a
p-Akt/Akt
1.5
1.0
0.5
0.0
DMSO
SF1670

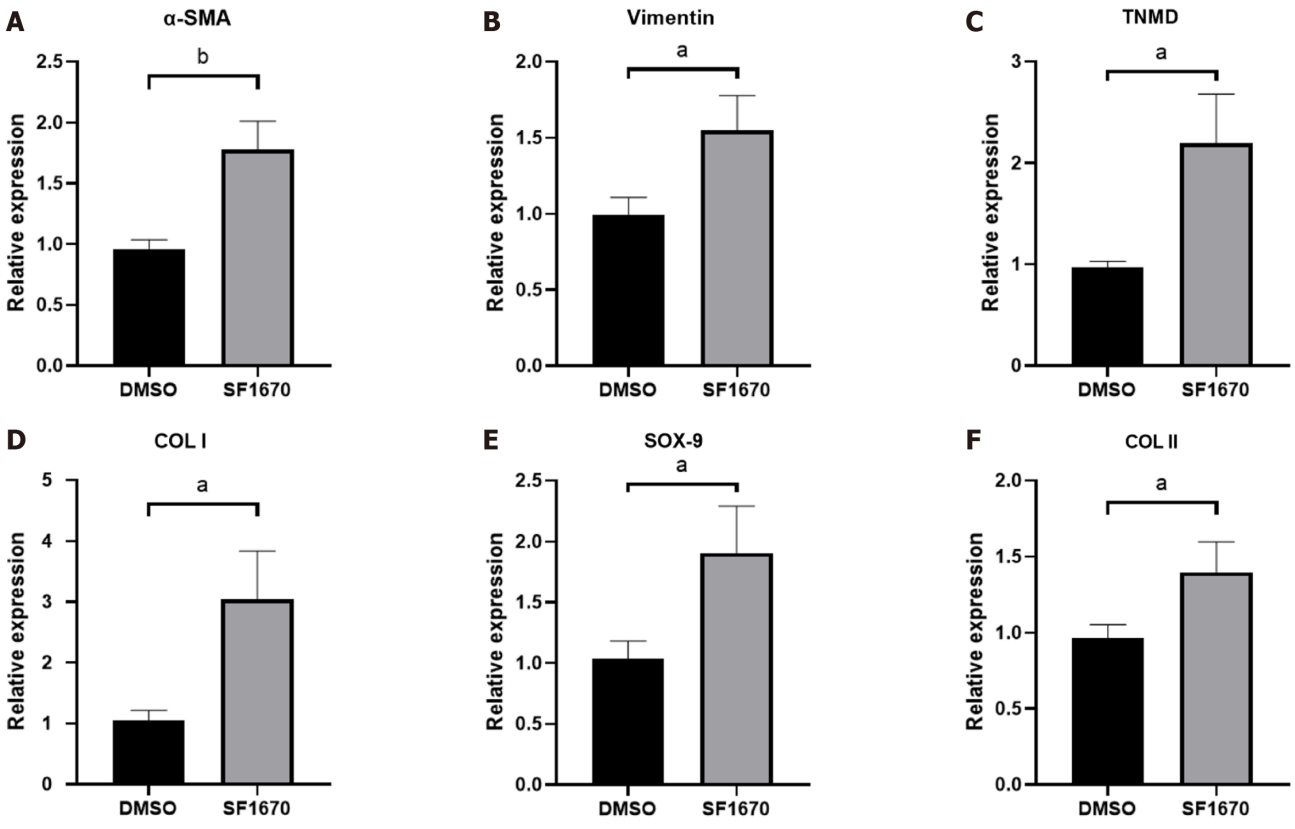

B
BrdU
Dapi
Merge
DMSO
SF1670
b
BrdU incorporation (Fold change)
6
4
2
0
DMSO
SF1670


C
0 h
24 h
DMSO
SF1670
DMSO
SF1670
Open wound area
1.0
0.8
0.6
0.4
0.2
0.0
a
0 h
24 h



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Figure 8 Inhibition of phosphatase and tensin homolog promoted cell proliferation, migration, and collagen synthesis. A: The protein levels of p-Akt and Akt in NIH3T3 cells were determined by western blot; B: Bromodeoxyuridine assay was performed to measure the proliferation ability; C: Wound healing assay was performed to measure the migratory capability; D: Collagen contraction assay was performed to evaluate the cell contractility. *n* = 3 per groups. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.



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Figure 9 Inhibition of phosphatase and tensin homolog change the gene expression of fibroblastic-, tenogenic-, and chondrogenic-related factors in NIH3T3 cells. The mRNA level of α-smooth muscle actin, vimentin, TNMD, Col I, Sox-9, and Col II in NIH3T3 cells treated with or without SF1670 was determined by quantitative real-time reverse transcription polymerase chain reaction. Data are shown as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$. α-SMA: α-smooth muscle actin.

ARTICLE HIGHLIGHTS

Research background

The normal tendon-bone insertion has a transitional structure consisting of four gradated layers including bone, mineralized fibrocartilage layer, non-mineralized fibrocartilage layer and tendon. This complex structure can disperse force from bone to tendon, preventing stress concentration. However, current therapeutic strategies cannot restore this structure, hence raising the risk of re-injury.

Research motivation

Recent evidence indicates that conditioned medium, primarily contains exosomes of mesenchymal stem cells (MSCs), can stimulate the activation of fibroblasts, thereby promoting tendon-bone healing. However, the underlying mechanism is not comprehensively understood. Moreover, the expression of exosome microRNA (miRNA) may vary depending on the status of MSCs, which presents difficulties in identifying the roles associated with MSC-derived exosomal miRNAs.

Research objectives

To identify overlapped bone marrow MSC (BMSC)-derived exosomal miRNAs in three GSE datasets and verify their effects as well as mechanisms on fibroblasts.

Research methods

BMSC-derived exosomal miRNAs data were downloaded from the Gene Expression Omnibus database. The candidate miRNAs were obtained by the intersection of different datasets. TargetScan was used to predict potential target genes. Functional and pathway analyses were conducted using the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases. Hub genes in the protein-protein interaction (PPI) network were analyzed using Cytoscape software. The transcripts of the target gene were assessed by dual luciferase reporter assays. BrdU, wound healing assay, collagen contraction assay and the expression of COL I and α-smooth muscle actin positive were applied to investigate the cell proliferation, migration and collagen synthesis.

Research results

Bioinformatics analyses showed two BMSC-derived exosomal miRNAs, has-miR-144-3p and has-miR-23b-3p, were overlapped in three GSE datasets. PPI network analysis and functional enrichment analyses indicated that both miRNAs regulated the PI3K/Akt signaling pathway by targeting phosphatase and tensin homolog (PTEN). *In vitro* experiments confirmed that both miRNAs stimulated proliferation, migration and collagen synthesis of NIH3T3 fibroblasts. Interfering with PTEN affected the phosphorylation of Akt and thus activated fibroblasts.

Research conclusions

BMSC-derived exosomes promote fibroblast activation possibly through the PTEN and PI3K/Akt signaling pathways, which may serve as potential targets to further promote tendon-bone healing.

Research perspectives

Further exploration is needed to pinpoint the specific functions of exosomal miRNAs. *In vivo* studies may better reveal the effect of exosomal miRNAs on tendon-bone healing.

FOOTNOTES

Author contributions: Li FQ, Chen WB, and Chen YS designed and performed most of the experiments, analyzed and interpreted the data, and wrote the manuscript; Luo ZW and Sun YY assisted during the acquisition, analysis, and interpretation of data and revised the manuscript; and all authors have reviewed and approved the final manuscript. Li FQ, Chen WB, and Luo ZW contributed equally to this study. Su XP (xiaopingsu2013@aliyun.com), Sun JM (sjm990205@163.com), and Chen SY (cshiyi@163.com) correspond to this study.

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Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Repetitive administration of cultured human CD34+ cells improve adenine-induced kidney injury in mice

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Abstract

BACKGROUND

There is no established treatment to impede the progression or restore kidney function in human chronic kidney disease (CKD).

AIM
To examine the efficacy of cultured human CD34+ cells with enhanced proliferating potential in kidney injury in mice.

METHODS
Human umbilical cord blood (UCB)-derived CD34+ cells were incubated for one week in vasculogenic conditioning medium. Vasculogenic culture significantly increased the number of CD34+ cells and their ability to form endothelial progenitor cell colony-forming units. Adenine-induced tubulointerstitial injury of the kidney was induced in immunodeficient non-obese diabetic/severe combined immunodeficiency mice, and cultured human UCB-CD34+ cells were administered at a dose of 1×10^6/mouse on days 7, 14, and 21 after the start of adenine diet.

RESULTS
Repetitive administration of cultured UCB-CD34+ cells significantly improved the time-course of kidney dysfunction in the cell therapy group compared with that in the control group. Both interstitial fibrosis and tubular damage were significantly reduced in the cell therapy group compared with those in the control group ($P < 0.01$). Microvasculature integrity was significantly preserved ($P < 0.01$) and macrophage infiltration into kidney tissue was dramatically decreased in the cell therapy group compared with those in the control group ($P < 0.001$).

CONCLUSION
Early intervention using human cultured CD34+ cells significantly improved the progression of tubulointerstitial kidney injury. Repetitive administration of cultured human UCB-CD34+ cells significantly improved tubulointerstitial damage in adenine-induced kidney injury in mice *via* vasculoprotective and anti-inflammatory effects.

Key Words: Chronic kidney disease; CD34+ cell; Adenine; Tubulointerstitial injury; Quality and quantity control culture; Umbilical cord blood

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Core Tip: There is no established treatment for retarding the progression or improving advanced chronic kidney disease (CKD). Here, we provided effectiveness of cord blood derived cultured human CD34+ cell on progressive CKD model in mice. Kidney function and pathological damage were improved by repetitive cell therapy along with the improvement of microvasculature and inhibition of inflammatory cell infiltration in the kidney. Enhanced cell potential (proliferation and increase of endothelial progenitor cell colony-forming unit) by culture might be a key factor for improving progressive kidney injury.

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INTRODUCTION

Chronic kidney disease (CKD) is a progressive disease that leads to end-stage renal disease (ESRD) with increasing incidence and mortality[1]. Incomplete recovery from acute kidney injury (AKI) can also lead to CKD (AKI-CKD transition) through tubular atrophy and failed tubular recovery[2]. CKD affected nearly 750 million people globally in 2016 and constitutes a serious public health burden worldwide[3]. Several treatment strategies, including pharmaceutical therapies, such as renin-angiotensin-aldosterone blockers, and diet control, including salt and protein restriction, have been applied to impede CKD progression. However, satisfactory outcomes have not been achieved. Considering the progressive nature of CKD, which is associated with high mortality rates, especially in late-stage CKD patients, including those undergoing dialysis, there is a pressing need to develop novel therapeutic strategies to treat CKD.

The efficacy of cell-based regenerative therapy in animal models of CKD has been tested, and preclinical studies have provided favorable results[4]. Cell-based therapy has been shown to improve functional and histological parameters of kidney injury and delay the development of CKD in animal

models[4]. However, clinical trials of cell-based therapy for CKD in humans are still limited, with only three clinical trials published[5-7]. These trials used single administration of autologous bone marrow-derived mesenchymal stem cells[5,6] or granulocyte colony-stimulating factor-mobilized autologous peripheral blood-derived CD34+ cells[7]. Although the safety and tolerability of cell therapy were confirmed, the efficacy in improving or stabilizing renal dysfunction was not established by these early phase trials.

Adenine-induced renal injury in mouse is used as a model of chronic kidney injury caused by 2,8-dihydroxyadenine (DHA), a metabolic product of adenine[8,9]. The primary site of adenine-induced renal injury is the tubulointerstitial region. Accumulation of DHA crystals in the renal tubules and interstitial tissue causes progressive degeneration of tubular epithelial cells and induces interstitial fibrosis. Moreover, adenine-containing diet induces chronic and progressive renal injury in mice in a dose-dependent manner. This animal model is suitable for evaluating the efficacy of cell-based therapy for progressive tubulointerstitial injuries. However, to evaluate the efficacy of xenotransplantation of human cells, the animal's immune response has to be avoided. Therefore, we stablished an adenine-induced renal injury model in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, which lack mature T, B, and natural killer cells.

Human CD34+ endothelial progenitor cells (EPC) have high angiogenic and anti-inflammatory potential, and thus used for this study[10,11]. However, because EPCs from patients with several diseases have impaired function and are insufficient, allogeneic sources are considered as good alternatives. We therefore used umbilical cord blood (UCB)-derived CD34+ cells. However, in our preliminary study, uncultured human UCB-derived CD34+ cell administration could not effectively retard the progression of chronic kidney injury in this model. Hence, we cultured UCB-CD34+ cells to enhance their vasculogenic potential using the quality and quantity culture (QQc) method, which was established for EPCs[12], and used in our previous report[13]. Once or twice administration of human UCB-QQc-CD34+ cells could not improve the time-course of kidney injury. However, administration of human UCB-QQc-CD34+ cells once a week for 3 consecutive weeks demonstrated a significant improvement of kidney function and reduction of renal histological damages. This is the first report of the application of human UCB-QQc-CD34+ cells as an effective regenerative therapy for CKD.

MATERIALS AND METHODS

Cell preparation

Human UCB-CD34+ cells were purchased from the RIKEN Bioresource Center (Tsukuba, Japan), plated at a density of 1 × 10^4 cells/well in a 24-well tissue culture plate (BD Falcon, Bedford), and cultured in QQc media for 7 d as previously described[12,13]. QQc uses a serum-free StemSpan SFEM media (Stem Cell Technologies, Vancouver, BC, Canada) with an optimized combination of growth factors as well as cytokines (20 ng/mL thyroid peroxidase, 20 ng/mL interleukine-6, 100 ng/mL SCF, 100 ng/mL Flt-3 Ligand, and 50 ng/mL vascular endothelial growth factor). All reagents were purchased from PeproTech Inc. (Rocky Hill, NJ, United States). This culture method significantly enhanced the vasculogenesis, proliferation, and colony-forming units of CD34+ cells[10]. UCB-CD34+ cells from five separate samples were cultured, and cell populations of pre- and post-QQc human UCB-CD34+ EPCs were evaluated using FACS analysis (FACSVerse™, BD, United States). FlowJo™ (version 10.6, BD, United States) was used for data analysis.

EPC colony-forming assay

EPC colony-forming units (EPC-CFUs) of pre-QQc and post-QQc human UCB-CD34+ cells were assessed using a colony-forming assay as previously described[12,13]. The EPC colony-forming assay was designed to evaluate the vasculogenic potential of EPCs and categorize total EPC-CFUs (TEPC-CFUs) into two different types of EPC-CFUs: Primitive (small cell-sized) and definitive (large cell-sized). Primitive EPC-CFUs (PEPC-CFUs) have greater potential for proliferation, whereas definitive EPC-CFUs (DEPC-CFUs) are predominantly vasculogenic populations with a greater differentiation potential. Briefly, 500 human UCB-CD34+ cells per dish were seeded into a 35-mm hydrophilic tissue culture dish. Ten to fourteen days later, total EPC colony-forming unit (TEPC-CFUs), PEPC-CFUs, and DEPC-CFUs were counted under light microscopy by two investigators who were blinded to the experimental conditions. Five samples of UCB-CD34+ cells were used for EPC colony-forming assay. All experiments were performed in triplicate.

Mice

Six-week-old male NOD/SCID mice were purchased from Charles River Laboratories Inc. (Tsukuba, Japan) and used for all experiments. All animal use was in accordance with institutional and ARRIVE guidelines (https://arriveguidelines.org). The experimental protocol was approved by the Administration Committee of Experimental Animals at Kamakura Techno-science, Inc. (No. 20-034, 20-079, Kanagawa, Japan).

Adenine-induced chronic tubulointerstitial kidney injury

The CKD animal model (adenine-induced chronic tubulointerstitial kidney injury) using NOD/SCID mouse was developed in collaboration with Kamakura Techno-science, Inc. First, several doses of adenine were used to optimize and induce progressive tubulointerstitial kidney injury. Kidney damage showed a dose-dependent pattern, and the optimized dose and period of adenine diet ingestion were finally determined as 0.14% and 3 wk, respectively.

The experimental design is shown in Figure 1. Thirty-five male NOD/SCID mice were used in this experiment. All mice had free access to tap water, and were fed a 0.14% (w/w) adenine-containing diet from day 0 to day 21. The adenine-containing diet was stopped on day 21 and changed to standard laboratory chow diet (CRF-1 powder diet, Oriental Yeast Co., Ltd., Tokyo, Japan) from days 22 to 28 in all mice. Body weight and food intake were measured weekly. Blood sampling from the tail vein was performed on days 0, 7, 14, 21, and 28. After evaluating the serum creatinine levels on day 7, the mice were divided into two groups, *i.e.*, control group (n = 17) and the cell therapy group (n = 18). After blood sampling on day 28, mice were anesthetized using isoflurane, and kidney tissues were collected for histological analysis. All animal experiments were performed at Kamakura Techno-science, Inc. (Kanagawa, Japan).

Cell transplantation

Mice in the control group were injected with 100 μL of vehicle (Iscove's modified Dulbecco's medium: IMDM), and those in the cell therapy group were injected with 1 × 10^6 UCB-QQc-CD34+ cells in 100 μL IMDM *via* the tail vein. UCB-QQc-CD34+ cells were administered three times on days 7, 14, and 21. Cell purity and viability were evaluated just before cell therapy.

Kidney function and renal histology

Serial blood samples were drawn from the tail vein on days 0, 7, 14, 21, and 28, and serum creatinine levels were immediately measured using DRI-CHEM CRE-P III (FUJIFILM Co., Ltd., Tokyo, Japan). Kidney tissues were harvested and fixed in 4% paraformaldehyde, transferred to 70% ethanol, and embedded in paraffin. From these paraffin blocks, 2 μm-thick sections were prepared and stained with periodic acid-Schiff (PAS) and Masson's trichrome (MT) stain for analysis of tubular damage and interstitial fibrosis, respectively. To examine the extent of tubulointerstitial injury, 10 non-overlapping fields from the entire cortical and outer medulla areas in PAS-stained specimens and MT-stained specimens were captured under 100 × magnification. Tubulointerstitial injury in PAS-stained sections were categorized as tubular dilatation with epithelial and tubular atrophy. The degree of injury was scored on a scale of 0 to 4 according to the percentage of the damaged area; 0: 0%, normal; 1, 0%-25%; 2, 25%-50%;3, 50%-75%; and 4, 75% or more. Interstitial fibrosis in MT-stained sections was quantified as fibrotic area per total area (%) and measured using an automatic image analyzer (cellSens, Olympus, Japan). Tubulointerstitial injury score and interstitial fibrosis area were evaluated in each mouse (n = 17 in the control group and n = 18 in the cell therapy group), and mean values in each mouse were used to calculate the group mean value for further comparison between the control group and cell therapy group.

CD31 and F4/80 immunohistochemistry

Since CD31 antigen is constitutively expressed on the endothelial cell surface, the loss of CD31-positive staining most likely reflects capillary loss. We performed CD31 immunostaining to evaluate peritubular capillary (PTC) loss using 3 μm-thick paraffin sections. PTCs were identified by immunostaining with a rabbit monoclonal anti-CD31 (PECAM-1) antibody (1:100; #77699, Cell Signaling Technology, Danvers, MA, United States). Immunostaining was performed using a BOND-III autostainer (Leica Biosystems, Wetzlar, Germany) according to the manufacturer's protocol. Briefly, after deparaffinization of paraffin sections, heat-induced epitope retrieval was performed using BOND Epitope Retrieval Solution 2 (prediluted, pH 9.0; Leica Biosystems, Wetzlar, Germany) for 20 min at 100 °C. Sections were incubated sequentially with endogenous peroxidase block for 5 min, primary antibody for 30 min, secondary detection polymer for 10 min, diaminobenzidine for 10 min, and hematoxylin for 5 min.

PTC density was evaluated according to a previously published method with some modifications [11]. Twenty randomly selected fields encompassing the renal cortex and outer medulla were randomly captured by digital imaging (× 400). Each image was divided into 270 squares using a grid, and the number of squares with CD31-positive staining was counted. The PTC density was represented as a percentage of CD31 positive squares per total number of squares.

Macrophages were identified using a rabbit polyclonal anti-F4/80 antibody (1:100; ab100790, Abcam, Cambridge, United Kingdom). Ten non-overlapping fields in the cortical and outer medulla areas were randomly selected and captured by digital imaging (× 100). The degree of macrophage infiltration was calculated using the 270-square grid method, as described above. The rate of macrophage infiltration was represented as a percentage of F4/80- positive squares relative to the entire cortical and outer medulla areas. Macrophage infiltration rate and PTC density were assessed in the control group and cell therapy group.



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Figure 1 Experimental design. The adenine-containing diet was fed from day 0 to day 21, and switched to a standard laboratory diet from day 22 to day 28. Vehicle (IMDM) or UCB-CD34+ cells in IMDM were administered on days 7, 14, and 21 *via* the tail vein in the control group and cell therapy group, respectively. IMDM: Iscove's modified Dulbecco's medium; UCB: Umbilical cord blood.

Statistical analysis

All data are expressed as the mean ± SE, unless otherwise specified. Comparison between two groups were performed using the Mann-Whitney *U* test. Differences in time-course of serum creatinine levels during the experimental period between the control group and the cell therapy group were analyzed using repeated measures analysis of variance (ANOVA). Statistical analysis was performed using SPSS version 11.0 software (SPSS Inc., Chicago, IL, United States) and a P value < 0.05 was considered statistically significant.

RESULTS

Effect of the QQc on CD34+ cell expansion and EPC-CFUs

The QQc significantly increased the number of CD34+ cells. CD34+ positivity per the 1 × 10^4 initially seeded cells was significantly increased in post-QQc-CD34+ cells compared with that in pre-QQc-CD34+ cells (353821 ± 122429 *vs* 7341 ± 947, P < 0.001) (Figure 2A). Cell viability of QQc-CD34+ cells was higher than that of pre-QQc CD34+ cells (93.6% ± 1.3% *vs* 87.0% ± 0.8%, P < 0.01). After seven days of culturing using the QQc method, the number of CD34+ cells showed almost a 60-fold increase on day 7 compared with that on day 0 (59.0 ± 10.4 *vs* 1-fold, P < 0.001) (Figure 2B). Post-QQc CD34+ cells formed more TEPC-CFUs than did pre-QQc CD34+ cells (1138.0 ± 124.0 *vs* 27.0 ± 4.5, P < 0.001) (Figure 2C). Moreover, both PEPC-CFUs and DEPC-CFUs showed a significant increase after QQc compared with pre-QQc (PEPC-CFUs: 17.4 ± 3.0 *vs* 666.0 ± 127.5, P < 0.001; DEPC-CFUs: 9.4 ± 2.5 *vs* 472.0 ± 112.0, P < 0.01).

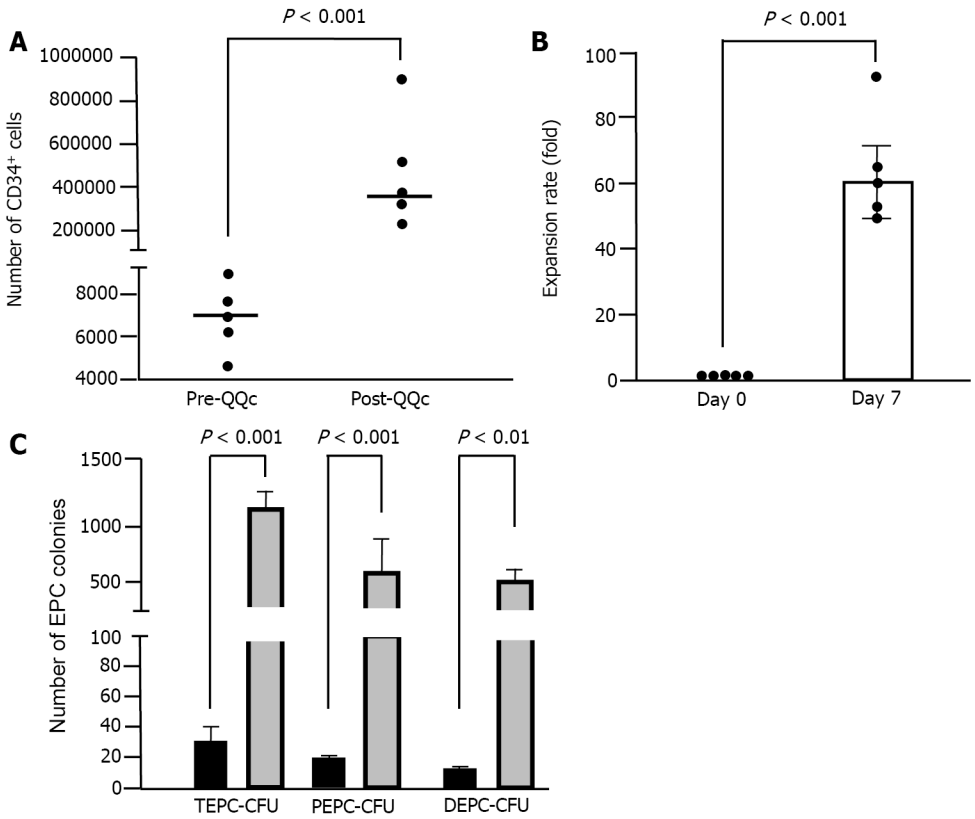
QQc-CD34+ cells improved kidney function

The body weight was not significantly different between the cell therapy and control groups on days 0, 7, 14, 21, and 28. Diet consumption was also comparable between the two groups throughout the experimental period (data not shown).

To evaluate the role of UCB-QQc-CD34+ cell therapy in renal injury, serum creatinine levels were determined in the cell therapy and control groups. The time-course in serum creatinine levels is shown in Figure 3A. Serum creatinine levels were not statistically different between the cell therapy and control groups (0.56 mg/ dL ± 0.02 mg/dL *vs* 0.61 mg/dL ± 0.03 mg/dL) on day 0. During adenine-containing diet feeding, serum creatinine levels showed a gradual and constant increase in the control group (1.51 mg/dL ± 0.14 mg/dL on day 21) demonstrating a state of renal dysfunction. After switching to the standard diet on day 22, serum creatinine levels in the control group decreased to 1.41 mg/dL ± 0.14 mg/dL on day 28. In the cell therapy group, serum creatinine levels increased until day 14 and then showed a gradual increasing trend on day 21 (1.29 mg/dL ± 0.09 mg/dL). After switching to the standard diet, serum creatinine levels decreased to 1.09 mg/dL ± 0.09 mg/dL on day 28 in the cell therapy group. Time-course of serum creatinine levels between the control and the cell therapy groups showed borderline significance (P = 0.06). However, the relative ratio of changes in serum creatinine levels from day 0 to day 28 showed a statistically significant difference between the control and the cell therapy groups (255.1% ± 25.9% *vs* 185.9% ± 18.5%, P = 0.035) (Figure 3B). Thus, repetitive administration of UCB-QQc-CD34+ cells significantly improved the time-course of adenine-induced chronic kidney injury.

QQc-CD34+ cells reduced interstitial fibrosis and tubular damage

The adenine-containing diet induced prominent tubular damage, including tubular dilatation and



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Figure 2 CD34+ cell and endothelial progenitor cell colony-forming units expansion by quality and quantity culture method. A: Number of total CD34+ cells. The plot graph demonstrates CD34+ positivity per 1 × 10^4 fresh UCB-CD34+ sample and post-culture CD34+ cell number per 1 × 10^4 initially seeded cells; B: Expansion rate of CD34+ cells. The expansion rate of CD34+ cells from fresh CD34+ cells (day 0) to cultured CD34+ cells (day 7); C: Endothelial progenitor cells (EPC) colony-forming unit. The number of TEPC-CFUs, primitive EPC-CFUs, and definitive EPC-CFUs of post- quality and quantity culture (QQc) CD34+ cells. Gray column: Significantly increased compared with that of pre-QQc CD34+ cells; black column: Statistical significances in Figure 2A-C were determined using Mann-Whitney *U* test. UCB: Umbilical cord blood; EPC: Endothelial progenitor cell; CFU: Colony-forming unit; QQc: Quality and quantity culture; TEPC: Total endothelial progenitor cell; PEPC: Primitive endothelial progenitor cell; DEPC: Definite endothelial progenitor cell.

tubular atrophy accompanied by interstitial fibrosis (Figure 4A-D). In line with the reduced time-course of renal injury in cell therapy mice, the interstitial fibrosis area was significantly reduced in the cell therapy group compared with that in the control group (8.1% ± 5.4% *vs* 16.3% ± 7.0%, $P < 0.01$) (Figure 4E). The tubular damage score was also significantly decreased in the cell therapy group compared with that in the control group (1.48 ± 1.27 *vs* 2.83 ± 1.00, $P < 0.01$) (Figure 4F).

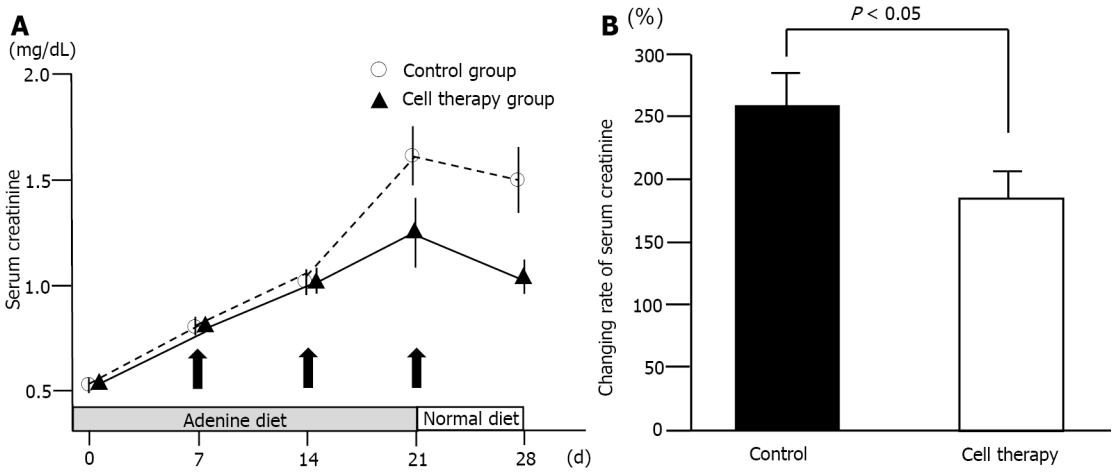
PTC density and macrophage infiltration

Representative images of PTC density as evaluated by CD31 immunohistochemical staining are shown in Figure 5. CD31+ staining was significantly decreased, especially around the dilated and degenerated tubules, in this model (Figure 5A). The cell therapy group showed significantly higher PTC density than did the control group (57.3% ± 6.4% *vs* 31.0% ± 6.1%, $P < 0.01$) (Figure 5B and C).

Macrophage infiltration is shown in Figure 6. Infiltration of F4/80-positive macrophages was mainly observed in the area with interstitial fibrotic changes and dilated and degenerated tubules in the control group (Figure 6A). However, macrophage infiltration was dramatically reduced in the cell therapy group compared with that in the control group (1.3% ± 0.3% *vs* 14.4% ± 1.1%, $P < 0.001$) (Figure 6B and C).

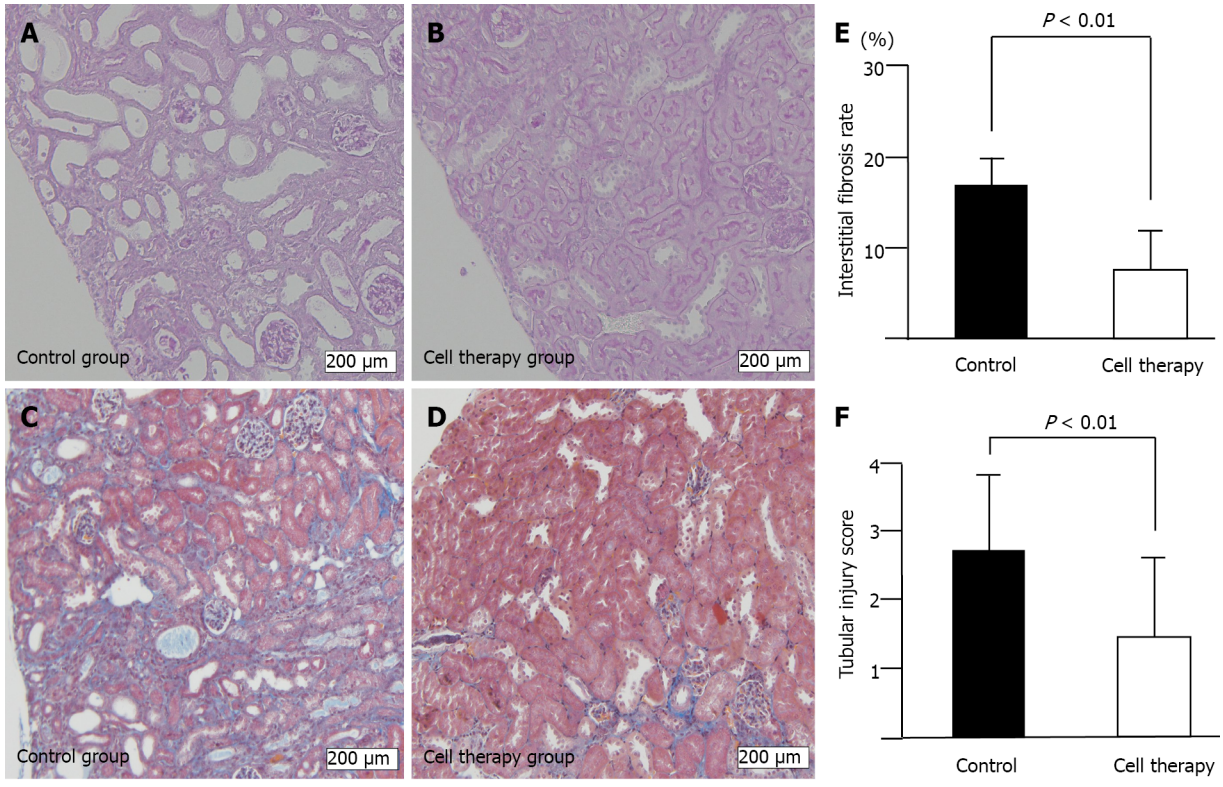
DISCUSSION

We demonstrated that repetitive administration of human UCB-QQc-CD34+ cells significantly improved the time-course of adenine-induced tubulointerstitial injury in mice. The relative changes in serum creatinine levels from baseline to day 28 were significantly improved in the cell therapy group compared with those in the control group. Interstitial fibrosis area and tubulointerstitial injury score were also significantly improved by cell therapy. The PTC network, visualized by CD31+ staining, was found to be severely damaged in adenine-induced kidney injury. However, PTC rarefaction was



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Figure 3 Changes in serum creatinine levels. A: Time-course of serum creatinine levels in the control group (n = 17) and cell therapy group (n = 18). The P value between the control group and the cell therapy group on day 28 was 0.06; B: Change rate of serum creatinine levels from baseline to day 28 in the control and cell therapy groups. Statistical significance in Figure 3A was determined using repeated measures analysis of variance. Statistical significance in Figure 3B was determined using Mann-Whitney U test.

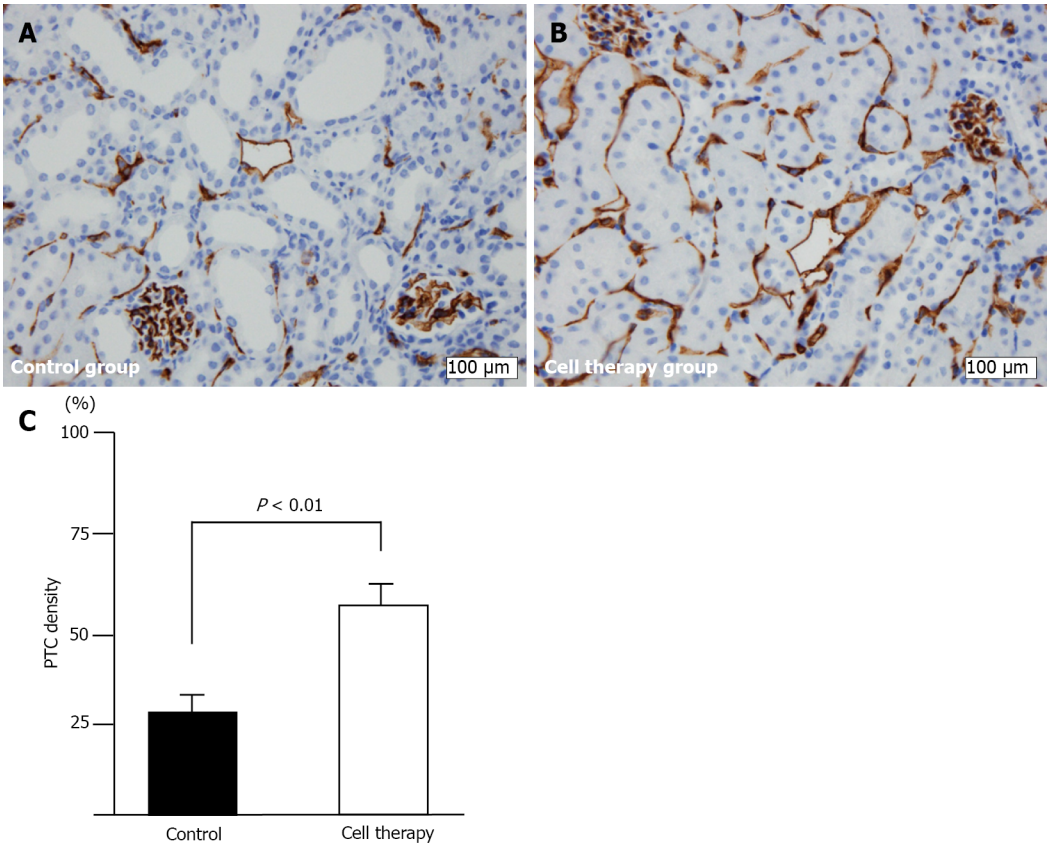


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Figure 4 Histological analysis of renal injury. A and B: Tubular injury including tubular dilatation, tubular epithelial atrophy, and tubular atrophy, evaluated using periodic acid-Schiff-stained renal tissue sections of control group (A) and cell therapy group (B); C and D: Interstitial fibrosis using Masson's trichrome stained sections of control group (C) and cell therapy group (D). Scale bar: 200 μm; E: Quantitative analysis of interstitial fibrosis rate, and F: semi-quantitative analysis of tubular injury score. Statistical significance of Figure 4E and F were determined using Mann-Whitney U test.

significantly improved by cell therapy. Furthermore, macrophage infiltration was dramatically reduced in the cell therapy group. These results might imply the significant potential of CD34+ cell-based therapy for clinical application in CKD.

CKD progression is caused by multiple factors, including diabetes, hypertension, and glomerulonephritis. Regardless of the diverse causes of CKD, tubulointerstitial damage is the final common pathway that results in its transition to ESRD[14]. Therefore, one major target of cell-based regenerative therapy for CKD is the arrest of chronic tubular and interstitial damage. Loss of integrity of the



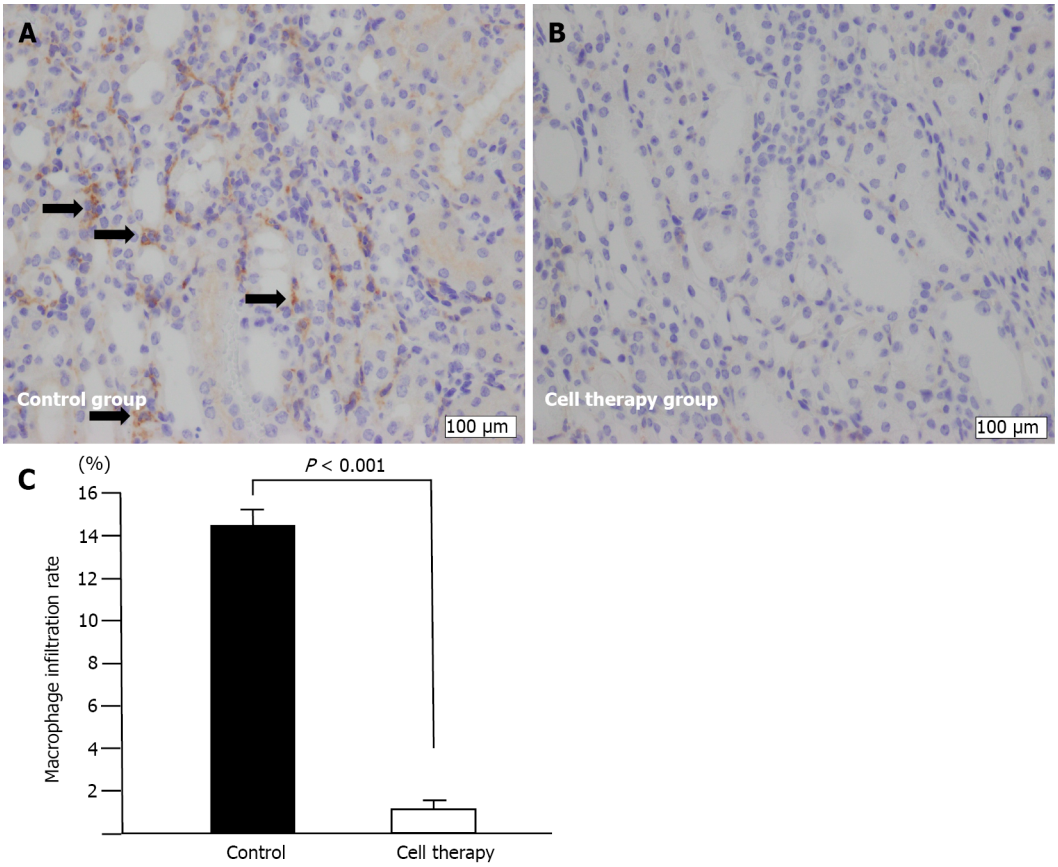
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Figure 5 Peritubular capillary density evaluation using CD31+ staining. A and B: CD31+ immunostaining of peritubular capillary (PTC) in control group (A) and cell therapy group (B); original magnification (× 400). Scale bar: 100 μm; C: Quantitative analysis of PTC density. Statistical significance in Figure 5C was determined using Mann-Whitney *U* test.

intrarenal microvasculature is also a major contributor to the progression of renal diseases[15-17]. Tubular damage, interstitial fibrosis, and microvasculature impairment are associates with each other and form a vicious cycle to promote CKD[18-24]. A close association between PTC rarefaction and tubulointerstitial fibrosis has been proven in the human kidney[20]. Hypoxia due to PTC rarefaction induces the production of local inflammatory cytokines[25], which in turn, worsen hypoxia in inflamed tissues. Thus, hypoxia also creates a vicious cycle of inflammation[26].

CD34+ cells have been used in regenerative therapy for several organ damages, including critical limb ischemia[27,28], myocardial ischemia[29], stroke[30], and acute kidney injury[31]. CD34+ cells possess two important properties that are considered necessary for regenerative therapy: Enhanced angiogenic and anti-inflammatory potential[10,11]. Externally administered CD34+ EPCs improved kidney injury such as endothelial cell damage in several animal models[13,15,19,32-35]. In cases of acute and severe renal endothelial cell injury, intrinsic repair may not function sufficiently[36]. Furthermore, endothelial disturbances related to ischemia induce permanent damage to PTCs[37]. Therefore, a strategy for vascular protection or angiogenesis might be important for the long-term preservation of kidney function. Recently, horizontal transfer of regenerative information *via* extracellular vesicles (EVs) between regeneration associated stem/progenitor cells and injured cells in the damaged organ was shown to be a major regenerative mechanism. Exosomes from human CD34+ cells, by themselves, have demonstrated angiogenic activity in *in vivo* and *in vitro* experiments[38,39]. Hence, CD34+ based cell therapy could be an excellent strategy to induce vasculogenesis and repair kidney function.

Regarding cell administration, frequency of transplantation as well as the number and regenerative potential of transplanted cells is thought to be very important. Multiple rather than single administration might show more advantages to improve organ damage. Lv *et al*[40] demonstrated that multiple administration of UCB-mesenchymal stem cells showed a superior therapeutic effect compared with that of single administration in an animal model of chemotherapy-induced premature ovarian failure. We also demonstrated that repetitive administration (3 times, once in a week) of cultured UCB-CD34+ cells significantly improved the time-course of adenine-induced chronic kidney injury. The causative signals for CKD, including oxidative stress, tissue hypoxia, and inflammatory cell infiltration with the release of inflammatory cytokines continuously injure the kidney. Therefore, multiple administration of regeneration-associated CD34+ cells at fixed time intervals might be a better treatment strategy for CKD. For the number of administered cells, 1 × 10^6 cells/20 g mouse is equivalent to about 50 × 10^6/kg



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Figure 6 Macrophage infiltration analysis. A and B: F4/80+ macrophage immunostaining (arrows) in control group (A) and cell therapy group (B); original magnification (× 400). Scale bar: 100 μm; C: Quantitative analysis of the rate of macrophage infiltration area. Statistical significance in Figure 6C was determined using Mann-Whitney *U* test.

in humans, an amount that is probably not achievable clinically. It is known that in cases of cell administration *via* the tail vein in mice, many cells are trapped in the lung, spleen, and bone marrow (extra-renal trap)[13]. Extra-renal trap might necessitate more cells to be administered in mice to deliver sufficient number of cells into the kidney. Therefore, future clinical trials should consider the route of cell administration. To get better effects in future clinical trials, cells should be designed for direct arterial administration.

Herein, we used human UCB-derived CD34+ cells. UCB-CD34+ cells can be used as an off-the-shelf treatment. Because EPCs from patients with several diseases have impaired function and are insufficient, allogeneic source is considered as a good alternative. Immune reaction against allogeneic cells might limit their use. However, a recent study reported the immunosuppressive effect of cultured human EPCs. The proliferation and activation of mouse T cells were significantly suppressed by co-culture with allogeneic human UCB-EPCs[41]. Therefore, cultured UCB-EPCs with enhanced potential might have a chance of emerging as a possible tool for allogeneic cell therapy. One major limitation to using UCB-CD34+ cells is the limited number obtained from one UCB donor. Regarding this, QQc of UCB-CD34+ cells significantly increased the number of CD34+ cells (almost 60 folds) in one week, and EPC-CFUs (total, primitive, and definite EPC-CFUs) were significantly increased. The increased UCB-CD34+ cell number enabled multiple administration. Furthermore, the enhanced vasculogenic and anti-inflammatory potential of UCB-CD34+ cells might be critical for preserving PTC integrity and reducing inflammatory cell infiltration.

There are several limitations to this study. First, the effect of tissue hypoxia was not evaluated to establish the relationship among tubular damage, interstitial fibrosis, PTC integrity, macrophage infiltration, and hypoxia in the kidney tissue of control and cell therapy-treated mice. Second, the cellular behavior of UCB-CD34+ cells (homing and staying in injured kidney) remain undetermined. Third, we could not compare the contents (mRNA, miRNA, and proteins, including growth factors and cytokines) of EVs of pre-QQc and post-QQc UCB-CD34+ cells. Therefore, the EV-mediated mechanism by which UCB-QQc-CD34+ cells showed an effective response in repairing kidney injury remains to be elucidated. We evaluated the effect of cell therapy during the progressive phase, and not the advanced ESRD stage. Therefore, it is unclear whether cell therapy might be effective at the ESRD stage. Stopping adenine diet induced recovery from dysfunction. However, it remains unknown whether stopping

adenine diet without cell therapy brings kidney function completely to normal, because we did not conduct a longer follow-up after switching adenine diet to normal chow. At the same time, we could not elucidate whether the effects of cell therapy were long-lasting since the animals were sacrificed only one week after the last dose of cells. These limitations should be addressed in future research.

CONCLUSION

In conclusion, although the more precise mechanisms should be clarified, this study provides a new insight in the field of regenerative medicine. Repetitive administration of human UCB-QQc-CD34+ cells improved kidney function and reduced histological damage in adenine-induced tubulointerstitial injury. Tubular damage and interstitial fibrosis were significantly improved by cell therapy in accordance with improved PTC integrity and reduced inflammatory cell infiltration. Hence, clinical trials using repetitive administration of regenerative CD34+ cells with enhanced proliferative and angiogenic potential might be expected for patients with progressive CKD.

ARTICLE HIGHLIGHTS

Research background

Chronic kidney Disease (CKD) constitutes a serious public health burden worldwide, and there is no established treatment to impede the progression or restore kidney function in human CKD.

Research motivation

To discover and establish a new effective treatment for CKD is urgently needed. Cell-based therapy has a potential as a new effective treatment for progressive CKD.

Research objectives

To evaluate the efficacy of cultured human cord-blood-derived CD34+ cells.

Research methods

Progressive tubulointerstitial injury with kidney dysfunction was made in mice. Human umbilical cord blood (UCB)-derived CD34+ cells were incubated in vasculogenic conditioned medium for 1 wk to increase the number of CD34+ cells and ability to form endothelial progenitor cell (EPC)-colony-forming units. These cultured human CD34+ cells were administered on days 7, 14, and 21 after the start of adenine diet.

Research results

Cell therapy significantly improved the time-course of progressive kidney dysfunction. Pathological injuries including tubular injury and interstitial fibrosis were significantly reduced in the cell therapy group compared with those in the control group. Preserved microvasculature integrity and decreased macrophage infiltration were shown in the cell therapy group.

Research conclusions

Early intervention using human cultured CD34+ cells significantly improved the progression of tubulointerstitial kidney injury.

Research perspectives

In future research, the efficacy of cultured human CD34+ cells for more advanced CKD such as end-stage renal disease (ESRD), cell behavior (homing and staying in injured kidney) after cell administration, differences in extracellular vesicles from pre- and post-incubated UCB-derived CD34+ cells, should be evaluated for further analysis of the efficacy of cell therapy. A future clinical trial of cell therapy for progressive CKD might be expected.

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FOOTNOTES

Author contributions: Ohtake T contributed to principal investigator, conception and design, provision of study material, collection and assembly of data, data analysis, data interpretation, and manuscript writing; Itaba S, Fujii S, and Kumagai H contributed to animal experiment and data analysis; Salybekov AA and Sheng Y contributed to cell culture and cell population analysis; Sato T contributed to cell preparation and cell population analysis; Yanai M and Imagawa M contributed to immunohistochemical analysis; Harata M contributed to cell preparation; Asahara T contributed to advisor of cell culture, conception and design, confirmation of analytical methods and results, data interpretation, advisor for content of manuscript, and final approval of manuscript; Kobayashi S contributed to conception and design, data interpretation, advisor for content of manuscript, and final approval of manuscript.

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REVIEW

How the interplay among the tumor microenvironment and the gut microbiota influences the stemness of colorectal cancer cells

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Abstract

Colorectal cancer (CRC) remains the third most prevalent cancer disease and involves a multi-step process in which intestinal cells acquire malignant characteristics. It is well established that the appearance of distal metastasis in CRC patients is the cause of a poor prognosis and treatment failure. Nevertheless, in the last decades, CRC aggressiveness and progression have been attributed to a specific cell population called CRC stem cells (CCSC) with features like tumor initiation capacity, self-renewal capacity, and acquired multidrug resistance. Emerging data highlight the concept of this cell subtype as a plastic entity that has a dynamic status and can be originated from different types of cells through genetic and epigenetic changes. These alterations are modulated by complex and dynamic crosstalk with environmental factors by paracrine signaling. It is known that in the tumor niche, different cell types, structures, and biomolecules coexist and interact with cancer cells favoring cancer growth and development. Together, these components constitute the tumor microenvironment (TME). Most recently, researchers have also deepened the influence of the complex variety of microorganisms that inhabit the intestinal mucosa, collectively known as gut microbiota, on CRC. Both TME and microorganisms participate in inflammatory processes that can drive the initiation and evolution of CRC. Since in the last decade, crucial advances have been made concerning to the synergistic interaction among the TME and gut microorganisms that condition the identity of CCSC, the data exposed in this review could provide valuable insights into the biology of CRC and the development of new targeted therapies.

Key Words: Colorectal cancer; Colorectal cancer stem cells; Tumor microenvironment factors; Tumor stroma; Gut microbiota; Cancer progression

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Core Tip: Colorectal cancer (CRC) represents one of the most prevalent tumors worldwide. The tumor microenvironment (TME) through its proinflammatory role, among others, actively participates in CRC progression and the disturbance of gut microbiota (dysbiosis) can influence this inflammatory process. CRC stem cells (CCSC) are a tumor cell subpopulation that drives CRC initiation, progression and treatment failure. The features and behavior of CCSC are modulated by several factors including TME and gut microbiota. Here, we will give an overview of the synergistic interaction among TME and intestinal microorganisms that condition the CRC environment and shape CCSC characteristics allowing CRC evolution.

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INTRODUCTION

Colorectal cancer (CRC) is a multi-step process where intestinal cells acquire malignant phenotypic characteristics that allow them to proliferate, migrate, invade and establish in new tissues[1]. In the last decades, screening strategies and treatments have been improved, decreasing the proportion of CRC patients by as much as 65%–88%[2]. However, this disease remains the third most prevalent type of cancer, having an incidence of 10% and ranking second in mortality (9.4% among all cancer deaths) according to global cancer statistics[3]. The leading cause of patient deaths and relapses is the appearance of new CRC subtypes and the acquired resistance to currently used therapies[4]. Moreover, a great number of CRC are diagnosed with distal metastases and these patients have a poor survival rate due to a lack of response to therapy[2]. One of the causes that affect the treatment of this type of tumor by inducing resistance and the appearance of recurrences, is the presence of a small subpopulation of cells called CRC stem cells (CCSC). This small number of cells have mutations in specific oncogenes that allow them to develop the ability to induce tumor initiation, self-renew, differentiate, dedifferentiate, and acquire multidrug resistance[1,5]. The origin of this cell subpopulation is still controversial. They may originate from colorectal normal cells, colorectal normal stem cells, or CRC cells by genetic alterations or by the influence of environmental factors that induce epigenetic changes[5].

It is known that in the tumor niche, different cell types, structures, and biomolecules coexist and interact with cancer cells favoring the growth and development of the tumor. Together these components constitute the tumor microenvironment (TME). In the last decades, several investigations have demonstrated that tumor surrounding ambiance through its proinflammatory role, among others, actively participates in the development, progression and chemoresistance of CRC[1,4].

Researchers have deepened the study of the influence of the complex variety of microorganisms that inhabit the intestinal mucosa, collectively known as the gut microbiota, on this inflammatory microenvironment. Besides contributing to innate and adaptive immune function, it has been observed that the imbalance in the species present in the intestinal microbiota and the consequent variation in microbial products can promote the development of CRC and compromise the efficacy of its therapy[6].

Since all the factors mentioned are involved in the CRC progression and therapy resistance and considering the great influence of CCSC in several events of this disease, this review aims to analyze the available literature that is focused on the interaction of TME and the intestinal microbiota that favors the development and maintenance of CCSC properties.

COLON CANCER STEM CELLS: FEATURES AND BEHAVIOR

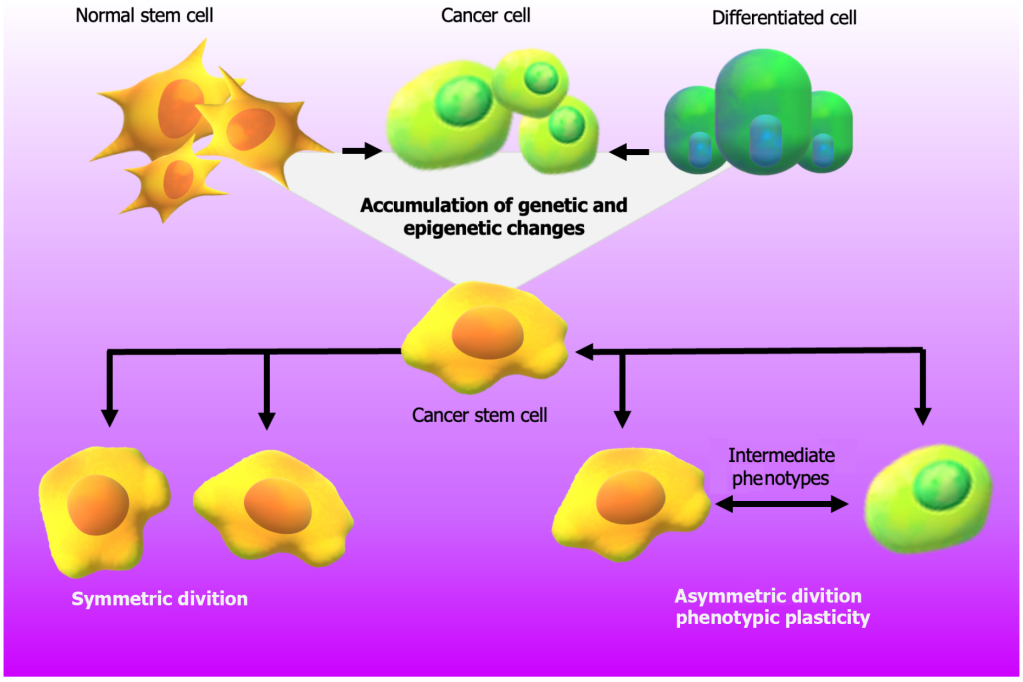
CRC is a heterogeneous pathology that has a variable clinical course and prognosis[7]. The etiology of this disease combines genetic alterations in colorectal epithelial cells with unhealthy lifestyles, such as smoking, alcohol consumption and poor nutritional habits[8,9]. In addition, it has been seen that sex, age, family history of CRC and the persistence of inflammatory processes or infectious agents in the intestinal tract, can be also considered risk factors[5,9-12]. In all these cases, the synergy among genetic mutations, epigenetic alterations and the influence of the TME and gut microorganisms promotes the acquisition of molecular and phenotypic features that allow tumor progression[5,6,11,13,14]. Therefore, within the tumor niche, cells present great heterogeneity but are still strictly organized. In the last 20 years, the focus has been on the study of cancer stem cells (CSC) derived from colorectal tissue (CCSC),

a subpopulation of cells that have a substantial tumorigenic capacity and maintain intestinal tumor growth[15]. CSC are responsible for resistance to multiple drugs maintaining a state of undifferentiation and slow cell division and also favoring the efficiency of desoxyribonucleic acid (DNA) damage repair mechanism[16]. Besides, they have similar features to normal stem cells, such as self-renewal, multipotency, cell cycle arrest, quiescence, and reversibility from their resting state[17,18]. As shown in Figure 1, the ability of CSC to maintain their population response to symmetric/asymmetric division, resulted in the first situation in two identical daughter stem cells and, in the second situation in two distinct cells with or without CSC properties[19,20]. In addition to the division theory, CSC undergo a bidirectional conversion process between stem and non-stem phenotype[20]. Although initially a hierarchical model has been established, in which CSC are the initiators of a monoclonal developmental hierarchy, emerging data highlight the concept of phenotypic plasticity of CSC. This new theory is supported by a dynamic state of interconversion between CSC and non-CSC that can be driven by the TME[21-23]. As the reader can see in Figure 1, during this phenomenon, cells can easily exchange their status within the tumor transforming from CSC to intermediate phenotypes to stemless states and vice versa[15,18,22,24]. Therefore, based on the data provided by the literature and shown in Figure 1, it can be concluded that any cell type is capable of initiating and promoting cancer development[24]. This model contributes with new concepts to the classical theory of the origin/behavior of CSC that highlight the importance of taking into account the study of phenotypic plasticity and the reversible state of this type of cells and that support the criterion that cancer cells with or without stem characteristics must be eradicated for successful therapy.

CCSC constitute about 2% of the cell population in the tumor nest and this percentage can be higher with tumor progression, particularly after chemotherapy or radiotherapy treatments[17,18,25]. Since an increase in the proportion of this cell subtype is an indicator of poor prognosis, in the last decades the identification and targeting of CCSC have become one of the key topics of study[26]. The recognition of CCSC is possible by the detection of typical phenotypic characteristics such as the expression of surface markers, membrane transporters and enzymes. Some of them are Prominin-1/cluster of differentiation 133 (CD133), a transmembrane glycoprotein that is associated with metastasis, invasiveness and chemoresistance in CRC[18]; cluster of differentiation 44 (CD44) a receptor of hyaluronic acid in extracellular matrix related to the epithelial to mesenchymal transition (EMT) program and poor survival in CRC patients[5,27]; cluster of differentiation 166 (CD166) and cluster of differentiation 24, both adhesion molecules whose expressions are associated with the aforementioned markers, and that contribute to stratify low, intermediate, and high-risk CRC cases[5,28]; leucine rich repeat containing G-protein coupled receptor 5 (LGR5) a key CCSC biomarker that decreases in advanced stages of CRC[20,29] and aldehyde dehydrogenase (ALDH), an intracellular enzyme found in high concentrations in most of CSC participating in self-renewal, differentiation and self-protection[20,30,31]. In addition, the study of the ATP-binding cassette transporter superfamily through Hoechst 33352 dye efflux is also employed to detect CCSC[15,32]. In experimental models, the identification and characterization of CCSC can also be performed by fluorescence-activated cell sorting, selection by cell culture properties, *in vivo* transplantation of cells derived from spheroids or organoids, and lineage tracing techniques with labeled CCSC[22]. The above mentioned markers are hallmarks of CCSC and are involved in CRC pharmacotherapy and pathophysiology[33,34], but can also be present in enterocytes and cells of other tissues[20]. Hence, to increase the detection sensitivity and specificity, it is essential to combine the analysis of different biomarkers with CCSC isolation techniques.

Another substantial aspect to consider in the study of CSC is their association with other cellular processes such as EMT, autophagy and the response to cellular stress[15]. In particular, EMT is a physiological process that is also involved in tumor progression. The activation of this program reduces intercellular adhesion and causes epithelial cells to acquire mesenchymal properties that increase the invasiveness and migration of tumor cells[35]. Several studies have reported a link between EMT and the acquisition of CCSC characteristics in both, *in vitro* and *in vivo* assays[35-38]. These investigations show that transcription factors and signaling pathways that are altered in the EMT program are also deregulated in CSC, generating this subpopulation to exhibit phenotypes like EMT[39]. However, recent evidence indicates that EMT may not be necessary to acquire CSC properties. Then, although these processes can go along with each other, they can also happen through independent paths[15]. One of the tumor events that is known to be related to EMT and CSC is the high metabolic demand of TME and the existence of a tortuous vasculature that promotes a hypoxic environment. This phenomenon induces the release of factors such as hypoxia-inducible factor 1α (HIF-1α) that promotes not only EMT but also autophagy associated with CSC. In CRC it was demonstrated that blocking this factor with the consequent inhibition of autophagy reduces cell proliferation and the acquisition of stem-like characteristics[40].

Another cause that has been reported that promotes a stem-like phenotype on several types of tumor cells is the cellular imbalance derived from oxidative stress[15]. In breast and lung cancer cell lines, studies demonstrate that oxidative stress upregulates the CSC marker SRY-box transcription factor 2 (Sox2) activity, and stem-like properties[41,42]. However, in CRC cells it was shown that the reduction of intracellular reactive oxygen species inhibits the formation of CRC stem-like cells[43]. Since this type of cellular stress is considered potentially cytotoxic, more studies are necessary to know the mechanisms by which it has a positive effect on the development of CCSC[15].



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Figure 1 Theory-based models of cancer stem cell. The ability of cancer stem cells (CSC) to maintain their population response to symmetric/asymmetric division, resulting in the first situation in two identical stem cells daughters and in the second situation in two distinct cells with or without CSC properties. In addition to the division theory, CSC undergo a bidirectional conversion process between stem and non-stem phenotype. During this phenomenon, cells can exchange their status within the tumor transforming from CSC to intermediate phenotypes to stemless states and vice versa. Also differentiated cells, normal stem cells or cancer cells through the accumulation of genetic and epigenetic changes are capable of initiating and promoting cancer development. These general theories are applicable to colon CSC.

Furthermore, it is important to note that like all processes and phenomena related to tumorigenesis and malignant progression, CCSC and their features are modulated by the aberrant activation of various signaling pathways. Wnt, NOTCH, hedgehog (HH), and transforming growth factor-β (TGF-β) are important cascades that are usually misregulated in CCSC and play a central role in the therapy resistance of these cells[5,44].

Thus, understanding CCSC features and all the events and factors associated with cell plasticity constitute a fundamental tool for the development of new target therapeutic strategies.

INFLUENCE OF THE TME ON CCSC FEATURES

It has been reported that multiple links exist between inflammatory processes and stemness in CRC[2]. In this context, the role of the tumor stroma is crucial. The TME in CRC is a physical shelter for CSC[5] composed of biomolecules from the extracellular matrix, an aberrant vasculature and multiple stromal and immune cell types. These cells include mesenchymal stem cells, cancer-associated fibroblasts (CAFs), endothelial cells (ECs), pericytes, and tumor infiltrating immune cells which comprehend: Macrophages, neutrophils, natural killer cells, Treg cells and cytotoxic T lymphocytes[2,4]. The interaction between CRC cells and the different types of cellular and non-cellular elements of TME involves complex and dynamic crosstalk by paracrine signaling[22]. Therefore, self-renewal, differentiation and properties of CRC cells and CCSC are modified by factors released by the surrounding stroma[1]. These factors are cytokines, growth factors and small nucleic acids, which have different mechanisms of action. Next, we will discuss those derived from TME that modulate CCSC properties and that are summarized in Table 1.

Cytokines have been shown to play a key role in CRC stemness. It was reported that TME-derived factors with a pro-inflammatory action such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β foster EMT phenotype and stem cell proliferation in human colon cancer cells[45,46]. Besides, it is known that CAFs, one of the most studied cells in the TME, produce IL-6, which promotes the expression of CCSC markers such as ALDH1 and LGR5[1,47].

The acquisition of a stem-like phenotype is also influenced by the expression and secretion of growth factors[48,49]. It was demonstrated that the epidermal growth factor and the insulin-like growth factor regulate and promote CCSC growth[50]. Moreover, Muñoz Galván *et al*[49] have proved that the treatment of CRC derived cells with hepatocyte growth factor (HGF) and/or macrophage migration

Table 1 Tumor microenvironment factors associated with stemness in colorectal cancer

TME factor	Action	Ref.
Growth/inducible factors		
Epidermal growth factor	Regulates and promotes CCSC growth	[50]
Insulin-like growth factor	Regulates and promotes CCSC growth	[50]
TGF-β	Participates in the initiation of the EMT, invasion, metastasis and initiation of angiogenesis associated to CCSC	[13,29,50]
Bone mophogenetic protein 4	Induces differentiation and decreases the tumorigenic potential of CCSC	[16,60,63]
Bone mophogenic protein 2	Stimulates the differentiation of CCSC inducting autophagic degradation of β-catenin	[44,63]
Hepatocyte growth factor	Activates Wnt signaling and the clonogenicity from CCSC	[53,54]
Macrophage migration inhibitory factor	Increases CCSC properties	[49]
Vascular endothelial growth factor	Promotes growth, epithelial to mesenchymal transition and stemness	[50,51]
Platelet derived growth factor	Promotes growth, epithelial to mesenchymal transition and stemness	[50]
Osteopontin	Regulates EMT and participates in the activation of the Wnt/β-catenin signaling pathway, promoting stemness	[4,156]
HIF-1A	Activates Wnt/β-catenin pathway inducing self-renewal of CCSC. Promotes survival and maintenance of CCSC	[40,157]
Citokines/immune associated proteins		
IL-1β	Modulates the expression of CCSC markers	[158]
IL-4	Facilitates the communication of CCSC with stromal cell, maintains their properties and evades the immune system	[5,44]
IL-6	Promotes the expression of the CCSC markers, ALDH1 and LGR5	[1,47]
IL-8	Induces stemness and EMT	[50,159]
IL-17A	Promotes invasiveness and self-renewal and increases CCSC properties	[12]
IL-22	Promotes invasiveness and self-renewal and increases CCSC properties	[12]
IL-33	Induces the expression of core stem cell genes in CRC-derived cells	[160]
Chemokine (C-C motif) ligand 2	Promotes CCSC properties	[4,49]
Tumor necrosis factor- α	Modulates CCSC features and induces cell death	[158,161]
Parathyroid hormone related-protein	Activates Wnt/β-catenin pathway and promotes events related to stemness	[162-164]
Non-coding RNA		
miR-135 a/b and miR-17	Promote stemness through the activation of Wnt/β-catenin signaling	[157]
miR-34 and miR-93	Inhibit stemness	[157]
miR-92a-3p	Promotes Wnt signaling activation and consequently the expression of β-catenin target genes related to stemness, the EMT program, and chemoresistance	[165]
miR-20a and miR-106 a/b	Repress TGF-β activity and stemness	[157]
miR-146 and Let-7	Affect stem cell fate or proliferation, activation of several stemness markers in a colon cancer cell line	[157]
miR-221/222 and miR-21	Induce the development and maintenance of CCSC	[157]
miR-21	Promotes the activation of the Wnt/β-catenin signaling pathway and increases the population of CCSC	[157]
miR-145	Represses miR-21 and its expression inversely correlates with that of CCSC markers	[157,166]
miR-137	Suppresses CCSC tumorigenicity	[167]
miR-147	Decreases the expression of CCSC markers	
miR-200, miR-203, miR-141 and miR-429	Regulate CCSC through negative modulation of EMT and self-renewal	[157]

lncRNA H19	Promotes CCSC phenotype and drug resistance	[168]
Signaling pathway ligands		
Wnt ligands	Increase CCSC characteristics and enhances tumor-initiating potential	[5,157]
Delta like canonical Notch ligand 4	Participates on CSC maintenance	[44]
Jagged1	Participates on CSC maintenance	[66]
SHH	Promotes CCSC survival, self-renewal and drug resistance	[67,68]
Enzymes		
Phospholipase D2	Promotes CRC stemness	[4,49]
Extra-cellular matrix components		
Tenascin, fibronectin, collagen type I, secreted protein acidic and rich in cysteine, galectin	Contribute to stemness and CCSC activities	[1]

EMT: Epithelial to mesenchymal transition; CCSC: Colorectal cancer stem cells; CRC: Colorectal cancer; lncRNA: Long non-coding ribonucleic acid; miR: Micro ribonucleic acid; SHH: Sonic Hedgehog protein; TGF-β: Transforming growth factor beta; IL: Interleukin.

inhibitory factor increases the number and size of colonospheras and significantly enhances the expression of putative markers like CD133[49].

Proangiogenic factors like vascular endothelial growth factor (VEGF) and platelet derived growth factor are also implicated in promoting growth and metastasis, both processes directly related to stemness[50]. Furthermore, it was demonstrated that clusters of ECs improve the survival of CCSC and promote their spread[51].

As it is known, all these TME factors modulate the activation of different signaling pathways, altering gene expression and thus modifying the molecular and phenotypic profile of tumor cells[5,44,50]. Wnt signaling is a key stem cell pathway involved in the maintenance of the CCSC and the TME[13,52]. One decade ago, Vermeulen *et al*[53] observed that high activity of the Wnt signaling was associated with CCSC features. Furthermore, this activity was mainly observed near fibroblasts in the tumor niche. Vermeulen *et al*[53] then demonstrated that HGF derived from CAFs activates Wnt signaling and the clonogenicity from CCSC[53]. This research had a great impact on the study of CSC and recently, Essex and collaborators replicated these studies and obtained similar results. They found that TME regulates the activation of the Wnt signaling pathway, increases CCSC characteristics and enhances tumor-initiating potential[54]. Regarding this, it is known that several Wnt ligands are secreted mostly by CAFs[53-56]. Moreover, other TME factors participate in the activation of the Wnt/β-catenin pathway (Table 1).

Some ligands from other signaling pathways are also related to stem cell phenotype. TGF-β is a growth factor that belongs to a superfamily of molecules including inhibins and bone morphogenetic proteins (BMP)[13]. It has the ability to promote or suppress tumor development depending on the interactions that take place in the TME[57]. As a pro-tumor factor, TGF-β regulates immune responses and participates in many neoplastic events such as proliferation, EMT and stemness[13]. TGF-β signaling pathway mutations and CCSC are linked[58] and in accordance with this, Zhou *et al*[29] found an association between TGF-β signaling and the expression of LGR5 biomarker in CRC[29]. Even more, Gu *et al*[59] have recently demonstrated that the expression of genes related to CCSC features like the carcinoembryonic antigen-related cell adhesion molecule alters TGF-β signaling and promotes CRC[59]. Some other members from the TGF-β family, like bone morphogenetic protein 4 and bone morphogenetic protein 2 (BMP4 and BMP2, respectively), have the capacity to induce CCSC differentiation and increase the response to standard chemotherapy[16,60-62]. Besides, the modulation of the BMP4 pathway by hormones like triiodothyronine was reported in CCSC, decreasing its tumorigenic potential [44,63,64]. This result suggests that CCSC features are modulated not only by local molecules from the TME but also by endocrine factors[44].

Notch signaling is also associated with the expression of CSC features in CRC cells[16,65]. In fact, it was reported that delta like canonical notch ligand 4 and jagged 1, both notch ligands, are overexpressed in this type of tumor providing essential signals for CCSC maintenance[44,66]. Moreover, since HH signaling is implicated in CRC development[20], in the last years several investigations were conducted on the association between this pathway and CCSC properties. Regan *et al*[67], have shown that the activation of the non-canonical HH pathway is required for CCSC survival and depends on sonic hedgehog protein (SHH) ligand[67]. Recently, it has been also observed that the modulation of HH-related proteins expressions by non-coding ribonucleic acids (ncRNAs) impacts on CCSC self-renewal capacity and drug resistance[68]. In line with this, Skoda and collaborators showed that treatment with HH pathway inhibitors such as vismodegib and sonidegib weakens the ability of CCSC [69]. Since no significant differences have been found in clinical trials[70], more studies are needed to determine the effects of the inhibition of this pathway in CRC patients.

Besides the aberrant activation of several signaling pathways, hypoxia is known as a hallmark of CCSC and TME interaction[5]. This is a condition in the tumor niche whose main cause is the poor vasculature associated with the tumor and the upregulation of HIF-1α, a factor released mainly by ECs [40,71,72]. This condition activates Wnt/β-catenin pathway inducing self-renewal and maintenance of CCSC[50,73]. Also, HIF-1α promotes cancer cell proliferation and CCSC survival[40].

Furthermore, short ncRNAs like microRNAs (miRs) and long ncRNAs are secreted not only by tumor cells but also by stromal cells in the TME[4]. In the last decades, the study of ncRNAs has gained importance in CRC. In the framework of factors and signaling pathways related to CCSC biology, these small nucleic acids have a key role[74]. miRs related to stemness in CRC are exposed in Table 1.

As previously mentioned, the interaction between CRC cells and their TME also involves non-cellular elements. Colonic stromal cells mediate the remodeling of the extra-cellular matrix favoring the healing or progress of the disease[75]. Recently, it has been demonstrated, by lineage tracing, that components of the extra-cellular matrix regulate dormancy in CCSC[76]. Tenascin, fibronectin, collagen type I, secreted protein acidic and rich in cysteine (SPARC), galectin and some other components of the tumor matrix are associated with stemness and CCSC activities[1].

Finally, another important concept to consider in the tumor nest is that CCSC also release various factors and cytokines that enable them to communicate with stromal cells, maintain their properties and evade the immune system, such as IL-4 and the cluster of differentiation 200[5,44].

The aforementioned data (and shown in Table 1) suggest that TME instructs the development, properties, plasticity, maintenance and dissemination of CCSC. In the last decade, the remarkable influence of the stroma on CRC development prompted the postulation of a novel classification of this disease based on its impact on tumor gene expression[5]. This CRC staging contains four consensus molecular subtypes (CMS) plus a group called "unclassified" since their features do not fit into the other CMS. All these subtypes are summarized in Table 2[1,5,77-79]. As the reader can see in this table, the influence of TME determines a low or high degree of immune and inflammatory response depending on the CMS, highlighting the importance of factors from TME in the distinctive characteristics of each CRC subtype. Taking into account that the mentioned inflammatory/immune process (that is relevant for CRC classification) can be influenced by the intestinal microorganisms, next we will discuss the interactions of this microbiota with tumor cells and their microenvironment that modulate the behavior and characteristics of CCSC since it is the focus of this review.

ESTABLISHED DYNAMICS BETWEEN THE GUT MICROBIOTA, THE TME AND CCSC

As we mentioned in this work, the inflammatory microenvironment contributes to promoting CRC initiation and progression. However, the role of the cell types involved in this process, including intestinal microorganisms, has not been completely understood yet.

The human microbiome, a concept that is mentioned throughout this section, represents microorganisms with their genetic elements and the interactions arising with the environment in which they are found[80]. Advances in the characterization of this human microbiome have led to the consideration that the role of the microbiota in metabolic functions and maintenance of homeostasis is more important than previously believed. Currently, the human is considered as a holobiont organism inhabited by millions of microorganisms including bacteria, archaea and fungi[81]. The gut microbiota is a complex ecosystem that contains more than 500 bacteria species involved in physiological processes like immune regulation and maintenance of human health[6] and its composition relies fundamentally on diet and lifestyle[74].

In physiological conditions, stromal and immune cells from the gut mucosa interact with this ecosystem to maintain intestinal equilibrium[82]. Cells from the immune system recognize antigens from foreign cells and generate memory and effector cells, which control or avoid the generation of diseases[82].

It has been observed that sustained shifts in this ecosystem, known as intestinal dysbiosis, have unfavorable repercussions on health[74,83]. In this sense, the presence of harmful microorganisms (“drivers”) could induce changes in the intestinal mucosa and favor the colonization by opportunistic bacteria (“passengers”)[84]. This model is known as driver-passenger[84] and could involve changes in the immune system allowing the advance of the damage in the intestinal epithelium tissue[85,86]. This imbalance of the local microbiota promotes the restructuring of the intestinal environment and alters the immune status of the host contributing to the appearance of malignant cells and a favorable niche for tumor development, invasion and metastasis[85,87,88]. The mechanisms of these microorganisms that influence directly the immune system are different and involve the synthesis of immunomodulatory compounds and metabolites, like short-chain fatty acids (SCFAs), polyamines and other fermentation products[89,90]. Moreover, it is known that the intratumoral composition of microorganisms affects T-cell-mediated cytotoxicity and anti-tumor immune surveillance[91]. The unfavorable changes in the intestinal microbiota can promote a pro-inflammatory environment and impair anti-cancer immunity [91]. In this context, cells from TME secrete factors like interferon-γ, TGF-β, IL-6, IL-8, CXCL1 and TNF-α,and favoring the differentiation of T helper 17 Lymphocytes to develop an adaptive immune

Table 2 Consensus molecular subtypes of colorectal cancer

	CMS1-immune (14%)	CMS2-canonical (37%)	CMS3-metabolic (13%)	CMS4-mesenchymal (23%)	Unclassified (13%)
General features	Hypermutated	Epithelial	Epithelial	TGF-β activation. Angiogenesis	Mixed phenotype of multiple CMS
	Microsatellite unstable	WNT and MYC signaling activation	Metabolic dysregulation	Upregulation of EMT	
Mutations	*BRAF, MSH6, RNF43, ATM, TGFBr2, PTEN*	*APC, KRAS, TP53, PIK3CA*	*APC, KRAS, TP53, PIK3CA*	*APC, KRAS, TP53, PIK3CA*	
TME	Decrease of CAFs	Decrease of CAFs	Decrease of CAFs	Increase of CAFs; Immunosuppressive signature	
	High immune and inflammatory signature	Low immune and inflammatory signature	Low immune and inflammatory signature		

This Table is based on Islas *et al*[1], 2022; Fidelle *et al*[79], 2020; Trinh *et al*[169], 2018; Becht *et al*[78], 2016; Guinney *et al*[77], 2015. *APC: Adenomatous polyposis coli gene; ATM: Ataxia telangiectasia mutated gene; BRAF: Serine/threonine-protein kinase B-raf gene*; CAFs: Cancer associated fibroblasts; CMS: Consensus molecular subtype; EMT, Epithelial to mesenchymal transition; *KRAS: Ki-ras2 kirsten rat sarcoma viral oncogene homolog gene; MSH6: MutS homolog 6 gene; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene; PTEN: Phosphatase and tensin homolog gene; RNF43: Ring finger protein 43 gene*; TGF-β: Transforming growth factor beta; *TGFBr2: Transforming growth factor beta receptor 2 gene*; TME: Tumor microenvironment; *TP53: Transformation-related protein 53 gene.*

response that contributes to immune-prone carcinogenesis and CRC development[79,87,92]. In this regard, increasing evidence suggests that gut microorganisms condition CRC patients response to immunotherapy, because they alter the expression of elements such as anti-programmed cell death protein 1 (PD-1) and its ligand (PD-L1) and anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4)[91,93]. PD-1/PD-L1 has been highly studied in the last years on the tumor-microbiome-immune axis[91]; in fact, several investigations provide evidence that PD-L1 is overexpressed on different tumor cells and stromal immune cells, allowing tumors to evade attacks *via* T-cell infiltration[91,94,95].

The increased expression of PD-L1 in CRC cells both *in vitro* and *in vivo* is a mechanism involved in the influence of certain pathogenic bacteria associated with an immunosuppressive TME[96]. In contrast, bacteria associated with healthy microbiota improve the efficacy of anti-PD-L1 therapy by enhancing the accumulation of cytotoxic T cells in the TME[97]. This suggests that TME reprogramming through manipulation of the microbiota can modulate the response to immunotherapies in CRC[98]. Concerning all this information, CRC could be considered as a bacterial-induced disease and disturbance in microbiota could be potentially useful as diagnostic biomarker, indicator of risk and predictor of response to therapies for this type of cancer[74,88].

On the other hand, CRC modifies the local metabolic environment[99]. In this context, it is important to mention that metabolites and factors derived from CRC cells and TME cells such as spermidine, L-valine, L-lysine or stearic acid confer an advantage for the growth and development of certain bacterial species, conditioning changes in the intestinal microbiota[99]. Although different factors produce changes in gut microbiota, recently it has been seen that the shift in the metabolome of tumor cells and TME cells is a key aspect in this event[86,99,100]. Thus, TME can be the consequence or the cause of intestinal dysbiosis.

The gut microorganisms cited below in this section are described in the available literature due to their role in CCSC development and maintenance. They are also summarized in Table 3.

Regarding CSC properties, some pathogenic bacteria such as *Helicobacter pylori* and *Porphyromonas gingivalis* can promote the expression of markers associated with stemness such as CD44 and CD133 in gastrointestinal tumors[101,102]. This association between the presence of certain bacteria genera in the gut and the expression of CSC markers has led to the study of the effects of microorganisms shifts and bacterial metabolites on CRC. Several models of tumorigenesis induced by bacteria have been proposed, suggesting how the interactions of host-microorganism promote the development and progression of this type of cancer[101]. In fact, it is known that the metabolites from the intestinal microbiota have the potential to act as tumorigenic factors. However, others can act as anti-tumorigenic factors since many of these microbiota-derived products are capable of inhibiting CRC progression[103]. Kim *et al*[43] have demonstrated that ursodeoxycholic acid, a secondary bile acid produced by Clostridium species, including *Clostridium absonum* and *Clostridium baratii*, regulates the oxidative stress suppressing CCSC growth and CRC cells proliferation[43]. Moreover, it has been observed that niacin, a product of the metabolism of some intestinal bacteria, such as *Lactobacillus acidophilus*, has different effects on CCSC. Depending on the dose, this vitamin can promote proliferation or death in this cell subtype[104]. Additionally, bowel microorganisms produce SCFAs such as butyrate, propionate and acetate[92]. It has been reported that these SCFAs favor beneficial bacteria proliferation and stimulate regulatory T cells to reduce inflammatory mediators, regulating immune response[105]. Butyrate participates in epithelial integrity maintenance and has antitumor effects. Several investigations show that in CRC, this product

Table 3 Microorganisms present in the intestinal mucosa associated with stemness in colorectal cancer

Microorganism	Action	Ref.
Bacterioides dorei, Bacterioides vulgatum, Parabacterioides distasonis, Lachnoclostridium sp., and Mordavella sp	Inhibit the action of factors related to CCSC phenotype. Inhibit CRC development and progression	[59, 114]
Bacterioides fragilis	Releases an enterotoxin that promotes immune TME cells activation with secretion of factors related to CCSC	[118]
Citrobacter rodentium	Protects the inflammatory CCSC niche	[121]
Clostridium septicum	Contributes to CRC development and to the activation of signaling pathways associated with CCSC	[59]
Enterococcus faecalis	Induces the expression of TGF-β, thereby activating signaling pathways associated with CCSC. Activates Wnt/β-catenin signaling and pluripotent transcription factors associated with CCSC	[113, 115]
Escherichia coli	Upregulates the expression of CCSC-associated genes. Releases genotoxin colibactin which induces the production of growth factors related to CCSC	[112, 117,79]
Fusobacterium nucleatum	Stimulates the secretion of immune factors related to CCSC	[79]
Helicobacter pylori	Promotes the expression of markers associated with stemness	[101, 102]
Lactobacillus acidophilus	Promotes proliferation or death in CCSC depending on dose	[104]
Porphyromonas gingivalis	Promotes the expression of markers associated with stemness	[101, 102]
Shigella, and Citrobacter	Upregulate the expression of CCSC-associated genes	[112]

CCSC: Colorectal cancer stem cells; CRC: Colorectal cancer; TGF-β: Transforming growth factor beta; TME: Tumor microenvironment.

inhibits events associated with CSC such as invasion and proliferation[6]. Interestingly, butyrate inhibits cell proliferation to a greater extent in CRC derived cells than in non-cancerous cells[92]. Although butyrate was reported as an anti-tumor and chemopreventive agent[92,106], other studies have shown that it has variable outcomes on CCSC[92]. So, more investigations are necessary to determine the mechanistic action of this type of fatty acid. Experiments with other SCFAs like acetate and propionate with similar results demonstrated that these acids have opposing effects[6,107]. Besides that, a large number of microbial products such as deoxycholic acid, lithocholic acid chenodeoxycholic acid, taurochenodeoxycholic acid and others, are associated with the promotion of gastrointestinal tumors including CRC[6,108]. Recent studies have found that in CRC patients the microbial composition of the colonic crypt is different from that of the intestinal lumen. In the environment of the crypt of the colorectal tumor, groups such as *Proteobacteria* and anaerobes, such as *Acinetobacter, Stenotrophomonas* and *Delftia* were found[109]. Therefore, specific microorganisms could have a role in the maintenance of CCSC, located in the crypt, through the production of specific metabolites[110]. However, more studies are needed in this field since the molecular mechanisms underlying the effects of intestinal microbial products on CCSC have not yet been fully elucidated.

Currently, the study of mechanisms involved in the communication between the microbiota, the tumor cells and their microenvironment has gained impact on CRC. One reported mechanism for this interaction is through pattern recognition receptors located on intestinal epithelial cells that have the ability to detect distinctive microbial macromolecular ligands called pathogen-associated molecular patterns such as lipopolysaccharides and peptidoglycans[111]. Congruently, a recent work documented an altered function of CSC in a CRC murine model due to intruding bacteria like *Escherichia, Shigella*, and *Citrobacter*. This effect results in the activation of a toll-like receptor (TLR), a class of pattern recognition receptors, and the consequent upregulation of stem cell-associated genes such as *Cd44v6* and *Lgr5*[110,112]. In line with this, the microorganisms are capable of activating several signaling pathways in tumor cells and/or TME cells inducing the secretion of factors associated with CCSC features. In this context, it has been observed in a murine model that microorganisms such as *Enterococcus faecalis* cause colitis after infection and induce expression of TGF-β, thereby activating the Smad signaling pathway[113]. A recent study has demonstrated an inverse correlation between the expression of molecules associated with TGF-β signaling pathway and stem cells- related genes in CRC. Moreover, the authors of this work have compared feces from mice with defects in TGF-β signaling with feces from wild-type (WT) mice, and have shown that the first ones had increased bacterial species associated with the development and progression of CRC, such as *Clostridium septicum*, and diminished amounts of favorable microorganisms including *Bacteroides vulgatus* and *Parabacteroides distasonis*[59]. Similar results were obtained by Wang and collaborators who showed that the amounts of beneficial species (*Bacterioides dorei*, *Lachnoclostridium sp*., and *Mordavella sp*.) are recovered in WT mice but not in those

with mutated TGF-β signaling after chemotherapy treatment[114]. These investigations demonstrate the close relationship between the microbiota, the production and release of TGF-β and CCSC in the tumor.

Concerning other signaling pathways, Wang *et al*[115] have shown that *Enterococcus faecalis* are capable of polarizing macrophages by activating Wnt/β-catenin signaling and pluripotent transcription factors associated with the dedifferentiation, reprogramming and development of CCSC such as cellular myelocytomatosis oncogene, Kruppel-like factor 4, octamer-binding transcription factor 4 (Oct4), and Sox2[115]. These events respond to the microbiota-induced bystander effect theory based on the fact that macrophages induce genetic mutations and chromosomal instability in intestinal cells[116].

Other signaling pathways associated with pro-inflammatory and growth factors can be activated in response to bacterial products. For instance, the unbalance in the amount of the gut bacteria *Escherichia coli*, correlates with CRC progression by producing the genotoxin colibactin[79]. This toxin accelerates tumor progression and involves the production of growth factors related to CCSC, such as the HGF and the consequent activation of its signaling pathway[79,117]. Also, the enterotoxin produced by *Bacteroides fragilis* promotes immune TME cells activation with the secretion of IL-17 which favors CCSC properties [118]. Furthermore, as we have previously mentioned, gut microorganisms shape the immune environment promoting tumor evolution and CCSC features. For example, *Fusobacterium nucleatum* stimulates IL-8 secretion by TME cells and the inhibition of T and NK cell functions[79]. This bacteria has been deeply studied, since clinical analysis of specimens from CRC patients showed that the levels of *F. nucleatum* are significantly higher in neoplastic tissues than in adjacent normal tissues, and correlate with tumor invasion and metastasis[119]. These results support the role of *F. nucleatum* in the regulation of CCSC plasticity and EMT[101]. Also, it is known that *F. nucleatum* and other microorganisms like Epstein–Barr virus are capable of incorporating human ncRNAs favoring microbial growth [74]. In this regard, Tarallo *et al*[120] found a human and microbial ncRNA signature in CRC in which many miRs associated with CSC features, are overexpressed including miR-21 and miR-200[74,120]. A recent study conducted by Wang showed that *Citrobacter rodentium* infection induces the inhibition of miR-34a, which protects the inflammatory CCSC niche[121]. These investigations suggest a close relationship between the intestinal microbiota and the regulation of ncRNAs involved in CCSC properties.

Finally, not only the shift in the number of microorganisms is responsible for stemness and CRC progression, but the interaction and collaboration between several types of bacteria in biofilm communities also participate in bowel inflammation and CRC. It was demonstrated that biofilms correlate with an increase in IL-6 secretion by TME cells playing a key role in proliferation, cell transformation and stemness[79].

The data in this section demonstrate a close interrelationship between the gut microbiota, the TME, and CCSC. This information highlights the relevance of further investigating the intestinal microbiota switch in patients with CRC and the associated mechanisms that lead to TME changes and promote stemness.

THERAPEUTIC TARGETING OF TME AND THE GUT MICROBIOTA: A KEY TOOL TO MODULATE STEMNESS IN CRC

Standard chemotherapeutic approaches for CRC are based on attacking the replicative mechanisms of tumor cells to induce tumor regression. However, considering CSC properties, this subpopulation usually results unharmed by the treatment because they present a low division rate as well as a great capacity to correct DNA defects[122]. This entails therapy resistance of CSC and the subsequent treatment failure and disease progression. It is interesting to note that in CRC, CSC represent around 2.5% of neoplastic cells but due to their phenotypic plasticity, they constitute a dynamic population[123, 124]. This fact, together with the lack of response to therapies, highlights the need of new clinical strategies targeting CCSC[125].

As we explain throughout this review, the influence of the TME and the intestinal microorganisms on CSC properties makes these factors a promising tool in therapy. Many therapeutic agents are capable of inhibiting those events associated with the maintenance of CCSC. For instance, Apatinib napabucasin, Bigelovin, Wogonin and Metformin are drugs whose mechanisms are associated with the inhibition of EMT or angiogenesis in CRC[1]. Moreover, it has been demonstrated that therapeutic agents such as Genistein cause the inhibition of CSC characteristics by glioma-associated oncogene1 signaling pathway [126]. Targeting the activation of those signaling pathways associated with CCSC can also be considered as a mechanism to reduce stemness in CRC tumors and thus improve the response to the therapy. LGK974, Foxy-5, PRI-724[127] and DKN-01[126] are agents that act targeting the Wnt/β-catenin pathway. However, the clinical application of most of these drugs is still under study.

The tumor protective niche also could be modified to eradicate CCSC and overcome chemoresistance. As we have mentioned in previous sections, in the TME, immune cells modulate cancer development and progression. For that reason, in the last decade the treatment of patients with immune checkpoint inhibitors such as CTLA-4 and PD-1/PD-L1 has been studied. Even though employing these drugs leads to various systemic and organic complications, immunotherapy may be promising in sorting these

obstacles and could ameliorate the response of CRC patients to the treatment[1,128]. In fact, coadjuvant therapy with FOLFOX (a combination of leucovorin, fluorouracil and oxaliplatin which are first-line chemotherapeutic drugs)[129], and PD-1/PD-L1 inhibitors had an objective response rate of 50% in clinical trials[130]. In addition, in a phase II trial in CRC metastatic patients, immune checkpoints inhibitors like nivolumab and nivolumab-ipilimumab show improvement in patients survival rate[130]. Moreover, monoclonal antibodies against CAFs and antifibrotic drugs were also tested in clinical studies[5]. Another type of antitumor therapy was accomplished through the production of a cell-based vaccine with specific antigens of CCSC[5].

In addition, plenty of compounds were designed in the last decade to target CCSC signaling pathways[5]. These strategies include the inhibition of HH signaling components, NOTCH pathway inhibitors, anti-angiogenic agents and Wnt ligand blockers. All these drugs are undergoing clinical trials [129]. Despite being an encouraging strategy, it still has limitations like the inhibition of signaling pathways involved in physiologic processes.

In the last years, the particularities exhibited by extracellular vesicles (EVs) have led researchers to consider them as a therapeutic delivery strategy of great value in CRC and other types of tumors. Within the different types of EVs are the exosomes, which are secreted by a variety of cells. These vesicles carry out the molecular content of donor cells and enable cellular communication over short and long distances. These EVs are loaded with coding nucleic acids, ncARNs and bioactive proteins which determine their functions. Exosomes can target a specific tissue and internalize in a cell type by the recognition of surface ligands/receptors[131]. In this regard, Han *et al*[132] investigated the delivery of human cord blood-derived MSC exosomes loaded with miRs as CRC targeted therapy. The results showed an inhibition of tumor growth *in vitro* and *in vivo*, as well as a selective increase of these ncRNAs in CRC cells[132]. The relation between miRs and CCSC was mentioned in previous sections so their delivery may be strong weapons to confront drug resistance and CCSC maintenance[5]. Circular RNAs are ncRNAs that exhibit cell-type and tissue-specific signatures. There has recently been considerable attention on these ncRNAs as they modulate miRs expression[129]. In CRC, recent studies have focused on their study as biomarkers. However, they have not been applied in patients' therapy yet[133, 134]. Moreover, the importance that these small molecules could have in CRC is unknown[129].

Foods containing biologically active ingredients are termed functional foods or nutraceuticals[135, 136]. In the past years, the influence of diet on CRC development and evolution was demonstrated. A diet with natural products like phytochemicals and nutritional herbs has shown protective effects in overcoming CRC associated dysbiosis[137,138]. Diets enriched in dairy are a major source of products that are known to have a protective effect on CRC development such as, calcium, vitamin D and folate [138]. Sulforaphane, a sulfur-rich compound found in cruciferous vegetables like broccoli, has been documented to diminish CSC markers and improve the chemotherapeutic efficacy of drugs commonly used in CRC treatment such as cisplatin, doxorubicin and fluorouracil[137]. It has been observed that dietary polyphenols like quercetin have similar effects[137,139]. Other polyphenols or flavonoids are known to target ABCG-2 transporters and miRs strictly associated with CCSC[139]. Curcumin is one of several substances present in turmeric plants. It has been demonstrated that this bioactive agent inhibits the activation of several signaling pathways related to CSC characteristics. The treatment with this natural product on a CSC model diminished the expression of CD44 and CD133 markers[137]. Moreover, some other natural products have been observed that interfere with intrinsic CSC pathways, like epigallocatechin-3-gallate (EGCG), resveratrol and genistein[140].

Diet can also manipulate the gut microbiota. Indeed, this is achieved by the administration of probiotics in the diet. As probiotics and their active metabolites can exert immunomodulatory and anti-tumorigenic effects[135], the study of them and their metabolites has gained ground in recent decades. Probiotics are live microorganisms, normally lactic acid bacteria, recognized as safe by the United States Food and Drug Administration[135]. Defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[141], they can improve health by administration along vegetable fibers and other prebiotics stimulating beneficial bacterial growth in the intestine[142].

Probiotics administration can be done by different routes, commonly through functional foods, but also by commercial supplements or vaccines[135,138]. It is known that probiotic oral vaccines promote mucosal immunity that prevents enteric infections and could complement the standard therapy in the patient[143]. Microorganisms administration including probiotics and synbiotics (pharmaceutical preparation that contains probiotics and prebiotics that implies a synergy between both) are a potential resource for prophylaxis and therapy in CRC[138,144]. In addition, the luminal cocktail of microorganisms in the bowel can be modified not only by dietary approaches but also with the use of antibiotics or fecal microbiota transplantation (FMT)[145,146]. In particular, FMT has gained considerable interest in recent years as a strategy to treat different gastrointestinal disorders[147-149]. It consists of introducing a healthy microbial population from a disease-free host into a diseased host that has a dysbiotic community to restore microbial homeostasis[150]. Although there are limited data on the use of FMT in the treatment of CRC, several studies are under development to answer relevant questions such as if CRC can be detected, treated or prevented with this method. Rosshart and collaborators observed that mice treated with this method improved their resistance against colorectal tumorigenesis induced by azoximetane[151]. Besides, it has been seen that FMT in Balb-c mice prevents intestinal damage, and chemotherapy-induced toxicity[152]. Interestingly, the fecal microbiota from CRC patients

has been shown to cause tumors in healthy and germen-free $Apc^{min/+}$ mice through the activation of the Wnt signaling pathway. In these mice, the intestinal barrier is also altered and the presence of pro-inflammatory cytokines is increased[153]. These data reveal that the composition of the microbiota may play a determinant role in TME conditions during tumorigenesis. Nevertheless, the subjacent mechanisms of all these treatments or how they ameliorate the side effects of chemotherapy is not clear yet.

In summary, we need a favorable and efficient clearance of tumor cells, all tumorigenic cells including CCSC and a restructuring of the TME for the complete eradication of CRC. Based on everything described in this review, a specific combination of techniques and therapies for each tumor and patient would be necessary to achieve this goal.

FUTURE PERSPECTIVE

According to the information stated in the previous sections, in CRC occurs an alliance between the TME, intestinal microorganisms and CCSC that favors tumor progression. In this scenario, it is emerging a new query regarding the direct effects of CCSC on gut microbiota. Perhaps the appearance of CCSC by spontaneous mutations favors (through paracrine signals and the release of specific factors) a dysbiotic and pro-inflammatory environment but in this regard, new investigations are necessary to evaluate the regulation of CCSC on CRC microbiota. So, there is great potential in the study of the interrelationship between these three components in the tumor niche, mostly for the development of new therapies aimed at the eradication of CCSC and non-stem cells, the restructuring of the TME and the growth induction of microorganisms that are beneficial to the intestinal mucosa.

Many of the therapies currently in use or under clinical evaluation are associated with systemic toxicity since they do not act on a well-defined target[137]. Therefore, the combination of radiotherapy and chemotherapy has still remained the strategy of choice in CRC[145] and not much attention is paid to nutritional accompaniment. Since the gut microbiota seems to be a pivotal factor in inflammatory disease and CRC development, overcoming therapy resistance could also improve with changes in diet. For this purpose, is crucial the development of foods containing compounds with anti-CCSC activity such as flavonoids but with better bioaccessibility and bioavailability[154]. Moreover, bacteriotherapy is a great opportunity to customize CRC treatment and the following tools that we will mention could be useful in this type of therapy. The modification of patient microbiome tending to resolve dysbiosis through the administration of beneficial bacteria could significantly improve conventional treatment [93]. Even more, considering that some microbial species exhibit tumor targeting specificity, this strategy could ameliorate cytotoxicity in non-tumor cells. Regarding bacterial products, given their low molecular weight and hydrophobicity, they can easily enter tumor tissues and exert their action[155]. These features result in the use of microorganisms with potential preventive or palliative action in CRC currently receiving special attention. In fact, microbe-based therapies, and bacteria-mediated modulatory strategies are studied to be used for the delivery of drugs to the tumor site and to produce anti-cancer vaccines[145,155]. However, the information about the toxins, metabolism of microbial-derived agents and complications from bacteriotherapy is still limited[155]. Thus, placing emphasis on clinical research that allows the use of these new therapies, overcoming the obstacles related to it, will be essential in the coming years.

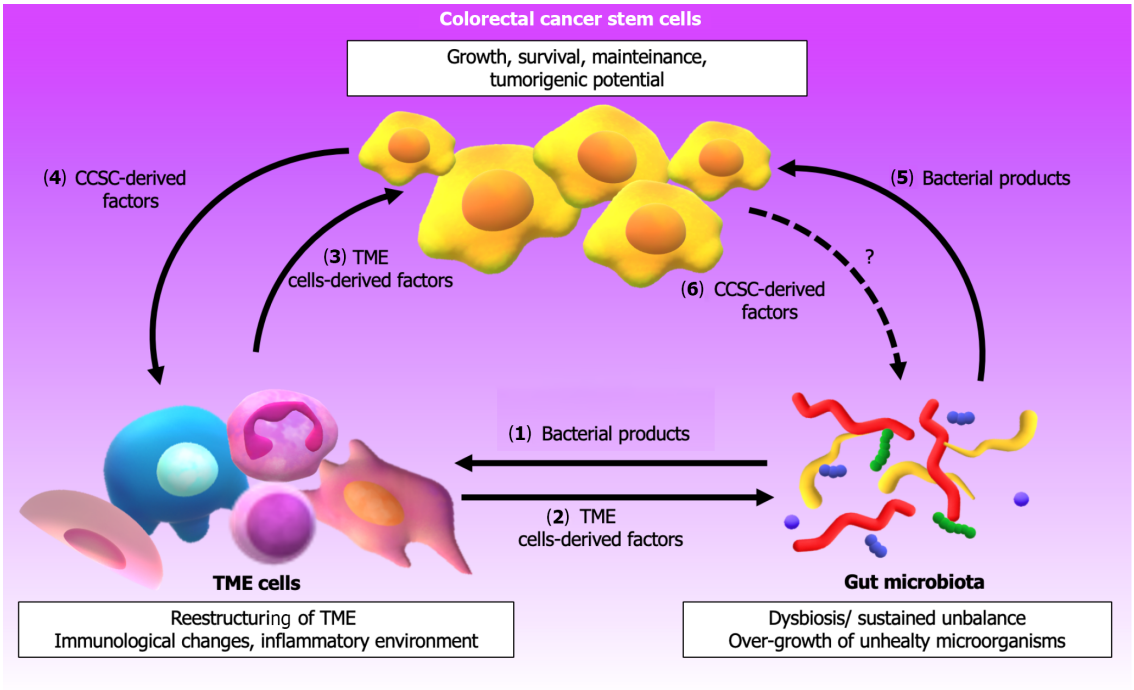
In addition, as we discussed in the previous section, it is also necessary to focus on the restructuring of the TME in favor of improving conventional CRC treatment. Restructuring the extracellular matrix, modulating the immune response with vaccines, antibodies, or inhibitory drugs, employing drugs that induce changes in the secretion profile of TME cells, switching macrophages polarization and inhibiting CAFs and processes like fibrosis and inflammation are some of the potential effective techniques under investigation[1,5,116,128].

The development of vaccines containing CSC-specific antigens is also under investigation[5]. However, since many of the antigens present in this cell subtype are also found in differentiated cells or normal stem cells, this is a challenge to overcome for successful therapy.

So, the combination of conventional therapies with new targeted inhibitors (*e.g.* inhibitors of signaling pathways or molecules derived from TME) plus an appropriate diet that favors beneficial colonic microbiota, as well as the use of targeting methods such as charged nanoparticles or specific bacterial species, could constitute a reliable alternative to fight with CRC chemoresistance and relapses. The use of different *in vitro* and *in vivo* preclinical models of CCSC such as colonospheras, organoids and xenografts, is essential to achieve this goal and bring it to clinical research.

In the near future, the challenge will be the development of selective and combined therapies to promote: (1) CSC eradication; (2) Eradication of cancer cells, owing to their phenotypic plasticity, even in the absence of CSC features; and (3) Reduction of the damage to cells outside the tumor bulk.

In any case, it is clear that the standardization of treatment protocols is not always effective for this disease. It is advisable to resort to a combined and personalized therapy that considers the needs and responses of each patient.



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Figure 2 The interplay among the tumor microenvironment and the gut microbiota influences the stemness of colorectal cancer cells. (1) Gut microorganisms and/or their derived products in a dysbiosis context influence the restructuration of tumor microenvironment (TME), favoring the release of several factors (growth factors, cytokines, non-coding ribonucleic acids and enzymes), immunological changes and an inflammatory environment; (2) The factors released by TME cells impact on intestinal microbiota promoting the growth of unhealthy microorganisms and their sustained unbalance; (3) Moreover, these TME factors can modulate the properties and behavior of colorectal cancer stem cells (CCSC) promoting effects such as their growth, survival, maintenance and tumorigenic potential; (4) In this context, CCSC response expressing factors that enable them to communicate with stromal cells and also influence a TME restructuration; (5) Microorganisms and/or their derived products can directly modulate the features and properties of CCSC, which in response; and (6) Probably affect the intestinal microbiota. All these associated events contribute to colorectal cancer progression. CCSC: Colorectal cancer stem cells; TME: Tumor microenvironment.

CONCLUSION

Figure 2 shows the interplay between the TME and the gut microbiota that influences the properties/behavior of CCSC. Besides, the reader can appreciate that CCSC influence on cells from TME favoring CRC progression but probably also on gut microbiota. The knowledge described in the present review provides data that may promote future research aimed at addressing the complexity of the components in the CRC-associated microenvironment and microbiota. Compounding such complexity, CRC is not an isolated neoplasm, but it's rather emerging as a dynamic pathology whose actors are capable, regrettably, of contributing to evasion mechanisms of the current therapeutic strategies. Although the incidence and mortality from CRC have decreased in recent years, a large number of patients still suffer from relapses due to resistance to treatment. The development of metastases and chemoresistance is undoubtedly one of the greatest challenges in CRC therapy. As we have seen in this work, the properties of the CCSC make this cell subtype have the main responsibility for the recurrences. The shift in the tumor niche and the intestinal microbiota favors the acquisition of CSC characteristics, promoting a worse prognosis of CRC. Although much is currently known about the interrelationship between components of the TME, the microorganisms present in the intestinal mucosa and CCSC, there is still much to be discovered in this field.

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FOOTNOTES

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REVIEW

Delineating the glioblastoma stemness by genes involved in cytoskeletal rearrangements and metabolic alterations

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Abstract

Literature data on glioblastoma ongoingly underline the link between metabolism and cancer stemness, the latter is one responsible for potentiating the resistance to treatment, *inter alia* due to increased invasiveness. In recent years, glioblastoma stemness research has bashfully introduced a key aspect of cytoskeletal rearrangements, whereas the impact of the cytoskeleton on invasiveness is well known. Although non-stem glioblastoma cells are less invasive than glioblastoma stem cells (GSCs), these cells also acquire stemness with greater ease if characterized as invasive cells and not tumor core cells. This suggests that glioblastoma stemness should be further investigated for any phenomena related to the cytoskeleton and metabolism, as they may provide new invasion-related insights. Previously, we proved that interplay between metabolism and cytoskeleton existed in glioblastoma. Despite searching for cytoskeleton-related processes in which the investigated genes might have been involved, not only did we stumble across the relation to metabolism but also reported genes that were found to be implicated in stemness. Thus, dedicated research on these genes in GSCs seems justifiable and might reveal novel directions and/or biomarkers that could be utilized in the future. Herein, we review the previously identified cytoskeleton/metabolism-related genes through the prism of glioblastoma stemness.

Key Words: Glioblastoma; Stemness; Cytoskeleton; Metabolism; Biomarkers; Therapy

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Core Tip: Glioblastoma stemness intensifies the resistance to treatment *via* increased invasiveness. Among the processes crucial for glioblastoma stem cells, metabolism is known to influence invasion. However, the cytoskeleton is currently negligent in glioblastoma stemness research, while it also regulates invasion. Herein, we review the link between stemness and cytoskeleton/metabolism-related genes that we previously identified in glioblastoma. These genes influence stemness *via* numerous biological processes; for some genes, clinical trials are currently ongoing. Others were connected to glioblastoma stemness for the first time. Future glioblastoma-related research should delve into the cytoskeleton since the concept is already encouraging.

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INTRODUCTION

Glioblastoma (GBM) has remained an incurable condition with increasing incidence in many countries [1,2]. Although GBM is less prevalent than breast, colon, or lung cancer, it outperforms other tumors by affecting patients in the prime of their lives and causing them to lose many years of life[3]. The initial intervention in newly diagnosed GBM includes a surgical approach, with post-surgery temozolomide (TMZ) and radiation therapy[4]. Adding tumor-treating electric fields (TTFields) to maintenance TMZ chemotherapy was found to prolong progression-free and overall survival, but is currently limited due to the lack of methods to predict or quantify the efficacy of TTFields (the imaging features associated with treatment response are unclear and there are no predictive neuroimaging markers). Moreover, the treatment device is required to be worn for a predetermined period (typically approximately 75% of the time) or until there is a clinical progression of the disease, which introduces a delay in getting used to the device and makes patients anxious with regard to the intended therapy effect[5]. Strong motivation to predict TTField efficacy in a patient-specific manner was provided[6]. Nevertheless, glioblastoma recurrence is practically inevitable which, combined with a grim prognosis and ineffective treatment, underlines the importance of further research into this deadliest tumor[3,7].

One of the GBM traits that implicate the lack of effective treatment is the heterogeneity that can be explained by both clonal evolution and the presence of stem cells[8]. Stemness refers to the molecular events that underlie the essential characteristics of self-renewal and differentiation into daughter cells [9]. On the cellular level, some processes were indicated as crucial for GBM stemness, namely epigenomic regulation, posttranscriptional regulation, and metabolism[10]. Glioblastoma stemness research in recent years has also bashfully introduced a key aspect of cytoskeletal rearrangements [11, 12] while it has been long time since this machinery is well-known for controlling two processes that influence cancer malignant behavior, *i.e.*, cellular division and invasion[13]. The stemness itself is also responsible for potentiating the resistance to treatment[14,15], *inter alia* due to increased invasiveness [16]. In addition, more recent studies have identified the role of metabolism in GBM invasion[17]. Although non-stem glioblastoma cells are less invasive than GBM stem cells (confirmed by sevenfold reduced cell migration through the Matrigel, or 3.8-times and 6.8-times lower expression of matrix metalloproteinase-14 and -16)[18], the same cells also acquire stemness with greater ease if they are characterized as invasive cells and not tumor core cells[19,20].

The above-mentioned data imply that GBM stemness should be further explored for any phenomena related to the cytoskeleton and metabolism, as they may provide the missing puzzle from the point-of-view of invasion. Moreover, the cytoskeleton and metabolism are related; for instance, the cytoskeleton is involved in carbohydrate metabolism[21] and at the same time the actin and tubulin require energy from nucleotide hydrolysis to maintain structural dynamics[22]. Cytoskeletal rearrangements and metabolic alterations are important not only for GBM cells but also for neuronal and glial progenitors. For example, cytoskeleton dynamics underlie the cellular asymmetry while metabolic reprogramming ensures a transition in energy production from glycolytic to oxidative[23,24]. Nevertheless, it is possible to discriminate normal glial cells from glioblastoma; the cancerous cells present decreased cortical but increased intracellular stiffness, and preferentially metabolized glucose into lactate despite the abundance of oxygen[17,25]. Stiffness and metabolic adaptations can also influence stem cell differentiation[26,27]. Moreover, the cellular cross-talk that utilizes cytoskeleton or metabolites affects physical dynamics and signaling of various cell types including astrocytes, neurons, and oligodendrocytes[28, 29]. In cancers, such cross-talk renders abnormal protrusions or extensions termed as tumor microtubes that contribute to glioma resistance[30]. These structures are rich in cytoskeletal proteins, such as actin and tubulin, and are known to modify energetic metabolism of the receiving cells *via* transport of

mitochondria[31].

Our previous research has proved that interplay between metabolic alterations and cytoskeletal rearrangements exists in GBM[32]. Of genes described below in the present review (some previously identified genes were not included if their implication in stemness was not found in the literature) (Supplementary Table 1)[33-37], the example of a relationship between metabolism and cytoskeleton can be visualized (Figure 1) based on the literature on methylenetetrahydrofolate dehydrogenase 2 (MTHFD2)[38-41] and ribonucleotide reductase subunit M2 (RRM2)[42-45]. In our previous research, despite searching for cytoskeleton-related processes in which the investigated genes might have been involved, not only did we stumble across the relation to metabolism, but we also reported some genes which were found to be implicated in glioblastoma stemness. Thus, the dedicated work on these genes in the GBM stem cells (GSCs) seems justifiable and might reveal novel therapeutic directions and/or biomarkers that could be utilized in the future. Herein, we review the previously identified cytoskeleton/metabolism-related genes through the prism of GBM stemness. Literature screening allowed the decision to split these genes based on whether their role in stemness is known from GBM or another tumor, the latter suggesting an urgent need to experimentally verify the observations in the glioblastoma context.

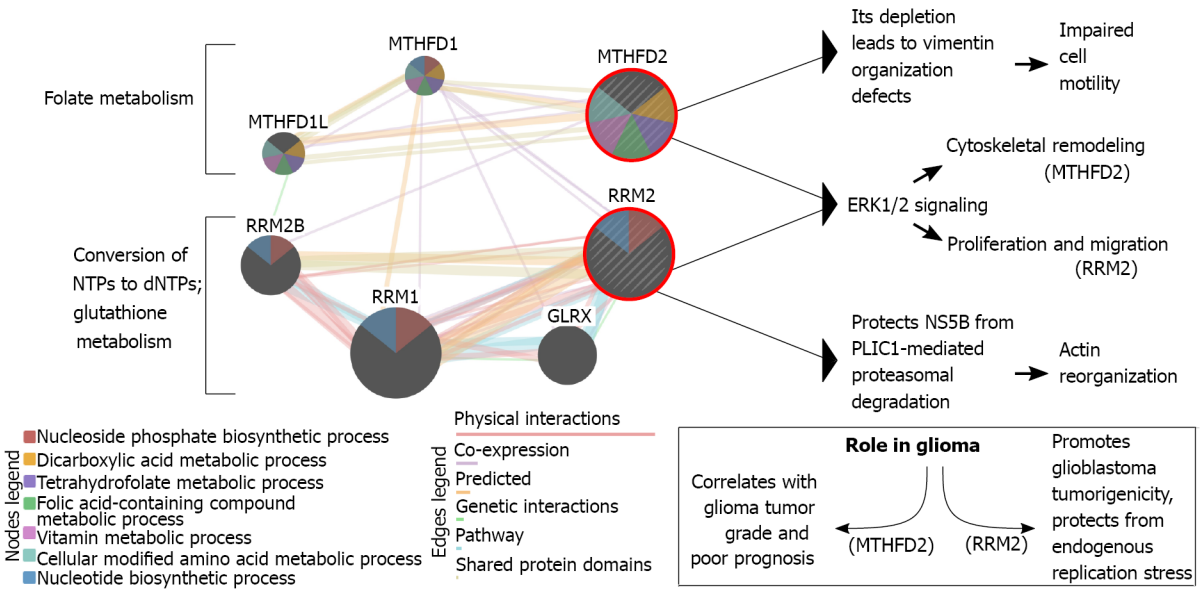
GENES WITH CONFIRMED ROLE IN GLIOBLASTOMA STEMNESS

Bone morphogenetic protein 4

Based on the literature abundance, the best-known from its implication in glioblastoma stemness is bone morphogenetic protein 4 (*BMP4*). The bone morphogenetic proteins are growth factors from the TGF-β superfamily that undergo expression during embryogenesis and control development. Initially denoted as crucial for osteogenesis, they are now described as regulators of gastrulation, neurulation, mesoderm patterning, proliferation, and differentiation in many tissues[46]. About 15 years ago, it was found that the signaling *via* BMPs and their cognate receptors (BMPRs) influenced the activity of normal brain stem cells but could also inhibit the cancer-initiating GBM stem-like cells[47]. Later the same year, these authors confirmed that *in vivo* delivery of *BMP4* blocked the tumor growth and associated mortality, which occurred in all mice following intracerebral grafting of human glioblastoma[48]. This protein was suggested as a non-cytotoxic therapeutic agent that can be utilized in combination with stem cell-based therapy[49]; this complements its usage as an agent used to differentiate GSCs into normal glial cells [50]. *BMP4* has been found promising to the extent that it entailed the development of novel therapies. For example, one that utilizes the oncolytic vaccinia virus was developed to alleviate glioblastoma and prevent its recurrence[51]. Later on, the cell-based treatment option of BMP4-secreting human adipose-derived mesenchymal stem cells was found to reduce proliferation and migration both *in vitro* and *in vivo*, as well as prolong survival in a murine model[52]. Still, Videla Richardson *et al*[53] admitted that little is known about this morphogen regarding triggered cellular events, which prompted the authors to establish several GSC-enriched cell lines growing as adherent monolayers and not floating neurospheres. Distinct lineage preferences were noticed depending on the expression pattern of BMP signaling-astrocyte fate or neuronal commitment was noticed and, under certain conditions, even a smooth muscle-like phenotype[53]. Providing new findings to the available data, *BMP4*-overexpressing neural stem cells were found to promote GSCs apoptosis *via* Smad1/5/8 signaling[54]. Moreover, recent studies indicate a formerly underestimated link between *BMP4* and metabolism or mechanotransduction which affects oxygen consumption or matrix stiffness[55]. The latter is known to be associated with cytoskeletal remodeling[56,57]. With regard to the cytoskeleton, *BMP4* was found to re-organize actin dynamics *via* activation of Rac1, Rho, and Cdc42[58]. The impact of *BMP4* in inducing asymmetric cell division was also noted, limiting the GSCs expansion[59]. The newest literature data on *BMP4* consider it on a broader scale, either evaluating other GBM aspects and referring to *BMP4*, or investigating upstream/downstream molecules. Ciechomska *et al*[60] explored *EGFR* alterations in glioblastoma since GSCs with various *EGFR* levels respond differently to therapy; the authors found that EGFR/FOXO3a/BIM signaling pathway determined chemosensitivity of BMP4-differentiated GSCs to TMZ. On the other hand, Wu *et al*[61] identified BIRC3 as an inducer of glioblastoma stemness *via* downstream *BMP4* inactivation. At last, the most recent paper by Verploegh *et al*[62] summarized the cellular viability variance in response to *BMP4* and proposed early-response markers for sensitivity to *BMP4*. Three cultures with the highest sensitivity for *BMP4* revealed a new cell subpopulation that presented a reduced cell proliferation but an elevation of apoptosis. These changes in composition correlated with treatment efficacy; the latter was predicted using *OLIG1/2* expression. Furthermore, upregulated *RPL27A* and *RPS27* were considered early-response markers. Interestingly, *RPS27* is one of the genes identified in our previous study that prompted us to investigate the aspects presented in this review. This gene will be described below in a separate subsection.

Glutamate ionotropic receptor NMDA type subunit 2B

Glutamate ionotropic receptor NMDA type subunit 2B (GRIN2B) encodes one subtype of glutamate-binding GluN2 subunit, which is a part of the N-methyl-D-aspartate receptor (NMDAR). Ionotropic



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Figure 1 Example of the interplay between cytoskeleton and metabolism using the biological function of methylenetetrahydrofolate dehydrogenase 2 and ribonucleotide reductase subunit M2 enzymes. Typically, methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) dehydrogenase is known for its activity in folate metabolism, whereas ribonucleotide reductase subunit M2 (RRM2) reductase is known for the conversion of ribonucleotide triphosphates to deoxyribonucleotide triphosphates which requires metabolic resources supplied by reduced glutathione. However, these two enzymes (encircled in red) are also involved in cytoskeletal rearrangements that are summarized on the right side of the figure. Literature data indicate that they also affect the same pathway (*i.e.*, ERK1/2 signaling) but render various outcomes. Moreover, their role in glioma has already been proposed (bottom-right panel). Figure created using Inkscape and GeneMania (*MTHFD2* and *RRM2* as query genes; five "resultant" genes included to highlight interconnectivity; exemplary metabolism-related processes included from the built-in functional analysis). NTP: Ribonucleotide triphosphates; dNTPs: Deoxyribonucleotide triphosphates; MTHFD: Methylenetetrahydrofolate dehydrogenase; RRM2: Reductase subunit M2.

glutamate receptors from this family mediate Ca^{2+}, *i.e.*, the permeable component of excitatory synaptic transmission in the central nervous system (CNS)[63]. NMDARs assemble from four subunits: two GluN1 and two GluN2. The former subunits are widely expressed in the nervous system, while four subtypes of GluN2 subunits (from "A" to "D") are characterized by various expression patterns[64]. *GRIN2B* encodes the GluN2B subunit, which is abundantly expressed in the prenatal period, then declines in most brain parts[65]. The presence of GluN2B in such an early stage implies that it contributes to brain development, circuit formation, synaptic plasticity, as well as migration and differentiation[66]. Glutamate-dependent synaptic transmission is frequently dysfunctional in gliomas[67], and regarding this specific subunit, an enrichment of expression was noticed in GSCs[68]. In our previous research, with the use of literature data, we related this gene with the cytoskeleton since GluN2B interacts with cytoskeletal protein α-actinin-2 *via* the carboxyl-terminal domain[63]. It might be of importance as α-actinin-2 is closely associated with multimerins which are possible markers and therapeutic targets in low-grade glioma[69]. Moreover, one of the multimerins encoded by the *MMRN1* gene was found to be correlated to stemness and chemoresistance, although these observations were based on the leukemia model[70]. Nevertheless, *GRIN2B* is confirmed to influence stemness not only in glioblastoma but also in lung cancer. She *et al*[71] identified *GRIN2B* expression to be higher in primary tumors than in normal tissues, and at the same time higher in metastatic lesions than in primary tumors which contributed to poorer prognosis. Moreover, the same authors observed inhibition of tumorsphere formation during *GRIN2B* silencing.

Homeobox protein A10 and A1

The homeotic genes, in vertebrates denoted as homeobox, are highly conserved and regulate the proper development of various body segments during ontogeny[72]. Homeobox protein A10 (*HOXA10*) is implicated in the embryogenesis of the uterine epithelium, stroma, and muscle[73]. In response to hormones, it undergoes periodical expression in the mature endometrium, controlling receptivity during the implantation window[74]. Concerning GBM stemness, the functionality of *HOXA10* was presented as a direct result of the activation of protein from the *Trithorax* family, which serves as a histone methyltransferase, *i.e.*, MLL. Afterward, *HOXA10* activated other *HOXA* genes, such as *HOXA7* and *HOXC10*[75]. In another study, *HOXA10* was marked as one of the strongest candidates (alongside the *HOX -A9*, *-C4*, and *-D9* genes), having value as a therapeutic target and biomarker for both GBM and GSCs[76]. Our previous research echoed the data that *HOXA10* facilitated cytoskeleton remodeling (*via CK15*)[77], promoted tumorigenesis in glioma[78], and regulated homologous recombinant DNA

repair and subsequently TMZ resistance in GBM[79]. Since stemness also contributes to treatment resistance[14], the last two events complement each other mutually. Another homeotic gene that we identified in our previous study was *HOXA1*, a homeobox that is abundantly expressed in the mesoderm and neuroectoderm at the level of the brainstem precursor[80]. Upregulation of *HOXA1* was noted in GBM, which inversely correlated with the survival of patients[81]. This homeotic member was also implicated in regulating the cytoskeleton *via* E-cadherin. Namely, *CDH1*-dependent signaling was found to increase *HOXA1* expression through Rac1, *i.e.*, the same pathway that regulates actin cytoskeleton at cadherin adhesive contacts[79]. With regard to GBM stemness, Schmid *et al*[82] observed upregulated HoxA locus (encompassing, *e.g.*, *HOXA1*) after they dedifferentiated murine astrocytes into GSCs *via* Rb knockout, Kras activation, and Pten deletion. These cells were sufficient to form GBMs in their transplant mouse model. Although the insights did not provide further mechanistic details, the regulation loop of *HOXA1* and HOXA transcript antisense RNA (HOTAIRM1) was found to be involved in stemness maintenance[81,83]. This was presented in colorectal carcinoma and uveal melanoma. Still, taking into account the study by Schmid *et al*[82], the profound investigation of *HOXA1* in GSCs in this aspect should be considered.

Matrix metalloproteinase 13

Matrix metalloproteinases are constituents of extracellular matrix (ECM) belonging to the zinc-containing endopeptidases family that encompasses 23 members[84]. Functionally, these calcium-dependent molecules are responsible for the degradation and remodeling of other proteins that constitute ECM. Moreover, their roles in various biological and physiological processes dependent on hormones, growth factors, and cytokines were described[85]. It is known that different ECM components modulate cancer stem cells' properties; regarding glioblastoma, the confirmed ones were type I collagen, laminin α2, fibronectin, periostin, decorin, and lumican[86]. Matrix metalloproteinase 13 (*MMP13*) is a collagenase almost universally upregulated in the pan-cancer view[87]; in GBM, its overexpression increases migration and invasion[88], as well as confers poor prognosis[89]. The relationships between *MMP13* and the cytoskeleton[33] or metabolism[90] are known. In terms of stemness, Inoue *et al*[91] suggested that highly invasive potential GSCs depended on MMP13 enzymatic activity; the authors also proposed MMP13 as a potential therapeutic target.

MTHFD2

The folate cycle is responsible for appropriate cellular metabolism by regulating ATP production, methylation reactions for DNA/protein synthesis, or developing immunomodulatory molecules that orchestrate signaling and cytotoxicity[92]. The differences between *MTHFD1* and *MTHFD2*, two enzymes implicated in the folate pathway, include the use of different co-enzyme (NADP *vs* NAD), functionality (*MTHFD1* has three distinct enzymatic activities while *MTHFD2* is bifunctional), and location (cytoplasm *vs* mitochondria). Compared to *MTHFD1*, which generates NADPH and formate for purine biosynthesis, *MTHFD2* is overexpressed in rapidly proliferating malignant tumors. It is considered the "main switch" that enables mitochondria to produce additional growth-facilitating one-carbon units and generates NADH necessary for protection from reactive oxygen species[93]. *MTHFD2* is also an excellent example to present the link between metabolism and cytoskeleton. Lehtinen *et al*[39] have found that *MTHFD2* depletion leads to vimentin organization defects, and identified this gene as a regulator of cell migration and invasion. Regarding glioma, *MTHFD2* was found to be associated with tumor grade and prognosis[38]. Inhibition of this enzyme in GSCs induced apoptosis and affected not only central carbon metabolic pathways (*e.g.*, glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle) but also unfolded protein response, highlighting a novel connection between one-carbon metabolism and reaction to cellular stress[94]. Nishimura *et al*[95] suggested that the purine synthesis pathway, as well as folate-mediated one-carbon metabolism, seem to be crucial for the maintenance of tumor-initiating cells. The same authors also concluded that EGF-induced expression of *MTHFD2* may be mediated by Myc, with the latter regulating the expression of metabolic enzymes for the maintenance of brain tumor-initiating cells.

Plant homeodomain finger-like domain-containing protein 5A

Alternative splicing maintains post-transcriptional gene regulation, which enables a single gene to be transcribed into various RNAs, diversifying the proteome. Abnormal splicing function can lead to tumor-related processes, *e.g.*, proliferation, angiogenesis, and metastasis[96]. Spliceosome, a dynamic machinery responsible for splicing, is made of small nuclear ribonucleoproteins (snRNPs; five molecules are known: U1, U2, U4, U5, and U6) and numerous non-snRNP proteins[97,98]. U2 snRNP comprises U2 snRNA, SF3a complex, and SF3b complex, which are responsible for recognizing branchpoint sequences during initial spliceosome assembly stages[99]. Splicing factors comprising the SF3b complex include plant homeodomain (PHD) finger-like domain-containing protein 5A (*PHF5A*), which facilitates interactions between the U2 snRNP and RNA helicases[100] but can also bind chromatin *via* its PHD that is composed of a small zinc finger structural fold[101,102]. The knockdown of *PHF5A* results in reduced GBM viability and cell cycle arrest[103]. Trappe *et al*[104] revealed that systematic deletion of its yeast homolog is lethal, showing that *PHF5A* is crucial for cell viability. The flagship paper on *PHF5A*

in brain tumor[105] indicates that the gene is required to expand GSCs and that in these tumor-initiating cells, but not untransformed neural stem cells, *PHF5A* contribute to the identification of exons having unusual C-rich 3′ splice sites in thousands of essential genes. The same authors inhibited *PHF5A*, which reduced GSCs-driven tumor formation *in vivo* and inhibited the growth of established GBM patient-derived xenograft tumors.

Ribosomal protein S27

One of the most dynamic and largest molecular motors (driven by a complex thermal ratchet translocation mechanism) are ribosomes[106]. Metallopanstimulin-1, also known as ribosomal protein S27 (*RPS27*), is a constituent of the human 40S ribosome that is mainly found in the cytoplasm while it can also relocate to the nucleus[107] or even extracellular space[108]. Regarding the nuclear location, it is able to interact with DNA *via* its C4-type zinc finger[109]. In glioblastoma, *RPS27* was found to be correlated with age in IDH-mutated glioma patients and with Ki67 in GBM patients. Interestingly, it is detected in astrocytic tumors but not in normal astrocytes unless the tissue was inflamed[109]. This allowed the same authors to emphasize that in comparison to inflammatory tissue (in which only a small number of macrophages were positive for *RPS27*), almost all macrophages in tumor tissue were distinctly enriched in *RPS27* expression. As for GSCs, the ribosomes and related proteins were generally found to reprogram glioma cells to induce plasticity and stemness[110]. Among these molecules, *RPS27* was considered oncogenic with higher expression at the GSC-dominant area[111]. Inquisitive findings revealed that *RPS27* is also detected in the microvascular proliferation area and pseudopalisading cells around necrosis[110]. It is worth underlining that aberrant vessels are crucial for the formation of pseudopalisading necrotic regions that provide shelter for residing cancer stem cells from anti-tumor agents, which enable these cells to expand and promote proliferation and growth[112]. As mentioned above, upregulated *RPL27A* and *RPS27* were considered to be early-response markers related to the presence of *BMP4*. This suggests a link that should be further investigated since the signaling of ribosome translation was found to be overexpressed during the response to stress in glioblastoma.

RRM2

A balanced supply of deoxyribonucleotide triphosphates (dNTPs) is a prerequisite of DNA synthesis. Still, *de novo* synthesis of dNTP is also possible *via* the reaction catalyzed by the ribonucleotide reductase (RR) that reduces the C2′-OH bond of the four ribonucleotides triphosphates to form corresponding dNTPs[113]. *RRM2* encodes the β subunit of RR; each RRM2 monomer contains the tyrosyl radical and non-heme iron[114]. Since a sufficient supply of dNTPs drives an uncontrolled DNA replication in cancer[115], it is not surprising that *RRM2* was frequently subjected to molecular therapy[116,117]. Currently, several RRM2 inhibitors have been developed, *e.g.*, radical scavengers, iron chelators, subunit polymerization inhibitors, or expression silencers[118-120]; this is to inhibit proliferation, division, but also invasion[32]. In glioblastoma, *RRM2* is responsible for the advancement of GBM tumorigenicity and protection from endogenous replication stress *via* the BRCA1-RRM2 axis[45]. For glioma in general, regulation of proliferation and migration *via* ERK1/2 and AKT signaling was noted[44]. Available literature also links the *RRM2* to the cytoskeleton *via* hPLIC1; the latter decreases during *RRM2* downregulation, which entails actin cytoskeleton re-organization[42]. Perrault *et al*[121] have suggested that *RRM2* can be a chemoresistance driver that dictates how GBM cells respond to TMZ. The same authors further verified that *RRM2*-overexpressing cells had enhanced DNA repair efficiency. Moreover, the use of a selective FDA-approved RRM2 inhibitor, 3-AP Triapine, enabled Perrault *et al* [121] to observe that in comparison to both TMZ and control, glioblastoma treated with the 3AP + TMZ formed fewer neurospheres that were also significantly smaller. Another group found that *RRM2* expression dramatically declined after 12 d of dasatinib treatment compared to naïve GSCs of the GSC8 cell line[122].

Serum amyloid A protein 2

In order to re-establish homeostasis, both adaptable and primordial mechanisms exist; the latter comprises the acute-phase response (APR) that is a set of changes that occur after inflammation, infection, or trauma[123]. During APR, the changes include the altered levels of serum proteins, with the most notable being C-reactive protein and serum amyloid A (SAA)[124]. Being an apolipoprotein, SAA is related to plasma high-density lipoprotein and is implicated in the cholesterol transport to the liver for excretion as bile[125]. Its other functions include regulation of amyloidogenesis, tumor pathogenesis, anti-bacterial events, and inflammatory response[126]. The role of SAA in tumor progression was suggested owing to its cytokine-like properties that influence the course of inflammation[127]. *SAA2* is one of the paralogs of the family and was investigated as a lung cancer biomarker a few years ago[128]. The description of its role in glioblastoma is limited, yet it is already known that *SAA2* increases GBM proliferation and invasion[129]. Knebel *et al*[130] have confirmed that SAA production occurs not only in the liver but also in tumor cells; the authors emphasized that exploring the SAA influence on the cytoskeleton and invasiveness using more complex assays is needed. In terms of GBM stemness, Adamski *et al*[131] recently have compiled the literature data and stated that *SAA2* is implicated in a drug-promoted cellular dormancy, with the latter being closely connected to stem cell characteristics.

The group also indicated the ability of *SAA2* to sustain inflammatory conditions in the brain, which consequently supports TMZ resistance and induces the expression of stemness markers in glioblastoma.

Wilms' tumor protein 1

The 5-methylcytosine (5mC) and its derivatives have altered patterns in a range of tumors. 5mC can be recognized and oxidized to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine by Ten-Eleven translocation (TET) enzymes[132,133]. One of the transcription factors that directly interacts with TET proteins is Wilms' tumor protein 1 (WT1): A master regulator essential for urogenital, epicardium, and kidney development that can act as a tumor suppressor or oncoprotein in multiple tumors[134, 135]. Initially cloned as a suppressor of Wilms' tumor, WT1 is now considered to be an oncoprotein in hematologic malignancies and a variety of solid tumors, as well as the protein with the highest potential for cancer immunotherapy[136-138]. According to the phase I/II clinical trial, WT1 peptide-based vaccine for glioblastoma patients was considered safe and induced cellular and humoral immune response[139]. This is important due to the fact that *WT1* is involved in GBM tumorigenicity *via* increasing proliferation and decreasing apoptosis[140]. As for the impact on the cytoskeleton, this protein was found to interact with actin both in the cytoplasm and nucleus, as well as supposedly binds to RNA in a cytoskeleton-dependent regulation manner[141]. Focusing on GBM stemness, Mao *et al*[142] found that *WT1* was expressed predominantly in mesenchymal GSCs which, compared to proneural stem cells subtype, are characterized by higher proliferation, greater radioresistance, and implication in worse patients' prognosis. Uribe *et al*[143] reviewed that mesenchymal GSCs develop tumors having more blood vessels, hemorrhagic lesions, and necrotic areas; the expression pattern in these stem cells generally facilitates inflammation, angiogenesis, migration, invasion, and glycolysis-mediated metabolism. Undoubtedly, more insights are needed concerning GBM molecular pathways in which *WT1* is implicated.

GENES WITH STILL UNCONFIRMED ROLE IN GLIOBLASTOMA STEMNESS

Chemokine-like factor superfamily 6

Cytokines are soluble proteins that are secreted by immune and non-immune cells in response to stimulants such as immunogens or mitogens; this allows them to maintain the immune response and homeostasis[144]. Chemokines constitute a specific type of small (8-13 kDa) cytokines that promote the directed chemotaxis of nearby cells[145]. Consisting of nine members, the chemokine-like factor superfamily (CMTM) is expressed throughout the human tissues and regulates immune, circulatory and muscular systems, as well as the hematopoiesis[146-149]. The aberrant *CMTM* expression is implicated in various diseases, *e.g.*, rheumatoid arthritis, atopic dermatitis, focal cerebral ischemia, male infertility, as well as tumorigenesis and metastasis[150-153]. The influence of *CMTM6* on glioblastoma is known, but the research in this entity seems to be in the initial state. Guan *et al*[154] revealed that the highest *CMTM6* expression was noted in the glioblastoma (WHO grade IV) compared with WHO grade II and III gliomas. Enrichment was also observed in both microvascular proliferation and hyperplastic blood vessels, which are both essential for tumor progression. In GBM, *CMTM6* was also associated with one of the genes of immune checkpoints, *i.e.*, *TIM-3*. From a broader glioma scale, the same authors summarized it as a molecule diminishing T-lymphocyte-dependent anti-tumor immunity, reducing patient survival and indicating poor prognosis. However, it is still yet to be elucidated what role *CMTM6* may play in the GBM stemness. Currently, its contribution to such characteristics is confirmed on the basis of data from head-and-neck squamous cell carcinoma. Chen *et al*[155] observed poorer patient prognosis during *CMTM6* overexpression that correlated with overactive Wnt/β-catenin signaling, *i.e.*, the pathway crucial for tumorigenesis, epithelial-to-mesenchymal transition (EMT) and cancer stem cells maintenance. Silencing of *CMTM6* led to PD-L1 downregulation, decreased tumor growth, and increased $CD8^+$ and $CD4^+$ T-cell infiltration. Eventually, the authors not only suggested the therapeutic suitability of *CMTM6* but also concluded that this protein is implicated in EMT, stemness, and T-cell dysfunction. Similar research in the glioblastoma context is advisable, especially since *CMTM6* can stabilize PD-L1 protein to impair T-cell function[156,157], as well as their combined expression had prognostic significance in pancreatic ductal adenocarcinoma and triple-negative breast cancer[158]. Nowadays, the role of PD-L1 in cancer and immunotherapy is unquestionable[159]; focusing on another protein related to this well-established molecule might bring novel strategies.

Dual specificity phosphatase 7

Signal transduction is based on phosphorylation and dephosphorylation events performed by kinases and phosphatases, leading to a cellular program relevant to the encountered stimulus[160]. Dual specificity phosphatases (*DUSP*) are responsible for the dephosphorylation of threonine and tyrosine residues on mitogen-activated protein kinases, rendering them inactive[161]. Even if *DUSP7* was only noted as downregulated in glioblastoma, whereas *DUSP1*, *DUSP5*, and *DUSP6* were induced within pseudopalisading and perinecrotic GBM regions[162], the role of *DUSP7* in preserving the pluripotency of non-cancerous stem cells was certified in a murine model[163]. However, its contribution could be

distinct from *DUSP1*, *DUSP5*, and *DUSP6* but similar to *DUSP2*, *DUSP8*, and *DUSP9* which were clustered together with *DUSP7* in the study of Mills *et al*[162]. At last, it is worth noting that *DUSP7* guides chromosome dynamics which is known for being regulated by cytoskeletal proteins[164,165]. The study linking this phosphatase to metabolism revealed that *DUSP7* knockout accelerates metabolic disorder and insulin resistance in mice with a high-fat diet[166].

Kinesin family member 20A

Cytoskeletal elements that act as scaffolds for intracellular cargo transport are microtubules. Motor proteins known as kinesins and dyneins orchestrate microtubule-related transport that is essential for cell differentiation or survival[167]. Kinesins constitute a large superfamily responsible for cargo trafficking, as well as controlling microtubule growth and stability[168]. Increased expression of kinesin superfamily representatives KIF4A, -9, -18A, and -23 was associated with poor prognosis in low-grade glioma and glioblastoma[169]. The pro-cancerous characteristics of *Kinesin family member 20A* (*KIF20A*) were noted more than 15 years ago in pancreatic cancer, which presented a reduction of proliferation once *KIF20A* was downregulated[170]. Currently, accumulating evidence shows that this kinesin is overexpressed in multiple tumors[171]. In glioblastoma, *KIF20A* downregulation induces cell cycle arrest and apoptosis *via* suppressing PI3K/AKT pathway[172]. Regarding cytoskeleton-related events, it is not only essential for cytokinesis but also interacts with Rab6 to regulate Golgi-related vesicle trafficking[173]. Although the role of *KIF20A* in GBM stemness has not yet been confirmed, it was suggested outside of the glioblastoma context in a study by Qiu *et al*[174]. The authors conceived the importance of *KIF20A* in controlling proliferation *vs* differentiation of tumor-initiating cells, based on both the fact that cancer stem cells share many mechanisms with neural progenitors, as well as their observations where *KIF20A* was implicated in balancing symmetric and asymmetric divisions during cerebral cortical development[175]. The *KIF20A* inactivation affected cortical neural progenitor cells that switched from proliferative to differentiative mode. During divisions, daughter cell-fate specification was controlled by *KIF20A* in coordination with *RGS39* and *SEPT710*[174,176].

Neurofibromatosis type 2 protein

Neurofibromatoses (type 1, type 2, schwannomatosis) are distinct, dominantly inherited disorders that have in common the occurrence of nerve sheath tumors[177]. Type 1 neurofibromatosis presents with neurofibromas, cafe-au-lait spots/macules, freckling, and optic gliomas, whereas type 2 neurofibromatosis is characterized by bilateral vestibular schwannomas, ependymomas, and meningiomas [178]. Each disease has a different underlying genetic alteration: Type 1 neurofibromatosis is related to the *neurofibromatosis type 1 protein* (*NF1*) gene, type 2 is linked to *NF2*, while schwannomatosis to integrase interactor 1 (*INI1*, also known as *SMARCB1*). The protein product of *NF2* has the same name as its gene but can also be referred to as Merlin. Although this tumor suppressor is not mutated in GBMs, it exhibited oncogenic properties in glioblastoma when phosphorylated at serine 518; this post-translational modification inactivates Merlin's anti-cancer capabilities, which affects the expression of EGFR or Notch1 and its downstream targets, *i.e.*, *HES1* or *CCND1*[179]. Other authors demonstrated that upon *NF2* re-expression, a regulation of YAP, cIAP1/2, and the Hippo signaling pathway led to the inhibition of glioma growth and progression[180]. Merlin is also known for regulating cell morphology or motility, and its loss renders dramatic changes in cellular adhesion and cytoskeleton organization [181,182]. Specifically, this protein is closely related to ezrin, radixin, and moesin (collectively denoted as "ERM"), *i.e.*, critical proteins that enable the anchorage between membrane proteins and cortical cytoskeleton[183]. Ultimately, the link between *NF2* and stemness might be related to CD44, the receptor of which cytoplasmic tail can interact with both Merlin and "ERM" proteins[184,185]. Literature data state that *NF2* exhibits tumor suppressor function *via* negative regulation of CD44[186], whereas this receptor has been repeatedly indicated as a marker of cancer stem cells in various tumors, such as leukemia and carcinoma of breast, colon, ovarian, prostate, or pancreas[187-191]. Knowing that CD44 is also an upstream regulator of the aforementioned Hippo signaling pathway[192], of which components regulate the stem cell niche, self-renewal, maintenance, and differentiation[193-196], one could investigate Merlin in the GBM stemness context taking into the account the NF2-ERM-CD44-Hippo regulation network.

Retinoid X receptor gamma

The signal transduction molecules being vitamin A derivatives are retinoids, they regulate cellular differentiation and proliferation *via* members of the nuclear receptors superfamily, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs)[197]. The RXR family members (RXRA, RXRB, and RXRG) form heterodimers within the superfamily, *e.g.*, with vitamin D, retinoic acid, or peroxisome proliferator-activated types of receptors[198,199]. RXRs have tumor suppressor properties and, as partners of *RARA* and *RARB*, they are implicated in the anti-proliferative effects of retinoic acid[197]. *RXRG* was found to modulate differentiation and apoptosis in various tumors, indicating its function in cancer pathogenesis[200]. Glioblastoma-related research certifies the general view that *RXRG* contributes to anti-neoplastic effect *via* its ligands; in study by Papi *et al*[201], the treatment of GBM with 6-OH-11-O-hydroxyfenantrene had anti-proliferative and anti-invasive effects. However, the literature

data on glioblastoma stemness seem to focus on RARs rather than RXRs. Ying *et al*[202] evaluated the cellular and molecular responses of GSCs to all-*trans* retinoic acid; this treatment changed cells morphology (*e.g.*, decreased neurosphere-forming capacity), caused growth arrest at G_1/G_0 to S transition, reduced cyclin D1 expression, and elevated p27 expression. Moreover, differentiation markers such as Tuj1 and GFAP were induced, while stem cell markers, such as CD133, Msi-1, Nestin, and Sox-2, had decreased expression. Friedman *et al*[203] provided similar observations with regard to Nestin level or neurosphere formation but also indicated that GBM differentiation induced by all-*trans* retinoic acid is executed *via* the ERK1/2 pathway. Evidently, retinoid-related research in the GBM context frequently focuses on all-*trans* retinoic acid while this isomer is bound only by RARs and not by both RARs and RXRs, as is the case with another retinoic lipid: *9-cis*[204]. Even if two of the best-known retinoid receptors (*RARA* and *RXRA*) are described in detail by Rodriguez *et al*[205] in the GBM stemness context, the data on *RXRG* is still lacking and should begin with evaluation of whether *9-cis* retinoid acid is able to manifest the anti-glioblastoma effects *via RXRG* and subsequently ERK1/2 pathway.

SPARC/Osteonectin, CWCV, Kazal-like domains 1

ECM is a component containing elastin, collagen, laminins, glycoproteins, fibronectin, and proteoglycans. Together, these elements bind *via* cell adhesion receptors and form a complex macromolecular network[206]. Matricellular proteins are made of matrix-binding proteins and cytokines that can be located within the cell or secreted outside[207]. SPARC/Osteonectin, CWCV, Kazal-like domains 1 (SPOCK1), also referred to as testican-1, is an ECM proteoglycan from a matricellular family of proteins that regulate matrix remodeling and affects tumor progression[208-210]. As the interplay between ECM and cytoskeleton is known[211], it is not surprising that changes in *SPOCK1* lead to alterations in cytoskeletal components. For example, Schulz *et al*[212] noticed that *SPOCK1* upregulation paralleled that of *EPB41L4B*, the latter being a cortical cytoskeleton protein underlying cellular membrane. With regard to brain tumors, testican-1 contributes to GBM metastasis and resistance to TMZ, as well as promotes glioma invasion, migration, and proliferation *via* Wnt/β-catenin and PI3K/AKT pathways[213,214]. Mediating TMZ chemoresistance *via SPOCK1* in GBM was independently confirmed by Sun *et al*[215]. Although not yet directly concluded by any scientific group, it is conceivable that the impact of *SPOCK1* on TMZ resistance renders a similar GSCs-related effect as *SAA2* which was described in one of the previous sections.

Ubiquitin-like with PHD and ring finger domains 1

The proteins' turnover and degradation depend on ubiquitination that is orchestrated by the ubiquitin-proteasome system (UPS)[216], of which alterations can lead to several tumor types[217,218]. One of the ubiquitin-protein ligases responsible for the UPS specificity is ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*)[219], a molecule also interacting with DNA methyltransferase 1, which together constitute the main regulatory axis of cellular senescence[220]. *UHRF1* was already identified as a novel oncogene and/or druggable epigenetic target for various tumors[221-223], and Jung *et al*[220] suggested its role as a switch molecule between senescence and cancer. In GBM, *UHRF1* is overexpressed by upstream CD47 and regulates downstream silencing of tumor suppressor gene $p16^{INK4A}$, leading to increased proliferation[224]. Regarding cytoskeleton, *UHRF1* contributes to microtubule organization through its downstream targets: *BRCA2*, *HOOK1*, *KIF11*, and *KIF18A*[225]. The role of *UHRF1* in different types of stem cells is documented but overlooks GSCs. Namely, it was found to be required for the proliferative potential of basal stem cells in response to airway injury[226], as well as regulate the transcriptional marks at bivalent domains in pluripotent stem cells[227]. On the other hand, *UHRF1* decrease was found to be a major cause of DNA demethylation in embryonic stem cells[228] and led to the activation of retroviral elements and delayed neurodegeneration[229]. It is evident that research in the glioblastoma context should be pursued in the future, especially since some epigenetic features, next to transcriptional ones, are unique in GSCs compared to neural stem cells and may include druggable targets for new therapeutic approaches[230].

DISCUSSION

Despite molecular advancements, there is still a considerable need for glioblastoma biomarkers[231], especially since the relatively ineffective treatment leaves the patients with a very dismal chance of survival[232]. One of the glioblastoma traits involved in the absence of effective treatment is tumor heterogeneity which can be explained by clonal evolution and the presence of stem cells[8].

Many independent studies on various tumor types have reported common genes as potential therapeutic or diagnostic biomarkers[233]. Al-Fatlawi *et al*[234] contemplated that biomarker signatures for different cancer types should be similar, due to the fundamental mechanisms shared between tumors, *e.g.*, survival, tumor growth, or invasion. Thus, we presume that our description of stemness-related genes, especially those still unconfirmed in GBM, adds significant value to the current knowledge and provide insights into novel therapeutic or diagnostic directions.

For clarity, a graphical presentation was prepared to emphasize the role of described genes specifically in stem cells, setting aside the rest of the information provided for each gene (Figure 2). At first glance, the most frequently regulated processes are proliferation and chemoresistance, followed by differentiation, tumor growth, invasion, and apoptosis. Except for *BMP4* (increase in asymmetric cell division and apoptosis), *NF2* (reduced self-renewal, tumor growth, stemness maintenance), *RXRG* (decrease in invasion and proliferation), and *DUSP7* (insufficient data for a definite conclusion), the remaining genes exhibit pro-cancerous properties. This corresponds to what was described in subsections, separately for each gene. Interestingly, two genes that promote invasiveness of stem cells (*SPOCK1*, *MMP13*) are known to affect the cytoskeleton[33,212] and, in terms of *MMP13*, also the metabolism[90]. Two genes that were also found to regulate both the cytoskeleton and metabolism were *MTHFD2* and *RRM2*. On the one hand, they control the organization of vimentin and actin; these proteins are known for influencing glioblastoma migratory potential[235,236]. On the other hand, the contribution of *MTHFD2* and *RRM2* to metabolism is related to folate and glutathione cycles that are implicated in the resistance of GBM to therapy[237,238].

In order to gravitate towards the link between metabolism, cytoskeleton, and GBM stemness, the appropriate representatives of each process (including the most frequently regulated processes that were mentioned above), were compiled into a cross-talk network. This allowed us to integrate the aim of our review with the main processes that are regulated by genes described in this work, additionally with the inclusion of GBM biomarkers (acquired from review by Sasmita *et al*[231]). Prevalent interaction types include co-expression and physical interaction between these representatives, there is also a high interconnectivity of the entire network, confirming that these molecular events are related. The cross-talk is visualized in Supplementary Figure 1, whereas the datasets used in the workflow are summarized in Supplementary Table 2.

The narrative of this review was intended to elaborate on the background of the biological machinery in which each successive gene is involved, then proceed with details regarding the regulation of glioblastoma, cytoskeleton/metabolism, and stemness (GBM-related or, if not present in the literature, any available). It is worth emphasizing that the herein described genes constitute more than half of the "top genes" that we established in our previous *in silico* study *via* a multi-stage methodology that included, *e.g.*, enrichment analysis, machine learning algorithm, and differential expression analysis [32]. The remainder was not presented due to a lack of stemness-related literature data (Supplementary Table 1). For the part available in this paper, the majority of genes (*BMP4*, *GRIN2B*, *HOXA10*, *HOXA1*, *MMP13*, *MTHFD2*, *PHF5A*, *RPS27*, *RRM2*, *SAA2*, *WT1*) were confirmed to influence GSCs. The attempt to associate *CMTM6*, *DUSP7*, *KIF20A*, *NF2*, *RXRG*, *SPOCK1*, and *UHRF1* with glioblastoma stemness revealed the promising implication in crucial biological processes that should be validated in future experiments. For *BMP4*, *WT1*, and *RXRG*, their contribution to novel therapeutic strategies was above-mentioned on the basis of literature data, prompting us to investigate whether any clinical trials utilize the products of described genes as drug components or targets. According to the ClinicalTrials website (https://clinicaltrials.gov/), cancer-related data can be found for six genes (Table 1); however, the seventh trial on *GRIN2B* was also included because it focused on brain research and highlights that selective *GRIN2B* antagonist is already developed. Moreover, the details on NF2-related intervention are not yet disclosed[239]. Collectively, these studies are in the early phases, certifying that there is still a room for further research.

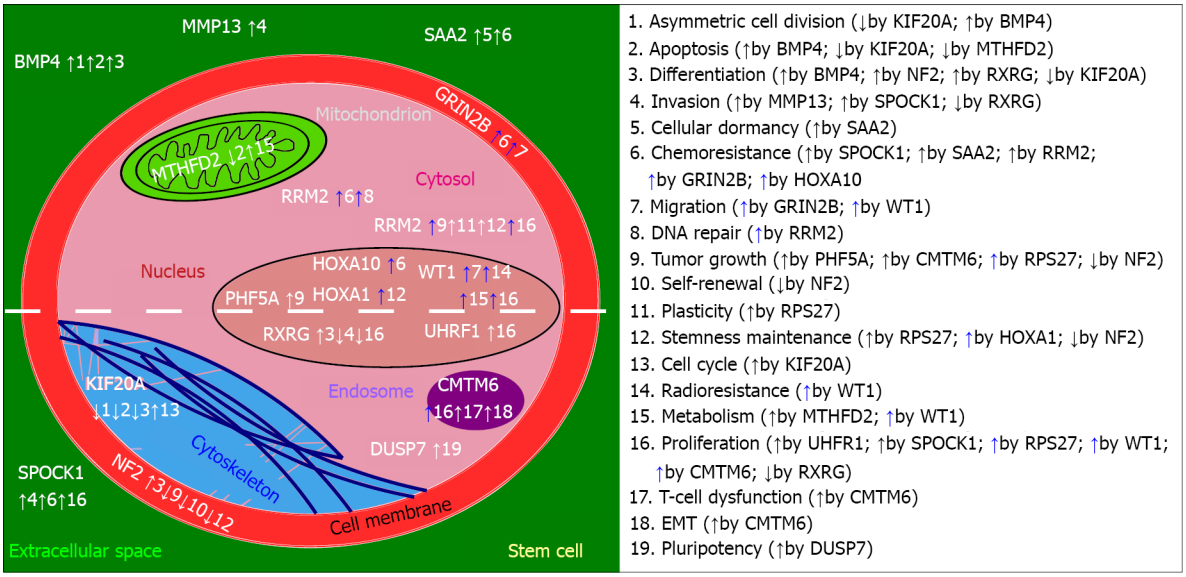
CONCLUSION

Taken together, a promising set of genes involved in cytoskeletal rearrangements and metabolic alterations were found to influence glioblastoma stemness *via* a plethora of biological processes. Most of the described genes exhibit pro-cancerous properties; among them, clinical trials on *GRIN2B*, *RRM2*, *WT1*, and *KIF20A* are ongoing and focus on selective inhibitors or peptide-based vaccines. Concerning tumor suppressors, the anti-cancer effect can also be achieved *via* delivery of recombinant proteins (*BMP4*), ligands for tumor suppressors (*RXRG*), or counteracting the pathways that become hyperactive following an anti-oncogene loss (*NF2*). The cytoskeletal phenomena currently linked to the described genes require experimental verification of their contribution to GSCs expansion. Future GBM stemness-related research should generally delve into cytoskeleton and related molecular events, since the concept is already encouraging.

Table 1 Clinical trials that utilize the products of described genes as drug components or targets

Gene	Compound	Condition	Trial number and phase	Intervention details
BMP4	hrBMP4	Glioblastoma	NCT02869243 (phase I)	Delivery of human recombinant BMP4
GRIN2B	EVT 101	Healthy volunteers (brain function assessment)	NCT00526968 (phase I)	Delivery of selective GRIN2B antagonist
RRM2	COH29	Solid tumors	NCT02112565 (phase I)	Delivery of ribonucleotide reductase inhibitor
WT1	DSP-7888	Gliomas (incl. GBM)	NCT02750891 (phase I/II)	Delivery of WT1 peptide-based cancer vaccine
KIF20A	KIF20A peptide	Small cell lung cancer	NCT01069653 (phase I)	Delivery of KIF20A peptide-based vaccination
NF2	IAG933	Solid tumors	NCT04857372 (phase I)	Not yet disclosed (the drug presumably counteracts the YAP/TAZ hyperactivity that occur following NF2 loss)
RXRG	*9-cis* retinoic acid	Breast cancer	NCT00001504 (phase I)	Delivery of RXRG ligand

NF2: Neurofibromatosis type 2 protein; BMP4: Bone morphogenetic protein 4; RXRG: Retinoid X receptor gamma; MMP13: Metalloproteinase 13; RRM2: Reductase subunit M2; SPOCK1: SPARC/Osteonectin; CWCV: Kazal-like domains 1; ECM: Extracellular matrix; WT1: Wilms' tumor protein 1; KIF20A: Kinesin family member 20A; GRIN2B: Glutamate ionotropic receptor NMDA type subunit 2B.



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Figure 2 Impact of described genes on biological processes related to stem cells. The "↑" or "↑" (blue) symbol indicates activation of the process while "↓"denotes inhibition. The impact of genes on processes (numbered from 1 to 19) is either directly confirmed (solid arrow next to the number) or recapitulated based on available data from various literature sources (dashed arrow next to the number). The "↓" (blue) symbol was not required as any gene inhibited the given process in an indirect manner. The white dashed line dividing the stem cell into two halves separates the genes with a confirmed role in glioblastoma stem cells (above the line) from those involved in cancer stemness outside the glioblastoma context (below the line). Figure created using Inkscape. NF2: Neurofibromatosis type 2 protein; BMP4: Bone morphogenetic protein 4; RXRG: Retinoid X receptor gamma; MMP13: Metalloproteinase 13; RRM2: Reductase subunit M2; SPOCK1: SPARC/Osteonectin; CWCV: Kazal-like domains 1; ECM: Extracellular matrix; CMTM: Chemokine-like factor superfamily.

FOOTNOTES

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REVIEW

Tissue-specific cancer stem/progenitor cells: Therapeutic implications

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Abstract

Surgical resection, chemotherapy, and radiation are the standard therapeutic modalities for treating cancer. These approaches are intended to target the more mature and rapidly dividing cancer cells. However, they spare the relatively quiescent and intrinsically resistant cancer stem cells (CSCs) subpopulation residing within the tumor tissue. Thus, a temporary eradication is achieved and the tumor bulk tends to revert supported by CSCs' resistant features. Based on their unique expression profile, the identification, isolation, and selective targeting of CSCs hold great promise for challenging treatment failure and reducing the risk of cancer recurrence. Yet, targeting CSCs is limited mainly by the irrelevance of the utilized cancer models. A new era of targeted and personalized anti-cancer therapies has been developed with cancer patient-derived organoids (PDOs) as a tool for establishing pre-clinical tumor models. Herein, we discuss the updated and presently available tissue-specific CSC markers in five highly occurring solid tumors. Additionally, we highlight the advantage and relevance of the three-dimensional PDOs culture model as a platform for modeling cancer, evaluating the efficacy of CSC-based therapeutics, and predicting drug response in cancer patients.

Key Words: Cancer stem cells; Therapy resistance; Tissue-specific cancer stem cell markers; Patient-derived organoids; Pre-clinical cancer models

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Core Tip: Therapeutic approaches targeting cancer stem cell (CSC) markers hold great promise toward developing effective anti-cancer treatment. Tissue-specific CSCs (TSCSCs) possess unique expression profile that allows for their identification, isolation, and targeting. TSCSCs, isolated from patient tumor tissues, were shown to form organ analogs or patient-derived organoids (PDOs) under specific culturing conditions *in vitro.* These models simulate the original tumor characteristics in a three-dimensional culture dish. As such, PDOs have the potential to be used in patient-specific *in vitro* drug clinical trials and proof-of-concept studies on CSC-targeted therapies.

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INTRODUCTION

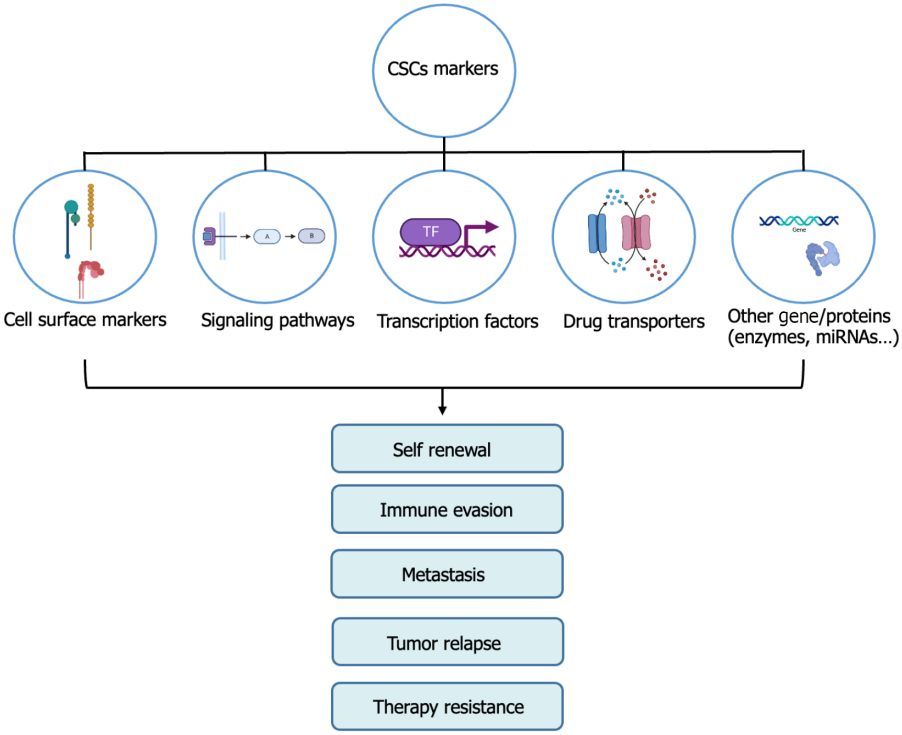
Cancer disease remains a leading cause of death worldwide. Despite significant progress directed toward developing anti-cancer therapies, the successful management of cancer remains impeded by multiple challenges, including metastatic dissemination, conventional-therapy resistance, and disease relapse[1,2]. Accumulating evidence suggests that the cancer stem cells (CSCs) subpopulation plays a vigorous role in sustaining the tumorigenic properties, thus contributing to tumor re-growth and progression[3] (Figure 1). This subpopulation of multipotent cells possesses unique properties of self-renewal and differentiation and is capable of extensively proliferating and generating different lineages of cancerous cells, which constitute the tumor bulk and contribute to the heterogeneous phenotype found in tumors[2,4].

CSCs may arise from the transformation of normal stem cells (SCs) found within tissues or from the de-differentiation of differentiated cells[5]. They were first identified in acute myeloid leukemia[6], and compelling evidence later showed that they exist in a variety of solid tumors where they act as key drivers of tumor progression and metastasis[7,8]

CSCs harbor multiple resistance mechanisms that enrich cancer hallmarks and result in the failure of conventional anti-cancer therapies. One underlying mechanism is the disrupted intracellular pathways that profoundly control CSCs behavior. For instance, overexpression of the Notch pathway plays a dual role that is context and cell-type-dependent, acting either as an oncogene or tumor suppressor[9-11]. In the context of CSCs, the Notch pathway has been implicated in proliferation, angiogenesis, metastasis, stemness maintenance, tumor immune evasion, and resistance to radiation[9,11-13]. Moreover, the Wnt pathway has been linked to the activation of dormant CSCs, their proliferation, maintenance, and inhibition of apoptosis. This pathway also plays a role in the metastasis and de-differentiation of CSCs [14,15]. Besides, the Hedgehog pathway is associated with increased proliferation, maintenance, and self-renewal of CSCs, as well as their migration, invasiveness, and resistance to chemotherapy[14,16,17]. Additionally, the NF-κB pathway is implicated in self-renewal, maintenance, and inhibition of apoptosis of CSCs, as well as regulation of epithelial to mesenchymal transition (EMT), angiogenesis, and metastasis[18]. Finally, the aberrant expression of the JAK/STAT3 pathway promotes cell survival and stemness properties, as well as metastasis and resistance to chemotherapy[14,19]. The intrinsic regulation of CSCs also occurs at the level of stemness-related transcription factors (TFs) such as OCT-4, SOX2, KLF4, c-MYC, STAT3, and NANOG, as well as epigenetics and epi-transcriptomics, which contribute to stemness maintenance and plasticity of CSCs[11]. Additionally, CSCs are regulated at an extrinsic level by their microenvironment, specifically by cancer-associated fibroblasts and tumor-associated macrophages. The tumor microenvironment is a major player in modulating CSCs resistance, metastasis, and heterogeneity[11,20].

The resistance mechanisms of CSCs further include their overexpression of DNA repair genes, resulting in resistance to radiotherapy and other DNA-damaging agents[21]. Also, they express upregulated multidrug efflux pumps such as ATP-binding cassette (ABC) transporters that mediate the active transport of chemotherapeutic drugs out of the cell[22]. CSCs were shown as well to overexpress aldehyde dehydrogenases (ALDHs) which are enzymes involved in the detoxification of aldehydes, chemotherapeutic agents, and reactive oxygen species[23]. Another mechanism that promotes the survival of CSCs is their ability to exist at a reversible quiescent state in the G_0 phase, which contributes to their drug resistance since most chemotherapeutic agents target highly proliferative tumor cells[24]. Thus, standard therapies succeed at reducing tumor size but tend to spare the highly resistant CSCs subpopulation. The successful elimination of tumors, therefore, necessitates targeting the residual dormant CSCs to yield long-lasting eradication of cancer and prevent relapse.

In this review, we provide a recapitulation of the main tissue-specific CSC (TSCSC) biomarkers in five of the most diagnosed solid tumors. Importantly, we highlight the beneficial role of these CSCs in



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Figure 1 Schematic presentation of cancer stem cell markers and their contribution to cancer development, progression, and resistance to therapy. Several cancer stem cell (CSC) markers and regulatory signaling pathways are involved in the sustenance and activation of self-renewal, immune evasion, and tumor metastasis, and contribution to tumor re-growth and therapy resistance. CSC markers serve as potential therapeutic targets for cancer treatment. CSCs: Cancer stem cells.

providing relevant preclinical cancer models and thus improving CSC-targeted therapies.

TISSUE-SPECIFIC CANCER STEM CELLS

Given the importance of CSCs in tumor progression and prognosis, several attempts were made to identify and isolate CSCs from the tumor mass based on the markers they express. CSCs express a wide spectrum of markers, some of them being more universal than others. Several markers, mostly located on the cell surface, are often used in combination to ensure a more tissue-specific isolation of targeted CSCs. Here we provide an updated overview of the most prominent TSCSC surface markers, focusing on five solid cancers (prostate, colon, bladder, breast, and lung). Refer to Table 1 for the full list of markers.

Prostate-specific cancer stem cells

The presence of prostate CSCs (PCSCs) was identified by Collins *et al*[25] using SCs markers (integrin $\alpha_2\beta_1$ and CD133) that were previously identified in the normal prostate epithelium[25,26]. This subpopulation of PCSCs isolated from human prostate cancer (PC) biopsies showed a high expression of CD44, CD133, and integrin $\alpha_2\beta_1$. The isolated cells exhibited high proliferative ability and were highly invasive on Matrigel™. Moreover, they possessed a high self-renewal ability and could also differentiate into cells expressing the same phenotype as PC cells, thus re-establishing the original heterogeneous tumor from which they were isolated[27].

CD133 (Prominin-1), a cell surface glycoprotein, remains one of the most used biomarkers to identify and isolate PCSCs either alone or in combination with other markers. In fact, $CD133^+$ PC cells that were isolated from human PC cell line exhibited self-renewal ability, which was correlated with their expression of stemness genes[28]. These cells could also generate a heterogeneous tumor mass when transplanted into immunocompromised mice. Moreover, they displayed high clonogenic abilities and led to the formation of tumor spheres (prostaspheres) that were more malignant than the ones formed by $CD133^-$ PC cells. Furthermore, the $CD133^+$ cells were chemo-resistant and demonstrated high proliferation[28]. Interestingly, a well-established combination of $CD133^+$ and $CD44^+$ PC cells allowed the isolation of PCSCs and the formation of spheroids characterized by heterogeneous PC cells[29].

Table 1 Summary of the most prominent biomarkers required to identify and isolate the tissue-specific cancer stem cells in prostate, colon, bladder, breast, and lung tumors

TSCSCs markers	PCSCs	CCSCs	BCSCs	BrCSCs	LCSCs	Ref.
CD24	-	+	+	-		[64,90,159,160]
CD26		+				[161]
CD29	+	+		+	+	[99,162-164]
CD44	+	+	+	+	+	[31,63,74,108,165]
CD47			+	+	+	[78,166,167]
CD49b (integrin α_2 or ITGA2)	+	+	-	+	+	[168-171]
CD49f (integrin α_6 or ITGA6)	+	+	+	+	+	[99,169,172,173]
CD51	+	+				[69,174]
CD61				+		[99]
CD66c		+	-			[84,175]
CD67LR			+			[84]
CD87					+	[116]
CD90		+	+	+	+	[99,110,176,177]
CD117	+			+	+	[38,116,178]
CD126		+		+	+	[179-181]
CD133	+	+	+	+	+	[28,51,87,99,107]
CD151	+					[35]
CD166	+	+		+	+	[46,104,182,183]
CD326 (EpCAM or ESA)	+	+	+	+	+	[48,56,116,184,185]
Integrin $\alpha_2\beta_1$	+	+				[27,186]
TRA-1-60	+	+				[35,187]
Trop2	+					[45]
CXCR4	+	+		+	+	[102,162,188,189]
ABCB5		+				[73]
ABCG2	+	+	+	+	+	[49,102,177,190,191]
MAGE-A3			+			[177]
GLDC					+	[102]
ALDH	+	+	+	+	+	[44,68,96,102,177]
BCMab1			+			[79]
Lgr5		+		+	+	[53,99,192]
Prox1	+	+				[70,193]
EMA (MUC1)	+	+	-			[77,194,195]
E-cadherin	+	+				[196,197]
ZEB-1	+	+		+	+	[198-200]
PSA	-					[201]
CK5	+		+	+	+	[117,202-204]
CK17			+			[89]
CK18	-		-	-		[89,205,206]

CK20		-	[89]
Ar-v7	+		[207]

+: Over-expressed; -: Under-expressed; Blank: Not found in the literature/controversial. TSCSCs: Tissue-specific cancer stem cells; PCSCs: Prostate cancer stem cells; CCSCs: Colon cancer stem cells; BCSCs: Bladder cancer stem cells; BrCSCs: Breast cancer stem cells; LCSCs: Lung cancer stem cells; EpCAM: Epithelial cell adhesion molecule; TRA-1-60: T cell receptor alpha locus; ALDH: Aldehyde dehydrogenase; EMA: Epithelial membrane antigen.

CD44 (also referred to as P-Glycoprotein 1) is a transmembrane glycoprotein that interacts with several extracellular matrix components, such as collagen, hyaluronic acid, osteopontin, and matrix metalloproteinases. It is one of the most conventional markers used to identify and isolate PCSCs. The expression of CD44 allowed the isolation of cells that were able to differentiate into all types of PC epithelium leading to complete reconstitution of the original tumor bulk when injected into immunocompromised mice[30]. Notably, $CD44^+$ PC-derived cells expressed elevated levels of several mRNAs associated with stemness[31]. This marker was also associated with several aspects of PC tumorigenesis including proliferation, invasion, adhesion, EMT initiation, metastasis, and therapy resistance[32].

T cell receptor alpha locus (TRA-1-60) is a carbohydrate addition to podocalyxinis, which is a cell surface antigen that belongs to the CD36 family. TRA-1-60 is expressed on pluripotent SCs conferring them the ability to induce differentiation. TRA-1-60 was shown to be overexpressed in PC cells as compared to the adjacent normal prostate tissue, which qualifies it as a favorable marker to specifically target PCSCs while sparing normal cells[33]. Moreover, it was detected in the peripheral blood of patients with metastatic PC[34]. The isolation of TRA-1-60^+ cells led to the generation of spheres and initiation of PC in a more efficient manner as compared to other known PCSCs markers. TRA-1-60 was then combined with two other markers of PCSCs (CD166 and CD151) leading to a more enhanced sphere-forming ability. Furthermore, the injection of the triple-marker-positive cells was able to form tumors with at least 5-fold more efficiency as compared to TRA-1-60^+ cells alone[35].

CD117 (also termed c-Kit) is a member of the Type-III tyrosine kinase receptors known to be involved in several cancer mechanisms by binding to its stem cell factor (SCF) ligand[36]. CD117 overexpression was detected in PC[37]. A recent study suggested that CD117 may be considered a potential marker for PCSCs because it was shown to display a broad spectrum of tumorigenic abilities[38]. In fact, CD117 stimulated PC cell proliferation and migration. Moreover, $CD117^+$ cells were able to form 1.35-fold larger prostaspheres as compared to $CD117^-$ cells. Most importantly, $CD117^+$ cells expressed stemness genes and their implantation into immunocompromised mice led to PC initiation[38].

CD49f (integrin α6 or ITGA6) is a transmembrane glycoprotein that was demonstrated to be a putative marker of PCSCs. $CD49f^{high}$ cells were shown to be tumor-initiating cells in the Pten-null PC model[39]. Moreover, CD49f was shown to be the most selective marker for targeting colony-forming cells[40]. Additionally, it was expressed on the surface as well as in the middle of prostatospheres[41]. Importantly, the expression of CD49f allowed the isolation of sphere-forming SCs[42].

In addition to the ones discussed above, there are several markers that can be used to target PCSCs including ALDH1A1 (ALDH 1 family member A1)[43,44], trop-2 (Tumor-associated calcium signal transducer 2)[45], CD166 (activated leukocyte cell adhesion molecule)[46,47], EpCAM (Epithelial cell adhesion molecule)[48], and ABCG2 (ATP binding cassette super-family G member 2)[49].

Colon-specific cancer stem cells

Colon CSCs (CCSCs) were first identified and isolated by Ricci-Vitiani *et al*[50] after the injection of colon cancer (CC) $CD133^+$ cells into immunocompromised mice, which led to the generation of the original tumor mass contrary to their $CD133^-$ counterparts. The $CD133^+$ cells were able to exponentially grow *in vitro* as undifferentiated spheres while preserving the same phenotypic properties of the initial colon tumor[50]. O'Brien *et al*[51] in 2007 also showed that all CC-initiating cells were $CD133^+$ cells that were able to either maintain themselves as undifferentiated CCSCs or to differentiate and therefore sustain the tumor heterogeneity[51].

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) (also recognized as FEX; HG38; GPR49; GPR67) is a seven-transmembrane G-protein coupled receptor. LGR5 is an "orphan" receptor abundantly expressed in active SCs of the intestinal crypts[52]. LGR5 was shown to be overexpressed in CC[53]. A growing body of evidence supports the idea that LGR5 is a main marker of CCSCs. For instance, human $LGR5^+$ CC cells were visualized as the CSC pool in proliferating CC tissue[54]. Furthermore, LGR5 was demonstrated to be a marker of tumor-initiating cells, where implantation of $LGR5^+$ cells was able to form colon tumors, indicating that LGR5 provides a dynamic stemness characteristic in CC[55]. Additionally, LGR5 was correlated with tumor proliferation due to the ability of $LGR5^+$ cells to form more multipotent spheres as compared to $LGR5^-$ cells[56]. Notably, LGR5 was shown to be involved in the colony formation capacity of CCSCs[56,57]. Importantly, LGR5 was found to have an essential role in CC metastasis where organoids derived from $LGR5^+$ cells led to liver cancer formation in the absence of a primary tumor[55]. In addition, LRG5 was selected to be the most suitable CSC marker that identifies immature cancer cells in regional lymph nodes of CC patients[58].

EpCAM (also known as CD326) is a Type-I transmembrane glycoprotein that serves as an epithelial cell adhesion molecule. Interestingly, EpCAM along with its reprogramming TFs were shown to be overexpressed in CC-initiating cells leading to a high self-renewal ability and increased invasiveness [59]. In fact, EpCAM was considered to be a robust CCSCs marker[60]. Indeed, it was used along with CD133 and CD44 to initiate CC in mice[61]. Furthermore, EpCAM provided more enhanced CSC-like properties when combined with LRG5 and CD44[56]. Moreover, EpCAM was proven to promote CC invasion and metastasis, as $EpCAM^{high}/CD44^{+}$ cells were visible in corresponding liver metastasis regions of CC patients[62].

CD44 was also shown to be a robust marker for CCSCs. In fact, a single $CD44^{+}$ cell was able not only to generate a sphere, but also to form a tumor with similar characteristics as the primary one from which it was isolated[63]. Moreover, the expression of CD44 was correlated with CC proliferation[4]. Furthermore, CD44 was reported as a stemness marker in spherical clusters[64]. In addition, CD44 was considered a reliable marker for the prediction of hepatic cancer metastasis in CC patients[65].

ALDH1 is also selected as a potential marker for CCSCs. ALDH1 expression increased during CC tumorigenesis and the implantation of only 25 $ALDH1^{+}$ cells into immunocompromised mice led to the generation of xenograft tumors even in the absence of other CCSCs markers such as CD133 and CD44 [66]. Furthermore, ALDH1 expression conferred high tumorigenic abilities and chemo-resistance to CC cell lines[67]. Interestingly, ALDH1 was linked to lymph node and vascular invasion in CC patients[68].

Among the most specific CSCs related to CC are LGR5, CD44 and EpCAM. However, the combination of multiple markers allows more accurate detection of CSCs which was proven when LGR5, CD44 and EpCAM resulted in more potent CSCs properties as compared to each marker alone [56]. Other markers are also attributed to CCSCs such as CD59[69], Prox1 a regulator of Notch-independent $LGR5^{+}$ SCs[70,71], CD24[4,64], CD166[72], and ABCB5 (ATP binding cassette super-family B member 5)[73].

Bladder-specific cancer stem cells

Bladder CSCs (BCSCs) were first isolated in 2009 by using markers for normal basal bladder SCs ($CD44^{+}$). It was found that the $CD44^{+}$ subpopulation of bladder cancer (BC) cells was 10 to 200 more likely to form tumors in immunocompromised mice in comparison with their CD44 counterparts[74]. Additionally, $CD44^{+}$ BCSCs efficiently maintained the heterogeneity of the initial tumor mass after serial transplantation[74].

Epithelial membrane antigen (EMA, also known as MUC1) is a membrane-bound glycoprotein that belongs to the family of mucins[75]. EMA^{+} bladder cells are usually located in the mature differentiated layer of the urothelium, whereas EMA^{-} cells are found in the basal layers, where SCs reside. It was demonstrated that EMA^{-} BC cells had a greater colony-forming ability when compared with the unsorted BC population[75,76]. BCSCs can thus be identified through the combination of EMA^{-} and $CD44^{+}$ BC cells[77].

CD47 (also known as integrin associated protein) is a transmembrane protein overexpressed on the surface of $CD44^{+}$ BCSCs compared to the $CD44^{-}$ subpopulation and was thus hypothesized to be a BCSCs marker[78,79]. CD47 acts like a "don't eat me" signal by interacting with the signal regulatory protein-1 receptor on the surface of macrophages and neutrophils. Thus, CD47 has an immunosuppressive role, protecting the BSCSC from phagocytosis[78,79], that makes it a promising target for cancer therapy[80,81].

ALDH1A1 has also been used to isolate BCSCs. In fact, $ALDH1A1^{+}$ cells retained the stem-cell ability to divide asymmetrically, yielding both $ALDH1A1^{+}$ and $ALDH1A1^{-}$ cells[82]. Additionally, $ALDH1A1^{+}$ BCSCs exhibited a greater tumorigenic potential both *in vitro* (sphere formation ability) and *in vivo* (xenografts in immunocompromised mice) compared to $ALDH1A1^{-}$ BC cells[82]. Knocking down the ALDH1A1 gene in BCSCs reduced their proliferation, confirming the key role played by the ALDH enzyme in BCSCs division and renewal[83]. Furthermore, ALDH1A1 BCSCs maintained the original tumor heterogeneity after sequential transplantations into immunocompromised mice[83]. Finally, $ALDH^{+}$ BCSCs demonstrated an enhanced ability to migrate and invade tissues contrary to $ALDH^{-}$ BC cells[82].

$67LR^{+}$ (67KDa Laminin Receptor)/ $CD66c^{-}$ (also known as CEACAM6) BC cells were demonstrated to have stemness properties. These markers, similar to CD44, are also present in normal bladder SCs[84]. He *et al*[85] showed that $67LR^{+}$ BCSCs were 5 to 10 times more potent in initiating tumors *in vivo* compared to $67LR^{-}$ ones[85]. In addition, $67LR^{+}$ BCSCs expressed a panel of genes involved in stemness and resistance to chemotherapy and radiation[85,86]. Similarly, $CD66c^{-}$ cells were demonstrated to be more tumorigenic than the $CD66c^{+}$ counterparts[85].

$CD133^{+}$ BC cells were shown to upregulate the expression of genes involved in pluripotency. This subpopulation of BC cells was also more resistant to the chemotherapeutic agent cisplatin and to radiation. Additionally, $CD133^{+}$ BCSCs exhibited a greater tumorigenicity both *in vitro* and *in vivo*, as well as a more aggressive proliferation in immunocompromised mice in comparison to $CD133^{-}$ BC cells [87].

Additional markers are also used for the identification of BSCSC namely MAGE-A3 (Melanoma antigen family A, 3)[88], BCMab1[79], and several members of the cytokeratin family of proteins ($CK5^{+}$, $CK17^{+}$, $CK18^{-}$, $CK20^{-}$)[89].

Breast specific cancer stem cells

The importance of breast CSCs (BrCSCs) markers was first demonstrated by Al-Hajj *et al*[90] only a subpopulation of human breast cancer (BrC) cells appeared to lead to the formation of tumors in immunocompromised mice. Al-Hajj *et al*[90] isolated $ESA^+CD44^+CD24^{-/Low}$ cells from human BrC tissue, and showed that as low as 200 of these cells were enough to initiate cancer in immunocompromised mice, whereas more than 50000 BrC cells with a different phenotype were unable to form tumors[90].

CD44 and CD24 are often used in combination to detect and isolate BrCSCs[91]. In addition to its key role in adhesion, cell survival, metastasis and angiogenesis, CD44 act as a TF to regulate metastasis and stemness of BrCSCs[92,93]. On the other hand, CD24 is a cell surface adhesion glycoprotein which plays a key role in cell-cell and cell-extracellular matrix (ECM) interactions[94,95]. Even though CD24 is overexpressed in a number of cancers (including BrC), only $CD44^+CD24^{-/Low}$ BrCSCs were able to form tumors in immunocompromised mice[90]. $CD44^+CD24^{-/Low}$ BrCSCs were also shown to be more resistant to chemotherapy[91].

ALDH1 has also been used to target BrCSCs, as it was shown that $ALDH1^+$ BrC cells were more resistant to chemotherapy and were able to form tumors in immunocompromised mice in comparison to $ALDH1^-$ cells[96]. ALDH1 is essential for the early development of the stemness properties of BrCSCs [97]. Interestingly, the subpopulation of BrC cells expressing ALDH1 is distinct from the $CD44^+CD24^{-/Low}$ BrCSCs, with minimal overlap between the two (approximately 1%)[91]. Moreover, $ALDH1^+/CD44^+$ BrCSCs were highly tumorigenic, with a higher metastatic potential, and greater resistance to cancer therapies[91].

To date, CD44, CD24 and ALDH1 remain the most used biomarkers to isolate BrCSCs. Although there is little overlap between $CD44^+CD24^{-/Low}$ and $ALDH1^+$ BrCSCs, cells that share all three markers were more tumorigenic[98]. Moreover, the CD44/CD24 markers were more associated with cell proliferation and tumorigenesis while the ALDH1 marker was positively correlated with tumor metastasis [98]. Nonetheless, other markers have been studied and found suitable for the identification of BrCSCs, such as CD133 (in triple negative BrC; TNBC), GD2 (ganglioside in TNBC), CD49f, $CD61^+$ (β3 integrin in Her2 BrC), CD29 (β1 integrin), CD90, and EpCAM[99-101].

Lung cancer stem cell markers

Lung cancer is histologically divided into non-small cell lung carcinoma cells (NSCLC) and small cell lung carcinoma (SCLC)[102]. Due to a higher incidence and the greater ease to obtain NSCLC tissue, NSCLC CSCs (referred to afterward as lung CSCs; LCSCs) markers have been better characterized.

CD166 (also known as ALCAM) has also been associated with stemness properties of NSCLC. CD166 is a member of the immunoglobulin superfamily of cell adhesion molecules and participates in both homophilic and heterophilic interactions. Additionally, CD166 plays an important role in migration and invasion of LCSCs[103]. CD166 was characterized by Zhang *et al*[104] as the most robust cell marker for isolating LCSCs among other candidates (CD44, EpCAM and CD133)[104]. In contrast to $CD166^-$ NSCLC cells which failed to form tumors *in vivo*, $CD166^+$ LCSCs were able to initiate tumors in immunocompromised mice. Furthermore, $CD166^+$ NSCLC cells had enhanced self-renewal properties and were able to consistently form spheres *in vitro*.

The $CD133^+$ subpopulation of NSCLC cells were able to indefinitely divide and form spheres in an *in vitro* setting, whereas $CD133^-$ NSCLC cells were characterized by a slow growth and an inability to form spheres[105]. These results also parallel the *in vivo* ability of $CD133^+$ LCSCs to form tumors in immunocompromised mice compared to $CD133^-$ cells; the $CD133^+$ xenografts were histologically similar to the initial cancer mass[105,106]. Moreover, the expression of CD133 in LCSCs was associated with increased resistance to chemotherapy and radiation[105,107]. Finally, $CD133^+$ LCSCs are more prone to metastasize than their $CD133^-$ counterparts, especially to lymphoid organs. In fact, detection of $CD133^+$ metastatic NSCLC in lymph nodes is indicative of a poor prognosis[107].

CD44 has also been studied as a marker to isolate LCSCs. Accordingly, $CD44^+$ NSCLC cells demonstrated a greater ability to form spheres *in vitro* and to initiate tumors in immunocompromised mice in comparison to $CD44^-$ cells. Additionally, $CD44^+$ LCSCs upregulated several stemness TFs to maintain their pluripotent properties. $CD44^+$ LCSCs were also more resistant to the chemotherapeutic agent cisplatin compared to $CD44^-$ cells[108]. Moreover, the expression of CD44 in LCSCs was associated with an enhanced ability to metastasize and invade tissues[20].

CD90 (also known as Thy-1) is a glycosylphosphatidylinositol-anchored surface protein that is involved in cell-cell as well as cell-ECM interactions[109]. Initial studies have shown that $CD90^+$ NSCLC cells demonstrated greater self-renewal and proliferative properties and expressed a higher level of stemness genes. Additionally, when compared to a control, as few as 5000 $CD90^+$ LCSCs were able to initiate tumors in immunocompromised mice, indicating the stronger tumorigenicity associated with CD90[110].

ALDH1 was also suggested to be a LCSCs marker. Indeed, $ALDH1^+$ LCSCs exhibited enhanced proliferative abilities and self-renewal properties[111,112]. Accordingly, knocking down the ALDH1A3 gene greatly reduced the tumorigenicity and clonogenicity of LCSCs[113]. In addition, $ALDH1^{high}$ LCSCs also showed greater resistance to chemotherapeutic drugs in comparison to $ALDH1^{low}$ cells[112]. Interestingly, the overexpression of the TAZ oncogene induces the formation of LCSCs by activating the

ALDH1 gene[114] ALDH1 also appears to play a key role in chemoresistance as its inhibition leads to the re-sensitization of LCSCs to cisplatin[115].

Of note, additional markers have been used to isolate LCSCs. These include but are not limited to CD47, CD87, CD117, EpCAM, and CK5[116,117].

TSCSCS' BENEFICIAL ROLE IN CANCER MODELING FOR THERAPEUTIC IMPLICATIONS

The conception of CSCs-targeted therapies relies on employing the above-mentioned CSCs' resistant characteristics and markers, which allows for CSCs' isolation, enrichment, characterization, and targeting[118]. CSCs-based therapeutic strategies include selectively targeting the stemness markers, such as the TSCSCs' surface markers, TFs, ABC transporters, and ALDHs[14,119]. As well as, the disrupted signaling pathways that enrich CSCs'-resistant features and contribute to their survival, proliferation, self-renewal, and differentiation. Also, targeting the tumor microenvironment components which acts as a foster niche in protecting CSCs[14,119].

In spite of the significant advances in CSCs' research and the great interest in drug discovery, there are currently few therapeutic approaches that have reached the late clinical stages. Many CSCs-targeting therapeutics performing remarkably *in vitro* and *in vivo* cultures have faced multiple hurdles in clinical trials[14,120]. One major reason behind this is the irrelevance of the preclinical cancer models being used[121-123]. Thus, more relevant CSCs models, that reflect the original tumor behavior of the individual patients, might strengthen the rationale for developing effective CSCs-targeted therapeutic modalities and complement more conventional cancer therapies.

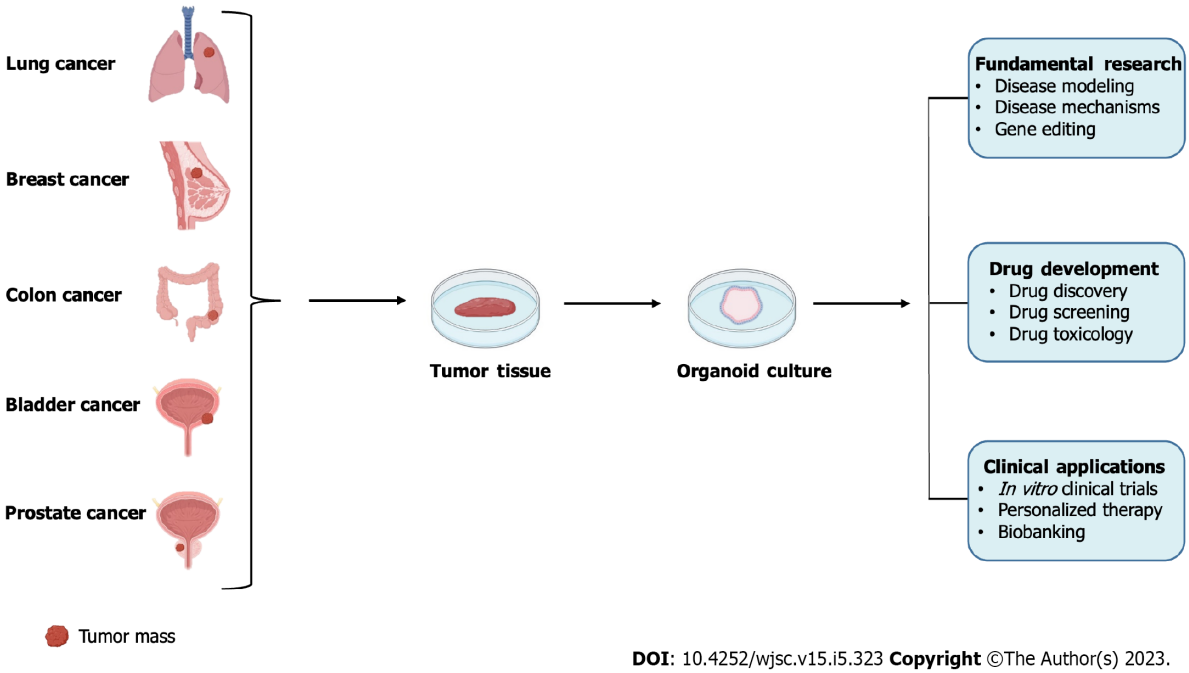
A new era of targeted and personalized anti-cancer therapies has evolved with the three-dimensional (3D) patient-derived organoids (PDOs)[124]. This versatile technique relies on the exclusive ability of SCs to give rise to organ-like structures known as organoids[125]. Sato *et al*[126] established the first organoid model with small intestinal crypt $LGR5^+$ SCs[126]. Subsequently, models of normal and cancer PDOs from multiple tissues were derived successfully[127-134].

The formation of the 3D microscopic organoids from patient tumor tissues is accomplished using specific culturing conditions that are designed to preserve the CSCs component of the patient's tumor [135]. The formed PDOs, hence, recapitulate the structural and functional complexity constituting the originating tumor, mediated by the CSCs' ability for self-renewal and differentiation into multiple cell types[136,137]. PDOs tool allows the modeling of human carcinogenesis in an *in vitro* culture dish[138, 139]. Precisely, the process followed to generate cancer PDOs includes utilizing a tumor tissue sample, surgically isolated from a cancer patient, and dissociating it into single-cell suspension using mechanical dissociation and enzymatic digestion methods. The heterogeneous population of cells obtained, containing TSCSCs, is then cultured in proper culturing conditions to allow the self-organization of cells into functional units or tissue-specific architectures; organ analogs. The suspended culturing system includes the usage of biological or synthetic hydrogel scaffolds that mimic the natural ECM components. In addition to using a specific culturing medium that contains a cocktail of growth factors and inhibitors to imitate the organ stem cell niche, allow the generation of distinct component lineages, and stimulate the long-term expansion of organoids[140,141].

As PDOs are CSCs-based structures and replicate faithfully the heterogeneity and histological characteristics of the original cancers, they gain superiority over other models in terms of mimicking tumor microenvironments, facilitating the formation of ECM, exhibiting adequate proliferation rates with representative cellular morphology, maintaining the expression of 'stemness-related' markers and genes, and demonstrating a realistic individualized drug response[142-144]. This nominates PDOs to be ideal preclinical drug-response models for providing perspectives for testing novel CSCs-targeted therapies and evaluating the potential drug effectiveness in cancer patients (Figure 2).

PDOs technique generally shares several main steps but differs in varying degrees depending on the type of tissue being processed. Scaffold-based techniques are mostly adopted in culturing PDOs, where Matrigel™ is commonly used. The latter is a mixture of heterogeneous and gelatinous proteins secreted by mouse sarcoma cells. It comprises mainly adhesive proteins such as laminin, collagen IV, entactin, and heparin sulphate glycoprotein, which resemble the ECM and provide interactive and structural support to the cells[145-148]. Moreover, the universal organoid medium used in the culturing system adopts the first protocol developed by Sato *et al*[126] which includes advanced DMEM/F12 medium supplemented with epidermal growth factor, Noggin (NOG), and Wnt agonist R-spondin-1[126,127]. Other factors were then added including anaplastic lymphoma kinase 3/4/5 inhibitor A83-01, dihydrotestosterone, fibroblast growth factor-10, fibroblast growth factor-2, prostaglandin E2, nicotinamide (NAM), and p38 inhibitor SB202190, N-acetylcysteine (NAC), B27 supplement and Rho kinase inhibitor Y-27632 to culture PDOs successfully[149].

To date, organoids derivation from multiple human tumors including prostate, colon, bladder, breast, and lung cancers has been described, with varying success rates[133,150-155]. The established PDOs are subjected to tissue-specific genes and lineage markers expression studies to confirm that they represent the original tumor of the patient. Importantly, the cancerous origin of these organoids is confirmed by



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Figure 2 Schematic presentation of patient-derived organoid applications in cancer research. Patient-derived organoid (PDO) models can be utilized in multiple fields of cancer research including fundamental research, drug development, and clinical application. Cancer PDOs have been used to simulate the tumor tissue *in vitro*, study the disease mechanisms and gene expression patterns, and expose them to different drugs for efficacy screenings and drug discovery validations. Organoids are further used as *in vitro* pre-clinical models for personalized medicine and the generation of 'living' organoid biobanks. PDO culturing system serves as an advanced tool in the implementation and development of precision medicine.

checking for the CSCs markers specific to each tumor tissue. The patient drug response to the therapy of interest can then be evaluated primarily by assessing the organoids' formation efficiency and size.

For example, a study done by Cheaito *et al*[150] established a minimum of 5-factor medium including NAC, NOG, A83-01, B27, and NAM to grow and maintain PC PDOs. Histopathological, transcriptomic, immunofluorescent, and immunohistochemical studies showed that the formed PDOs mimicked the histological architecture and prostate lineage profiles of their corresponding tissue specimens. This was confirmed by the presence of both prostate epithelial lineages, as the organoids stained positive for the luminal- (CK8, AR, and PSA) and basal- (CK5, CK14, and p63) specific markers. In addition, an intermediate cell population, co-expressing luminal CK8 and basal CK5 markers was also detected. Interestingly, CSCs markers, CD44 and CD49f, positive staining demonstrated the existence of putative stemlike cells within the bulk of the PDOs. Furthermore, differential drug response, between different patient samples, was recognized upon treatment with chemo-, radio-, and androgen-deprivation therapies[150]. In another study, Monzer *et al*[151] succeeded in establishing and propagating PDOs that model CC disease. The formed organoids recapitulated the architecture and the characteristics of CC tissues as revealed by the co-expression of the epithelial marker lineage CK19 and the CSC surface marker CD44. The organoids derived from different patients showed to exhibit different responses to Diiminoquinone treatment tested alone or in combination with Fluorouracil (5FU) chemotherapeutic drug. Similarly, Al Bitar *et al's* study showed different responses to individual and combination treatments of radiation and Thymoquinone in CC PDOs[152].

Moreover, Yu *et al*[153] utilized BC PDOs to evaluate chimeric antigen receptor (CAR)-T cell-mediated cytotoxicity against BC. Analysis was done to confirm that the established organoids recapitulate the heterogeneity and the key features of the parental BCs. Based on a set of luminal (CK20, uroplakin II, and GATA3) and basal markers (CK5, P63, and CD44), the formed organoids were classified into luminal or basal subtypes, respectively. All the BC PDOs and their corresponding tumors expressed Ki67 and E-cadherin, confirming their epithelial origin and high proliferative ability. Additionally, the specific surface antigen profiling of each tumor sample was analyzed, and the MUC1 antigen was shown to be highly expressed among all tested antigens, in both the cancer tissues and their derived organoids. MUC1 was then used as a putative target to test the efficacy of second-generation CAR-T cells in BC PDOs[153]. Furthermore, a promising study done by Chen *et al*[154], showed the significance and applicability of using BrC PDOs as pre-clinical models for broader cancer studies, and more specifically as a tool to provide personalized therapy recommendations for patients with advanced refractory disease. This study focused mainly on deriving PDOs from specimens isolated from patients with advanced clinical features, including drug-resistant and metastatic BrC. The histopathological, immunohistochemical, and genomic characteristics were shown to be well inherited by the formed PDOs from the drug-treated as well as treatment-naïve tumors. Distinctive drug

responses were also observed[154]. Furthermore, Kim *et al*[133] demonstrated the distinctive therapeutic responses of LC and normal bronchial PDOs, derived from patient tissues comprising five histological subtypes of LC and non-neoplastic bronchial mucosa. The differential responses to the tested drugs were shown to be affected by the individual genomic alterations profile. The PDOs were also proved to duplicate the tissue architecture and maintain the genomic alterations of the parental lung tumors during long-term expansion *in vitro*[133].

CONCLUSION

In this review, we have discussed briefly some of the CSC features that are known to account for cancer resistance and relapse and make CSCs promising anti-cancer targets. Additionally, we have summarized the updated list of the TSCSC molecular markers in prostate, colon, bladder, and lung tumors that are significant to selectively isolate and therapeutically target the CSCs subpopulation. Besides, we highlighted the advantage of utilizing the CSC-based PDO models to simulate carcinogenesis and predict patient-specific drug responses *in vitro*.

Despite the present challenges[156,157], PDOs are highly credible models that possess more physiological and pathological relevance than traditional ones. This robust method proved to faithfully maintain the histological, genetic, and stemness characteristics of their respective native tissues. Interestingly, the CSCs profile mimicked by the PDOs can serve as a platform for testing CSCs-targeted therapeutics. To our knowledge, there are no clinical trials discussing cancer PDOs in a preclinical context for testing CSC-targeted therapeutics[158].

Indeed, PDOs have prospective applications in patient-specific *in vitro* drug clinical trials and proof-of-concept studies on CSC-targeted therapies and -resistance mechanisms. If remarkable advancements are made, cancer patients will ultimately benefit from this radical technology.

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REVIEW

Advancements in adipose-derived stem cell therapy for skin fibrosis

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Abstract

Pathological scarring and scleroderma, which are the most common conditions of skin fibrosis, pathologically manifest as fibroblast proliferation and extracellular matrix (ECM) hyperplasia. Fibroblast proliferation and ECM hyperplasia lead to fibrotic tissue remodeling, causing an exaggerated and prolonged wound-healing response. The pathogenesis of these diseases has not been fully clarified and is unfortunately accompanied by exceptionally high medical needs and poor treatment effects. Currently, a promising and relatively low-cost treatment has emerged-adipose-derived stem cell (ASC) therapy as a branch of stem cell therapy, including ASCs and their derivatives-purified ASC, stromal vascular fraction, ASC-conditioned medium, ASC exosomes, *etc.*, which are rich in sources and easy to obtain. ASCs have been widely used in therapeutic settings for patients, primarily for the defection of soft tissues, such as breast enhancement and facial contouring. In the field of skin regeneration, ASC therapy has become a hot research topic because it is beneficial for reversing skin fibrosis. The ability of ASCs to control profibrotic factors as well as anti-inflammatory and immunomodulatory actions will be discussed in this review, as well as their new applications in the treatment of skin fibrosis. Although the long-term effect of ASC therapy is still unclear, ASCs have emerged as one of the most promising systemic antifibrotic therapies under development.

Key Words: Adipose-derived stem cell; Cicatrix, hypertrophic; Keloid; Scleroderma, localized; Stromal vascular fraction; Exosomes

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Core Tip: Pathological scarring and scleroderma are the most common conditions of skin fibrosis with high medical needs and poor therapeutic effects. Adipose-derived stem cell (ASC) therapy has emerged as a promising treatment for skin fibrosis. Here, we discuss the possible mechanism of skin fibrosis as well as the latest research about the mechanism of ASC therapy and its application in treating these conditions. ASC therapy provides a brand-new insight into the treatment of skin fibrosis.

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INTRODUCTION

Skin fibrosis is characterized by fibroblast proliferation and extracellular matrix (ECM) deposition. In severe cases, it can lead to pathological changes in the skin, such as keloid and hypertrophic scars (HS), systemic sclerosis (SSc), and scleroderma[1,2]. The fact that there are no practical disease-modifying therapies for those diseases and current treatment is mainly toward managing symptoms and relieving complications calls for a new therapy[3,4].

Since 2001, when adipose-derived stem cells (ASCs) were first characterized, ASCs have been broadly studied and applied as the most promising sources of cells with regenerative and multilineage characteristics[5]. In recent years, various ASC derivatives, which are rich in not only ASCs but also other cellular and tissue components, have been seen as possible alternatives to ASCs and have received increasing attention for exploring their potential applications. Due to their immunomodulatory properties and abundance of growth factors[6,7], ASCs and their derivatives have become new remedies in the treatment of skin fibrosis[8-10].

In this review, we discuss the mechanism of skin fibrosis and the mechanism of ASC therapy. We then summarize the application of ASCs and their derivatives in skin fibrosis. Finally, we retrospectively describe the safety of ASC therapy and predict the future of skin fibrosis treatments.

MECHANISM OF SKIN FIBROSIS

Many fundamental studies exploring the molecular mechanisms underlying fibrosis have revealed a large number of genes, molecules, and cell types that may contribute to this problem[11,12].

Keloid and HS

The pathogenesis of keloids and HS is not fully understood due to the complex dynamic process of wound healing. However, among all the factors that stimulate fibroblasts to differentiate into myofibroblasts and produce excessive amounts of collagen and ECM, the role of the inflammatory response is increasingly considered important[1,11,13]. Downregulation of proinflammatory cytokines such as interleukin 6 (IL-6) and IL-8[14] and upregulation of anti-inflammatory cytokines such as IL-10 may reduce scar tissue formation[15]. Additionally, inflammatory cells such as macrophages, T cells, and mast cells, all increase and take part in a variety of biological activities in keloids and HS[1,16]. Although multiple intracellular signaling pathways such as Smad, signal transducer and activator of transcription 3, and extracellular signal-regulated kinase 3, are involved in hypertrophic scar formation, transforming growth factor-β (TGF-β)/Smad is thought to be a driving force[17,18]. Thus, the basic purpose of current prevention and therapy methods is still to reduce inflammatory processes[19].

SSc and scleroderma

SSc is an immune-mediated rheumatic disease that is characterized by excessive collagen from myofibroblasts in the skin and some internal organs, microangiopathy, and impairment of the humoral and cellular immunity system[20,21]. Scleroderma features, without the involvement of internal organs, are similar to SSc[4]. SSc pathogenesis involves early vasculopathy and innate and adaptive immune system dysfunction[12]. Initial vasculopathy and immune system dysfunction are both involved in SSc pathogenesis and cause SSc inflammation and tissue fibrosis[22]. Immune cells, endothelial cells, and fibroblasts interact with each other and release cytokines and growth factors[21]. Workers are convinced that type-1-interferon and interferon-inducible genes play a role in SSc pathogenesis[23]. Additional important factors include platelet-derived growth factor, endothelin 1, insulin-like growth factor 1, and TGF, which is thought to be a major regulator of fibrosis pathways[24]. Combined treatment that targets epigenetic/genetic, vascular, and immunologic defects and progressive fibrosis is urgently needed[12,

21,25].

MECHANISM OF ASC THERAPY

ASCs have long been thought to have immune privileges as mesenchymal stromal cells, which do not induce a severe allogeneic response when injected into another organism[26,27]. However, they have been demonstrated to evoke cellular and humoral responses in vivo, which may lead to the rapid elimination of transplanted cells[27]. However, ASCs function primarily through a "hit-and-run mechanism" with consequently a small effect on therapeutic efficacy, at least in the short or middle term [28,29]. Indeed, most ASCs do not require cell-to-cell contact to function but rather function through paracrine mechanisms that release cytokines, growth factors, and extracellular microvesicles in the surrounding environment[30]. As reported, the therapeutic effect of ASCs and their derivatives depends on paracrine secretion[31-34]. ASC-secreted active substances such as cytokines[35], growth factors[36], chemokines[37], and extracellular vesicles[38], regulate the microenvironment around fibroblasts and themselves[39,40] (Figure 1).

Regulation of the microenvironment

Immunomodulation and anti-inflammatory: After injection, ASCs activate adaptive cellular responses, secreting IL-1, prostaglandin E2 (PGE2), IL-4 and IL-10, and TGF-β, which modulate and stimulate innate immune cells[41]. It was reported that ASCs suppress $CD4^+$ and $CD8^+$ T-cell expansion and differentiation while promoting regulatory T-cell proliferation and enhancing their immunosuppressive activity[42]. Additionally, ASCs secrete immunosuppressive substances such as nitric oxide, PGE2, hepatocyte growth factor (HGF), and indoleamine 2,3-dioxygenase, which downregulate TGF-β in skin fibrosis and attract bone marrow (BM) cells involved in tissue repair[43,44].

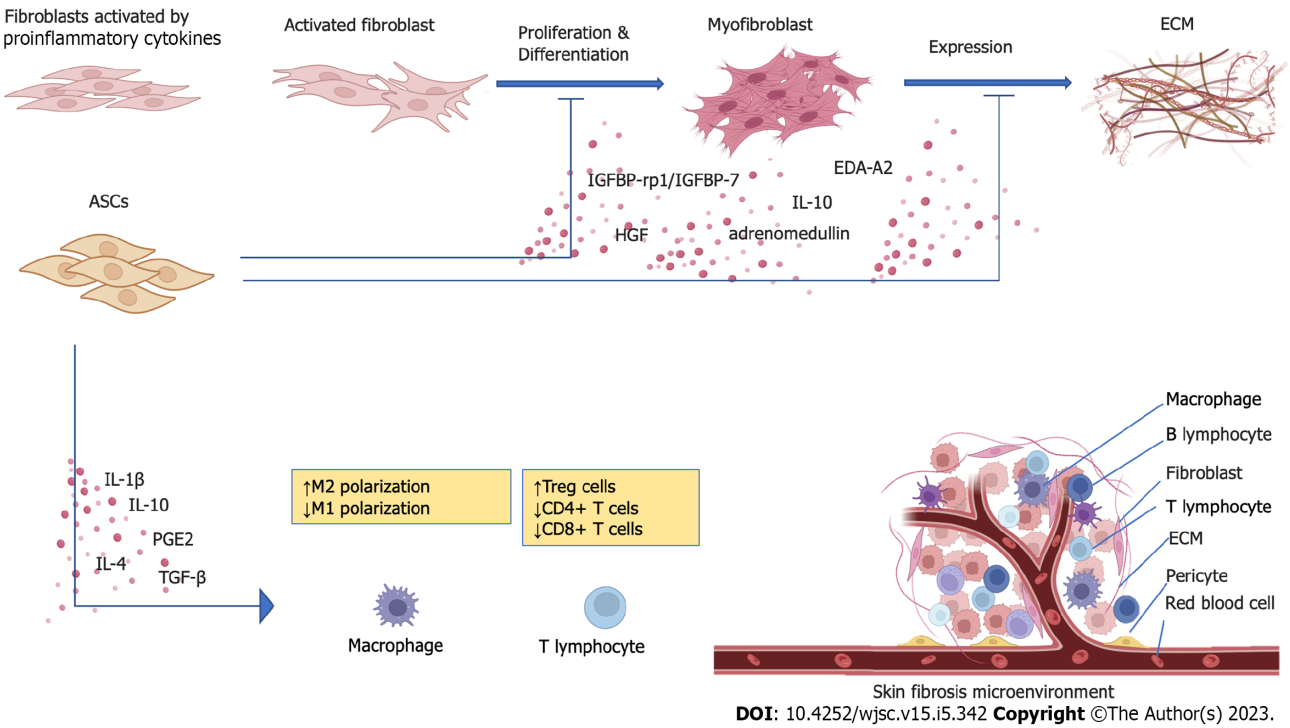
Despite their immunomodulatory ability, the anti-inflammatory effects of ASCs have been gaining increasing attention. ASCs can drive anti-inflammatory M2 macrophage polarization and ameliorate macrophage infiltration[34,45]. Additionally, in a rabbit model of HS, ASCs mediated the inhibition of M1-polarized macrophages and defection of inflammation. Moreover, the expression of inflammatory cytokines and proteins such as IL-6 and monocyte chemotactic protein-1, which affect inducible nitric oxide synthase and cyclooxygenase-2, was notably decreased in the treated groups[46-48].

Angiogenic effects: The angiogenic effects of ASCs have been broadly discussed with regard to myocardial infarction, nerve injury, and tissue transplantation[49-52]. The secretion of vascular endothelial growth factor (VEGF) as well as the transcription of angiogenic genes are improved by ASCs [52,53]. ASC transplantation greatly improves revascularization and tissue perfusion in ischemic scars by stimulating endotheliocyte proliferation in blood vessels, hastening the resumption of blood circulation, providing oxygen and nutrition, and improving scar texture[54]. There is also an interplay between ASCs and endothelial precursor cells (EPCs). Growth factors produced by ASCs, such as VEGF, increase the migration and survival of EPCs, while EPC-produced platelet-derived growth factor BB stimulates ASC proliferation and migration[36].

Regulation of fibroblasts

Proliferation and differentiation: Activated dermal fibroblasts change their phenotype into myofibroblasts in response to injury or stress, which increases their expression of α-smooth muscle actin (α-SMA) and contractile ability[55,56]. Previous studies have demonstrated that ASC conditioned medium (ASC-CM) contains abundant growth factors and cytokines, such as IL-10, adrenomedullin, and HGF[7,57]. HGF, proven to inhibit fibroblast differentiation into myofibroblasts, contributes to limiting the profibrotic functions of myofibroblasts[58,59]. It has also been reported that ectodysplasin-A2, insulin-like growth factor binding protein-related protein-1/insulin-like growth factor-binding protein-7 (IGFBP-rp1/IGFBP-7), and thrombospondin-1 are increased in concentration in serum-starved ASC-CM, which could play a role in the inhibition of fibrosis[60]. These ASC-secreted immunosuppressive substances suppress fibrosis by various mechanisms, including reducing the expression of TGF-β1 and collagen and promoting the expression of matrix metalloproteinases (MMP), thus significantly repressing the activity of fibroblasts *in vitro* and *in vivo*[34,61].

Expression of ECM: The synthesis of collagen, hyaluronic acid, and fibronectin by myofibroblasts, in particular, is essential for the prolonged and excessive formation of ECM constituents[56,62]. Inhibition of HS-derived fibroblast (HSF) proliferation and reduction in α-SMA, type I collagen, and type III collagen expression can partly explain the molecular mechanism of the effects of ASCs on HSs[46,63,64]. In another study, ASC-CM reduced the synthesis of collagen and the expression of connective tissue growth factor, fibronectin, and α-SMA[65]. However, in a coculture model of ASCs and normal human dermal fibroblasts, ASCs increased the formation of collagen types I, III, and VI in the ECM[66]. It appears that ASCs could target abnormal fibroblasts and reduce pathological deposition of ECM.



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Figure 1 The brief mechanism of adipose-derived stem cell therapy in skin fibrosis. ECM: Extracellular matrix; ASC: Adipose-derived stem cell; HGF: Hepatocyte growth factor; IGFBP-rp1/IGFBP-7: Insulin-like growth factor binding protein-related protein-1/Insulin-like growth factor-binding protein-7; EDA-A2: Ectodysplasin-A2; IL-10: Interleukin 10; IL-4: Interleukin 4; IL-1β: Interleukin 1β; TGF-β: Transforming growth factor beta; PGE2: Prostaglandin E2. Figure 1 is created with BioRender.com.

ASC THERAPY APPLICATION

ASC therapy, including the application of ASCs and their derivatives, can be roughly divided into ASC-based therapy and stem cell-free therapy. ASC-based therapy is mainly composed of various ASCs and stromal vascular fractions (SVFs), which have been broadly studied and applied in the clinic (Figure 2). Stem cell-free therapy, such as exosomes and ASC-CM, is increasingly popular, with fewer moral and safety concerns.

ASCs

One of the most promising stem cell groups, ASCs, are abundant in adipose tissue, easy to extract, and have few adverse effects. Compared to BM-mesenchymal stem cells, ASCs exert potent anti-inflammatory and remodeling properties with similar therapeutic effects[30].

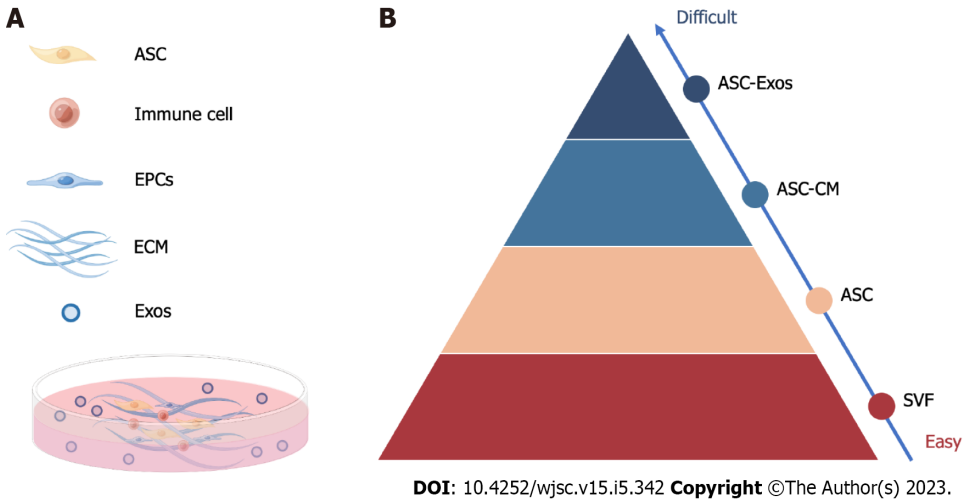
Intralesional injection of ASCs reduces the formation of scars while improving color quality and scar pliability, potentially leading to an effective and novel anti-scarring therapy[59,67,68]. These studies revealed that ASCs not only inhibited fibroblast proliferation and migration but also reduced the expression of molecules such as TGF-β1 and Notch-1. The antifibrotic effect on fibroblasts was most likely mediated by the inhibition of multiple intracellular signaling pathways[18,65].

As they are inherently heterogeneous, different ASC subgroups have been studied in the hope of finding suitable subgroups for specific diseases.

A subpopulation of ASCs that are positive for $CD74^+$ possesses enhanced antifibrotic abilities both *in vitro* and *in vivo*. Additionally, $CD74^+$ ASC-assisted fat grafts reduce dermal thickness and fibrosis in radiation-induced fibrosis mouse models[69]. Another $CD73^+$ ASC subpopulation has expressed significantly lower levels of procollagen lysyl hydroxylase 1, a potent stimulator of fibrosis, showing better therapeutic effects on wound healing[70].

To modify or enhance some properties of ASCs and overcome the limitations of curative effects of ASCs only, ASCs are coated or activated with small molecule drugs or genetically overexpressing molecules that are involved in fibrosis formation.

After overexpressing MMP-3, ASCs-MMP-3 possess not only the ability of ASCs to accelerate wound healing but also the capability of MMP-3 to reduce scarring[71]. Compared with mASCs alone, migration ability and HGF production are significantly higher in mASCs activated with LMWH, showing higher anti-inflammatory and anti-fibrotic capability, and might be a promising candidate for SSc treatment[72]. IL-10-ASCs have been proven to have the capacity to suppress the development of HS by reducing inflammation during wound healing as well as the proliferation and migration of HSFs that produce ECM[73]. Poly(3-hydroxybutyrate-cohydroxy valerate) loaded with ASCs contains the



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Figure 2 The culture and classification of adipose-derived stem cells and their derivatives. A: Culture of adipose-derived stem cells (ASC) and their derivatives; B: Classification of ASC and their derivatives. EPCs: Endothelial precursor cells; ECM: extracellular matrix; Exo: Exosomes; ASC: Adipose-derived stem cell; ASC-CM: ASC-conditioned medium; ASC-Exo: ASC exosomes; SVF: Stromal vascular fraction. Figure 2 is created by Figdraw.

bioactive cues required to improve wound healing and scarring[74].

SVF

SVF is an aqueous fraction that contains ASCs, EPCs, endothelial cells, macrophages, smooth muscle cells, lymphocytes, pericytes, and preadipocytes, among other components. The advantages of SVF over ASCs are thought to be in two areas. First, the heterogeneous cellular composition of SVF may be responsible for the superior therapeutic results seen in comparative animal studies. Second, in contrast to ASCs, SVF can be obtained significantly more quickly without the need for cell separation or special cultivation conditions. As a result, the therapeutic cellular product is relatively safe and is only required to meet minimal regulatory requirements[75]. However, it should be emphasized that whereas ASCs are useful for both allogeneic and autologous treatments, SVF is only appropriate for autologous treatments because it contains a variety of cell types that are known to trigger immunological rejection. hASCs have seemed to be more effective than SVF in HS, related to their higher levels of MMP-2 and MMP-2/tissue inhibitors of metalloproteinase-2 ratio, as well as higher expression of TGF-3 and HGF[76]. Whether SVF is indeed superior to ASCs in skin fibrosis treatment needs further research[6,77].

In addition to HS and keloids, SVF is also broadly applied clinically to scleroderma and SSc. SVF gel has superior anti-inflammatory and antifibrotic effects on scleroderma[78]. Moreover, SSc does not impair SVF's ability to heal vascular damage, hence justifying the use of this novel autologous biotherapy[79]. SVF injection is a potentially effective treatment that seems to last for at least one year. Quality of life, Raynaud's phenomenon, finger edema, and hand impairment and discomfort were significantly improved[80-83].

Stem cell-free therapy

The secretome of ASCs, with a focus on exosomes, appears to be a suitable and safe alternative with more effectiveness and fewer adverse effects due to restrictions on the use of stem cells in cell-based treatment. Moreover, the ability to biobank the ASC secretome is a significant benefit of cell-free therapy. In this review, we concentrate on the current understanding of the secretome of ASCs, such as ASC exosomes (ASC-Exos) and ASC-CM, used in skin fibrosis stem cell-free therapy (Figure 3).

ASC-Exo: As one of the components of paracrine signaling, ASC-Exos are small, single membranous secretory organelles rich in proteins, lipids, nucleic acids, and carbohydrate conjugates[26,84,85]. Among other research discoveries, they are thought to have a variety of activities, such as reshaping the ECM and transmitting signals and molecules to other cells. In addition, they are not rejected by the immune system, have homing effects, and the dose is easily controlled[86,87]. Compared to ASCs, ASC-Exos offer a great opportunity to create new cell-free therapeutic techniques that could circumvent the challenges and dangers related to using natural or synthetic stem cells[86,88].

ASC-Exos release miR-29a-3p, which can suppress the expression of several profibrotic, antiapoptotic, remodeling, and methylase genes[89]. ASC-Exos are now a viable new option for the systemic treatment of keloids. They significantly suppress the development of ECM in keloids by decreasing collagen synthesis and impairing the microvessel structure, enhancing the expression of TGF-3 while inhibiting the protein expression of Smad3 and Notch-1[84]. By suppressing the expression of the TGF-1/Smad pathway, ASC-EXOs may prevent keloid fibroblasts from proliferating and migrating and consequently promoting death[90].

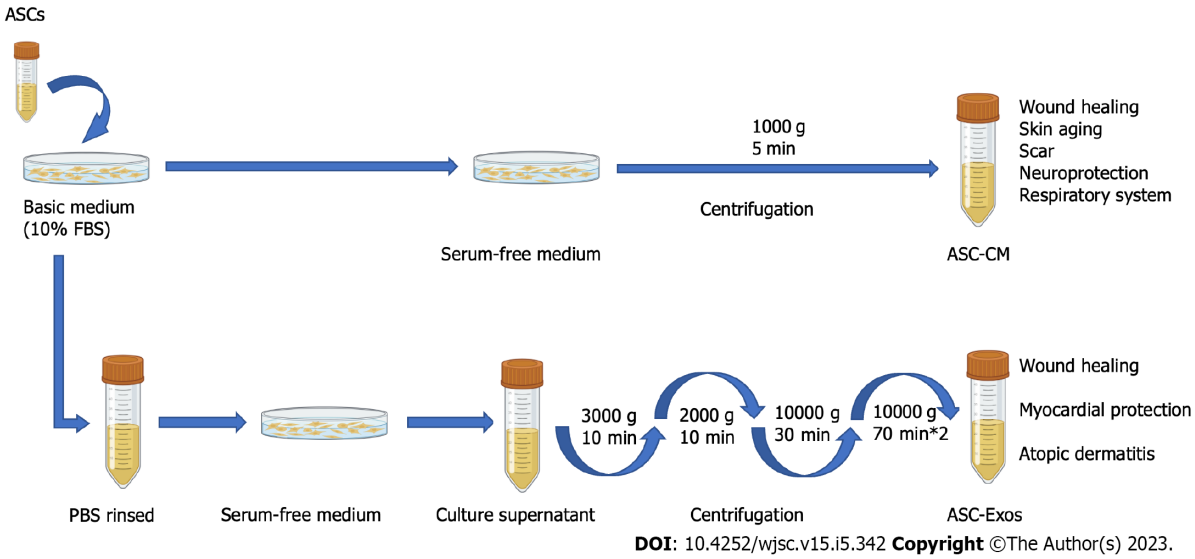


Figure 3 Adipose-derived stem cell conditioned media, exosomes, and adipose tissue extracts synthesis and therapeutic application. ASC: Adipose-derived stem cell; ASC-CM: ASC-conditioned medium; ASC-Exo: ASC exosomes; FBS: fetal bovine serum; PBS: Phosphate-buffered saline. Figure 3 is created with BioRender.com.

In hypertrophic scar fibrosis, ASC-exosomal miR-192-5p targeted IL-17RA to control the Smad pathway, and miR-29a inhibited the TGF-2/Smad3 signaling pathway, which could be responsible for the antifibrotic effects[91,92]. Another postoperative study showed that hASC-Exo therapy inhibited collagen deposition and myofibroblast aggregation *in vivo* and reduced the development of HS[93].

ASC-CM: Active chemicals released by ASCs, such as cytokines, exovesicles, exosomes, DNA, and RNA, are found in ASC-CM and can facilitate tissue healing and control immunity. ASC-CM can lower treatment costs and avoid the safety issues associated with stem cell therapy[94]. One disadvantage of CM over stem cells is the short life of active components. Stem cells can anchor inside a tissue or organ after local administration and function there for a long time, but CM-containing substances such as growth or enzyme factors are rapidly diluted and eliminated by diffusion[95,52].

ASC-CM may reduce collagen deposition and scar formation, inhibiting the p38/mitogen-activated protein kinase signaling pathway can have an anti-scarring effect, and the use of ASC-CM may offer a unique therapeutic approach for the treatment of HS[96]. According to *in vitro* and *ex vivo* experiments, chyle fat-derived stem cell-CM reduced the expression of type I collagen (Col1), type III collagen (Col3), and SMA, which prevents fibrosis in HSFs[63]. ASCs-CM dramatically elevated MMP-1 expression and dose-dependently decreased cell survival, expression of fibrosis markers, tissue inhibitor of metalloproteinases-1, the amount of collagen produced, and the ratio of Col1/Col3. These findings show that ASC-CM efficiently blocks fibrosis-related factors and controls ECM remodeling in HSF[64]. Combining ASC-CM with therapeutic therapies is another development. A histologic study revealed that ASC-CM increased the density of cutaneous collagen and elastin and arranged them in a certain order. A good combination therapy for treating atrophic acne scars and skin rejuvenation is ASC-CM with FxCR[97]. Stronger antifibrotic effects of CD74+ ASC-conditioned media may have resulted from increased production of HGF, FGF2, and TGF-3 and lower levels of TGF-β1[69]. ASC-CM and polysaccharide hydrogels might cross-bind in situ, which could significantly improve the therapeutic results by reducing scar proliferation, offering a promising alternative for the prevention of HS[98].

UPDATES ON THE CLINICAL APPLICATIONS OF ASC THERAPY

To evaluate the effectiveness of ASCs, numerous clinical trials have been carried out; however, they have largely focused on SSCs. More research is required to determine the long-term safety of ASCs, detailed mechanisms of effect, and the capacity to translate experimental results into clinical practice.

ASCs are used to treat secondary-progressive multiple sclerosis in 30 individuals. However, assessments of treatment efficacy revealed a mild tendency toward effectiveness. Establishing the possible therapeutic benefit of this technique would require larger studies and presumably treatment at earlier stages[99].

To compare the effectiveness of an injection of ASC-SVF derived from adipose tissue with placebo in decreasing hand disability in 40 SSc patients. This research demonstrated a gradual improvement with no evidence that the AD-SVF was superior. Given the limitations of this trial, a study with a larger

group of patients is urgently needed to accurately determine the value of ASC-SVF therapy[100]. It was revealed through a randomized controlled trial that regional adipose tissue grafting is beneficial in repairing ischemia digital ulcers in SSc[101].

This study investigates the safety and efficacy of administering autologous SVF cells to SSc patients. Early evaluations at six months suggest a possible efficacy that has to be confirmed in a larger population randomized placebo-controlled trial. Quality of life, Raynaud's phenomenon, finger edema, and hand impairment and discomfort are significantly improved[83]. A sequential 12-mo follow-up showed significant improvement in the vascular suppression score, skin sclerosis, motion and strength of the hands, and finger edema. The decrease in hand discomfort was statistically significant. A benefit was found in daily tasks, housework, and social activities, according to the questionnaire[82].

An open cohort study found that ASCs dramatically reduced the consequences of orofacial fibrosis in SSc. With the inhibition of fibroblast proliferation and important fibrogenesis regulators, including TG-1 and CTGF, ASCs may alleviate skin fibrosis[95].

SAFETY ASSESSMENT

ASCs overcome the ethical issues associated with embryonic stem cells and are therefore considered safe. However, as a stem cell therapy, ASCs still have problems with storage and transport, as well as the risk of inducing tumors and malformations[102]. Further studies on their efficiency are yet needed, taking into account the host environment and patient-related factors. Importantly, a long-term follow-up is needed to supervise cancer recurrence rates in the context of previous malignancy[103].

CONCLUSION

While the underlying mechanism of skin fibrosis is still unclear, ASC therapy plays multiple roles in the treatment of skin fibrosis, with a combination of aesthetic and therapeutic outcomes. Different ASC derivatives show various properties, which might be further explored in clinical trials. In the future, ASC therapy is likely to become an indispensable part of combined treatment in skin fibrosis.

FOOTNOTES

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REVIEW

Modulation of stem cell fate in intestinal homeostasis, injury and repair

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Abstract

The mammalian intestinal epithelium constitutes the largest barrier against the external environment and makes flexible responses to various types of stimuli. Epithelial cells are fast-renewed to counteract constant damage and disrupted barrier function to maintain their integrity. The homeostatic repair and regeneration of the intestinal epithelium are governed by the $Lgr5^{+}$ intestinal stem cells (ISCs) located at the base of crypts, which fuel rapid renewal and give rise to the different epithelial cell types. Protracted biological and physicochemical stress may challenge epithelial integrity and the function of ISCs. The field of ISCs is thus of interest for complete mucosal healing, given its relevance to diseases of intestinal injury and inflammation such as inflammatory bowel diseases. Here, we review the current understanding of the signals and mechanisms that control homeostasis and regeneration of the intestinal epithelium. We focus on recent insights into the intrinsic and extrinsic elements involved in the process of intestinal homeostasis, injury, and repair, which fine-tune the balance between self-renewal and cell fate specification in ISCs. Deciphering the regulatory machinery that modulates stem cell fate would aid in the development of novel therapeutics that facilitate mucosal healing and restore epithelial barrier function.

Key Words: Intestinal stem cell; Epithelial repair; Homeostasis; Regeneration; Self-renewal; Apoptosis

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Core Tip: The homeostatic repair and regeneration of the intestinal epithelium upon injury are governed by the Lgr5$^+$ intestinal stem cells (ISCs) located at the base of crypts, which fuel rapid renewal and give rise to different epithelial cell types. We review the current understanding of the intrinsic niche signaling and extrinsic stimulating factors that control homeostasis and regeneration of the ISCs. Deciphering the regulatory machinery that modulates stem cell fate, and formulating strategies for better repair and regeneration would aid in the development of novel therapeutics that facilitate mucosal healing and restore epithelial barrier function.

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INTRODUCTION

Intestinal epithelium serves as the first line of defense against the external environment. As an outward single-layered epithelial structure, the intestinal mucosa withstands continuous mechanical, physico-chemical, and biological insults[1,2]. To counteract intestinal injury and preserve their barrier function, epithelial cells are renewed every 2–5 d in most adult mammals[3]. The epithelial turnover is coordinated by Lgr5$^+$ intestinal stem cells (ISCs) residing at the base of the crypts where they are kept in a multipotent state and produce transit amplifying (TA) progenitor cells. TA cells will undergo several cycles of division before migrating to the villi and ultimately differentiate into multiple lineages[4,5]. The disrupted barrier function and defective mucosal healing are the predominant biological features of intestinal pathology, and particularly, chronic gastrointestinal inflammation such as inflammatory bowel disease (IBD), which is represented by ulcerative colitis and Crohn's disease[6-8]. Current clinical strategies focus on the symptomatic relief and blockade of inflammatory progression[9,10], while better solutions should emphasize the motivation of regenerative response orchestrated by ISCs for complete mucosal healing.

Mucosal healing is an integrated network initiated by a series of biological processes and signals[11]. Intestinal homeostasis is characterized by constant regeneration which demands a fine-tuned balance between ISC proliferation and differentiation[12,13]. In response to diverse insults, the cellular response, combined with the stem cell niche adaptions, synthetically modulates the fate of ISCs to restore homeostasis by replenishment of damaged epithelial cells, or to hasten cell demise by impairment of cell function and vitality[14,15]. Therefore, understanding the cellular response and niche adaptations during injury-induced intestinal regeneration is therefore of importance for ISC biology.

Constant efforts have been made to exploit the regulatory mechanisms of critical components that seal the fate of ISCs. In this review, we give an overview of ISCs and review the adaptations and signals required for homeostasis maintenance. We focus on the cell fate specification and biological alterations of ISCs upon diverse insults and provide insights into intestinal regeneration.

INTESTINAL STEM CELL

Two distinct ISC populations located at the crypts have been proposed: Crypt base columnar (CBC) cells, the active cycling stem cells that facilitate homeostatic self-renewal[16], and +4 cells, the quiescent stem cells reserved for injury-induced repair[17].

CBC cells have been the centerpiece of stem cell research since they were initially identified in 1974 as continuously cycling cells at the base of the crypts[18]. Radionucleotide labeling and autoradiography have been used to state that the cells derived from the crypts migrate upward along the villi to be extruded at the villus tips[19,20]. This conveyor belt mechanism confirms stem cell fueling this rapid self-renewal process resides at the base of the crypts[21]. The generation of Lgr5$^{EGFP-IRES-CreERT2}$ mice reveals that Lgr5, a receptor for WNT signaling-associated R-spondins, is a highly suitable candidate for CBC cell recognition and specification[3]. Single-sorted Lgr5$^+$ stem cells are also able to form these crypt–villus organoids and the Lgr5 hierarchy is maintained in organoids[22]. Recent studies have identified that p27 and Mex3a label the slowly cycling subpopulation of Lgr5$^+$ ISCs based on single-cell transcriptome profiling[23,24].

In addition to Lgr5$^+$ CBC cells, Bmi1$^+$ cells localized at the fourth position of the crypt base and discovered by *in vivo* lineage tracing and transcriptome analyses, are a possible candidate stem cell population[4,25]. Functionally distinct from Lgr5$^+$ ISCs, the quiescent +4 stem cells are considered reserved stem cells that replenish the continuously cycling CBC cells pool when required, and are highly resistant to radiation and insensitive to Wnt signal[17,26]. In face of chemoradiotherapy, Lgr5$^+$

stem cells are vulnerable to chemical- or irradiation-induced injury, due to their predominantly cycling nature[24,27]. Bmi1$^+$ cells quickly revert to ISCs and the *de novo*-generated Lgr5$^+$ ISCs are vital for epithelial regeneration[28]. The evidence summarizes the relationship between active and quiescent stem cells and identifies Lgr5$^+$ stem cells as a substantial contributor to homeostatic regeneration[29].

The identification of new ISC markers and the dedication each subpopulation of ISCs commit to regeneration have improved the understanding of stem cell biology during homeostasis and disease. The emergence of new technologies has promoted the decoding of many key problems in intestinal diseases and tumors[4].

INTESTINAL STEM CELL NICHES

The niche in which ISCs reside can be defined as the microenvironment essential for self-renewal and stemness maintentance[13]. ISCs are strongly linked with adjacent cells of both epithelial and mesenchymal origin. These components, along with their communications, comprise the ISC niche[30,31]. The specific instructive microenvironment offers a native source of signals that fuel ISCs to maintain tissue homeostasis[32]. Various cell types of the niche elaborate typical paracrine signals containing Wnt, R-spondin, Notch, mammalian target of rapamycin (mTOR), bone morphogenetic protein (BMP), epidermal growth factor (EGF) and Hippo, which fine-tune the balance between differentiation and proliferation of ISCs, and ensure the production of an adequate number of cells in homeostatic and injury conditions[1,33] (Figure 1).

Wnt signaling

The canonical Wnt signaling acts as the prominent driver for ISC proliferation. Synchronous Wnt binding to Frizzled and to LRP5/6 suppresses APC-related ubiquitination of β-catenin which mediates its nuclear translocation, the association with lymphoid enhancer binding factor/T cell factor transcription factors, and the succeeding transactivation of Wnt target genes[34-36]. Multiple Wnts such as Wnt2b, Wnt4, and Wnt5a are abundantly expressed in intestinal stroma[30]. A subset of Foxl1$^+$ mesenchymal stromal cells that form a subepithelial plexus around the crypt is a crucial source of intestinal Wnt[37]. Genetic elimination of Foxl1$^+$ cells triggers the loss of Wnt family expression in the epithelium and an abrupt cessation of proliferation of both epithelial stem cell and TA progenitor cell populations, but not Paneth cell[38]. Wnt2b is highly expressed in Gli1$^+$ or αSMA$^+$ subepithelial stromal cells, which is sufficient to restore epithelial integrity when injected into mice which is devoid of Wnt secretion[39]. Gli1$^+$ subepithelial cells are essential contributors to the integrity of the colonic epithelium for Lgr5$^+$ ISC self-renewal in the colon[40]. As a noncanonical Wnt ligand, Wnt5a deficiency causes a failure to develop new crypts at the wound site and limits the proliferation of crypt cells after injury in a transforming growth factor (TGF)β-dependent manner[41]. These findings reveal the essential role of Wnt signal for the stemness and proliferation of ISC and highlight the contribution of stromal cells in the ISC niche.

The R-spondins comprise one of crucial elements of the niche. R-spondins are secretory glycoproteins which firmly cement the capacity of Wnt ligands for the activation of β-catenin-dependent transcription and canonical Wnt signaling, while R-spondins themselves have no intrinsic Wnt signaling activity[29,42]. Overexpressed R-spondins *in vivo* forcefully induce the expansion of ISCs and maintain the epithelial integrity against damage induced by the chemotherapeutic agent 5-fluorouracil, dextran sulfate sodium (DSS), or irradiation[43-45]. Wnt proteins are reported to be insufficient to directly regulate ISC self-renewal, while alternatively grant a fundamental competency through motivating R-spondin ligands to actively motivate ISC[46].

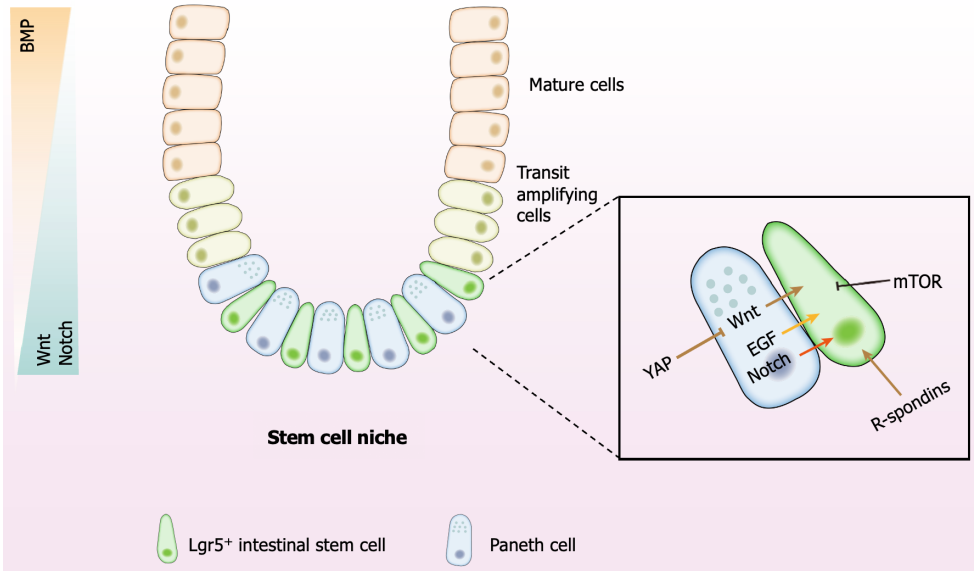
Notch signaling

Notch signaling plays a dominant role in the stem cell niche by preserving the quiescent state of ISCs [47]. Integration between Notch ligands (Notch1–4) and receptors (Jag1–2 and Dll1–4) in adjacent cells is required for Notch activation[48]. Different from Wnt signaling that is mainly generated from a stromal microenvironment, Notch signaling may function *via* neighboring epithelial cells or even stromal subpopulations contacting with ISCs, thus featuring an epithelial niche[49].

Disruption of Notch activity leads to the exhaustion of ISC and differentiation from proliferating TA cells to secretory cells[50]. Simultaneous Notch1/2 deletion recapitulates the global Notch inhibition phenotype of Lgr5$^+$ ISC loss, while the single deletion does not change ISC activity, which suggests the synthetical effect of Notch1/2 in stemness maintenance[51,52].

mTOR signaling

The mTOR signaling is a vital pathway for cellular development and metabolism in mammals. mTOR signaling directly modulates stemness and proliferation of ISCs, functioning as a crucial determinant of cell status within the ISC lineage and modulating differentiation in a nutrient-dependent way[53]. Inhibiting mTOR signaling helps to maintain stemness of ISCs, whereas activation of mTOR facilitates ISCs differentiation and proliferation[54,55]. In the case of caloric restriction, the activity of mTOR



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Figure 1 The intestinal stem cell niche and regulatory signals. The intestinal epithelium consists of crypts and villi. The crypts generate a constant stream of new cells that differentiate and migrate upward into the villi. These Lgr5+ intestinal stem cells reside at the bottom of the crypts and are wedged between Paneth cells, which protect and nurture stem cells. Above the stem cell zone is the lineage-committed progenitor cells, also known as the transit amplifying zone, divided to fuel the rapid epithelial cell turnover. Mature epithelial cells originate from the crypt and move up toward the villus tip. The Wnt and Notch signaling exhibit high activity in the stem cell niche. Activation of the signals decreases along with the increased distance from the crypt bottom. While the BMP activity stands in the opposite direction. WNT, R-spondin, epidermal growth factor, and mammalian target of rapamycin are secreted by Paneth cells or mesenchymal cells. YAP works through the Wnt signaling to maintain the crypt–villus integrity. Besides, Paneth cells provide essential Notch signals to stem cells by expressing Notch ligands. The signaling network in the niche establishes the baseline for self-renewal, fate determination, proliferation, and differentiation of intestinal stem cells. BMP: Bone morphogenetic protein; EGF: epidermal growth factor; mTOR: Mammalian target of rapamycin.

complex 1 is inhibited in Paneth cells, resulting in the paracrine release of cyclic ADP ribose that increases self-renewal of ISCs at the cost of differentiation[56]. In high relevance to diet, the mTOR pathway controls stem cell fate possibly by regulating mitochondrial metabolic states.

BMP signaling

BMP signaling acts as an initiator of differentiation in the crypt. Wnt and BMP signalings are deemed as opposite forces along the crypt–villus axis with counteractive gradients of activity[57]. BMP activity is lower in the bottom and higher towards the top of the villus[58]. To offset the inhibitory effects of BMP signaling on ISC fate, BMP antagonists like Noggin, Gremlin-1, and Gremlin-2 are highly expressed in the crypts, permitting the proliferation of ISCs. The BMP antagonists that enhance ISCs self-renewal are secreted by intestinal subepithelial myofibroblasts and smooth muscle cells[59,60].

EGF signaling

EGF is a vital component of the ISC niche[61]. The EGF receptor is abundantly expressed in CBCs, whereas its ligands are expressed in Paneth cells[62]. The activity of ErbB signaling is monitored by the negative regulation of Lrig1, a transmembrane protein coexpressed with Lgr5 in CBCs[62]. Loss of Lrig1 leads to the activation of receptors and a concomitant rapid expansion of crypts and cell numbers. Blockade of EGF signaling in intestinal organoids drives proliferative ISCs into quiescent state and stops organoid budding[63]. The evidence suggests the requirement of EGF in epithelial regeneration.

Hippo signaling

The Hippo pathway, a highly conserved signaling first described in *Drosophila* as an organ size control pathway, is comprised of a core kinase cascade, Mst1/2 and Lats1/2, which phosphorylate and suppress transcriptional coactivators Yes1 associated transcriptional regulator (YAP) and Tafazzin (TAZ), thereby modulating TEA domain transcription factor 1 (TEAD)-mediated transcriptional activation[64,65]. YAP/TAZ are the core components for stem cell-based regeneration. YAP overexpression in mice accelerates the self-renewal of colonic epithelium, and augments the number of proliferative cells and the cell migration along the crypt-villus axis, as detected by BrdU marker[43]. While YAP depletion causes a significant decrease in crypt proliferation, extensive crypt loss and consequently regeneration failure upon DSS or irradiation[66,67]. Loss of YAP activity contributes to higher sensitivity of ISCs to apoptosis and lower proliferative capacity during regeneration[68]. Moreover, the core Hippo kinases Lats1/2 are essential to maintain ISC activity and their deletion leads to the loss of ISCs[69]. This demonstrates their essential effects on epithelial proliferation and tissue regeneration.

YAP may actively block Wnt signaling and thus apply negative feedback on Wnt signaling *via* β-catenin inhibition[43], indicating the complex interaction between YAP and other niche signals which may need further investigation.

ENDOGENOUS AND ENVIRONMENTAL STIMULI

The intestinal epithelium is exposed to a hostile luminal environment, thus resulting in Lgr5$^+$ ISCs continuously encountering sources of stress to maintain dynamic homeostasis. We summarize the major endogenous and environmental stimuli that influence stem cell regenerative potential (Figure 2).

Endogenous factors

Niche signals: Damage to the crypts can result from infections, chronic inflammation, chemoradiotherapy, or traumatic injury and motivate a series of actions in the stem cell niche[31]. The microenvironment monitors the regenerative response *via* regulating a series of signaling pathways, such as Wnt, Notch, BMP, and Hippo. This has been discussed above.

Extracellular matrix: The state of stem cells largely depends on the properties of the extracellular matrix (ECM)[31]. The integrin complex assists cells in sensing the stiffness of the ECM and directs the fate of ISCs *via* adhesion signaling[70,71]. In this way, the ECM affects cellular behavior, including proliferation and differentiation. ECM stiffness is also a vital endogenous factor of mesenchymal stem cells to differentiate into osteoblasts, myoblasts, or neurons[72]. In particular, YAP/TAZ lay the foundation for ECM stiffness sensing and play a prominent role in intestinal repair and regeneration[73]. High matrix stiffness significantly enhances ISC expansion in a YAP-dependent manner[74]. These clues speculate that ECM sensation is capable of modulating self-renewal in ISCs.

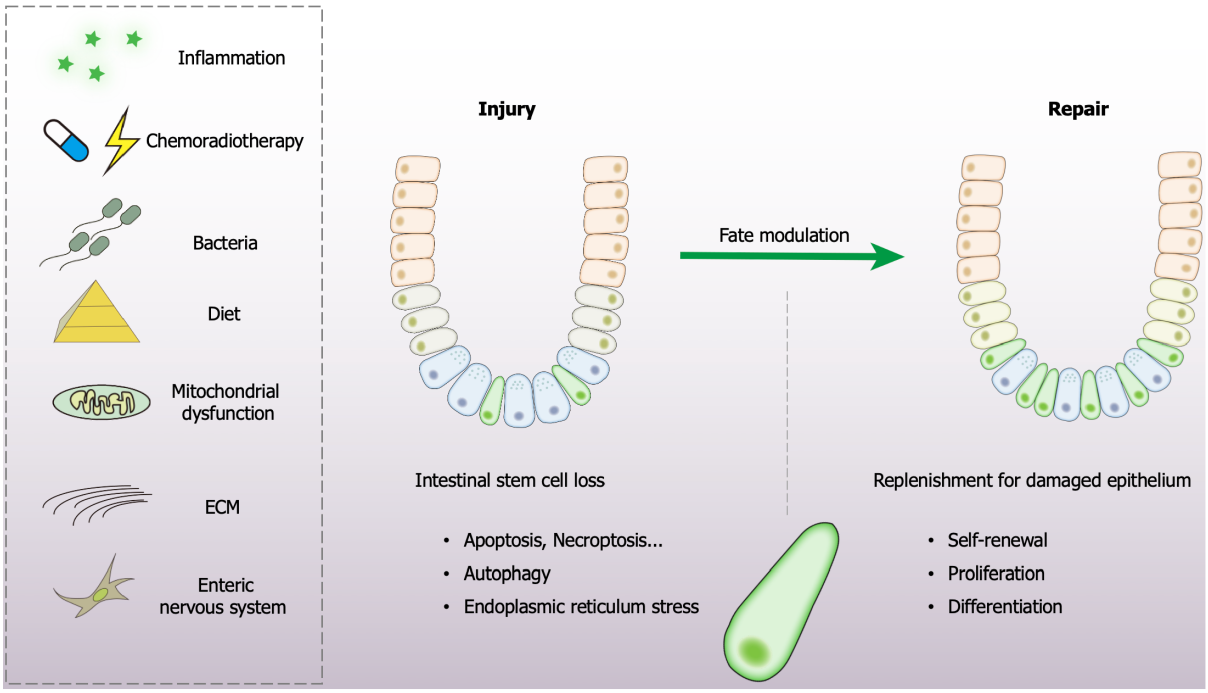
Mitochondrial function: Mitochondrial function emerges as a central player in cell fate determination and extensive control of cellular stress responses, metabolism, immunity, and apoptosis[75]. Mitochondria are the center of energy metabolism and the regulator of stem cell homeostasis[76,77], and oxidative phosphorylation is particularly important to maintain the function of stem cells[78]. For example, pyruvate oxidation in the mitochondria works as a metabolic checkpoint of ISC self-renewal and/or stemness maintenance[77]. Regulators of mitochondrial signals such as ATP, reactive oxygen species (ROS), the mitochondrial unfolded protein response, and AMP-activated protein kinase signaling, will in turn affect stemness and cell cycle progression[75].

Enteric nervous system: In spite of the limited studies into the underlying relationship between the nervous system and intestinal epithelial regeneration, some novel indications are pointed out that the enteric nervous system exerts a potential role with great value. Enteric glial cells are closely connected with the intestinal epithelium and depletion of enteric glial cells will exacerbate DSS-induced injury [79]. The administration of hepatocyte growth factor from neural cells of the enteric nervous system attenuates the hostile effects of DSS[80]. Several reports also unveil a potential effect of the enteric glial cells in mucosal healing through the release of the specific niche factors like glial-derived neurotropic factor, TGF-β1 or 15-deoxy-12,14-prostaglandin J2[41,81,82].

Extrinsic factors

Diet: The biological behavior of stem cells is largely affected by nutritional state[31]. The mTOR signaling is responsible for sensing the nutritional state[83]. Mice fed with a calorie-restricted diet exhibit an augmented function in Lgr5$^+$ ISCs and Paneth cells compared with mice with a normal diet [56,84]. Moreover, the calorie-restricted diet diminishes mTOR activity in quiescent stem cells, improving their resistance to radiation damage and promoting intestinal repair[85]. In contrast, a high-fat diet can increase ISC activity despite decreasing Paneth cells activity. ISCs of high-fat diet mice exhibit higher resistance to irradiation and more efficient organoid budding potential than control mice. The high-fat diet activates Wnt signaling in ISCs dependent on the nuclear peroxisome proliferator-activated receptor δ[86]. Another study also reveals that an obesogenic diet induces ISCs and progenitor cells hyperproliferation, triggers ISC differentiation and cell turnover, and alters the regional characteristics of ISCs and enterocytes in mice[87]. Acute fasting has been shown to lead to transient phosphatase and tensin homolog (PTEN) phosphorylation within quiescent ISCs and render quiescent ISCs functionally poised to contribute to the regenerative response during refeeding[88].

Microorganisms: Microorganisms play an indispensable role in gut homeostasis, but the underlying mechanisms are complicated and elusive. Small molecules and metabolites produced by gut microbiota significantly contribute to the host intestinal development, function, and homeostasis[89]. Lactate from lactic acid-producing bacteria plays a pivotal role in promoting ISC proliferation and epithelial development[90]. Butyrate within the crypts conveys a growth-inhibiting effect on Lgr5$^+$ ISCs *via* Forkhead box O3[91]. The bacterial product muramyl dipeptide has been reported to decrease the level of ROS in ISCs, and promote intestinal organoid growth and tissue repair[92,93]. *Salmonella* can enter



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Figure 2 Modulation of stem cell fate determination and epithelial repair by various sources of stress. Both exogenous (inflammation, chemoradiotherapy, and diet) and endogenous factors (mitochondrial dysfunction, extracellular matrix, and enteric nervous system) have vital effects on stem cell fate modulation. The self-renewal capacity, the balance of proliferation and differentiation are tightly controlled in the process of epithelial repair and regeneration. ECM: Extracellular matrix.

the crypts during infection and cause a significant decrease in Lgr5$^+$ ISCs[94,95]. The enteric pathogen rotavirus specifically invades and deteriorates differentiated cells at villus tips, and then motivates Lgr5$^+$ ISCs, crypt expansion, and hyperproliferation[96]. Gut pathogens are thus distinctive elements capable of tuning the stem cell fate.

Inflammatory signaling: In addition to the local niche signals and physicochemical stimuli, the activation of the immune system is also involved in the interactions between epithelial cells and the niche to guarantee proper initiation and continuation of the regenerative response. Interleukin (IL)-22, which derives from the intestine, contains group 3 innate lymphoid cells that reside in close proximity to intestinal crypts and are upregulated after injury and support subsequent epithelial regeneration[97]. Recombinant IL-22 has been shown to directly target ISCs, thus facilitating the growth of human and mice intestinal organoids and promoting ISC self-renewal[98,99]. A recent study also indicates that the symmetric division of ISCs can be triggered by inflammatory signals to prevent excessive expansion in the process of epithelial repair[100].

Chemoradiotherapy: Intestinal mucosal damage occurs in 40%–60% of patients receiving chemotherapy or radiotherapy[101]. Chemotherapy- or radiotherapy-induced cellular apoptosis can be the primary factor initiating the gastrointestinal syndrome[102,103]. The injury response of intestinal epithelium after chemoradiotherapy has been the most extensively characterized model of Lgr5$^+$ ISC loss and proliferation to date, due to its hypersensitivity to radiation and chemotherapy. Targeting p53-dependent stem cell death is the core strategy for intestinal chemo- or radioprotection[102,104,105]. The Toll-like receptor 4 signaling pathway[106], Slit guidance ligand 2 (Slit2)/ Roundabout guidance receptor 1 (Robo1) signaling[45], gut microbiota[90,107], and dietary components such as green tea derivative (-)-epigallocatechin-3-gallate[108], aspartate[109], pectin[110], and vitamin D[111] have been shown to mitigate the loss of ISCs and alleviate intestinal injury. The deletion of CREPT suppresses the proliferation and differentiation of ISCs and reduces Lgr5$^+$ cell numbers after X-ray irradiation[112]. Therapeutic strategies based on the inhibition of ISC apoptosis without compromising the efficacy of cancer treatment are of great potential.

MODULATION OF STEM CELL FATE

The injury response of intestinal epithelium is critical to restore epithelial integrity upon diverse insults [13]. The immediate response of intestinal damage is the loss of Lgr5$^+$ ISCs, while it is generally adaptive

modulation since the reserved subpopulations are activated to replenish the defects. However, excessive damage may cause ISC depletion and militate against epithelial regeneration. Critical cellular adaptations have been made to restore homeostasis in ISCs.

Apoptosis

As the best-understood form of programmed cell death, apoptosis has been largely clarified in the field of stem cells. Lgr5$^+$ ISCs are more vulnerable to apoptosis than Bmi1$^+$ stem cells are[113]. Considering the critical role of mitochondria in stemness maintenance, regenerative capacity determination, and modulation between self-renewal and cell death programs, mitochondrial function is the vital determinant of stem cell fate[75]. For Lgr5$^+$ ISCs, mitochondrial dysfunction is the major cause of apoptosis. A series of molecules such as Bcl-2, Puma, Survivin, Phosphoribosyl pyrophosphate synthetase 1 (PRPS1), and X-linked inhibitor of apoptosis have been characterized for modulating ISC apoptosis. Other biological processes, including immune response, hormone response, post-translational modification, and signaling such as Hippo and G protein coupled receptor, are crucial to controlling Lgr5$^+$ ISC apoptosis. The pivotal molecules regulating stem cell apoptosis are shown in Table 1. The excavation of novel strategies based on ISC survival is of great significance to epithelial regeneration.

Necroptosis

Necroptosis is also involved in crypt damage. The loss of SETDB1 in ISCs, a histone methyltransferase that induces the trimethylation of histone H3 at lysine 9, triggers Z-DNA-binding protein 1-dependent necroptosis, which irreversibly disrupts the integrity of the epithelial barrier and promotes the progression of IBD[114]. Intestinal organoids lacking ATG16L1 are more prone to initiate tumor necrosis factor (TNF)α-mediated necroptosis, and therapeutic blockage of necroptosis through TNFα or RIPK1 inhibition ameliorates the severity of IBD[115]. TNFα exacerbates necroptosis of differentiated cells and mediates the expansion of LGR5$^+$ ISCs[116]. Therefore, necroptosis inhibitors could be used to promote mucosal healing in IBD patients.

Autophagy

Autophagy is a highly conserved process during evolution in eukaryotes, by which the cytoplasmic materials are degraded inside the autolysosome. Three distinct forms of autophagy, including microautophagy, chaperone-mediated autophagy, and macroautophagy have been described. Autophagy has been demonstrated crucial in modulating the interactions between gut microbiota and innate and adaptive immunity, in host defense against intestinal pathogens, and in maintaining intestinal homeostasis[117].

In the *Drosophila* intestine, autophagy downregulates the sensitivity of differentiated enterocytes to ROS when exposed to commensal bacteria. Mechanistically, the autophagic substrate Ref (2)P/p62 accumulates upon autophagy deficiency, thus inactivating Hippo signaling and leading to stem cell over-proliferation[118]. Autophagy can also protect ISCs against irradiation-induced oxidative stress by preserving mitochondrial health and function. Accordingly, stem cell-based intestinal regeneration after radiotherapy is impaired in mice with Atg5 deficiency. Another recent work has highlighted the role of ATG16L1-dependent autophagy in protecting ISCs from irradiation-induced ROS[92,119]. In a *Drosophila* model, Atg6 deficiency impairs the inhibitory effect of metformin on ISC aging[120]. A recent study has confirmed the role of Atg7 in maintaining epithelial integrity against DNA damage and cell death[121]. With the rapid progress of Lgr5$^+$ ISC isolation and detection, the role of autophagy in ISCs will be further elucidated.

Endoplasmic reticulum stress and unfolded protein response

As the primary organelle for protein folding and quality control, endoplasmic reticulum (ER) is sensitive to multiple intrinsic cellular disturbances and extrinsic environmental changes, which would alter ER homeostasis and cause misfolded protein accumulation, leading to activation of unfolded protein response (UPR)[122]. Previous studies have shown that UPR exerts a significant role in the pathogenesis and progression of IBD[123]. Human genetic studies of IBD have identified primary genetic abnormalities in several genes, including *Xbp1*, *Agr2*, and *Ormdl3*, that encode proteins associated with ER stress[124-126]. More importantly, the control of ISC fate is coordinated by ER stress and UPR. Activation of ER stress leads to the loss of stemness of ISCs in a PERK–eIF2α-dependent manner[127]. XBP1, a stress sensor involved in the UPR, acts as a signaling hub to regulate stem cell function and epithelial DNA damage responses in a p53–DDIT4L-dependent manner[128]. XBP1 is also demonstrated to maintain ISC quiescence and control ISC activity[129]. Intestinal epithelium-specific deletion of glycoprotein 96, an ER-resident master chaperone, causes rapid destruction of stem cell niche, followed by complete eradication of the mucosal layer and epithelial cell death[130]. In summary, UPR is indispensable for stemness maintenance and fate determination of ISCs.

Table 1 Apoptosis regulation of intestinal stem cells

Biological process or signaling	Molecule	Role	Evidence
Mitochondrial dysfunction	Puma	Pro-apoptotic	Puma depletion reduces chemoradiotherapy- induced apoptosis in a p53-dependent manner [102,136]
	Bcl-2	Anti-apoptotic	Bcl-2 is highly expressed in ISCs and alleviating radiation-induced damage[137]
	Survivin	Anti-apoptotic	An essential guardian of ISC during mucosal healing[138]
	PRPS1	Pro-apoptotic	PRPS1 deficiency exhibit resistance against intestinal damage in a manner dependent upon Lgr5+ ISCs[139]
Immune response	IL-22	Anti-apoptotic	IL-22 deficiency led to increased crypt apoptosis, depletion of ISCs[97]
	NOD2	Anti-apoptotic	Nod2 stimulation triggers stem cell survival against oxidative stress-mediated cell death[93]
Hippo	YAP	Anti-apoptotic	Loss of YAP activity results in sensitivity of crypt stem cells to apoptosis and reduced cell proliferation during regeneration[140]
GPCR	β-Arrestin1/2	Anti-apoptotic	βArr reduced the chemotherapy- induced Lgr5+ stem cell apoptosis by inhibiting endoplasmic reticulum stress[141,142]
Hormone	GLP-2	Anti-apoptotic	GLP-2 expanded intestinal organoids and downregulated apoptosis-related genes[143]
	Ghrelin	Anti-apoptotic	Ghrelin treatment accelerated the reversal of radiation-induced epithelial damage and defective self-renewing property of ISCs[144]
Methylation	Mettl14	Anti-apoptotic	Specific deletion of the Mettl14 gene resulted in colonic stem cell apoptosis[145]
	GsdmC	Anti-apoptotic	GsdmC N6-adenomethylation protects mitochondrial homeostasis and is essential for Lgr5+ cell survival[146]
Glycosylation	HYOU1	Anti-apoptotic	HYOU1 glycosylation modulated by FUT2 protects ISCs against apoptosis[147]

ISCs: Intestinal stem cells; PRPS1: Phosphoribosyl pyrophosphate synthetase 1; GPCR: G protein coupled receptor.

FUTURE DIRECTIONS FOR STEM-CELL-BASED THERAPY

Stem-cell-based therapy holds great promise for the complete mucosal healing of gastrointestinal diseases. Related studies have applied exogenous stem cells such as mesenchymal stem cells and placental-derived stem cells for treating intestinal inflammation and injury[131,132], and achieved encouraging outcomes. With the boost of research in the field of ISCs, the intestinal organoid models, especially those of human origin, offer a unique platform to explore the mystery of ISC fate decisions and lineage specification in physiological and pathological conditions[14], and excavate novel strategies to facilitate the regenerative capacity of ISCs. Integration with novel nanomaterials can provide a more effective strategy for facilitating intestinal repair targeting at ISCs, such as grape exosome-like nanoparticles[133], polydopamine nanoparticles[134], and carbon nanoparticles[135]. Thus, one important future direction in the ISCs field is to precisely tune the fate of stem cells for better regeneration.

CONCLUSION

Intestinal epithelial regeneration is a complex network that is based on the function of ISCs. The dynamic balance between stemness and self-renewal is fine-tuned by stem cell niche and various endogenous or extrinsic factors. Great strides have been made in our understanding of the function and fate specification of ISCs in health and disease. In this review, we summarize the different components and signals that function in ISCs in the process of intestinal epithelial injury and repair. Cellular adaptations including apoptosis, necroptosis, autophagy, and UPR have been extensively investigated. Modulating the essential niche signaling or facilitating beneficial elements in the stem cell microenvironment provides novel insights into the regenerative process and opens an avenue for stem cell-based therapies for diseases caused by intestinal epithelial injury.

FOOTNOTES

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REVIEW

Stimulating factors for regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells

Jia-Qi Zhou, Hao-Yang Wan, Zi-Xuan Wang, Nan Jiang

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Abstract

Mesenchymal stem cells (MSCs), distributed in many tissues in the human body, are multipotent cells capable of differentiating in specific directions. It is usually considered that the differentiation process of MSCs depends on specialized external stimulating factors, including cell signaling pathways, cytokines, and other physical stimuli. Recent findings have revealed other underrated roles in the differentiation process of MSCs, such as material morphology and exosomes. Although relevant achievements have substantially advanced the applicability of MSCs, some of these regulatory mechanisms still need to be better understood. Moreover, limitations such as long-term survival *in vivo* hinder the clinical application of MSCs therapy. This review article summarizes current knowledge regarding the differentiation patterns of MSCs under specific stimulating factors.

Key Words: Mesenchymal stem cells; Differentiation; Osteogenic; Chondrogenic; Literature review.

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Core Tip: Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating in specific directions. The differentiation process of MSCs depends on diverse specialized external stimulating factors. The results from recent studies have revealed other underrated roles in the differentiation process of MSCs. However, several questions remain to be solved prior to stable and effective clinical treatment. Our review explores the differentiation patterns of MSCs and summarizes the relevant research according to stimulus types. Finally, future prospects are discussed with regard to their clinical applications.

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INTRODUCTION

Mesenchymal stem cells (MSCs), which were originally identified in the bone marrow, are adult stem cells with multilineage differentiation potential. Under specific induction conditions, MSCs could differentiate into bone, adipose, muscle, neural, and endothelial tissue cells[1]. With the development of research, MSCs have been obtained from other tissues, including adipose, peripheral blood, umbilical cord blood, and periodontal membrane tissue[2-5]. Due to their multilineage differentiation potential and rich tissue sources, the application of MSCs in research on regenerative medicine is virtually limitless[6]. However, a specific number of MSCs are necessary for tissue regeneration; hence, there is a requirement for MSC amplification before therapy[7]. The question of how the differentiation of MSCs are controlled *in vitro* and *in vivo* remains unanswered, which has limited the effectiveness of MSCs in the application of regenerative medicine research. Recently, various external stimulus factors, such as biochemical stimuli, hypoxia, physical stimuli, material properties, and exosomes, have been found to have an impact on the differentiation process of MSCs (Figure 1). The purpose of this review is to discuss a variety of recent findings regarding the important external stimulus factors that influence the self-renewal and osteogenic and chondrogenic differentiation potential of MSCs.

BIOCHEMICAL STIMULI

Growth factors, cytokines, and miRNAs are examples of biochemical stimuli that have typically been employed to control the destiny of MSCs. Growth factors and cytokines bind to the corresponding receptors and transfer signals, while miRNAs degrade mRNAs or inhibit the translation of mRNAs to regulate gene expression and thus influence the differentiation fate of MSCs. Numerous studies have examined the effects of various growth factors, cytokines, and miRNAs on the proliferation and differentiation of MSCs into other cellular phenotypes (Table 1).

Growth factors

Growth factors, including fibroblast growth factor (FGF), transforming growth factor (TGF), platelet-derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor and bone morphogenetic protein (BMP), are a class of peptides that regulate cell growth and other cell functions by binding to specific cell membrane receptors[8].

FGF-2, also known as basic bFGF, has been the subject of the majority of FGF research to date. In a concentration-dependent manner, bFGF might promote the proliferation of MSCs from several tissue sources, including bone marrow peri-adipocyte cells[9], synovial MSCs[10], adipose-derived stem cells (ADSCs)[11], umbilical cord-derived MSCs[12], and bone MSCs (BMSCs)[13,14]. Ramasamy *et al*[12] reported that cell proliferation increased accordingly with increasing bFGF concentrations in the range of 0-40 ng/mL. However, Ma *et al*[11] and Wang *et al*[14] observed that the proliferation efficiency of cells at 5 ng/mL of bFGF was higher than that at 10 ng/mL. As a result, the use of 5 ng/mL of bFGF appeared to be an appropriate choice to promote the proliferation of different MSCs. In addition to enhancing MSC proliferation, bFGF has been shown to maintain stemness, support cartilage differentiation, and influence osteogenic differentiation[9,10,13]. Intriguingly, Wang *et al*[14] reported that bFGF pretreatment inhibited osteogenic differentiation at the early stage, but promoted it in the medium phase[13]. This finding might indicate that the addition of different growth factors at different phases of osteogenesis induction could successfully promote osteogenic differentiation. Therefore, more studies are needed to clarify the mechanism of action of bFGF at different stages of osteogenic differentiation, as well as to identify the best combination of growth factors to effectively promote the osteogenic differentiation of MSCs.

Previous research has demonstrated the involvement of TGF-β in inducing chondrogenic differentiation[5]. However, while promoting cartilage differentiation, TGF-β also led to early hypertrophic maturation and the eventual formation of nonfunctional fibrocartilage[2,15]. In addition, TGF-β was also found to promote the proliferation of MSCs and their effect on osteogenic differentiation[16,17]. MSC osteogenic differentiation was influenced by TGF-β in a dose-dependent manner. According to research by Xu *et al*[17], low concentrations of TGF-β (1 ng/mL) promoted the osteogenic development of BMSCs, whereas high concentrations (10–50 ng/mL) of TGF-β inhibited osteogenic differentiation. Igarashi *et al*[18] showed that 5 ng/mL of TGF-β regulated the phenotypic differentiation of BMSCs toward osteoblasts but seemed to inhibit osteogenic differentiation at the late stage, suggesting that

Table 1 Growth factors, cytokines, and their effects on the differentiation of mesenchymal stem cells

Factors	Amount/types	Concentration	Cell dource	Results	Ref.
FGF	FGF-2	10 ng/ml	BM-PACs	FGF-2 did not lead to cell differentiation into a chondrogenic lineage	Endo *et al*[9]
	bFGF	5 ng/ml	SMSCs	Promoted SMSCs chondrogenic differentiation	Okamura *et al*[10]
	bFGF	0-40 ng/ml	UC-MSCs	bFGF did not alter osteogenic nor adipogenic differentiation potential	Ramasamy *et al*[12]
	bFGF	20 ng/ml	BMSCs	bFGF pretreatment inhibited osteogenic differentiation of BMSCs at early stage, promoted it in the medium phase, and maintained it in the later stage during osteogenic induction	Wang *et al*[13]
TGF-β	TGF-β3	10 ng/ml	SF-MSCs	Increased the expression levels of COL2A1, SOX9, ACAN, COL10A1	Jia *et al*[15]
	TGF-β	10 ng/ml	ADSCs	Promoted ADSCs chondrogenic differentiation but led to early hypertrophic maturation	Hesari *et al*[2]
	TGF-β1	1, 10, 20 or 50 ng/ml	BMSCs	Low concentration of TGF-β1 (1 ng/ml) promoted osteogenic differentiation of BMSCs while high concentration of TGF-β1 (10 to 50 ng/ml) significantly inhibited osteogenesis	Xu *et al*[17]
	TGF-β	5 ng/ml	BMSCs	Promoted osteogenic differentiation of BMSCs but suppressed the maturation of ostroblastic MSC differentiation at the last stage of osteogenic process	Igarashi *et al*[18]
	TGF-β3	10 μg/L	PDLSCs	Induced chondrogenesis	Choi *et al*[5]
IL	IL-6	100 ng/mL	BMSCs	Promoted BMSCs osteogenic differentiation	Xie *et al*[21]
	IL-17A	5-40 ng/ml	BMSCs	Promoted the neuronal-associated gene expression of BMSCs	Chen *et al*[24]
	IL-17	50 ng/mL	Mouse MSCs	Enhanced the osteogenic differentiation of mMSCs	Liao *et al*[22]
	IL-6	100 ng/mL	hMSCs	IL-6/soluble IL-6R promoted chondrogenic differentiation of MSCs	Kondo *et al*[20]
	IL-17A	50 ng/ml	BMSCs	Inhibited osteogenic differentiation of BMSCs	Wang *et al*[23]
	IL-22	10 ng/ml	MSCs	Upregulated osteogenic and adipogenic transcription factors	El-Zayadi *et al*[25]

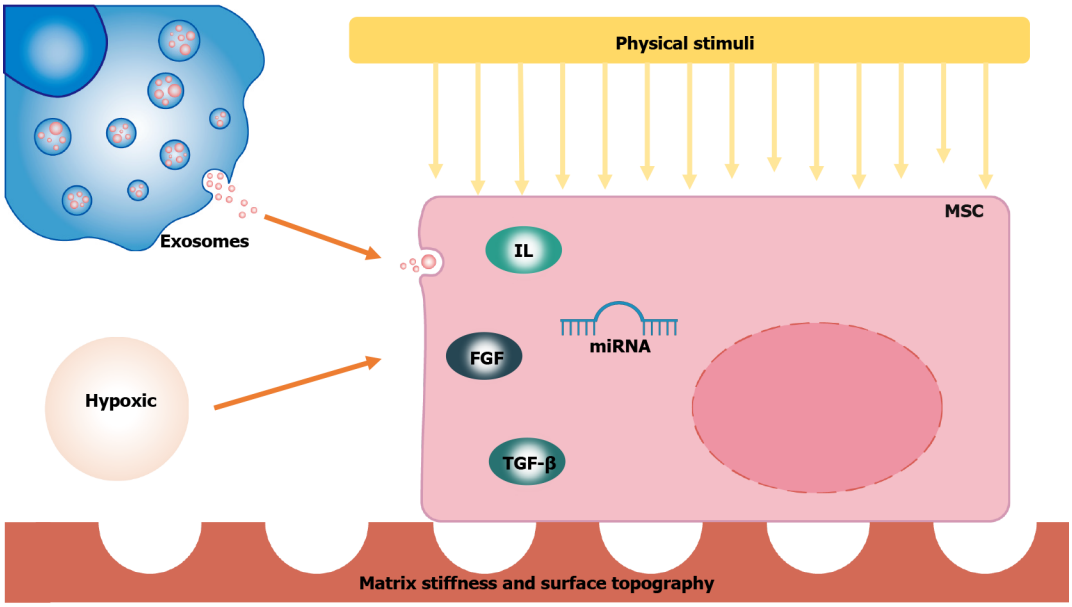
FGF: Fibroblast growth factor; FGF-2/bFGF: Basic fibroblast growth factor; TGFβ: Transforming growth factor β; IL: Interleukin; BMSCs: Bone mesenchymal stem cells; BM-PACs: Bone marrow peri-adipocyte cells; ADSCs: Adipose-derived stem cells; hMSCs: Human mesenchymal stem cells; SMSCs: Synovial mesenchymal stem cells; UC-MSCs: Umbilical cord-derived mesenchymal stem cells; SF-MSCs: Synovial fluid-derived mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; COL2A1: Collagen type II alpha 1 chain; SOX9: Sex-determining region Y-box 9; ACAN: Aggrecan protein; COL10A1: collagen type X alpha 1 chain.

additional cellular signals were necessary for the osteogenic differentiation of some types of MSCs. Therefore, it is crucial to determine how to prevent hypertrophy during TGF-β promoted cartilage differentiation.

Cytokines

The fate of MSCs might be influenced by many cytokines, such as interleukin (IL), tumor necrosis factor (TNF) and interferons (IFN). Studies have previously examined how various cytokines affected osteogenic differentiation. IL-10, IL-11, IL-18, and IFN-γ promoted osteogenesis, while TNF-α, TNF-β, IL-1α, IL-4, IL-7, IL-12, IL-13, IL-23, IFN-α and IFN-β inhibited osteogenesis[19]. In this article, we focus on recently discovered cytokines such as IL-6, IL-17, and IL-22 that have the potential to affect the fate of MSCs.

MSCs both produced IL-6 and reacted to it. Furthermore, the gradual reduction in IL-6 secretion by MSCs during chondrogenic differentiation suggested that IL-6 was one of the distinguishing characteristics of undifferentiated MSCs[20]. Nevertheless, the addition of exogenous IL-6 was found to be



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Figure 1 Overview of stimulating factors in differentiation of mesenchymal stem cells. MSC: Mesenchymal stem cell; IL: Interleukin; FGF: Fibroblast growth factor; TGF-β: Transforming growth factor-β.

effective in promoting the osteogenic differentiation and chondrogenic differentiation of MSCs[20,21]. In contrast to previous studies, Xie *et al*[21] discovered that IL-6 secretion by BMSCs increased rather than decreased with osteogenic differentiation. The effect of IL-17A on the osteogenic differentiation of MSCs also seemed to be contradictory. Liao *et al*[22] reported that IL-17A inhibited the osteogenic differentiation of MSCs as well as pre-osteoblast cell lines. However, the study by Wang *et al*[23] showed the opposite. The appearance of these phenomena might be due to different microenvironments and cellular sources. Additionally, different concentrations of IL-17A have been shown to promote neuronal differentiation, with the best effect at 20 ng/mL[24]. The effect of IL-22 on the proliferation and differentiation of MSCs was first reported by scholars in 2017, which showed that IL-22 alone could upregulate the levels of osteogenic and lipogenic transcription factors but needed to be combined with IFN-γ and TNF to promote the proliferation of MSCs[25].

Cytokines must bind to specific receptors to transmit signals. The amount of the relevant receptor for cytokines appeared to be the rate-limiting element regulating the differentiation of MSCs[20]. Therefore, more studies are required to determine how cytokines affect the growth and differentiation of MSCs. Moreover, a fresh approach will be to look for factors that may raise the number of cytokine receptors on the surfaces of MSCs.

miRNAs

Small non-coding RNAs (approximately 20–25 nucleotides) called miRNAs are a subclass that could bind to complementary target sites in mRNA molecules to inhibit translation or decrease mRNA stability, which controls gene expression[26]. In this case, miRNAs could regulate the expression of key genes during the differentiation of MSCs in specific lineages to influence the direction of differentiation of MSCs (Table 2).

The osteogenic differentiation of MSCs was found to be regulated by micro RNA-1286[27], micro RNA-223-3p[28], micro RNA-346-5p[29], micro RNA-21[4] and micro RNA-130a[30], whereas the chondrogenic differentiation of MSCs was found to be regulated by micro RNA-130b[31], micro RNA-218[32], micro RNA-495[33] and micro RNA-30a[34]. In addition to this, some miRNAs also exhibited roles in regulating the adipogenic differentiation[30], endothelial differentiation[26], neuronal differentiation[35], and myocardial differentiation[36,37] of MSCs.

In conclusion, investigating the impact of biochemical stimuli on the growth and differentiation of MSCs has aided our understanding of the patterns of the aberrant differentiation of MSCs in diseased situations and aided in identifying novel therapeutic targets. It appears to be a promising avenue to examine the impact of the combination of diverse biochemical stimuli on the fate of MSCs, since distinct biochemical stimuli in the microenvironment in which MSCs are positioned function in a compound manner. Additionally, since the functions of cytokines and growth factors are dependent on binding to the appropriate receptors and some studies have indicated that receptor expression might be the rate-limiting factor, it would be preferable to determine methods to boost receptor expression as opposed to raising cytokine and growth factor concentrations.

Table 2 Micro RNA and their effects on the differentiation of mesenchymal stem cells

Amount/types	Expression	Cell source	Results	Ref.
micro-RNA-1286	Over expression	hMSCs	↓ Osteogenic differentiation	Zhou *et al*[27]
micro-RNA-223-3p	Low expression	BMSCs	↑ Osteogenic differentiation	Long *et al*[28]
micro-RNA-346-5p	Over expression	BMSCs	↓ Osteogenic differentiation	Zhang *et al*[29]
micro-RNA-21	Over expression	hucMSCs	↑ Osteogenic differentiation	Meng *et al*[4]
micro-RNA-130a	Over expression	BMSCs	↑ Osteogenic differentiation ↓ adipogenic differentiation	Lin *et al*[30]
micro-RNA-130b	Low expression	BMSCs	↑ Chondrogenic differentiation	Zhang *et al*[31]
micro-RNA-218	Over expression	SDSCs	↑ Chondrogenic differentiation during the eraly stage	Chen *et al*[32]
micro-RNA-495	Over expression	hMSCs	↓ Chondrogenic differentiation	Lee *et al*[33]
micro-RNA-30a	Over expression	BMSCs	↑ Chondrogenic differentiation	Tian *et al*[34]
micro-RNA-145	Low expression	ADSCs	↑ Endothelial differentiation	Arderiu *et al*[26]
micro-RNA-124	Over expression	ADSCs	↑ Neuronal differentiation	Mondanizadeh *et al*[35]
micro-RNA-10-5p	Low expression	BMSCs	↑ Myocardial differentiation	Li *et al*[36]
micro-RNA-499a-5p	Over expression	BMSCs	↑ Cardiomyogenic differentiation	Neshati *et al*[37]

↑: Increase; ↓: Decrease; hMSCs: Human mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; hucMSCs: Human umbilical cord mesenchymal stem cells; SDSCs: Synovium-derived mesenchymal stem cells; ADSCs: Adipose-derived stem cells.

PHYSICAL STIMULI

In addition to the previously mentioned biochemical stimuli, physical stimuli such as electromagnetic fields (EMF), microgravity (MG), fluid shear stress (FSS), and hydrostatic pressure (HP) could also have an impact on the proliferation and differentiation of MSCs (Table 3). EMF, a non-invasive biophysical therapy, is a combination of electric and magnetic fields and has been widely used in the treatment of bone diseases[38,39]. Exposure to sinusoidal EMF (1mT,15Hz,4h/d) promoted the proliferation and osteogenic and chondrogenic differentiation of BMSCs[40]. In contrast, Wang *et al*[41] found that EMF also promoted the osteogenic differentiation of MSCs but did not inhibit their proliferation under the same parameters. With the exception of 75 Hz square EMF, Asadian *et al*[42] discovered that EMFs of various frequencies and waveforms (25, 50 Hz square, and sinusoidal waveform EMFs) enabled the suppression of BMSC growth. This might imply that MSCs from different sources had different sensitivities to EMFs. Distinct EMFs had different responses to MSCs. It is crucial to investigate the most appropriate EMF parameters for the proliferation or directed differentiation of MSCs from various sources. For instance, MSCs exposed for a brief period of time to low-amplitude and low-frequency pulsed EMF could be encouraged to differentiate into chondrogenic cells[43], while sinusoidal EMF at 1 mT, 15 Hz, 4 h/d was favorable for MSCs to differentiate into osteogenic cells[40,41], and higher-frequency EMF could also encourage MSCs to differentiate into neuronal cells[42].

Another independent factor influencing the destiny of MSCs has been identified as MG. Most of the research was thus for only conducted in a simulated MG (SMG) environment produced by a clinostat or rotating vessel, since examining the proliferation and differentiation patterns of MSCs in an actual MG environment led to some technical and budgetary challenges[44]. Quynh *et al*[45] found that SMG inhibited the proliferation of human umbilical cord MSCs by blocking the cell cycle; in contrast, a study by Nakaji-Hirabayashi *et al*[46] revealed a proliferative effect. The various SMG action times could be responsible for this circumstance. Shorter SMG treatments appeared to inhibit osteogenesis[47-49] and promote endothelial cell differentiation[48], neuronal differentiation[44,48], and adipogenic differentiation[48,49]. However, extended SMG decreased the potential for chondrogenic differentiation in MSCs[50] and encouraged their differentiation toward osteogenesis[46,48]. Different SMG action times had different effects on the cytoskeleton and could even lead to the aforementioned changes through different signal transduction pathways. In this regard, further studies are needed to determine the appropriate SMG treatment time in regulating the specific lineage differentiation of MSCs.

FSS refers to the mechanical force caused by the friction of fluid flow on the apical cell membrane. It has been demonstrated that the proliferation and differentiation of MSCs are significantly influenced by the strength, timing, and rate of FSS. Jing *et al*[51] discovered that the proliferation of BMSCs could be effectively induced by 0.06 dyn/cm^2 of FSS stimulation, but as the intensity of the FSS increased, cell proliferation gradually decreased or was even inhibited. Meanwhile, Zhao *et al*[52] revealed that FSS

Table 3 Physical stimuli and their effects on the differentiation of mesenchymal stem cells

Physical stimuli	Parameters	Cell source	Results	Ref.
EMF	1 mT, 15 Hz, 4 h/day	BMSCs	BMSCs pretreated with EMF exhibited stronger osteogenic and chondrogenic differentiation potential and weaker adipogenesis capacity	Tu *et al*[40]
	25, 50, 75Hz square and sinusoidal waveform EMF	BMSCs	EMF induced BMSCs differentiation to neuron cells in all treatment groups	Asadian *et al*[42]
	1 mT, 15 Hz, 4 h/day	Rabbit MSCs	EMF enhanced the osteogenic potential of MSCs	Wang *et al*[41]
	PEMF	MSCs	Brief exposure to low amplitude PEMFs enhanced the ability of MSCs to produce and secrete paracrine factors capable of promoting cartilage regeneration	Parate *et al*[43]
SMG	30 g for 72 h or 10 days	Adult rat MSCs	A shorter period of SMG promoted MSCs to differentiate into endothelial, neuronal and adipogenic cells. In comparison, a longer period of SMG promoted MSCs to differentiate into osteoblasts	Xue *et al*[48]
	10 rpm, 72 h, 0.001 G	BMSCs	Inhibited osteogenic differentiation of MSCs	Liu *et al*[47]
	30 rpm clinorotation, 3 d	Adult rat MSCs	Promoted the neuronal differentiation of rat MSCs	Chen *et al*[44]
	7 rpm, 21 d	hMSCs	Lowered the chondrogenic potential of hMSCs	Mayer-Wagner *et al*[50]
Microgravity	0.001 G	hMSCs	microgravity-cultured hMSCs showed a better ability to differentiate into osteoblasts and adipocytes compared to cells cultured under natural gravity conditions	Nakaji-Hirabayashi *et al* [46]]
Spare microgravity		hMSCs	Spare microgravity reduced the osteogenic differentiation of hMSCs and shifted the osteogenesis of hMSCs into adipogenesis, even during ostergenic induction	Zhang *et al*[49]
FSS	0.375 dyn/cm^2, 2 h/d	BMSCs	Promoted osteogenesis-related genes and proteins in BMSCs	Jiang *et al*[54]
	0.06 dyn/cm^2, 6 h/d	BMSCs	Proper FSS stimulation obviously enhanced BMSCs osteogenesis, while the expressions of osteogenic genes decreased with higher intensity of FSS	Jing *et al*[51]
	0.5, 0.8 Pa, 3 h/d	MSCs	Promoted MSCs ostegenesis	Jiao *et al*[55]
	3-7 dynes/cm^2	hMSCs	Enhanced osteogenic differentiation	Zhao *et al*[52]
	4.2 dynes/cm^2	hMSCs	FSS could lead to the osteogenic differentiation of hMSCs	Liu *et al*[53]
	ΔSS from 0 dyn/cm^2 to 10 dyn/cm^2	MSCs	Fast ΔSS (0–0') profits the chondrogenic differentiation, while Slow ΔSS (0–2') advances osteogenic differentiation	Yue *et al*[57]
	ΔSS from 0 dyn/cm^2 to 10 dyn/cm^2	MSCs	Fast ΔSS (0–0') profits the chondrogenic differentiation, while Slow ΔSS (0–2') advances osteogenic differentiation	Lu *et al*[56]
HP	10 MPa, 1 Hz, 4 h/d, 5 d/w, 3 w	BMSCs	HP promoted BMSCs chondrogenic differentiation	Steward *et al*[60]
	0-0.5 MPa, 0.5 Hz	hMSCs	HP promoted the differentiation of the hMSCs toward osteogenesis	Huang *et al*[59]
	270 kPa, 1 Hz, 1 h/d, 5 d/w, 3 w	BMSCs	HP promoted chondrogenic differentiation of BMSCs	Luo *et al*[64]
	100 psi	ADSCs	HP significantly increased osteogenic differentiation of AMSCs	Ru *et al*[65]
	90 kPa, 1 h	BMSCs	HP promoted chondrogenic differentiation of BMSCs	Zhao *et al*[61]
	90 kPa, 1 h	BMSCs	HP promoted the expression of marker genes for early osteogenic differentiation and chondrogenic differentiation of the BMSCs	Zhao *et al*[62]

BMSCs: Bone mesenchymal stem cells; EMF: Electromagnetic field; PEMF: Pulsed electromagnetic field; ADSCs: Adipose-derived stem cells; SMG: Simulated microgravity; hMSCs: Human mesenchymal stem cells; hucMSCs: Human umbilical cord mesenchymal stem cells; FSS: Fluid shear stress; ΔSS: Rate of fluid shear stress; HP: Hydrostatic pressure.

regulated cell proliferation in a rate- and time-dependent manner, with high FSS (9–20 dyn/cm^2) and the continuous effect of low FSS both inhibiting MSC proliferation, but the intermittent effect of low FSS (1–9 dyn/cm^2) appeared to have little or no effect. Liu *et al*[53] shown that FSS (4.2 dyn/cm^2) could promote the proliferation of MSCs implanted on 3D poly(lactic-co-glycolic acid) scaffolds. Although the

effects of FSS on the proliferation of MSCs were differently stated, its promotion of osteogenic differentiation[52-55] seemed to be consistent. Regarding how the rate of FSS (ΔSS) affects MSCs, it was observed that quick ΔSS (From 0 dyn/cm^2 in 0 min) was more beneficial for MSCs' chondrogenic development, whereas slow ΔSS (From 0 dyn/cm^2 in 2 mins) encouraged their osteogenic differentiation [56,57]. Clearly, more research is required to confirm the impact of FSS on MSC proliferation, as well as the appropriate stimulus parameters for osteogenic differentiation and MSC proliferation.

HP, unlike other physical stimuli, applies homogeneous tension without causing cellular deformation [58]. Physiological load (0.1-10 mPa) did not affect the proliferation of MSCs[59,60], whereas a load of 90 kPa effectively promoted the proliferation of MSCs[61,62], a possibility that resulted from the initiation of the cell cycle by HP[62]. Studies conducted in the past have indicated that HP at low loads (1–50 kPa) has an osteogenic impact on MSCs, whereas HP at physiological loads efficiently promoted chondrogenic differentiation[63]. This concept was also supported by several recent research works[60,64]. Some investigations, however, discovered a facilitative effect of physiological loading on MSCs' osteogenic differentiation[59,65], and a chondrogenic effect of low loading on MSCs[61,62]. Additionally, the study by Zhao *et al*[62] discovered that HP (70 kPa) could not only stimulate RhoA activation, which in turn promoted the expression of early osteogenic differentiation genes in BMSCs, but could also upregulate Rac1 and downregulate RhoA, which further promoted cartilage development in BMSCs. These findings suggested that further studies are needed to determine the effects of different loads of HP on the spectral differentiation of MSCs and their complex mechanisms.

Overall, physical stimuli did influence MSCs' proliferation and differentiation to varying degrees, but there is still no consensus on the parameters that are most conducive to the proliferation and specific lineages' differentiation of MSCs. Cell heterogeneity, various stemness potentials, culture conditions, and techniques that simulated physical stimulation might all be contributing factors in this issue. Therefore, more studies are needed to determine the appropriate parameters of physical stimuli that promote the differentiation of MSCs. In fact, the actual microenvironment in which cells were exposed was multifactorial. Therefore, some studies are now starting to consider the effect of compound factors [50,55,61,66] on the behavior of MSCs. Compound factors could have synergistic effects that increase the benefits for MSCs or counteract the drawbacks of a single factor. This might emerge as a new trend.

HYPOXIA

In most studies, MSCs were cultured under atmospheric oxygen tension (20%-21% O_2)[67]. However, MSCs in different ecological niches encounter oxygen concentrations that are significantly lower than 20% (Table 4). For instance, the O_2 concentration that MSCs experienced varied from 1% to 5%[68] in adipose tissue and from 1% to 7%[69] in bone marrow. As a result, MSCs from different tissue sources were in a hypoxia microenvironment *in vivo*. Hypoxia activated various signaling pathways within a cell, which could lead to either cell death or cell adaptation[70]. Theoretically, culturing MSCs at physiological oxygen concentrations facilitated their proliferation, differentiation, and the secretion of cytokines and growth factors. Ciapetti *et al*[71] discovered that hypoxic circumstances greatly boosted BMSCs' proliferation and colony-forming capacity, as well as the expression of genes relevant to bone, such as alkaline phosphatase and osteocalcin, supporting the above idea. In contrast, in a study by Xu *et al*[72], hypoxia inhibited the osteogenic differentiation of BMSCs by activating the Notch pathway. Therefore, we focus on the effect of hypoxia on the behavior of MSCs and try to explain the contradictory findings in different studies.

The two primary techniques used nowadays to create *in vitro* hypoxic settings are anaerobic chambers [73] and simulation utilizing different chemicals[74]. In a study by Elabd *et al*[75], moderate hypoxia (5% O_2) circumstances promoted the chondrogenic and adipogenic differentiation of BMSCs but had no effect on proliferation or osteogenic differentiation. At the same oxygen concentration, Lee *et al*[76] showed that hypoxia promoted MSC proliferation and increased the chondrogenic differentiation potential. The proliferation of MSCs was also effectively promoted at a 5.5%-6.5% O_2 concentration simulated by 10 μM $CoCl_2$ and 4.0 mmol/L Na_2SO_3[74]. In contrast, Yu *et al*[77] demonstrated that a 50 M $CoCl_2$-simulated hypoxia environment appeared to prevent the growth of MSCs. Consistently, the osteogenic differentiation of MSCs was promoted in hypoxia environments simulated using different concentrations of $CoCl_2$[74,77]. Cicione *et al*[78] investigated the changes in the trilineage differentiation potential of BMSCs under severe hypoxia (1% O_2) and showed that the trilineage differentiation potential of BMSCs was inhibited to different degrees. Additional research demonstrated that the activation of the Notch pathway may be responsible for the suppression of the osteogenic differentiation of MSCs by severe hypoxia (1% O_2)[3,72]. In addition, Kim *et al*[79] found that hypoxia could inhibit the osteogenic differentiation of ADSCs by upregulating insulin-like growth factor binding-protein-3. Hypoxia has also been shown to encourage the tendon[73] and neural[80] differentiation of MSCs.

Compared to the laboratory culture environment (20%-21% O_2), hypoxia is more representative of the oxygen concentration in the ecological niche of MSCs. Varied oxygen concentrations had extremely different impacts on MSCs. Moderate hypoxia environment enhanced MSCs' proliferation, osteogenic differentiation, and chondrogenic differentiation. The differentiation capability of all three lineages of

Table 4 Hypoxia and their effects on the differentiation of mesenchymal stem cells

Conditions	Cell source	Results	Ref.
Hypoxic culture (5%O_2)	BMSCs	↑ Chondrogenic differentiation; ↑ adipogenic differentiation	Elabd *et al*[75]
Hypoxic culture (5.5%-6.5%O_2)	Balb/c mouse clonal MSCs	↑ Osteogenic differentiation	Kim *et al*[74]
Hypoxic culture (50 μM $CoCl_2$ simulation)	Mice MSCs	↑ Osteogenic differentiation	Yu *et al*[77]
Hypoxic culture (5%O_2)	ADSCs	↑ Chondrogenic differentiation	Lee *et al*[76]
Hypoxic culture (1%O_2)	PBMSCs	↑ Osteogenic differentiation	Yang *et al*[3]
Hypoxic culture (1%O_2)	BMSCs	↓ Osteogenic differentiation; ↓ adipogenic differentiation; ↓ chondrogenic differentiation	Cicione *et al*[78]
Hypoxic culture (1%O_2)	BMSCs	↑ Neuronal differentiation	Wang *et al*[80]
Hypoxic culture (1%O_2)	BMSCs	↓ Osteogenic differentiation	Xu *et al*[72]
Hypoxic culture (2%O_2)	ADSCs	↑ Tenocyte differentiation	Yu *et al*[73]
Hypoxic culture (2%O_2)	ADSCs	↓ Osteogenic differentiation	Kim *et al*[79]
Hypoxic culture (2%O_2)	BMSCs	↑ Osteogenic differentiation	Ciapetti *et al*[71]

↑: Increase; ↓: Decrease; BMSCs: Bone mesenchymal stem cells; MSCs: Mesenchymal stem cells; ADSCs: Adipose-derived stem cells; PBMSCs: Peripheral blood mesenchymal stem cells.

MSCs was, however, somewhat hindered under severe hypoxia. The contradictory behavior in the aforementioned research might potentially be connected to the cell source of MSCs and whether they were differentiated under hypoxia conditions. In view of current studies generally focusing on hypoxia exposure either in the phase of expansion or differentiation, which have not been fully grasped, further research is necessary to comprehend the effects on MSCs specifically in these two culture forms.

MATRIX STIFFNESS AND SURFACE TOPOGRAPHY

Two crucial material physical characteristics that have a significant impact on MSC behavior are matrix stiffness and surface topography. Matrix stiffness is a passive mechanical parameter that the cell can not directly sense. By exerting traction pressures on the cytoskeleton through focal adhesion, cells might deform the extracellular matrix (ECM), reflecting matrix stiffness[81]. Materials with ECM properties are currently being designed to simulate the actual microenvironment of cells. The ECMs of different native tissues, such as bone, cartilage, nerves, or blood vessels, are composed of micro- and nanoscale topographic patterns[82]. As a result, an increasing number of researchers have begun to look into how the substrate surface topography affects MSC behavior. Size and surface roughness are the two most fundamental parameters of surface topography[83], and the effects of these two factors, as well as substrate stiffness, on the proliferation and differentiation of MSCs are also mainly explored here.

Matrix stiffness

Stiffness is one of the most common metrics in assessing a material's mechanical properties[81], and it is typically expressed in terms of Young's modulus. Matrix stiffness has been shown in many studies to affect the proliferation and differentiation of MSCs. MSCs exhibited higher proliferative behavior under a higher substrate stiffness, and Winer *et al*[84] found that MSCs inoculated in 250-Pa polyacrylamide gels that mimicked the elasticity of bone marrow and adipose tissue exhibited cell cycle arrest, but these arrested cells re-entered the cell cycle when a stiff substrate was present[84]. In comparison to lower-stiffness gels, higher-stiffness matrices could increase the number of cells by a factor of 10[85]. With fibronectin-coated polyacrylamide hydrogels, Sun *et al*[86] controlled the mechanical environment of BMSCs and discovered that BMSCs' proliferation increased with increasing stiffness. However, as opposed to firmer substrates, Lin *et al*[87] discovered that MSCs cultivated on softer substrates had greater cell proliferation rates. Gelma hydrogels with different concentrations not only had different hardness, but also showed different porosity as well. Moreover, the pore size also seemed to be one of the influencing factors for the proliferation and differentiation of MSCs. Indeed, many studies have focused on the effect of matrix stiffness on the direction of differentiation of MSCs. MSCs exhibited the upregulation of biomarkers matching tissue stiffness on polyacrylamide gels of different stiffness, such as neurogenic (0.1-1 kPa, brain), myogenic (8-17 kPa, muscle), and osteogenic (25-40 kPa, bone) markers

[88]. BMSCs could be driven to develop into an osteogenic phenotype and expressed increased quantities of bone-derived biomarkers including Runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and bone-bridging proteins when grown on polyacrylamide hydrogels (62-68 kPa) [86]. Rowlands *et al*[85] found that the osteogenic differentiation of MSCs occurred mainly on polyacrylamide gels of 80 kPa stiffness and that RUNX2 was also expressed at high levels. This might be due to the fact that the 80 kPa collagen I coating could well simulate the microenvironment of the bone tissue. Without an induction medium, the stiffness of the hydrogel itself had a substantial impact in controlling MSC differentiation early on, with softer substrates encouraging the adipogenic differentiation of MSCs, while harder substrates encouraged the osteogenic differentiation of MSCs[89]. However, this effect seemed to be gradually attenuated by biochemical effects in the culture medium, implying that the effects of different factors on the differentiation behavior of MSCs might occur at different stages of differentiation. On 22 kPa gels, as opposed to softer matrices, MSCs produced larger quantities of ALP, which was consistent with the effect of matrix stiffness on osteogenic fractionation shown in the previous work[90]. Although more disagreement has emerged regarding the effect of softer matrices on the differentiation fate of MSCs, such as adipogenic differentiation[84,90-92], myogenic differentiation[85,88], neurogenic differentiation[88], and endothelial differentiation[87], there seems to be a consensus on the osteogenic role of harder matrices for MSCs. The Stiffer matrix enabled cells to produce more cytoskeletal tension and sent differentiation signals *via* transmembrane proteins such as integrins[81,85], which promoted osteogenic differentiation. Furthermore, the nuclear localization of Yes-associated protein (a key mediator of mechano-transduction) and RUNX2 could be impacted by the substrate stiffness[89,90].

Surface topography

Zhao *et al*[93] produced nanotubes of various sizes and micro- and nano-hybrid topographies with ECM-like micro/nanostructures and examined their effects on the proliferation and osteogenic differentiation of MSCs. They discovered that larger-sized nanotubes hindered the early proliferation of MSCs, but the micro- and nano-morphology group had a greater cell number. Additionally, they discovered that MSC osteogenic differentiation might be induced by micro/nanotopographies, even in the absence of osteogenic inducers[93]. Similar results were obtained by Chen *et al*[94], who discovered that the micron/submicron hybrid topography of Ti surfaces promoted osteogenic and chondrogenic differentiation in the early stages of induced differentiation. By introducing nanoengineered topographic glass substrates with different surface roughness, Qian *et al*[95] investigated the impact of surface morphology on the osteogenic differentiation of MSCs. They found that surface roughness could replace the osteogenic inducer dexamethasone and worked in concert with ascorbic acid and β-glycerophosphate to jointly promote the osteogenic differentiation of MSCs[95]. In the past, it was generally agreed that surface roughness seemed to have a positive effect on osteogenic differentiation[95-97]. The osteogenic differentiation of MSCs, however, was more strongly influenced by the nanopore size than by the surface roughness, according to several recent studies[83,98]. The frequent coupling of size and surface roughness in many studies makes it difficult to state the degree of influence of each factor on the behavior of MSCs[83]. Moreover, the methods used to prepare rough surfaces in these studies differ, such as randomly rough surfaces produced by treatments such as mechanical polishing, acid etching, *etc*., where cells form focal attachments that differ from those seen on surfaces of the same roughness [98]. Therefore, more research is required to demonstrate how size and surface roughness affect MSC proliferation and differentiation, respectively. Through a variety of pathways, including the control of adhesion, cytoskeletal tension, and nuclear localization of transcription factors[95], MSCs appeared to be able to detect and respond to the surface topography, which in turn influenced their behavior such as proliferation and differentiation. At this stage, it has been reported that micro- and nano-surface topographies inhibit the proliferation of MSCs and promote osteogenic differentiation to some extent. However, there is no detailed elaboration on their respective effects on MSCs in terms of size and surface roughness.

EXOSOMES

Various cells jointly create the microenvironment by secreting functional molecules, which leads to the sharing of stimuli between multiple cell lineages[99]. In addition to the ECM and growth factors, exosomes were considered to be an important component of the microenvironment[100]. Exosomes are small vesicles with a diameter of 30-150 nm that are released by cells through cytosolic action. The released exosomes could interact with target cells and translocated proteins, lipids, mRNAs and miRNAs to the cytoplasm of target cells[101]. Crosstalk existed between MSCs-osteoblasts and monocytes-macrophages and researchers used this to regulate bone homeostasis[99]. In vitro, BMSCs' behaviors were influenced variably by cell-conditioned media produced by variously polarized macrophages[102]. Previous studies had suggested that cytokines were the main contributors to the function exercised by macrophages. However, Song *et al*[103] found that lipopolysaccharide (LPS)-activated macrophage-derived exosomes inhibited the osteogenic differentiation of BMSCs by

mediating inflammatory stimuli. Therefore, the effect of exosomes secreted by monocytes-macrophages on the differentiation of MSCs should be considered (Table 5).

According to Liu *et al*[104], miR-21a-5p found in M1 macrophage-derived exosomes directed BMSCs toward an osteoblastic fate during the early stages of osteogenesis[104]. In their investigation of the effects of MO, M1, and M2 macrophage-derived exosomes on BMSCs, Xia *et al*[105] discovered that M1 macrophage-derived exosomes efficiently enhanced the proliferation, osteogenic differentiation, and adipogenic differentiation of BMSCs, but M2 macrophage-derived exosomes were harmful to the proliferation of BMSCs and, curiously, all three hindered the chondrogenic differentiation of BMSCs. Xiong *et al*[106] noticed that miRNA-5106, enriched in M2 macrophage-derived exosomes, promoted the osteogenic differentiation of BMSCs by suppressing the expression of salt-inducible kinase 2 (SIK2) and SIK3, which was consistent with the role of M2 macrophage-derived exosomes in promoting osteogenesis in a study by Li *et al*[107]. Kang *et al*[108] demonstrated that M0 and M2 macrophage-derived exosomes were positive for BMSC osteogenesis while M1 macrophage-derived exosomes lowered BMP expression and inhibited the osteogenic differentiation of BMSCs[108]. Despite being enriched in distinct miRNAs, primary extraction M2 macrophages[109] and RAW264.7 mouse monocyte-macrophage leukemia cell[107] derived exosomes both showed osteogenesis-promoting and lipogenic differentiation-inhibiting effects. Current research has indicated the impact of exosomes produced from monocytes[110], osteoclasts[111], and osteoblasts[112] on BMSCs, in addition to exosomes released by macrophages. Ekström *et al*[110] found that exosomes released from LPS-stimulated monocytes could be ingested by MSCs and encouraged the osteogenic differentiation of MSCs. Liang *et al*[111] showed that osteoclast-released exosomes promoted osteogenic differentiation and facilitated osteogenic mineralization by inhibiting Rho GTPase activating protein 1. This might imply that active osteoclasts release large amounts of extracellular vesicles during the resorption phase, promoting the osteogenesis of MSCs for better stabilization and bridging the transition between bone resorption and formation. The addition of osteoblast exosomes could further enhance the expression of RUNX2 and osterix, thereby promoting osteogenic differentiation, and, in addition, osteoblast exosomes could even alter adipocyte ECM-mediated lineage differentiation[112].

Exosomes, one of the recently identified microenvironment components, have unique benefits, such as a nano size, non-toxicity, low immunogenicity, biocompatibility, and versatility of use, drawing widespread attention[113]. The current work appeared to demonstrate the beneficial influence of M2 macrophage-derived exosomes on the osteogenic differentiation of MSCs. As for MO and M1 macrophage-derived exosomes, further research is required to understand their impacts on MSC differentiation and the processes at play. At the same time, research has been conducted progressively on the influence of exosomes released by cells in the same microenvironment as BMSCs on the differentiation of BMSCs, which might represent a new avenue.

CONCLUSION

MSCs play important roles in pathological and physiological processes because of their self-renewal, migration, and pluripotency. Especially due to their multi-differentiation potential, MSCs have been considered as a new therapeutic agent in regenerative medicine. Since the detailed mechanisms involved in these regulation processes has not been fully revealed, research on intrinsic and extrinsic factors regulating MSCs' differentiation may provide new methods in manipulating the cell fate of MSCs. Here, we discussed multiple chemical and mechanical factors affecting the osteogenic and chondrogenic differentiation of MSCs, including typical differentiation promoting patterns, cell environmental factors, and other interesting research areas, such as material morphology and exosomes. After sensing these differentiation-stimulating factors, MSCs from various sources are able to differentiate into specific cell lineages. With the rising demand for MSCs in clinical treatment, noble strategies have been developed that aim at inducing the stable and directional differentiation of stem cells, and further providing efficient methods of MSC regulation in basic research and clinical application.

Meanwhile, there is much more to discover in stem cell research. Due to some limitations of MSCs, such as homing efficiency and long-term survival *in vivo*, most of the research has achieved its results at the cellular level in vitro. Moreover, discrepancies remain between single-factor experiments and synergistic effects by multiple factors. At present, extensive research on factors stimulating MSCs' differentiation has promoted our understanding of cell functional alterations. However, mechanisms involved in manipulating MSCs' cell fate have so far been incomplete. With the deepening of stem cell research alongside technology improvements, the synergistic effect of multiple factors inducing MSC differentiation is increasingly likely to be clarified, as well as providing new patterns in clinical stem cell therapy.

Table 5 Exosomes of different cell sources and their effects on the differentiation of mesenchymal stem cells

Source and kind	Specific cargo	Target	Results	Ref.
M1 macrophages-EVs	miRNA-21a-5p	BMSCs	↑ Osteogenic differentiation	Liu *et al*[104]
M0 macrophages-EVs		BMSCs	↓ Chondrogenic differentiation	Xia *et al*[105]
M1 macrophages-EVs		BMSCs	↑ Osteogenic differentiation; ↑ adipogenic differentiation; ↓ chondrogenic differentiation	
M2 macrophages-EVs		BMSCs	↓ Chondrogenic differentiation	
M2 macrophages-EVs	miRNA-5106	BMSCs;SIK2 and SIK3	↑ Osteogenic differentiation	Xiong *et al*[106]
M2 macrophages-EVs	miRNA-690	BMSCs	↑ Osteogenic differentiation; ↓ adipogenic differentiation	Li *et al*[107]
M0 macrophages-EVs		MSCs	↑ Osteogenic differentiation	Kang *et al*[108]
M1 macrophages-EVs	miRNA-155	MSCs	↓ Osteogenic differentiation	
M2 macrophages-EVs	miRNA-378a	MSCs	↑ Osteogenic differentiation	
M2 macrophages-EVs	miRNA-26a-5p	BMSCs	↑ Osteogenic differentiation; ↓ adipogenic differentiation	Bin-bin *et al*[109]
Macrophages-EVs		BMSCs	↓ Osteogenic differentiation	Song *et al*[103]
Monocytes-EVs		MSCs	↑ Osteogenic differentiation	Ekström *et al*[110]
Osteoclasts-EVs	miRNA-324	BMSCs	↑ Osteogenic differentiation	Liang *et al*[111]

↑: Increase; ↓: Decrease; EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; SIK2/SIK3: Salt-inducible kinase 2/3.

FOOTNOTES

Author contributions: Zhou JQ and Wan HY contributed equally to this study; Jiang N conceived and designed the study; Zhou JQ searched the literature; Zhou JQ and Wan HY drew the figure; Zhou JQ and Wang ZX drafted the article; Wan HY and Jiang N made critical revisions; all the authors approved the final version of the submitted article.

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REVIEW

Cell transplantation therapies for spinal cord injury focusing on bone marrow mesenchymal stem cells: Advances and challenges

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Abstract

Spinal cord injury (SCI) is a devastating condition with complex pathological mechanisms that lead to sensory, motor, and autonomic dysfunction below the site of injury. To date, no effective therapy is available for the treatment of SCI. Recently, bone marrow-derived mesenchymal stem cells (BMMSCs) have been considered to be the most promising source for cellular therapies following SCI. The objective of the present review is to summarize the most recent insights into the cellular and molecular mechanism using BMMSC therapy to treat SCI. In this work, we review the specific mechanism of BMMSCs in SCI repair mainly from the following aspects: Neuroprotection, axon sprouting and/or regeneration, myelin regeneration, inhibitory microenvironments, glial scar formation, immunomodulation, and angiogenesis. Additionally, we summarize the latest evidence on the application of BMMSCs in clinical trials and further discuss the challenges and future directions for stem cell therapy in SCI models.

Key Words: Spinal cord injury; Bone marrow derived mesenchymal stem cells; Neuroprotection; Axon; Myelin; Inhibitory microenvironment

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Core Tip: In this work, we review the specific mechanism of bone marrow-derived mesenchymal stem cell (BMMSC) in spinal cord injury (SCI) repair mainly from the following aspects: Neuroprotection, neuronal circuit, axon sprouting and or regeneration, myelin regeneration, inhibitory microenvironment, glial scar formation, immunomodulation, and angiogenesis. Additionally, we also summarize the latest evidence on application of BMMSC in clinical trials and further discuss the challenges and future directions for stem cell therapy in SCI models.

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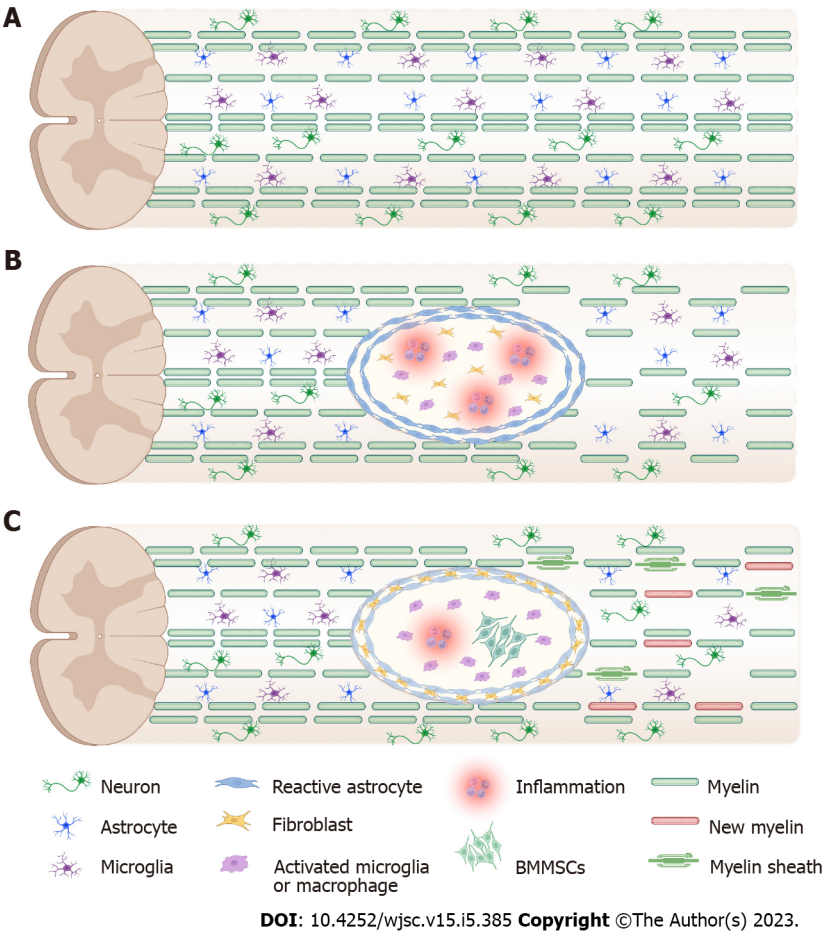
INTRODUCTION

Spinal cord injury (SCI) is a serious neurological disorder that often results in paralysis during the reproductive years, causing temporary or permanent changes in normal motor, sensory, and autonomic functions, with significant impacts on individuals, families, and socioeconomic systems[1]. It has been reported that more than 27 million patients worldwide experience long-term disability due to SCI[2], with 541 cases per 100000 people[3]. Complex pathophysiology and time sensitivity in particular limit the therapeutic effects of SCI[4]. In incomplete SCI, there is less hemorrhage in the gray matter and no change in the white matter 3 h after injury; 6-10 h after injury, the hemorrhagic foci gradually expand, and the neurological tissue becomes edematous, which gradually subsides after 24-48 h. As the degree of incomplete SCI differs between mild and severe injuries, milder injuries only have small foci of necrosis in the center, and most of the nerve fibers are preserved. Severe injuries may have foci of necrosis and softening in the center of the spinal cord and are replaced by gliosis or scarring, and only a small portion of the nerve fibers are preserved. Most posttraumatic tissue degeneration is caused by multiple secondary injuries, including blood-spinal cord barrier (BSCB) disruption, free radical formation, ion imbalance, apoptosis, demyelination, and inflammatory response (Figure 1). Spontaneous recovery occurs within a limited time window because the subacute phase of SCI is thought to be detrimental to axonal regeneration and functional recovery[5]. Currently, clinical treatment includes surgical decompression, stabilization of the spinal cord, relief of spasticity and rehabilitation care, which consists primarily of supportive care and injury management. Frustratingly, the effectiveness of these treatments is limited because they are not effective in stimulating repair of the injured spinal cord.

CLINICAL CHARACTERISTICS AND PATHOLOGY OF SCI

The spinal cord consists of the gray and white matter, which contains nerve cell bodies and ascending and descending fibers. Thus, the different locations and levels of SCI can cause various degrees of disability, from partial loss of motor or sensory function to complete paralysis below the injured location. The resulting lifelong devastating deficits associated with impaired mobility (weakness or paralysis), sensation and autonomic dysfunction, and disabled neurological conditions lead to permanent and severe impacts on patients' daily lives and their caregivers.

Pathophysiology following SCI comprises interrelated multicellular, multimolecular interactions and multiphasic events[6]. Classically, the pathophysiology of SCI is divided into two phases: Primary injury and secondary injury. SCI commonly occurs after sudden trauma because of direct and immediate mechanical insult to the spinal cord from vertebral fractures and dislocation with features of bone fragments and spinal ligament tearing. This was accompanied by extensive bleeding with further compression and interruption of the spinal cord blood supply. Thus, the primary injury mainly includes compression, contusion, shear, laceration, and acute stretching. This is followed by disruption of the neural parenchyma, shearing of the axonal network, destruction of the glial membrane, and vascular disruption[7,8]. Secondary injuries consist of ischemia, spinal cord ischemia-reperfusion injury, vascular dysfunction, edema, excitotoxicity, formation of free radicals, glial and neuronal apoptosis, and the inflammatory response[7]. This secondary damage is divided into three phases: The acute phase, which is accompanied by vascular and cell membrane damage and the secretion of different proinflammatory factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), with microglial activation. The subacute phase is accompanied by edema and vascular damage, inflammatory cytokine and glutamate secretion, astrogliosis, and demyelination for a few days. The chronic phase has symptoms such as the formation of a cavity in the spinal cord[8]. Based on further study of the spinal cord



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Figure 1 Pathophysiology of spinal cord injury and related mechanisms of bone marrow-derived mesenchymal stem cell in spinal cord injury. A: The normal spinal cord contains axons wrapped in the myelin sheath, neuron bodies, microglia, fibroblasts, and astrocytes; B: Secondary injuries following primary injury include glial and neuronal apoptosis, axon rupture, inflammatory response, ischemia, spinal cord ischemia-reperfusion injury, vascular dysfunction, edema, inhibitory microenvironments, excitatory toxicity, and free radical formation; C: Bone marrow-derived mesenchymal stem cell promote the mechanism of spinal cord injury repair, including neuroprotection, axon growth, myelination, immune regulation, microenvironment regulation, inhibition of scar formation, and promotion of angiogenesis. BMMSCs: Bone marrow-derived mesenchymal stem cells.

microenvironment, pathophysiological changes are divided into tissue, cellular and molecular levels. The tissue level involves hemorrhage and ischemia, glial scar formation, demyelination and remyelination[9]. The cellular level involves the activation of astrocytes, the differentiation of endogenous neural stem cells, oligodendrocyte progenitors and microglia, and the infiltration of macrophages. The molecular level involves the expression of neurotrophic factors and their pro-peptides, cytokines, chemokines, and ion imbalance. There is an imbalance between promoting and inhibiting growth molecules in the microenvironment of SCI, where growth inhibitors occupy the dominant position.

THE THERAPEUTIC POTENTIAL OF STEM CELLS

Cell transplantation has come to the forefront in SCI regenerative strategies due to its potential neuroprotective effects. Many cell types have been widely investigated in SCI treatment, including oligodendrocyte precursor cells, Schwann cells, olfactory ensheathing cells, neural stem cells, and mesenchymal stem cells (MSCs)[10,11]. MSCs have the capacity for self-renewal and multilineage differentiation potential and can differentiate into osteoblasts, adipocytes, and chondrocytes[12]. Moreover, MSCs express surface markers (CD105, CD73 and CD90) and do not show expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, or human leukocyte antigen type DR surface molecules[13]. MSCs mainly exist in bone marrow and can also be isolated from other tissues and organs, such as umbilical cord, adipose tissue and muscle. Among these, bone marrow-derived MSCs (BMMSCs) are the most widely studied cell type in SCI application due to their low immunogenicity, easy isolation, few ethical concerns and reduced tumorigenesis risks[14]. According to the current research progress at home and abroad, BMMSC-based treatment has extraordinary prospects in the field of SCI.

In this review, we will summarize the applications of BMMSCs in SCI based on the most frequently proposed mechanisms: Neuroprotection, neuronal circuit, axon sprouting and/or regeneration, myelin regeneration, inhibitory microenvironments, glial scar formation, immunomodulation, and angiogenesis. A better understanding of these mechanisms could allow the identification of more targeted therapies.

NEUROPROTECTIVE EFFECTS OF BMMSCS

Neurons are postmitotic, without the ability to proliferate, and strategies developed to promote neuronal protection and regeneration have long-term benefits. Neuroprotective measures are crucial not only for the preservation of further injury for optimal neuron survivability but also for the restoration of injured nerve cells during pathological progression. BMMSCs reestablish the injured spinal cord *via* neuroprotection, neural regeneration, and remyelination in SCI[15]. MSCs can release growth and neurotrophic factors, including brain-derived neurotrophic factor (BDNF)[16], vascular endothelial growth factor (VEGF)[17], glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), fibroblast growth factor (FGF), neurotrophin-3, and epidermal growth factor, which can enhance regeneration and repair in damaged tissues[18,19]. miR-22, regulated by Gasdermin D mRNA, plays a role in inhibiting the occurrence of pyroptosis[20]. miR-22-modified BMMSCs suppress pyroptosis-mediated inflammation and neuronal injury during SCI[20]. Moreover, MSC-derived exosomes enhance the survival of neurons and repair of nerve fibers by inhibiting Nod1 inflammasome activation, suppressing pyroptosis in pericytes, preserving the integrity of the BSCB[21], inhibiting neuronal apoptosis through the Wnt/beta-catenin signaling pathway[22] and eventually improving functional recovery. The overexpression of miR-338-5p in exosomes was shown to profoundly increase the expression levels of neurofilament 200 and growth-associated protein-43 and decrease those of myelin-associated glycoprotein (MAG) and glial fibrillary acidic protein (GFAP), which provided neuroprotective effects through the cannabinoid receptor 1/Rap1/Akt pathway after acute SCI[23]. cAMP-mediated Rap1 activation plays an important role in apoptosis reduction and neuronal survival induced by the PI3K/Akt pathway[23].

Despite the inhibitory environment in the adult mammalian central nervous system, neuronal-intrinsic mechanisms are sufficient to support significantly extensive axonal growth and synapse formation after SCI, resulting in the formation of new neuronal circuits that restore electrophysiological activity and behavior[24]. When stem cells are cotransplanted with a supportive fibronectin matrix containing growth factors, axons form connections with host axons over significantly long distances. Even when crossing the inhibitory white matter, they elongate rapidly at a rate of 1-2 mm per day[24]. In addition, axons from the host spine regenerate into neural stem cell grafts, and this bidirectional growth contributes to the recovery of hindlimb mobility[24]. BMMSCs were initially thought to be similar to neural stem cells in their ability to multidirectionally differentiate into neurons and glial cells, but this theory was gradually discarded. In fact, the mechanism of action of BMMSCs may be cell fusion or trans-differentiation rather than cell differentiation[25]. In summary, transplanted BMMSCs after SCI exhibit significant autocrine and paracrine activities, which in turn stimulate the proliferation and differentiation of other cells and themselves, promoting tissue repair and functional recovery[26].

AXON GROWTH

The axon is a unique cellular structure that maintains communication between neurons[27]. An important cause of persistent dysfunction after SCI is axonal disruption, and therefore promoting axonal regeneration and plasticity is very promising. However, previous studies have shown that the percentage of injected MSCs aggregating in the CNS is 0.75%-18.5%[28] and 6.7%[29], and thus it is conceivable that only a fraction of the cells reach the site of SCI. The Nakano *et al*[30] found that bone marrow stromal cell transplantation *via* cerebrospinal fluid was effective in acute, subacute and chronic spinal cord injuries in rats, and although the transplanted cells did not survive more than 7 d, a large number of axons crossed longitudinally through the astrocyte-deficient connective tissue. Transplantation *via* the CSF is a more clinically preferred modality because it does not cause secondary injury to preserved spinal cord tissue. From another perspective, Okuda *et al*[31] demonstrated that transplantation of BMMSC sheets after SCI prompted Tuj-1-positive axons to span a specially designed cell sheet without requiring a scaffold, offering a permissive microenvironment for damaged axons[31].

BMMSC therapy has been shown to play a positive role in rodent models of SCI[32], but evidence of its long-term therapeutic efficacy and effectiveness in human clinical trials is limited. Gene modification of MSCs, for example, by overexpression of neurotrophic or growth factors, is one of the ways to enhance their well-known beneficial effects. Overexpression of IL-13, an inducer of M2 microglia/macrophages, in BMMSCs significantly ameliorates axonal retraction caused by axonal-attacking macrophages[33]. In addition, the combination of neurotrophic factors and physical stimuli is often used to enhance the effects of BMMSCs. Cografting stromal-derived factor-1-overexpressing BMMSCs with

neural stem cells (NSCs) at day 9 after SCI resulted in better axonal density enhancement than BMMSCs alone[34]. Furthermore, physical factor therapy has also shown a synergistic effect, as low-intensity pulsed ultrasound-optimized BMMSCs transplanted one week after SCI showed a good promotion effect on axonal regeneration[5].

Although the transplanted cells can reach the site of injury, they cannot survive long enough to integrate with the host spinal cord tissue, thus showing that these cells do not act as scaffolds for tissue repair. The use of biomaterials can provide transplanted cells with an environment closer to their physiological state, maintaining and regulating stem cell properties and protecting them from the harsh local microenvironment. Permissive bridging material allows nerve fibers with regenerative growth potential or collateral sprouting to pass through nonpermissive physical spaces[35]; therefore, tissue-engineered grafts loaded with cells and growth factors have become popular as bridging therapy for SCI. After spinal cord injuries in rats and dogs, NT-3/fibroin-coated gelatin sponge scaffolds were shown to continuously release NT-3 for up to 28 d, maintain the cell activity of MSCs, promote axonal regeneration, and attenuate the inflammatory response[36]. The multichannel poly lactic-co-glycolic acid scaffolds implanted with Schwann cells and BMMSCs were shown to effectively connect the injury gap of rats with complete SCI, and the cell combination strategy promoted the survival and neuron-like characteristics of BMMSCs and finally facilitated axonal regeneration and functional recovery[37]. The short half-life and rapid clearance challenges posed by the innate immune system have hampered the popularity of extracellular vesicle therapy[38]. Wang *et al*[39] synthesized an F127-polycitrate-polyethyleneimine hydrogel (FE) with sustainable and long-term extracellular vesicle release (FE@EVs), and its *in situ* administration after SCI inhibited the inflammatory response and promoted myelination and axonal regeneration. There is no shortage of biomaterials in clinical trials for cancer, but no clinical trials on biomaterials for SCI repair have been registered on the ClinicalTrials.gov website. Possible reasons for this include inconsistency between injury models from preclinical studies and actual injury models in the clinic (thoracic SCI models are often used in preclinical studies, but cervical SCI is more common in humans[40]), unpredictable residual degradation products in the body, and potentially low payloads [41]. Caution is needed in drawing conclusions about axonal regeneration because the current consensus is that it refers to regrowth of axons after transection[42], whereas there is a significant degree of axonal preservation in incomplete spinal cord injuries, and the best model for exploring axonal regeneration is complete SCI.

REMYELINATION

Demyelination in traumatic SCI can lead to loss of function, and poor myelination of preserved nerve fibers may lead to permanent functional impairment. Myelin loss is accompanied by oligodendrocyte apoptosis, and replacement of lost oligodendrocytes and myelin improves conductivity and protects axons from degeneration[43]. The process of transplanted cells producing myelin around axons that have lost myelin sheaths is a mechanism that enhances recovery after SCI[43]. Conditioned medium from MSCs not only improves the survival of oligodendrocytes *in vitro* in culture but also increases levels of Olig2, a transcription factor that plays a key role in the differentiation of oligodendrocytes, in SCI[44]. Intravenous infusion of MSCs during the chronic phase in a severely injured SCI model promotes remyelination of axons[45]. BMMSCs can act as catalysts for the differentiation of NSCs into oligodendrocytes by regulating Id2 and Olig1/2[46]. However, the genetically engineered cells showed a more satisfactory therapeutic effect than the original cells. BMMSCs secrete various trophic factors, including VEGF, insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), basic FGF, GDNF [47,48], and certain extracellular matrix molecules, such as laminin and type IV collagen[49]. Given the positive effects of IGF-1 on oligodendrocyte differentiation and survival during normal development [50], IGF-1-overexpressing BMMSCs better protect the integrity of myelin sheaths[51]. Similarly, GDNF is a potential target for axon enhancement and is directly involved in axonogenesis and dopaminergic neuronal differentiation *via* the Ras/MARK pathway and P13K signaling pathway[52].

In addition to the abovementioned cytotrophic factors, myelination-related graft factors can also be used as targets for genetic engineering. Silencing Nogo-66 receptor expression was shown to promote neurite outgrowth after BMMSC differentiation and increase myelinated nerve fibers after SCI[53]. There are benefits from the effects of neuroprotective drugs themselves, which have antioxidant effects, inhibit intracellular calcium overload, regulate γ-aminobutyric acid receptors and inhibit apoptosis, and combining these drugs can also improve the therapeutic effect of BMMSCs. Currently, the combination of BMMSCs and propofol, a neuroprotective anesthetic, has dramatically increased the number of myelinated and nonmyelinated fibers after SCI[54]. Transplanted cells with the ability to myelinate do not always promote functional recovery, and sufficient myelination needs to occur to achieve significant results[55].

IMMUNOMODULATION

The inflammatory response is a critical component in the secondary injury cascade, which can persist for weeks to months after SCI, during which microglia/macrophages and leukocytes are recruited to the injury site. A certain inflammatory response in the injured spinal cord is required for clearing neurotoxic cellular debris and limiting tissue damage. However, macrophage clearance can promote regeneration after SCI. In addition, overactivation of the inflammatory response can aggravate tissue damage[56]. In the acute phase of SCI, these cells produce proinflammatory cytokines such as IL-6, IL-β, and TNF-α. Reactive oxygen species (ROS), matrix-metalloproteinase (MMP), and inducible nitrous oxide synthase are released by neutrophils, macrophages and microglia, which can exacerbate the inflammatory response[57]. Microglia/macrophages have been regarded as an important cell type in the innate and adaptive immune responses after SCI[58]. Homeostatic macrophages are the main phenotype in the normal spinal cord, but resting macrophages are activated into different phenotypes. Some macrophages produce proinflammatory cytokines that aggravate inflammation and inhibit axon regeneration, while other macrophages produce anti-inflammatory cytokines that promote functional recovery, such as IL-10 and transforming growth factor-β (TGF-β)[59]. Therefore, immunomodulatory therapeutic approaches are mainly focused on inducing macrophage polarization from the proinflammatory phenotype to the anti-inflammatory phenotype following SCI.

Many studies have shown that BMMSC transplantation after SCI can exert therapeutic effects by attenuating detrimental inflammation or enhancing beneficial inflammation. Yagura *et al*[60] found that human BMMSC implantation significantly increased CCL5 expression and enhanced macrophage polarization. Similarly, another study analyzed the tissue expression levels of IL-1β, TNF-α, and Toll-like receptor 4 (TLR4) in a rat SCI model after intravenous BMMSC injection[61]. This study demonstrated that BMMSCs could attenuate spinal cord inflammation by inhibiting the TLR4-mediated signaling pathway and decreasing the expression levels of IL-1β and TNF-α. Similar situations were observed in another study that investigated the efficacy of grafted BMMSCs after SCI[62]. Interestingly, most recent studies have focused on the anti-inflammatory roles of exosomes derived from BMMSCs in SCI treatment[20,63,64]. For example, Sheng *et al*[63] confirmed that the application of BMMSC-derived exosomes promoted the phagocytosis ability of macrophages by upregulating the expression of MARCO, an important phagocytic receptor. In addition to modulating the phagocytic capacity of macrophages, BMMSC-derived exosomes could also affect the balance of macrophage polarization through the nuclear factor-kappaB pathway[65].

BMMSC transplantation exerts immunoregulatory effects following SCI mainly by inducing the formation of anti-inflammatory immune cells, modulating the expression levels of TLRs, and inhibiting the inflammatory response in the injured spinal cord, thereby promoting functional recovery[61]. Immediately after SCI, the organism enters an immunosuppressive state due to shock and stress. The first cells to mobilize at the injured site are the myeloid cells of the innate immune system, such as neutrophils and macrophages, which phagocytose debris. Then, adaptive immune cells such as B- and T-lymphocytes are recruited to the injured spinal cord[66,67]. As previously described, microglia, which participate in the clearance of apoptotic and myelin debris, are resident immune cells[68]. In the acute stage after SCI, microglia extend their processes toward the injured site and are biased toward the activated subtype, which leads to a further loss of neurons and promotes scar formation[69]. During this period, although microglia have beneficial effects on tissue recovery by clearing cellular and myelin debris, they have also been reported to aggravate secondary tissue damage and axonal retraction[67,70]. Homeostatic microglia in the injured spinal cord may play a protective role in tissue repair. Therefore, therapeutic strategies for targeting immunomodulation should be directed at modulating cytokine levels and other factors in the microenvironment and balancing activated/homeostatic microglia levels.

IMPROVING THE INHIBITORY MICROENVIRONMENT

In mammals, damaged axons in the spinal cord are unable to regenerate at the lesion site and to reestablish synaptic connections with their destination due to “natural barriers” and diminished intrinsic regenerative capacity[71,72]. This barrier consists of a lumen and a nonpermissive environment. Nogo-A, MAG and oligodendrocyte myelin glycoprotein (OMgp) are well-defined myelin breakdown products[73], but knockdown of the MAG and OMgp genes did not lead to neuronal growth after SCI, suggesting that Nogo-A plays a dominant role in inhibiting axonal regeneration, while MAG and OMgp play secondary roles[74]. In addition, several types of cells, including astrocytes, glial cells, and microglia/macrophages, migrate to the center of the injury, leading to the formation of glial scars and impeding the progression of axonal growth cones[75]. Glial scarring does not always play a negative role; on the one hand, it initiates the injury repair process, limits lesion expansion and inhibits the spread of the inflammatory response, and on the other hand, it acts as a physical barrier to nerve regeneration[76].

Few oligodendrocyte markers were found to be expressed in SCI centers transplanted with BMMSCs, while the number of axons was significantly increased, indicating that the transplanted cells provided a suitable environment for the regeneration and neural differentiation of endogenous neural stem cells [77]. BDNF in overexpressed BMMSCs further promotes axonal remyelination by affecting oligodendrocytes[78]. Zhao *et al*[79] found that the association of BDNF-overexpressing BMMSCs with platelet-rich plasma promoted more axonal remyelination and oligodendrocytes, probably due to astrocyte migration to the lesion region and increased graft BDNF-BMSCs, which could provide a favorable substrate for stabilizing regenerating axons in an inhibitory environment. However, damaging properties of the microenvironment (such as increased ROS) result in extensive stem cell death and dysfunction, severely impairing stem cell therapy for SCI.

INHIBITING GLIAL SCAR FORMATION

The spinal injury scar is generally classified into two components: The lesion core and the lesion border [80]. The lesion core is primarily composed of stromal-derived fibroblasts and inflammatory immune cells and is commonly regarded as the fibrotic scar. The lesion border is formed by microglia and reactive astrocytes, which surround the core and are generally considered the glial scar. The glial scar (mainly astrocytic) strongly upregulates the expression of intermediate filament proteins such as vimentin, GFAP, and chondroitin sulfate proteoglycans (CSPGs)[9]. At the acute stage after SCI, the glial scar separates healthy tissue from inflammatory cells and limits the spread of inflammation. However, the glial scar creates a long-lasting physical and molecular barrier that hinders axon regeneration and outgrowth during the chronic period[81]. Over the past decade, accumulating evidence has attributed the failure of axon regeneration to diminished intrinsic neuronal plasticity and the presence of glial scars and myelin-associated growth inhibitors.

Numerous experimental studies in SCI animal models have shown that BMMSC transplantation can suppress glial scar formation. Okuda *et al*[31] explored whether BMMSC sheets are permissive for injured nerve fiber elongation and the extension of astrocyte processes. They found that GFAP-positive astrocyte processes penetrated into the BMMSC-transplanted site, which is an indicator of a permissive environment for axon outgrowth[31]. Another study showed that transplantation of human BMMSCs in an SCI rat model largely reduced the inflammatory reaction and the expression of collagen type IV, one of the markers of fibrotic scars[82]. The application of biomaterials to improve the survival rate and efficacy of implanted cells has attracted attention in recent years. Some biomaterials are primarily used as scaffolds to support the growth of BMMSCs[83,84], while a few biomaterials are capable of modulating the harsh microenvironment following SCI[85,86]. For instance, Li *et al*[84] investigated the efficacy of a ROS-responsive hydrogel cotransplanted with BMMSCs in a rat transection SCI model. They performed double immunofluorescence staining to identify the formation of two distinct scars: The glial scar was labeled by GFAP, and the fibrotic scar was labeled by platelet-derived growth factor receptor-β. These results revealed that this BMMSC-encapsulated hydrogel could significantly alleviate the formation of both glial and fibrotic scars at the injury site[84]. In addition, CSPGs, which are enriched in glial scars and secreted by reactive astrocytes, are potent inhibitors of axonal outgrowth. Takeuchi *et al*[87] found that SCI mice treated with chondroitinase ABC, a kind of CSPG-digesting enzyme, can promote axon regeneration and improve functional outcome. Indeed, another study explored the efficacy of chondroitinase ABC plus BMMSCs in the repair of SCI. Notably, the application of chondroitinase ABC combined with BMMSCs significantly reduced GFAP expression at the injury site, and the scar tissue area was much smaller than that in the model group[88].

To date, the specific molecular mechanisms of scar formation have been widely studied. TGF-β initiates signaling after binding to transmembrane type I and type II receptors, and then the type I receptor leads to the recruitment and phosphorylation of Smad2 and Smad3 proteins[89]. After the formation of a heteroprotein complex together with the co-Smad protein SMAD4, this complex can translocate into the nucleus, where it acts as a transcription factor to regulate target gene expression [90]. There is a growing body of evidence to suggest that the TGF-β/Smad signaling pathway plays an important role in collagen deposition[91]. Studies have shown that administration of BMMSCs after SCI can inhibit scar formation by downregulating TGF-β and collagen expression[92,93]. Furthermore, studies have shown that the activation and proliferation of astrocytes can be suppressed after inhibiting the JAK/STAT3 or JNK/c-Jun pathway, thus reducing scar formation and promoting functional recovery after SCI[94]. Kim *et al*[95] suggested that acute transplantation of BMMSCs can modulate astrogliosis through the MMP2/STAT3 pathway.

In summary, BMMSC transplantation can suppress glial scar formation and provide a favorable environment for axon regeneration after SCI. However, there has been some debate in the field on the role of scar formation in recovery following SCI[96,97]. Although the glial scar has an important protective role in separating healthy tissue from pathology after injury, it has been acknowledged that scar formation has an inhibitory role, as it is associated with failed axon regrowth. Recent evidence suggests that the phenotypes of reactive glial cells are considered the key regulators of the dual nature of the spinal injury scar[98]. In addition, the opposing roles of the scar matrix cannot be ignored, which

contains beneficial molecules that are required for the formation of scar borders and inhibitory molecules such as CSPGs, tenascin, ephin B2, and slit proteins[99]. Therefore, therapeutic strategies for targeting the spinal injury scar should be directed at reducing scar formation or blocking inhibitory molecules associated with the scar.

PREVENTING BLOOD VESSEL LOSS AND IMPROVING ANGIOGENESIS

After SCI, immediate loss of spinal vascular support occurs, which induces local hypoxia around the injury epicenter, followed by a series of molecular cascades that lead to increased microvascular permeability and BSCB disruption. Angiogenesis, which is the process of forming new vasculature, plays an important role in the proliferation phase of wound healing[100,101]. Therefore, stabilizing the provisional vessels and forming a permanent vascular network are necessary for tissue repair following SCI. Angiogenesis is a multistep process that requires endothelial proliferation and differentiation, crosstalk among endothelial cells and extracellular matrix components, and the interplay of multiple proangiogenic and antiangiogenic factors[102]. VEGF, which is an important proangiogenic factor, is upregulated after hypoxia stimulus and induces blood vessel morphogenesis by binding to the VEGF receptor. Other critical angiogenesis-related proteins include FGF, TGF-β, angiopoietin-1, and angiopoietin-2[103].

Accumulating evidence has documented that BMMSC engraftment can promote angiogenesis and vascular stability in the treatment of different diseases, especially ischemic diseases, such as myocardial infarction and cerebral infarction[104-109]. Similarly, enhancement of angiogenesis by implanted BMMSCs has been demonstrated after SCI in animals[108]. In addition, *in vitro* studies also confirmed that BMMSC transplantation promotes vascular formation and vasoprotection[110,111]. In general, there is a close relationship between angiogenesis and enhanced functional recovery following SCI. Emerging evidence has shown that improved angiogenesis and BSCB integrity can promote motor function recovery[112,113]. A recent study found strong correlations between the level of angiogenesis and the number of surviving BMMSCs at the injury site. This study demonstrated that the expression of occludin and ZO-1 was significantly upregulated, which indicates the maturation and sealing of newly formed vasculature[114]. BMMSCs can produce FGF and VEGF-A, which can enhance the proliferation, migration, and vascular tube formation of microvascular endothelial cells[115]. BMMSCs also secrete specific factors, including IGF-1, HGF, VEGF, NGF, and TGF-β1, which can provide a favorable environment for angiogenesis after SCI[116]. For instance, Cantinieaux *et al*[117] investigated the efficacy of conditioned medium from BMMSCs in SCI treatment in a rat model and found that blood vessels displayed larger diameters in the conditioned medium-treated group, indicating enhanced regional blood perfusion at the lesion epicenter.

BMMSC engraftment can promote revascularization, enhance blood supply and increase BSCB integrity, which will attenuate secondary injury and promote axon growth, thereby improving functional recovery following SCI. Improved functional outcome after SCI is closely related to successful revascularization. First, a well-vascularized injury site can provide a regeneration-permissive microenvironment for the transplanted cells to survive. Additionally, blood vessels may act as a scaffold to guide transplanted cell migration and axon sprouting after injury. Emerging evidence has demonstrated a significant interaction between vascular regrowth and nerve repair. For example, some neurotrophins, such as NGF and NT-3, can control the sympathetic innervation of blood vessels, and VEGF-A, secreted by neurons and glial cells, can enhance vascular regrowth. To date, treatments based on revascularization for SCI include gene modulation, proangiogenic factor administration, cell therapy and biomaterial application. However, many aspects of the process of blood vessel formation remain unclear, and the therapeutic effect is limited. Therapeutic strategies for targeting angiogenesis after injury should be focused on the identification of combined strategies.

CLINICAL TRIALS OF BMMSCS FOR SCI PATIENTS

During past decades, cell transplantation has been regarded as a promising therapy after SCI. There have been not only many animal and preclinical studies but also a considerable number of clinical studies, and several systematic reviews/meta-analyses have proven the effects of cell transplantation in patients with SCI[118-122]. Among them, MSC transplantation is the most widely used and promising therapeutic approach for treating SCI. MSCs are mainly derived from adipose tissue and bone marrow sources and are accordingly divided into adipose tissue-derived MSCs and BMMSCs. The main stem cell type used in clinical trials to treat SCI is the BMMSC, which has lower immunogenicity and a widely available source and has been proven to be overall safe, well tolerated and valid in SCI patients, with a particular effectiveness in chronic and complete injuries.

After assessing the relevant literature, we found 38 clinical studies containing 1090 participants that provided overall evidence of the safety and efficacy of BMMSC cell transplantation for SCI patients, which was mainly represented by ASIA score improvement in at least one segment, and both sensory

and motor improvements were observed according to different previous studies. Some studies have shown that up to 70% of patients with complete cervical SCI and 33% of patients with thoracic SCI could recover at least one spinal cord level within 1 year after injury by spontaneous recovery[123,124]. Chhabra *et al*[125] indicated that most of the spontaneous neurological recovery in AISA subjects was likely to occur within the zone of partial preservation, which was less likely related to cell therapy. Due to the complicated process of neuroregeneration, minor therapeutic effects at the anatomical/histological level were difficult to detect in clinical trials, which might be ignored. Some novel assessments may provide further insights into the recovery of neuroregeneration after SCI in future clinical research. Furthermore, an increasing number of studies have tended to certify the same efficacy on bladder and gastrointestinal functions by slightly improved maximum capacity and decreased bladder pressure and residual urine volume, which are still unsatisfactory.

Considering the various therapy effects, there were related issues: Ambiguities in the selection of patients, timing of intervention, injection doses and routes of stem cell transplantation in different clinical trials. The optimal dose of cell transplantation has not yet been determined, and cell numbers between 10^6-10^8 seemed to be more beneficial[126-128]. Transplant routes included intrathecal, scaffold-loaded, intralesional, venous, arterial, and subdural administration, with intrathecal injection as the most widely used.

However, there were still some potential adverse events (AEs) observed, such as neuropathic pain, muscle spasm, and fever. Some of these AEs were slight and without further injury, while other more potentially serious AEs required a longer follow-up visit. Most of the current studies have a small number of samples, are low quality, lack control groups, and represent single-arm, early-stage clinical trials with the main purpose of evaluating the safety of stem cells. Nonetheless, prospective, well-designed randomized trials in larger cohorts with extensive follow-up are still awaited to confirm and update the findings.

CONCLUSION

Based on the results of previous studies, the effects that can be achieved with a single BMMSC treatment are limited, and combination therapy is an important future development direction. Combination therapy using various molecules or factors (including gene modulation, *etc.*) can enhance the effect of cellular therapy and achieve multi-effective and superimposed effects. In addition, most preclinical studies are currently designed with observation periods of 4 and 8 wk, and longer observation periods are important for the clinical translation of stem cell therapy to explore and address certain adverse effects when possible. Finally, given the current encouraging preclinical trial results, some treatments have been translated into clinical practice. BMMSC transplantation has been shown to be safe in SCI patients, and partial efficacy has been seen in some cases, but most clinical studies are still in phases I and II, and the results of phase III trials have extraordinary implications for the clinical translation of stem cell therapy for SCI. In conclusion, although many problems and challenges remain, researchers have been working to optimize preclinical studies and actively translate them to the clinic, and these efforts will pave the way for the field of SCI.

FOOTNOTES

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REVIEW

Different priming strategies improve distinct therapeutic capabilities of mesenchymal stromal/stem cells: Potential implications for their clinical use

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Abstract

Mesenchymal stromal/stem cells (MSCs) have shown significant therapeutic potential, and have therefore been extensively investigated in preclinical studies of regenerative medicine. However, while MSCs have been shown to be safe as a cellular treatment, they have usually been therapeutically ineffective in human diseases. In fact, in many clinical trials it has been shown that MSCs have moderate or poor efficacy. This inefficacy appears to be ascribable primarily to the heterogeneity of MSCs. Recently, specific priming strategies have been used to improve the therapeutic properties of MSCs. In this review, we explore the literature on the principal priming approaches used to enhance the preclinical inefficacy of MSCs. We found that different priming strategies have been used to direct the therapeutic effects of MSCs toward specific pathological processes. Particularly, while hypoxic priming can be used primarily for the treatment of acute diseases, inflammatory cytokines can be used mainly to prime MSCs in order to treat chronic immune-related disorders. The shift in approach from regeneration to inflammation implies, in MSCs, a shift in the production of functional factors that stimulate regenerative or anti-inflammatory pathways. The opportunity to fine-tune the therapeutic properties of MSCs through different priming strategies could conceivably pave the way for optimizing their therapeutic potential.

Key Words: Mesenchymal stromal/stem cells; Mesenchymal stromal/stem cell therapeutic properties; Mesenchymal stromal/stem cell paracrine effects; Mesenchymal stromal/stem cell priming; Pro-inflammatory priming; Hypoxic priming, 3D culture priming

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Core Tip: Mesenchymal stromal/stem cells (MSCs) have demonstrated promising therapeutic results in the field of regenerative medicine. However, due to their heterogeneity, the application of MSCs in clinical trials has shown moderate or poor efficacy. Here, we review data on the principal priming approaches for enhancing the therapeutic potential of MSCs. We found that different priming strategies can modify MSC properties and, in this case some therapeutic effects on different disease models can be obtained in relation to dose and/or combination of the priming factors used. The production of priming type-specific functional factors in MSCs could pave the way toward implementing new MSC-based therapies.

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INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) are multipotent adult stem cells involved in the homeostasis of tissue regeneration and, because of their therapeutic potential, have been extensively investigated in various clinical conditions[1-6]. Though MSC treatment was initially thought to promote tissue regeneration thanks to MSC multipotency of differentiation[7-9], recent evidence has revealed that the efficacy of MSC-based therapies is, at least in part, linked to the production of functional paracrine factors. These cells are able to secrete numerous products, *e.g.*, growth factors, cytokines, chemokines, and extracellular vesicles (EVs), which can regulate many pathophysiological processes, such as fibrosis, immune dysregulation, angiogenesis, and stimulation of tissue resident stem cells, in order to coordinate both tissue regeneration and functional recovery[10-12]. In injured tissue, MSC engraftment is limited because they undergo cell death, and their beneficial effects are exerted through secretion of various functional factors that not only enhance the function of resident cells, but also attract immune and progenitor cells, contributing to the coordination of tissue repair[13,14]. Therefore, considering the importance of the paracrine component in mediating MSC functions, there is growing interest in the molecular basis of MSC secretion involved in the therapeutic function of these cells.

Over the years, a large number of tissues, including placenta, adipose, umbilical cord, dental pulp, bone marrow, synovial membrane, liver and others, have been used as a source of MSCs[15-20]. It is quite clear that MSCs derived from all these sources possess a wide variety of functional effects, which they apply physiologically to their own original tissue, regulating homeostasis and regeneration. Interestingly, these effects may be useful for therapeutic applications of MSCs[3,21]. Currently, there are 1487 clinical trials registered at clinicaltrials.gov aimed at studying MSC therapeutic efficacy in the treatment of several clinical disorders, including lung, liver, kidney, orthopedic, cardiovascular, neurodegenerative, and immune diseases. In different clinical settings, MSC-therapies have been tested, showing tolerable safety, and demonstrating therapeutic benefits, and this has led to regulatory approvals of some MSC-based therapeutic products in several countries. In 2012, Cartistem, a MSC product based on the use of umbilical cord-derived MSCs for the treatment of traumatic or degenerative osteoarthritis, was approved by Korea's Ministry of Food and Drug Safety[22]. Moreover, Remestemcel-L, based on the use of bone marrow-derived MSCs (BM-MSCs), has been investigated in a phase 3 clinical trial in patients with steroid-refractory acute graft-versus-host disease (GVHD)[23]. Recently, due to the immunomodulatory properties of Remestemcel-L, which are able to work against cytokine storm linked to several inflammatory conditions, this therapy has also been tested for the treatment of coronavirus disease 2019-associated multisystem inflammatory syndrome[24]. The increasing interest in the clinical applications of MSCs as a cellular therapy has also been evidenced by the burgeoning of several companies that sell MSC therapies to United States clinics[25]. However, this has highlighted that in some cases the propensity for economic gain has outweighed the clinical advantages, despite the lack of solid scientific evidence that supports the broad use of MSCs in treating various human disorders. Indeed, in many clinical trials it has been shown that MSCs have moderate or poor efficacy, and the results from some studies are controversial[26-31]. In particular, due to both the inconsistent criteria used for the MSC identity across studies, and MSC heterogeneity, which depends on the different MSC origin[32] and the diverse harvesting and culture strategies[33], the clinical results obtained after MSC therapy are frequently variable. This makes it very difficult to obtain reliable conclusions regarding MSC therapeutic efficacy. Thus, while MSCs demonstrate a good margin of safety as cellular treatment, they have usually been therapeutically ineffective in humans[21].

These issues have underscored the urgent need to optimize the clinical use of MSCs or enhance MSC therapeutic effects. After determining the most appropriate cell source to use both in terms of invasiveness for cell isolation and cell yield, specific standardized production methods are needed to

ensure MSC therapeutic abilities and, therefore, their clinical efficacy. MSCs can be considered a key regulatory component in the tissue stem cell niche and, starting with the physiological role that these cells play in regulating tissue regeneration following injury[3,4,6,34-39], specific priming strategies can be understood and adapted for MSC clinical application. In this regard, much attention has been paid to the opportunity of MSC pre-conditioning to prime the cells before their clinical use. In this case, the therapeutic properties of MSCs can be modulated by pre-treatment of cells with hypoxia, cytokines, as well as growing MSCs under three-dimensional (3D) culture. In those instances, in response to MSC priming, the phenotype of MSCs was switched toward an anti-inflammatory, pro-trophic and more regenerative potential, which results in an enhanced therapeutic function of the cells[3,40-45].

In this review, we summarize the principal priming methods aimed at improving MSC efficacy as a therapeutic product. We would also like to highlight the fact that specific priming strategies can be considered more suitable for some types of diseases, leading to new therapeutic approaches that could be used to develop more powerful and predictable MSC therapies.

THE SECRETION OF PARACRINE FACTORS MEDIATE THE THERAPEUTIC FUNCTION OF MSCs

The secretion of functional products is central to MSC-based therapy, as demonstrated in numerous studies. Indeed, individual components of MSC secretome, such as functional proteins and EVs, are involved in the regulation of various biological processes, including angiogenesis, immunoregulation, wound healing, and tissue repair/protection[14,46-49]. Among the MSC-derived functional products, exosomes (EXOs), belonging to EVs, are anuclear particles ranging from 50 to 200 nm in size that are constitutively released from the endosomal compartment of MSCs. They contain a plethora of functional protein and other molecules, including microRNAs (miRNAs), which mediate several MSC properties [15,50,51]. EXOs are key components of intercellular communication, because they are released into the intercellular space where they exert local paracrine or distal systemic effects[52]. In fact, EXOs are able to regulate numerous biological processes, including angiogenesis[53], cell proliferation[54], and the activation/inhibition of immune cells[55]. Interestingly, EXO content can be changed by various priming stimuli[3,40,55]. Recently, it has been revealed that EXO-derived miRNAs play a critical role in mediating EXO effects[56]. MiRNAs are 19-22-nucleotide-long non-coding RNAs that regulate mRNA translation, and are involved in many cellular processes[56,57]. Therefore, even if some therapeutic functions of the MSCs are mediated by cell-to-cell contact, the secretion of paracrine factors can be considered the main mechanism by which MSCs elicit functional responses in target cells[3,40,58,59]. In many *in vitro* and *in vivo* disease models, MSC-derived products have been identified as responsible for therapeutic effects[60-63]. For example, promising preclinical therapeutic effects have been obtained using MSC-derived EVs. In particular, regarding BM-MSC-derived EVs, Haga *et al*[64] found that these functional factors were able to reduce hepatic injury by modulating cytokine expression in a mouse model of fulminant hepatic failure. Reis *et al*[65] demonstrated that the administration of EXOs in a rat model of gentamycin-induced kidney injury, was able to improve the kidney injury score. Moreover, it has been shown that EXOs derived from umbilical cord-derived MSCs were able to accelerate wound healing in a rat skin burn model[66], and EXOs derived from BM-MSCs overexpressing hypoxia-inducible factor (HIF)-1α accelerated bone regeneration and angiogenesis in a rabbit model of steroid-induced avascular bone necrosis[67].

MSCs can also secrete a number of cytokines/chemokines that control both the innate and adaptive immune responses, resulting in immunoregulation and the induction of tolerance[68]. Indeed, it has been shown that MSCs can produce both anti- and pro-inflammatory factors which, depending on their ratio, regulate the pro- or anti-inflammatory activity of MSCs[69]. In this case, final immunoregulatory properties may be affected by cell culture conditions that can prime/enhance MSC properties[3,70,71]. MSCs also have the ability to roll and adhere to post-capillary venules, and migrate to injured tissues, contributing to tissue repair/regeneration[72]. In this case, once MSCs reach the site of the injury, these cells put in place an active regulation by producing paracrine factors that impact tissue survival/repair, and activate tissue resident stem cells[3,73,74]. The secretion of various soluble factors has also been found to be responsible for the pro-angiogenic and anti-apoptotic effects of MSCs[75]. Though not well understood, the beneficial effects of conditioned media (CM) derived from MSCs have been clearly demonstrated by various experimental findings, supporting the concept of paracrine effects[76]. Several preclinical studies have tested the efficacy of CM in different diseases models. MSC-derived CM has been shown capable of improving cell viability and reducing inflammation in both *in vitro* and *in vivo* models of lung ischemia/reperfusion injury (IRI)[59,77]. Moreover, it has been demonstrated that BM-MSC-derived CM was able to reduce lung inflammation and edema in a mouse model of lipopolysaccharide-induced lung injury[78], and to improve renal tissue pathology in a mouse model of cisplatin-induced kidney injury[79]. Youdim *et al*[80], in a rat model of fulminant hepatic failure, found that the CM derived from BM-MSCs reduced leukocytic infiltrates and hepatocellular death. The CM derived from the same cells, in a mouse model of antigen-induced arthritis, was also able to reduce joint swelling, cartilage loss, and tumor necrosis factor (TNF)-α secretion[81]. In a rat model of lung fibrosis

and hypertension, using CM derived from adipose MSCs (AdMSCs), demonstrated the ability of secretome to reduce collagen deposition and improve lung blood flow[82]. In a rabbit model of surgical bone lesion, Linero and Chaparro[83] found that the CM produced from AdMSC cultures induced bone regeneration.

THE SECRETION OF MSC PARACRINE FACTORS CAN BE MODULATED BY VARIOUS PRIMING STRATEGIES

Given the heterogeneity of results supporting the efficacy of MSCs in the treatment of different human disorders, there is a need to improve the therapeutic properties of MSCs, and the best way might be that of preconditioning/priming. Though this approach has been widely used in the field of immunology, has also been effectively applied to MSCs[3,84,85]. Among commonly used priming strategies, leading approaches can be attributed to three main categories: (1) MSC priming with inflammatory molecules; (2) MSC priming with hypoxia; and (3) MSC priming with 3D cultures. These priming signals activate potential MSC mediators, including surface receptors and ligands, signalling molecules that induce survival/growth, regulatory molecules such as miRNAs, and transcription factors, which can modify the MSC phenotype[86-89], with a consequent boosting of MSC therapeutic functions (Figure 1).

Priming with inflammatory molecules

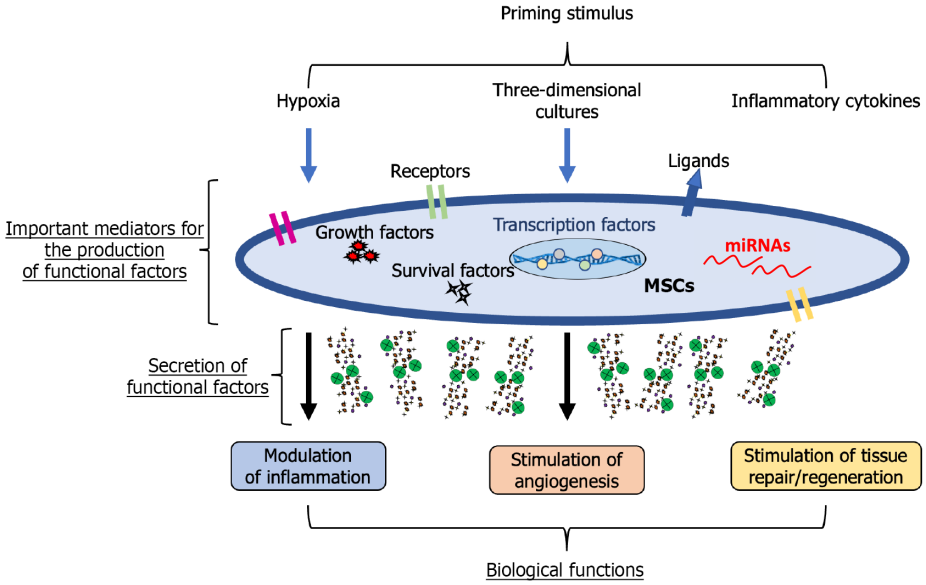
Numerous studies have revealed that the immunosuppressive properties of MSCs are not intrinsically possessed, but require priming of MSCs by inflammatory factors[90-92]. Depending on the inflammatory conditions, it has been demonstrated that MSC phenotypes can be polarized into MSC type 1 (with pro-inflammatory properties) and MSC type 2 (with immunosuppressive properties)[93,94]. Several strategies have been implemented to modulate/enhance the secretion of functional molecules in MSCs. As shown in Figure 2, the treatment of MSCs with inflammatory cytokines, including interferon-gamma (IFN-γ), interleukin (IL)-1α/β, IL-25, IL-6, TNF-α, and IL-17 enhanced the immunomodulatory properties of MSCs[40,95-112]. These treatments increase the production/secretion of functional factors, including hepatocyte growth factor (HGF), transforming growth factor-β, IL-6, prostaglandin E2 (PGE2), leukemia inhibitory factor, granulocyte colony-stimulating factor, IL-10, macrophage inflammatory protein-1α, indoleamine 2,3-dioxygenase (IDO), intercellular adhesion molecule, programmed death ligand 1-2, monocyte chemoattractant protein (MCP)-1, monokine induced by IFN-γ, induced protein 10, and macrophage inflammatory protein-1β, which in turn confer more paracrine immunomodulatory properties to MSCs (Figure 2). It has been demonstrated that CM enriched with the above-described factors was able to inhibit T cell proliferation/activation, reduce the secretion of inflammatory mediators, and induce monocyte polarization towards anti-inflammatory the M2 phenotype[40,102,105-112]. It has been shown that the treatment with inflammatory cytokines was also able to improve the immunomodulatory capabilities of EXOs, and these effects appear to be mediated by specific miRNAs, such as miR-21, miR-23a, miR-26b, miR-125b, miR-130b, miR-140, miR-146a, miR-203a, miR-223, miR-224, and miR-320a[40,109,111,113].

Priming with hypoxia

Differently from inflammatory cytokines, hypoxic treatment of MSCs seems to stimulate primarily the secretion of functional factors involved in the processes of angiogenesis and tissue proliferation/regeneration (Figure 2). Hypoxic preconditioning was able to promote angiogenic potential of MSCs *via* the activation of the HIF-1α-GRP78-Akt axis, and the overproduction of vascular endothelial-derived growth factor (VEGF) and HGF[114]. Lee and Joe[115] demonstrated that hypoxia priming induces an increase in HIF-1α expression and consequent VEGF production, improving the ability of MSCs to stimulate migration and tube formation of human umbilical vein endothelial cells (HUVECs). Moreover, Bader *et al*[116] found that hypoxic preconditioning induces the anti-apoptotic and pro-angiogenic effects of MSCs compared with untreated cells. In particular, Bcl-xL, BAG1, and VEGF were overexpressed after hypoxic priming, enhancing HUVEC proliferation and migration. Hypoxic MSCs are also able to produce numerous factors related to tissue remodelling, including matrix metallopeptidase 1 (MMP1), MMP2, and MMP9[117-119], as well as crucial factors such as IL-8 and MCP-1, involved in the chemotaxis and activation of innate immune responses[120,121]. Also with regard to EVs, hypoxic priming has been shown to have important effects. Xue *et al*[122] discovered that EXOs derived from hypoxia-treated MSCs were able to increase migration and tube formation of HUVECs through the PKA signalling pathway. Moreover, Ge *et al*[123] demonstrated the efficacy of hypoxic MSC-derived EXOs in enhancing angiogenesis. In particular, they showed that hypoxic EXOs containing miR612 promoted, through HIF-1α activation, the production of VEGF in human brain microvascular endothelial cells, inducing proliferation, migration, and angiogenic activities of these cells.

Priming with 3D culture of MSCs

Various *in vitro* strategies have been applied for the production of MSCs, with improved therapeutic



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Figure 1 Potential mechanisms mediating mesenchymal stromal/stem cell-primed therapeutic properties. Mesenchymal stromal/stem cells (MSCs) can be primed through different signals, including hypoxia, three-dimensional cultures, and inflammatory cytokines to obtain a therapeutic phenotype. The potential mediators of this new phenotype comprise a plethora of regulatory molecules within MSCs, including surface receptors and ligands, signalling molecules inducing survival/growth, regulatory molecules such as microRNAs, and transcription factors regulating several pathways. Thus, primed MSCs can modulate inflammation, stimulate angiogenesis, and promote tissue repair/regeneration. MSCs: Mesenchymal stromal/stem cells; miRNAs: MicroRNAs.

properties, and priming with inflammatory factors may impact the expression of HLA-DR, thus altering allogeneic therapeutic possibilities[124-126]. MSC priming through 3D culture techniques, which allows for the generation of MSC spheroids, strictly recapitulates the *in vivo* MSC niche and enhances the phenotypic profile of MSCs, increasing both trophic and immunomodulatory functionalities. MSC spheroid action is exerted by the paracrine secretion of functional factors that possess anti-inflammatory, angiogenic, anti-fibrotic, anti-apoptotic, and mitogenic properties (Figure 2)[127]. Recently, through omics approaches, such as RNA sequencing and analysis of DNA methylation, it has been demonstrated that, compared with conventional 2D culture, MSC spheroids were able to modify their transcriptome profile by overexpressing genes that can regulate proliferation/differentiation, as well as immunomodulatory and angiogenic processes[128]. Concerning immunomodulatory and regenerative effects, 3D culture of MSCs seems to have more intermediary functions than the above-mentioned priming strategies (priming with inflammatory molecules or hypoxia) (Figure 2). 3D MSC spheroids have been shown to be capable of secreting multiple functional factors. For example, it has been found that various regenerative and immunomodulatory factors, such as stromal cell-derived factor-1α, growth-related oncogene α, MCP-1/3; IL-4, IL-10; epidermal growth factor (EGF), leukemia inhibitory factor, placental growth factor-1, VEGF-A/D, HGF, insulin like growth factor 1, TNFAIP6, STC1, platelet-derived growth factor B, transforming growth factor-β, PGE2, and IDO were up-regulated in 3D MSC spheroids compared with those of the MSCs cultivated under conventional 2D conditions[43,44,59,73,128-131] (Figure 2). The paracrine effects of 3D MSC appear to be also mediated by EVs. In particular, EXOs derived from MSC 3D cultures have been shown to have higher yields and enhanced activity. Indeed, compared with 2D cultures, EXOs isolated from CM of MSC spheroids were able to inhibit T cell proliferation and stimulate angiogenesis *in vitro*[44], as well as attenuate inflammation and periodontitis *in vivo*[132], and stimulate tissue regeneration in both *in vitro* and *in vivo* models[133].

THERAPEUTIC PROPERTIES OF PRIMED MSCs IN PRECLINICAL MODELS

Principal priming strategies to treat chronic immune-related disorders

By virtue of their immunomodulatory properties, MSCs are being studied to treat numerous chronic conditions, including GVHD and inflammatory bowel disorders, in order to attenuate inflammation and induce tissue recovery (Table 1). As already mentioned, treating MSCs with inflammatory factors enhances their immunomodulatory properties, and renders these cells able to inhibit T cell proliferation/activation and induce monocytes toward an anti-inflammatory phenotype. This quality makes these cells more clinically effective when applied to chronic inflammatory-related diseases (Figure 2). Indeed, several experimental studies have demonstrated that the treatment of MSCs with inflammatory factors, such as IFN-γ, IL-1β, and IL-25, enhanced MSC therapeutic effects in *in vivo* models of chronic

Table 1 Representative priming strategies of mesenchymal stromal/stem cells and their application in preclinical studies

MSCs	Dose	Priming treatments	Study model	Observed therapeutic effects	Ref.
AMSCs	1×10^5 MSCs/5×10^5 PBMCs	IFN-γ	*In vitro* model of T cell activation and monocyte M1/M2 polarization	Regulation of T cell activation/anergy and induction of M2-like polarized phenotype in monocytes	[40]
BM-MSCs	0.5×10^6 MSCs/mouse	IFN-γ	*In vivo* model of chronic colitis	Attenuation of inflammation and colitis	[96]
BM-MSCs	NA	IFN-γ; TNF-α	*In vitro* model of MLR	Inhibition of allogeneic MLR	[97]
CB-MSC-derived EVs	NA	IFN-γ	*In vivo* model of acute kidney injury and *in vitro* model of T cell activation	Regulation of T cell activation and amelioration of kidney injury with unprimed MSCs only	[100]
BM-MSCs and CB-MSCs	1×10^6 MSCs/mouse	IFN-γ	*In vivo* model of GVHD	Reduction of the symptoms of GVHD	[101]
BM-MSCs	1×10^4 MSCs/2×10^3 macrophages	IFN-γ; LPS; TNF-α	*In vitro* model of monocyte M1/M2 polarization	Induction of monocyte polarization toward an anti-inflammatory M2 phenotype	[102]
UC-MSCs	1×10^6 MSCs/mouse	IFN-γ; TNF-α	*In vivo* model of GVHD	Reduction of the symptoms of GVHD	[103]
BM-MSCs	2.5×10^5 MSCs/5×10^5 macrophages	IFN-γ; IL-1β	*In vitro* model of monocyte M1/M2 polarization	Induction of monocyte polarization toward an anti-inflammatory M2 phenotype	[105]
BM-MSC-derived CM	NA	IFN-γ; IL-1α/β; TNF-α	*In vitro* model of LPS-injured microglial cells	Reduction in the secretion of inflammatory factors	[106]
AdMSCs; BM-MSCs; CB-MSCs.	NA	IFN-γ	*In vitro* model of T cell activation	Suppression of T cell proliferation	[110]
BM-MSCs	NA	IFN-γ; spheroids	*In vitro* model of T cell activation	Suppression of T cell activation and proliferation	[112]
BM-MSCs	2×10^6 MSCs/mouse	IFN-γ	Autoimmune encephalomyelitis	Attenuation of pathologic manifestations	[134]
BM-MSCs	1×10^6 MSCs/mL	IFN-γ	*In vitro* model of T cell activation and *in vivo* model of colonic wounds	Regulation of T cell activation and acceleration of healing of colonic mucosal wounds	[135]
UC-MSCs	2×10^6 MSCs/mouse	IL-1β	*In vivo* model of chronic colitis	Attenuation of inflammation and colitis	[98]
UC-MSCs	1×10^6 MSCs/mouse	IL-1β	*In vivo* model of sepsis	Increase in survival rate	[109]
MSC-derived EVs	40 μg/mouse	IL-1β	*In vitro* model of monocyte M1/M2 polarization and *in vivo* model of sepsis	Induction of monocyte M2 polarization and amelioration of sepsis	[111]
AdMSC-derived CM	20 μL/rat	TNF-α	*In vivo* model of wound healing	Acceleration of wound closure and angiogenesis	[99]
BM-MSCs	1.6×10^6 MSCs/mouse	TNF-α	*In vivo* model of peritonitis	Attenuation of inflammatory responses	[136]
BM-MSCs	5×10^6 MSCs/rat	IL-25	*In vivo* model of chronic colitis	Attenuation of inflammation and colitis	[95]
BM-MSCs	1×10^6 MSCs/mL	IL-6	*In vivo* model of liver fibrosis	Reduction of liver injury and fibrosis	[104]
BM-MSCs	3.91×10^4 MSCs/3.91×10^6 T cells	IL-17	*In vitro* model of T cell activation	Suppression of T cell proliferation/activation and Th1 cytokines	[108]
AdMSCs	5×10^5 MSCs/mouse	Hypoxia	*In vivo* model of hindlimb ischemia	Improvement of angiogenesis	[114]
BM-MSC-derived CM	100 μL/mouse	Hypoxia	*In vivo* model of wound healing	Acceleration of skin wound healing	[120]
BM-MSCs	2.5×10^5 MSCs/mouse	Hypoxia	*In vivo* model of pancreatic islet transplantation	Reversion of impaired glucose tolerance	[121]
BM-MSCs	5×10^5 MSCs/mouse	Hypoxia	*In vivo* model of hindlimb	Improvement of angiogenesis	[139]

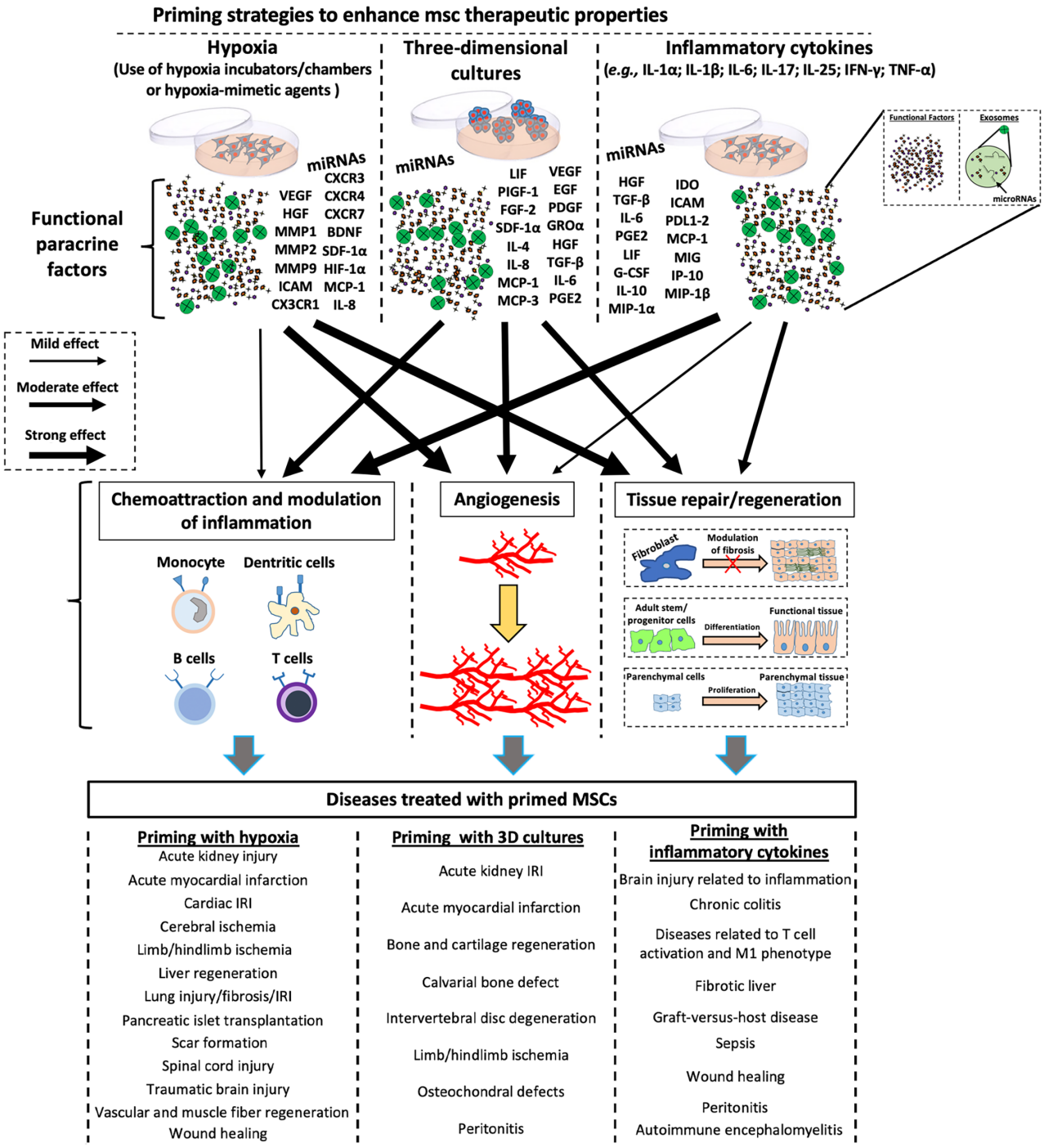
			ischemia		
AdMSCs	5 × 105 MSCs/mouse	Hypoxia	*In vivo* model of hindlimb ischemia	Improvement of functional recovery and neovascularization	[140]
AdMSC-derived CM	NA	Hypoxia	*In vivo* model of partial hepatectomy	Enhanced liver regeneration	[142]
AdMSCs	2 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of acute kidney injury	Improvement of angiogenesis and inhibition of ROS generation	[145]
AdMSC-derived CM	100 μL/mouse	Hypoxia	*In vivo* model of acute kidney injury	Improvement of renal function and reduction of inflammation	[146]
BM-MSCs	1 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of lung IRI	Attenuation of pathologic lung injury score by inhibiting inflammation and generation of ROS and anti-apoptotic effects	[147]
BM-MSCs	NA	Hypoxia	*In vivo* model of radiation-induced lung injury	Improvement of antioxidant ability	[148]
BM-MSCs	1 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of myocardial infarction	Improvement of angiogenesis and function	[150]
BM-MSCs	1 × 10^6 MSCs/mouse	Hypoxia	*In vivo* model of myocardial infarction	Prevention of apoptosis in cardiomyocytes	[151]
BM-MSC-derived EVs	1 μg of EVs/mouse	Hypoxia	*In vivo* model of myocardial infarction	Reduction of cardiac fibrosis	[152]
BM-MSC-derived EVs	50 μg of EVs/rat	Hypoxia	*In vivo* model of cardiac IRI	Reduction of IRI and improvement of cardiomyocyte survival	[153]
BM-MSC-derived EVs	200 μg of EVs/20 g	Hypoxia	*In vivo* model of myocardial infarction	Improved cardiac repair by amelioration of cardiomyocyte apoptosis	[154]
BM-MSCs	1 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of cerebral ischemia	Enhanced angiogenesis and neurogenesis	[157]
BM-MSC-derived CM	100 μg of CM/kg	Hypoxia	*In vivo* model of traumatic brain injury	Improved neurogenesis, motor and cognitive function	[158]
UC-MSCs	1 × 10^5 MSCs/rat	Hypoxia	*In vivo* model of spinal cord injury	Increase in axonal preservation and decrease of apoptosis	[159]
PMSC-derived CM	100 μL/mouse	Hypoxia	*In vivo* model of scar formation	Reduction of scar formation	[162]
BM-MSCs	5 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of partial hepatectomy	Enhanced liver regeneration	[164]
DP-MSCs	N.A.	Hypoxia	*In vivo* model of dental pulp injury	Regeneration of dental pulp with a rich vasculature	[167]
AF-MSC-derived CM	N.A.	Hypoxia	*In vivo* model of wound healing	Acceleration of skin wound healing	[168]
AMSC-derived CM and EVs	200 μL CM and 5 μg EVs/1 × 10^5 PBMCs, and 100 μL CM and 5 μg EVs/1 × 10^4 HUVECs	3D cultures/spheroids	*In vitro* model of T cell activation and HUVEC cells	Induction of angiogenesis and inhibition of T cell proliferation	[44]
AMSCs	250 μL CM/ 1.5 × 10^5 alveolar epithelial cells	3D cultures/spheroids	*In vitro* model of lung IRI	Attenuation of IRI side effects by improving the efficacy of in vitro EVLP	[59]
AMSC-derived CM	50 μL CM/ 1 × 10^4 liver cells	3D cultures/spheroids	*In vitro* model of liver IRI	Attenuation of IRI side effects by inhibiting inflammation and apoptosis	[131]
BM-MSCs	3 × 10^6 MSCs/mouse	3D cultures/spheroids	*In vivo* model of peritonitis	Production of anti-inflammatory cytokines	[137]
BM-MSCs	1.5 × 10^6 MSCs/mouse	3D cultures/spheroids	*In vivo* model of peritonitis	Attenuation of inflammatory responses	[138]
CB-MSCs	1 × 10^7 MSCs/mouse	3D cultures/spheroids	*In vivo* model of hindlimb ischemia	Improvement of survival and angiogenesis	[141]
AdMSCs	2 × 10^6 MSCs/rat	3D cultures/spheroids	*In vivo* model of acute kidney injury	Reduction of apoptosis and tissue damage, promotion of vascularization, and amelioration of renal	[143]

				function	
UC-MSC-derived EVs	200 μg of EVs/mouse	3D cultures/spheroids	*In vivo* model of acute kidney injury	Attenuationof pathological changes and improvement of renal function	[144]
BM-MSCs	2×10^6 MSCs/rat	3D cultures/spheroids	*In vivo* model of myocardial infarction	Promotion of cardiac repair	[155]
BM-MSCs	5×10^5 MSCs/rat	3D cultures/spheroids	*In vivo* model of myocardial infarction	Stimulation of a vascular density and improvement of cardiac function	[156]
AdMSCs	1×10^7 MSCs/mouse	3D cultures/spheroids	*In vivo* model of hindlimb ischemia	Improvement of angiogenesis	[163]
AdMSCs	2×10^6 MSCs/rabbit	3D cultures/spheroids	*In vivo* model of disc degeneration	Induction of disc repair	[169]
BM-MSCs	NA	3D cultures/spheroid	*In vivo* model of bilateral calvarial defects	Induction of bone regeneration	[170]
SMSCs	NA	3D cultures/spheroid	*In vivo* model of osteochondral defects	Induction of cartilage regeneration	[171]

MSCs: Mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; AMSCs: Amnion-derived mesenchymal stem cells; UC-MSCs: Umbilical cord-derived mesenchymal stem cells; AdMSCs: Adipose-derived mesenchymal stem cells; CB-MSCs: Cord blood-derived mesenchymal stem cells; WJ-MSCs: Wharton's Jelly-derived mesenchymal stem cells; PMSCs: Placenta-derived mesenchymal stem cells; AF-MSCs: Amniotic fluid derived mesenchymal stem cells; SMSCs: Synovial derived mesenchymal stem cells; EVs: Extracellular vesicles; CM: Conditioned medium; NA: Not available; GVHD: Graft-versus-host disease; IRI: Ischemia-reperfusion injury; 3D: Three-dimensional; IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin; MLR: Mixed lymphocyte reactions; LPS: Lipopolysaccharide; HUVEC: Human umbilical vein endothelial cell.

colitis[95,96,98]. Rafei *et al*[134], in a mouse *in vivo* model of autoimmune encephalomyelitis, found that treatment with allogeneic MSCs primed with IFN-γ reduced clinical signs in a dose-dependent manner. In this study the authors showed that, though the priming treatment induced the increase of CCL2 and MHCI/II expression in IFN-γ-primed MSCs, it inhibited manifestations of autoimmune encephalomyelitis while keeping their immunogenicity low. The use of IFN-γ- or TNF-α-primed MSCs has also been shown to attenuate symptoms of GVHD[101,103]. In these cases, in the first study it was shown that therapeutic effects of MSCs were mediated by overproduction of IDO induced through the IFN-γ-JAK-STAT1 pathway[101]. In the second study, the therapeutic function of MSCs was activated by TNF-α, which induced overexpression of Chi3 L1 and consequent suppression of T-helper 17 cells[103]. Recently, it has been revealed that the priming of MSCs with IL-1β relieved the side effects of sepsis[109,111]. In particular, Song *et al*[109] demonstrated that IL-1β makes MSCs more effective in inducing macrophage polarization toward an anti-inflammatory M2 phenotype, and this effect was mediated, at least in part, through overproduction of EXOs containing miR146a. Similar results on M2 macrophage polarization were also obtained by Yao *et al*[111], who revealed the ability of IL-1β to stimulate the production of MSC-derived EXO containing miR21. The therapeutic efficacy of MSCs primed with IFN-γ was also found in an *in vivo* model of colonic wounds. Particularly, García *et al*[135] showed that these cells were able to enhance healing of colonic mucosal wounds in both immunocompromised and immunocompetent mice. Similar results were also obtained using MSCs primed with TNF-α, which were able to accelerate wound closure and angiogenesis in an *in vivo* model of wound healing[99]. The priming with inflammatory cytokines seems to also be effective for the treatment of chronic liver diseases. Indeed, treatment with IL-6 improved the ability of MSCs to reduce liver injury[104]. The study reported that in a mouse *in vivo* model of liver fibrosis, treatment with IL-6-primed MSCs reduced both fibrosis and apoptosis, and improved liver functions[104]. Moreover, TNF-α-primed MSCs were also able to attenuate inflammation in an *in vivo* model of peritonitis[136]. In this study, the authors demonstrated that TNF-α induced the overproduction of the anti-inflammatory factor TSG-6, generating a mechanism that reduces inflammation in an *in vivo* model of zymosan-induced peritonitis[136]. Interestingly, in a similar experimental model, Bazhanov *et al*[137] found that after intraperitoneal injection MSCs formed 3D aggregates, and stimulated the production of anti-inflammatory cytokines, such as IL-10 and PGE2. In this regard, Bartosh *et al*[138] showed that the priming of MSCs with 3D culture decreased inflammation in an *in vivo* model of peritonitis[138]. In particular, the authors suggest that MSC spheroids overexpressed TSG-6, and these cells were more effective than conventional MSCs as therapy for diseases characterized by unresolved inflammation.

Overall, the above-mentioned studies suggest that treatment with pro-inflammatory cytokines or the 3D culture of MSCs represents promising priming strategies for enhancing the MSC immunoregulatory phenotype, making these cells more suitable for clinical disorders related to exacerbated immune responses (Figure 2).



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Figure 2 Schematic representation of the molecular effects after priming of mesenchymal stromal/stem cells. Mesenchymal stromal/stem cells (MSCs) can be primed through various stimuli, including hypoxia, three-dimensional cultures, and pro-inflammatory cytokines to enhance their therapeutic potential. Each priming method induces the production of specific factors (*e.g.*, trophic factors, angiogenetic factors, chemokines, cytokines, and exosomes containing both proteins and microRNAs), which induce the activation of biological processes such as angiogenesis, tissue repair/regeneration, chemoattraction, and modulation of inflammation. Each priming strategy seems to stimulate the production of functional factors in a different way, thus eliciting different responses. miRNA: MicroRNA; VEGF: Vascular endothelial-derived growth factor; CXCR: Chemokine receptor; HGF: Hepatocyte growth factor; MMP: Matrix metallopeptidase; BDNF: Brain-derived neurotrophic factor; SDF: Stromal cell-derived factor; HIF: Hypoxia-inducible factor; ICAM: Intercellular adhesion molecules; MCP: Monocyte chemoattractant protein; IL: Interleukin; LIF: Leukemia inhibitory factor; PIGF: Placental growth factor; EGF: Epidermal growth factor; FGF: Basic fibroblast growth factor; PDGF: Platelet-derived growth factor; GRO: Growth-related oncogene; TGF: Transforming growth factor; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase; PDL1-2: Programmed death ligand 1-2; MIG: Monokine induced by interferon-gamma; G-CSF: Granulocyte colony-stimulating factor; IP-10: Induced protein 10; MIP: Macrophage inflammatory protein; IRI: Ischemia/reperfusion injury; MSCs: Mesenchymal stromal/stem cells; 3D: Three-dimensional.

Main priming strategies for treating acute injury

Priming strategies for MSCs have been considered a crucial tool for enhancing their therapeutic effects, making these cells more suitable for application in the field of regenerative medicine[3,85]. However,

while the priming of MSCs with pro-inflammatory cytokines potentially represents the principal strategy modulating inflammation in chronic immune-related disorders (or, in any case, conditions in which the inflammation is exacerbated), the priming of MSCs with hypoxia is thought to represent the more appropriate priming strategy for boosting MSC effects for the stimulation of tissue function recovery after acute injury (Figure 2). This has been demonstrated in numerous study models, and on different organs (Table 1). For example, hypoxia pre-conditioning significantly improved blood flow recovery in mouse models of hindlimb ischemia. Rosová *et al*[139] demonstrated that hypoxic MSCs better migrate to the injured site compared with non-hypoxic MSCs, thus speeding up the restoration of blood flow. The authors demonstrated that the observed effects were likely mediated by the HGF-cMET axis. It has been shown that hypoxia helps MSCs to better integrate in the damaged tissue. Han *et al*[140] revealed that hypoxic priming enhanced survival and proliferation of transplanted MSCs, thus improving the regeneration of hindlimb ischemic tissues. After MSC treatment, the authors observed inhibition of apoptosis and promotion of neovascularization and, as they showed the increased expression of the normal cellular prion protein upon hypoxia pre-conditioning, they identified this prion as a potential target for MSC therapy. In a similar manner, Lee *et al*[115] recently identified GRP78 as new potential target for the development of functional MSCs. GRP78 has been shown to be induced by hypoxia, thus increasing transplanted-MSC survival and proliferation in a mouse model of hindlimb ischemia. Moreover, the authors found that the HIF-1α-GRP78-Akt axis regulates the suppression of cell death signals, and increases angiogenic cytokine secretion, thus strongly improving tissue recovery from the damage[114]. Recently, it has been found that mild hypoxia can be induced in MSCs when they are cultured as spheroids. Various studies have clearly demonstrated that 3D culture conditions induce hypoxia in the core of the spheroid, thus stimulating the production of both growth and pro-angiogenic factors, which in turn stimulate the fast recovery of damaged tissues in mouse models of hindlimb ischemia[141,142]. Interestingly, it has also been shown that the CM derived from MSCs primed by 3D culture attenuated injury and inflammation in two IRI *in vitro* models of both lung and liver[59,131]. 3D pre-conditioning has been shown to also be effective for other type of diseases, such as acute kidney injury (AKI). Xu *et al*[143] found that 3D pre-conditioned MSCs, when transplanted in mice with AKI, are more viable than the 2D cultured cells, and exhibit higher paracrine secretions, as evidenced by the increased levels of VEGF and TSG-6. Furthermore, the authors show that the paracrine secretion, which also includes basic fibroblast growth factor, insulin like growth factor, and EGF, significantly improved renal function and reduced tissue apoptosis, thus speeding up the regeneration of renal tissues upon injury[143]. Recently, the secretome of 3D MSCs transplanted for the treatment of AKI was furtherly investigated. For example, Cao *et al*[144] found that the paracrine effect on AKI was mediated not only by soluble factors, such as anti-inflammatory cytokines, but also by EXOs, whose production is increased after 3D pre-conditioning. Furthermore, by using a cisplatin-inducing AKI model in mice, the authors showed that the increased number of EXOs upon 3D culture enhanced the renoprotective and anti-inflammatory efficacy of MSCs[144]. Treatment of AKI with MSC therapy has been implemented in recent years by defining new protocols of MSC pre-conditioning. Along with 3D culturing, hypoxia priming has been used for the treatment of IRI-inducing AKI in animal models, and Zhang *et al*[145] demonstrated that hypoxia priming enhanced angiogenic and antioxidative MSCs properties in a rat model of renal IRI. In addition, in the same model, the authors found that transplanted MSCs attenuated renal apoptosis by reducing cleaved caspase3 activation. Notably, hypoxia also enhanced MSC therapeutic potential in a cisplatin-induced mouse model of AKI. Overath *et al*[146] found that hypoxic conditions increased the efficacy of transplanted MSCs in attenuating renal damage upon injury both by reducing creatinine and N-GAL serum levels, and decreasing pro-inflammatory cytokine release. MSC hypoxia pre-conditioning has also been found to be strongly effective for the treatment of IRI in the lung. For example, MSC infusion in lung perfusates demonstrated that hypoxic MSCs quickly migrate from the pulmonary artery to the lung tissue, where they attenuate parenchymal damage by reducing oxidative stress, inflammation, and apoptosis, and by stimulating cell proliferation and survival[147]. In a similar manner, MSC hypoxia has been found to have important effects also for radiation-induced lung injury (RILI). A mouse model of RILI was recently established by exposing the lungs of mice to irradiation, thus generating tissue damage. Upon irradiation, the authors demonstrated that hypoxic MSCs reside for longer in the injured tissue compared with normoxic MSCs. In addition, Li *et al*[148] showed that hypoxia-primed MSCs enhanced cell viability and proliferation, as well as anti-oxidative and anti-apoptotic capabilities in lung parenchymal cells. Finally, the authors highlighted the role of HIF-1 in modulating resistance to lung hypoxic stress induced by RILI, thus promoting tissue repair and regeneration upon injury.

The use of MSCs as cellular therapy has also been shown to be effective for the treatment of acute myocardial injury in several preclinical models (Table 1). Also in this case, to ameliorate the therapeutic effects of MSCs various priming strategies have been evaluated. In particular in myocardial infarction (MI), it has been widely believed that tissue injury is related to ischemia and the hypoxic environment. Therefore, the *in vitro* hypoxic condition was tested to improve MSC therapeutic effects in MI animal models[149]. In a mouse model of MI, it was found that intramyocardial injection of hypoxia-preconditioned MSCs reduces infarct size, influences heart remodelling by modulating vasculogenesis, and improves heart functions, promoting cell survival[150,151]. Of note, expression analysis in hypoxic MSCs has revealed an increase in expression of pro-survival and pro-angiogenic factors, including HIF-

1α, ANGPT1, VEGF, Flk-1, Bcl-2, Bcl-xL, and these proteins can act in a paracrine manner on MI, inducing functional recovery[150]. It has also been observed that hypoxic MSCs influence the expression of specific miRNAs that can be secreted through EVs. In particular, Feng *et al*[152] demonstrated that after hypoxic treatment of MSCs an increase of miR22 was observed in EXOs, and this miRNA was considered responsible for targeting Mecp2, with beneficial effects on survival of cardiomyocytes exposed to ischemia. Similarly, EVs derived from hypoxic MSCs overexpressing miR26 were able to reduce the damage from ischemia/reperfusion in a rat model[153]. In the same way, in an MI mouse model the intracardial injection of hypoxic-preconditioned MSC-derived EXOs was able to positively regulate cardiomyocyte proliferation and survival, and this effect was ascribable to the overexpression of miR125b[154]. In addition to the use of hypoxia priming, the use of 3D culture has also been shown to be effective in the improvement of MSC therapeutic effects on the treatment of acute myocardial injury. You *et al*[155], in an acute MI rat model, found that treatment with 3D-primed MSCs resulted in a retention of MSCs at the epicardium, where MSCs exerted cardiac protection/repair, and functional recovery. Moreover, in the same animal model, Wang *et al*[156] revealed that 3D MSCs were able to stimulate vascular density and improve cardiac function after MI.

Over the last decade, MSCs have also been intensively studied for their potential use in the treatment of neurological acute injury, including cerebral ischemia, traumatic brain injury, and spinal cord damage. For example, in an *in vivo* model of cerebral ischemia, it has been shown that hypoxic-preconditioned MSCs enhanced angiogenesis and neurogenesis after ischemia[157]. In an *in vivo* model of traumatic brain injury, Chang *et al*[158] demonstrated that the priming of MSCs with hypoxia improved their therapeutic function, and resulted in an amelioration of neurogenesis, and motor and cognitive functions. Moreover, in a rat model of spinal cord injury, hypoxic MSCs were also able to increase axonal preservation and decrease apoptosis[159].

Principal priming strategies for stimulating tissue regeneration

MSCs are involved in tissue homeostasis, which is necessary for physiologically coordinating regeneration/repair of tissue, also after injury[3,6,36]. Thus, the use of MSCs in regenerative therapies is garnering great interest due to their potentially numerous clinical applications.

In the complex process of cutaneous wound healing, a central role is played by fibroblasts, which contribute, through the interaction with surrounding cells, to the production of ECM, glycoproteins, adhesive molecules, and various growth factors[160]. Recent evidence suggests that CM produced by primed MSCs from different sources, such as bone marrow[120], adipose tissue[160], amnion fluid[161], and placenta[162] enhanced the migration and proliferation of fibroblasts *in vitro*, and accelerated wound healing in *in vivo* models (Table 1). In all these cases, hypoxia treatment represented the chosen priming strategy for driving MSCs in increasing secretion of various angiogenic factors, cytokines, and chemokines. Therefore, the priming of MSCs with hypoxia might well represent the main approach to improving the therapeutic effects of MSCs to be applied in the stimulation of tissue regeneration (Figure 2). This idea has also been supported by other studies (Table 1). Indeed, in both hepatectomized mouse and rat models, it has been demonstrated that hypoxic MSCs produce crucial functional molecules, including HGF and VEGF, which were considered responsible for the induction of liver regeneration[163,164]. Kuo *et al*[165] showed that systemic infusion of MSCs restored liver function and promoted liver regeneration in rodents. In this regard, in a rat massive hepatectomy model, Yu *et al*[164] found that hypoxia-conditioned MSCs secreted significantly more VEGF than normoxia-conditioned cells, and the infusion of primed MSCs promoted proliferation of hepatocytes and liver regeneration. Several studies have focused on the signalling pathways up-regulated by MSC during liver regeneration. Lee *et al*[163] using a partially hepatectomized mouse model, found that treatment with hypoxic MSC-derived CM increased the viability of hepatotoxic hepatocytes, and enhanced liver regeneration through JAK/STAT3 signalling. These data were also confirmed by Lee *et al*[166], who confirmed the activation of JAK/STAT3 signalling induced by MSC CM during mouse liver generation. Hypoxic MSCs that secrete high level of VEGF were also able to regenerate pulp-like tissues and vasculature similar to the native pulp in a rat model of pulp repair[167]. HGF and VEGF produced by hypoxic MSCs were considered by Chang *et al*[158] to be responsible for improvement of neuronal proliferation. Moreover, Zhilai *et al*[159] demonstrated that both HGF and VEGF produced by hypoxia-primed MSCs facilitated axonal survival in a rat model of spinal cord injury. Han *et al*[140], in a murine hindlimb ischemia model, found that the expression levels of EGF, VEGF, fibroblast growth factor, and HGF were significantly higher in ischemic tissue treated with hypoxic MSCs, where an improvement of neovascularization was observed. The efficacy of hypoxic MSCs was also tested in reducing scar formation and inducing wound healing in various *in vivo* models[120,162,168].

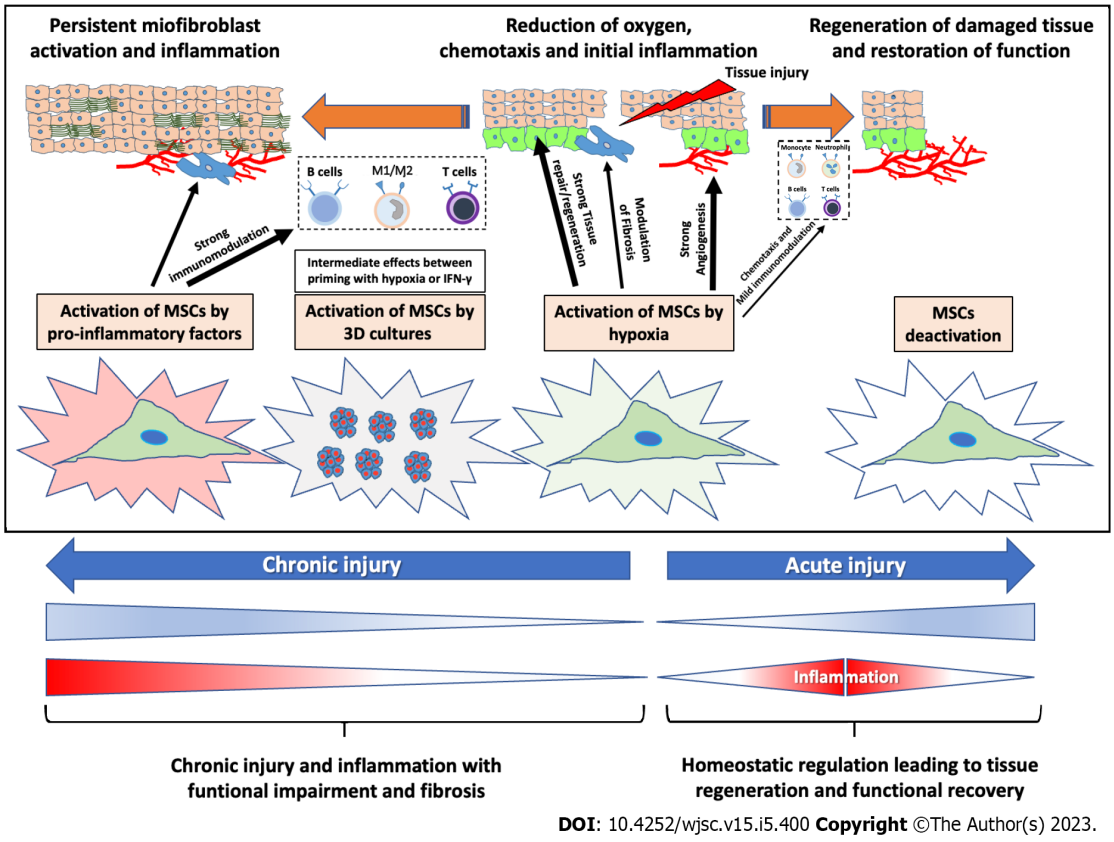
Despite the fact that the principal MSC priming strategy used for both *in vitro* and *in vivo* regeneration experiments was hypoxia treatment, 3D culture of MSCs as priming strategy has also been investigated in tissue regeneration (Figure 2). In fact, MSC spheroids have also shown therapeutic abilities with regard to both bone and cartilage regeneration. In particular, it has been found that treatment with MSC spheroids was effective in inducing disc repair in an *in vivo* model of disc degeneration, bone regeneration in an *in vivo* model of bilateral calvarial defects, and cartilage regeneration in an *in vivo* model of osteochondral defects[169-171].

CONCLUSION

The therapeutic effects of MSCs have been demonstrated in both *in vitro* and *in vivo* studies. Nevertheless, due to their heterogeneity related mainly to tissue source, which can impact MSC functional properties[85,172], the application of MSCs in clinical trials has shown moderate or poor efficacy. MSCs are considered key regulators of tissue repair and, in this case, different stimuli are crucial in modulating the functional properties of these cells. In fact, it is believed that inflammation and low oxygen levels are essential signals for triggering MSC activity in a suitable manner. Moreover, it has recently been shown that different priming approaches can eliminate the functional heterogeneity of MSCs[173]. Therefore, specific priming strategies have been implemented to improve the regenerative and immunomodulatory properties of MSCs. In this review, we have explored data regarding the principal priming approaches used to enhance the therapeutic potential of MSCs. The above-mentioned data underscore that several factors play a role in the ability to modify MSC properties. Moreover, some therapeutic effects, on different disease models, can be obtained in relation to dose and/or combination of the priming factors used.

Several diseases have in common tissue injury and repair processes, in which inflammation plays a central role in coordinating different pathways that regulate tissue regeneration and functional recovery. Indeed, after acute injury, a low level inflammation (acute inflammation) occurring after specific triggers, is crucial in stimulating wound healing and tissue repair, facilitating the resolution of inflammation and restoring tissue structure/function (inflammation drives regeneration). On the other hand, in the case of abnormal damage repair, chronic unregulated inflammation can lead to pathological processes, including hormonal metabolic changes, which culminate in the onset of specific diseases, including cancer and fibrosis[174,175]. Therefore, the regulation of both acute and chronic inflammation is essential for a proper restorative response and, in this scenario, MSCs can have a crucial physiopathological role. In fact, it has been shown that when MSCs coordinate damaged tissue for repair, they undergo local stimuli such as inflammatory cytokines, and hypoxia, which in turn boost and direct the reaction of MSCs to orchestrate tissue regeneration[85,176]. In Figure 3, we depict a hypothetical model that occurs during physiopathologic tissue injury and repair. In this model, MSCs are activated differently by various microenvironment stimuli to manage tissue functional recovery. One of the first factors that arises after tissue injury is the establishment of a hypoxic and weakly inflammatory microenvironment, which in turn activates local cells to protect/regenerate tissues[3,177]. Hypoxia rapidly up-regulates the level of intercellular adhesion molecule-1 in local-inflamed endothelium, promoting MSC migration to injured tissues[178,179]. Moreover, a mild inflammation may stimulate MSCs to release chemokines for attracting immune cells and amplifying immune responses[180]. Once MSCs reach the site of injury, the paracrine properties of MSCs to release chemotactic and angiogenic factors is significantly amplified under hypoxic conditions[181]. In this case, naïve MSC are activated to recruit neutrophils and stimulate the formation of new blood vessels. Neutrophil action is followed by monocyte/macrophage activity that ensures sustained release of pro-inflammatory cytokines and potentiation of the fibroproliferative response[182,183]. If these processes are not adequately regulated, a state of chronic inflammation occurs. Thus, cytokines such as IFN-γ, TNF-α, and IL-1 accumulate in the injured tissues, and the inflammatory environment becomes central in affecting the regulatory role of MSCs that exhibit immunosuppressive capacities[184]. The MSC phenotype is switched into a lower regenerative potential and a higher anti-inflammatory phenotype (Figure 3). Thus, high amounts of pro-inflammatory cytokine confer a dramatic immunomodulatory ability to MSCs[40,91,124,125,185,186] which, in turn, act as a homeostatic regulator to control the inflammatory response. Overall, this scenario describes what occurs when MSCs are exposed to low levels of both oxygen and inflammation, and their phenotype is potentially inclined to low immunomodulation and high stimulation of tissue regeneration. Otherwise, high levels of inflammation can imprint a MSC phenotype inclined toward high immunomodulation and weak stimulation of tissue regeneration (Figure 3). In this regard, Vigo *et al*[87] found that IFN-γ can orchestrate MSCs functions in a dose-manner, and this is reflected in the opportunity to modulate MSC properties before their use in clinical practice. In addition, considering the heterogeneous immune regulatory functions of MSCs due to intrinsic characteristics of individual clones, the priming of MSCs with pro-inflammatory factors can equally amplify immune therapeutic properties of MSCs, and eliminate the variances among different MSC clones[173].

Priming with inflammatory signals polarizes MSCs toward an anti-inflammatory and pro-trophic phenotype allowing, on the one hand, the regulation of inflammatory responses, and on the other the final remodelling and recovery of damaged tissue. Likewise, different priming strategies can be used to direct the therapeutic effects of naïve MSCs toward specific pathological processes. As also highlighted by the studies we have noted in this review, while hypoxic priming of MSCs could be used mainly to treat acute disease, to principally stimulate angiogenesis and tissue regeneration, inflammatory cytokines could be used mainly to prime MSCs for treating chronic immune-related disorders. The change of perspective from regeneration to inflammation implies in the MSCs the shift in the production of functional factors that stimulate regenerative or anti-inflammatory pathways (Figure 2). Interestingly, the 3D culture of MSCs as priming strategy appears to be an intermediate functional priming between the two mentioned above. The production of priming type-specific functional factors in MSCs could well pave the way for optimizing their therapeutic potential, aimed at a greater effectiveness as an



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Figure 3 Schematic illustration of the physiological role and biological action of mesenchymal stromal/stem cells primed *in vivo* in a model of tissue injury and repair. During tissue injury and repair, mesenchymal stromal/stem cells (MSCs) are differently activated by various microenvironment stimuli to orchestrate tissue repair and functional recovery. First, naïve MSC activation (hypoxic activation) leads to the release of both angiogenic factors and chemokines, which stimulate the formation of new blood vessels, the recruitment of neutrophils, and the expression of adhesion molecules. Neutrophil action is followed by macrophage activity, which ensures sustained release of pro-inflammatory cytokines, and potentiation of the fibroproliferative response. If this process is not adequately regulated, a state of chronic inflammation occurs; the MSC phenotype is switched into an anti-inflammatory phenotype. MSCs: Mesenchymal stromal/stem cells; 3D: Three-dimensional.

advanced therapy medicinal product.

FOOTNOTES

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REVIEW

Communication between bone marrow mesenchymal stem cells and multiple myeloma cells: Impact on disease progression

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Abstract

Multiple myeloma (MM) is a hematological malignancy characterized by the accumulation of immunoglobulin-secreting clonal plasma cells at the bone marrow (BM). The interaction between MM cells and the BM microenvironment, and specifically BM mesenchymal stem cells (BM-MSCs), has a key role in the pathophysiology of this disease. Multiple data support the idea that BM-MSCs not only enhance the proliferation and survival of MM cells but are also involved in the resistance of MM cells to certain drugs, aiding the progression of this hematological tumor. The relation of MM cells with the resident BM-MSCs is a two-way interaction. MM modulate the behavior of BM-MSCs altering their expression profile, proliferation rate, osteogenic potential, and expression of senescence markers. In turn, modified BM-MSCs can produce a set of cytokines that would modulate the BM microenvironment to favor disease progression. The interaction between MM cells and BM-MSCs can be mediated by the secretion of a variety of soluble factors and extracellular vesicles carrying microRNAs, long non-coding RNAs or other molecules. However, the communication between these two types of cells could also involve a direct physical interaction through adhesion molecules or tunneling nanotubes. Thus, understanding the way this communication works and developing strategies to interfere in the process, would preclude the expansion of the MM cells and might offer alternative treatments for this incurable disease.

Key Words: Multiple myeloma; Mesenchymal stem cells; Bone marrow microenvironment; Soluble factors; Extra-cellular vesicles; Cells adhesion molecules; Tunnelling-

nanotubes

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Core Tip: Mesenchymal stem cells (MSCs), the main cell population of the bone marrow (BM) stroma, can influence BM microenvironment through their paracrine activity, involving both soluble factors and extracellular vesicles, but also through direct communication. Being the BM the predominant localization of multiple myeloma cells (MM), finding the appropriate conditions at this niche, is key for the survival and expansion of tumour cells and thus, for the progression of the disease. Since the activity of BM-MSCs could determine the fate of MM cells at BM, these cells could be interesting targets for the design of new antitumor drugs.

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INTRODUCTION

Multiple myeloma (MM) is one of the most common hematological diseases, only second to non-Hodgkin lymphoma[1]. MM affects mainly older adults, with the median age of diagnosis being around 69 years. Only in 2020, 32270 new cases and 12830 deaths in the United States were estimated by the American Cancer Society Statistics Centre. In global terms, the cases would reach 160000, accounting for 0.9% of all cancer diagnosis. Importantly, incidence of MM has risen 126% globally, and hence, there is an increasing need to find new effective treatments for this incurable disease[2,3].

Besides the initial treatments for MM, consisting in alkylating agents often combined with corticosteroids, the last couple of decades have seen an important advance in the available treatments for this disease. We first saw the introduction of proteasome inhibitors (Bortezomib), histone deacetylase inhibitors (Panobinostat) and drugs such as Selinexor, with a nuclear export inhibition activity. In recent years monoclonal antibodies such as Daratumumab (anti-CD38) or Elotuzumab (anti-SLAMF7), and more recently the use of chimeric antibody receptor (CAR) T-cell products, has introduced immunotherapy as a viable approach to MM treatment[4]. According to data from the National Cancer Institute (Bethesa, MD, United States), all these treatments have had a deep impact on patients' survival, substantially raising the survival rate to 55% in the period between 2011 and 2017. More recently, the use of small molecules, with a molecular weight smaller than 1kDa, has also improved treatments, since it offers important advantages compared to the former therapies, as the easy cell entry, the simplicity of the molecules, and a much lower production cost than other drugs[5]. However, despite these advancements, there are still limitations to existing treatment options. Some patients may not respond to or may develop resistance to certain medications, many patients can become refractory to treatment and thus, there is a high risk of relapse. This promotes the search for new treatments to handle relapsed or refractory MM.

MM is caused by aberrant plasma cells (PC) proliferation in the bone marrow (BM). The premalignant states, known as monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM, transition under specific conditions to the malignant state of PC leukemia or extramedullary myeloma [6]. A key characteristic of MM is the infiltration into and the colonization of the BM, one of the two primary lymphoid organs[7]. This colonization produces typical lytic bone lesions that would be present in approximately 80% of patients with newly diagnosed MM and are the major source of morbidity[8]. The bone lesions, resulting from the stimulation of bone resorption by B-cell plasmacytomas, are associated with hypercalcemia and often, severe bone pain and bone fractures[8,9].

While the initiation of a tumor mainly depends on the accumulation of genetic defects, the transition from a premalignant to a malignant state highly relies on the interaction of the tumor cell with a permissive microenvironment that would support the malignant transformation and the proliferation of the tumor cells, aiding them to evade apoptosis. The relevance of tumor microenvironment in disease progression was first discussed in the "seed and soil hypothesis" formulated by Stephen Paget in 1889, where the establishment of tumor metastatic sites is influenced by the cross-interaction between the seeds (cancer cells) and the soil (a particular microenvironment)[10]. This is not different in MM[11,12]. The progression to MM, which would occur in approximately 50% of patients diagnosed with MGUS [6], requires multiple genomic events, but also a permissive BM microenvironment[13]. MM cells proliferate almost exclusively within the BM niche, highlighting the role of this microenvironment in

supporting cancer growth. In fact, there is also mounting evidence indicating this BM microenvironment is not only key for PCs survival, but also has a crucial role in resistance to treatment and disease recurrence[14,15].

The MM cells infiltrating the BM will encounter a complex microenvironment formed by cellular and non-cellular components. Amongst the non-cellular components influencing the BM microenvironment, it is important to consider the extracellular matrix (ECM) proteins as well as a milieu of cytokines, chemokines, and growth factors. Many of these factors can have a positive effect on MM cells, boosting their proliferation and survival and the resistance to different types of drugs. A good example of these cytokines supporting MM progression are interleukin (IL)-6 and ligands of the B-cell maturation antigen, such as a proliferation-inducing ligand and B-cell activating factor (BAFF)[16,17]. Regarding the cellular components of the BM niche, many different types of bone cells (osteoblasts, or bone forming cells, and osteoclasts, or bone resorbing cells, and osteocytes) and cells from the immune system (macrophages, natural killer cells and regulatory T-cells) share this niche. Other cells present here are fat cells (adipocytes), fibroblasts, endothelial cells and two multipotent stem cells, BM mesenchymal stem cells (BM-MSCs), which differentiate into different mesodermal cell lineages, and hematopoietic stem cells (HSCs), that would differentiate into hematological lineages, including the myeloid lineage that would give rise to osteoclasts. MM cells are likely to interact with all the cells in the BM niche and elicit mutual influence[18]. In fact, it is known that communication between MM cells and BM-MSCs is essential in the progression of MM[19]. Once MM cells infiltrate the BM, their presence in the BM niche alters the activity of many of the cells found there, including those involved in bone homeostasis such as osteoclasts[20,21] and osteoblasts[22-24]. While in normal bone homeostasis, the activities of osteoblast and osteoclasts are carefully balanced to ensure a correct bone regeneration, the influence of MM cells disrupts this balance increasing both the resorptive activity of osteoclast and their numbers, and decreasing osteoblasts numbers as well as their osteogenic capacity[25], overall leading to an increase in bone destruction and the appearance of the aforementioned osteolytic lesions typical of this disease. Other cells at the BM niche which activity is highly influenced by MM cells are BM-MSCs. The presence of MM cells at the BM niche alters the MSCs behavior in different ways. In fact, changes in the expression of certain microRNAs (miRNAs) in BM-MSCs leading to important alterations of their secretory profile and osteogenic differentiation potential have been observed after co-cultivation of BM-MSCs and MM cells[26,27]. These changes at the BM niche upon MM invasion produce a microenvironment that would support disease progression. Indeed, there is strong evidence indicating that is precisely this interaction what leads to the formation of the lytic bone lesions[28]. One of the characteristics of this permissive microenvironment is the high presence of pro-inflammatory cytokines that would favor the progression of neoplasia[29]. The crosstalk between MM cells and the BM-MSCs at the BM niche is key to sustain this pro-inflammatory microenvironment and thus, to allow MM cell persistence and growth[30]. It is important to clarify, that this pro-inflammatory microenvironment would be the result of the action not only of the infiltrated MM cells but also of other cells residing at the BM niche, including BM-MSCs.

MSCs, have a key role in regulating the BM microenvironment through their paracrine activity, but also through direct cell-to-cell interaction. Regarding their paracrine activity, these cells produce a plethora of soluble biomolecules and vesicular components, known altogether as "secretome", that exert multiple actions on other cells at the BM microenvironment[31]. BM-MSCs role in MM disease development and progression has been reported as having both inhibitory[32] and supportive roles[33,34]. Sadly, the latter is the most frequent. Once at the BM niche, MM will exert their influence on resident MSCs, altering their signaling and gene expression pattern and thus, also their secretion pattern. After interaction with MM cells, MSCs will produce a secretome rich in pro-inflammatory cytokines. In fact, it has been previously described how MSCs react to IL-1 produced by the myeloma PCs by producing large quantities of IL-6, a cytokine that would in turn stimulate the survival of the MM cells[35,36]. Therefore, the soluble part of this secretome has a key role in the progression of tumor. Moreover, in the last few years, several molecules (miRNAs) that are present in the cargo in the extracellular vesicles (EVs) produced by BM-MSCs upon MM cells stimulation also seem to have a key role in the disease promotion. Although the soluble proteins and EVs produced by the BM-MSCs are the main actors in the communication between BM-MSCs and MM cells, other ways of communication have also been implicated. This will be discussed in the following sections.

Current available treatments for MM patients mainly target MM cells but have none or limited effect on other cells in the BM or de BM microenvironment. Knowledge of the different interactions between BM-MSCs and MM cells is key to understand how MM cells behave and grow within the BM and how osteolytic lesions are formed. In this work, we will address key aspects of the different ways of communication between MSCs and MM cells as well as the outcome of this crosstalk.

SOLUBLE FACTORS IN THE COMMUNICATION BETWEEN BM-MSCs AND MM CELLS

The multiple cellular interactions taking place in the BM, make this microenvironment a dynamic compartment with a myriad of soluble factors that would affect the behavior of the various cell types

concurring at that microenvironment. Although many of those cells have paracrine activity, BM-MSCs are the ones that have a stronger impact in the BM microenvironment due to the wide variety of soluble and non-soluble factors secreted by these cells. Various constituents of the, so called, BM-MSC secretome orchestrate the fate of the MM cells, from the first step encompassing the homing of those cells to the BM, onwards.

Role of soluble factors in the homing of MM cells to the BM

A key factor in the communication between BM cells and MM cells during the first stages of BM colonization, is the cytokine stromal cell derived factor 1α (SDF1α), also known as CXCL12. This factor, produced by BM-MSCs, works as a chemoattractant, being responsible of the homing of HSCs to the BM once they abandon the fetal liver during development[37]. SDF1α activity is mediated by the binding to a specific G-protein 7-span transmembrane receptor (CXCR4) at the target cells. CXCR4 is expressed at the surface of different cells in the BM microenvironment[38], and also at the surface of MM cells and other tumor cells[39]. Thus, SDF1α/CXCR4 interaction might have a relevant role in directing de metastasis of hematopoietic malignancies. Similar to its effect on HSCs, the interaction of SDF1α with its receptor at the MM cells, increases their migration, homing and adhesion towards the BM, in fact, knock down of CDCR4 in BM-MSCs or the use of the CXCR4 inhibitor AMD3100 (AnorMED), that blocks the binding of SDF1α to its receptor[40], seems to inhibit the migration of MM towards the BM[41]. The binding of SDF1α to its receptor at the MM cells, also triggers the activation of the phosphatidylinositol 3-kinase (PI3K) and the MAPK kinase (MEK)-extracellular signal regulated kinase (ERK, MEK/ERK) pathways, inducing a rearrangement in the cytoskeleton of MM cells that facilitates BM colonization [41]. SDF1α has also been described to act in a more indirect way, not mediated by the binding to CXCR4. SDF1α interacts with other molecules including matrix metalloproteinases (MMPs), integrins or growth factors such as hepatocyte growth factor (HGF), insulin like growth factor-1 (IGF-1) or molecules of the GTPases family. All of these effects elicited by SDF1α, in one way or another, lead to a promotion in MM cells migration, homing or adhesion into the BM[38].

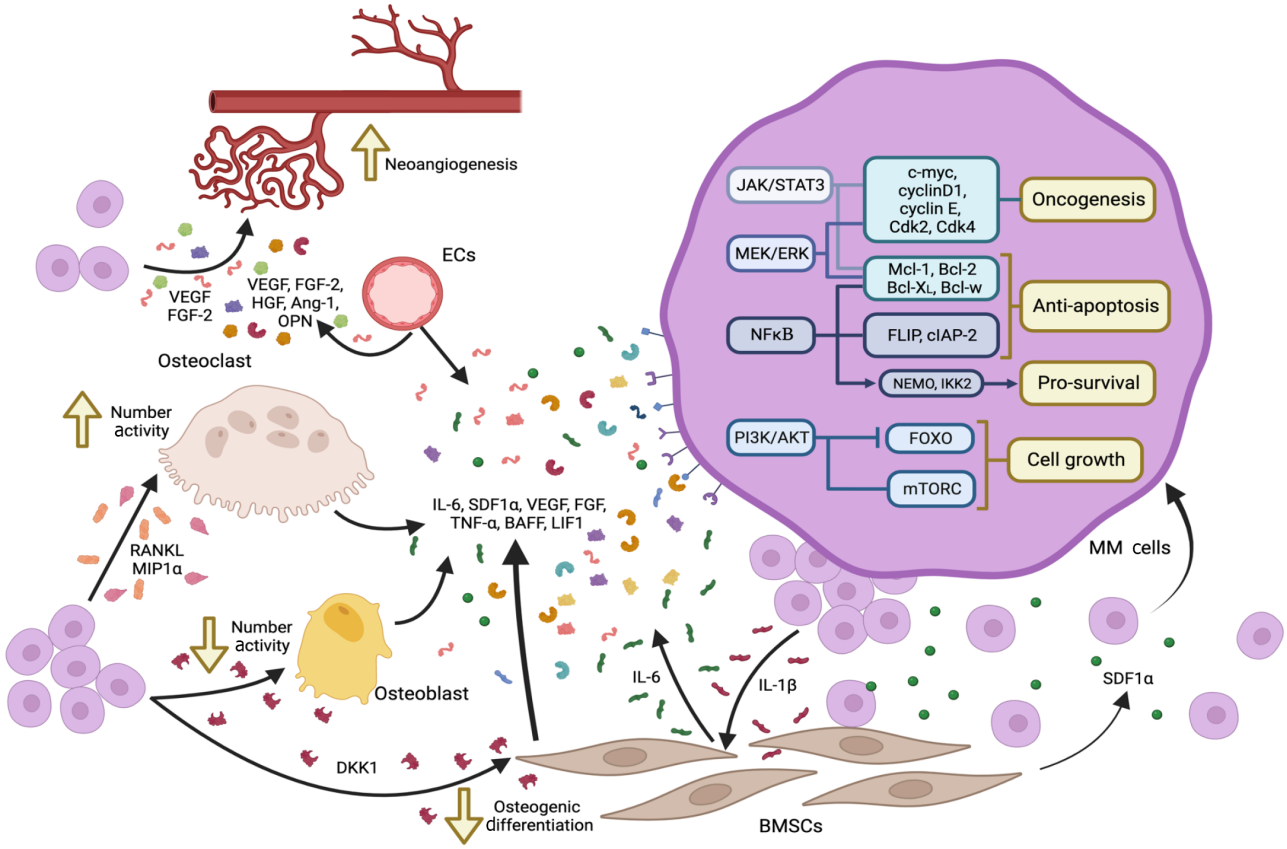
Role of soluble factors in the promotion of proliferation and MM cell survival

Many of the factors secreted by BM-MSCs and by other cells of the BM microenvironment, activate key signaling pathways in the MM cells that would increase their chances to survive and proliferate in the BM microenvironment. A summary of these factors as well as the signaling pathways involved in this communication are shown in Figure 1. In fact, some mutations activating those pathways have also been found in patients with MM. We will address some of those key pathways in this section.

Once in the BM, for the tumor to progress further, MM cells would need a permissive microenvironment. This microenvironment would be created by multiple soluble factors secreted by the different cell types present at the BM. The soluble factors produced by the BM-MSCs seem to be the main, but not the only, effectors of the changes elicited in the MM cells. Besides SDF1α, BM-MSCs seem to secrete other important soluble factors such as IL-6, IL-17, vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), tumor necrosis factor-α (TNF-α), BAFF or leukemia inhibitory factor-1; osteoclasts mainly secrete IL-6 and VEGF; and vascular endothelial cells secrete cyclophilin-A[42,43]. These factors will activate specific signaling pathways in the MM cells such as PI3K/Akt, MEK/ERK, Janus kinase 2 (JAK2)-signal transducer and activator of transcription 3 (STAT3, JAK2/STAT3) pathways, related to cell survival, proliferation and drug resistance[43]. It is important to highlight that this communication is bi-directional, since MM cells would also produce cytokines such as IL-1β, VEGF, and transforming growth factor-beta (TGF-β) that would exert their effect on BM-MSCs, activating the nuclear factor kappa-B (NFκB) pathway and thus, inducing further secretion of cytokines by the BM-MSCs into the BM microenvironment, particularly IL-6[44,45].

IL-6 is the main activator of the JAK2/STAT3 pathway, known to be implicated in the pathogenicity of cancer. JAK2/STAT3 pathway activation promoted by IL-6 leads in MM cells to the expression not only of potent proto-oncogenes such as c-myc and cyclin D1, but also of anti-apoptotic genes like Mcl-1, Bcl-XL and Bcl-2. Moreover, STAT3 activation has also a immunosuppressive effect since it regulates T-cell mediated cytotoxic immune response[46], contributing to the establishment of a immunosuppressed microenvironment that would contribute to the survival and proliferation of the MM cells in the BM. On the other hand, IL-6 activation of JAK2/STAT3 pathways, also has an important role in bone destruction, a hallmark of MM. IL-6/JAK2/STAT3 axis induces the expression of the receptor activator of NFκB ligand (RANKL)[36,47] whose binding to its receptor at the surface of pre-osteoclasts, promotes their differentiation towards mature osteoclasts, activating bone resorption and thus, promoting the formation of osteolytic lesions.

It is important to highlight that the NFκB signaling pathway also has an important role in the survival of MM cells and in the maintenance of the tumorigenic microenvironment at the BM. Both canonical and non-canonical NF-kB pathways are activated by different factors present in the BM microenvironment, including IL-6, IGF-1, TNF-α or BAFF[48]. While IGF-1 is able to activate NFκB pathway, inducing the expression of anti-apoptotic, caspase-8 inhibitors FLIP and cIAP-2[49], TNF-α has a pro-survival effect through NFκB pathway mediators such as NFκB (NEMO) and IκB kinase subunit 2[44]. On the other hand, BAFF activates NFκB non-canonical pathway upregulating the expression of antiapoptotic proteins including Mcl-1, Bcl-XL, Bcl-w and Bcl-2[50]. There is also evidence indicating that IL-6 is



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Figure 1 Schematic representation of the main factors involved in the bidirectional communication between multiple myeloma cells and cells in the bone marrow microenviroment (bone marrow mesenchymal stem cells, osteoclasts, osteoblast, *etc.*). The main signaling patwthays activated by these factors are also depicted (Created with Biorender.com). VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factors; HGF: Hepatocyte growth factor; OPN: Osteopontin; ECs: Endothelial cells; IL: Interleukin; SDF1α: Stromal cell derived factor 1α; TNF-α: Tumor necrosis factor-α; BAFF: B-cell activating factor; DKK-1: Dickkopf-1; MM: Multiple myeloma; BM-MSC: Bone marrow mesenchymal stem cells; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; NFκB: Nuclear factor kappa-B; PI3K: Phosphatidylinositol 3-kinase; RANKL: Receptor activator of NFκB ligand; Ang-1: angiopoietin-1; MEK: MAPK kinase; ERK: Extracellular signal regulated kinase; LIF1: Leukemia inhibitory factor-1. Created with BioRender.com.

linked to the expression of VEGF in MM cells, being some of the VEFG isoform expression driven by the NFκB pathway[51,52].

The MEK/ERK pathway is the signaling pathway most found activated in MM patients, with a prevalence in between 43% and 53% of the patients[53]. Changes in MEK/ERK pathway have important effects in cell cycle, due to the alteration in the expression of molecules such as cyclin D1, cyclin E, Cdk2 and Cdk4 and in apoptosis prevention by the induction of the phosphorylation of the pro-apoptotic protein Bim. This phosphorylation results in the release of anti-apoptotic molecules such as Mcl-1, Bcl-XL and Bcl-2, also related to Akt pathway[54]. In the absence of mutations that activate this pathway, the stimulation of the MEK/ERK pathway in the MM cells might also occur by the action of different soluble factors present in the BM microenvironment such as BAFF, IL-6, SDF1α, VEGF or TNF-α among others[42]. As with other relevant signaling pathways that become activated in MM, the MEK/ERK is also studied as a potential therapeutic target.

PI3K/Akt signaling pathway also has a relevant role in cell proliferation, cell cycle and apoptosis. Alteration of the PI3K/Akt/mTOR pathway due to genetic modifications or its hyper-activation contributes to carcinogenesis, metastasis, invasion, proliferation and drug resistance of tumor cells. However, no activating mutations have been described in MM cells yet. Despite this fact, PI3K/Akt/mTOR pathway is important for MM cells survival[55,56].

Role of soluble factors in angiogenesis and bone homeostasis

Up to this point, we have mentioned some of the effects of the pro-tumorigenic microenvironment in the BM on the MM cell survival and growth. However, once modified by the BM microenvironment, MM cells will start to release different soluble factors that will not only perpetuate that tumorigenic microenvironment, but also will have a deep impact in angiogenesis and bone homeostasis.

Neovascularization in the bone is an essential feature for MM progression and the presence of high density of micro-vessels in the BM microenvironment is characteristic in MM. Cells residing at BM, such as BM-MSCs, osteoblasts, HSCs, or endothelial precursor cells, commonly express various angiogenic

factors, such as VEGF, FGF-2, TNF-α, HGF, IL-6, BAFF, SDF-1α, angiopoietin-1 or osteopontin (OPN). Also, MM cells are able to directly produce VEGF stablishing a VEGF autocrine loop where the produced VEGF would stimulate MM cells proliferation through the MEK-1/ERK pathway[57,58]. FGF-2 is another key pro-angiogenic molecule that would be produced by both MM cells and BM-MSCs[59]. However, contrary to VEGF, which is produced by all MM cells, FGF-2 production by MM does not seem to be a general feature in all MM cases[59]. Other molecules with pro-angiogenic activity such as MMPs[60,61] or OPN, also produced by MM cells, have also a relevant role in promoting micro-vessels formation in the BM microenvironment. The overall increase in the production of such angiogenic factors is elicited by the MM cells. The activation of angiogenesis linked to tumor progression is known as "angiogenic switch"[62] .

Bone homeostasis is a dynamic process driven by osteoclasts, osteoblast and osteocytes. Alterations in the balance between these cell types will lead to the remodeling of the bone. The characteristic bone lesions found in MM derive from the disruption of bone homeostasis initiated by the activation of JAK2/STAT3 pathway by IL-6 and the subsequent induction of RANKL expression by MM cells. Not only this but, as will be discussed later, cell-to-cell interaction of MM cells with BMSCs also induce the expression of the macrophage inflammatory protein (MIP)-1α[63]. Both RANKL and MIP-1α are mediators in the bone destruction driven by MM as they have an both in the number of osteoclasts and in their activity. MIP-1α is a chemoattractant for osteoclasts and stimulates osteoclast formation[64], while RANKL after being recognized by its receptor RANK, will induce the commitment of the macrophage/monocyte precursor cells to the osteoclast lineage[65].

Secreted by MM cell in response to the activation of the JNK pathway, Dickkopf-1 (DKK-1) is also a disruptor in bone homeostasis[66]. DKK-1 is an extracellular inhibitor of the Wnt pathway. DKK-1 interacts with membrane receptors as transmembrane proteins Kremen 1/2 and the human low-density lipoprotein receptor-related protein 5/6, thus competing with Wnt[67]. As one of the main regulatory pathways for osteogenic differentiation of BM-MSCs into osteoblasts[68], the inhibition of the Wnt/β-catenin pathway by DKK-1 will result in a reduced number of osteoblasts. By the action of these factors, RANKL, MIP-1α and DKK-1, the balance between bone formation and bone resorption driven by osteoblasts and osteoclasts is disrupted, resulting in the characteristic bone lesions present in MM patients.

A table summarizing the latest scientific evidence regarding key factors involved in MM/BM-MSCs communication and their effect is shown (Table 1).

EVs-MEDIATED COMMUNICATION BETWEEN BM-MSCs AND MM CELLS

Under non-pathological conditions, BM homeostasis is maintained by cell-to-cell contact, soluble molecules, and EVs. Whereas, over the years solid evidence has accumulated about the relevance of the first two, the involvement of EVs-mediated communication in the maintenance of BM homeostasis has started to be contemplated only in the last few decades[69]. Despite being a fairly new field, important advances have been made in the knowledge of EVs, such as their classification, in terms of their size and biogenesis, into three major categories (exosomes, micro-vesicles, and apoptotic bodies) and the fact that its content varies according to the state of their parental cells[31].

As we have previously discussed, MM cells have the capacity to alter the environment in which they reside[70] as well as the characteristics of cells present in that microenvironment. Thus, it is not surprising that the EVs produced by MM cells also play a key role in disease progression. In fact, it has recently been shown that, exosomes (a particular class of EVs) produced by both BM-MSCs and MM cells are largely responsible for MM pathogenesis[71]. This recent demonstration of the relevance of EVs in MM progression has resulted in several studies in the lats few years, however, the multitude of agents and interactions involved in the development and progression of this disease has made it difficult to fully understand the molecular mechanisms involved. In this section we aim to gather the available information so far.

Effect of MM-EVs on the BM-MSCs and bone homeostasis

As previously mentioned, osteolysis, one of the main hallmarks of MM disease, is linked to the negative effect of MM cells on cells responsible for bone homeostasis, such as MSCs, osteoblasts and osteoclasts [72]. In particular, myeloma bone disease (MBD) has a unique feature compared to other diseases that encompass bone destruction, since in MBD osteoblast activity is also severely impaired[24]. Several authors have suggested that an essential part of this bone damage is related to EVs directly produced by MM cells (MM-EVs). Zhang *et al*[73] demonstrated that the cargo of MM-EVs was enriched in various molecules which negatively regulate osteogenesis. They confirmed that MM-EVs induced high expression of miR-103a-3p in BM-MSCs, which led to impaired osteogenesis *in vitro*. Moreover, they showed that injection of MM-EVs in mouse tibia resulted in defective bone formation. Interestingly, *in vitro* assays also revealed that MM-EVs were also able to influence MM cells increasing viability and IL-6 production, known to regulate MM cell proliferation thus, establishing an autocrine feedback. MM-EVs also increased miR103a-3p expression in MM cells however, in those cells the increased prolif-

Table 1 Summary for the key soluble factors involved in multiple myeloma/bone marrow mesenchymal stem cells communication

Soluble factors	Origin	Function	Ref.
SDF1α	BMSCs	Chemoattractant of MM cell towards the BM microenvironment	[38]
IL-1β	MM cells	Act over BMSCs inducing the secretion of soluble factors, mainlyIL-6	[45]
IL-6	BMSCs	Closely related with cancer pathogenicity due to it proto-oncogenic and anti-apoptotic effect over MM cells	[46,47]
		Immunosuppressive effect over T cells	
		Also related with bone destruction by inducing the expression of RANKL by the MM cells	
VEGF	BM cells, MM cells	Promotes bone neovascularization, essential for tumour progression	[58]
RANKL	MM cells	Induce the commitment of the macrophage/monocyte precursor cells to the osteoclast lineage. Promoting indirectly bone destruction	[63]
DKK-1	MM cells	Disruptor of bone homeostasis by inhibiting BMSCs differentiation into osteoblasts	[66,67]

SDF1α: Stromal cell derived factor 1α; BM-MSC: Bone marrow mesenchymal stem cells; MM: Multiple myeloma; IL: Interleukin; RANKL: Receptor activator of NFκB ligand; VEGF: Vascular endothelial growth factor; DKK-1: Dickkopf-1.

eration of MM cells after exposures to MM-EVs does not seem to be related to miR103a-3p but to other miRNAs also present in the MM-EVs cargo, such as miR107 and miR181a-3p[24].

Among the different biomolecules found as part of the exosome cargo, long non-coding RNAs (lncRNAs) and miRNAs have been the focus of attention due to their key regulatory roles. Various miRNAs found in MM-EVs have been studied for their involvement in the disruption of osteogenesis. miR-129-5p was identified as a player in vesicle-mediated bone disease[74]. In particular, miR-129-5p seemed to inhibit the transcription factor specificity protein 1, leading to a reduction of ALPL, both at the mRNA and protein levels, during the early osteogenic differentiation of MSCs. On the other hand, the long non coding RNA Long Intergenic Non-Protein Coding RNA 461, found as part of the MM-exosomes cargo, has also been found to inhibit osteoblast differentiation by reducing the activity of Wnt/β-Catenin pathways, responsible for osteoblast proliferation, differentiation and activity[75]. Other molecules, such as soluble proteins present in the MM-EVs cargo also showed anti-osteogenic activity Faict *et al*[72] revealed that Wnt/β-Catenin inhibitor DKK-1 is present in MM-EVs and observed a lower expression of Osterix (OSX), Collagen 1A1 and alkaline phosphatase in differentiated MC3T3-E1 cells after MM-EVs treatment.

Runx2 is the master regulator of early osteogenic differentiation, and therefore a possible target for the anti-osteogenic effect of MM-EVs. In fact, lncRNA RUNX2-AS1 present in the MM-EVs cargo was identified as a bioactive molecule able to reach MSCs and form a transcriptionally repressed RNA duplex with RUNX2 premRNA, reducing the osteogenic activity[76]. In addition, a MM-EVs impact in osteoblastic differentiation through reduction of Runx2, together with OSX and OCN, has been described by Liu *et al*[77]. These authors also record increased levels of IL-6 secretion *via* APE1/NF-kB which, as aforementioned, is an important survival factor of MM cells.

Once the EVs produced by MM cells reach the BM-MSCs, their cargo modifies the BM-MSCs behaviour in the benefit of MM cells**.** A clear example of this is miR-146a which acts in a positive loop to favor disease progression[19]. Once this miRNA targets BM-MSCs, it produces an increase in the secretion of several cytokines and chemokines from those cells, including CXCL1, IL-6, IL-8, inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), and CCL-5, which, in turn, once released into the BM microenvironment, would favor MM cell viability and migration. In addition, MM-EVs cargo miR-146a and miR-21, participate in proliferation and transformation of MSCs into cancer associated fibroblasts (CAFs). This is a type of cell which could contribute to a tumour-supportive microenvironment through secretion of cytokines, including IL-6 and TGF-β[78].

Interestingly, it has been shown that conventional chemotherapeutic agents including melphalan, and anti-proteases such as bortezomib and carfilzomib can stimulate a considerable MM-EVs release. The EVs produced under these circumstances are called "chemoexosomes". These chemoexosomes are characterized by the high presence of the heparanase enzyme in their surface. This heparanase is implied in several cellular changes leading to chemoresistance and the subsequent relapse of the patient. Heparanase EVs content is delivered in MM cells and activate ERK pathway as well as TNF-α production by macrophages, matrix degradation and migration promotion[71].

Effect of EVs produced by BM-MSCs on MM cells and MM progression

So far, we have analyzed the influence of MM-EVs on BM-MSCs, however, this communication, as previously mentioned, is bidirectional. In 2016, Wang *et al*[69] showed that BM-MSC-EVs from MM patients contained a lower level of the tumor suppressor miR-15a, and higher levels of oncogenic

proteins, cytokines, and adhesion molecules, when compared to EVs from healthy BM-MSCs. Cytokines such as IL-1ra, interferon-IP-10, MCP-1, MIP-1α, MIP-1β, and SDF1α were detected in murine BM-MSC-EVs. They confirmed that BM-MSC-EVs from MM patients act on MM cells activating proliferation, survival, and migration, as well as drug resistance to bortezomib, a widely used clinical drug for MM treatment.

In a similar study, a reduction of mir-15a levels in the cargo of BM-MSCs-EVs from MM patients was also detected. This change was shown to promote cell proliferation and dissemination or metastasis to other niches, which is a hallmark of MM. The same authors also revealed the importance of some of the proteins present in BM-MSCs-EVs cargo, as they detected higher content levels of IL-6, CCL2, γ-catenin and fibronectin, which are key to MM pathogenesis[70]. Other miRNAs cargo were also implicated in these processes. miR-483-5p was found packed in BM-MSCs-EVs and was responsible for promoting MM cell proliferation and reduced apoptosis *via* the miR-483-5p/TIMP2 axis[79]. Umezu *et al*[80] highlighted the role of miR-10a in MM disease since its transference *via* BM-MSC-EVs promoted cell proliferation in several MM cell lines (RPMI 8226, KMS-11, and U266) compared to BM-MSC-EVs with miR-10a blocked. Moreover, Gao *et al*[81] studied miR-155 present in BM-MSC-EVs cargo, which turned out to be involved in viability, stemness and drug resistance in MM cells. The role of miR-155 was underscored by the fact that incubation of the MM cells line mitochondrial pyruvate carrier 11 (MPC-11) with miR-155-mimics for 24 h resulted in a significantly reduced cell apoptosis *in vitro* and augmented expression of stemness maintenance markers OCT-4 and Nanog and drug resistance-associated proteins MRP1, ABCG2 and P-g.

As in the previous section, a table summarizing the main works referred to the relevance of communication between MM cells and BM-MSCs through EVs and the role of their cargo is shown (Table 2).

The resistance to treatment is precisely one of the major problems in MM at the clinical level, as this is directly responsible for the relapses. Some studies investigating the mechanisms behind this resistance have highlighted the implication of the activation of several signaling pathways, including p38, p53, c-Jun N-terminal kinases and Akt through the assessment of bortezomib treatment. The role of BM-MSC-EVs in interfering with the antitumor effect developed by bortezomib in MM was confirmed through different experiments. BM-MSC-EVs were able to alter apoptosis-related proteins Bcl-2, Bax, caspase-8, caspase-9, and caspase-3 promoting an antiapoptotic profile in both murine and human cells. These EVs blocked the significant reduction of Bcl-2 expression caused by bortezomib and reduced cleaved caspase-9, caspase-3, and PARP either in the absence or presence of bortezomib. Moreover, the use of GW4869, a neutral sphingomyelinase inhibitor of the formation of exosomes by the ceramide pathway, in combination with bortezomib treatment led to a significant effect on tumor load reduction[71,82].

In conclusion, the two-way communication between MM cells and BM-MSCs mediated by EVs is extremely intricate and plays a pivotal role in the progression of the disease. Since BM-MSCs-EVs have a key role in supporting MM development, this could become a key target to develop new therapies for the treatment of this hematological disease.

COMMUNICATION THROUGH CONTACT DEPENDENT MECHANISMS

As well as the already described interactions through paracrine secretion of different cytokines and EVs, MM cells also interact with BM-MSCs by direct cell-to-cell contact. These cell-to-cell interactions are not restricted to MM and BM-MSCs since MM cells also interact with other cells of the BM microenvironment such as osteoclasts and osteoblasts, endothelial cells, and lymphocytes. It is known that these contacts are also key to protect MM cells against chemotherapy, helping them to accumulate inside the BM[83], to adhere to endothelium, and to spread into the bloodstream[84], although the detailed mechanisms involved in those processes have not been completely elucidated[85].

Cell adhesion molecules in MM/BM-MSCs communication

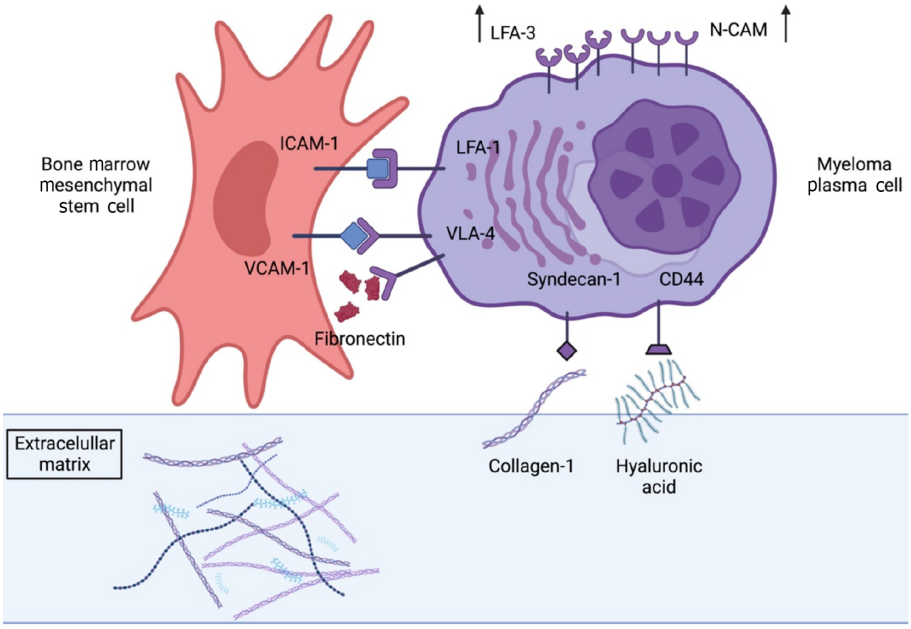
Direct cell-to-cell adhesion and communication mechanisms have been known for more than 40 years [86,87]. These cell-to-cell communication is mediated by Cell Adhesion Molecules (CAMs), a subcategory of adhesion proteins located at the cell surface, involved in binding either to other cells, or in attaching cells to proteins of the ECM[88], suchas fibronectin, laminin or collagen (Figure 2). While it has been well documented that the ECM promotes the survival of different types of tumors, much less is known about the influence of the direct contact of BM-MSCs in their progression.

CAMs play a central role in cell communication and the maintenance of tissue homeostasis[89]. There are different superfamilies or groups of CAMs with different specificities and distributions. These families would include the Immunoglobulin superfamily CAMs (IgCAMs), integrins, cadherins and one superfamily of proteins that contain a C-type lectin-like domain (C-type lectin domain proteins or CTLDs)[89]. Following other criteria, CAMs can be classified into calcium-independent or calcium-dependent molecules[90], meaning that these molecules would need Ca^{2+} ions binding to different domains of the protein in order to rigidify their extracellular domains and enable interaction[91]. Integrins and IgCAMs belong to the calcium independent group whereas CLTDs and selectins belong to

Table 2 Summary of evidence about the relevance of different cargo molecules in the extracellular vesicles of multiple myeloma cells and bone marrow mesenchymal stem cells s to the progression of multiple myeloma

	Function	Ref.
MM-EVs cargo		
lncRNA RUNX2-AS1	Form a RNA duplex with RUNX2 premRNA, reducing the osteogenic activity in MSCs	[76]
miR-146a	Increase the secretion of several cytokines in BM-MSCs that favor MM cell viability and migration and induce CAF transformation	[78]
DKK-1	Lower expression of OSX, COL1A1 and ALP in osteoblast precusor cell line (MC3t3-E1)	[72]
MSC-EVs cargo		
mir-15a	Promote MM cell proliferation and dissemination to other niches	[70]
miR-483-5p	Induce MM cell proliferation and reduced apoptosis	[79]
miR-155	Reduce MM cell apoptosis and augment expression of stemness maintenance and drug resistance markers	[81]

EVs: Extracellular vesicles; MM: Multiple myeloma; BM-MSC: Bone marrow mesenchymal stem cells; CAF: Cancer associated fibroblast; DKK-1: Dickkopf-1; OSX: Osterix; COL1A1: Collagen 1A1; ALP: Alkaline phosphatase.



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Figure 2 Schematic representation of the main cell adhesion molecules in multiple myeloma cells and bone marrow mesenchymal stem cells. The main interactions between cell adhesion molecules (CAMs) of these two types of cells as well as the interactions of these CAMs with proteins of the extracellular matrix are displayed (Created with Biorender.com). ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cell adhesion molecule-1; LFA-1: Leukocyte function-associated antigen 1; N-CAM: Neural cell adhesion molecule; VLA: Very late antigen. Created with BioRender.com.

calcium dependent group[92]. Cell adhesion molecules bind to different ligands. Cadherins, selectins and IgCAMs are associated with the cell-to-cell contact, while integrins are involved in the attachment of MM cells to the ECM[93]. All these molecules are integral in modeling cellular mechanisms such as growth, contact inhibition and apoptosis. In fact, changes in cell adhesion, involving these molecules, can be the defining event in a wide range of diseases, including cancer development[94], as lower intercellular adhesiveness allows malignant cells to scape from their site, thus, destroying the architecture of the original tissue, commonly the first step leading to cancer[94].

As well as the already described interactions through paracrine secretion of different cytokines and EVs, MM cells also interact with BM-MSCs by direct cell-to-cell contact. In fact, recent studies revealed that many of the changes undergone by BM-MSCs supporting the progression of MM, are acquirable by physical contact with MM cells[95]. In MM, this cell-to-cell interactions are not restricted to MM and BM-MSCs since MM cells also interact with other cells of the BM microenvironment such as osteoclasts and osteoblasts, endothelial cells, and lymphocytes. All these interactions are regulated by CAMs. It is

known that these contacts are key to protect MM cells against chemotherapy, helping them to accumulate inside the BM[83], to adhere to endothelium, and to spread into the bloodstream[84], although the detailed mechanisms involved in those processes have not been completely elucidated[85].

The most relevant role of CAMs in MM pathophysiology is related to the homing of malignant PCs to the BM. To complete the process of homing, mediated by CXCL12, MM cells need to adhere to either ECM proteins or BM-MSCs. This is mediated by CAMs such as very late antigen (VLA)4, VLA5, CD44, leukocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule 1 (ICAM-1), MPC-1 and syndecan (Figure 2).

One way of ensuring adhesion of MM to the ECM is the binding of its integrin VLA-4 to fibronectin, a common component of the ECM. VLA-4, which is in fact a heterodimer of two integrins CD49d(a4) and CD29(b1), also mediates the interaction of MM cells with BM-MSCs, through the vascular cell adhesion molecule-1 (VCAM-1), located at the BM-MSCs[96]. This interaction activates the secretion of MIP-1α and MIP-1β in MM cells, leading to an increase of osteoclastogenic activity[97]. Moreover, the direct contact of these two types of cells through VLA-4 also induces the production of DKK-1 by MM cells, which inhibits osteoblastic differentiation of BM-MSCs. Thus, these two actions, promotion of osteoclastogenesis and inhibition of osteogenesis, would have a detrimental effect on bone structure, contributing to the typical osteolytic lesions in MM. In addition, BM-MSCs unable to undergo osteoblastic differentiation would produce higher levels of IL-6, a cytokine that would stimulate the proliferation of DKK-1-secreting MM cells[25]. Moreover, it has been observed that VLA-4–fibronectin binding is an essential step that supports the IL-6-mediated induction of PCs in normal BM, since antibodies against VLA-4 were found to inhibit the secretion of IL-6 in co-cultures of MM cells and BM-MSCs cells[96,98,99].

The interaction between MM cells and BM-MSCs is also mediated by ICAM-1 (CD54) and LFA-1 (CD11α/CD18) expressed in BM-MSCs and MM respectively. The glycoprotein ICAM-1 is the main ligand for b2 integrins and its expression is induced in response to an inflammatory microenvironment [100], such as the one resulting in the BM following the colonization by MM cells. The ICAM-1/LFA-1 interaction seems to have a key role in the progression of MM since the blocking of LFA-1 through the use of monoclonal antibodies, inhibits the production of IL-6 by BM-MSCs. Thus, this interaction is focus of various studies aimed to the development of treatments for MM[101].

Syndecan (CD138) is the principal transmembrane proteoglycan expressed in the surface of MM cells and has in fact been used as a marker for the detection of this pathology. Syndecan has multiple functions in MM. This molecule mediates de adhesion of MM cells to the collagen in the ECM through direct interaction with collagen molecules but can also mediate myeloma cell-cell adhesion[102]. Syndecan-1 also plays a broad role in cells signaling since heparan sulfate chains on syndecan-1 can bind to and sequester growth factors and cytokines, regulating their availability to cells. Also, a recent study has shown that syndecan contributes to the survival of mature MM cells by enhancing IL-6 signaling[103]. Finally, the binding of syndecan to VEGF and other angiogenic factors, has been shown to promote angiogenesis in MM[104].

Finally, CD44, a transmembrane glycoprotein, interacts mainly although not exclusively with hyaluronic acid in the ECM[105]. CD44 signaling has been shown to activate various signaling pathways in different types of cancer including PI3k/AKT, MAPK/ERK and NF-kB[106], which, as we have seen, promote MM cell survival.

Although normal PC and MM cells express basically the same set of CAMS, some of these molecules were found to be more significantly overexpressed in MM cells when compared to healthy patients. In this group we can include, leukocyte adhesion molecule LFA-3 (CD58)[107] and neural cell adhesion molecule (CD56)[108]. MM cells can also express the lymphocyte function-associated antigen LFA-1 (CD11α/CD18) which was associated with tumor growth and homotypic tumor cell adhesion or aggregation[109]. It is also worth mentioning that some homing molecules could not be detected on MPCs: Selectin molecule L-selectin and collagen receptor VLA-2[89]. Although this study provides relevant information, for this information to be biologically relevant, ligands of these receptors had to be available within the tumor environment.

Overall, given the importance of some of these CAMs in the process of MM cells homing, these molecules could be important targets for designing antitumoral treatments. Several approaches have been explored, including antibodies specifically targeting these molecules on the cell surface, as well as small molecule inhibitors that interfere with the binding of the CAMs to their ligands. Moreover, receptor-blocking antibodies against most of these CAMs (VLA-4, CD56, MPC-1, CD21) were found to partially block MM cells adhesion to the BM stroma. This partial effect could be attributed to an additional adhesion mechanism yet to be discovered[110,111].

In MM a specific type of drug resistance seems to be mediated by CAMs, the so called, CAMs mediated drug resistance[112]. CAMs can activate intracellular pathways that promote cell survival, promote cancer cell adhesion to the ECM and regulate the expression of drug transporters that could pump chemotherapy drug out of cancer cells and reduce their efficacy. It is also important to highlight that MM spreading in the last stages of the diseases also involve important changes in cell adhesion. MM can abandon de BM microenvironment and become stroma independent because of different processes involving changes in the expression levels of CAM and certain cytokines. Once this happens, cells can be found to spread extramedullary at different sites such as lungs, liver, or pleural fluid[113, 114].

Role of tunneling nanotubes in MM/BM-MSCs communication

As we have seen, communication between MM and BM-MSCs cells can take place through mechanisms that can be classified as contact-dependent and/or contact-independent mechanisms[115]. While the previous section has been dedicated to direct communication mediated by cell adhesion molecules, in this last section we will briefly discuss transport *via* tunneling nanotubes (TNTs), another form of contact-dependent interaction.

TNTs are transient intercellular structures formed by the polymerization of F-actin which provide an important and general mechanism of cell-to-cell communication[116,117] and constitute a reliable infrastructure for vesicle and protein trafficking[118]. Numerous examples of communication between MSCs and malignant hematological cells such as B cells, MM and chronic lymphocytic leukemia, are already known, as well as the effects of this communication, such as increased drug resistance. This has already been demonstrated in acute myeloid leukemia (AML), B-cell precursor acute lymphocytic leukemia (ALL) or CML[119,120]. Therefore, TNTs are considered one of the key pharmacological targets in current research.

The role of TNTs is to deliver autophagosomes, mitochondria and other lipophiles to MSCs. This induces the secretion of specific cytokines, including interferon-γ-IP-10, CXCL10, IL-8, MCP-1 and CCL2 and other growth factors which, in turn, induce tumor cell survival, enhanced growth and even drug resistance[121]. This has been checked in AML, where increased survival of cells against chemotherapy treatments is observed by means of mitochondrial transfer from MSCs routed by TNT. In this case, the mitochondrial transfer translates into an increase of up to 14% in mitochondrial mass in co-cultures of tumor cells with MSCs and a 1.5-fold increase in mitochondrial adenosine triphosphate production (ATP), making them less prone to mitochondrial depolarization and thus resulting in increased survival against chemotherapy treatments[122].

Numerous lines of treatment are currently under development for various hematological diseases that reduce the formation of TNT by blocking actin polymerization. This inhibits the cellular communication that promotes disease progression. Those treatments include cytochalasin D, cytarabine, latrunculin A and B, daunorubicin, everolimus, metformin, nocodazole CK-666, ML-141 or 6-thio-GTP [123]. In addition, vinca alkaloids or taxanes are also being targeted because of their role in the polymerization of microtubules[124].

Although the fate of mitochondria transferred into tumor cells remains unclear, there is evidence indicating that MSCs play a key role in the progression of AML, ALL, MM and mitochondrial transfer chemoresistance. It is well known that the initiation of cancer requires metabolic adjustments, since rapid proliferation cancer cells have high metabolic requirements. This mitochondrial and/or mitochondrial DNA transfer to cancer cells increases mitochondrial content and enhances the mitochondrial process of oxidative phosphorylation (Oxphos), which generates a larger quantity of ATP than glycolysis, thus, promoting cell proliferation and invasion[125]. Therefore, targeting mitochondrial respiration and Oxphos is also a treatment option, FOXM1 is known to regulate myeloma cell metabolism by increasing glycolysis and Oxphos. NB73 is a FOXM1 inhibitor that promotes FOXM1 degradation and thus growth of MM cells, making it a potential drug targeting Oxphos[126].

Studies to date have elucidated that mitochondrial transfer dynamically induced resistance occurs between MM cells and other cells in the BM microenvironment *via* TNT, providing a starting point for the development of new targeted therapies[127]. An example of this line of treatment for MM is the use of anti-CD38 monoclonal antibodies[128]. This antibodies have different mechanisms of action, including cell apoptosis[129]. Moreover, their administration in mice has shown inhibition of mitochondrial transfer, a reduction in tumor volume and, in general, increased survival[1]. However, it should be noted that, although patients who have received this treatment show increased survival, it has been observed that resistance to these treatments can be acquired in the long term.

CONCLUSION

Conditions at the BM microenvironment are essential for the establishment and progression of MM. The complex BM microenvironment encompasses hematopoietic cells, immune cells, and cells involved in bone homeostasis such as osteoclasts, osteoblasts and BM-MSCs. Thus, it is understandable that the disruption of microenvironment homeostasis by MM cells results in angiogenesis, osteolysis, immune suppression and anemia[69].

As key regulators of this microenvironment, BM-MSCs play an important role in the progression of the disease. The crosstalk between MM cells and BM-MSCs takes place at different levels, through soluble cytokines, EVs, and direct cell-to-cell contact.

The interaction between these two cell types can have both positive and negative effects on the proliferation and survival of MM cells. The communication between MM cells and BM-MSCs can promote tumor growth. The survival and proliferation of MM cells once they reach the BM is associated with immune suppression, eliminating the possibility of an effective antitumor response. Although it is the interaction between all cells in the BM what produces this immunosuppressive microenvironment, BM-MSCs have a relevant role in the construction of this particular microenvironment due not only to their

important paracrine activity, but also to their ability to establish direct communication with other cells in that microenvironment. All these direct or indirect interactions activate a pleiotropic proliferative and antiapoptotic cascades favoring disease progression.

On the other hand, the communication between MM cells and BM-MSCs can also have a negative impact on cancer cell growth and survival. BM-MSCs can secrete factors that inhibit the growth and survival of MM cells.

Currently, therapeutic advances in the treatment of this disease are based on targeted therapies using monoclonal antibodies or CAR-T. These treatments have improved patient prognosis, although long-term resistance is still observed, and further research is needed into the specific mechanisms by which cells acquire this resistance. In the quest for new effective treatments for MM, the importance of communication between MM cells and BM-MSCs cannot be overstated. Understanding the molecular mechanisms involved in this two-way communication can provide valuable insights into MM pathogenesis and help identify key targets involved in the survival and proliferation of MM cells in the BM microenvironment and thus, opening new opportunities for the design of targeted therapies to avoid disease progression.

FOOTNOTES

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MINIREVIEWS

Molecular signaling in cancer stem cells of tongue squamous cell carcinoma: Therapeutic implications and challenges

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Abstract

Head and neck squamous cell carcinoma is the seventh most common cancer worldwide with high mortality rates. Amongst oral cavity cancers, tongue carcinoma is a very common and aggressive oral cavity carcinoma. Despite the implementation of a multimodality treatment regime including surgical intervention, chemo-radiation as well as targeted therapy, tongue carcinoma shows a poor overall 5-year survival pattern, which is attributed to therapy resistance and recurrence of the disease. The presence of a rare population, *i.e.*, cancer stem cells (CSCs) within the tumor, are involved in therapy resistance, recurrence, and distant metastasis that results in poor survival patterns. Therapeutic agents targeting CSCs have been in clinical trials, although they are unable to reach into therapy stage which is due to their failure in trials. A more detailed understanding of the CSCs is essential for identifying efficient targets. Molecular signaling pathways, which are differentially regulated in the CSCs, are one of the promising targets to manipulate the CSCs that would provide an improved outcome. In this review, we summarize the current understanding of molecular signaling associated with the maintenance and regulation of CSCs in tongue squamous cell carcinoma in order to emphasize the need of the hour to get a deeper understanding to unravel novel targets.

Key Words: Head and neck squamous cell carcinoma; Cancer stem cells; Signaling; Tongue squamous cell carcinoma

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Core Tip: Tongue squamous cell carcinoma is one of the most common and aggressive oral cavity carcinomas, particularly among the Indian population. Despite various treatment strategies employed, the survival rates of the patients remain poor. A rare population *i.e.*, cancer stem cells (CSCs), plays an important role in resistance, recurrence as well as metastasis which are factors responsible for the poor survival outcome. In this review, we discuss the recent findings regarding cell signaling pathways and markers associated with the CSCs and the need to gain a deeper understanding on the properties of the CSCs.

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INTRODUCTION

Global cancer statistics by GLOBOCAN in 2020 showed that 2.0% of new cancer cases reported worldwide were lip and oral cavity cancers, while 1.8% of the total cancer-related deaths were of lip and oral cavity cancers. Lip and oral cavity cancers are the most commonly diagnosed cancers that are responsible for most cancer-related deaths in India[1]. Most cases of oral squamous cell carcinomas (OSCCs) are presented at advanced stages, *i.e.*, stages III or IV [tumor-node-metastasis (TNM) staging], where the 5-year survival of the patients is less than 50%. Further, 40% of the oral carcinomas are presented as tongue carcinomas[2].

Head and neck squamous cell carcinomas (HNSCCs) are carcinomas of the oral cavity, nasopharynx, oropharynx, larynx, and hypopharynx[3]. The oral cavity carcinomas comprise the anterior 2/3rd of the tongue, buccal mucosa, retromolar trigone, lower and upper alveolar ridge, hard palate, and floor of the mouth[3].

The poor survival observed in HNSCCs is primarily attributed to loco-regional/ distant metastasis and therapy resistance. Therefore, understanding the molecular mechanism underlying these properties of tumors has become very crucial. Current treatments for HNSCCs include surgery, chemo-radiotherapy, and targeted therapy.

The first evidence of cancer stem cells (CSCs) was observed in acute myeloid leukemia[4], where it was reported that only 10000 cells expressing $CD34^+/CD38^-$ could give rise to leukemia in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice. These cells possess high tumorigenic potential, which are termed as CSCs. CSCs exhibit stem cell-like properties such as self-renewal, slow cycling, and the ability to divide and differentiate into various sub-populations[5]. Further, CSCs were isolated from solid tumors such as breast cancer, HNSCCs, colorectal cancer, ovarian cancer, lung cancer, *etc*[5].

Owing to their unique properties, these CSCs escape the current treatment regimes, thereby adversely affecting patient survival. Therefore, to design an effective treatment regime in order to achieve better efficiency and treatment outcome, it is crucial to understand the molecular mechanism involved in maintaining these CSCs within the tumor. In this review, we focus on the tongue squamous cell carcinoma (TSCC) and have summarized the known molecular markers of CSCs, molecular signaling involved in the regulation of CSCs, the inhibitors used in clinics for treatment, and the ones that are in clinical trials.

CSC MARKERS FOR TSCC

CD44 and variants

CD44 is a single-chain proteoglycan transmembrane glycoprotein expressed on human embryonic stem cells at the developmental stages of cell types such as bone marrow and connective tissue. CD44 interacts with molecules such as hyaluronic acid (HA), collagen, osteopontin, fibronectin, chondroitin and serglycin/sulfated proteoglycan. CD44 has variant isoforms such as CD44, CD44s, CD44v3, CD44v6, CD44v8-10[6] with HA as the most specific ligand for CD44 and its isoforms[6].

The first report of isolation and characterization of CSCs from HNSCC showed that 5000 $CD44^+/Lin^-$ cells gave rise to *in vivo* tumors in NOD-SCID mice[7]. CD44 expression is co-related with the expression of known stem cell marker BMI-1 in HNSCC cells.

Recently, CD44v3 is reported to be overexpressed in HNSCC tumors as compared to the cut margin. Transfection of CD44v3 in the HNSCC cells increases cell migration[8]. Tumor cell growth, migration, matrix metalloproteinase activity, and lymph node metastasis in patients are associated with CD44v3

overexpression in HNSCC cells[9]. Decreased expression of CD44v9 co-relates with poor overall survival (OS) in TSCC[10]. Overexpression of CD44 co-relates with tumor invasiveness and epithelial-mesenchymal transition (EMT). Expression of CD44 in invasive margins of OSCC was associated with samples showing poor histopathological differentiation, high tumor budding activity, and single-cell invasion[11]. Further, increased expression of CD44, together with increased expression of NANOG was associated with poor survival in HNSCC patients as compared to those showing low expression of CD44 and NANOG[12].

Cells overexpressing CD44 ($CD44^+$) showed self-renewal property with high tumorigenic potential, metastasis, and chemo-resistance. Therefore, $CD44^+$ cells in HNSCC tumors are considered as CSC-rich population. CD44, paired with the overexpression of other stem cell markers, such as aldehyde dehydrogenase (ALDH) and CD133, are being used for the isolation of CSCs from HNSCC tumors[13]. In TSCC, $CD44^+/CD133^+$ cells showed stem cell-like properties such as high proliferation, invasion, and migration with high tumorigenicity[13].

There have been recent reports linking $CD44^+$ cells in HNSCC tumors to early angiogenesis[14], lymph node metastasis[15], and occult metastasis[16]. Moreover, overexpression of CD44 in adjacent normal epithelia of TSCC co-related with clinical stage and nodal metastasis in patients[17]. CD44 mRNA expression did not show any co-relation with age, sex, smoking history, size of the tumor, or 5-year survival rate[18].

ALDH

ALDH is an enzyme superfamily which converts aldehydes to carboxylic acids that are involved in drug resistance and detoxification. The human ALDH1A subfamily is involved in the retinoic acid pathway, which regulates gene expression and cell development in both normal and cancer cells. The enzymes belonging to ALDH1A subfamily viz., ALDH1A1, ALDH1A2, and ALDH1A3 are located in the cytosol that catalyze the irreversible conversion of retinaldehyde to retinoic acid.

Amongst the ALDH1A subfamily, ALDH1A1 is overexpressed in the CSCs of HNSCC. Overexpression of ALDH has co-related with overexpression of other stem cell markers such as OCT-3/4, NANOG, STELLA, SNAIL, and BMI-1 in HNSCC. $ALDH^{high}$ cells have also been shown to have increased *in vitro* sphere formation ability and *in vivo* tumorigenesis ability[19,20]. Higher expression of ALDH also co-related with poor patient survival. Importantly, 500 $ALDH^{high}$ cells isolated from HNSCC tumors showed a higher tumorigenic potential upon *in vivo* serial transplantations as compared to $ALDH^{low}$ cells[21]. High expression of ALDH1A1 in oropharyngeal carcinoma co-related with poor differentiation in tumors and poor OS patterns in patients[22]. $ALDH^{high}$ TSCC cells showed serum independency and a higher ability to form tumorospheres than $ALDH^{low}$ cells. $ALDH^{high}$ cells also exhibited overexpression of stem cell-related genes such as NOTCH2[23]. ALDH1A1 expression was directly co-related with OS and lymph node metastasis in HNSCC[24]. The study showed a co-relation of ALDH1A1 expression with TWIST1 expression in primary tumor tissues and lymph node metastases. Recent reports have demonstrated the involvement of ALDH isoforms in cisplatin resistance in HNSCC. Treating cells with ALDH inhibitors showed decreased cell viability and reduced tumor burden *in vivo* when given in combination with cisplatin as compared to only cisplatin treatment. This study also showed that treating cells with the ALDH3A1 activator along with cisplatin increased cell survival[25]. Overexpression of ALDH1A1 in HNSCC tissues co-relates with poor survival as compared to low ALDH1A1 expression[26]. Additionally, the expression of ALDH1 increased from epithelial dysplasia to oral carcinoma, that co-related with poor survival rates in OSCC patients[27]. In addition, low ALDH1A1 in the HNSCC patients showed significantly better OS as compared to high ALDH1A1 expression[25].

CD133

CD133/AC133/prominin-1 is a 97 kDa pentaspan transmembrane glycoprotein encoded by the prominin 1 (*PROM1*) gene. CD133 protein has an intracellular C-terminal domain, an extracellular N-terminal domain, and five transmembrane segments[28].

Spheroids obtained from HNSCC patient tumor cells showed higher CD133 expression than normal epithelial cells[29]. High expression of CD133 with high expression of CD44 and CD117 was observed as marker of CSCs in OSCC cells[30]. TSCC cells overexpressing CD133 showed a higher *in vitro* and *in vivo* tumorigenicity as compared to cells with low expression of CD133[31]. Recent reports showed that $CD133^+$ OSCC cells exhibit properties such as self-renewal, drug resistance, higher tumorigenic potential, and higher growth rate as compared to $CD133^-$ cells. Further, increased expression of stem cell markers such as NANOG, OCT4, SOX2, and ALDH1A1 in $CD133^+$ cells suggested that CD133 is a potential CSC marker for OSCC CSCs[32]. TSCC-derived spheroids were reported to overexpress CD133[33].

Other markers

Stemness markers such as OCT-3/4, NANOG, SOX2, KLF-4, and BMI-1 have been associated with characteristics such as self-renewal, pluripotency, and development of embryonic stem cells are overexpressed in CSCs in TSCCs.

Invasive TSCC cells overexpressing CD44 and SOX9 showed a higher expression of SOX2 and OCT-4 [34]. SOX2 overexpression in TSCC tissues co-related with poor OS in patients[35]. In addition, SOX2 overexpression co-related with tumor size, cell differentiation, nodal metastasis, and clinical TNM stage. In TSCC cells, the knockdown of SOX2 showed a decrease in cell proliferation, cell migration and invasion, and colony forming, which was reversed with overexpression of SOX2[36]. Moreover, an increased SOX2 expression was associated with poor OS, disease-specific survival, and disease-free survival (DFS) in TSCC[37]. Downregulation of SOX2 by MTA-3 was reported to repress CSC properties and tumor growth in TSCCs, and patients exhibiting $MTA3^{low}/SOX2^{high}$ showed the worst prognosis [38]. Additionally, SOX2 regulated HEY1, which in turn regulates NOTCH4 expression, followed by increased EMT in HNSCC cells[39].

The expression of both OCT-3/4 and NANOG was high in side population cells that co-related with distant metastasis[40]. Also, high OCT-4 expression in TSCC samples and NANOG in adjacent cut margin tissues have been reported as indicators of lymph node metastasis and worse prognosis[12]. Furthermore, TSCC showed an association between BMI-1 overexpression and increased proliferation, nodal metastasis, and decreased OS in patients. Further, knocking down BMI-1 in TSCC cells showed a reduction in cell proliferation and migration, increased cell apoptosis, senescence, and cisplatin sensitivity[41]. Ectopic overexpression of BMI-1 increased susceptibility of tongue carcinogenesis after exposure to 4-nitroquinoline-1-oxide in mice. The ectopic expression of BMI-1 was shown to regulate the pathways such as mTOR signaling, EIF2 signaling, and p70S6K signaling[42]. Additionally, high expression of OCT4 and BMI-1, along with ALDH1, co-related with poor survival in OSCC patients[27].

The *TRIM* (tripartite motif) gene family have ubiquitin ligase function that plays an important role in various human diseases such as muscular dystrophies and atrophies and HIV infections *etc*[43]. A recent report showed that overexpression of TRIM14 induces CSC-like properties with an increased sphere formation ability and cisplatin resistance in TSCC cells. Further, on inhibition of TRIM14 by miR-15b, these characteristics were reversed, implying that TRIM14 might play an important role in the maintenance of these properties[44].

MOLECULAR SIGNALING

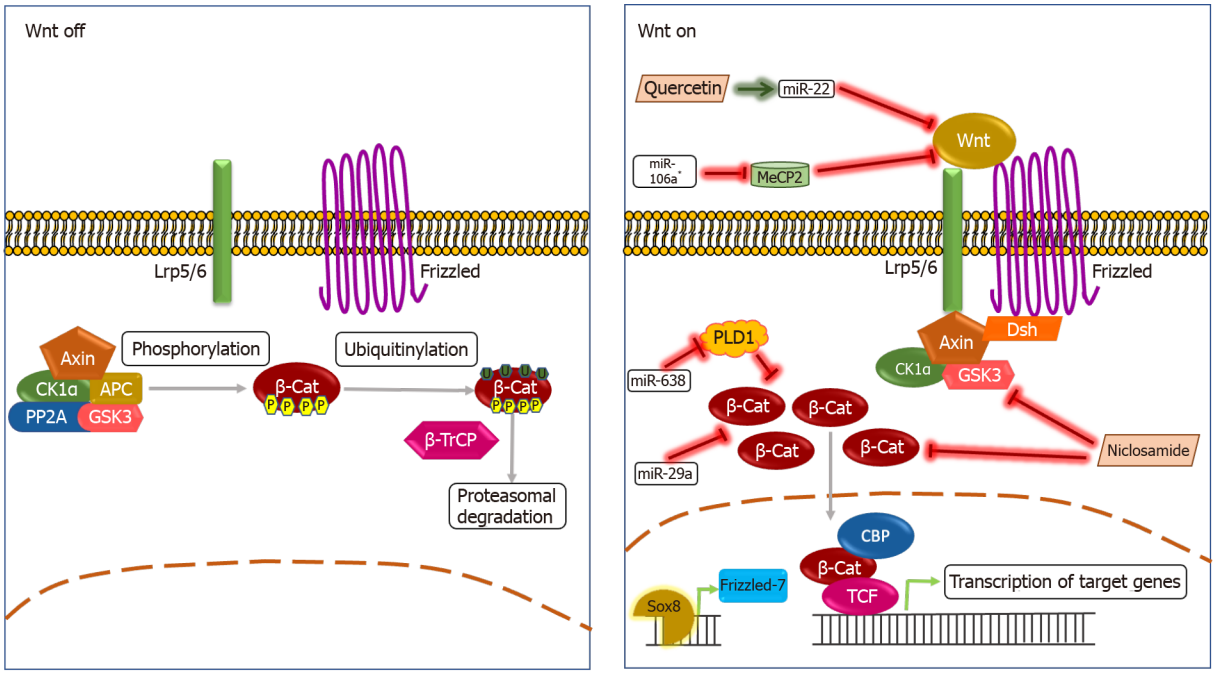
The Wnt pathway

The Wnt signaling consists of the Canonical (involving β-catenin) and non-canonical pathways. Canonical Wnt signaling initiates when Wnt ligand binds to FRZ receptor and low-density LRP5/6 co-receptors. In the absence of the Wnt ligand, a complex of Axin, APC, GSK3, and CK1 phosphorylates β-catenin, leading to ubiquitination and subsequent proteasomal degradation of β-catenin. When present, Wnt ligand binds to FRZ receptor leading to FRZ-induced LRP5/6 phosphorylation followed by activation of the scaffold protein DVL. Activated DVL recruits Axin to receptors, which then inhibits the phosphorylation of β-catenin. Subsequently, β-catenin translocates into the nucleus promoting the transcription of Wnt target genes by interacting with T cell-specific factor/lymphoid enhancer-binding factor. Proper functioning of the Wnt signaling pathway is important for embryonic development and self-renewal of normal stem cells[45]. Deregulated Wnt signaling is involved in the development of various cancers such as colorectal cancer, epidermal cancer[46], hepatocellular carcinoma, breast cancer, glioma, *etc*[47].

Recent studies showed that the suppression of the Wnt signaling inhibits the progression of OSCC. Micro RNAs such as miR-29a[48], miR-638[49], miR-106a*[50], *etc.*, have been shown to suppress tumor progression by regulating the Wnt signaling. The miR-638 and miR-106a* regulate Wnt through downregulating oncogenes PLD1 and MeCP2, while miR-29a caused reduction in β catenin levels. In addition, chemical compounds such as quercetin (bioactive flavonoid) and niclosamide (anthelminthic) were reported to inhibit tumor progression by affecting the Wnt signaling in OSCC. Quercetin induced miR-22 expression, thereby inhibiting the Wnt1/β catenin axis[51], while Niclosamide downregulated the expression of β-catenin, DVL2, phosphorylated GSK3β, and Cyclin D1[52]. SOX8 was shown to regulate chemo-resistance and EMT in TSCC cells by activating the Wnt pathway suggesting that it might play a crucial role in the maintenance of tongue CSCs[53]. Furthermore, the spheroid forming ability and expression of CSC markers (CD44 and ALDH) was negatively impacted in the presence of the Wnt antagonist sFRP4 in tongue carcinoma cells. In presence of the Wnt ligand, WNT3a, these properties were reverted[54]. The Wnt on and off pathway and its role in TSCC is shown in (Figure 1).

The Hedgehog pathway

The Hedgehog pathway has three different types of ligand proteins in mammals such as Sonic-Hedgehog (SHH), Indian-Hedgehog, and Desert-Hedgehog. The ligand binds to the receptor PTCH1 and removes the inhibition on the transmembrane protein Smoothened (SMO). This further leads to SMO accumulation in the cytoplasm. Subsequently, the translocation of glioma-associated oncogene (Gli) proteins into the nucleus initiates the transcription of target genes that are involved in intercellular communication, organogenesis, regeneration, and homeostasis[55].



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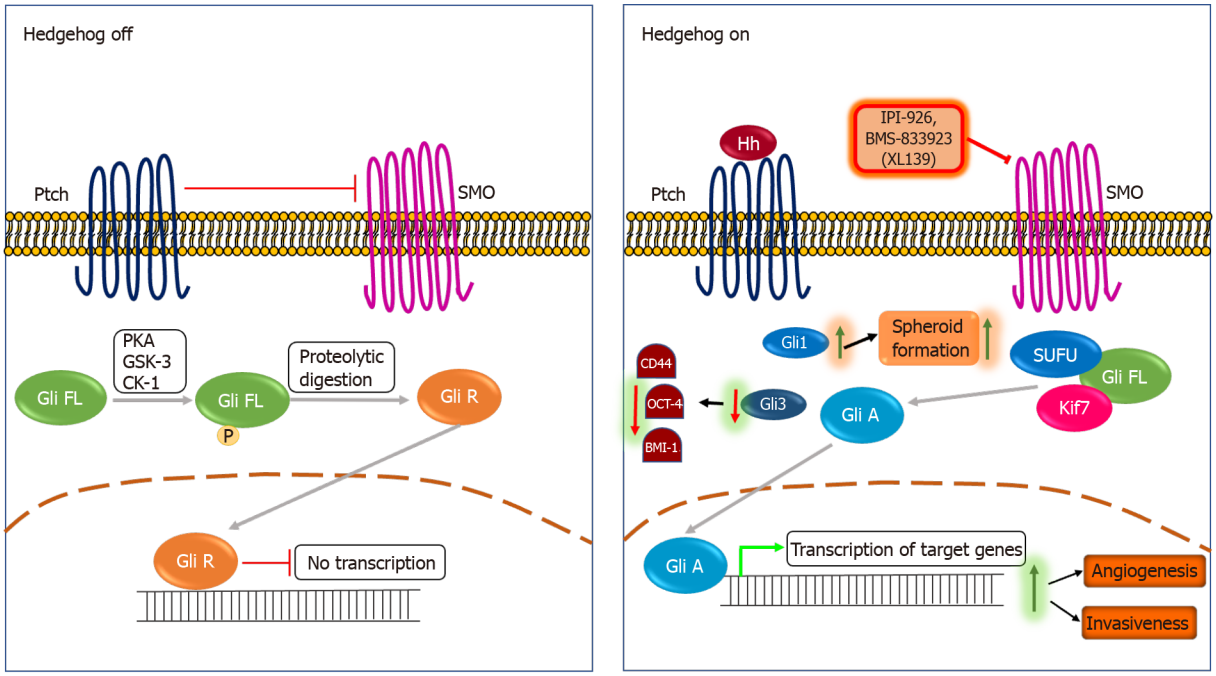
Figure 1 The Wnt signaling pathway and cancer stemness. In the absence of a Wnt ligand, in the canonical pathway,, a complex of Axin, CK1α, APC, PP2A, and GSK3 (termed destruction complex) phosphorylates β-catenin targeting it for ubiquitinylation, that leads to its proteasomal degradation. When the Wnt ligand binds to the Frizzled receptor and LRP5/6 co-receptor, the destruction complex gets localized to the receptor, preventing the degradation of β-catenin that localizes to the nucleus that further activates transcription of the target genes. It has been reported that miRNAs such as miR-29a, miR-638, and miR-106a* reduce levels of β-catenin and Wnt ligand. The miR-638 targets PLD1, a generally accepted oncogene, leading to the reduction β-catenin levels[49]. The miR-29a also directly causes a reduction in β-catenin levels in TSCC cell lines[48]. The miRNA miR-106a* causes a reduction in MeCP2 (a gene expression regulator and oncogene) levels that inhibit the binding of Wnt ligand to the receptor that in turn causes downregulation of the Wnt pathway[50]. Chemical compounds such as Quercetin and Niclosamide downregulate the Wnt signaling pathway. Quercetin causes an increase in levels of miR-22 that in turn inhibits the Wnt1/β-catenin axis [51], while Niclosamide directly binds to DVL2, phosphorylated GSK3β, and Cyclin D1 that reduces levels of β-catenin[52]. Sox8 is shown to activate the Wnt pathway by inducing the expression of Frizzled-7[53].

Hedgehog pathway activation promotes angiogenesis in OSCC. Overexpression of SHH ligand in human TSCC and expression of PTCH1, Gli1, and Gli2 proteins in microvascular cells have been observed in the tumor invasive front[56]. The involvement of Hedgehog pathway has been shown in angiogenesis by macrophages and endothelial cells[57]. Hedgehog and TGFβ signaling are involved in bone invasion and destruction. Gli2 expression is associated with bone invasion. Silencing of Gli2 showed a reduction in invasiveness in orthotopic mice models[58]. Gli3 knockdown in TSCC cells have resulted in the downregulation of the CSC markers such as CD44, OCT-4, and BMI-1 genes and a reduction in CSCs[59]. Further, increased expression of Gli1 has been shown in spheroid forming cells in TSCC cell line[60]. The Hedgehog pathway and its role in TSCC have been shown along with inhibitors in clinical trials for various cancers except for HNSCC (Figure 2).

The Notch pathway

The Notch pathway has four receptors such as NOTCH 1, NOTCH 2, NOTCH 3, and NOTCH 4. The ligands are of two types, viz. Delta-like ligands (DLL1, DLL3 and DLL4) and Jagged ligands (JAG1 and JAG2). Notch pathway also involves proteolysis by metalloprotease, tumor necrosis factor-α-converting enzyme (TACE), and γ-secretase. The binding of the ligand to the receptor releases the extracellular domain by TACE activity, which then binds to the receptor on an adjacent cell, while the intracellular domain is cleaved by γ-secretase activity that further gets translocated into the nucleus, which acts like a transcriptional factor for the activation of the target genes (HES family, HEY, NF-κB, VEGF, and c-MYC) [61].

Notch signaling has been shown to induce EMT in OSCC cells. Activation of Notch signaling is directly co-related with the expression of markers such as E-cadherin and Vimentin and increased invasiveness of OSCC cells[62,63]. Decreasing NOTCH1 in the TSCC cells showed a reduction in the invasiveness of the cells and decreased expression of MMP-2 and MMP-9 (associated with metastasis and invasion) in TSCC[64]. Additionally, activation of the notch intracellular domain in TSCC cell line co-related with stemness characteristics such as spheroid formation and expression of stemness markers viz. OCT4, SOX2 and CD44. The knockdown of NOTCH1 co-related with chemo-sensitization and loss of spheroid-forming ability. Further, high expression of NOTCH1 showed a significantly poor OS as well as DFS in HNSCC patients[65]. Further, NOTCH4 expression promoted cell-cycle dysregulation,



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Figure 2 The Hedgehog signaling pathway and cancer stemness. In the absence of Hedgehog ligand, Patched inhibits smoothened (SMO), leading to the full-length Gli protein that gets phosphorylated by PKA, GSK-3 and CK-1 and converted into Gli repressor through proteolytic digestion. The Gli repressor further inhibits the Hedgehog pathway. When the Hedgehog ligand binds to the Patched receptor, the inhibition on SMO is released, leading to the dissociation of Gli from SUFU and Kif7 that lead to the activation of the Gli protein (Gli A). Further, Gli A translocates to the nucleus that further activates transcription of target genes. Activation of the Hedgehog pathway promotes angiogenesis and invasiveness *in vivo*[57,58]. Downregulation of Gli3 reduces expression of stemness markers such as CD44, OCT-4, and BMI-1 in tongue carcinoma (TSCC) cells[59], while upregulation of Gli1 increases the spheroid formation ability of TSCC cells[60]. IPI-926 and BMS-833923 (XL139) are SMO inhibitors used to therapeutically target the Hedgehog pathway. IPI-926 is a semi-synthetic derivative of cyclopamine, while BMS-833923 (XL139) is a small molecule inhibitor of SMO. Ptch: Patched; SMO: Smoothened; Hh: Hedgehog ligand; Gli FL: Full length glioma associated oncogene; Gli A: Gli activator; Gli R: Gli repressor; IPI-926, BMS-833923 (XL139): Smoothened inhibitors.

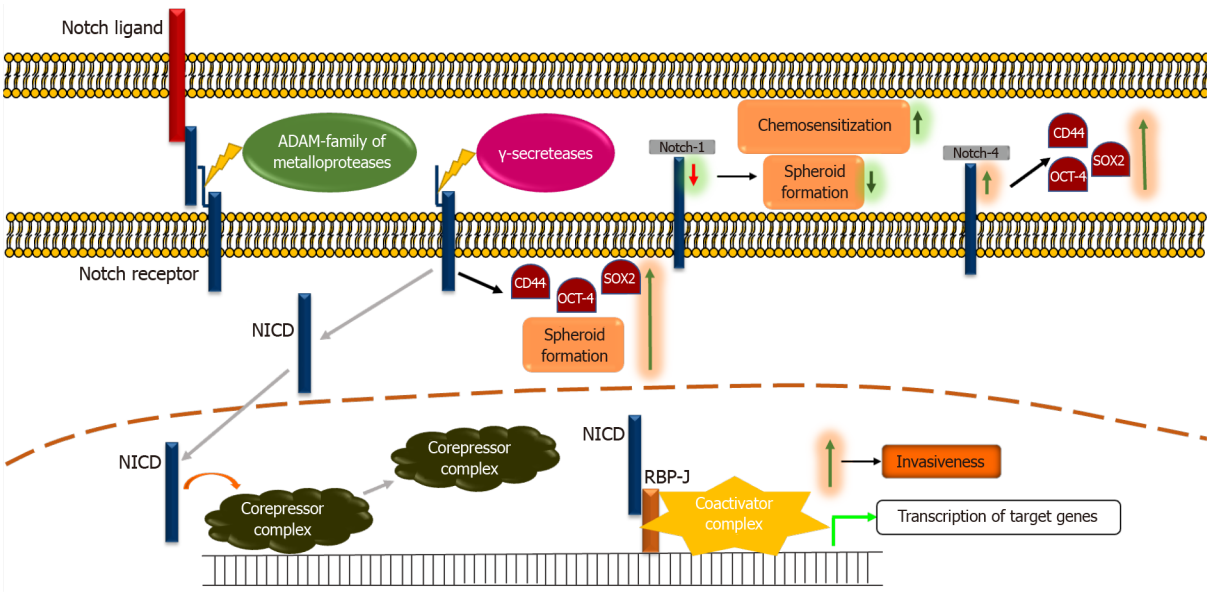
cell proliferation, drug resistance, and inhibition of apoptosis. Elevated expression of NOTCH4 along with HEY1 co-related with OCT4, SOX2, and CD44 overexpression that showed increased migration and invasion in TSCC cells[66]. Recently, it was reported that a STAT3-activated long non-coding RNA, hepatocyte nuclear factor 1 homeobox A antisense RNA 1, promoted tumorigenesis by activating the Notch pathway in OSCC cells[67]. Further, high expression of NOTCH1 and JAG1 have been shown to be predictors of poor OS as well as DFS in oral carcinoma patients[68]. The notch signaling pathway and its role in TSCC is shown in (Figure 3).

The HGF/c-MET pathway

The HGF/c-MET pathway is involved in tumorigenesis in various cancers such as HNSCC, non-small cell lung cancer, hepatocellular carcinoma, ovarian cancer, bladder cancer, cervical cancer, *etc.*[69]. The binding of ligand HGF to the kinase receptor c-MET leads to the dimerization of two subunits. The dimerization results in the auto-phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor, which then creates a docking site for various adaptor proteins that regulate pathways such as PI3K/AKT pathway and Wnt pathway[69].

HGF treatment has been shown to increase the expression of CSC markers and sphere forming ability of HNSCC cells, which were decreased upon c-MET knockdown. The transcriptional level of c-MET was higher in cells with high ALDH activity (one of the HNSCC CSC markers). Moreover, c-MET knockdown in the HNSCC stem-like cells resulted in better survival in *in vivo* orthotopic tongue xenograft models[70].

Significant co-relation has been observed in TSCC between the c-MET expression and tumor stage, nodal status, clinical stage, locoregional recurrence, and distant metastasis. In addition, high expression of c-MET and autocrine motility factor receptor (AMFR) was associated with worse DFS. The study suggested that c-MET and AMFR expression can be potent prognosis marker that targets to decrease metastasis in OSCC[71]. Immunostaining for the c-MET showed a significant co-relation with lymph node metastasis, recurrence, and pathological stage of TSCC[72]. High c-MET expression was co-related with lymph node metastasis, greater depth of invasion, decreased patient survival, increased invasion & migration in *in vitro* and subcutaneous *in vivo* mice model injected with TSCC cells[73]. Further, the knockdown of c-MET has shown to reduce cervical lymph node metastasis and improve survival patterns in *in vivo* models[74]. Overexpression of c-MET was shown to co-relate with occult metastasis



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Figure 3 The Notch signaling pathway and cancer stemness. In absence of the Notch ligand, the Notch pathway is in the inactivate state. The binding of ligand to the Notch receptor leads to cleavage by ADAM-family metalloproteases releasing the extracellular domain of the receptor. Further, the receptor is cleaved by γ-secretase leading to the formation of the Notch intracellular domain (NICD), thereby activating it. The NICD then translocates to the nucleus and releases inhibition on the target genes by dissociating the corepressor complex and forms a complex with RBP-J and co-activator complex thereby activating the transcription of target genes. Further, knockdown of Notch-1 expression has been shown to increase chemo-sensitization and decrease the spheroid formation ability of tongue squamous cell carcinoma (TSCC) cells[64]. An increase in Notch-4 levels increases stemness markers such as CD44, SOX2, and OCT-4 in TSCC cells[66]. NICD activation has been shown to increase levels of CD44, OCT-4, and SOX2 in TSCC cells[65]. Activation of the Notch pathway increases the invasiveness in TSCC cells[62,63,66,67]. NICD: Notch intracellular domain.

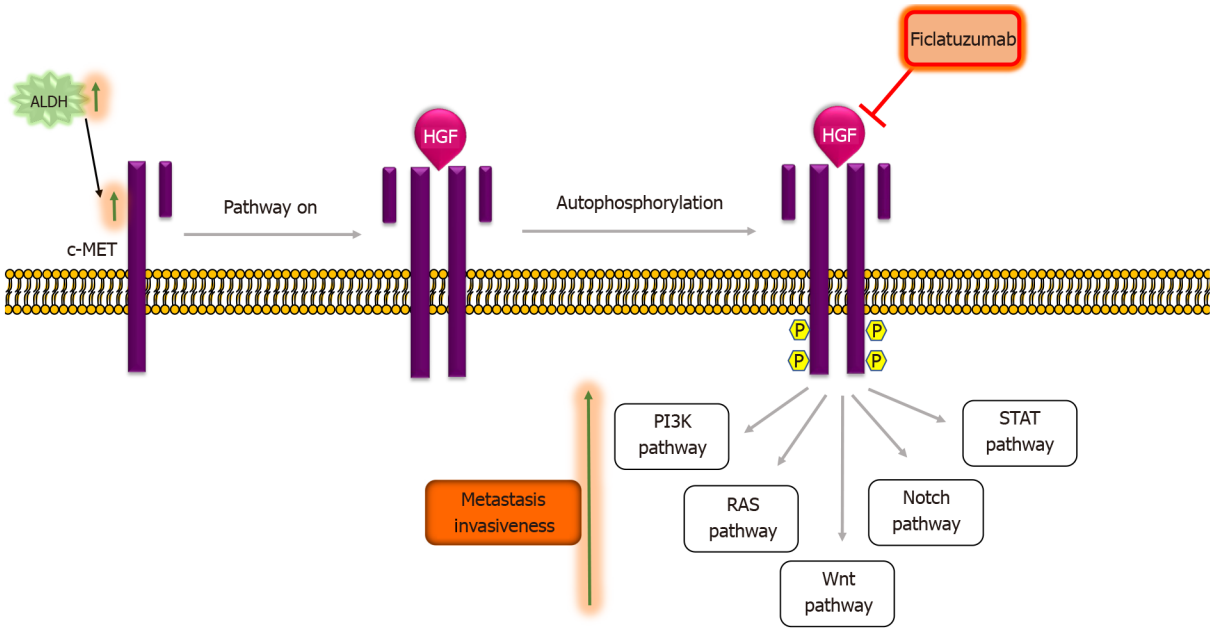
in TSCC[75]. The HGF/c-MET pathway and its role in TSCC is shown along with inhibitor in clinical trials for various cancers except for HNSCC (Figure 4).

Other pathways

The transcription factor Nrf2 has been shown to induce the expression of genes involved in cellular antioxidant and anti-inflammatory responses. Normally, Nrf2 is located in the cytoplasm. Upon activation, it translocates to the nucleus, forming heterodimers with proteins such as c-JUN and small musculoaponeurotic fibrosarcoma protein that bind to antioxidant response element, which regulate the expression of around 200 genes that regulate anti-inflammatory and antioxidant response. Nrf2 is involved in the regulation of mitochondrial biogenesis pathways[76]. A compound named Plumbagin is shown to suppress EMT and stemness characteristics by regulating redox homeostasis and inducing reactive oxygen species (ROS) generation within the cell by suppressing the Nrf2-regulated pathways [77].

The Hippo/transcriptional coactivator with PDZ-binding (TAZ) signaling pathway is also involved in the regulation of properties such as cell proliferation, apoptosis, invasion, migration *etc.* in TSCC. When the pathway is off, the Yes-associated protein (YAP) and TAZ motif translocate to the nucleus, thereby inducing the transcription of various genes by binding to the TEA domain family proteins and other transcription factors. LATS1 is activated by MST1 with Salvador through phosphorylation, which then phosphorylates the YAP/TAZ, retaining it in the cytoplasm, which then binds to 14-3-3 and gets degraded[78]. Factors such as HIF-1α[79] and epigallocatechin-3-gallate[80] affect the Hippo pathway to modulate proliferation, apoptosis, invasion, and migration in TSCC cells.

Approximately 90% HNSCCs overexpress the EGFR pathway[81]. The EGFR is a receptor tyrosine kinase of the ErbB family. The ligands of the EGFR are EGF, heparin-binding EGF, and TGFα. Upon receptor-ligand binding, the inactive monomer of the receptor dimerizes, either with another monomer of EGFR forming a homodimer or with another ErbB family receptor forming a heterodimer. This active dimer then auto-phosphorylates the C-terminal domain of the receptor providing a docking site for the phospho-tyrosine binding domain and Src homology 2 domain resulting in the activation of several signaling pathways such as MAPK, PI3K/Akt pathway, and phospholipase Cγ pathways[82]. Stimulation of OSCC cells by EGF showed an induction of EMT in the cells, which revealed morphological changes with the downregulation of E-cadherin and the upregulation of vimentin in the cells. In addition, stimulated cells showed enrichment of stem-like population ($CD44^+/CD24^-$) with an increase in CSC markers such as ALDH1 and BMI-1, suggesting that EGF may be responsible to induce CSC properties in OSCCs[83].



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Figure 4 The HGF/c-MET signaling pathway and cancer stemness. In the absence of the HGF ligand, the HGF/c-MET pathway remains inactivated. The binding of the HGF ligand to the c-MET kinase receptor results in the dimerization of two subunits that further leads to auto-phosphorylation of the tyrosine residues in the cytoplasmic domain of the receptor creating a docking site for adaptor proteins, which regulate pathways, such as PI3K, RAS, Wnt, Notch and STAT pathway. Transcriptional levels of c-MET were high in ALDH^{high} cells[70]. Activation of c-MET results in an increase in invasiveness and metastasis *in vitro* as well as *in vivo*[73, 74]. Ficlatuzumab is a humanized IgG1 monoclonal antibody targeting HGF used to therapeutically target the HGF/c-met pathway. ALDH: Aldehyde dehydrogenase; Ficlatuzumab: HGF/c-MET pathway inhibitor.

Ephrin (EPH) receptors and their ligands play important roles in controlling the actin cytoskeleton and cellular responses, including attraction/repulsion, migration, and cell positioning during developmental stages[84]. Recent report has shown regulation of the expression of stemness markers by EPHA10. EPHA10 and its ligand EFNA4 increased cell migration, sphere formation, and expression of markers such as SNAIL, NANOG, and OCT4 in OSCC cells. It was also reported that high mRNA levels of EFNA4 with NANOG or OCT4 co-related with poor survival patterns in OSCC patients[85].

CURRENT TREATMENT REGIME

The current treatment regime for HNSCCs is dependent on the TNM staging of the carcinoma based on T (tumor size considering the depth of invasion), N (nodal metastasis considering extranodal extension or ENE), and M (presence of distant metastasis)[86]. For early stage of cancer, single modality treatment, in which mostly surgery, is preferred. With the progression of disease manifested either through larger tumor dimensions or nodal metastasis, multimodality in treatment is employed, wherein surgery is followed by adjuvant chemotherapy or radiotherapy. In case of distant metastasis, where surgical intervention is difficult, chemotherapy is the preferred choice. Further, administration of adjuvant chemo or radiation therapy following surgery is shown to exhibit better patient survival[87]. Chemotherapeutic agents commonly administered are cisplatin, carboplatin, docetaxel, 5-fluorouracil, methotrexate, and paclitaxel. Radiotherapy is also employed in cycles of 1.8-2 Gy/day with a total dose of 66-72 Gy[88]. Targeted therapies, specifically acting on certain upregulated pathways, such as EGFR, are administered. For example, cetuximab targets the EGFR pathway in HNSCC patients or anti-PD1 agents for immunotherapy in HNSCC. Agents such as pembrolizumab and nivolumab, which target PD-1, have shown promising results in clinical trials[87]. Although, recent reports highlighted that owing to heterogeneity in PD-L1 expression throughout tumors and utilization of different methods and antibodies, there might arise errors in immuno-histochemical assessment of PD-L1 prior to therapy decisions[89-92]. In the course of currently existing assessment methods across various cancers, Marletta *et al*[91] observed that in HNSCC, the registration trial utilized the 22C3 clone (Dako) on Agilent autostainer link 48 while the European Medicines Agency granted administration of immunotherapy regardless of antibody, and the instrument used. The reports emphasized on the establishment of a standardized uniform protocol considering the heterogeneity of expression as well as the antibodies and platforms used for the assessment of PD-L1 before deciding whether immunotherapy should be administered to the patients[89-92].

Table 1 Clinical trials currently active for head and neck squamous cell carcinoma

Region	Drug name	Target	Phase	NCT number
Worldwide	Everolimus	Inhibitor of mTOR	I	NCT01637194
	Cetuximab	Monoclonal anti-EGFR antibody		
	Bevacizumab (with fluorouracil and hydroxyurea)	Anti VEGF-A antibody	I	NCT00023959
	Trastuzumab (with IL-12)	Monoclonal anti-EGFR antibody	I	NCT00004074
	Erlotinib	Tyrosine kinase receptor (EGFR)	I, II	NCT00101348
	Cetuximab	Monoclonal anti-EGFR antibody		
	With or without bevacizumab	Anti VEGF-A antibody		
	Erlotinib hydrochloride	Tyrosine kinase receptor (EGFR)	I, II	NCT00101348
	Cetuximab	Monoclonal anti-EGFR antibody		
	Erlotinib hydrochloride	Tyrosine kinase receptor (EGFR)	I	NCT00397384
	Cetuximab	Monoclonal anti-EGFR antibody		
	Zalutumumab (after radiotherapy)	Monoclonal anti-EGFR antibody	III	NCT00496652
	Temsirolimus	mTORC1 inhibitor	II	NCT01256385
	With or without cetuximab	Monoclonal anti-EGFR antibody		
	Cetuximab	Monoclonal anti-EGFR antibody	II	NCT00939627
	Sorafenib tosylate	Tyrosine kinase inhibitor		
	Cetuximab	Monoclonal anti-EGFR antibody	II	NCT01316757
	Erlotinib hydrochloride	Tyrosine kinase receptor (EGFR)		
	Varlilumab	Monoclonal anti-CD27 antibody	I, II	NCT02335918
	Nivolumab	Monoclonal anti-PD-1 antibody		
	MEDI7247	Monoclonal anti-ASCT2 antibody conjugated with pyrrolobenzodiazepine dimer	I	NCT03811652
	Cetuximab with lenalidomide	Monoclonal anti-EGFR antibody	I	NCT01254617
	Durvalumab	Monoclonal antibody that blocks PD-1/PD-L1 interaction	I	NCT03144778
	With or without tremelimumab	Monoclonal antibody against CTLA-4		
	Sitravitinib	Inhibitor of receptor tyrosine kinases	Early phase I	NCT03575598
	Nivolumab	Monoclonal anti-PD-1 antibody		
	Nivolumab	Monoclonal anti-PD-1 antibody	I	NCT03229278
	Pembrolizumab with trigriluzole	Monoclonal anti-PD-1 antibody		
	BKM120	PI3K inhibitor	I, II	NCT01816984
	Cetuximab	Monoclonal anti-EGFR antibody		
	FATE-NK100		I	NCT03319459
	Cetuximab	Monoclonal anti-EGFR antibody		
	Trastuzumab	Monoclonal anti-EGFR antibody		
	Nivolumab with SBRT	Monoclonal anti-PD-1 antibody	II	NCT02684253
	BYL719	PI3K inhibitor	II	NCT03292250
	Poziotinib	EGFR inhibitor		
	Nintedanib	Angiokinase inhibitor		
	Abemaciclib	CDK4 and CD6 inhibitor		
	Durvalumab	Monoclonal antibody that blocks PD-1/PD-L1 interaction		

	Tremelimumab	Monoclonal anti-CTLA-4 antibody		
	Nivolumab and tadalafil	Monoclonal anti-PD-1 antibody	Early phase I	NCT03238365
Involving Indian institutes	Gefitinib (Iressa) with cisplatin and radiotherapy	EGFR inhibitor (tyrosine kinase inhibitor)	II	NCT00229723
	Lapatinib	EGFR inhibitor (tyrosine kinase inhibitor)	II	NCT00371566
	Gefitinib with methotrexate	EGFR inhibitor (tyrosine kinase inhibitor)	III	NCT00206219
	P276-00 with EBRT	CDK inhibitor	I, II	NCT00899054
	P276-00	CDK inhibitor	II	NCT00824343
	Lapatinib with chemoradiation	EGFR inhibitor (tyrosine kinase inhibitor)	III	NCT00424255
	Lapatinib with chemoradiation	EGFR inhibitor (tyrosine kinase inhibitor)	II	NCT00387127
	MEDI4736	Monoclonal antibody blocking interaction between PD-L1 with its receptors	III	NCT02551159
	Tremelimumab with chemotherapy	Monoclonal anti-CTLA-4 antibody		

SBRT: Stereotactic radiation therapy.

Recently, a clinical trial consisting of 13 TSCC patients showed that immunotherapy of cytokine-induced killer cells after chemotherapy helped in the reversion of immunosuppression caused during chemotherapy and surgery. Twelve out of thirteen patients survived for more than 90 mo post-therapy. No immune system related toxicities were reported in the surviving patients. No other side effects of the treatment were observed except complaints of aphthous ulcers by two patients[93]. This showed that multiple modality treatments might improve the survival and quality of life of the patients. However, for further improvement in treatment, more targets specific to CSCs need to be explored.

There have been few clinical trials worldwide as well as those involving Indian institutes for newer molecules targeting EGFR pathway, immune checkpoints, PD-L1, cyclin-dependent kinases, *etc.*, as single treatment agent or in combination with other chemotherapeutic agents/radiation in HNSCC (Table 1). However, these trials are still in the early phases and do not particularly target CSCs. Therefore, further detailed study is essential in finding newer targets specific to CSCs in HNSCC.

CONCLUSION

The CSC population plays an important role in therapy resistance, recurrence, and metastasis. These factors adversely affect patients' survival. In spite of several years of research, most of the treatment regimes employed currently target the tumor bulk. CSCs possess self-renewing property, slow cycling, aberrant cell signaling, dynamic ABC transporter system, DNA repair mechanism, epigenetic modifications, and metabolic regulation, *etc.*, which enable CSCs to escape this therapy and thereby play an important role in recurrence, therapy resistance, and loco-regional/distant metastasis.

A better understanding of the aberrant signaling pathways involved in poor prognosis and maintenance of the CSC population would be more effective in improving treatment outcome. Such an understanding would also be important in the prognosis, prediction, and designing of treatment regime that not only reduce the bulk of the tumor but also effectively eliminate the CSC population thereby improving patient survival.

Molecules specifically targeting signaling pathways that regulate the CSC population administered in combination with conventional therapy or as a single treatment modality need to be studied in TSCC. Uncovering the signaling pathways for CSCs, and targeting them would enable better clinical outcomes.

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FOOTNOTES

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MINIREVIEWS

Human pluripotent stem cell-derived extracellular vesicles: From now to the future

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Abstract

Extracellular vesicles (EVs) are nanometric particles that enclose cell-derived bioactive molecules in a lipid bilayer and serve as intercellular communication tools. Accordingly, in various biological contexts, EVs are reported to engage in immune modulation, senescence, and cell proliferation and differentiation. Therefore, EVs could be key elements for potential off-the-shelf cell-free therapy. Little has been studied regarding EVs derived from human pluripotent stem cells (hPSC-EVs), even though hPSCs offer good opportunities for induction of tissue regeneration and unlimited proliferative ability. In this review article, we provide an overview of studies using hPSC-EVs, focusing on identifying the conditions in which the cells are cultivated for the isolation of EVs, how they are characterized, and applications already demonstrated. The topics reported in this article highlight the incipient status of the studies in the field and the significance of hPSC-EVs' prospective applications as PSC-derived cell-free therapy products.

Key Words: Pluripotent stem cells; Extracellular vesicles; Exosome; Cell-free therapy

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Core Tip: The research on extracellular vesicles (EVs) derived from different cell types, such as adult stem cells, has shown potential in the treatment of various pathologies. However, little has been explored regarding EVs derived from human pluripotent stem cells (hPSC-EVs). In this review, we provide an overview of studies carried out on these EVs, highlighting methodologies used for the culture of hPSCs for isolating EVs, their characteristics, and potential applications. We note the potential of hPSC-EVs as future acellular therapies. However, studies are in the infancy, and more research is needed to confirm their benefits.

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INTRODUCTION

Extracellular vesicles (EVs) are nanometric particles that are enclosed by a lipid bilayer and released by all cell types. They lack a functional nucleus and are therefore unable to replicate[1]. EVs are composed of bioactive factors such as lipids, proteins, and nucleic acids, including mRNAs and non-coding RNAs [2]. EV is an umbrella term that encompasses a heterogeneous population of membrane vesicles generated through a variety of mechanisms. The two major EV subpopulations include microvesicles (MVs) and exosomes (EXOs). EXOs are intraluminal vesicles of endosomal origin released when multivesicular bodies fuse with the plasma membrane, whereas MVs or ectosomes are generated from the outer budding of the plasma membrane[3]. Due to their distinct biogenesis, MVs are generally larger (up to 1000 nm in diameter) than EXOs (less than 200 nm). However, these vesicle populations overlap not only in terms of size but also in composition[4]. Recently, other nomenclatures were described in the "Minimal information for studies of extracellular vesicles 2018" guidelines (MISEV2018) based on the physical characteristics of EVs, for example, size (< 200 nm, small EVs; > 200 nm, medium or large EVs) or density (low, middle, or high)[1].

Potential uses of EVs, such as for the diagnosis and treatment of pathologies or as potential drug carriers, have been investigated. In the field of regenerative medicine, the secretomes of adult stem cells, primarily mesenchymal stem/stromal cells (MSCs), including their EVs, are of great interest as they have been shown to act mainly in a paracrine manner rather than their potential for differentiation[5]. An interesting list of advantages and disadvantages of the use of EVs instead of stem cells has been presented by Öztürk *et al*[6]. Among the advantages of using EVs cited by them and others are low immunogenicity and toxicity; minimal risk of malign transformation; minimal risk of getting trapped in the lung or causing vasculature obstruction; avoidance of contamination with undesired cell types; avoidance of uncontrolled cell division; the ability to manipulate EVs in order to obtain potential improvements; optimization of MSC culture to obtain a higher amount of EVs; and their ability to cross the blood-brain barrier, among others[4,6]. In addition, EVs mimic the beneficial effects of MSCs in cell therapies in a wide range of animal models for different diseases[7-9].

MSC-derived EV (MSC-EV) has been extensively studied and has demonstrated several promising effects, as reviewed by Gowen *et al*[10], Tieu *et al*[11], Fuloria *et al*[12], Kou *et al*[13], and Yudintceva *et al* [14]. However, despite the high potential of MSC-EVs, several factors limit their use. Recently some reviews highlighted the difficulty of establishing criteria to define the specific characteristics of MSC-EV and discussed the great variation in the MSC-EV preparations[15,16]. Disadvantages of MSCs as a source for EVs include the variability between cells derived from different tissues, the variability between different donors, their limited ability to proliferate, the fact that they enter senescence, and genomic instability after a few passages[17]. This raises the question of whether pluripotent stem cell (PSC) derived EVs have a similar to or better therapeutic potential than adult stem cell-derived EVs.

In this context, our objective is to show, using a non-systematic search, studies that use or characterize EVs derived from human PSC (hPSC-EVs) to understand the advances in the area. We also aim to identify the conditions in which the cells are cultivated for the isolation of EVs, how these are characterized, and any demonstrated applications (*in vivo* or *in vitro*).

HPSC-EVS

Overview of hPSCs

hPSCs are characterized by unlimited proliferation and the potential to generate specialized cell lineages [18]. Human embryonic stem cells (hESC) were first isolated from human blastocysts in 1998 by Thomson *et al*[19], and to date, hundreds of hESC lineages have been established worldwide. hESC-based therapeutic technologies have applications in many diseases and conditions, such as spinal cord injuries, age-related tissue degeneration, and diabetes[20]. However, ethical issues related to using cells from embryos have hindered the application of hESCs in research and treatment, leading to the development of the induced PSC (iPSC) technology by Takahashi and Yamanaka[21] and Takahashi *et al* [22]. Since the generation of the first iPSC, many research groups have developed human iPSC (hiPSC) lineages reprogrammed from different adult cells, and obtained lineages very similar to hESC in terms of morphology and differentiation potential[23]. For more information about hPSCs, see Karagiannis *et al*[24], Liu *et al*[25], and Yamanaka[26].

Especially after the discovery of hiPSCs, pluripotent cells represented a promising alternative for regenerative medicine, transplants, disease modeling, and many other research applications[27-29]. The possibility of generating pluripotent cells from patients and, from them, differentiated cells for tissue repair may mitigate common transplant issues, such as immunologic rejection. Nevertheless, the immunogenicity of pluripotent cells remains controversial[30], and the potential for tumorigenesis hinders the wide application of these cells in clinics. The risks of contaminating the differentiated cell populations with remaining pluripotent or proliferative cells, as well as the transmission of active pluripotency transcription factors or the acquisition of mutations by the pluripotent cells during *in vitro* culture[26,31], limit the acceptance of hPSC-based therapies. Therefore, cell-free therapeutic approaches, including EVs, offer promising possibilities for applying hPSC-derived products[32].

It seems that the role of the secretomes of these cells has only recently begun to be investigated, possibly due to the difficulties still encountered in using hPSCs in the clinic. Some interesting studies show that EVs from ESCs could help with embryo implantation[33] and maintaining ESC stemness[34], while others have investigated the biogenesis of ESC-EVs[35,36], although they used murine PSCs. We will focus this review on studies with hPSCs due to their potential clinical applications.

HPSC-EVs: Isolation and characterization methodologies

The first investigation on the isolation of EVs from hPSC dates from 2015. In this initial approach, EVs were isolated from hiPSC cultured in Essential 8™ medium using differential centrifugation (DF)/ ultracentrifugation (UC). It was shown that the hiPSC-derived EVs (hiPSC-EV) contain a variety of microRNAs (miRNAs) (such as miR-382, miR-611, and others) related to pathways such as focal adhesion, Wnt, PI3K-Akt, and MAPK signaling, as well as proteins related to processes involved in signal transduction, receptor binding, and others. In addition, the EVs positively affected the metabolism, proliferation, apoptosis rate, and differentiation capacity of cardiac MSCs. Better results were obtained when cells were exposed for only 22 h to EVs[37]. This initial attempt demonstrated how hPSC-EVs could be beneficial and of interest for future acellular therapy applications.

Despite the potential of hPSC-EVs, we observed that the number of publications in this area is still low, and most of the existing publications date from the last five years (Figure 1A, Table 1). Some studies evaluate EVs that were isolated during the differentiation process or from cells that differentiated from PSCs, such as hiPSC-derived keratinocytes[38]; hPSC-derived cardiac progenitors or cardiomyocytes[39-41]; hPSC-derived MSCs[42-44]; hiPSC-derived neurons[45-47]; and hESC-derived chondroprogenitor cells[48]. However, our review explores studies that isolated EVs from undifferentiated hPSCs.

Using a non-systematic search, we found 36 studies that isolate hPSC-EVs mainly from the hiPSC lineages (Figure 1B). Table 1 summarizes these studies, highlighting the cell culture medium used to culture the PSC, time of conditioned medium collection, EV isolation method, and EV mean size. The most common culture media were commercial, with defined components (Figure 1D). The two most common media used were mTeSR™1 (StemCell Technologies) and Essential 8™ medium (Thermo Fisher) (Table 1). A study published by Luo *et al*[49] aimed to optimize culture conditions for isolation of hiPSC-EVs. Using DMEM with different concentrations of EV-depleted KnockOut™ Serum Replacement (ED-KSR), they observed that cells remained viable at a 0.5% ED-KSR concentration and were able to isolate EVs from PSCs cultured in this condition efficiently. However, after 5 d of culture, there was a reduction in the expression of some pluripotency markers. Thus, although it may be cheaper than commercial media, it is necessary to consider the additional step of centrifugation of the KSR to remove particles, as well as the effects of the change in pluripotency-related parameters on the composition and potential of the EVs.

The biggest variations in EV isolation methods relate to the collection time of the conditioned medium: Many studies do not state the conditioning time. In most studies, however, the EVs were isolated after 24 h of cell culture or every 24 h for 3-5 consecutive days (Table 1), avoiding exceeding the 80%-90% cell confluence in the cell cultures. This collection time is possibly related to the nature of PSCs, as the culture medium must be changed daily, and cells must not reach 100% confluence to guarantee their viability and pluripotency.

Other relevant aspects of EVs are their size, morphology, and estimated particle concentrations. Most studies presented the information listed in MISEV2018, including positive and negative protein markers in EVs, usually using the western blot technique (31/36 articles) and performing a single EV analysis mainly using transmission electron microscopy (31/36 articles) to verify EV morphology and nanoparticle tracking analysis (20/36 articles) to verify their mean size and concentration (Figure 1C). The greatest number of studies used small EVs/EXOs, with sizes up to 200 nm (small EVs) (Table 1).

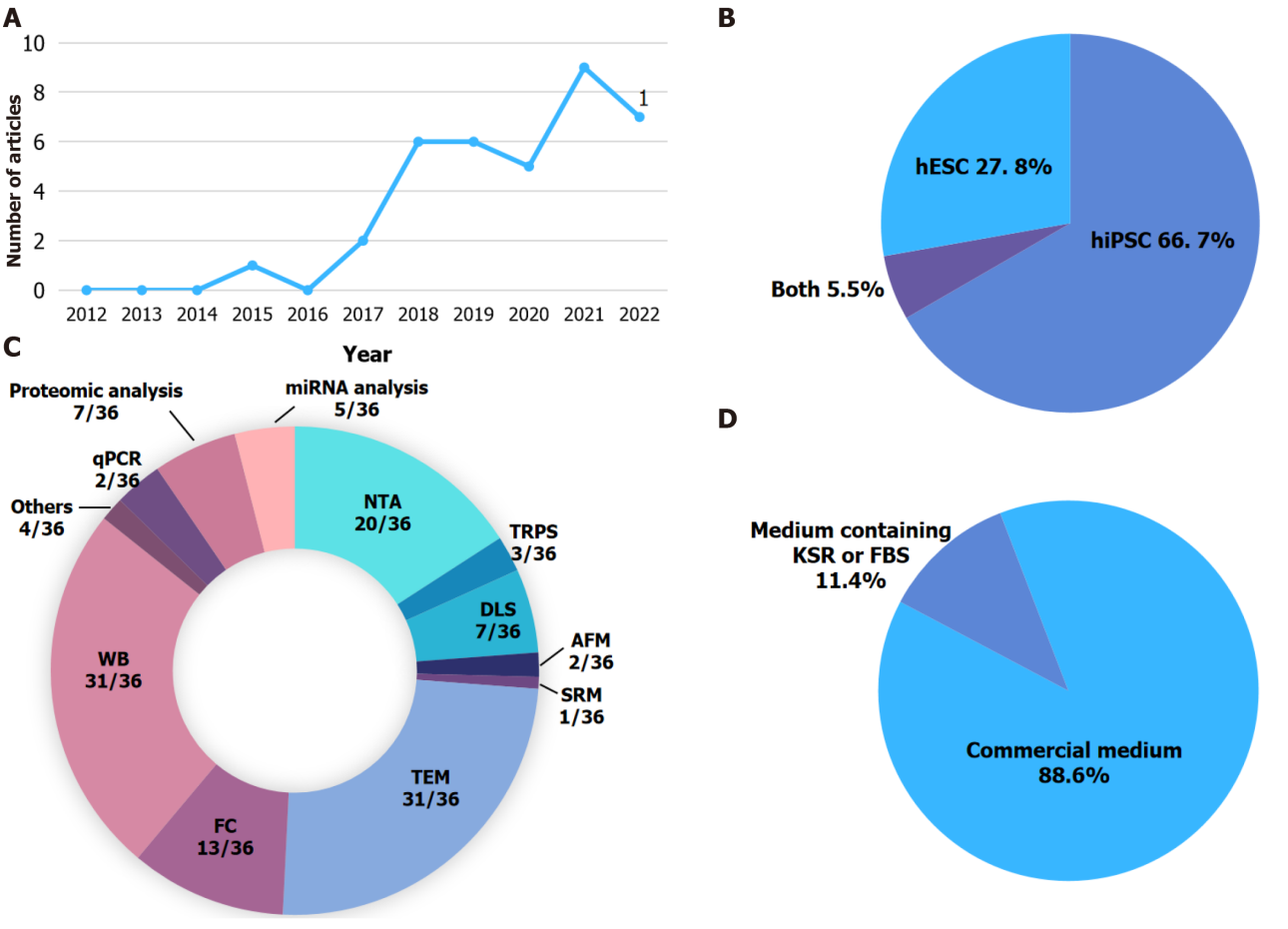
The most common method for hPSC-EV isolation is DF (here defined as the initial centrifugations to remove cellular debris and apoptotic bodies) followed by UC (Table 1). Although this is the most common method used, it is unsuitable for isolating EVs from large-scale experiments and clinical trials. Using a large-scale 2D culture, Andrade *et al*[69] isolated hPSC-EVs using tangential flow filtration (TFF) with or without subsequent UC (TFF + UC). The isolated EVs presented a size of approximately 100 nm, regardless of whether UC had been performed, with similar particle concentration, although TFF + UC resulted in a smaller number of proteins. The effect of different culture conditions (hypoxia - 1% O_2, physiological hypoxia - 5% O_2, and normoxia) on the therapeutic potential of hPSC-EVs was also

Table 1 Human pluripotent stem cell-derived extracellular vesicles: Methods of isolation and vesicle size

Ref.	Culture medium	EV collection time	EV isolation method	EV mean size (nm)
Bobis-Wozowicz *et al*[37], 2015	Essential 8™ medium	NI (cells in 70%-90% confluency)	DC + UC	146
Ju *et al*[50], 2017	PSCeasy medium (Cellapy)	24 h	DC + UC	122, 132
Zhou *et al*[51], 2017	mTeSR™-1 medium	NI (cells in 60%-90% confluency)	DC + 0.22 μm filter + UC	101
Ding *et al*[52], 2018	mTeSR™-1 medium	48 h	DC + UC	103.1
Kaur *et al*[53], 2018	Essential 8™ Flex medium	48 h	DC + UC or miR-CURY™ Exosome Isolation Kit (Exiqon A/S)	100-200
Kobayashi *et al*[54], 2018	DMEM-F12 + NEAA, 200 mM L-gln, KSR, 0.1 M BME	2-3 d before passage	MagCapture Exosome Isolation Kit PS (Wako)	100
Oh *et al*[55], 2018	Essential 8 medium	Daily, from day 2 to day 5	0.45 μm filter + ExoQuick-TC kit	85.8
Peng *et al*[56], 2018	mTeSR™-1 medium	24 h (cells in about 80% confluency)	MV: DC + 16500 g, 1 h; EXO: DC + 120000 g, 2 h	MV = 200-600; EXO = 40-80
Saito *et al*[57], 2018	mTeSR™-1 medium	NI	DC + concentration in 100-KDa filter + MagCapture™ Exosome Isolation Kit PS	179
Chen *et al*[58], 2019	mTeSR™-1 medium	NI	DC + UC	50-150
Liu *et al*[59], 2019	Essential 8™ medium	Daily, for 3-5 d	DC + concentration in 100-kDa filter + SEC	150
Marzano *et al*[60], 2019	mTeSR™-1 medium	Daily, for 4 d	0.22 μm filter + concentration in 100-kDa filter + Total Exosome Isolation Reagent (Thermo Fisher) or DC + UC	about 240
Povero *et al*[61], 2019	NI	24-48 h	DC + UC	300-400
Sun *et al*[62], 2019	mTeSR™-1 medium	48 h	DC + concentration in 100-kDa filter + Exosome Isolation Kit (PureExo) + UC + 0.22 μm filter	70-100 (cell-dependent)
Zhu *et al*[63], 2019	mTeSR™-1 medium	48h (cells 80%-90% confluency)	DC + 0.22 μm filter + UC	70.2
Collino *et al*[64], 2020	mTeSR™-1 medium	24 h	DC + UC	119
Hu *et al*[65], 2021	ncEpic hPSC medium	NI	DC + 0.22 μm filter + UC	72.4 ± 21.3
Kurtzwald-Josefson *et al*[66], 2020	DMEM/F12 Ham 1:1 + 20% KSR, 1% NEAA, 1% L-gln, 0.2% BME, 4 ng/mL rhFGF basic	24 h (cells in about 80% confluency)	Total exosome isolation reagent (Thermo Fisher Scientific)	115 ± 7
Liu *et al*[67], 2020	mTeSR™-1 medium	NI	DC + UC	50-75
Wang *et al*[68], 2020	PGM1 medium	NI	DC + 0.22 μm filter + UC	30-120
Andrade *et al*[69], 2021	mTeSR™-1 mediuma	Daily, for 4-5 days	TFF with or without subsequent UC	103-109
Ashok *et al*[39], 2021	StemMACS medium with 10 μM ROCK inhibitor and 0.2% Pluronic F68	Days 3, 4, and 5 prior to differentiation	DC + 0.22 μm filter + UC + SG	50
Hu *et al*[65], 2021	ncEpic hPSC medium	NI	DC + 0.22 μm filter + UC	-100
Karnas *et al*[70], 2021	Essential 8™ medium	NI	DC + UC	215.7
Ke *et al*[71], 2021	Exo-depleted FBS	48 h	MV: DC + 16500 g, 60 min; Exo: DC + 120000 g, 120 min	MV = 200-600; Exo = 40-80
Luo *et al*[49], 2021	DMEM/F12 + KSR (0.5%, 2.5%, 5%, or 20%)	Daily, for 5 d	DC + 0.45 μm filter + concentration in 10-kDa filter + 0.22 μm filter + UC or ExoQuick-TC kit (SystemBioscience)	187.8, 168.2
Saito *et al*[46], 2021	StemFit AK-03N medium (Ajinomoto)	NI	15000 × g, 30 min + 0.22 μm filter + UC	70

Wang *et al*[72], 2021	mTeSR™-1 medium	NI	DC + UC	120-140
Xia *et al*[73], 2021	Nuwacell hiPSC/hESCs medium	24 h	DC + 0.22 μm filter + UC	50-150
Bi *et al*[74], 2022	ncTarget medium (Nuwacell. Ltd, China)	24 h (cells in about 80% confluency)	DC + UC + 0.22 μm filter + UC	hESC = 133.1; hIPSC = 157.7
Gu *et al*[75], 2022	mTeSR™-1 medium	NI	0.45 μm filter + concentration in 100-kDa filter + GC + 0.22 μm filter + UC	143.5
Gupta *et al*[76], 2022	StemFlex™ medium	48 h	DC + one-step sucrose cushion UC	123.6 ± 60
Hsueh *et al*[77], 2023	StemFlex™ medium	48 h	DC + UC	136.8
Li *et al*[78], 2023	ncEpic hPSC medium	NI	DC + UC	74.70 ± 20.77
Li *et al*[79], 2022	mTeSR™-1 medium	NI	DC + 0.22 μm filter + UC	50.75-105.7
Pan *et al*[80], 2022	mTeSR™-1 medium	24 h (cells in about 80% confluency)	DC + UC	142.2 ± 64.1

DC: Differential centrifugation; EXO: Exosome; FBS: Fetal bovine serum; MV: Microvesicle; NI: Not informed; SEC: Size exclusion chromatography; SG: Sucrose gradient; TFF: Tangential flow filtration; UC: Ultracentrifugation; hPSC: Human pluripotent stem cell; hESC: Human embryonic stem cells; hIPSC: Human induced pluripotent stem cell; KSR: KnockOut™ Serum Replacement.



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Figure 1 Overview of studies on human pluripotent stem cell-derived extracellular vesicles published between 2012 and 2022. A: Timeline of published articles on human pluripotent stem cell-derived extracellular vesicles (hPSC-EVs). [1]Two articles were published online in 2022 but published in print in 2023; B: Analysis of the percentage of articles that use human embryonic stem cells, human induced pluripotent stem cell, or both cell types to isolate EVs; C: Methods used in the studies to characterize hPSC-EVs. The graphic depicts the number of articles using certain techniques/total number of articles included in the analysis; D: Analysis of media used to culture hPSC to isolate the EVs. AFM: Atomic force microscopy; DLS: Dynamic light scattering; FC: Flow cytometry; NTA: Nanoparticle tracking analysis; qPCR: Quantitative polymerase chain reaction; SRM: Super-resolution microscopy; TEM: Transmission electron microscopy; TRPS: Tunable resistive pulse sensing; WB: Western blot; hESC: Human embryonic stem cells; hIPSC: Human induced pluripotent stem cell.

investigated. The results showed that EVs derived from hPSC cultured in 1% O_2 (hypoxia) had greater angiogenic potential than those derived under other conditions and that better results were achieved when obtaining EVs using TFF[69].

Another highly discussed topic about PSCs is the possible formation of teratomas, as well as the biodistribution of these cells when applied in *in vivo* models. These concerns also extend to PSC-EVs. To clarify these points, Gu *et al*[75] evaluated the safety and biodistribution of hiPSC-EVs. They used several approaches to show that PSC-EVs are safe, have no adverse effects on cells (*e.g.*, do not cause hemolysis), are not genotoxic, and can be administered by different routes (nasal, intramuscular, or intravenous) without generating adverse effects (*e.g.*, inflammation at the site or pathological changes in the organs of rats).

Potential therapeutic applications of hPSC-EVs

Although few investigations have been carried out with hPSC-EVs, we notice that almost all of them have already applied hPSC-EVs to different disease models, both *in vitro* and *in vivo*. PSC-EVs have been described as having: Protective effects in *in vitro* and *in vivo* models of ischemia-reperfusion kidney injury[64]; neural protective abilities[60]; the capacity to modulate neuroinflammation and protect against ischemic stroke through Treg cell expansion[73]; antifibrotic effects *in vivo* and in *in vivo* models of liver injury[61,72]; and reduced cartilage degradation in an osteoarthritis model[77]. They have shown improvements in wound closure, angiogenesis, and increased nerve fiber density in a wound-healing diabetic mouse model[54,79]; and improved recovery of ovarian function in a premature ovarian failure mouse model[67]. EVs were also associated with acellular nerve grafts, demonstrating their potential to repair peripheral nerve defects[80].

It was also demonstrated that MVs, but not EXOs, dedifferentiated Müller cells into retinal progenitor cells *in vitro*[71]. Other studies showed the ability of PSC-EVs to promote regeneration of diseased or damaged retinas[56] and to accelerate corneal epithelium defect healing *in vivo*[68]. Other potential uses cited for PSC-EVs are: In antitumoral activity[51,63]; in angiogenesis stimulation[69]; as a gene delivery vector[50]; to increase the functional properties of cord blood-derived hematopoietic stem and progenitor cells[70]; and to improve the number of beating EBs depending on the hiPSC origin[66].

One noteworthy effect shown in some studies is the capacity of PSC-EVs to "rejuvenate" different cell types, such as senescent endothelial cells[52,58], senescent human dermal fibroblasts[55], senescent chondrocytes[77], and others. Considering this potential, the hPSC-EVs, hESC-EVs, and hiPSC-EVs were investigated as therapeutic tools for age-related diseases. Regarding neurological diseases, the hPSC-EVs showed potential in recovery of senescent hippocampal neural stem cells in rats with vascular dementia - partially through the transfer of miRNAs that inhibit mTORC1 activation - resulting in an improvement in disease status (*e.g.*, reverse cognitive impairment)[81]. Furthermore, using mice of varying ages, hPSC-EVs were found to rejuvenate hippocampal neural stem cells partly through the transfer of SMAD proteins that activate myelin transcription factor 1 (MYT1), which is reduced in senescent cells, and activates a signaling cascade in the MYT1-Egln3-Sirt1 axis[81].

In an ischemic stroke model, hPSC-EVs reduced the expression of inflammatory cytokines and leukocyte infiltration, and increased the number of regulatory T cells and other immunomodulatory effects that alleviate neurological deficits[73]. They also reduced blood-brain barrier damage in aged stroke mice through blood-brain barrier rejuvenation, partially through the transfer of AKT1 and CALM from EVs to endothelial cells leading to activation of the endothelial nitric oxide synthase-Sirt1 axis[78]. Therefore, hPSC-EVs could be a promising cell-free therapy to treat age-related diseases associated with cellular senescence.

In order to evaluate the benefit of hPSC-EVs compared to other EVs, one interesting study demonstrated that both hiPSC-EVs and hMSC-EVs, isolated through size exclusion chromatography (Table 1), could improve the proliferation of senescent MSCs and alleviate cellular aging in a replicative aging model, possibly modulating reactive oxygen species production with peroxiredoxins presented in EVs. However, despite the similar effects, EVs derived from iPSCs enter target cells more efficiently, and the production of hiPSC-EVs was about 16-fold higher than that of MSC-EVs (using the same culture medium)[59].

hPSC-EV composition

Even though many articles described the effects of hPSC-EVs, few made deeper characterizations of, for example, the protein and miRNA content of these EVs. Some performed proteomic analysis to help explain some of the effects[59] or as a control (time 0) to study the differentiation process[39,46]. In one interesting approach using high-density lectin microarray, Saito *et al*[57] demonstrated that rBC2LCN, a specific lectin for hPSCs, bound to hiPSC-derived EVs but not to adipose-derived stem cell-, hemodiafiltration-, or chondrocyte-derived EVs, which suggests a particular glycan-signature for hiPSC-EVs, resembling the glycome signature of the cell surface.

One recent study that provided a detailed description of the contents of hPSC-EVs was conducted by Bi *et al*[74]. The proteomics of hESC-, hiPSC-, and hMSC-EXOs showed that the main enriched proteins were related to distinct pathways between vesicles of pluripotent and multipotent cells. In hPSCs, EXO content was more focused on development, metabolism, and anti-aging properties, and in hMSCs, it was related to immune regulation. Another study in 2022 also indicated that hMSC-EV content is

strongly related to immune regulation while hPSC-EV content does not present many of the proteins related to this function[76]. Actually, 79 proteins were found to be shared between hMSC- and hPSC-EVs, yet the main biological processes related to them were DNA regulation, signal transduction and cell communication[76]. Liu *et al*[59] also compared the protein content of hiPSC-EVs and hMSC-EVs and described more than 1100 proteins shared between the different EVs, allowing to identify proteins that could be responsible for the anti-senescent effect observed in the study.

Considering the protein content of hESCs and hiPSC-EXOs, Bi *et al*[74] suggested that hESC-EXOs are more prone to regulate development and pluripotency pathways, and hiPSC-EXOs have a stronger correlation with metabolism. Regarding the most enriched miRNAs for both hPSC-EVs, it was shown that they were related to cell cycle and metabolism regulation. Interestingly, miRNAs found in both hESC-EXOs and hiPSC-EXOs were involved in cell differentiation, development, and cell cycle, even though the hiPSC-EXO set of miRNAs seemed to play a less significant role in these functions than the hESC-EXO set[74].

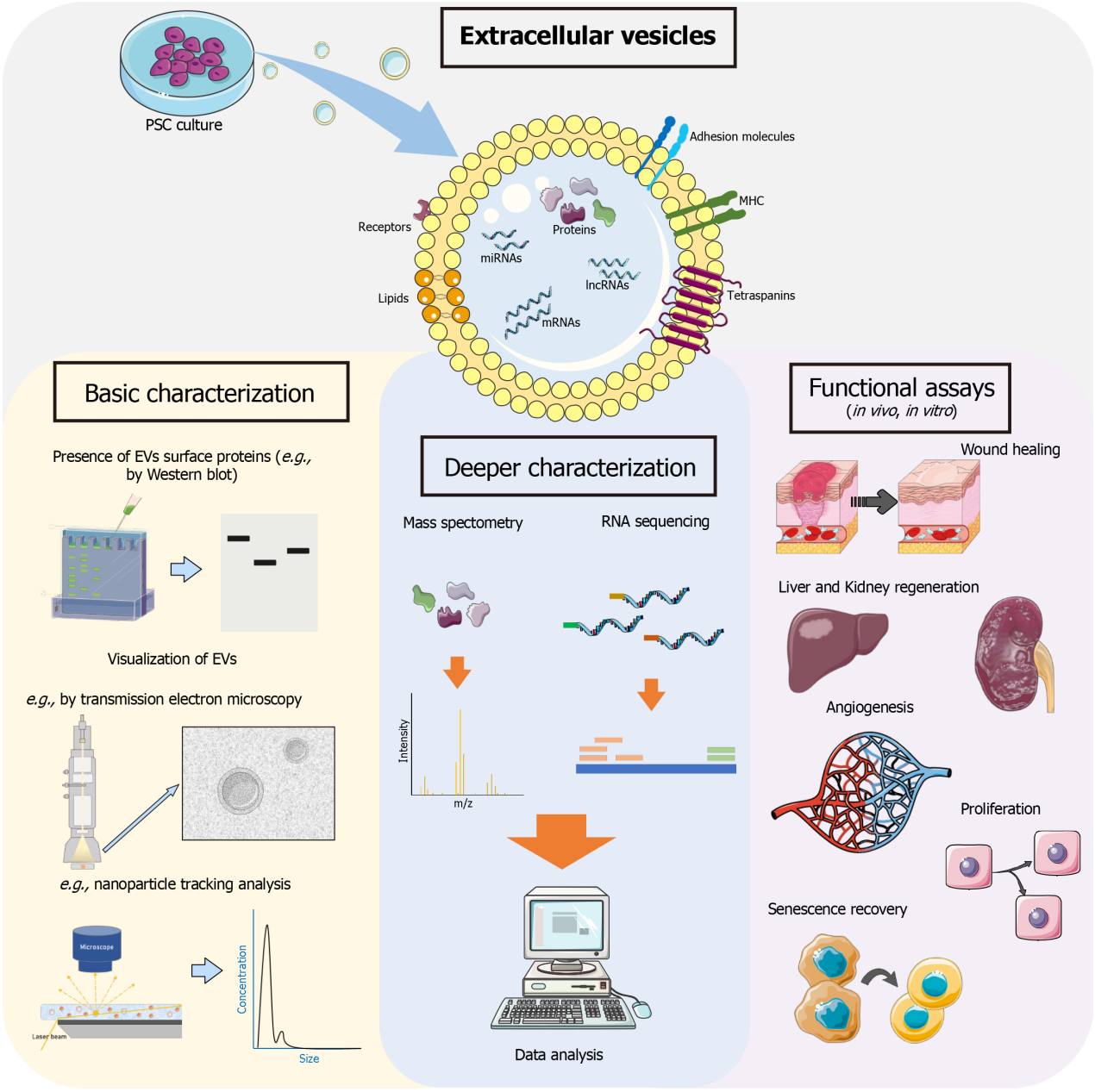
In order to explore whether apoptosis-linked gene 2-interacting protein X (ALIX), a protein present in the endosomal sorting complex required for the transport and biogenesis of EXOs, could regulate the protein content of EV, Sun *et al*[62] isolated EVs from hiPSCs in which ALIX was overexpressed (using lentiviral transduction) or were knocked out (using CRISP-Cas9 system). EVs isolated from these cell lineages were of similar size, although EVs generated from knockout cells were slightly larger. The evaluation of protein content in EVs showed that those derived from knockout cells had fewer proteins, while EVs from overexpressing cells presented a higher number of proteins. These differences could be related to the differences demonstrated in functional assays, *e.g.*, cell viability, apoptosis inhibition, and formation of capillary-like structures, where EVs from overexpressing cells had better effects. So, EVs with different protein profiles could have different therapeutic applications.

CONCLUSION

Although hPSC cultivation has been carried out for some time, the requirements for *in vitro* culture of these cells are very specific, as many factors are necessary to maintain them in their undifferentiated state. This, together with the cost, could be one of the reasons why secretomes and isolation of hPSC-EVs have not been extensively studied so far. Commercial media are now defined with a few components that are no longer as expensive as before, which may have contributed to the increase in publications in recent years.

An overview of the hPSC-EV studies is shown in Figure 2, which illustrates the potential use of these EVs for regenerative medicine. Regarding EV characterization, we observed in the publications that hPSC-EVs follow the basic requirements described in MISEV2018. However, despite the recent increase in research in this area, further characterization of the content of these EVs needs to be carried out. In addition, studies with modified cells aimed to enrich the content of EVs with some specific protein or miRNA may be of great interest. One interesting approach requiring more extensive discussion is the possible use of hPSC-EVs in reprogramming adult cells into PSCs. A recent study used EVs derived from ESCs undergoing cardiac differentiation to transdifferentiate fibroblasts to cardiomyocyte-like cells with relatively high efficiency[82].

Our review shows that hPSC-EVs have therapeutic potential, although no publications demonstrate that they are effectively better than other EVs, such as hMSC-EVs. hPSC can be obtained from different sources (embryonic or reprogrammed from adult cells) and, despite showing some heterogeneity between lineages, they are highly similar in their main characteristics: They are pluripotent and with a high proliferative capacity. The latter makes it possible to obtain a large number of EVs. It should be noted that PSC-EV derived from different hPSC lineages may show some variability in their content. But considering the fact that we can isolate EVs from a single source (a homogenous culture), this can possibly bring less variability between batches compared to other common EV sources. However, studies in this area are still needed as current results are highly variable. Alternatives to EVs include the use of cell-engineered nanovesicles generated by serial extrusion of hiPSCs, as described by Lee *et al*[83], which presented similar results to PSC-EVs, but with higher production yield. However, more studies are needed to verify the viability of this method for future applications. Thus, challenges that remain are the large-scale production of EVs, which in the case of hPSC cultivation can be expensive, and the investment in efficient methodologies for EV isolation that could be used in good manufacturing practices for future acellular therapies.



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Figure 2 Diagram of pluripotent stem cell-derived extracellular vesicle isolation, its most common characterizations, and the applications described or indicated for these extracellular vesicles. EV: Extracellular vesicle; miRNA: MicroRNA; lncRNA: Long noncoding RNA; PSC: Pluripotent stem cell. The images were obtained from Servier Medical Art (http://smart.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License.

FOOTNOTES

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MINIREVIEWS

Single-cell RNA sequencing in cornea research: Insights into limbal stem cells and their niche regulation

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Abstract

The corneal epithelium is composed of stratified squamous epithelial cells on the outer surface of the eye, which acts as a protective barrier and is critical for clear and stable vision. Its continuous renewal or wound healing depends on the proliferation and differentiation of limbal stem cells (LSCs), a cell population that resides at the limbus in a highly regulated niche. Dysfunction of LSCs or their niche can cause limbal stem cell deficiency, a disease that is manifested by failed epithelial wound healing or even blindness. Nevertheless, compared to stem cells in other tissues, little is known about the LSCs and their niche. With the advent of single-cell RNA sequencing, our understanding of LSC characteristics and their microenvironment has grown considerably. In this review, we summarized the current findings from single-cell studies in the field of cornea research and focused on important advancements driven by this technology, including the heterogeneity of the LSC population, novel LSC markers and regulation of the LSC niche, which will provide a reference for clinical issues such as corneal epithelial wound healing, ocular surface reconstruction and interventions for related diseases.

Key Words: Cornea; Limbal stem cells; Single cell RNA sequencing; Heterogeneity; Novel markers; Niche regulation

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Core Tip: Limbal stem cells (LSCs), a cell population that resides at the limbus in a highly regulated niche. With the advent of single-cell RNA sequencing, our understanding of LSC characteristics and their microenvironment has grown considerably. This review focuses on the current research on single cell sequencing in LSCs. We highlight the heterogeneity, novel specific markers and niche regulation of LSCs.

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INTRODUCTION

The cornea is a unique transparent tissue in the human body exposed to the external environment and is the window for sight[1,2]. Specifically, the corneal epithelium acts as a protective barrier on the ocular surface and is constantly regenerating. This unique property of the corneal epithelium is dependent on self-renewing epithelial stem cells located at the limbus, known as limbal stem cells (LSCs)[3-5]. LSCs reside in the "palisades of Vogt" (also known as limbal epithelial crypts) and are critical for corneal epithelial regeneration and wound healing. LSCs respond to corneal epithelial cell renewal or wound healing by differentiating to produce limbal progenitor cells (LPCs) and transient amplifying cells (TACs), which then migrate to the central cornea to replenish the corneal epithelium[6-9]. This process was summarized as the XYZ hypothesis[10] and explained the balance of cell numbers and homeostasis in the corneal epithelium maintained by LSCs.

Like the stem cells in other tissues, the surrounding microenvironment or limbal niche strictly supports and regulates the functional behaviors of LSCs[11,12]. The limbal niche has unique characteristics and components, including mesenchymal cells, immune cells, melanocytes, vascular cells, extracellular matrix and signaling molecules (*e.g.*, growth factors and cytokines)[13-16]. Significant pathology involving any component of the limbal niche can lead to the dysfunction of LSCs or even result in limbal stem cell deficiency (LSCD), a disease that is characterized by impaired wound healing or blindness[17,18].

Various studies have identified numerous markers of LSCs but identifying definitive molecular signatures to distinguish LSCs and other corneal epithelial cells is still challenging. The unclear internal heterogeneity of the LSC population can increase the difficulty in efficiently isolating pure LSCs for clinical transplantation. In addition, emerging evidence supports that reconstruction of the limbal niche may be introduced to treat LSCD. Therefore, understanding the function and niche regulation of LSCs is needed to discover novel therapies for ocular surface disease.

With the development and maturity of sequencing technology, more and more genomic, transcriptomic, epigenetic and proteomic sequencing technologies have been applied to studying eye tissues [19-22]. In recent years, single-cell RNA sequencing (scRNA-Seq) technology has provided a powerful tool for discovery of new cell types and for dissecting their potential heterogeneity in unprecedented resolution[23-25]. For multicellular organisms, cell heterogeneity is defined by differences in genetic background, transcriptomic and proteomic profiles[26]. Compared to other traditional techniques for detecting the average expression of genes in multiple cells, single-cell sequencing can detect differential signals between individual cells, improve the resolution of research from the tissue to the cellular level [27-29]. A single-cell atlas has been compiled for several ocular tissues, such as the uvea[30], retina[31-34], iris[35,36], sclera[37,38] and human cornea[39,40]. In this review, we summarize the current advances on LSCs derived from single-cell studies to better understand the features and functions of LSCs and the precise cellular and molecular mechanisms of niche regulation. Overall, this review presents key points from recent discoveries to enrich our knowledge on LSC biology and ocular surface homeostasis reconstruction or other clinical problems.

HETEROGENEITY OF THE LSC POPULATION

LSCs are located in the basal layer of the corneal epithelium. As previously mentioned, they are characterized by a high proliferative potential, small size, high nucleoplasmic ratio and slow cell cycle[41,42]. LSCs are scarce, and finding bona-fide markers to distinguish them from other basal epithelial cells is challenging. In addition, few studies have investigated the heterogeneity and hierarchy of LSCs. Understanding the heterogeneity of LSCs is important for comprehending the function to effectively

isolate them for clinical transplantation.

Dou *et al*[43] performed scRNA-Seq on human limbal tissues and identified four subclusters of stem/progenitor cells after single-cell transcriptome analysis. In this study, the authors annotated eight cell types, including prominent limbal epithelial cells, stromal cells and other rare cell populations. The authors then subclustered limbal epithelial cells and resolved their heterogeneity, including limbal stem/progenitor cells (LSPCs), limbal suprabasal cells and limbal superficial cells. To further explore the LSC population, the authors then subclustered LSPCs and obtained four subpopulations (Figure 1) including: (1) A subpopulation with the classical LSC marker *TP63*[44]; (2) A subpopulation with high expression of *CCL20*, which is a chemokine that can induce cell migration and proliferation[45]; (3) A subpopulation with specific expression of *GPHA2*, a marker recently identified in quiescent LSCs (qLSCs) from humans and mice[46,47]; and (4) A subpopulation with high expression of *KRT6B*, which is associated with rapid keratinocyte division and contributes to inhibiting the migration of mitotic cell populations from the basal layer[48]. The authors then investigated the differences in stemness and differentiation status and observed that *TP63*$^+$ and *CCL20*$^+$ cells presented a high stemness state, whereas *GPHA2*$^+$ and *KRT6B*$^+$ showed a high differentiation state.

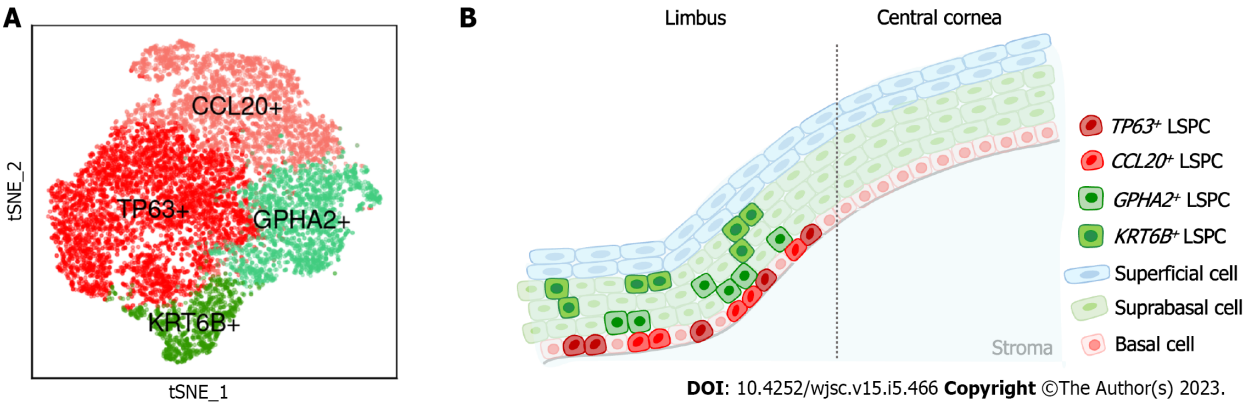
Another study by Li *et al*[49] annotated five subtypes from the limbal basal epithelium of the human cornea. They characterized terminally differentiated cells (TDCs), post-mitotic cells, TACs, LPCs and LSCs. Furthermore, the authors discovered that these five subtypes represented the major stages and trajectories of human LSC proliferation and differentiation (from LSCs, LPCs, TACs and post-mitotic cells to TDCs), and they were spatially situated in different regions from the limbus to the central cornea. In TDCs, corneal epithelium-specific differentiation markers and keratinocyte keratinization markers were expressed at the highest levels, while the LSC differentiation markers had the lowest expression.

LSCs in mice are also heterogeneous and behave differently than human LSCs. Altshuler *et al*[46] combined scRNA-Seq and quantitative lineage tracing for in-depth analysis of mouse limbal epithelium. The authors revealed the presence of two distinct subpopulations of mouse LSCs that were in separate and well-defined spatial locations called the "inner" and "outer" limbus (Figure 2). The inner limbus contains active LSCs, which maintain the homeostasis of the corneal epithelium. The outer limbus contains qLSCs that have a significantly lower rate of division and are involved in wound healing and border formation. Spectral tracking experiments displayed that qLSCs can quickly exit the dormant state and enter the cell cycle in response to injury, suggesting that qLSCs are a reservoir for tissue regeneration. In addition, their circumferentially extended clonal growth model and continuous localization on the border highly indicates that these cells play a crucial role in border maintenance. Notably, this finding was also confirmed by a study utilizing the two-photon live imaging approach [50]. Collectively, LSCs are highly heterogeneous in both humans and mice, unlike stem cells in other tissues. Further studies are needed to investigate the self-renewal and differentiation mechanisms of LSCs.

NOVEL MARKERS FOR LSCS

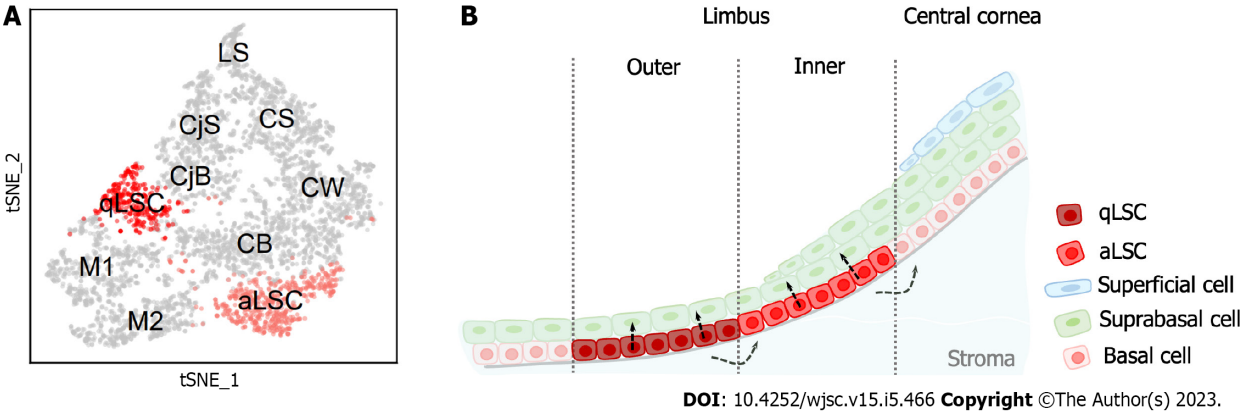
Since 1989, when LSCs were discovered[4], a series of markers have been found to identify these cells, such as *TP63, KRT3, KRT12*. However, the marker pattern typically labels the broad limbal basal cell population. Accurately distinguishing LSCs from other epithelial cells is still challenging and is still an active area of research. Altshuler *et al*[46] discovered a novel set of markers to accurately identify LSCs. They applied *in situ* hybridization probes for *Krt4* and *Krt12* to label mouse conjunctival and corneal basal and suprabasal cells, respectively. *Gpha2* staining could obviously demarcate the outer LSCs (also known as qLSCs), while the inner LSCs (also known as active LSCs) were labeled as *Atf3*$^+$. Then, they used immunofluorescence staining to confirm that the outer limbal epithelial basal cells were *Krt15*$^+$/*Ifitm3*$^+$/*Cd63*$^+$, and the inner limbal epithelial basal cells were *Atf3*$^+$/*Mt1-2*$^+$. Next, the authors explored the correlation between mouse and human LSC markers. Immunofluorescence images revealed that *KRT15*, *IFITM3* and *GPHA2* were expressed in human limbus epithelial basal cells. *Ifitm3* was found to be restricted to cellular vesicles in the cytoplasm of undifferentiated limbal cells, which was consistent with a previous study's findings[51]. *Ifitm3* knockdown led to a differentiation phenotype and a reduced colony-forming capacity. These experiments suggest that *Ifitm3* and *Gpha2* can be used to identify LSCs, and *Ifitm3* mediates the undifferentiated state.

Gpha2 has been frequently studied in human LSCs. Dou *et al*[43] explored the four subclusters of LSPCs, which were identified by *TP63*, *CCL20*, *GPHA2* and *KRT6B*. Collin *et al*[47] identified several novel genes, one of which was *GPHA2*, using an unbiased approach to recognize marker genes that were highly expressed in human LSCs relative to other corneal epithelial cells. High and specific expression of *GPHA2* was observed in the limbus crypts, which was consistent with the findings of Altshuler *et al*[46]. Moreover, the authors used RNA interference (RNAi) to downregulate *GPHA2* and observed a significant reduction in cell proliferation and differentiation efficiency, indicating an important role of GPHA2 in maintaining the undifferentiated state of human LSCs. The authors also performed flow activated cell sorting analysis with colony forming efficiency assays to confirm the



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Figure 1 Heterogeneity of limbal stem cells in humans. A: The *t*-distributed stochastic neighbor embedding plot of four subpopulations of limbal stem cells; B: Schematic diagram of the heterogeneous limbal stem cells in the human limbus. LSPC: Limbal stem/progenitor cell.



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Figure 2 Heterogeneity of limbal stem cells in mice. A: The *t*-distributed stochastic neighbor embedding plot of the corneal epithelial cell subpopulations in the mouse limbus. The limbal stem cells are highlighted in red; B: Schematic diagram of the heterogenous limbal stem cells in the mouse limbus. aLSC: Active limbal stem cell; CB: Corneal basal cell; CjB: Conjunctival basal cell; CjS: Conjunctival suprabasal cell; CS: Corneal superficial cell; CW: Corneal wing cell; LS: Limbal superficial cell; M1/M2: Cells in mitosis; qLSC: Quiescent limbal stem cell.

RNAi data.

Other LSC markers have also been identified. Li *et al*[49] identified *TSPAN7*$^+$ and *SOX17*$^+$ cells distributed in a scattered pattern in human limbus epithelium basal cells. The authors established an *in vitro* model of epithelial cells and discovered *TSPAN7* and *SOX17* were not strongly expressed in human limbal epithelial cells. However, mRNA and protein expression levels were significantly activated after injury, especially during cell migration and growth. The authors also utilized RNAi to downregulate *TSPAN7* and *SOX17* and observed inhibited cell proliferation and significantly delayed epithelial regeneration during wound healing. Overall, the discovery of novel markers of LSCs (Table 1) can help us to better distinguish LSCs from other cells to further understand the function and state of LSCs and provide a more effective strategy for the isolation, culture and clinical application of LSCs.

NICHE REGULATION OF THE LSCS AT THE LIMBUS

LSC proliferation, migration and differentiation are inseparable from the regulation of the limbal niche microenvironment. The stem cell niche is the local microenvironment directly promoting or protecting stem cell populations[52-54]. The LSC niche provides a sheltered environment that protects LSCs from stimulation[55-58]. If the LSC niche is involved in pathological damage, then LSC dysfunction can occur. Therefore, the study of the LSC niche is essential.

Collin *et al*[47] investigated the interaction between LSCs and the limbal niche by single-cell analysis. The authors combined scRNA-Seq and ATAC-Seq and performed CellPhoneDB analysis[59]. They identified multiple significant interactions between human LSCs and regulatory factors of immune cells such as proinflammatory cytokines [tumor necrosis factor, interleukin (IL)-1β, IL-6, IL-17A, interferon γ, and oncostatin M], proinflammatory cell surface receptor (triggering receptor expressed on myeloid cells 1), proinflammatory cytokine expression (adaptor complexes 1) and regulators of inflammatory

Table 1 Novel limbal stem cell markers identified by single-cell RNA sequencing

LSC subtype	Marker	Species	Ref.
LSPC with high stemness	*TP63, CCL20*	Human	[43]
LSPC with high differentiation	*GPHA2, KRT6B*	Human	
LSC	*TSPAN7, SOX17, SELE, ECSCR, RAMP3, RNASE1, NPCD1, NNMT, SLC2A3, KLF2, PDK4*	Human	[49]
Limbal progenitor cell	*DCN, PLIN2, DEGS1, MMP10, IFITM3, SLC6A6, LTB4R, SLP1*	Human	
qLSC	*Gpha2, Cd63, Ifitm3*	Mouse	[46]
aLSC	*Atf3, Socs3, Mt1, Prdm1*	Mouse	

aLSC: Active limbal stem cell; LSC: Limbal stem cell; LSPC: Limbal stem/progenitor cell; qLSC: Quiescent limbal stem cell.

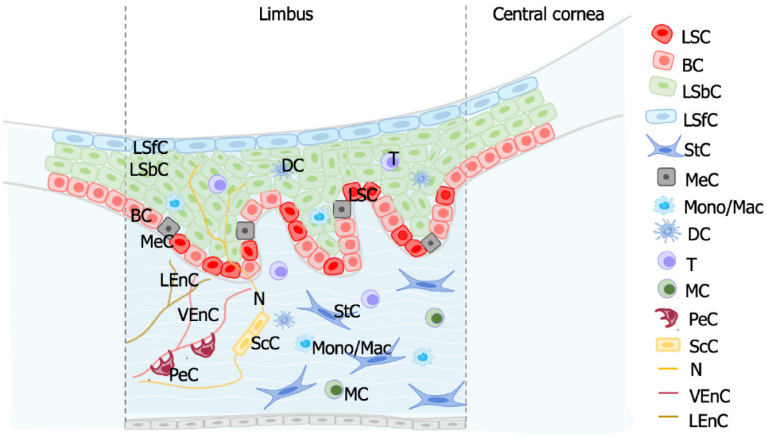
responses (nuclear factor kappa B, RELA, colony-stimulating factor 2, phosphoinositide 3-kinase, extracellular signal-regulated kinase 1/2, and F2). The authors verified that limbal epithelial cells were significantly reduced in cell culture medium containing tumor necrosis factor-α and IL-1β. This suggested that proinflammatory cytokines produced by immune cells were involved in the apoptosis of limbal epithelial cells[60], thus mimicking the central corneal defect and stimulating the proliferation of LSCs[61]. This was also consistent with other reports showing that the addition of proinflammatory factors to limbal epithelial cell cultures can directly affect the expression of LSC markers and their colony forming efficiency capacity[60,62-64].

Dou *et al*[43] systematically explored intercellular communication between LSPCs and other cell populations based on ligand-receptor analysis. By correlating the corresponding receptor-ligands in human LSPCs and their niche cells, the authors observed that LSPCs were regulated by the limbal niche as well as by other cells in the limbal niche. The Notch signaling pathway was also involved in cell-cell interaction between LSPCs and their niche cells. NOTCH1-4 receptors were expressed in LSPCs, and their relevant ligands were primarily identified in niche cells, such as Schwann cells, stromal cells, pericytes and LSPCs. Likewise, the WNT7A, WNT7B and WNT5A ligands, which participate in the Wnt/β-catenin signaling pathway, were detected on LSPCs. Their corresponding receptors were primarily detected on limbal epithelial cells, stromal cells, immune cells, Schwann cells and LSPCs. The presence of multiple chemokines, such as CCL4, CCL4L2, IL-1β and IL-24, on LSPCs and their paired receptors indicated that immune cell interactions may potentially regulate LSPCs.

Altshuler *et al*[46] revealed that T cells acted as niche cells and served its function in the maintenance of quiescence, epithelial thickness control and wound healing. By studying the limbus of the severe combined immunodeficiency (SCID) and non-obese diabetic SCID mice, which are unable to make mature T and B lymphocytes, it was observed that the GPHA2 protein was substantially decreased to almost undetectable levels. In contrast, the expression of *Ifitm3* did not rely on the existence of immune cells, implying that it was regulated by other niche cells. When T cells were inhibited by topical application of the corticosteroid dexamethasone, LSCs showed a dramatic reduction in *Cd63* and *Gpha2* expression levels and increased cell proliferation, demonstrating that T cells played a crucial role in regulating qLSCs. Finally, corneal epithelial debridement followed by epithelial closure by fluorescein dye infiltration revealed delayed epithelial wound healing in mice lacking T cells.

In addition, other niche cells were determined to be important for the microenvironment regulation of LSCs. Oxidative stress can lead to a variety of eye diseases, such as keratitis, cataracts and retinal diseases, which are subject to varying degrees of oxidative damage[65,66]. Recently, studies found that melanocytes in the limbal niche (as antioxidant systems) protected LSPCs from UV-induced oxidative damage and reduced oxidative stress through the transfer of melanosomes[67,68]. Moreover, by ligand analysis, Dou *et al*[43] identified the intercellular communications between melanocytes and LSCs. NAMPT, as a ligand, was highly expressed in melanocytes and had been reported to act as a critical switch in melanoma cells. CD44 acted as a receptor and was also highly enriched in melanocytes.

Vascular endothelial cells are also one of the important niche cells of LSCs. It has been reported that vascular endothelial cells were highly correlated with the classic Wnt signaling pathway involved in the regulation of the corneal limbal niche[69,70]. Furthermore, Dou *et al*[43] performed a differential expression analysis with the integration of the scRNA-Seq datasets from the limbus and the skin and observed that the vascular endothelial cells from the limbus highly expressed anti-vascular factors compared to that from the skin, consistent with characteristics of corneal angiogenic privilege. Above all, these studies have shown that the regulation of the LSC niche (Figure 3) occupies a key role in the growth, development, proliferation and differentiation of LSCs.



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Figure 3 Structure and cellular compositions in the limbal stem cell environment. Niche cells regulate limbal stem cells. LSCs: Limbal stem cells; BC: Basal cell; DC: Dendritic cell; LEnC, Lymphatic endothelial cell; LSbC: Limbal suprabasal cell; LSfC: Limbal superficial cell; MC: Mast cell; MeC: Melanocyte; Mono/Mac: Monocyte/macrophage; N: Nerve; PeC: Peripheral cell; ScC: Schwann cell; StC: Stromal cell; T: T cell; VEnC: Vascular endothelial cell.

CONCLUSION

The first Drop-Seq experiments were performed on mouse retina in 2015[23]. Since this revolutionary experiment, single-cell sequencing technology has been widely used in many fields, including ophthalmology, and gene expression has been studied at an unprecedented resolution in multiple ocular tissues. Corneal transparency is essential for normal vision; thus, comprehension of the mechanisms related to corneal wound healing and regeneration is crucial for the treatment of patients suffering from corneal disease. Currently, corneal epithelial regeneration is a relatively satisfactory approach and has the potential to treat corneal superficial scars. However, for multiple corneal basal scars or endothelial disease, corneal transplantation remains the only option to restore clear vision[71-73]. Unfortunately, corneal clouding remains one of the leading causes of blindness worldwide due to the lack of corneal donor tissue or the limited availability of corneal surgery[74,75]. Although most studies support corneal regeneration through LSC therapies[76,77], the study of LSCs is particularly important.

This review focused on the current research on single-cell sequencing in LSCs. We highlighted the heterogeneity of LSCs and presented several novel specific markers of LSCs and the role of niche regulation of LSCs. LSCs can be identified in both humans and mice, and several markers, such as *GHPA2* and *IFITM3*, can be highly and specifically expressed on LSCs. Moreover, both T cell regulation in mice studied by Altshuler *et al*[46] and immune cell regulation in humans studied by Collin *et al*[47] and Dou *et al*[43] suggest that niche regulation is of vital importance for LSCs.

Future research can still benefit from RNA-Seq technology as it can aid in acquisition of further knowledge on the functions and characteristics of LSCs, including in the discovery of more novel highly specific expression markers and more niche regulated components that can promote or inhibit the proliferation and differentiation of LSCs. These discoveries should be translated into better prevention and treatment strategies to treat blindness and improve the clinical prognosis of patients with LSCD and other LSC-related diseases.

FOOTNOTES

Author contributions: Dou SQ and Shi WY designed the report; Sun D collected the data and wrote the paper; Dou SQ and Shi WY reviewed and edited the manuscript; and all authors discussed the study's results and provided important intellectual comments on the manuscript.

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ORIGINAL ARTICLE

Basic Study

Exosomes from circ-Astn1-modified adipose-derived mesenchymal stem cells enhance wound healing through miR-138-5p/SIRT1/FOXO1 axis regulation

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Abstract

BACKGROUND

Wound healing impairment is a dysfunction induced by hyperglycemia and its effect on endothelial precursor cells (EPCs) in type 2 diabetes mellitus. There is increasing evidence showing that exosomes (Exos) derived from adipose-derived mesenchymal stem cells (ADSCs) exhibit the potential to improve endothelial cell function along with wound healing. However, the potential therapeutic mechanism by which ADSC Exos contribute to wound healing in diabetic mice remains unclear.

AIM

To reveal the potential therapeutic mechanism of ADSC Exos in wound healing in diabetic mice.

METHODS

Exos from ADSCs and fibroblasts were used for high-throughput RNA sequencing (RNA-Seq). ADSC-Exo-mediated healing of full-thickness skin wounds in a diabetic mouse model was investigated. We employed EPCs to investigate the therapeutic function of Exos in cell damage and dysfunction caused by high glucose (HG). We utilized a luciferase reporter (LR) assay to analyze interactions among circular RNA astrotactin 1 (circ-Astn1), sirtuin (SIRT) and miR-138-5p. A diabetic mouse model was used to verify the therapeutic effect of circ-Astn1 on Exo-mediated wound healing.

RESULTS

High-throughput RNA-Seq analysis showed that circ-Astn1 expression was increased in ADSC Exos compared with Exos from fibroblasts. Exos containing high concentrations of circ-Astn1 had enhanced therapeutic effects in restoring

EPC function under HG conditions by promoting SIRT1 expression. Circ-Astn1 expression enhanced SIRT1 expression through miR-138-5p adsorption, which was validated by the LR assay along with bioinformatics analyses. Exos containing high concentrations of circ-Astn1 had better therapeutic effects on wound healing *in vivo* compared to wild-type ADSC Exos. Immunofluorescence and immunohistochemical investigations suggested that circ-Astn1 enhanced angiopoiesis through Exo treatment of wounded skin as well as by suppressing apoptosis through promotion of SIRT1 and decreased forkhead box O1 expression.

CONCLUSION

Circ-Astn1 promotes the therapeutic effect of ADSC-Exos and thus improves wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as a potential therapeutic option for the treatment of diabetic ulcers.

Key Words: Adipose-derived mesenchymal stem cells; Circular RNA astrotactin 1; Diabetic; Exosomes; Angiogenesis

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Core Tip: Circular RNA astrotactin 1 (circ-Astn1) promoted the therapeutic effect of adipose-derived mesenchymal stem cells-exosomes and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as a potential therapeutic alternative for diabetic ulcers.

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INTRODUCTION

Diabetes affects 30 million children as well as adults in the United States, *i.e.* one out of every eleven people in the United States, which leads to $327 billion costs each year. Consequently, it is important to develop a new method of diabetes treatment. Interventions that improve healing rates and decrease diabetic ulcer size could lower the infection incidence, amputation rate, and care cost[1]. Diabetic foot (DF) is a severe complication of type 2 diabetes mellitus (T2D). DF infection is the main reason for DF development and deterioration, and controlling infection plays an important role in disease treatment. Previous studies have found that diabetes is associated with hyperglycemia, one of the most important causes of oxidative stress. Endogenous antioxidants are able to destroy the reactive species and create a balance between antioxidants and free radicals[2,3]. The impaired function and senescence of endothelial progenitor cells (EPCs) and high glucose (HG)-induced reactive oxygen species likely exacerbate DFs[4].

Accumulated evidence shows that mesenchymal stem cell (MSC)transplantation promotes angiogenesis and accelerates diabetic wound healing[5,6]. Adipose-derived mesenchymal stem cells (ADSCs) therapy provides potentially new therapeutic options to improve diabetic wound healing[7], and autologous stem cell transplantation reduces the cost of drug development, which in turn reduces financial costs. However, the mechanism is not clear.

Stem cells live in niches, which are complicated microenvironments that exert important functions in directing the division, differentiation, and activity of stem cells. However the direction of differentiation is affected by hypoxia, cytokines, trophic factors, chemical and pharmacological agents, and physical factors[8]. Considering the safety of *in vivo* transplantation, some investigations have suggested that exosomes (Exos) from ADSCs play a similar functional role to ADSCs in promoting diabetic wound healing. Exos are tiny endosomal membrane-bound vesicles, 50–200 nm in length, that have a variety of contents including protein and nucleic acids which vary with cell or tissue origin. They play their full role by fusing with selected cells and releasing their cargo that could contain bioactive molecules including lipids, proteins, non-coding-RNA (ncRNA)[9-11] and mRNAs. Previous studies have found that Exos can regulate the epithelial–mesenchymal transition and disease progression in different cancers[12,13]. Exos secreted from ADSCs attenuate diabetic nephropathy by promoting autophagy flux

and by inhibiting apoptosis in podocytes[14]. Exos from nuclear factor erythroid 2–related factor 2-overexpressing ADSCs accelerate cutaneous wound healing by promoting vascularization in a DF ulcer [4]. Exos from linc00511-overexpressing ADSCs accelerate angiogenesis in healing DF ulcers by suppressing progestin and adipoQ receptor family member 3-induced Twist1 degradation[15]. However, it remains largely unknown if Exos from ncRNA-modified ADSCs can improve wound healing.

The ncRNAs include circular RNA (circRNA), long non-coding RNA (lncRNA), and microRNA (miRNA). circRNA activity is indispensable during the regulation of gene expression, demonstrating that circRNAs function not only as candidate therapeutic agents but also as diagnostic markers. circRNA 5′ and 3′ extremities are linked to form an integrated circular structure, which makes circRNAs more resistant to RNA exonuclease degradation. as well as more stable 8than linear RNAs[16,17]. A previous study found that circRNAs possess activity and potential clinical benefits in skin wound healing[18].

To identify relevant circRNAs as therapeutic targets, we used high-throughput sequencing detection to identify the function of mmu_circ_0000101 (circ-Astn1), which acts as the key factor in delivery by ADSC Exos. Exos from circ-Astn1-modified ADSCs improve wound repair in diabetic rats through miR-138-5p/SIRT1 pathway regulation. The present study verified the effect of treatment with Exos from circ-Astn1-overexpressing ADSCs on HG-induced EPC dysfunction. The abundance and simple methods of sampling of ADSC-Exos make it safer in terms of trauma and other adverse reactions.

MATERIALS AND METHODS

Ethics statement

The Animal Care and Use Committee of Peking Union Medical College Hospital approved the investigation protocol (No: XHDW-2020-01; Beijing, China). We carried out all postoperative animal care along with surgical interventions following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All surgeries and euthanasia were performed under sodium pentobarbital anesthesia (30 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering.

High-throughput and strand-specific RNA sequencing library construction

Total RNA from ADSCs and fibroblast Exos was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Our team prepared about 3 μg total RNA *per* sample using the VAHTS Total RNA-seq (H/M/R) Library Prep Kit from Illumina (Vazyme Biotech Co., Ltd., Nanjing, China) to isolate the ribosomal RNA and remove other RNAs such as ncRNA and mRNA. We then performed RNA purification using RNase R (Epicenter, 40 U, 37 °C for 3 h) followed by TRIzol. An RNA sequencing RNA-Seq) library was prepared using the KAPA Stranded RNA-Seq Library Prep Kit (Roche, Basel, Switzerland) and they were exposed in order following extensive codifying with Illumina HiSeq 4000 from Aksomics, Inc. (Shanghai, China).

Cell treatment

To investigate endothelial precursor cell (EPC) dysfunction as well as apoptosis, we cultivated EPCs at 37 °C with 5% carbon dioxide in EPC medium (Gibco, Carlsbad, CA, United States) and processed them after 1 d using 5.5 or 30 mmol/L glucose. We harvested EPCs for detection of apoptosis as well as to test their response to Exo therapy. In order to study the protective function of Exos on EPCs, we added 100 μg/mL Exos to cultures following 80% EPC fusion to evaluate the protective function against damage caused by prior HG treatment with various glucose concentrations.

ADSC isolation and identification

We isolated ADSCs from adipose tissue following the method used in a previous study[4]. We observed no uninduced differentiation in cultural expansion. We induced osteogenic differentiation *via* a 3-wk culture of ADSCs in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 mmol/L β-glycerophosphate. We induced adipogenic differentiation through culturing ADSCs for 2 weeks in DMEM supplemented with 10% FBS, 10 μM insulin, 0.5 mmol/L isobutylmethylxanthine, 200 μM indomethacin, and 1 μM dexamethasone. We also investigated the osteogenic or adipogenic differentiation of ADSCs through Oil-Red O and alkaline phosphatase staining.

Identification and isolation of ADSC-derived Exos

We isolated ADSC-derived Exos when cells reached 80%-90% confluence. Our team rinsed ADSCs from various groups with phosphate-buffered saline (PBS), and then cultured them in FBS-free endothelial cell growth medium (EGM)-2MV, which was supplemented with 1 × serum replacement solution (PeproTech, Rocky Hill, NJ, United States) for another 2 d. Then, we collected conditioned culture medium and centrifuged it at 300 × *g* for 10 min to remove cells and at 2000 × *g* for another 10 min to

remove apoptotic cells and cellular debris. Following centrifugation at 10000 × *g* for 30 min, we filtered the supernatant through a 0.22 μm filter (Millipore, Billerica, MA, United States), then transferred 15 mL supernatant to the Amicon Ultra-15 Centrifugal Filter Unit (100 kDa) and centrifuged it at 4000 × *g* to concentrate to approximately 1 mL. The ultrafiltration unit was washed twice with PBS centrifuged it again at 100000 × *g*, and the supernatant was aspirated. All processes were conducted at 4 °C. We resuspended the Exo pellets obtained in 500 μL PBS. Finally, the Exo protein content was evaluated using the Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). We stored Exos at -80 °C until subsequent use for experiments and identified Exos by western blotting and transmission electron microscopy.

Diabetic wound induction

We utilized Balb/c mice and induced diabetes through a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 4.5). Three days after STZ administration, we confirmed diabetes development by measuring fasting blood glucose levels in blood samples obtained from the tail vein. We considered a mouse with fasting blood glucose levels > 250 mg/dL diabetic, which we maintained for 1 mo and employed for subsequent analyses of posterior blood glucose stabilization. Following diabetes validation, we anesthetized mice through intramuscular injection with ketamine hydrochloride and xylazine cocktail at 80 and 10 mg/kg, respectively. Once anesthesia was established, hair was shaved from the dorsal leg area and the region was sterilized using povidone iodine solution. A sterile biopsy punch was used to generate a full-thickness 4 mm excisional wound. Then we allocated mice randomly to subcutaneous injection with 100 μL PBS containing 200 μg ADSC Exos or equivalent amount of PBS without Exos at four sites near the wound (25 μL/site). We euthanized mice and harvested skin specimens for histopathological validation.

RNA overexpression or interference

RNA overexpression or interference was induced by transfection of miR-138-5p mimics or inhibitor, circ-Astn1 and SIRT1 overexpression vector, and siRNA against circ-Astn1 (si-circ-Astn1) obtained from RiboBio (Guangzhou, China). Our team performed transfection using Lipofectamine 2000 (Thermo Fisher Scientific) following a method previously described[19].

Quantitative polymerase chain reaction

We isolated total RNA from skin tissue or cells from wounds using a TRIzol reagent kit. Our team synthesized cDNA to amplify with TaqMan miRNA Reverse Transcription Kit. Our team then performed quantitative polymerase chain reaction (qPCR) using a TaqMan Human miRNA Assay Kit, using the $2^{-\Delta\Delta CT}$ approach to detect fold changes with respect to expression. We used *U6* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal references. Primers utilized were: Circ-Astn1, F: 5′-CTGGACCCTTGTGAACACCAATG-3′, R, 5′-GGATCATCACCAGGCACAAGATG-3′; FOXO1, F: 5′-AAGGCCATCGAGAGCTCAGC-3′, R, 5′-GATTTTCCGCTCTTGCCTCC-3′; miR-138-5p, F: 5′-GCTGGTGTTGTGAATCAG-3′, R: 5′-GAACATGTCTGCGTATCTC-3; U6, F: 5′-AGTAAGCCCTTGCTGTCAGTG-3′, R: 5′-CCTGGGTCTGATAATGCTGGG-3′; GAPDH: F: 5′-GTCTCCTCTGACTTCAACAGCG-3′, R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′, and were designed by Gene Pharma (Shanghai, China).

Apoptosis detection

To assess apoptosis, we collected cells into centrifuge tubes and centrifuged them at 1000 rpm for 5 min. We resuspended cells in PBS at 4 °C and removed the supernatant following centrifugation. We resuspended the cell pellet at 1–5 × 10^6/mL in 1 × binding buffer, then 100 μL cell suspension was mixed with 5 μL Annexin V/fluorescein isothiocyanate in the dark at room temperature for 5 min. Lastly, we added 10 μL propidium iodide (PI) and 400 μL PBS to stain the cells. We analyzed data using the FlowJo package.

Immunofluorescence and immunohistochemical assays

We fixed skin tissue samples in 10% formalin solution, embedded them in paraffin, and sectioned them at 5 μm. Our team stained tissue sections with hematoxylin and eosin (HE) for histological detection, and cluster of differentiation 31 (CD31) immunofluorescence staining was used to detect histopathological changes associated with angiogenesis. We performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to identify apoptotic cells. Our team visualized sections using fluorescence (Nikon, Tokyo, Japan) or light microscope (Zeiss, Oberkochen, Germany), and photographed them using a digital camera.

Western blot analysis

Skin tissues were lysed, and lysates were centrifuged at 12000 rpm at 4 °C following addition of a protease inhibitor. The protein concentration was determined using the Pierce BCA kit (Thermo Fisher). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to PVDF membranes. The primary antibodies used to assay protein expression were SIRT1

(1:600), forkhead box O1 (FOXO1) (1:600) (all from Santa Cruz Biotechnology, Santa Cruz, CA, United States), and anti-GAPDH (1:1000; Sigma-Aldrich, St. Louis, MO, United States), followed by a horseradish peroxidase-conjugated secondary antibody (1:1000; Abcam, Cambridge, MA, United States). An enhanced chemiluminescence kit (Millipore, Burlington, MA, United States) was used to read the bands.

Luciferase reporter assay

We created and cloned wild-type (WT) and 3'-UTR mutant (MUT) *SIRT1*, as well as WT and MUT *circ-Astn1* into pMIR firefly luciferase-expressing vectors. We co-transfected the vectors into HEK293T cells once they reached 70% confluence, using 500 ng pMIR-SIRT1-wt/pMIR-SIRT1-Mut or pMIR-*circ-Astn1*-wt/pMIR-*circ-Astn1*-Mut combined with 50 nM miR-138-5p mimics using a Lipofectamine 2000 Transfection Kit for the luciferase assay. We assayed luciferase activity using a Dual-Luciferase Reporter System (Promega, Madison, WI, United States). We performed five independent assays.

Tube formation assay

We performed an EPC tube formation assay using Matrigel (BD Biosciences, Franklin Lakes, NJ, United States). Matrigel solution was mixed with ECMatrix diluent buffer then spread on μ-Slide plates and incubated at 37 °C for 1 h for the matrix solution to solidify. Next, we added various treatments to the EPCs (2×10^4 cells/well) to wells containing solid matrix and cultured them with EGM-2 medium at 37 °C for a period of 12 h. Our team detected tube formation under an inverted light microscope (100 ×) and evaluated three independent representative fields from each well to determine mean tube number.

Cell Counting Kit (CCK)-8 assay

EPC proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) (BD Biosciences). Our team cultivated transfected cells in 96-well plates with Exos in HG conditions for 1 d in wells to which 10 μL CCK-8 reagent and 90 μL fresh culture medium was previously added. Absorbance was detected at 450 nm using a microplate reader following incubation at 37 °C for 2 h.

Statistical analyses

We denoted continuous parameters by the mean ± SD and employed one-way variance of analysis (ANOVA) to compare data using GraphPad Prism (GraphPad, La Jolla, CA, United States). $P \leq 0.05$ indicated a statistically significant difference.

RESULTS

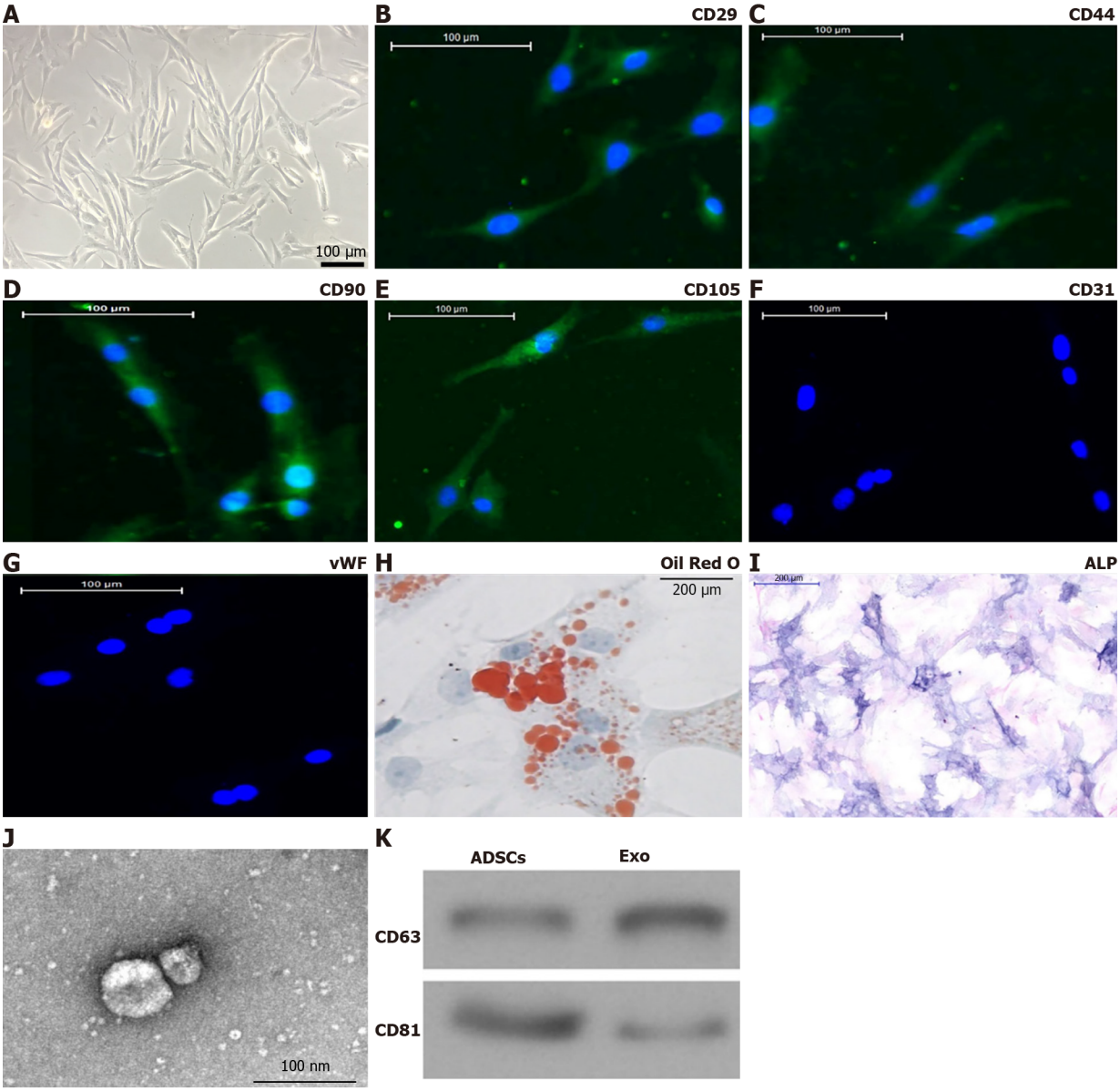
ADSC and Exo characterization

Isolated ADSCs have classical cobblestone-like morphology (Figure 1A). Immunofluorescence staining showed that ADSCs from mouse adipose tissue samples were positive for expression of the mesenchymal cell surface markers CD29 (Figure 1B), CD44 (Figure 1C), CD90 (Figure 1D), and CD105 (Figure 1E), but negative for expression of the endothelial cell marker CD31 (Figure 1F) as well as von Willebrand Factor (Figure 1G). The results of Oil Red O staining (Figure 1H) together with alkaline phosphatase staining (Figure 1I) verified that isolated ADSCs possessed both osteoblastic and adipocytic differentiation capacity. We concluded that ADSCs have the potential for multidirectional differentiation[20].

Exos were isolated by ultra-high-speed centrifugation. Transmission electron microscopy revealed that ADSC Exos had spherical or cup-shaped morphology with a diameter ranging from 50 to 120 nm (Figure 1J) as previously reported[21]. Western blotting suggested that ADSC Exos were positive for the Exo markers CD81 and CD63, which are cellular components (Figure 1K).

Exos derived from circ-Astn1-modified ADSCs play important roles in the restoration of EPC function by decreasing apoptosis under HG conditions

To determine the role of circRNAs in ADSC Exo-mediated restoration of EPC function under HG conditions, circRNA expression in ADSCs and fibroblast Exos was explored by RNA-Seq. The results verified that the contents of mmu_circ_0000101, mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 were all significantly upregulated in ADSC Exos compared with fibroblast Exos (Figure 2A). RT-qPCR analysis confirmed that mmu_circ_0000101, mmu_circ_0008040, mmu_circ_0008061 and mmu_circ_0008099 expression in EPCs decreased after exposure to HG conditions (Figure 2B), with expression of mmu_circ_0000101 in particular decreasing most significantly. Consequently, mmu_circ_0000101 was selected for subsequent study. Mmu_circ_0000101 originated from *Astn1* gene exon 5, so mmu_circ_0000101 was also known as circ-Astn1. The entire mature spliced sequence length was 967 bp. The gene is on chromosome 1: 160432178-160441253 (Figure 2C).



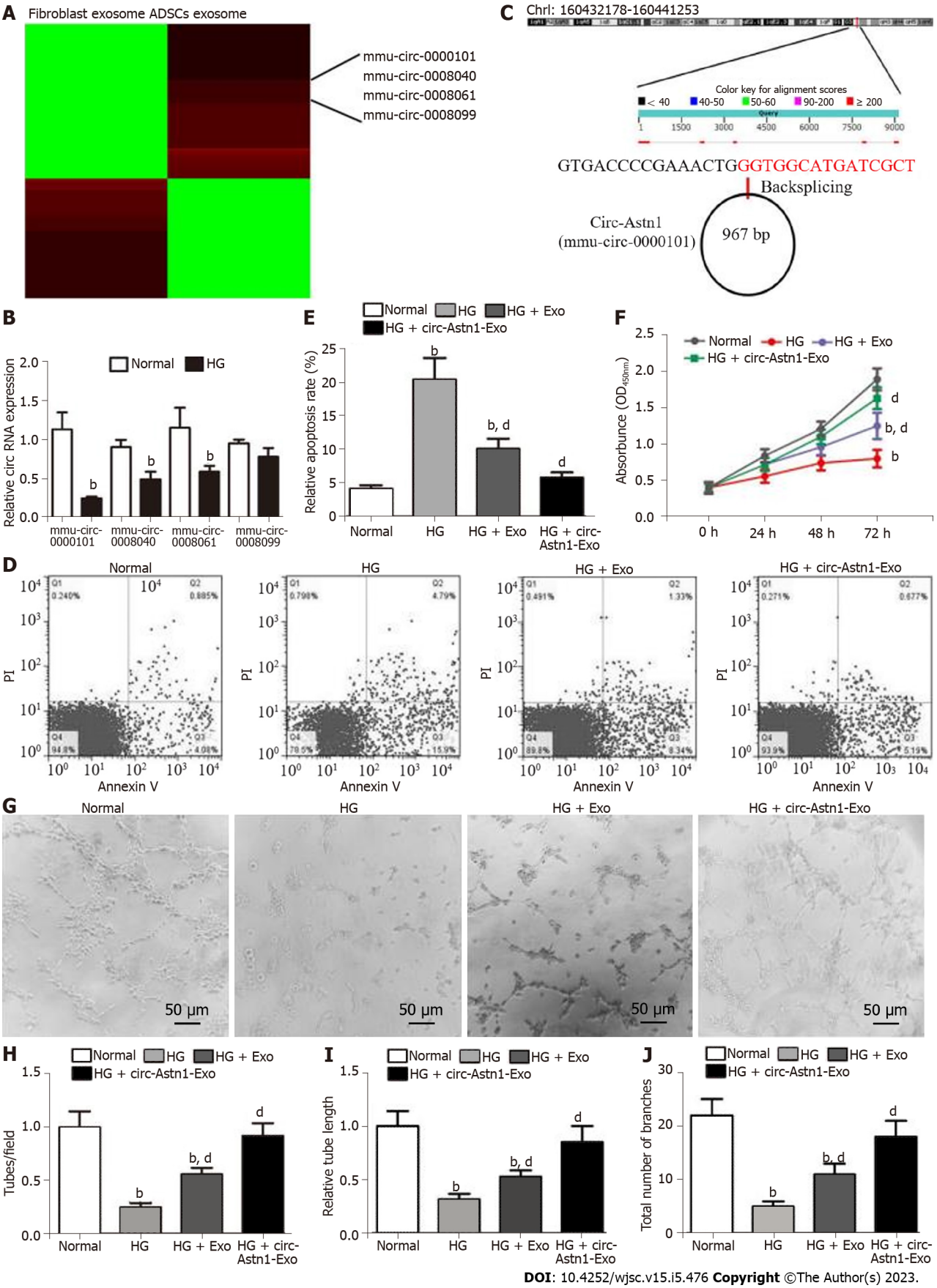
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Figure 1 Characterization of adipose-derived mesenchymal stem cells and exosomes. A: Adipose-derived mesenchymal stem cells (ADSCs) showed a typical cobblestone-like morphology. Scale bar: 100 μm; B–G: Immunofluorescence staining of cell surface markers. ADSCs exhibited positive expression of cluster of differentiation 90 (CD90), CD29, CD44, and CD105, but not von Willebrand factor or CD34. Scale bar: 100 μm; H and I: The differentiation potential of ADSCs assessed by Oil Red O (H) and alkaline phosphatase (I) staining. Scale bar: 200 μm; J: Transmission electron micrographs demonstrated ADSC exosome morphology. Scale bar: 100 nm; K: Western blotting detection of CD81 and CD63 expression in exosomes and ADSCs.

Flow cytometry investigations have shown that HG (30 mmol/L glucose) treatment promotes EPC apoptosis. Treatment with Exos from WT ADSCs suppressed HG-induced EPC apoptosis, and treatment with Exos from ADSCs overexpressing circ-Astn1 had a more significant effect in suppressing HG-induced apoptosis of EPCs than Exos from WT ADSCs (Figure 2D and E), suggesting that circ-Astn1 played an important role in ADSC-Exo-mediated EPC protection under HG conditions. CCK8 detection confirmed that treatment with Exos containing high levels of circ-Astn1 had a greater effect in restoring the proliferative ability of EPCs under HG conditions (Figure 2F). We used tubule formation by EPCs in Matrigel-coated culture wells as an *in vitro* angiogenesis model, and evaluated their potential by counting the branch numbers formed. HG conditions suppressed angiogenesis, and treatment with Exos containing high levels of circ-Astn1 was more effective in promoting angiogenesis of EPCs under HG conditions (Figure 2G-J).

The circ-Astn1-mediated miR-138-5p/SIRT1/FOXO1 signaling pathway protects EPCs under HG conditions by promoting angiogenesis

Bioinformatics data showed that circ-Astn1 regulates SIRT1 expression *via* inhibition of miR-138-5p. SIRT1 functions critically in promoting angiogenesis by activating the FOXO1 signaling pathway[22]. To



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Figure 2 Exosomes derived from circular RNA astrotactin 1-modified adipose-derived mesenchymal stem cells function importantly in endothelial precursor cell function restoration by decreasing apoptosis under high glucose conditions. A: Heat map regarding all differentially expressed circular RNAs (circRNAs) between adipose-derived mesenchymal stem cells (ADSCs) exosomes and fibroblast exosomes; B: Quantitative polymerase chain reaction giving mmu_circ_0000101 (circular RNA astrotactin 1), mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 expression in endothelial

precursor cells (EPCs) with or without high glucose (HG) treatment. Data are denoted by the mean ± SD; $^{b}P < 0.001$ *vs* normal; C: The genomic loci of circ-Astn1; D and E: We pretreated EPCs with ADSC exosomes before treatment with exosomes for 1 d under HG conditions. Our team assayed EPC apoptosis *via* flow cytometry after annexin V-FITC staining. $^{b}P < 0.001$ *vs* normal. $^{d}P < 0.001$ *vs* HG; F: EPC proliferation under different treatments, determined by Cell Counting Kit-8 assay. $^{b}P < 0.001$ *vs* normal. $^{d}P < 0.001$ *vs* HG; G-J: Representative photomicrographs of tube-like structures. Scale bar: 50 μm. Technician-counted tube branch points (H), relative tube length (I) and the total number of branches were calculated. $^{b}P < 0.001$ *vs* normal. $^{d}P < 0.001$ *vs* HG. PI: Propidium iodide.

validate the interaction among circ-Astn1, SIRT1, and miR-138-5p, we created a luciferase reporter (LR) vector. The candidate miR-138-5p-binding sites on circ-Astn1 as well as sites with point mutations inserted to prevent binding are shown in Figure 3A. Luciferase activity assay using 293T cells, which we transfected with MUT or WT circ-Astn1, verified that miR-138-5p suppressed circ-Astn1 activity (Figure 3B). RT-qPCR analysis suggested that circ-Astn1 overexpression suppressed miR-138-5p expression in EPCs (Figure 3C). Meanwhile the tubule formation assay showed that upregulation of circ-Astn1 restored angiogenic differentiation ability under HG conditions, but miR-138-5p overexpression destroyed the protective effect of circ-Astn1 (Figure 3D-G).

Next, we created the LR vector. Candidate miR-138-5p-binding sites on SIRT1 3'-UTR and those with point mutations inserted to prevent binding were constructed (Figure 3H). We transfected 293T cells with MUT or WT SIRT1 3'-UTR, which verified that WT miR-138-5p suppressed SIRT1 activity (Figure 3I). RT-qPCR analysis illustrated that miR-138-5p overexpression suppressed FOXO1 and SIRT1 expression at both mRNA and protein levels relating to EPCs (Figure 3J and K). However, overexpression of SIRT1 promoted SIRT1 and downregulated FOXO1 expression even after miR-138-5p overexpression. Analysis of tubule formation verified that miR-138-5p upregulation decreased angiogenic differentiation ability, but overexpression of SIRT1 restored the angiogenic differentiation ability of EPCs (Figure 3L-O).

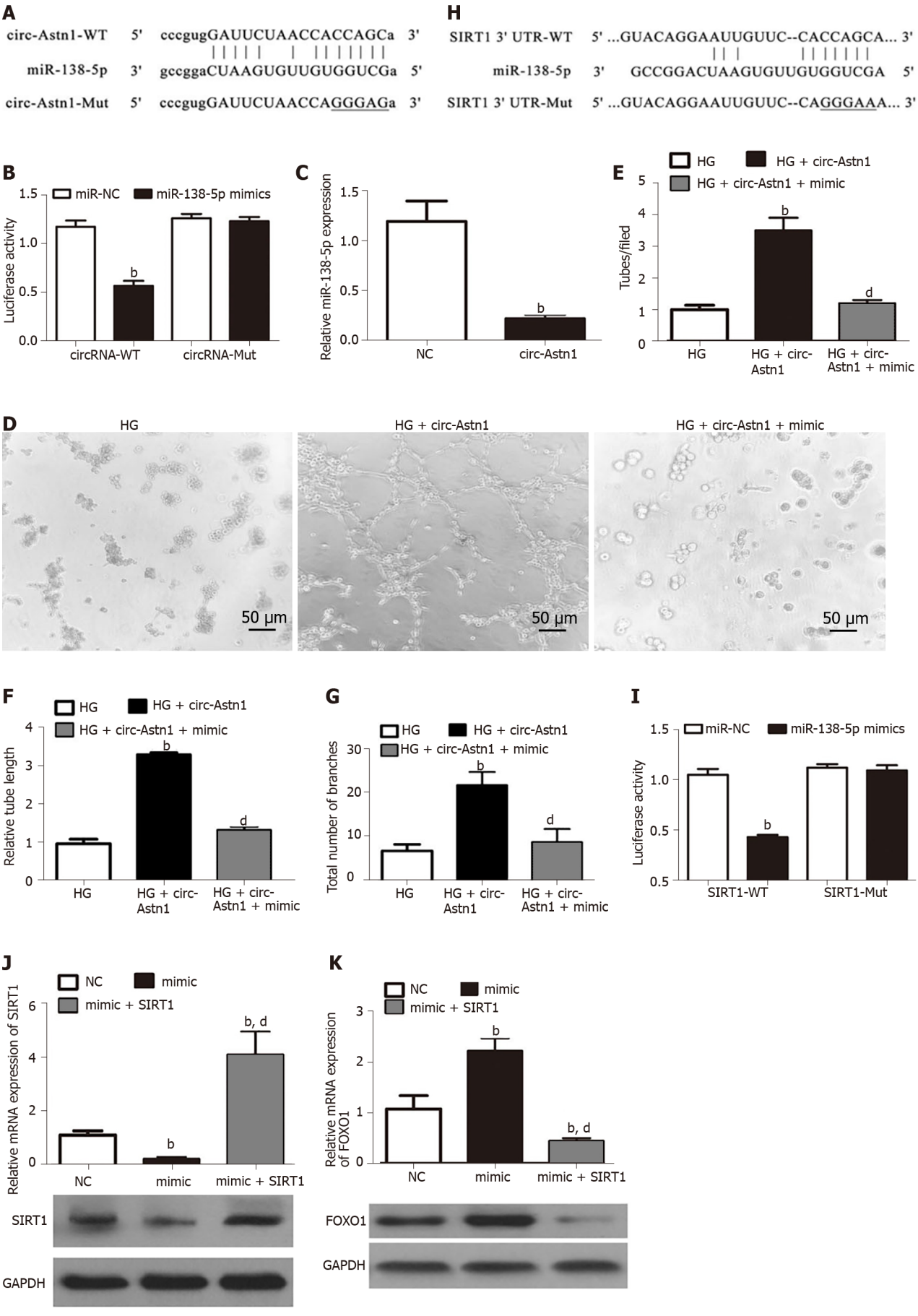
Exos from circ-Astn1-modified ADSCs possess high therapeutic effect, enhancing wound healing

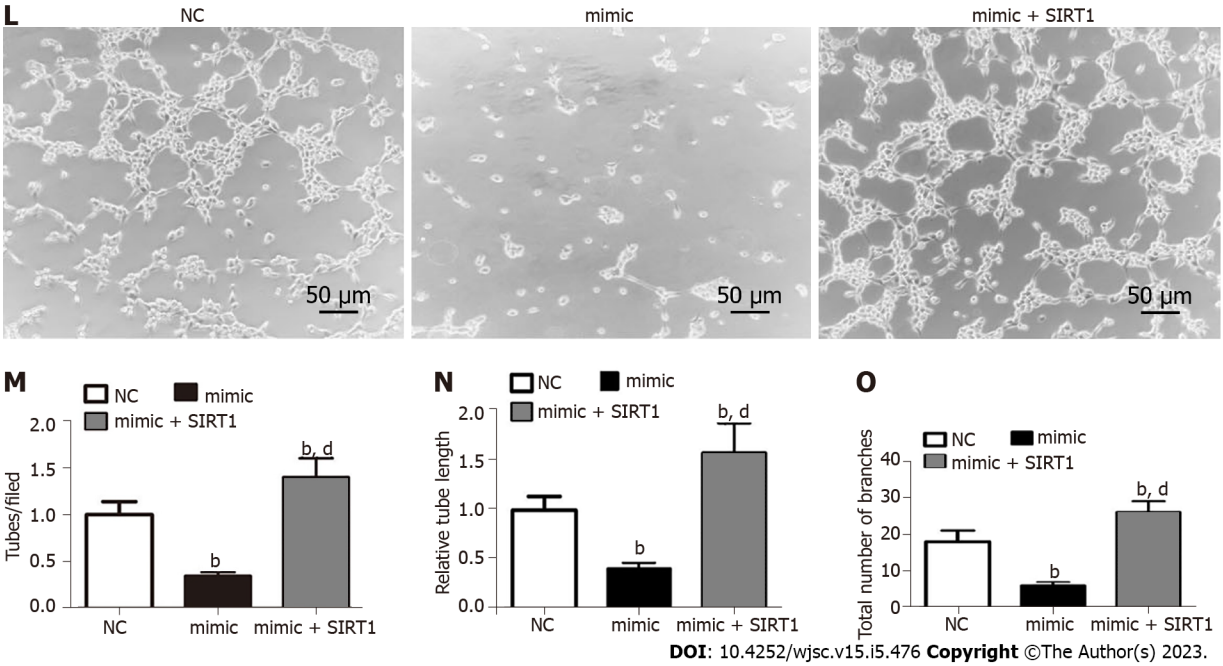
We investigated the influence of ADSC Exos on wound healing in full-thickness cutaneous wounds in mouse feet in a model of STZ-induced diabetes. Mice were treated by subcutaneous injection of Exos from WT or circ-Astn1-modified ADSCs, or an equivalent volume of PBS Exo diluent. Exos with high circ-Astn1 concentration accelerated wound closure significantly compared to PBS-treated control mice. The wounds treated with high circ-Astn1-containing Exos were almost closed by 14 d, while large areas of scarring were visible in both controls and circ-Astn1-knockdown-Exo-treated wounds (Figure 4A). Immunofluorescence with CD31 staining verified that microvascular development was more extensive with Exo treatments, specifically with high-circ-Astn1-containing Exos compared with the control group. However, circ-Astn1-knockdown suppressed the therapeutic effect of Exos (Figure 4B and C). TUNEL staining suggested that circ-Astn1 Exos significantly suppressed skin tissue apoptosis compared with control treatment, but circ-Astn1-knockdown suppressed the therapeutic effect of Exos (Figure 4D and E). Hematoxylin and eosin staining also showed that circ-Astn1 Exos treatment significantly promoted skin tissue wound healing compared with control treatment, but circ-Astn1-knockdown suppressed the therapeutic effect of Exos (Figure 4F). RT-qPCR analysis confirmed that circ-Astn1 Exos significantly suppressed miR-138-5p expression (Figure 4G) but promoted SIRT1 (Figure 4H) and decreased FOXO1 (Figure 4I) expression at both the mRNA and protein levels compared with controls.

DISCUSSION

Vascular deficits are fundamental factors regarding diabetes-related traits. Although former investigations have revealed that the proangiogenic wound healing phase is blunted by diabetes, detailed knowledge of factors regulating skin revascularization as well as capillary stabilization in diabetic wounds was missing[23]. Previous investigations revealed that Exos derived from ADSCs promote diabetic wound healing by regulating the disease microenvironment[4,20]. There is also evidence that circRNAs belong to a new RNA family that has been found to be broadly expressed, and have indispensable biological activities in regulating skin wound healing[18]. In this study, we found a series of circRNAs, which RNA-Seq detection showed were abnormally expressed in ADSC Exos compared with fibroblast Exos. Among the abnormally expressed circRNAs, expression of mmu_circ_0000101 (circ-Astn1), mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 was all increased significantly in ADSC Exos. Further study showed that circ-Astn1 decreased more significantly in EPCs after exposure to HG conditions. This suggesting that ADSC Exos protected EPCs from HG-induced damage related to circ-Astn1 delivery.

Our *in vitro* experiments revealed that HG conditions promoted EPC apoptosis and destroyed the ability of EPCs to differentiate into blood vessels. Transplantation of ADSC Exos exerted a protective effect in reversing HG-induced EPC damage. Increasing the circ-Astn1 content of Exos increased the protective effect. Bioinformatics analyses identified miR-138-5p as the circ-Astn1 downstream target, and this was confirmed by luciferase reporter (LR) experiments. A previous study revealed that overex-


A
circ-Astn1-WT 5' cccgugGAUUCUAACCACCAGCa 3'
miR-138-5p 3' gccggaCUAAGUGUUGUGGUCGa 5'
circ-Astn1-Mut 5' cccgugGAUUCUAACCAGGGAGa 3'
H
SIRT1 3' UTR-WT 5' ...GUACAGGAAUUGUUC--CACCAGCA... 3'
miR-138-5p 3' GCCGGACUAAGUGUUGUGGUCGA 5'
SIRT1 3' UTR-Mut 5' ...GUACAGGAAUUGUUC--CAGGGAAA... 3'
B
miR-NC
miR-138-5p mimics
Luciferase activity
circRNA-WT
circRNA-Mut
C
Relative miR-138-5p expression
NC
circ-Astn1
E
HG
HG + circ-Astn1
HG + circ-Astn1 + mimic
Tubes/filed
D
HG
HG + circ-Astn1
HG + circ-Astn1 + mimic
50 μm
F
Relative tube length
G
Total number of branches
I
SIRT1-WT
SIRT1-Mut
J
NC
mimic
mimic + SIRT1
Relative mRNA expression of SIRT1
SIRT1
GAPDH
K
Relative mRNA expression of FOXO1
FOXO1
GAPDH



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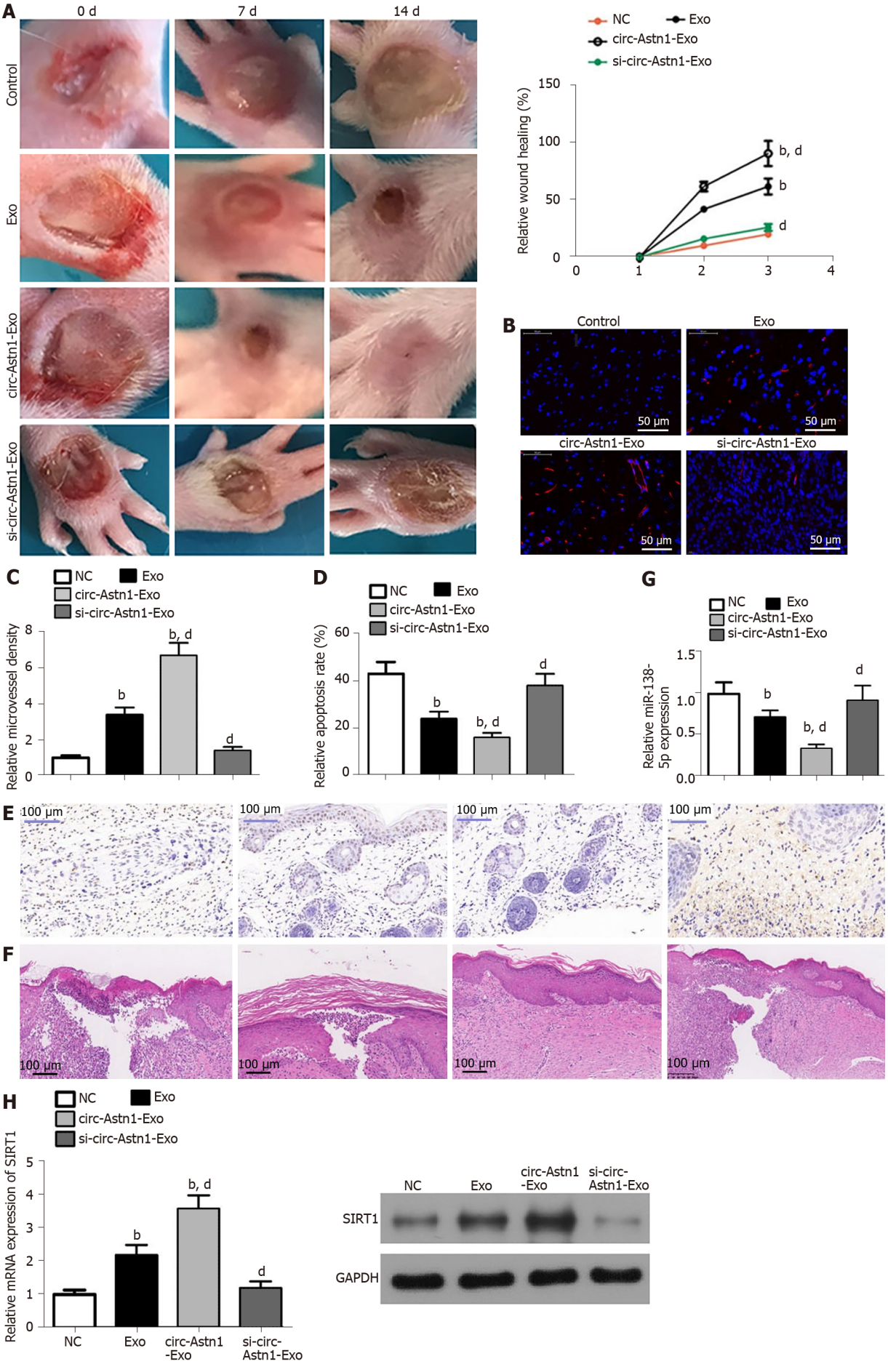
Figure 3 The circular RNA astrotactin 1-mediated mi-138-5p/SIRT1/forkhead box O1 signaling pathway plays an important protective role in endothelial precursor cells under high glucose conditions by promoting angiogenesis. A and B: Luciferase expression levels in HEK293 cells transfected with cloned circular RNA astrotactin 1 (circ-Astn1) wild-type (WT) or mutant (MUT) vector and miR-138-5p mimics. Data are denoted by the mean ± SD. $^{b}P < 0.001$; C: Quantitative polymerase chain reaction (qPCR) detection suggested that miR-138-5p expression was reduced after transfection with circ-Astn1-overexpressing vector in endothelial precursor cells (EPCs). Data are denoted as the mean ± SD. $^{b}P < 0.001$ *vs* NC; D-G: Representative photomicrographs of tube-like structures of EPCs under high glucose (HG) conditions after transfection with negative control or circ-Astn1-overexpressing vector. $^{b}P < 0.001$ *vs* HG. $^{d}P < 0.001$ *vs* circ-Astn1; H and I: Luciferase expression level in HEK293 cells transfected with cloned SIRT1 WT- or MUT-3' UTR vector and miR-138-5p mimics. Data are denoted by the mean ± SD. $^{b}P < 0.001$; J and K: qPCR and western blot analysis indicated that SIRT1 and forkhead box O1 expression were reduced after transfection with miR-138-5p overexpression vector in EPCs. Data are expressed as the mean ± SD. $^{b}P < 0.001$ *vs* NC. $^{d}P < 0.001$ *vs* miR-138-5p mimics; L-O: Representative photomicrographs of EPC tube-like structures under HG conditions after transfection with miR-138-5p mimics combined with or without SIRT1 overexpression vector. Data are denoted by the mean ± SD. $^{b}P < 0.001$ *vs* NC. $^{d}P < 0.001$ *vs* miR-138-5p mimics.

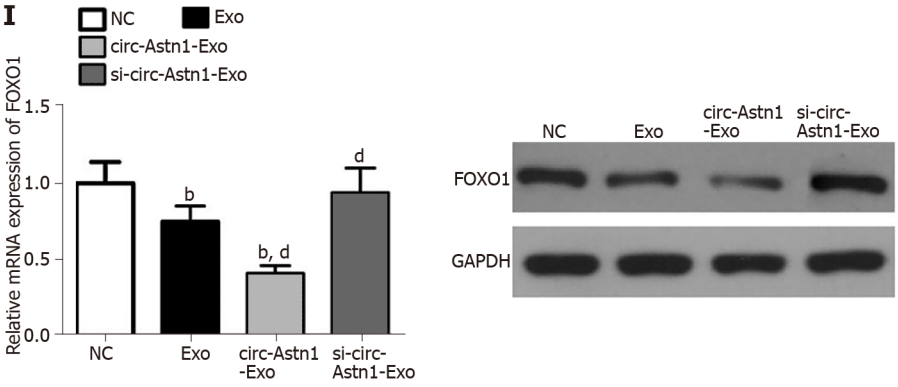
pression of miR-138 aggravates HG-induced vascular cell damage[24]. Our current investigation also found that circ-Astn1 overexpression decreased miR-138-5p expression. Meanwhile miR-138-5p overexpression reduced vascular EPC differentiation, suggesting that circ-Astn1 protected against HG-induced EPC damage by miR-138-5p adsorption.

Additional bioinformatics results showed that SIRT1 was also a miR-138-5p downstream target and this was verified by LR experiments. SIRT1 is a highly conserved nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase, which plays a regulatory role in metabolism and aging[25]. miR-138-5p overexpression reduced SIRT1 expression. Overexpression of SIRT1 restored vascular differentiation of EPCs after miR-138-5p upregulation. Previous studies have suggested that the SIRT1/FOXO1 pathway activity improves the stress microenvironment[26-28]. SIRT1 correlates to and deacetylates FoxO1. Moreover, previous studies have confirmed that SIRT1, a deacetylase that suppresses FoxO1 acetylation which is a crucial negative blood vessel development regulator, restrains anti-angiogenic activity[22,29,30]. Recently, it was reported that oxidative stress induces FoxO1 nuclear translocation which plays an important role in apoptosis regulation[26]. *In vivo* experiments have confirmed that Exos originating from circ-Astn1-modified ADSCs function indispensably in restoring EPC function and promoting wound healing by promotion of angiogenesis and suppression of apoptosis. RT-qPCR analysis demonstrated that treatment with Exos containing high levels of circ-Astn1 reduced miR-138-5p expression and promoted SIRT1. This increase in SIRT1 level suppressed FOXO1 expression, suggesting that Exos derived from circ-Astn1-modified ADSCs enhanced wound healing in a diabetic mouse model *via* miR-138-5p/SIRT1/FOXO1 axis regulation.

CONCLUSION

In conclusion, our research indicated that Exos derived from circ-Astn1-modified ADSCs enhanced wound healing in a diabetic mouse model *via* miR-138-5p/SIRT1/FOXO1 axis induction. Our study verified the therapeutic effects of circ-Astn1-Exos on an STZ-induced diabetic wound healing model. However, more in-depth studies are required to determine the actual role of miR-138-5p/SIRT1/FOXO1 in wound healing.


A
0 d
7 d
14 d
Control
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative wound healing (%)
150
100
50
0
0
1
2
3
4
b, d
b
d
B
Control
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
50 μm
C
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative microvessel density
8
6
4
2
0
b
b, d
d
D
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative apoptosis rate (%)
60
40
20
0
b
b, d
d
G
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative miR-138-5p expression
1.5
1.0
0.5
0.0
b
b, d
d
E
100 μm
F
100 μm
H
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative mRNA expression of SIRT1
5
4
3
2
1
0
b
b, d
d
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
SIRT1
GAPDH



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Figure 4 Exosomes from circular RNA astrotactin 1-modified adipose-derived mesenchymal stem cells have greater therapeutic effect in promoting wound healing in a diabetic mouse model. A: Representative images of full-thickness skin defects after treatment with adipose-derived mesenchymal stem cell (ADSC) exosomes or circular RNA astrotactin 1-modified ADSC exosomes for 0, 1, and 2 wk after wounding; B and C: Microvascular formation evaluated by immunofluorescence staining with cluster of differentiation 31. $^{b}P < 0.001$ *vs* control. $^{d}P < 0.001$ *vs* exosomes; D and E: We assayed apoptosis level *via* terminal deoxynucleotidyl transferase dUTP nick end labeling staining. $^{b}P < 0.001$ *vs* control. $^{d}P < 0.001$ *vs* exosomes; F: Hematoxylin and eosin staining shows wound changes; G-I: Quantitative polymerase chain reaction and western blot analysis showing mi-138-5p (G), SIRT1 (H), and forkhead box O1 (I) expression. $^{b}P < 0.001$ *vs* control. $^{d}P < 0.001$ *vs* exosomes.

ARTICLE HIGHLIGHTS

Research background

Wound healing impairment is a dysfunction induced by hyperglycemia and its effect on endothelial precursor cells (EPCs) in type 2 diabetes mellitus. There is increasing evidence showing that exosomes (Exos) derived from adipose-derived mesenchymal stem cells (ADSCs) exhibit the potential to improve endothelial cell function along with the wound healing process.

Research motivation

The potential therapeutic mechanism of ADSC Exos in wound healing in diabetic mice remains unclear.

Research objectives

To verify the effect of treatment with Exos from circular RNA astrotactin 1 (circ-Astn1)-overexpressing ADSCs on high glucose (HG)-induced EPC dysfunction.

Research methods

In this study, Exos from ADSCs and fibroblasts were used for high-throughput RNA sequencing (RNA-Seq). ADSC-Exo-mediated healing of full-thickness skin wounds in a diabetic mouse model was investigated. We utilized EPCs to investigate the therapeutic function of Exos in cell damage and dysfunction caused by HG. We utilized a luciferase reporter (LR) assay to detect interactions among circ-Astn1, SIRT1 and miR-138-5p. We employed diabetic mice to verify the therapeutic effect of circ-Astn1 on Exo-mediated wound healing.

Research results

High-throughput RNA-Seq detection showed that circ-Astn1 expression was increased in ADSC Exos compared with Exos from fibroblasts. Exos containing high concentrations of circ-Astn1 had enhanced therapeutic effect in restoring EPC function under HG conditions by promoting SIRT1 expression. Circ-Astn1 expression enhanced SIRT1 expression through miR-138-5p adsorption, which was validated by LR assay along with bioinformatics analyses. Exos containing high concentrations of circ-Astn1 had better therapeutic effect on wound healing *in vivo* compared to wild-type ADSC Exos. Immunofluorescence and immunohistochemical investigations suggested that circ-Astn1 enhanced angiopoiesis through Exo treatment of wounded skin as well as suppressing apoptosis through promotion of SIRT1 and decreased FOXO1 expression.

Research conclusions

In summary, we concluded that circ-Astn1 promoted the therapeutic effect of ADSC-Exos and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as a potential therapeutic alternative for treatment of diabetic ulcers.

Research perspectives

More in-depth studies are required to determine the actual role of miR-138-5p/SIRT1/FOXO1 in wound healing.

FOOTNOTES

Author contributions: Wang Z and Wang YB designed the project and drafted the paper based on feedback from the other authors; Feng C, Liu H, and Meng T performed all experiments and analyses; Huang WQ and Song KX took part in the analyses and draft revision.

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Institutional animal care and use committee statement: The Animal Care and Use Committee of Peking Union Medical College Hospital approved the investigation protocol (No: XHDW-2020-01). We carried out all postoperative animal care along with surgical interventions following the NIH Guide for Care and Use of Laboratory Animals. All surgery and euthanasia were performed under sodium pentobarbital anesthesia (30 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: The datasets used or/and analyzed during the current study are available from the corresponding author on reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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ORIGINAL ARTICLE

Basic Study

Stromal cell-derived factor-1α regulates chondrogenic differentiation *via* activation of the Wnt/β-catenin pathway in mesenchymal stem cells

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have been applied to treat degenerative articular diseases, and stromal cell-derived factor-1α (SDF-1α) may enhance their therapeutic efficacy. However, the regulatory effects of SDF-1α on cartilage differentiation remain largely unknown. Identifying the specific regulatory effects of SDF-1α on MSCs will provide a useful target for the treatment of degenerative articular diseases.

AIM

To explore the role and mechanism of SDF-1α in cartilage differentiation of MSCs and primary chondrocytes.

METHODS

The expression level of C-X-C chemokine receptor 4 (CXCR4) in MSCs was assessed by immunofluorescence. MSCs treated with SDF-1α were stained for alkaline phosphatase (ALP) and with Alcian blue to observe differentiation. Western blot analysis was used to examine the expression of SRY-box transcription factor 9, aggrecan, collagen II, runt-related transcription factor 2, collagen X, and matrix metalloproteinase (MMP)13 in untreated MSCs, of aggrecan, collagen II, collagen X, and MMP13 in SDF-1α-treated primary chondrocytes, of glycogen synthase kinase 3β (GSK3β) p-GSK3β and β-catenin expression in SDF-1α -treated MSCs, and of aggrecan, collagen X, and MMP13 in SDF-1α-treated MSCs in the presence or absence of ICG-001 (SDF-1α inhibitor).

RESULTS

Immunofluorescence showed CXCR4 expression in the membranes of MSCs. ALP stain was intensified in MSCs treated with SDF-1α for 14 d. The SDF-1α treatment

promoted expression of collagen X and MMP13 during cartilage differentiation, whereas it had no effect on the expression of collagen II or aggrecan nor on the formation of cartilage matrix in MSCs. Further, those SDF-1α-mediated effects on MSCs were validated in primary chondrocytes. SDF-1α promoted the expression of p-GSK3β and β-catenin in MSCs. And, finally, inhibition of this pathway by ICG-001 (5 μmol/L) neutralized the SDF-1α-mediated up-regulation of collagen X and MMP13 expression in MSCs.

CONCLUSION

SDF-1α may promote hypertrophic cartilage differentiation in MSCs by activating the Wnt/β-catenin pathway. These findings provide further evidence for the use of MSCs and SDF-1α in the treatment of cartilage degeneration and osteoarthritis.

Key Words: Stromal cell-derived factor-1α; Mesenchymal stem cells; Chondrogenic differentiation; Wnt/β-catenin; C-X-C chemokine receptor 4

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Core Tip: In this study, we investigated the effect of stromal cell-derived factor-1α (SDF-1α) on the differentiation of bone marrow mesenchymal stem cells (MSCs) and primary chondrocytes *in vitro*. We demonstrated that SDF-1α promotes the chondrogenic differentiation of MSCs, and similar results were observed in primary chondrocytes. In addition, SDF-1α also activates the Wnt/β-catenin pathway to regulate chondrocyte hypertrophy and maturation in MSCs.

Citation: Chen X, Liang XM, Zheng J, Dong YH. Stromal cell-derived factor-1α regulates chondrogenic differentiation *via* activation of the Wnt/β-catenin pathway in mesenchymal stem cells. *World J Stem Cells* 2023; 15(5): 490-501

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INTRODUCTION

Osteoarthritis (OA) is a chronic, multifactorial disease characterized by progressive degradation of articular cartilage[1]. The underlying molecular mechanism responsible for the pathogenesis of OA is not yet fully elucidated; as such, a disease-modifying therapy remains elusive[2], although a potential therapeutic strategy of cell-based cartilage regeneration using mesenchymal stem cells (MSCs) has been proposed[3,4]. It is known that following cartilage injury, MSCs undergo proliferation to form new cartilage and repair damage. During this process, chemokines play a role in targeted cell recruitment[5]. The chemokine stromal cell-derived factor-1α [SDF-1α, also known as C-X-C chemokine ligand (CXCL) 12 α][6] binds to the CXC receptor 4 (CXCR4) present in synovial fluid and cartilage tissues[7]. SDF-1α plays an important role in the targeted recruitment and chemotaxis of MSCs[8], and increased SDF-1α levels promote the entry of CXCR4-positive MSCs into damaged cartilage[9]. In addition, MSC recruitment mediated by the SDF-1α/CXCR4 axis has been shown to play an important role in other tissue repair processes[10]. Indeed, a previous study showed that intra-articular injection of meniscus progenitor cells promoted cartilage regeneration and improved OA *via* the SDF-1α/CXCR4 axis and by inducing progenitor cell homing[11]. Earlier, Hitchon *et al*[12] had reported the finding of upregulated expression levels of CXCR4 mRNA and protein in chondrocytes of rats with post-traumatic OA, while Kanbe *et al*[13] reported high SDF-1α expression in human chondrocytes of rheumatoid arthritis and OA joint fluid. This latter study also indicated that synovectomy significantly reduced SDF-1α and matrix metalloproteinase (MMP) concentrations in serum. Finally, Xiang *et al*[14] reported their study of human OA cartilage and *in vitro* SDF-1-induced OA chondrocytes, which demonstrated that inhibition of SDF-1α signaling was able to attenuate OA.

MSCs can differentiate into chondrocytes, which are characterized by SRY-box transcription factor 9 (Sox9), aggrecan, and collagen II expression[15]. *In vivo*, human MSCs used for cartilage repair undergo hypertrophic differentiation, which is characterized by an increase in cell volume and in the expression levels of several markers of hypertrophy, including runt-related transcription factor 2 (RUNX2), collagen X, MMP13, Indian hedgehog homolog, and alkaline phosphatase (ALP)[16]. Under physiological conditions *in vivo*, hypertrophic chondrocytes exhibit endochondral ossification. Furthermore, SDF-1α mediates several changes in the bone and cartilage[17], with roles in both physiologic and pathogenic processes. For example, SDF-1α/CXCR4 signaling regulates the bone morphogenetic protein-2-induced chondrogenic differentiation of MSCs and enhances chondrocyte

proliferation and maturation[18]. However, it also increases the expression of MMP3 in chondrocytes, leading to mechanical destruction of the bound matrix[19]. Therefore, despite its role in MSC recruitment, the direct effect of SDF-1α on cartilage differentiation by MSCs requires further clarification.

The present study focused on the direct role of SDF-1α in chondrocyte differentiation and demonstrated that SDF-1α participated in chondrocyte differentiation in MSCs. In addition, the Wnt/β-catenin pathway mediated the effects of SDF-1α on cartilage differentiation.

MATERIALS AND METHODS

MSC isolation and culture

MSCs were obtained from Sprague-Dawley (SD) rats. Ten male 4-8-wk-old SD rats weighing 150-200 g were housed in standard housing conditions with a 12-h light/dark cycle. The rats were euthanized using 20 mg/kg of ketamine intraperitoneally. Bone marrow was flushed from femurs of the SD rats using a 10-mL injector filled with Dulbecco's modified eagle medium (DMEM) and Ham's F12 medium containing 10% fetal bovine serum (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, United States), 100 IU/mL penicillin, and 100 IU/mL streptomycin (Boster Biological Technology, Pleasanton, CA, United States). The cultures were maintained at 37 °C in an atmosphere of 5% CO_2. The cells were grown for 48 h, and the medium was replaced. The cells were allowed to reach 70%-80% confluence and passaged by trypsinization using 0.05% trypsin/ ethylene diamine tetraacetic acid (Boster Biological Technology). The culture medium was replaced every 2 d. Rat MSCs cultured to passage 3 were used for the experiments.

Isolation and culture of primary chondrocytes

Ten male 3-d-old SD rats were euthanized by intraperitoneal ketamine, and their cartilage samples were soaked in a beaker containing 75% alcohol for 15 min. The cartilage surface of the proximal tibia was removed to a depth of 1.0-1.5 mm^3 using the micro-shear method and digested with 0.25% trypsin at 37 °C for 30 min. Following 10 min of centrifugation at 500 × *g*, the tissue pieces were collected and incubated with 0.25% collagenase II (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37 °C for 24 h. After a second centrifugation, the chondrocytes were cultured under the same conditions as described for the MSCs.

Multilineage differentiation of MSCs

To confirm that the isolated cells were MSCs, their differentiation into bone, cartilage, and adipose cell lineages was induced. For bone differentiation, passage 3 cells were cultured with osteogenic medium (RASMX-90021; Cyagen Biosciences, Inc., Santa Clara, CA, United States). After 21 d, the cells were stained with 0.5% alizarin red S at room temperature. In brief, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and then stained with alizarin red S solution for 30 min at room temperature. Morphology was evaluated using an inverted microscope (Leica DM IRM; Leica Microsystems, Wetzlar, Germany). Chondrogenic differentiation was achieved by pelleting 2.5 × 10^5 passage 3 cells in a 15-mL centrifuge tube at 500 × *g* for 5 min then resuspending the cells in 0.5 mL of chondrogenic induction medium [DMEM high-glucose, 100 nmol/L dexamethasone, 10 ng/mL transforming growth factor (TGF)-β 3, 50 mg/mL ascorbic acid 2-phosphate, 100 mg/mL sodium pyruvate, 40 mg/mL proline and insulin transferrin selenous acid-supplement][20]. The medium was replaced every 3 d. After 21 d, the pellets were fixed with 4% paraformaldehyde for 1 h at room temperature, then embedded in paraffin, cut into 5-μm sections, and stained with Alcian blue. Adipogenesis of MSCs was induced by culturing the cells in 6-well culture plates containing adipogenic medium (Cyagen Biosciences, Inc.). After 21 d, the cultures were fixed with 4% paraformaldehyde, stained with oil red O working solution (60% of 0.5% oil red O/ isopropanol in distilled water) for 1 h at room temperature, and observed using light microscopy (Leica DM IRM; Leica Microsystems).

Fluorescence staining

MSCs cultured in 12-well plates were prepared for immunofluorescence analysis (performed at room temperature). First, MSCs were fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were then permeabilized by incubating in 0.1% Triton (Boster Biological Technology, Inc.) in PBS for 10 min. After the cells were blocked with 3% bovine serum albumin (BSA; Boster Biological Technology, Inc.) in 0.1% Triton/PBS for 1 h at room temperature. The cells were initially incubated with anti-CXCR4 antibody (1:200; Abcam, Cambridge, United Kingdom) overnight at 4 °C and subsequently with an fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody (H + L) (1:200; Beyotime Institute of Biotechnology, Jiangsu, China) for 30 min at room temperature. The labeled cells were mounted with 4',6-diamidino-2-phenylindole (DAPI) at room temperature and observed by fluorescence microscopy.

MSC micromass culture

MSCs were first resuspended in F12-DMEM medium containing 10% fetal bovine serum, 0.25% penicillin-streptomycin, and 0.25% L-glutamine, and plated at a density of 2.5 × 10^5 cells/10 μL. After incubation for 4 h, a micromass culture medium supplemented with 1 mmol/L β-glycerophosphate and 0.25 mmol/L ascorbic acid with or without SDF-1α (PeproTech, Inc., Rocky Hill, NJ, United States) was added. The cells were cultured in chondrogenic induction medium that was replaced every other day. On day 7, the cells were stained with Alcian blue, and the absorbance of the supernatant was measured at 600 nm.

Chondrogenic differentiation assays

MSCs and primary chondrocytes were seeded in 6-well plates containing the chondrogenic induction medium. The following three conditions were assessed: Control (cytokine-free); 50 ng SDF-1α; and 100 ng SDF-1α[21]. The expression levels of collagen II, collagen X, aggrecan, MMP13, Sox9, and RUNX2 were determined. The expression levels of Wnt/β-catenin were measured in cells incubated for 24 h with 100 ng SDF-1α and ICG-001, an inhibitor of the Wnt/β-catenin pathway in MSCs.

Protein isolation and western blotting

Collagen II (1:2000), collagen X (1:2000), aggrecan (1:2000), MMP13 (1:1000), Sox9 (1:5000), and RUNX2 (1:2000) antibodies were purchased from Abcam, whereas the p- glycogen synthase kinase 3β (GSK3β) (1:2000), GSK3β (1:2000) and β-catenin (1:2000) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, United States). Secondary mouse IgG (1:10000) or rabbit IgG (1:10000) antibodies were purchased from Abcam, and the anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000) antibody was from Boster Biological Technology. Protein was extracted from the cells using 100 mL radio immunoprecipitation assay buffer (Boster Biological Technology, Inc.) supplemented with protease and phosphatase inhibitors. After microcentrifugation for 20 min at 10000 × *g*, the lysates were prepared as described above. The cell protein concentration was detected with a bicinchoninic acid kit (Boster Biological Technology, Inc.). Briefly, a total of 20 μg of cellular protein *per* sample was loaded onto a 10% Bis-Tris gel according to the protocol provided by the manufacturer. The separated proteins were then transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific), which were blocked for 1 h at room temperature with 5% BSA (Boster Biological Technology, Inc.) in Tris-buffered saline containing 0.1% Tween-20 (TBST). The blots were probed overnight at 4 °C with rabbit antibodies against GAPDH, collagen II, collagen X, aggrecan, MMP13, Sox9, RUNX2, p-GSK3β, GSK3β and β-catenin. Following three washes with TBST, the blots were incubated for 1 h at room temperature with anti-mouse or anti-rabbit IgG-horseradish-peroxidase-labeled secondary antibodies and washed three times with TBST. Finally, immunoreactivity was detected with enhanced chemiluminescence, and densitometry was performed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, United States). The results were summarized as mean ± standard deviation. Every experiment contained ≥ 3 replicate and was performed three independent times, unless otherwise stated. One-way analysis of variance and Fisher's least significant difference post hoc test were performed to compare differences between multiple groups. $P < 0.05$ indicated a statistically significant difference. we use 1 to express $P < 0.05$ and 2 to express $P < 0.01$.

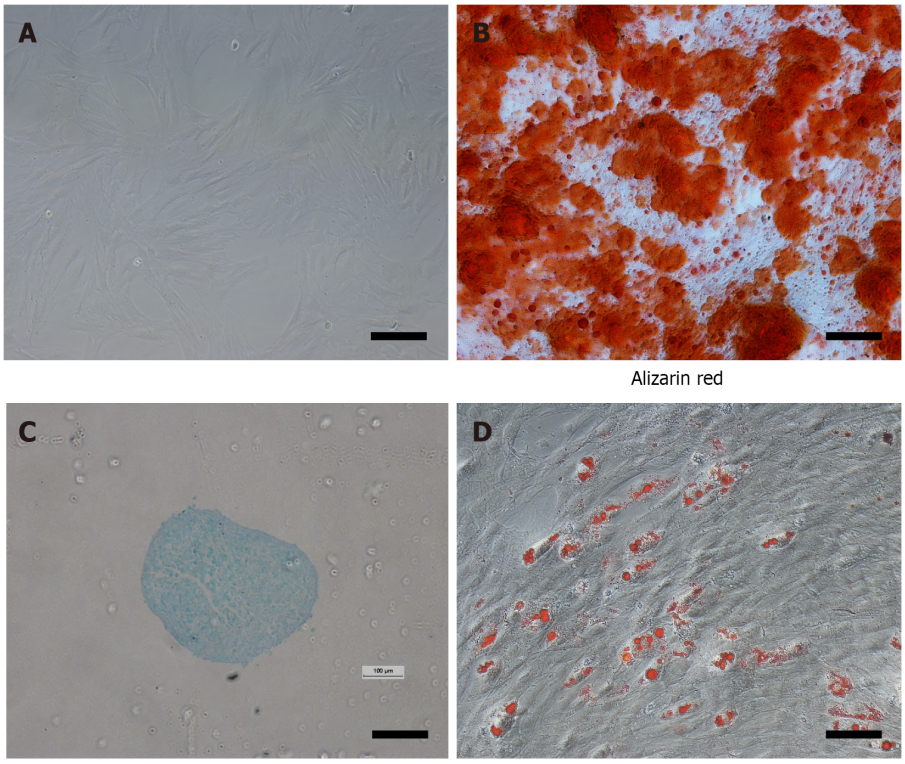
RESULTS

MSC culture and multilineage differentiation potential

The cells were initially quiescent but began to proliferate rapidly after day 3. Growth yielded a monolayer structure, composed of fibroblasts (Figure 1A). At passage 3, the isolated cells were successfully differentiated into the three skeletal cell lineages: Bone, cartilage, and adipose tissue. After culture in the osteogenic medium, nodules formed that were positive for alizarin red S staining, indicating calcium-bearing mineral deposits (Figure 1B). After culture with cartilage induction medium, cartilage microspheres were positive for Alcian blue staining. Blue granules were also noted in MSCs (Figure 1C). After culture in the adipogenic induction medium, lipid accumulation in the form of lipid droplets was noted in some of the cells, which were stained red by oil red O (Figure 1D).

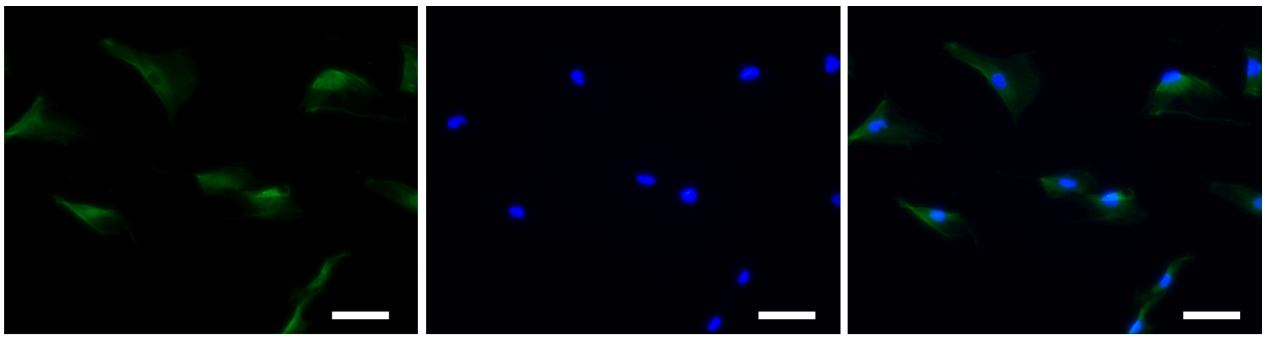
Expression of CXCR4 in rat MSCs

CXCR4 expression was detected in the membrane of the rat MSCs, while DAPI staining was confined to the nuclei of the MSCs (Figure 2).



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Figure 1 Characterization of mesenchymal stem cells. A: At passage 3, the cells resembled fibroblasts. Scale bar = 100 μm; B: Differentiation into bone cells was demonstrated by alizarin red S staining. Scale bar = 100 μm; C: Alcian blue staining indicated that the cells had successfully transformed into chondrocytes. Scale bar = 500 μm; D: Oil red O staining confirmed differentiation of the cells into adipose cells. Scale bar = 100 μm.



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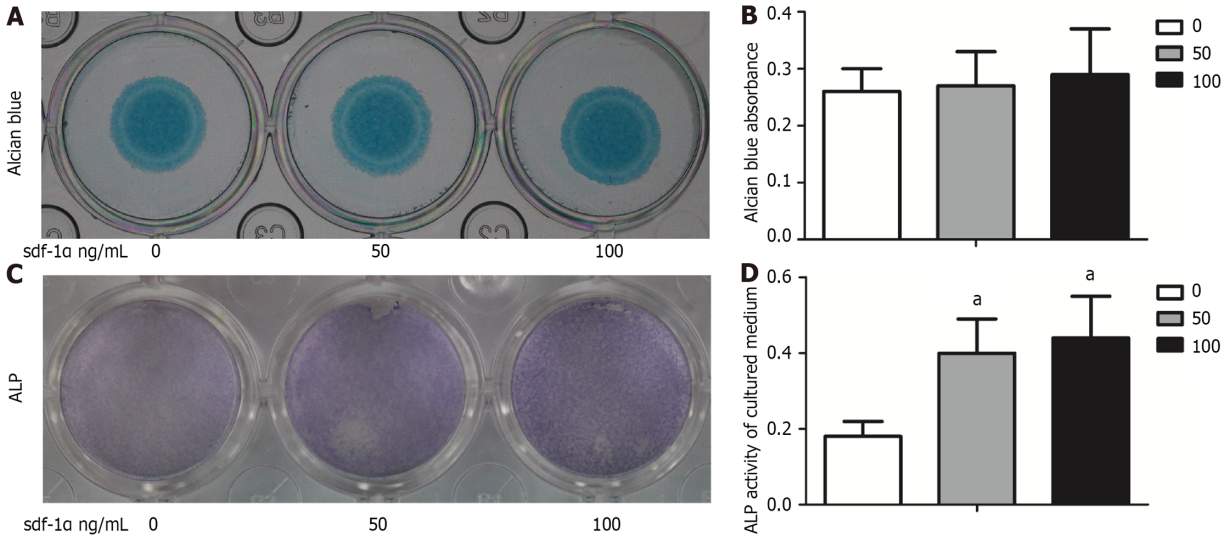
Figure 2 Expression of C-X-C chemokine receptor type 4 on rat mesenchymal stem cells. Representative image of the expression of C-X-C chemokine receptor type 4 (green fluorescence) on mesenchymal stem cell membranes. CXCR4: C-X-C chemokine receptor type 4; DAPI: 4',6-diamidino-2-phenylindole.

SDF-1a exerted no effect on early cartilage formation of MSCs but enhanced hypertrophic differentiation

No significant differences were noted between control (untreated) cells and cells treated with 50 ng SDF-1α or 100 ng SDF-1α in regards to the size of the cartilage micelles or the absorbance of Alcian blue (Figure 3A and B). ALP expression and activity levels were increased after 14-d SDF-1α treatment compared to control cells (Figure 3C and D).

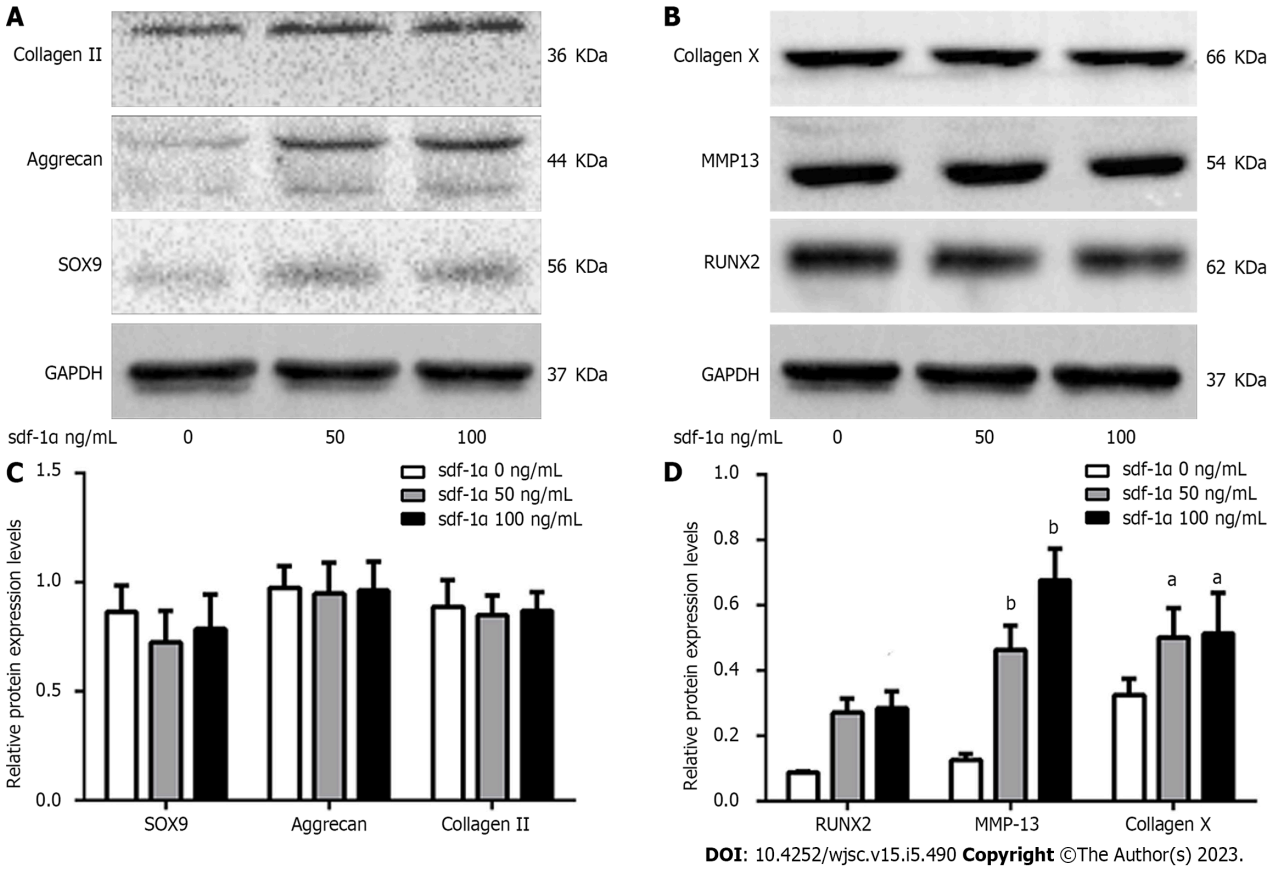
Effect of SDF-1a on MSCs during cartilage differentiation

Western blotting indicated no significant differences in the expression levels of early chondrocyte differentiation markers (Sox9, aggrecan, and collagen II) between MSCs treated with SDF-1α and untreated MSCs on day 7 (Figure 4A). On day 14, however, the expression levels of chondrocyte hypertrophy markers (RUNX2, collagen X, and MMP13) were increased in a dose-dependent manner in the SDF-1α-



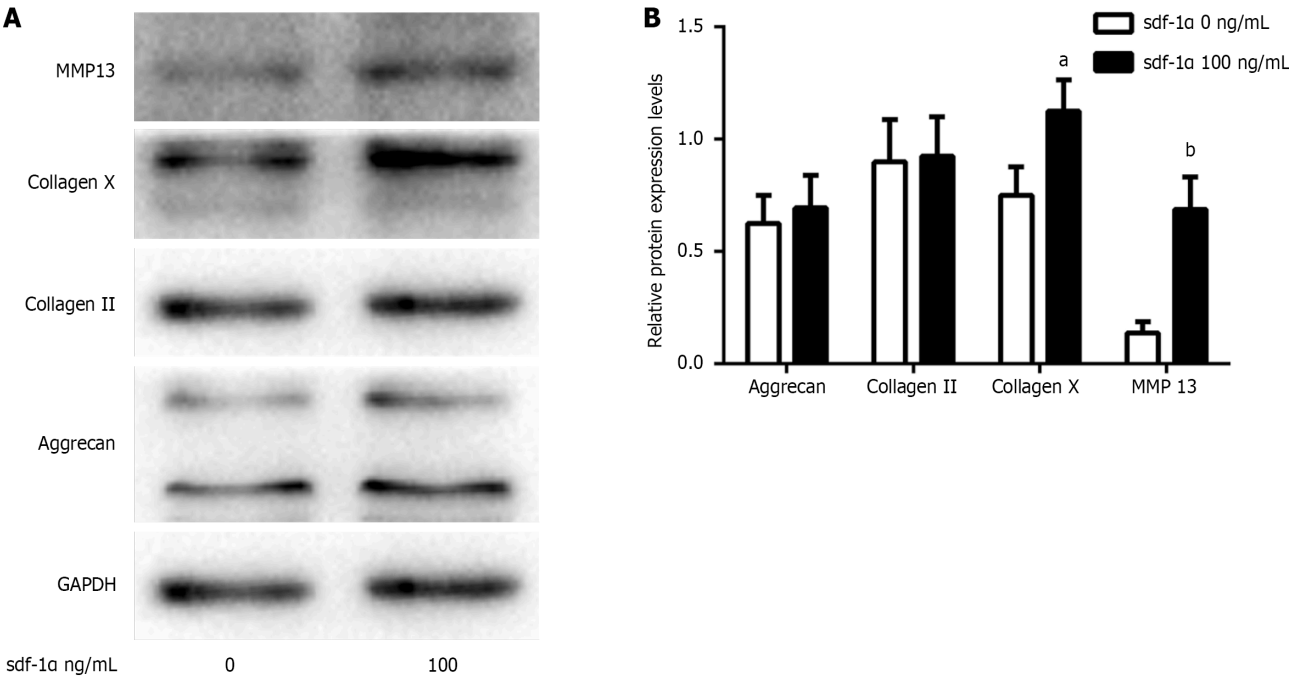
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Figure 3 Alkaline phosphatase activity levels in mesenchymal stem cells treated with stromal cell-derived factor-1α were noted in the absence of an effect on cartilage formation. A: Mesenchymal stem cells (MSCs) were cultured *in vitro* and stained with Alcian blue following 7 d of culture with or without stromal cell-derived factor-1α treatment; B: Alcian blue staining was measured after chemical extraction by measuring the absorbance of the supernatant at 600 nm; C: MSCs were positive for alkaline phosphatase (ALP; light purple staining); D: ALP expression was quantitatively analyzed. The values were representative of the mean ± standard deviation (n = 3). $^{a}P < 0.05$ *vs* control. ALP: Alkaline phosphatase; sdf-1α: Stromal cell-derived factor-1α.



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Figure 4 Effects of stromal cell-derived factor-1α on cartilage differentiation of mesenchymal stem cells. Representative images of western blot analysis of rat mesenchymal stem cells treated with stromal cell-derived factor-1α. A: No changes in the expression levels of SRY-box transcription factor 9 (Sox9), aggrecan, and collagen II were observed; B: Increased expression levels of Runt-related transcription factor 2 (RUNX2), collagen X, and matrix metalloproteinase 13 (MMP13) were observed; C: Relative Sox9, aggrecan, and collagen II protein expression; D: Relative RUNX2, collagen X, and MMP13 protein expression. $^{a}P < 0.05$ *vs* control (Student's *t*-test). $^{b}P < 0.01$ *vs* control (Student's *t*-test). sdf-1α: Stromal cell-derived factor-1α; Sox9: SRY-box transcription factor 9; RUNX2: Runx family transcription factor 2; MMP13: Matrix metalloproteinase 13.



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Figure 5 Effects of stromal cell-derived factor-1α on the cartilage phenotype of primary rat chondrocytes. A: Expression levels of collagen II, aggrecan, collagen X, and matrix metalloproteinase 13 (MMP13) were determined by western blotting in primary chondrocytes treated with stromal cell-derived factor-1α (100 ng/mL); B: Relative collagen II, aggrecan, collagen X, and MMP13 protein expression. $^{a}P < 0.05$ *vs* control (Student's *t*-test), $^{b}P < 0.01$ *vs* control (Student's *t*-test). MMP13: Matrix metalloproteinase 13; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; sdf-1α: Stromal cell-derived factor-1α.

treated group (Figure 4B).

Effects of SDF-1a on the cartilage phenotype of primary chondrocytes.

Western blotting showed that SDF-1α treatment did not affect the expression levels of collagen II and aggrecan in primary chondrocytes, whereas it significantly increased the expression levels of collagen X and MMP13 in the MSCs (Figure 5).

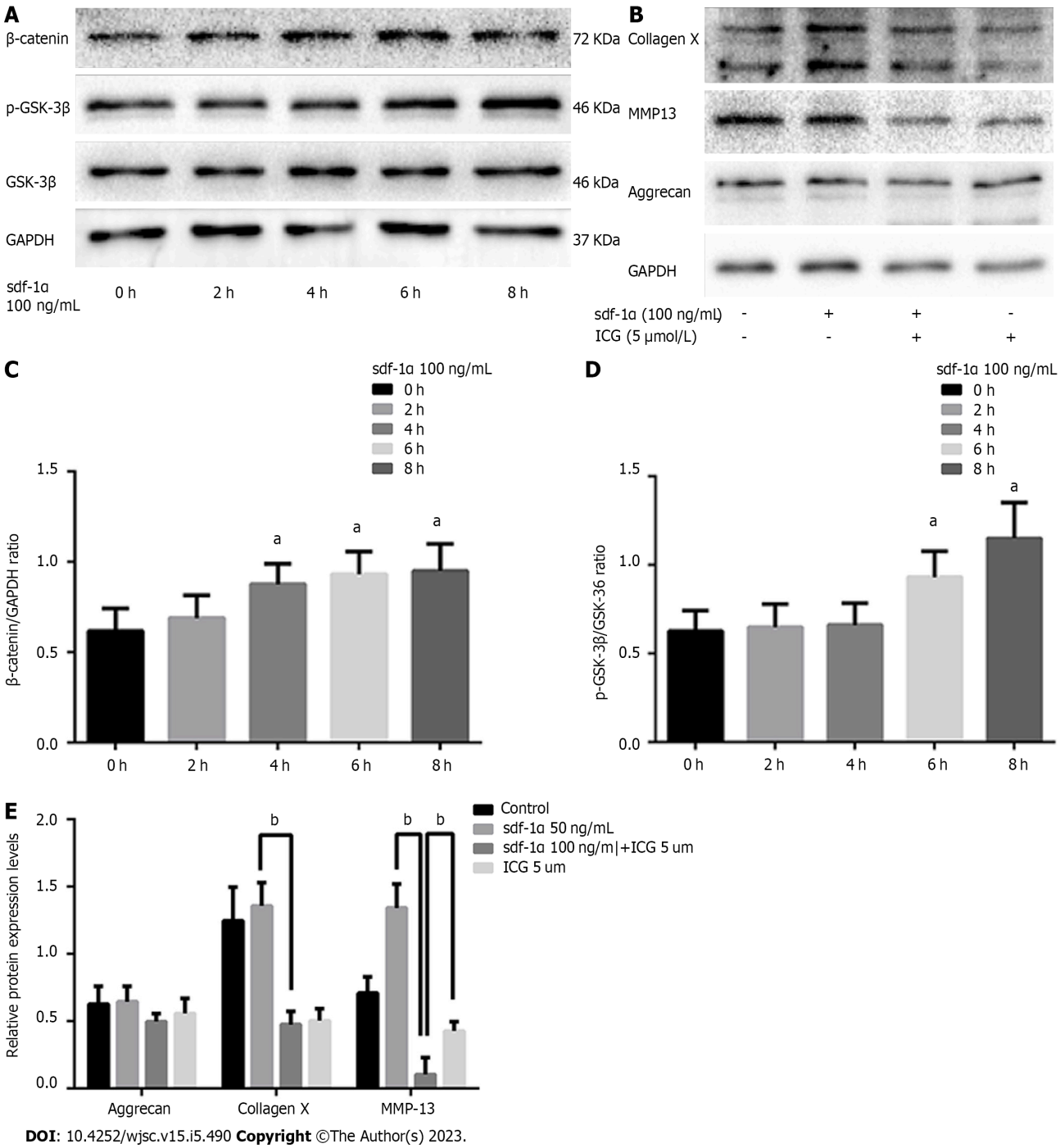
Wnt/β-catenin pathway was involved in the effect of SDF-1a on cartilage differentiation.

SDF-1α promoted the expression of p-GSK3β, decreased degradation of β-catenin, and a gradual increase in β-catenin expression were demonstrated (Figure 6A). Upon blockade of the Wnt/β-catenin pathway *via* ICG-001, the SDF-1α-mediated increase in the expression levels of collagen X and MMP13 was neutralized (Figure 6B).

DISCUSSION

In the present study, rat MSCs, which were successfully differentiated into the three skeletal cell lineages and were positive for the expression of the CXCR4 receptors on the cell membrane, were used to assess the effects of SDF-1α on cartilage formation. The results indicated that the size of the cartilage micromass, the absorbance of Alcian blue, and the expression levels of Sox9, aggrecan, and collagen II did not significantly change in response to SDF-1α. However, the expression and activity levels of ALP and the expression levels of RUNX2, collagen X, and MMP13 were significantly increased. These results demonstrated that SDF-1α promoted hypertrophic cartilage differentiation in MSCs, while not affecting the early differentiation of cartilage. Similar results were obtained in primary chondrocytes. The data further indicated that SDF-1α caused a gradual increase in the expression levels of p-GSK-3β *in vitro* and activated the Wnt/β-catenin pathway, leading to increased collagen X and MMP13 expression levels. These findings demonstrated that the SDF-1α/CXCR4 axis was required in the cartilage differentiation process. Previous studies have implicated other chemokine types, including CXCL8 and CXCL1, as capable of promoting chondrocyte hypertrophy and calcification[22,23].

The Wnt/β-catenin pathway is a classical Wnt signaling pathway involved in tissue development and cell proliferation, differentiation, and apoptosis[24,25]. The signal transduction of the Wnt/β-catenin pathway is well defined and proceeds as follows. Initially, the extracellular Wnt proteins (Wnt-3a, Wnt-4, Wnt-8c, and Wnt-9a) combine with the frizzled and LRP proteins on the cell membrane to form an activation complex. Subsequently, the phosphorylation of GSK-3β blocks the phosphorylation and



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Figure 6 Wnt/β-catenin pathway involvement in the effects of stromal cell-derived factor-1α on cartilage differentiation. A: Expression levels of β-catenin, p-glycogen synthase kinase 3β (p-GSK-3β), and GSK-3β by western blotting; B: Blockage of the Wnt/β-catenin pathway with ICG-001 inhibited the expression levels of collagen X and matrix metalloproteinase 13; C: Relative β-catenin protein expression; D: Ratio of relative protein expression of p-GSK-3β to relative protein expression of GSK-3β (p-GSK-3β/GSK-3β); E: Relative aggrecan, collagen X, and MMP13 protein expression. $^{1}P < 0.05$, $^{b}P < 0.01$, Student's *t*-test. p-GSK-3β: p-glycogen synthase kinase 3β; GSK-3β: Glycogen synthase kinase 3β; MMP13: Matrix metalloproteinase 13; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; sdf-1α: Stromal cell-derived factor-1α.

degradation of β-catenin. Finally, β-catenin enters the cell nucleus and modulates T cell factor/Lymphoid enhancer factor binding, which initiates the transcription of downstream genes, thus causing biological changes[26-30]. Several Wnt signaling components regulate the hypertrophic maturation of chondrocytes. Specifically, Wnt can induce the accumulation of β-catenin, which then enters the nucleus and binds to cell factor/lymphoid enhancer-binding factor to promote the transcription of the collagen X and MMP13 genes. Ultimately, P-GSK3β can add phosphate groups to the serine/threonine residues at the β-catenin N terminus to promote its degradation[4.31].

Overexpression of the Wnt receptor frzb-1 was shown to hinder chondrocyte maturation and mineralization[32]. In a subsequent study, knock-out of the secreted frizzled-related protein 1, a Wnt signaling antagonist, led to a reduced height of the growth plate and increased calcification of

hypertrophic areas, indicating that activation of the Wnt signaling pathway accelerated endochondral ossification[33]. The findings of the present study are consistent with the collective previous results indicating that the SDF-1α/CXCR4 axis activates the Wnt/β-catenin signaling pathway in MSCs, which in turn increases the production of collagen X and MMP13. Conversely, when we treated the MSCs with the Wnt/β-catenin inhibitor ICG-001, the effects of SDF-1α were no longer observable, which confirmed the regulatory role of Wnt/β-catenin. Thus, the present study indicates that SDF-1α does not promote the early stages of cartilage differentiation nor increase the expression of Sox9, which is similar to the results of Kim *et al*[34].

Hypertrophic differentiation of chondrocytes is the primary barrier preventing the use of MSCs in therapeutic cartilage repair[35,36]. Hypertrophy is sometimes noted in OA[37,38]. However, SDF-1α also mediates MSC recruitment and can exert a positive role in OA[31]. The identification of cytokines that block cartilage hypertrophy caused by SDF-1α, promote physiological endochondral ossification, prevent mineralization of the extracellular matrix, and mediate chondrocyte apoptosis will contribute to an improved understanding of the pathogenesis of OA and provide targets for development of future treatment strategies for this disease[39].

There were some limitations in this study, which must be considered when seeking to generalize our findings. First, measuring the stimulation with SDF-1α in MSCs is challenging because the only verification technique is overexpression or knockdown of the CXCR4 receptor. Second, this study primarily used cell experiments and lacked an *in vivo* perspective to the experimental research. Regardless, through this study, we were able to adequately demonstrate effects of SDF-1α on cartilage differentiation in MSCs and primary chondrocytes.

CONCLUSION

The present study demonstrated a role of SDF-1α in promoting hypertrophic cartilage differentiation in MSCs and primary chondrocytes *in vitro*. SDF-1α activated the Wnt/β-catenin pathway in MSCs. Identification of the novel molecular mechanism by which SDF-1α promotes cartilage differentiation in MSCs suggests a therapeutic approach to OA and cartilage repair.

ARTICLE HIGHLIGHTS

Research background

Stromal cell-derived factor-1α (SDF-1α) has a chemotactic effect on mesenchymal stem cells (MSCs), and SDF-1α and MSCs are used together to treat cartilage degeneration and cartilage defects. The specific effects of SDF-1α on cartilage differentiation in MSCs need to be clarified.

Research motivation

Understanding the effects of SDF-1α on MSCs will provide a new theoretical basis for the use of MSCs in the repair of cartilage degeneration.

Research objectives

To explore the role and mechanism of SDF-1α on cartilage differentiation in MSCs and primary chondrocytes.

Research methods

MSCs were treated with SDF-1α and subsequently stained for alkaline phosphatase and with Alcian blue to demonstrate chondrogenic differentiation. Western blot analysis was used to examine the expression of cartilage differentiation-related and Wnt/β-catenin pathway proteins in MSCs and primary chondrocytes.

Research results

After extraction and incubation with the appropriate differentiation media, MSCs differentiated into the three skeletal lineages. SDF-1α exerted no effect on early cartilage formation but enhanced hypertrophic differentiation in MSCs. SDF-1α had no effect on the expression of SRY-box transcription factor 9, aggrecan, and collagen II but increased the expression of runx family transcription factor 2, collagen X, and matrix metalloproteinase 13 in MSCs and primary chondrocytes. SDF-1α increased the expression of p- glycogen synthase kinase 3β and β-catenin.

Research conclusions

SDF-1α enhanced hypertrophic differentiation in MSCs and primary chondrocytes. This effect was achieved by activating the Wnt/β-catenin pathway.

Research perspectives

These findings provide a new theoretical basis for the treatment of cartilage degeneration with MSCs.

FOOTNOTES

Author contributions: Chen X acquired the data; Zheng J and Dong YH designed the experiments; Chen X and Liang XM analyzed the data and wrote the manuscript; Liang XM and Chen X supervised the study; all authors read and approved the final manuscript.

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REVIEW

Adipokines regulate mesenchymal stem cell osteogenic differentiation

Zhong-Hua Xu, Chen-Wei Xiong, Kai-Song Miao, Zhen-Tang Yu, Jun-Jie Zhang, Chang-Lin Yu, Yong Huang, Xin-Die Zhou

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Abstract

Mesenchymal stem cells (MSCs) can differentiate into various tissue cell types including bone, adipose, cartilage, and muscle. Among those, osteogenic differentiation of MSCs has been widely explored in many bone tissue engineering studies. Moreover, the conditions and methods of inducing osteogenic differentiation of MSCs are continuously advancing. Recently, with the gra-dual recognition of adipokines, the research on their involvement in different pathophysiological processes of the body is also deepening including lipid metabolism, inflammation, immune regulation, energy disorders, and bone homeostasis. At the same time, the role of adipokines in the osteogenic differentiation of MSCs has been gradually described more completely. Therefore, this paper reviewed the evidence of the role of adipokines in the osteogenic differentiation of MSCs, emphasizing bone formation and bone regeneration.

Key Words: Mesenchymal stem cells; Adipokines; Adipose tissue; Osteogenic differentiation; Osteogenesis; Bone regeneration

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Core Tip: Bone tissue supports and protects the organs of the human body. There is a close relationship between the immune system and bone homeostasis. Adipose tissue is an essential accessory tissue around bone tissue, which regulates bone homeostasis through the secretion of adipocytokines. There are many types of adipokines, but only some have been studied in detail. Different adipokines affect the behavior and differentiation of mesenchymal stem cells under different local microenvironments and surrounding inflammation, thus coordinating and participating in the regulation of bone homeostasis.

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INTRODUCTION

Adipose tissue is currently considered an endocrine organ[1] and comprises adipose cells, endothelial cells, fibroblasts, and immune cells[2]. Adipokines are factors secreted by adipose tissue and have multiple functions[3] involving various biological processes including immune responses, inflammation, glucose metabolism, insulin secretion, sensitivity regulation, regulation of blood pressure and myocardial contractility, blood vessel growth, and lipid metabolism[3,4]. Therefore, adipokines regulate different biological processes in different organs including the brain, liver, muscles, blood vessels, heart, and pancreas[5]. The function, characterization, molecular targets, and potential clinical disease correlation of adipokines are still unclear and the main focus of future adipokine research.

Mesenchymal stem cells (MSCs), pluripotent stem cells derived from the mesoderm, were identified by surface markers such as CD29, CD37, CD44, CD90, CD105, and CD166[6]. MSCs can be readily extracted from many tissues including bone marrow, umbilical cord, placenta, fat, liver, and skin[7]. However, the most well-studied source is bone marrow. MSCs have been shown to differentiate into mature cells of various tissues including cartilage, bone, tendon, ligament, and adipose tissue[8]. Due to its multipotential nature, MSCs have been used to treat many diseases including tumors, central nervous system disease, liver disease, graft-versus-host disease, inflammation, immune system disease, and bone regeneration[9-12]. In this review, we focus on the osteogenic differentiation of MSCs.

Bone is a rigid organ that supports and protects the other vital organs in the body. In adults, bones are renewed approximately every 7 years[13], and bone formation by osteoblasts and bone resorption by osteoclasts play a significant role. Osteoclasts originate from hematopoietic stem cell precursors, and osteoblasts originate from MSCs[14]. The dynamic balance of the two processes maintains the stability of bone metabolism, whereas the destruction of balance leads to various diseases including osteoporosis [15], osteopenia[16], and bone nonunion[17]. Osteoblasts promote the deposition of calcium salts in the bone matrix and stimulate bone remodeling and osteoblast differentiation of MSCs. It can be verified by the detection of runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteopontin (OPN). Therefore, the biological characteristics of MSC osteogenic differentiation have been widely used in bone tissue engineering to treat bone defects caused by trauma, infection, and tumor surgery[18-20]. As a common progenitor of both adipocytes and osteoblasts, MSCs are in a delicate equilibrium state during differentiation, whereas adipose-inducing factors inhibit the osteogenic differentiation of MSCs. In contrast, bone-inducing factors inhibit the adipogenic differentiation of MSCs[6]. As an important active secretion of fat, the position and role of adipokine in the osteogenic differentiation of MSCs are worth further consideration. Therefore, we reviewed the role of adipokines in the osteogenic differentiation of MSCs.

CYTOKINE AND CYTOKINE-LIKE PROTEINS

Interleukin (IL) is an essential inflammatory adipokine that plays a vital role in the differentiation of MSCs in the early stage of bone reconstruction[21]. Lacey *et al*[22] found that low-dose IL-1β (0.001-1 ng/mL) inhibited ALP activity, reduced RUNX2 and procollagen expression, and inhibited the degree of mineralization of MSCs in mice. IL-6 is a multifunctional lymphoid factor with pro-inflammatory and anti-inflammatory effects[23]. At the same time, it can be secreted by osteoblasts to stimulate the secretion of osteoclasts and participate in bone homeostasis. IL-6 induces osteogenic differentiation in human bone marrow-derived MSCs (BMSCs) *via* mitogen-activated protein kinase signaling[24]. IL-10 can reduce the synthesis of pro-inflammatory cytokines and chemokines and inhibit the expression of IL-1 and tumor necrosis factor alpha (TNF-α)[25]. In mice, IL-10 inhibits the osteogenic differentiation of MSCs prior to ALP expression[26]. IL-17 cytokines act by binding to the IL-17 receptor family[27]. In the early stage of bone injury, IL-17 secretion increases, promoting the transformation of MSCs into bone

progenitor cells or osteoblasts. In some cases, IL-17 can also act as an anti-osteoblast factor, leading to bone loss[28,29].

TNF-α is a pro-inflammatory cytokine that can bind to the TNF receptor superfamily and participate in the regulation of a variety of biological processes. Different doses of TNF-α showed different osteogenic differentiation activity of MSCs. Wang *et al*[30] showed that a high dose of TNF (50 ng/mL) could stimulate the upregulation of some osteogenic factors in MSCs, including vascular endothelial growth factor and insulin growth factor. Lacey *et al*[22] cultured BMSCs with different doses of TNF-α and found that low-dose TNF-α (0.1-10 ng/mL) inhibited the mineralization and activation of ALP and OPN in cultured MSCs.

Monocyte chemotactic protein 1 (MCP-1), also known as C-C motif chemotactic factor ligand 2, can influence monocyte migration and subsequent macrophage polarization[31]. Xie *et al*[32] showed that in the process of osteogenic differentiation, MSCs from patients with ankylosing spondylitis secreted more MCP-1 than MSCs from healthy people. Enhanced MCP-1 secretion promoted monocyte migration, increased classical macrophage polarization, and enhanced TNF-α secretion[32]. Other adipokine-related cytokines, such as progranulin and resistin, have not been reported to correlate with MSC osteogenic differentiation.

Transforming growth factor β (TGF-β) has a unique correlation with the differentiation of adult MSCs [33]. Through the precise matching of ligands, receptors, and cell signaling molecules, TGF-β is involved in the lineage transformation process of the differentiation of various stem cells such as lipids, osteoblasts, chondrogenic and myogenic cells[34]. Tang *et al*[35] confirmed that TGF-β1 induced the migration of MSCs to the bone resorption site of mice by activating the activin receptor-like kinase 5-Smad2/3-Smad4 pathway and restricted the further recruitment of osteoclasts but did not induce osteogenic differentiation. However, other studies have reported that TGF-β inhibits osteogenic differentiation through Wnt signaling interactions and inhibits RUNX2 through the activation of Smad3[36,37]. However, TGF-β has also been reported to promote the osteogenic differentiation of MSCs[38,39]. However, further research needs to be carried out in the future.

Chemerin is a secreted protein derived from adipocytes and liver cells involved in physiological processes including inflammation, angiogenesis, and calcium mobilization[40,41]. Epidemiological studies have reported that patients with osteoporosis have higher circulating chemerin[42], and the knockout of chemerin or its receptor CMKLR1 inhibits lipogenesis and promotes the osteogenic differentiation of MSCs[43]. Li *et al*[41] showed that chemerin promoted the osteogenic differentiation of C3H10T1/2 cells and MSCs through Akt/Gsk3β/β-catenin signaling. However, Akt inhibitors (MK2206) inhibited chemerin's promotion of osteogenic differentiation and active β-catenin.

PROTEINS OF THE FIBRINOLYTIC SYSTEM

Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor of the fibrinolytic system[44]. Adipose-derived PAI-1 is associated with various pathologic conditions including inflammation, diabetes, cancer, and obesity[44]. Takafuji *et al*[45] studied the role of PAI-1 in the osteogenic differentiation of MSCs using wild-type and PAI-1-deficient mice and found that the loss of PAI-1 significantly weakened the expression of BMSC osteogenic genes, such as bone morphogenetic protein 2 (BMP-2) and ALP.

Tissue factor, another adipokine that plays a crucial role in the clotting process[46], whose overexpression in the body can lead to multiple forms of thrombosis[47]. In a study aimed at improving coagulation activity, Rangasami *et al*[48] found that pluronic micelle-mediated tissue factor silencing could effectively induce the higher differentiation of MSCs in osteogenic and lipid-forming media.

COMPLEMENT AND COMPLEMENT-RELATED PROTEINS

Adipsin was the first adipocyte-secreted protein to be identified[49] and is currently named complement factor D[50]. Fat cells produce it through the activation of peroxisome proliferator-activated receptor gamma[51]. More recently, adipsin was shown to promote insulin secretion by pancreatic β cells and prevent β-cell death[52]. By activating Wnt signaling, adipsin initiates adipogenesis from BMSCs[53]. Experiments on BMSCs of adipsin knockout mice showed the increased expression of mineralized nodules and osteoblast markers including RUNX2, COL1A1, and osteocalcin compared with MSCs of normal origin[53].

Complement and complement-related proteins from adipose tissue include complement component 1q and TNF-related protein family, complement factor B, and acylating simulation protein[3,54]. However, it has not been reported whether they induce or inhibit the osteogenic differentiation of MSCs.

ADIPOKINES

Leptin, a hormone derived from adipose tissue, is involved in pathophysiological processes such as food absorption, energy metabolism, inflammation, immunity, and bone homeostasis[55-58]. Leptin binds to its leptin receptor, a marker specific to BMSCs[59]. Leptin has been shown to cross-regulate BMP-9 signaling through the JAK/STAT signaling pathway in MSCs, thereby enhancing BMP-9-induced osteogenesis[60].

Adiponectin plays a vital role in anti-inflammation, glucolipid metabolism, and insulin resistance regulation[61,62]. Wang *et al*[63] reported that adiponectin regulates BMSC osteogenic differentiation and osteogenesis through the Wnt/β-catenin pathway. Similar results have also been reported in other studies[64-66].

Visfatin is commonly produced by visceral adipose tissue and is also known as nicotinamide phosphoribosyltransferase (Nampt) or pre-B cell cluster enhancer. It is strongly expressed in osteogenic differentiation[67] and promotes the proliferation and mineralization activity of osteoblasts[68]. Visfatin induces the secretion of IL-6, IL-8, and MCP-1 during the osteogenic differentiation of MSCs and significantly increases matrix mineralization during osteogenic differentiation, while the expression of type I collagen is decreased[69].

Nicotinamide adenine dinucleotide (NAD) is involved in energy metabolism and protein modification[70]. Nampt has recently been identified as a novel adipokine[71]. Nampt is a rate-limiting enzyme and participates in all-around MC3T3 E1-osteogenesis prior to the cell differentiation process of NAD salvage pathways. Knocking out Nampt, or adding its specific inhibitor, Fk866, resulted in decreased intracellular NAD concentration and decreased osteogenic ability[67]. Thus, Nampt can be used as a specific marker for the osteogenic differentiation of MSCs[72].

Visceral adipose tissue-derived serine protease inhibitor (vaspin), an adipose-derived hormone, attenuates osteogenic differentiation of the preosteoblast cell line MC3T3-E1[73] and antagonizes the osteogenic differentiation of rat osteoblasts. However, the role of vaspin in the osteogenic differentiation of MSCs has not been reported[74].

BMPs, the largest component of the TGF-β ligand family, regulate multiple organogenetic pathways, fat formation, and energy metabolism[75,76]. BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7 all strongly promote osteogenesis. Short-term addition of BMP-2 increases osteocalcin expression[77], and BMP-7 induces the increased expression of ALP, a marker of osteoblast differentiation, and accelerates calcification[78]. The absence of BMP-2 and BMP-4 results in severely impaired osteogenic function, but the limb skeleton still develops normally without BMP-4[79]. BMP-3 regulates adult bone mass by limiting the differentiation of bone progenitor cells into mature osteoblasts[80]. It is important to note that BMP-7 has been marketed and used in surgery to aid fracture healing, with no reported local or systemic adverse events[81]. The effects of BMP-5[82,83] and BMP-6[84,85] on the osteogenic differentiation of MSCs have also been reported.

Nesfatin-1 is a novel anorexia polypeptide that has a wide range of biological effects including energy metabolism, gastrointestinal function, anxiety and depression, and the regulation of cardiovascular and reproductive function[86-88]. The role of nesfatin-1 in the osteogenic differentiation of MSCs has not been reported, but it can promote the expression of osteogenic genes such as ALP and RUNX2 in newly derived rat stem cells[89]. Therefore, we speculate that Nesfatin-1 has a similar role in the osteogenic differentiation of MSCs, but this conclusion still needs to be confirmed by further studies.

Cathepsins are an important category of enzymes located within the lysosomes[90]. Cathepsins are produced by various tissues, which also include adipose tissue[91]. Cathepsin K is a crucial enzyme in the degradation of the organic bone matrix, and its expression in bone formation-related cells, including fibroblasts, osteoblasts, and MSCs, has also been confirmed[92,93]. Zhang *et al*[94] showed that knockout or inhibition of cathepsin K can promote the regeneration of BMSCs of jaw bone through glycolysis, thus promoting alveolar bone regeneration. Similarly, cathepsin S deficiency alters the balance between adipocyte and osteoblast differentiation, increases bone turnover, and alters bone microstructure[95].

Apelin is an endogenous ligand of the G protein-coupled apelin receptor[96]. Besides being an adipokine, apelin is also expressed in skeletal muscle, the central nervous system, the heart, and other tissues, and is involved in lipolysis, glucose metabolism, cell proliferation, and angiogenesis[97]. Exogenous addition of apelin protein or overexpression of apelin promotes postpositional MSC osteoblast differentiation by activating the Wnt/β-catenin signaling pathway[98].

Omentin-1 is the adipokine most commonly expressed in omental adipose tissue and is also abundant in plasma[99]. Omentin-1 is involved in the physiological processes of inflammation, insulin, and cardiovascular functions[99,100]. For bone effects, a study of postmenopausal women found a negative correlation between omentin-1 levels and lumbar bone density[101]. Tang *et al*[102] found that omentin-1 has a dose-dependent effect on the viability of MC3T3-E1 cells, which can significantly increase the expression of members of the TGF-β/Smad signaling pathway, and also significantly increase the expression levels of BMP-2, RUNX2, OPN, osteocalcin, and other proteins, thus promoting osteogenesis.

Lipocalin 2 (LCN2) is a protein involved in host defense, autoimmunity, insulin resistance, skin healing, tumor, and infection[103,104]. LCN2 disrupts osteoclast formation in bone tissue by negatively regulating the proliferation and differentiation of osteoclast precursors[105]. As a secretory bone factor,

LCN2 positively affects the osteogenic differentiation and *in vivo* osteogenesis of MC3T3-E1[106].

Melatonin is an indoleamine that is synthesized and secreted primarily by the pineal gland in mammals but is also secreted by adipose tissue[107]. Melatonin mainly affects the circadian rhythm and sleep-wake cycle and is also involved in immune regulation and inhibition of tumor growth[108,109]. Melatonin is also involved in MSC differentiation, which is involved in developing and regenerating bone, muscle, and fat tissues. In BMSCs, melatonin enhances osteogenesis and inhibits lipogenesis. Melatonin also differentiates bone marrow progenitors from adipocytes to osteoblasts[110,111].

Gremlin-1 is a highly conserved glycoprotein, mainly distributed in the extracellular matrix, with a small amount in the endoplasmic reticulum[112]. As an adipokine, gremlin-1 plays an important role in adipose tissue homeostasis[113]. At the same time, studies have shown that gremlin-1 is a BMP protein inhibitor, which can inhibit their binding to BMP receptors on the cell membrane by binding to BMP-2, BMP-4, and BMP-7[112]. Specific overexpression of gremlin-1 in mouse bone tissue results in severe osteoporosis; however, conditional knockout of gremlin-1 increases trabecular volume and bone formation[114]. Gremlin-1 has also been shown to inhibit the viability and osteogenic differentiation of human BMSCs[115].

LIPID TRANSPORT

Apolipoprotein E (ApoE), one of the main components of plasma very low-density lipoprotein[116], regulates lipid homeostasis by regulating lipid transport between tissues and cells. ApoE4 is associated with hyperlipidemia and hypercholesterolemia, leading to coronary heart disease, stroke, and atherosclerosis[117-119]. BMP-2 can upregulate the ApoE level of the mouse mesenchymal progenitor cell line (C3H10T1/2), leading to enhanced osteogenic differentiation. At the same time, ApoE is also expressed *in vitro* in mouse cranial primary osteoblasts with advanced osteoblast sequences[120].

ENZYMES

Dipeptidyl peptidase 4 (DPP-4) is a protein secreted in the salivary glands, prostate, seminal vesicles, endometrium, small intestine, and decidual membrane, and has recently been identified in adipose tissue as well[121]. DPP-4 is an important drug target in type 2 diabetes and directly induces insulin resistance in adipocytes and skeletal muscle[121]. DPP-4 not only reflects but also promotes adipose tissue dysfunction. Choi *et al*[122] found that DPP-4, when overexpressed, could restrict the induction of osteogenic differentiation of heart artery flap-derived mesenchymal cells by the autocrine insulin-like growth factor-1 signaling pathway, but this result has not been verified on MSCs.

Tissue inhibitors of metalloproteinases (TIMPs) have four main members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4, and are primarily responsible for degrading most proteins in the extracellular matrix[123,124]. TIMPs are generally considered to be inhibitors of matrix metalloproteinases (MMPs) through the action of their terminal N-domain[125]. Meanwhile, TIMPs exist in the extracellular matrix in a soluble form and preemptively bind to the extracellular matrix, thus inhibiting the effect of MMPs[126]. TIMPs can selectively inhibit different MMPs, metalloproteinase and a disintegrin and metalloproteinase with thrombospondin motifs[125,126]. Inhibition of endogenous TIMP-1 can inhibit the proliferation, metabolic activity, and osteogenic differentiation ability of MSCs by activating Wnt/β-catenin signaling [127]. However, Liang *et al*[128] found in the process of MSC osteogenic differentiation that TIMP-1 knockdown increased the deposition of calcium nodules, ALP activity, and the expression of osteocalcin protein by activating Wnt/β-catenin signaling. The conclusions here are contradictory and need further confirmation by other studies. Studies targeting TIMP-3 have shown that increased expression of TIMP-3 can significantly promote osteogenic differentiation of MSCs in the fracture model of diabetic rats [129].

CONCLUSION

The formation and regeneration of bone tissue usually require regulation of the local microenvironment. The balance between bone resorption and bone regeneration is essential for bone tissue regeneration. Adipokines are exogenous immune regulatory substances secreted by adipose tissue, and are widely involved in pathophysiological processes of surrounding tissues, including bone homeostasis and bone regeneration. Not all human adipokines have been identified, but the current literature has revealed that the surface adipose tissue secretes more than 600 factors or proteins involving many processes of human pathophysiology[130]. There are many types of adipokines, including cytokines[22], fibrinolysin[44], complement and related proteins[49], enzymes[121], lipid transport systems[116], endocannabinoids [131], and angiotensinogen[132] (Table 1).

Table 1 Key activities of factors released by adipose tissue

Classification	Appellation	Mechanism	Ref.
Cytokine and cytokine-like proteins	Interleukin	IL-6 induces osteogenic differentiation in human bone marrow-derived MSCs *via* MAPK signaling. IL-10 inhibits osteogenic differentiation of MSCs prior to ALP expression. IL-17 promoting the transformation of MSC into bone progenitor cells or osteoblasts	[24,26,28]
	TNF-α	High dose of TNF could stimulate the upregulation of some osteogenic factors in MSCs, including VEGF and insulin-like growth factor. Low-dose TNF-α inhibited the mineralization and activation of ALP and OPN in cultured MSCs	[22,30]
	MCP-1	Influencing monocyte migration and subsequent macrophage polarization	[31]
	TGF-β	Through the precise matching of ligands, receptors, and cell signaling molecules, TGF-β is involved in the lineage transformation process of the differentiation of various stem cells, such as lipid, osteoblast, chondrogenic, and myogenic	[34]
	Chemerin	Chemerin promotes lipogenesis and inhibits osteogenic differentiation of MSCs	[42]
Proteins of the fibrinolytic system	PAI-1	Loss of PAI-1 significantly weakened the expression of bone marrow-derived MSC osteogenic genes, such as BMP-2 and ALP	[45]
	Tissue factor	Tissue factor silencing could effectively induce higher differentiation of MSCs in osteogenic and lipid-forming media	[48]
Complement and complement-related proteins	Adipsin	Adipsin initiates adipogenesis from bone marrow MSCs by activating Wnt signaling	[53]
Adipokines	Leptin	Leptin has been shown to cross-regulate BMP-9 signaling through the JAK/STAT signaling pathway in MSCs, thereby enhancing BMP-9-induced osteogenesis	[60]
	Adiponectin	adiponectin regulates BMSC osteogenic differentiation and osteogenesis through the Wnt/β-catenin pathway	[63]
	Visfatin	Promoting the proliferation and mineralization activity of osteoblasts	[68]
	Nicotinamide	Nampt is a speed-limit enzyme and participates in the all-around MC3T3-E1. Osteogenesis prior to the cell differentiation process of NAD salvage pathways	[67]
	Visceral	Attenuating the osteogenic differentiation of preosteoblast cell line MC3T3-E1	[73]
	Bone morphogenetic proteins	BMP-7 induced increased expression of ALP, a marker of osteoblast differentiation, and accelerated calcification. The absence of BMP-2 and BMP-4 resulted in severely impaired osteogenic function. BMP-3 regulates adult bone mass by limiting the differentiation of bone progenitor cells into mature osteoblasts	[78-80]
	Nesfatin-1	Promoting the expression of osteogenic genes such as ALP and RUNX2 in rats' newly derived stem cells	[89]
	Cathepsins	Knockout or inhibition of cathepsin K could promote the regeneration of bone marrow MSCs of jaw bone through glycolysis. Cathepsin S deficiency alters the balance between adipocyte and osteoblast differentiation, increases bone turnover, and alters bone microstructure	[94,95]
	Apelin	Promoting postpositional MSC osteoblast differentiation by activating the Wnt/β-catenin signaling pathway	[98]
	Omentin-1	Increasing the expression of BMP2, RUNX2, OPN, and osteocalcin	[102]
	Lipocalin 2	Disrupting osteoclast formation in bone tissue by negatively regulating the proliferation and differentiation of osteoclast precursors	[105]
	Melatonin	Differentiating bone marrow progenitors from adipocytes to osteoblasts	[111]
	Gremlin-1	BMP protein inhibitor	[112]
Lipid transport	ApoE	Enhancing osteogenic differentiation of the mouse mesenchymal progenitor cell line	[120]
Enzymes	DPP-4	Restricting the induction of osteogenic differentiation of heart artery flap-derived mesenchymal cells by the autocrine insulin-like growth factor-1 signaling pathway	[122]
	Tissue inhibitors of metallo-	Inhibition of endogenous TIMP-1 can inhibit the proliferation, metabolic	[127]

proteinases	activity, and osteogenic differentiation ability of MSCs by activating the Wnt/β-catenin signal

ALP: Alkaline phosphatase; ApoE: Apolipoprotein E; BMP-2: Bone morphogenetic protein 2; DPP-4: Dipeptidyl peptidase 4; IL: Interleukin; JAK: Janus kinase; MAPK: Mitogen-activated protein kinase; MCP-1: Monocyte chemotactic protein 1; MSC: Mesenchymal stem cell; Nampt: Nicotinamide phosphoribosyltransferase; OPN: Osteopontin; PAI-1: Plasminogen activator inhibitor-1; RUNX2: Runt-related transcription factor 2; TIMP-1: Tissue inhibitors of metalloproteinase; TGF-□: Transforming growth factor beta; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

In summary, this paper reviewed the current research on the regulation and influence of adipokine in the osteoblast differentiation of MSCs. However, this review did not include all currently discovered adipokines but only included published studies involving osteogenic differentiation of MSCs. Most of the included studies were conducted in BMSCs, with a small number involving osteoblast precursor cells, progenitor cells, and a small number of other tissue-derived stem cells. Our review suggests that different adipokines have different effects on the outcome of osteogenic differentiation, bone regeneration, and bone remodeling of MSCs. The progress of related research provides a good reference for subsequent preclinical and clinical studies and a new reference for treating osteogenic disorders and diseases of osteoblastic homeostasis.

FOOTNOTES

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REVIEW

Advances of nanotechnology applied to cancer stem cells

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Abstract

Cancer stem cells (CSCs) are a small proportion of the cells that exist in cancer tissues. They are considered to be the culprit of tumor genesis, development, drug resistance, metastasis and recurrence because of their self-renewal, proliferation, and differentiation potential. The elimination of CSCs is thus the key to cure cancer, and targeting CSCs provides a new method for tumor treatment. Due to the advantages of controlled sustained release, targeting and high biocompatibility, a variety of nanomaterials are used in the diagnosis and treatments targeting CSCs and promote the recognition and removal of tumor cells and CSCs. This article mainly reviews the research progress of nanotechnology in sorting CSCs and nanodrug delivery systems targeting CSCs. Furthermore, we identify the problems and future research directions of nanotechnology in CSC therapy. We hope that this review will provide guidance for the design of nanotechnology as a drug carrier so that it can be used in clinic for cancer therapy as soon as possible.

Key Words: Cancer stem cells; Nanotechnology; Nanoparticles; Nanodrug delivery systems; Drug resistance; Therapy

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Core Tip: Cancer stem cells (CSCs) have the potential to self-renew, proliferate, and differentiate. CSCs play a key role in the occurrence, development, recurrence, and metastasis of tumors. Due to the good compatibility and biodegradability of nanomaterials, they are applied to target CSCs for drug delivery, photothermal therapy, and magnetic hyperthermia to treat cancer.

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INTRODUCTION

Cancer is a major threat to people's health and life worldwide[1]. One of every eight deaths is caused by cancer[2,3]. Current cancer treatments mainly include surgical intervention, radiation therapy, and chemotherapy, which often kill healthy cells and are harmful to patients. Therefore, researchers are seeking better ways to eliminate cancer cells, with less side effects. Some researchers are working on the use of various macrocyclic ligands for cancer therapy, making ruthenium an ideal choice over other transition metals due to its special chemical properties[4]. However, what plagues most cancer treatments is the presence of a small number of cancer stem cells (CSCs) in tumor tissues[5,6], which have the potential for self-renewal, unlimited proliferative capacity, and multidirectional differentiation [5,7]. These cells are in the G0 phase and hypoxic microenvironment and play a key role in tumorigenesis, progression, recurrence, and metastasis. The presence of CSCs in solid tumors such as breast cancer (BC)[8,9], human leukemia[10,11], colorectal cancer[12,13], glioblastoma multiforme (GBM)[14], and ovarian cancer[15] has been reported, and it has been confirmed that CSCs play an important role in the development of tumors.

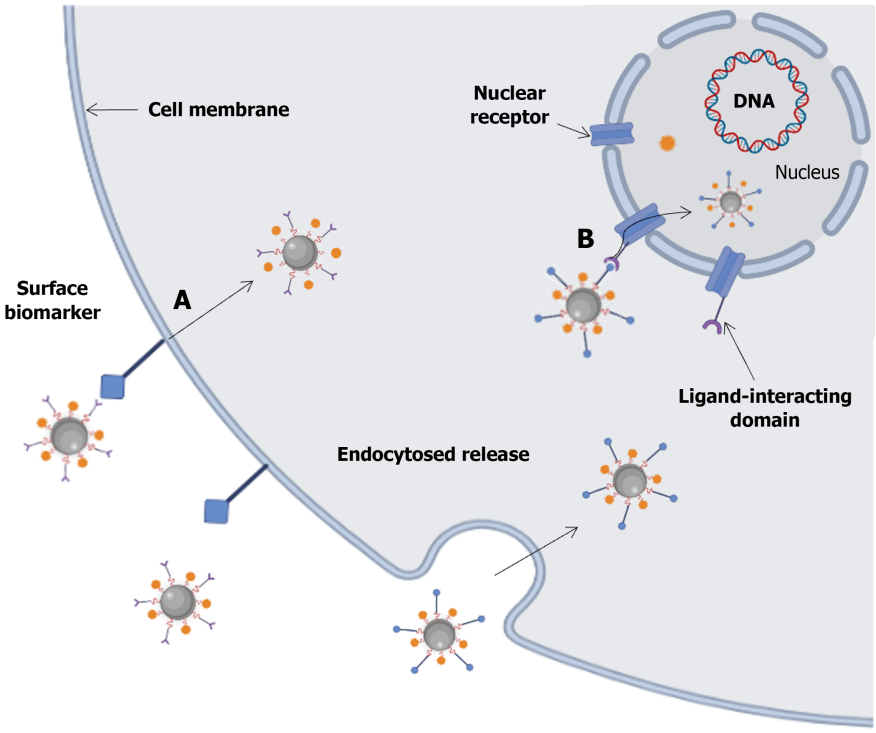
CSCs have inherent properties such as phenotypic plasticity, drug efflux transporters, overexpression of antiapoptotic proteins, an efficient DNA repair system and a persistent stemness profile that make them resistant to conventional therapies such as chemotherapy and radiation[16-18]. In general, CSC resistance mainly occur through stem cell pathways including the hedgehog[19,20], Notch[21,22], Wnt/β-linked protein[23,24], Nanog[25,26], nuclear factor kappa B (NF-*k*B)[27], and epidermal growth factor receptor pathways[28]. They express ATP-binding cassette (ABC) transporter proteins that can abrogate potential drug damage. CSCs also activate DNA repair capacity within tumor cells and are resistant to cell death[29], which helps to prevent the recruitment of apoptotic factors[5,30]. Therefore, the development of effective anticancer strategies to specifically kill tumor cells and tumor stem cells will be central to cancer therapy.

In recent years, the popularity of nanotechnology has promoted the development of nanodrug delivery systems (NDDS), and various nanodrug carriers have been applied to the treatment of tumors. Due to their small size, biocompatibility, and biodegradability, nanoparticles (NPs) help to fully exploit the function of NDDSs as drug delivery systems/drug carriers, including as imaging agents and for photothermal therapy (PTT), recognition, and drug and gene delivery[31]. As the carrier of active drugs in the drug delivery model, NDDSs can ensure the specific release of active drugs in the patient's body, improve drug solubility and bioavailability and prolong maintenance to improve drug efficacy[32]. Nanocarriers offer remarkable specificity in targeted delivery through active and passive targeting mechanisms (Figure 1)[33,34]. In active targeting, NPs are conjugated to antibodies, peptides, aptamers, and other small molecules[34]. Drug delivery using NP targeting reduces toxicity in healthy cells, prevents drug degradation, and has the advantages of better specificity, biocompatibility, less cytotoxicity, extended half-lives, controlled drug release, and high drug loading capacity for NP-based cancer treatments compared to traditional chemo-cancer treatments[35]. In passive targeting, enhanced permeability and retention (EPR) effects result in NPs circulating slowly in the tumor microenvironment and being more concentrated there than in healthy tissue[36]. Some commonly used nanocarriers (Figure 2) include lipid and micelle-based NPs, polymer/non-polymer NPs, nanobinding, carbon nanotubes (CNTs), graphene oxide (GO), nanocapsules, dendritic macromolecules, polymer micelles, and quantum dots (QDs), which are used to enhance the effectiveness of therapeutic interventions by delivering nontoxic large payloads[37-39]. Recent advances in nanotherapeutics have led to the development and exploration of various nanomaterial carriers for efficient drug/therapeutic delivery.

CSCs have been identified as playing a central role in the setbacks currently faced in clinical trials and research. Therefore, designing a system that can target them at the cellular and system levels is the most promising avenue in the evolution of therapeutic design. By reviewing the application of nanotechnology in CSCs, we hope to provide guidance for the design and in-depth study of nanotechnology drug carriers so that they can be applied in clinic to treat cancer.

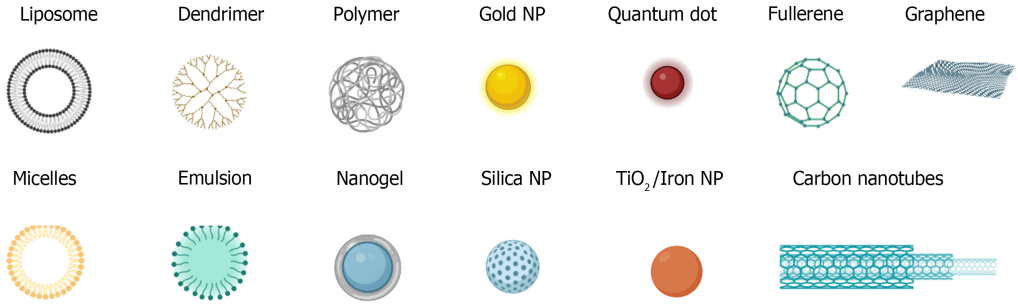
NANOTECHNOLOGY FOR CSC SORTING

To better understand the molecular basis of the contribution of CSCs to tumor progression, metastasis, and treatment resistance, many studies have identified biomarkers on the surface of CSC populations to distinguish them from the majority of tumor cells. Magnetic-activated cell sorting (MACS) is a CSC



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Figure 1 Different mechanisms of nanocarriers. A: Targeting through surface biomarkers; B: Targeting through the ligand-interacting domain on the nuclear receptor (created with BioRender).



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Figure 2 Different types of nanoparticles (created with BioRender). NPs: Nanoparticles.

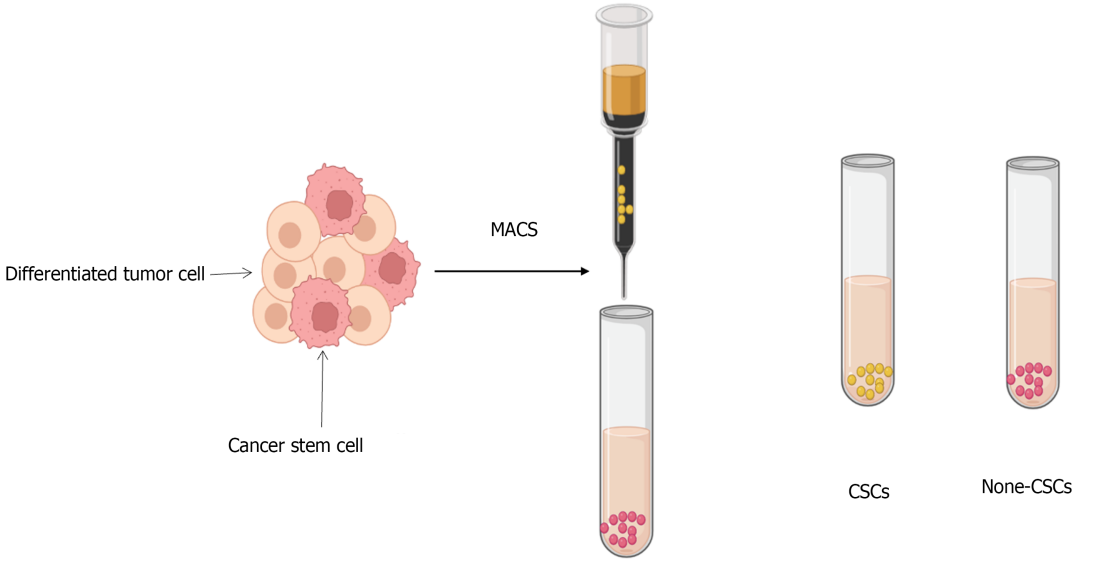
sorting technique. Magnetic NPs have unique magnetic activity and are one of the most actively studied NPs, usually ranging in diameter from 1 to 100 nanometers. Basically, magnetic NPs are classified as magnetically manipulated substances, consisting mainly of iron oxides or other metals (iron, nickel, or cobalt). MACS microbeads are superparamagnetic particles coupled to highly specific monoclonal antibodies. The cell surface-specific antigens are combined with stem cell markers such as CD44, CD133, and epithelial cell adhesion molecule (EpCAM), connected to the magnetic bead, and the cells labeled with the conjugated magnetic bead is separated by providing a uniform magnetic field to sort out the corresponding CSC population (Figure 3).

KINDS OF NANODRUG DELIVERY SYSTEMS

To date, nanomedicine has been focused on identifying alternatives to tumor therapy, with researchers focusing on the design of various nanocarriers, which have been used to load various anticancer drugs and herbal medicines to target tumor cells. In fact, according to the National Institutes of Health, there have been clinical trials involving the use of nanotechnology in CSC therapy (Table 1). Because the mechanisms of multidrug resistance are very complex and varied, targeting one mechanism alone does not address clinical needs. Nanocarriers have good stability, a high encapsulation rate and a high drug

Table 1 Clinical trials of advances in nanotechnology applied in cancer stem cells

Identifier	Trial name	Enrollment
NCT04907422	Cluster of differentiation 24-gold nanocomposite expression using quantitative polymerase chain reaction	60
NCT04907422	Nonconjugated cluster of differentiation 24 expression using quantitative polymerase chain reaction	60



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Figure 3 Magnetic-activated cell sorting (created with BioRender). CSCs: Cancer stem cells; MACS: Magnetic-activated cell sorting.

loading rate and have been proven to be effective carriers for genes and drugs delivered to tumor cells. This delivery induces apoptotic pathways and inactivates resistance genes for targeting tumor tissue to eliminate CSCs. According to the classification of nanotechnology used to target CSCs, they can be divided into Polymeric NPs(PNPs), liposomes, gold (Au) nanorods (GNRs), QDs, CNTs, GO, PTT, and magnetic fluid hyperthermia (Table 2).

PNPs

PNPs can enhance the therapeutic effects of drugs, reduce the drug resistance of CSCs, and improve the therapeutic effects of chemotherapy drugs. The following is a summary of the classification of PNPs through different antibody-ligand recognition (Figure 4), mesoporous silica ($mSiO_2$) NPs (MSNs), and other nanodrug delivery systems.

CD44

CD44[40,41] is a non-kinase transmembrane glycoprotein that is overexpressed in several cell types, including CSCs. Hyaluronic acid (HA) has become a research hotspot in drug release due to its simple chemical structure and inherent properties of targeting CD44. Kesharwani *et al*[42] designed a novel HA copolymerized styrene maleic acid and the effective anticancer agent 3,4-difluoromethylcurcumin to form nanomicelles. CD44+/CD133+/EpCAM+ pancreatic CSCs showed better uptake of HA-engineered nanomicelles and a better anticancer effect on CD44+ pancreatic CSCs. Furthermore, these nanomicelles significantly inhibited the expression of NF-κB, thereby reducing its proliferation and invasion. Debele *et al*[43] conjugated HA with hydrophobic 6-mercaptopurine (MP) and introduced doxorubicin (DOX) into colon cancer cells and colon CSCs through ligands. The inhibitory effect of the synthesized bisensitive polymer drug conjugate (HA-SS-MP) micelles on tumor growth was significantly higher than that of free drugs. In *vitro* cytotoxicity of HA-SS-MP and DOX-loaded HA-SS-MP micelles was great for CSCs (HCT116-CSCs). Gu *et al*[44] prepared mineralized HA-SS-tetracylecyl nanocarriers (M-HA-SS-TA) from oily, hydrophobic, and unstable sulforaphane (SFN), which showed a good response to highly reduced and weakly acidic tumor niches. The SFN nanomaterials (SFN/M-HA-SS-TA) can release SFN rapidly. Compared to free SFN, SFN/M-HA-SS-TA rapidly releases SFN in response to tumor niches, showing stronger inhibition of breast CSC (BCSC)-like properties (invasions, self-renewal, and tumor growth) *in vitro* and *in vivo*. However, magnetic fluid hyperthermia (MFH) mediated by anti-CD44 antibody-modified superparamagnetic iron oxide NPs (SPIONPs) can kill CSCs, and significantly inhibit the growth of transplanted Cal-27 tumors in mice[45].

Table 2 Nanocarrier systems for cancer stem cells

Nanocarrier	Therapeutic agent	Cancer type	Delivery model	Cell line	CSC marker	Ref.
PNPs	CDF	Pancreas	HA-SMA could be engineered to form nanomicelles with a potent anticancer agent, CDF	MiaPaCa-2, AsPC-1	CD44	[42]
	DOX	Colon	HA-SS-MP	HCT116	CD44	[43]
	SFN	Breast	SFN/M-HA-SS-TA	MDA-MB-231, Hs578t, MCF7, MCF10A	CD44	[44]
	SN-38	Colon	CD133Ab-NP-SN-38	HCT116	CD133	[47]
	ITGA5	Breast	ITGA5-targeting NPs	MDA-MB-231	-	[48]
	DOX, tariquidar	Breast	mSiO2-dPG	MCF-7	-	[55]
	DOX, tariquidar	Cancer cell	TTNV	Hela, A547	CD44	[56]
	DS	GMB	PLGA	U87MG, U251MG, U373MG	ALDH	[57,58]
	miR-148a, miR-296-5p	GMB	nano-miRs	GBM1A	Oct4/Sox2	[60]
	TPZ	Breast	MSN	MCF-7	CD133	[54]
	Cyclopamine	Prostate	HPMA	RC-92a/hTERT	CD133	[57]
	HPI-1	Liver, pancreas	HPI-1 was loaded with PLGA-PEG NPs	Huh7, Pa03C	CD133	[58]
	RNA drugs	Liver	ET-tMNV	hep3B	EpCAM	[52]
	DOX, Cyc	Breast	HA-SS-PLGA	MCF-7 MDA-MB-231	CD44	[64]
	LDN193189	Liver	Fe_3O_4-OA-DHCA-PEI-HA	-	CD44	[61]
	DOX	Breast	Gold NPs coupled to adriamycin by nitrogen condensation bond	sk-3	-	[62]
	ZnS	Breast	ZnS	MCF-7	CD44	[63]
	DOX, all-trans retinoic acid	Liver	PLGA-b-PEG	Hepa1-6	EpCAM	[51]
	Epiampicin, arsenic trioxide	Liver	Nanomicelles	hepG2	CD44	[97]
	Resveratrol	Oral	NP	H-357	-	[69]
	Cisplatin	Liver	PEI-modified MSN	Huh7	CD133	[53]
Liposomes	SAL, DOX	Liver	Nanoliposomes	HepG2	CD133	[73]
	TRAIL, SAL	Cancer cell	Liposomes	CSCs	-	[72]
	DOX	Liver	HLs	HepG2	EpCAM, CD133	[74]
	DOX, SAL	Liver	Redox-triggered dual-targeted liposomes	Huh7	CD133EpCAM	[48]
	DTXPL, TEL	Lung	DOX loaded with polyethylene glycolized liposomes	NCI-H460	CD133	[76]
Gold nanorods	-	Head, neck	SPIONPs	Cal-27	CD44	[45]
	PKF, SAHA	Breast	PKF and SAHA loaded on the corona of GNPs	MCF-7	-	[81]
	-	Liver	CD133-targeting aptamers modified on the surface of quantum dots and gold NPs with partially complementary paired	Huh7	CD133	[82]

			RNA (ssRNA)			
	DOX	Liver	EpCAM antibody conjugated onto lipophilic Au-NR	Hepa 1-6	EpCAM	[51]
	siRNA	Breast	Glu-NP	MDA-MB-231	GLUT1	[84]
	SAL	Breast	SAL-conjugated gold NPs, SAL-AuNPs	MCF-7	CD44	[86]
	HA	Breast	HA-capped AuNPs	MDA-MB-231	CD44	[86]
	Teleglenastat	Brain	Au-PEG-CD133-CB-839	GBM-1, NCH-644	CD133	[83]
GO	SAL	Ovarian	rGO-Ag	A2780	ALDH, CD133	[93]
CNTs	Paclitaxel	Breast	Multiwalled carbon nanotubes	HMLER	CD44	[45]
	-	brain	CD133 monoclonal antibody onto chitosan-modified CNTs	GBM tissues	CD133	[89]
	Paclitaxel, SAL	Breast	CD44 antibody hydrazone-linked onto SWCNT with pH-activated release system	MDA-MB-231	CD44+	[91]
	SAL	Glioblastoma	SAL-SWCNT-CHI-HA	AGS	CD44+	[90]

CD: Cluster of differentiation; CDF: 3,4-Difluorobenzylidene curcumin; anti-CD133 antibody-conjugated SN-38-loaded nanoparticles CNTs: Carbon nanotubes; CSCs: Cancer stem cells; Cyc: Cyclophosphamide;CB-839:telaglenastat; dPG: Dendritic polyglycerol; DOX: Doxorubicin; DTXPL: Docetaxel liposome; EpCAM: Epithelial cell adhesion molecule; ET: EpCAM-targeted; Fe_3O_4-OA-DHCA-PEI- HA: Mgnetic nanocubes were synthesized and modified with PEI and HA; GBM: Glioblastoma multiforme; GO: Graphene oxide; GLS1: Glutaminase 1; Glu-NP: Glucose-installed sub-50-nm unimer polyion complex-assembled gold nanoparticle; HA: Hyaluronic acid; HA-SMA: Hyaluronic acid conjugate of copoly (styrene maleic acid); HA-SS-MP: Hyaluronic acid-SS-mercaptopurine; HPMA: N-(2-hydroxypropyl) methylacrylamide; HLs: Hybrid lipo plastids;ITGA5: Integrin subunit alpha 5; MNVs: Milk-derived nanovesicles: M-HA-SS-TA: Mineralized HA-SS-tetracylecyl nanocarrier; miR: MicroRNA; $miSO_2$: Mesoporous silica; MSNs: Mesoporous silica nanoparticles; MWCNTs: Multiwalled carbon nanotubes; NPs: Nanoparticles; PEG: Polyethylene glycol; PKF: PKF118-310; PLGA: Poly(L-lactide-co-glycolide); PNPs: Polymeric nanoparticles; PDT: Photodynamic therapy; ROS: Reactive oxygen species; rGO-Ag: Reduced graphene oxide–silver nanocomposite; SAHA: Vorinostat; SAL: Salomycin; SFN: Sulforaphane; siRNA: Small interfering RNA; SWCNT: Single-walled carbon nanotubes; SPIONPs: Superparamagnetic iron oxide NPs; SAL-SWCNT-CHI-HA:CHI-coated SWCNTs loaded with SAL and functionalized with HA;TEL: Telmisartan; TPZ: Tirapazamine; TRAIL: Tumor necrosis factor-associated apoptosis-inducing ligand; TTNV: Targeted theranostic nano vehicle; ZnS: Zinc sulfide.

CD133

The CD133 antigen is a five-fold transmembrane single-chain glycoprotein that exists on the surface of tumor stem cells. It is a key molecule that regulates the fate of stem cells and a functional marker of stem cells. It can be used to detect and isolate CSCs in various solid tumors[46]. NPs with SN-38 (anti-CD133 antibody-conjugated SN-38-loaded nanoparticles (CD133Ab-NPs-SN-38)), a topoisomerase inhibitor conjured by anti-CD133 antibody, targets CD133+HCT116 cells and inhibits colony formation. The CD133-targeted NP delivery system can eliminate CD133-positive cells[47]. The Wnt/β-catenin pathway plays critical roles in CSC generation and maintenance as well as in normal stem cells. Integrin subunit alpha 5-targeting NPs attenuate β-catenin and significantly reduce triple-negative BC (TNBC) metastasis and may provide a facile and unique strategy of specially attenuating β-catenin *in vivo* for treating metastatic TNBC[48]. Codelivery of DOX and salomycin (SAL) REDOX-triggered double-targeted liposomes CEP-LP@S/D can be used for the synergistic treatment of liver cancer. The system is based on the binding of CD133- and EpCAM-targeting peptides to form Y-shaped CEP ligands that anchor to the liposome surface and allow selective targeting of CD133EpCAMlCSC[49].

EpCAM

EpCAM, considered to be a homogenous cell-cell adhesion glycoprotein, is expressed in epithelial and circulating tumor cells (CTCs), as well as CSCs[50], and is involved in the regulation of cell adhesion, proliferation, migration, dryness, and the epithelial-to-mesenchymal transition (EMT) of cancer cells. Locatelli *et al*[51] coloaded GNRs and adriamycin (Adr) to label EpCAM by targeting the surface of CSCs and killed CSCs under laser ablation. Ishiguro *et al*[52] used RNA nanotechnology to pair milk source nanocapsules (MNVs) with synthetic oligonucleotide aptamers that could bind to EPCAM with high affinity and specificity and loaded small interfering RNA (siRNA) onto β-catenin. The EpCAM-targeted (ET) therapeutic MNV has been prepared. These ET-TMNVs can target EPCAM-positive stem cell populations and effectively release siRNAs within cells that inhibit β-catenin expression and tumor growth. For polymer nanomicelles (GNRS-1/curc@Pms) made from biocompatible poly(L-lactide-co-

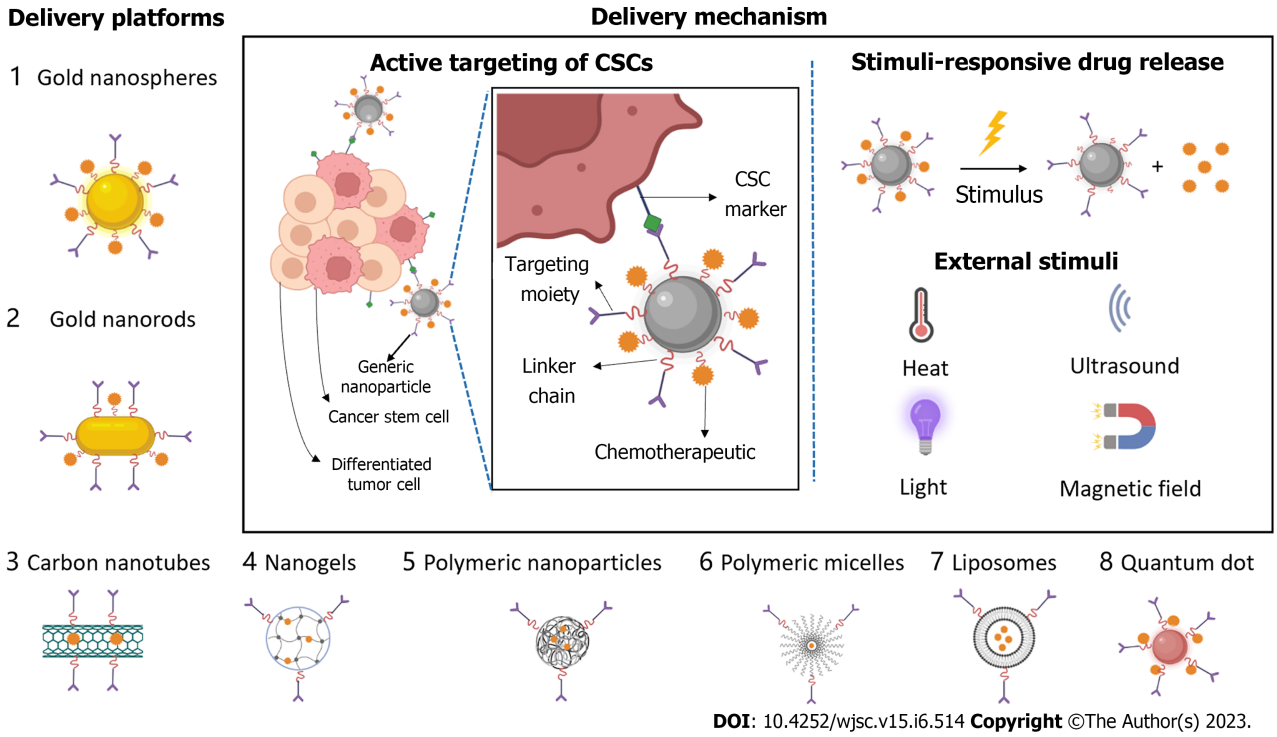


Figure 4 Nanoparticle-mediated targeted drug delivery to cancer stem cells (created with BioRender). CSCs: Cancer stem cells.

glycolide)-block-poly(ethylene glycol) (PLGA-b-PEG) copolymer as drug carriers for Adr and GNRs, when Adr/GNRs@Pms-antiEpCAM with EpCAM antibodies are modified, they are delivered to specific tumor stem cells and increase the drug concentration at the tumor site, thereby killing the entire tumor stem cell population[51].

MSN

MSN drug loading[53] can significantly enhance the cytotoxicity of anticancer drugs with low potency. Therefore, the positive polymer polyethylenimine (PEI) is usually coated and chemically modified to introduce a positive charge on the surface of MSNs, which can effectively bind the DNA structure, siRNAs, and other nucleic acids, thus enhancing their uptake by cells. The PEI-modified MSN was used for double delivery of the chemotherapy drug cisplatin, and the DNA encoding the hepatocyte nuclear factor 4 alpha transcription factor was used for gene therapy of liver cancer. This therapy inhibited the proliferation of CD133+ HUH7 cells, reduced the proportion of CSCs, and reduced the expression of dry-related genes. The nuclear targeting system of MSNs by Li *et al*[54] can directly target CSCs and enter the nucleus through anti-CD133 surface modification and heat-triggered exposure of the TAT polypeptide under an alternating magnetic field (AMF). Combined with hyperthermia and hypoxia-activated chemotherapy, the release of nuclear-targeted drugs eventually leads to complete apoptosis of CSCs. CSC-specific targeting of $mSiO_2$-dendritic polyglycerol (dPG) nanocarriers delivered the chemotherapy drug DOX and the P-glycoprotein (P-gp) inhibitor tariquidar (Tar) to reverse multidrug resistance (MDR) and enhance chemotherapy efficacy in bCSCs[55]. A targeted theranostic nano vehicle (TTNV) was designed using manganese-doped MSNs with an ideal surface area and pore volume for loading optimized ratios of antitumor DOX and the drug efflux inhibitor Tar. This strategically framed TTNV, which is chemically coupled with folic acid and HA, as a dual-targeted entity to promote folate receptor (FR)-mediated cancer cells and CD44-mediated CSC uptake, respectively[56].

Other nanodrug delivery systems

The N-(2-hydroxypropyl) methylacrylamide (HPMA) cyclopamine delivery system, as a selective macromolecular therapy for CSCs, has improved drug solubility and reduced systemic toxicity, allowing it to effectively remove CD133+ tumor stem cells in prostate tumors. HPI-1 was loaded with PLGA-PEG NPs to solve the problem of poor water solubility and effectively eliminate CD133+ CSCs in pancreatic and liver cancer[57,58].

GBM stem cells (GSCs) are the leading cause of chemotherapy failure in GBM. PLGA NP-encapsulated disulfiram effectively inhibited *in situ* and subcutaneous GSC xenografting in mouse models[59]. Although we are increasingly understanding GBM at the molecular level, treatment options are still limited. We have developed bioreducible poly(β-aminoester) NPs that exhibit high intracellular delivery efficacy and low cytotoxicity that have the ability to escape from endosomes and facilitate the

release of cytoplasmic environment-triggered cargo for the delivery of microRNAs to tumor-reproducing human CSCs[60]. In the study by Wang *et al*[61], a high temperature thermal breakdown approach was used to create composite magnetic nanocubes modified by PEI and HA. The ferric oxide nanocubes recognized hepatocellular carcinoma (HCC) stem cells *via* receptor-ligand binding of HA and CD44 (HA receptor), while loading small molecule LDN193189 inhibited the expression of stemness-related genes octamer-binding transcription factor 4 and Nanog. Double pH-sensitive polymeric drug-conjugated NPs showed enhanced inhibition of the progression of drug-resistant SK-3 CSCs, whereas AuNPs conjugated to Adr *via* nitrogen reduction bonds overcame resistance by avoiding P-gp efflux, thereby delivering more DOX to tumor stem cells. This mechanism resulted in the elimination of all tumor cell subpopulations and prevented the potential reaggregation of CSCs[62]. Tran *et al*[63] inhibited the transfer of MCF-7-SCs by inhibiting the EMT process, revealing the potential role of nanozinc sulfide in inhibiting the migration and invasion of bCSCs, which opened up a new way of thinking and provided a potential approach for the treatment of BC.

It has also been shown that the constructed amphiphilic polymer, HA-cystine-PLGA, can be used to deliver DOX and cyclopamine to CD44-high-expressing bCSC subpopulations and a large number of BC cells, and allow on-demand release. The dual delivery particles effectively reduce the number and size of tumor spheroids, and HA shows targeting effects on bCSCs[64].

Gao *et al*[65] proposed a novel intravenous photodynamic therapy (PDT) platform based on stem cell simulation of SUCNPs@mSiO2 for tumor targeting and enhanced PDT efficacy. Due to the coating of the stem cell membrane, the prepared nano SUCNPs@mSiO2 has good stability and biocompatibility. Moreover, it has the ability to be intravenously injected and escape immunity, extend the blood circulation time, and improve the tumor targeting function of stem cells, paving the way for the development of photosensitizers with bioactive cell components, such as SUCNPs@mSiO2, as a platform for targeting PDT. Sorafenib and glucose oxidase were integrated into the N-acetylgalactosamine-modified zeolite imidazolate framework (ZIF-8), designated SG@GR-ZIF-8, and this nano preparation exhibited significant antimetastatic HCC activity against C5 WN1 cells, a liver CSC-like cell line with tumorigenic and lung metastatic activity[66]. PT chemotherapy synergy was achieved by loading crocodile-based PT agents with natural cytotoxic heat shock protein (HSP) inhibitors that had high potency biradical characteristics into a redox-sensitive chitosan (CHI) matrix. Within solid tumors, PEG shells that prevent nano-assembled mono nuclei from phagocytosing were cleared quickly to expose the positively charged CHI, and the isolated peptide iRGD was further activated. This step drives tumor penetration of CHI NPs and allows CSC targeting by selective identification of CD44 proteins. Due to the inhibition and chemosensitization of HSPs, the designed nano assembly can completely eliminate CSCs and non-CSCs, thereby inhibiting tumor growth and metastasis[67]. Chen *et al*[68] used PLGA/d-alpha-tocopherol PEG 1000 succinate (TPGS) NPs for the first time with the combination of chemotherapy drugs and ATP-binding cassette (ABC) transporter inhibitors (ATIs) and used TPGS and PLGA to prepare NPs. Due to the overexpression of ABC transporters in CSCs, the combination of ATIs and chemotherapy drugs can overcome the multidrug resistance of CSCs. PLGA/TPGS NPs were prepared for the codelivery of DOX and extracellular lipopeptide composite to reach the tumor site with an optimized synergistic ratio, and resveratrol NP reduces cancer activity and decreases inflammation in CSC-rich oral cancer[69]. In addition to active targeting strategies, relying on intelligent changes in nanodrug size to penetrate deep into tumor tissues and improve the clearance rate of CSCs is also an important strategy for efficient reversal of MDR. On this basis, a special morphologically tunable nanodrug was developed, which integrated chemotherapy and immune checkpoint blocking therapy for large tumor cells and CSCs into drug delivery systems. As NPs are transferred from circulation to tumor tissue, particle size shrinks, favoring pharmacokinetics and cellular uptake while enabling sequential drug release when needed. The nanomedicine reduced the proportion of CSCs and enhanced the therapeutic effect on tumors, thereby prolonging the survival time of mice[70].

Liposomes

Liposomes are spherical vesicles consisting of one or more concentric phospholipid bilayer layers that enclose a water core. Liposomes are both nontoxic and biodegradable, making them powerful drug delivery systems. They improve the therapeutic effect of drugs by stabilizing compounds, overcoming barriers to cell and tissue uptake, and increasing the biological distribution of drugs at target sites in the body while minimizing systemic toxicity[71].

Tumor necrosis factor-associated apoptosis-inducing ligands (TRAILs) have received much attention for their favorable ability to activate apoptosis in cancer cells by interacting with death receptors (DRs). However, CSC-like cells lack or express low levels of the death receptor DR and are highly resistant to apoptosis mediated by TRAIL, limiting therapeutic efficacy. The liposomal component of the plasmid DNA encoding TRAIL and SAL enables cancer cells to express TRAIL as protein generators, and more importantly, to upregulate DR expression through SAL-induced CSCs, making drug-resistant CSCs sensitive to TRAIL-triggered apoptosis. This liposome-based programmable drug codelivery system shows the potential to effectively eliminate CSCs and inhibit CSC-rich tumor growth in mouse models of colon tumors *in situ*[72].

Gong *et al*[73] prepared and characterized SAL-loaded nanoliposomes (SLNs), DOX-LNs (DLNs) and SAL and DOX simultaneously delivered nanoliposomes (SAL/DOX). Novel SDLNs and SLN-DLNs are used to deliver SAL and DOX to HCC cells and CSCs. Hybrid lipo plastids (HLs) are nanosized liposome particles that can be prepared by the ultrasonic mixing of capsule and micelle molecules in buffer solution. The inhibitory effect of HLs on the growth of the CSC subpopulation of HCC cells (HepG2) has proven that HLs are a new type of nanomaterial that can be used to target CSCs in the treatment of HCC[74]. Dual-targeted liposomes CEP-LP@S/D selectively target CD133EpCAMlCSCs. Upon arrival at CSCs, CEP-LP@S/D liposomes undergo cytoplasmic endocytosis, in which high concentrations of glutathione break the disulfide bonds, thereby degrading the liposomes[75]. The combination of docetaxel liposome (DTXPL) and telmisartan (TEL) increased the cytotoxicity of H460 WT 3D cells two-fold. In H460 WT and DTX-resistant CD133+ xenograft tumor models, tumors treated with the combination of DTXPL and TEL showed reduced tumor volume, increased apoptosis, and downregulated CSC marker expression[76].

Lipid nanocapsule (LNC) encapsulated with paclitaxel and SAL can induce apoptosis in bCSCs, which is enhanced by the codelivery of paclitaxel and SAL. Synergistic cytotoxic effects on cells, non-bCSCs, and bCSCs, as well as effective reduction in tumor mammary globular growth by encapsulating both paclitaxel and SAL, suggest that LNCs have potential for the treatment of BC[77].These studies demonstrate the great potential of nanoliposome-targeted drug delivery to tumor stem cells.

GNRs and QDs

GNRs are pseudo-one-dimensional rod-like NPs, which have become one of the emerging materials of interest in recent years due to their anisotropic shape and adjustable plasma properties[78]. QDs, also known as nanocrystals, are NPs composed of II-VI or III-V elements that are rich in energy electrons and quantum-confined holes[79]. QDs are widely studied as biomedical imaging probes due to their unique optical and electronic properties. They are usually nanoscale semiconductor microcrystals and are widely used to improve the efficacy of fluorescent markers in bioimaging[80].

PKF118-310 (PKF) and vorinostat (SAHA) loaded on GNP corona Protein corona (PC), a AuNP system with protein corona coating for simultaneous delivery of PKF and SAHA resulted in a reduction of stem cell populations and Snail marker in MCF7 bCSCs[81]. Coloaded with GNRs and Adr, EpCAM was labeled by targeting the surface of CSCs to kill CSCs under laser ablation[54]. A novel fluorescent on nano aptamer sensor for the quantitative detection of CD133 has also been designed. By hybridization of CD133-targeting aptamers modified on the surface of QDs and AuNPs with partially complementary paired RNA (single-stranded RNA), the distance between the QDs and AuNPs is shortened, resulting in fluorescence resonance energy transfer between them so that the fluorescence of the QDs is quenched by AuNPs. The QD fluorescence recovery aptamer sensor is a sensitive and reliable sensor for the detection of CD133, providing a simple and promising detection tool for CSC markers[82]. Inhibition of glutamine decomposition may be an effective anti-CSC strategy. The glutaminase 1 (GLS1) inhibitor telaglenastat (CB-839) was loaded into Au pegylated NPs (Au-PEG-CD133-CB-839) equipped with covalently coupled CD133 aptamer. In an *in vitro* exposure to a CD133-positive brain tumor model, Au-PEG-CD133-CB-839 reduced the activity of CD133-positive cancer cells in a dose-dependent manner [83]. Glucose-installed-targeted NPs (Glu-NPs) demonstrated higher cellular uptake of siRNA payload in globular BC (MBA-MB-231) cell cultures compared to glucose-uncoupled control NPs (MeO-NPs). Glu-NPs, a promising nanocarrier design for CSC-targeted cancer therapy, caused significantly enhanced gene silencing in CSC-rich MDA-MB-231 tumor tissue in situ after systemic administration to tumor-bearing mice[84]. Liu *et al*[85] reported that SAL was conjugated with biocompatible AuNPs coated with PEG showed specific targeting ability and high antitumor efficacy against CD24 Low/CD44high subsets in BC cells. The biodegradable naturally negatively charged polysaccharide HA is used to synthesize AuNPs, while HA can act as a capping agent based on its hydroxyl group, thereby stabilizing newly produced AuNPs. HA-functionalized AuNPs exhibit excellent physical properties and high cell uptake and have a strong inhibitory effect on MDA-MB-231 cells and CSCs. In particular, synergistic chemothermal therapy with HA-capped AuNPs combined with NIR irradiation has shown more effective therapeutic results in terms of cytotoxicity, apoptosis, and necrosis compared with chemotherapy alone[86].

The disadvantage of metallic nanomaterials lies in their toxicity. Reactive oxygen species(ROS) generation, influence on cell structures and other characteristics of metallic NPs toxicity are similar to other NPs, and the toxicity is related to size, shape, dimensionality, surface charge. Therefore, metallic NPs should be carefully examined before used in clinic.

CNTs

The high surface-to-volume ratio, enhanced electrical conductivity, strength, biocompatibility, ease of functionalization, and optical properties of CNTs have led to their consideration as novel drug and gene delivery vehicles. CNTs are cylindrical tubes formed from sp2 hybrid carbon atoms, which can range in size from 1 nanometer to several microns. CNTs can be divided into single-walled CNTs (SWCNTs) and multiwalled CNTs (MWCNTs) according to the number of layers formed in them[87].

BCSCs have strong resistance to traditional hyperthermia, while PTT mediated by amino-modified multiwalled CNTs on the surface can effectively kill bCSCs[88]. CD133 is a currently recognized CSC marker for GBM. CNTs can be targeted to CD133-positive cells of GBM (GBM-CD133+) through a CD133 antibody. Wang *et al*[89] grafted a CD133 monoclonal antibody onto CHI-modified CNTs. Then, CSCs were effectively killed by PTT. The gastric CSC-specific targeted drug delivery system (SAL-SWNT-CHI-HA complex) is also based on CHI-coated SWCNTs loaded with SAL and functionalized with HA to selectively eliminate gastric CSCs[90]. SWCNTs facilitate active targeting due to their needle shape, significant transmembrane penetration, EPR effects, high drug loading capacity, and ease of functionalization of biological agents (*i.e.* antibodies). Surface functionalization with polymers such as PEG helps overcome the limitations of the original NTs, providing good water solubility, prolonging blood circulation, and reducing the toxic effects of SWCNT-based nanocarriers. The potential therapeutic effect of the combination of paclitaxel and SAL in BC and CSCs is mediated by a pH-responsive release mechanism near the acidic tumor microenvironment *via* a hydrazone junction[91].

GO

Driven by the achievements of CNTs, graphene, and GO are new types of drug nano carriers used to support a variety of therapeutic drugs, anticancer drugs, insoluble drugs, antibiotics, antibodies, *etc.*

GO is alleged to specifically target CSCs rather than normal cells and to induce CSC differentiation and inhibit tumor sphere formation in multiple cell lines, including breast, ovarian, prostate, lung, pancreas, and GBM cell lines, by inhibiting several key signaling pathways, including the Wnt, Notch, and signal tranducer and activator of transcription signaling pathways[92].

Choi *et al*[93] synthesized reduced graphene-silver nanocomposites (rGO-Ag) using the R-phycoglobin biomolecular mediated method. These composites have a toxic effect on ovarian CSCs (OvCSCs) and can reduce the survival rate of OvCSCs by decreasing the mitochondrial membrane potential and expression of apoptotic genes, leading to mitochondrial dysfunction and possibly apoptosis. RGO-Ag may be a novel nanotherapeutic molecule for specifically targeting highly tumorigenic ALDH+CD133+ cells and clearing CSCs.

PTT

PTT uses metal NPs to eradicate CSCs and stimulate a hyperthermal physiological response by converting light into heat[94]. MSNs under an alternating magnetic field eliminate CSCs by blocking the hypoxia signaling pathway and heating, thus effectively inhibiting tumor growth[56,95]. Burke *et al*[86] found that bCSCs have strong resistance to traditional hyperthermia, and PTT mediated by amino-modified multiwalled CNTs on the surface can effectively kill bCSCs. Wang *et al*[87] grafted CD133 monoclonal antibody onto CHI-modified CNTs. CD133 is currently recognized as a CSC marker for GBM, and CNTs can be targeted to GBM-CD133+ through the CD133 antibody. Then the CSCs were effectively killed by PTT. NPs loaded with bimodal metal cages and photodynamic therapys (PDT) PDTs target CSCs by reducing cell mobility under laser irradiation[96]. Researchers developed a CSC-specific-targeted, retinoic acid (RA)-loaded Au nanostar-dPG nanoplatform for the efficient eradication of CSCs. The nanocomposites possess good biocompatibility and exhibit effective CSC-specific multivalent-targeted capability due to HA decorated on the multiple attachment sites of the bioinert dPG. With the help of CSC differentiation induced by RA, the self-renewal of bCSCs and tumor growth were suppressed by the high therapeutic efficacy of PTT in a synergistic inhibitory manner[97]. Based on PTT properties of CNTs and metallic materials, nanoplatform functions with chemotherapy and PTT can be designed to produce synergistic effects.

Researchers have utilized MnO_2@Ce6 NPs and a PDT-based approach that improved tumor microenvironment-related therapeutic resistance by modulating the tumor microenvironment with excess hydrogen protons and water, resulting in subsequent radiation of CSCs[98]. Haldavnekar R *et al*. introduced nickel-based functionalized nanoprobe-facilitated surface-enhanced Raman scattering for the prediction of cancer dissemination by CSC-based surveillance[99]. MoS_2 nanosheets and a moderate PTT treatment were applied to target a CSC surface receptor (*i.e.* CD44) and modulate its downstream signaling pathway. The treatment showed attenuated self-renewal capacity, more response to anticancer drugs, and less invasiveness[100].

Although PTT can inhibit tumor growth by eliminating tumor stem cells, it is usually difficult to completely eradicate tumors due to the limited penetration depth of near-infrared (NIR) light. Therefore, combining PTT with other therapies is expected to overcome these challenges.

Magnetic fluid hyperthermia

MFH uses the good magnetic thermal conversion ability of magnetic NPs under the influence of an external alternating magnetic field to rapidly heat the internal tumor, forming a high-temperature zone, to kill tumor cells or induce their apoptosis[101].

When anti-CD44 antibody-modified SPIONPs are prepared, SPIONP-mediated hyperthermia can kill CSCs, and MFH significantly inhibits the growth of transplanted Cal-27 tumors in mice[59]. Nuclear targeting systems coated with MSNs of superparamagnetic iron oxide-based NPs can directly target CSCs and can be used to combine thermotherapy and hypoxia-activated chemotherapy with nuclear-

targeted drug release under an alternating magnetic field, ultimately leading to complete apoptosis of CSCs[73]. Biomimetic magnetic NPs induce apoptosis of stress-escape CSCs and inhibit their proliferation and metastasis *in vitro* and *in vivo* by the combined therapeutic effects of DOX chemotherapy and magnetic MSNs MFH under the action of an alternating magnetic field[102]. Antibody-modified NPs targeting lung CSCs enhance cellular uptake *in vitro* and prolong tumor accumulation *in vivo*. Due to the combined effects of hyperthermia and chemotherapy treatment, up to 98% of lung CSCs are killed by AMF within 30 min of application outside the body. In *in vivo* models, this combination therapy significantly inhibited tumor growth and metastasis in mice carrying lung CSC xenografts with minimal side effects and adverse reactions[103]. In summary, MFH shows great potential in targeting tumor stem cells.

FUTURE PERSPECTIVES AND CHALLENGES

The discovery of CSCs has made us gradually realize the complexity of tumors. CSCs are the roots of tumor occurrence, drug resistance, and postoperative recurrence. Therefore, the eradication of CSCs is of great significance for the treatment of cancer. At present, theoretical research on tumor stem cells is still in the initial stage, and many problems have not been solved. For example, CSCs and normal stem cells have very similar self-renewal, multidirectional differentiation, signaling pathways, and cell surface markers. How to effectively kill CSCs without damaging normal stem cells needs further research. Some regulatory mechanisms and biological behaviors of tumor stem cells have not been fully clarified. It is believed that with the continuous deepening of CSC research, more targets and a theoretical basis will be provided for clinical treatment.

In addition to effective drugs targeting CSCs, it is also necessary to consider the heterogeneity of CSCs to eliminate tumor cells and CSCs more effectively, inhibit recurrence and improve the survival rate of patients. Although great progress has been made in research on the molecular mechanism of cancer, cancer detection and treatment, and the treatment methods have been continuously improved, there is still a lack of effective treatments for cancer. The targeting, slow release, good biocompatibility, and stability of nanomaterials will play a huge role. In addition, nanotargeting technology is used to track the biological characteristics of CTCs[104]. The physical and chemical properties of each component of the tumor microenvironment are different from those of normal tissues, and the tumor microenvironment plays a huge role in the process of tumor occurrence and development, which makes the tumor microenvironment an important target for nanomaterial-targeted therapy[105]. Although there are still several difficulties in the wide application of nanomaterials in clinical practice, the most important of which is biosafety, there is still no convincing evidence that nanomaterials can be effectively metabolized in the body without accumulation and causing toxic side effects. In addition, how to improve the linking efficiency of targeted molecules and nanomaterials, the activity of targeted molecules after linking, the stability of the binding of targeted substances and drug carriers, and the metabolic pathways and toxicity of nanomaterials *in vivo* have not yet been solved. However, the strong ability shown in the early stages makes us hypothesize that nanomaterials for CSC-targeted therapy have broad prospects as a new generation of tumor treatment. With the continuous deepening of CSC research and the rapid development of nanotechnology, these fields will potentially overlap and provide a strong guarantee for cancer treatment.

CONCLUSION

Cancer is a huge barrier for researchers due to its high mortality rate and resistance to treatments. For example, multidrug resistance, recurrence, and the spreading nature of cancer cells make cancer extremely difficult to treat. CSCs are the main reason for inducing the characteristics of drug resistance and the regenerative ability of tumor cells. Therefore, the targeted system of cancer treatment began to turn to stem cell research. As an emerging field, nanotechnology is mainly applied to materials and carrier structures with diameters between 1 and 100 nanometers. Because nanomaterials have similar dimensions, they differ in composition, structure, hydrophobicity, magnetism, immunogenicity, and other properties. CSC therapies based on these unique properties have been extensively studied, but only a few have entered clinical trials. To better improve clinical translation, further research on targeted drug delivery of nanocarriers is needed to reduce toxicity, enhance permeability and retention, and minimize the shielding effect of the protein corona. By rationally designing and constructing new NDDS to accurately target CSCs that have developed drug resistance, the efficiency of reversing multidrug resistance and inhibiting tumor growth can be effectively improved, providing a tremendous opportunity to improve cancer treatment or prognosis, which will ultimately improve the survival rate of cancer patients.

FOOTNOTES

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REVIEW

Neural lineage differentiation of human pluripotent stem cells: Advances in disease modeling

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Abstract

Brain diseases affect 1 in 6 people worldwide. These diseases range from acute neurological conditions such as stroke to chronic neurodegenerative disorders such as Alzheimer's disease. Recent advancements in tissue-engineered brain disease models have overcome many of the different shortcomings associated with the various animal models, tissue culture models, and epidemiologic patient data that are commonly used to study brain disease. One innovative method by which to model human neurological disease is *via* the directed differentiation of human pluripotent stem cells (hPSCs) to neural lineages including neurons, astrocytes, and oligodendrocytes. Three-dimensional models such as brain organoids have also been derived from hPSCs, offering more physiological relevance due to their incorporation of various cell types. As such, brain organoids can better model the pathophysiology of neural diseases observed in patients. In this review, we will emphasize recent developments in hPSC-based tissue culture models of neurological disorders and how they are being used to create neural disease models.

Key Words: Induced pluripotent stem cells; Astrocytes; Oligodendrocytes; Microglia; Brain organoids; Assembloids

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Core Tip: This review discusses recent advances in the field of disease modeling using human-induced pluripotent stem cell-derived neural cell types as well as organoids. It also discusses challenges that exist with current approaches, in addition to considerations for possible improvements that will further advance the field of disease modeling.

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INTRODUCTION

Human pluripotent stem cells (hPSCs) include both human embryonic stem cells (hESCs) and human-induced PSCs (hiPSCs). Current *in vitro* disease models that use hiPSCs begin with skin or blood cells that have been reprogramed with the four transcriptional elements octamer-binding transcription factor 4, SRY-box 2, Krüppel-like factor 4, and MYC[1]. Through differentiation, these hPSCs are the starting material to create models for different organs including the brain[2], kidney[3], liver[4,5], lung[6], and pancreas[7]. These models are able to simulate a "disease-in-a-dish", mimicking different disease phenotypes *in vitro*[8,9]. Both genetically modified hiPSCs and patient-derived hiPSCs can generate disease models[10-12]. These models are advantageous because of their accessibility, quick processing, and species-specific human attributes. Patient-derived hiPSCs can also be used to test personalized medicine approaches to effectively model gene mutations and chromosomal abnormalities. To study neurological diseases, scientists have generated multiple neural cell types from hPSCs including neurons[13], astrocytes[14], and oligodendrocytes (OLs)[15]. In the past decade, advancements in disease modeling and tissue engineering have also led to the "brain organoid" model[16]. Brain organoids are self-assembled structures that resemble the fetal human brain and are composed of progenitor, neuronal, and glial cells. A related system is the spheroid, a circular aggregate of cells that may reflect biological properties of an organ system but ultimately lacks structural complexity. Perhaps the most cutting-edge form of modeling technology in stem-cell research is the assembloid, which are three-dimensional (3D) structures made by fusing and integrating two or more cell types or organoids from different organ culture protocols[17]. These assembloids can model the organ crosstalk interactions that occur across physiological systems in the human body.

In this review, we discuss recent advancements in the field of disease modeling using hiPSC-derived neural cell types as well as organoids. We also discuss challenges that exist with current approaches, in addition to considerations for possible improvements that will further advance the field of disease modeling.

NEURAL CELL TYPE DIFFERENTIATION FROM HPSCS

Neural progenitors

The development of the mammalian brain initially occurs at the gastrula stage when the ectoderm differentiates to form the neural tube. This process is called neural induction, wherein neural tube cells become neural progenitors[18,19]. These progenitors subsequently give rise to specific neuronal subtypes along the rostral-caudal axis and dorsal-ventral axis[20]. Protocols for neural progenitor differentiation from hPSCs have been developed that reflect this neural induction principle (Table 1). In 2001, the first protocol of neural progenitor differentiation from hPSCs was developed using the embryoid body (EB) method, which was a combined two-dimensional (2D) monolayer and 3D suspension culture [21]. Without extrinsic factors, the EB method mainly derived dorsal forebrain cortical neurons. In 2008, advances in the EB method eventually gave rise to a complete 3D culture system called the serum-free floating culture of EB-like aggregates with quick aggregation (SFEBq)[22]. The SFEBq method generated neural tissues with self-organized structure using hPSCs, paving the way for the development of more complex systems such as brain spheroids and organoids. Following this advance, in 2009 the dual SMAD inhibition method was developed, which successfully directed over 80% of hESCs to induce neural differentiation[23]. The dual SMAD inhibition method was initially intended for 2D monolayer culture by inhibiting the bone morphogenetic protein (BMP) and transforming growth factor beta (TGF-β) signaling pathways, but it has been widely applied to 3D culture for neural progenitor differentiation

Table 1 Comparison of methods for neural induction from human pluripotent stem cells

Method	Neural induction outcomes	Significance	Ref.
Embryoid bodies; selected neural rosettes; 2D and 3D culture	Neural tube-like rosettes stained with Nestin, Musashi-1 and NCAM; positive neuronal markers MAP2 and TUJ1 expression	First study of neural progenitor differentiation from hPSCs	Zhang *et al*[22], 2001
SFEBq aggregate; sorting cells; 3D culture	Self-organized structure with four distinct zones: ventricular, early and late cortical-plate, and Cajal-Retzius cell zones	Pure 3D culture, provides the basis for the brain organoid method	Eiraku *et al*[23], 2008
Dual SMAD inhibition; 2D monolayer culture	Complete neural conversion of > 80% of hESCs	Mostly wild used method; also enables neural induction in 3D culture	Chambers *et al*[24], 2009
Dual SMAD inhibition combined with retinoid signaling; 2D monolayer culture	More than 95% of hPSCs were PAX6 and OTX1/2 cortical progenitor cells in 15 d	Improved the dual SMAD inhibition protocol and higher neural induction efficiency	Shi *et al*[62], 2012
Cortical organoid/spheroid; 3D culture	Form layered structure tissues partially mimicking human cerebral cortex	Mostly brain-like tissue with some functions	Lancaster *et al*[17], 2013; Paşca *et al*[26], 2015; Qian *et al*[27], 2016
Dual SMAD inhibition combined with Wnt, FGF and Notch inhibition	Generate functional cortical neuron in 16 d	Improved the dual SMAD inhibition protocol and accelerated neural induction	Qi *et al*[28]

EB: Embryoid body; FGF: Fibroblast growth factor; hPSCs: Human pluripotent stem cells; MAP2: Microtubule-associated protein 2; NCAM: Neural cell adhesion molecule; OTX: Orthodenticle homeobox; PAX6: Paired box 6; SFEBq: Serum-free floating culture of EB-like aggregates with quick aggregation.

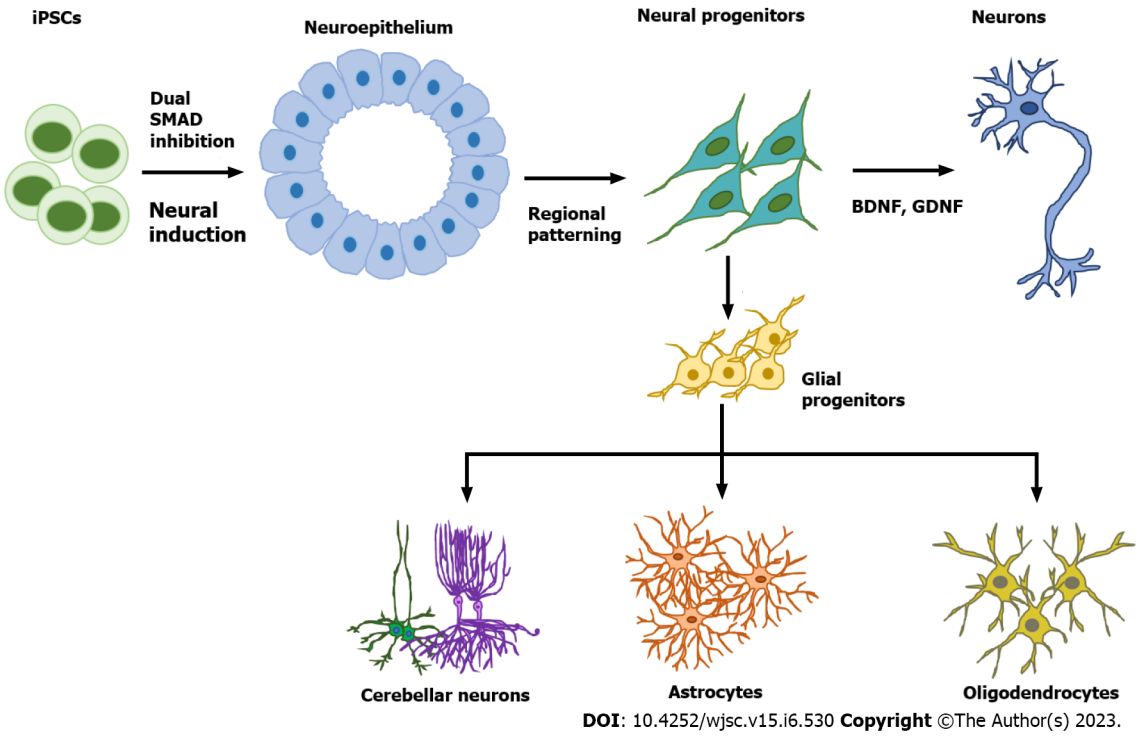
from hPSCs. It is worth noting that both the SFEBq and dual SMAD inhibition methods can enable the generation of cortical spheroids and organoids[24-26]. In 2017, Studer's group modified the dual SMAD inhibition protocol to also block mitogen-activated protein kinase (MAPK), fibroblast growth factor (FGF), and Notch signaling, thereby accelerating forebrain cortical neuron derivation[27]. Although these protocols yield primarily deep-layer cortical neurons, deriving upper layer cortical neurons such as L2/3 and L4 cells is still a challenge.

Astrocytes

Astrocytes are star-shaped populations of glial cells that help maintain homeostatic balance and support neuron growth within the central nervous system. There are two distinct groups of astrocytes: The highly branching protoplasmic astrocytes of the grey matter and the fibrous astrocytes found in the white matter that interact with OLs and axons[28]. Activated astrocytes can release neuroinflammatory cytokines and chemokines that mediate intercellular communication with microglia and invoke various neuroinflammatory responses. Similar to neurons, there are many subtypes of astrocytes depending on their location, morphology, molecular signature, and physiological function. The differentiation of glia cells from hPSCs usually takes more time and is more complicated than differentiating a neuron (Figure 1).

During brain development, astrocytes differentiate from radial glia or neural progenitors at the subventricular zone. It is currently unknown what signaling regulates the regional identity of astrocytes. The differentiation of astrocytes is usually initiated by inhibition of dual SMAD signaling using small molecules or by the EB method to generate neuroepithelial cells. Glial progenitors expressing nuclear factor IA (NFIA), S100β, and cluster of differentiation 44 (CD44) are derived from these neuroepithelial cells[29]. Ultimately, mature astrocytes are generated from radial glia by activating the signal transducer and activator of transcription 2 signaling pathway using ciliary neurotrophic factor. The most common marker for astrocytes is glial fibrillary acidic protein (GFAP)[30]. Mature astrocytes express aldehyde dehydrogenase family 1 member L1, aldolase C, glutamate transporter-1, and aquaporin 4[31]. In 2011, the first reported protocol for hPSC-derived astrocytes in a chemically defined system required long-term culture of up to 6 mo[29,32]. This protocol used the EB method and supported differentiation through the addition of the factors FGF2 and epidermal growth factor (EGF). To attenuate the culture time, shorter 4-6 wk long accelerated protocols for generating functional astrocytes through overexpression of the transcription factors SOX9 and NFIB were developed[33,34]. In 2017, the Pasca lab found a method to derive functional astrocytes using 3D cortical organoids. However, this protocol required up to 590 d, limiting its application[30]. The majority of recent studies use commercially available astrocyte differentiation medium to differentiate astrocytes from neural progenitor cells[35,36].

Astrocytopathies including Alexander disease[37,38], Aicardi-Goutières syndrome (AGS)[39], and vanishing white matter disease[40] can be effectively modeled with hiPSC-derived astrocytes[41]. Neurodegenerative diseases including Alzheimer's disease (AD)[36], Parkinson's disease (PD)[42], and Huntington's disease have also been modeled using similar methods. The familial presenilin-1 (*PS1*)



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Figure 1 Neural cell subtype differentiation from human pluripotent stem cells. The first step of neural cell differentiation is neural induction to generate neuroepithelial cells, usually by the dual SMAD inhibition method. Specific neural progenitors can be generated by tuning different signaling pathways such as Sonic Hedgehog, Wingless/integrated, retinoic acid, and bone morphogenetic protein. Neural progenitors can then be directed to become mature neurons through induction with neurotrophic factors such as brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor or derived into glial progenitors through treatment with the growth factors fibroblast growth factor 2 and epidermal growth factor. Glial progenitors can give rise to either astrocytes or oligodendrocytes. BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor.

mutation along with PD familial leucine-rich repeat kinase 2 (*LRRK2*) G2019S mutations were both modeled using hiPSC-derived astrocytes. The results showed the crucial role of astrocytes in the disease pathogenesis of AD and PD, respectively[36,42]. When co-cultured with neurons, astrocytes generated from Huntington's disease patient-derived hiPSCs displayed decreased electrophysiological activity and diminished neuroprotection consistent with Huntington's disease[43]. Regarding *in vitro* stroke modeling, ischemia-like conditions can be simulated by replacing normal oxygen (O_2)/carbon dioxide (CO_2) conditions with nitrogen (N_2)/CO_2 and subjecting cells to glucose deprivation[44,45]. However, cultures in 2D cannot effectively model stroke due logistical difficulties in restricting oxygenation as well as maintaining nutrition deprivation. However, Wevers *et al*[46] used neurovascular unit on-a-chip, which included a triculture of brain vascular cells, hiPSC-astrocytes, and hiPSC-neurons to model ischemic stroke. The study used antimycin-A, an inhibitor of complex III of the electron transport chain, to induce hypoxic conditions[45]. Modeling the motor neuron pathology linked to amyotrophic lateral sclerosis (ALS) was achieved using hiPSC-derived astrocytes from a patient who had the C9ORF72 mutation[46,47]. Recent studies also reported generation of ventral spinal cord-like astrocytes, which better reflect ALS pathophysiology[48]. Zika virus targeting of astrocytes has also been studied using hiPSC-derived astrocytes, which corroborated the reactive oxygen species imbalance, mitochondrial abnormalities, and DNA damage observed after Zika virus infection[49]. Astrocytes derived from hiPSCs are also beneficial in modeling neurodevelopment disorders including Down's syndrome[50-53], Rett syndrome[54-57], and schizophrenia[58,59]. Rare genetic diseases such as the lysosomal storage disorder Gaucher disease can be modeled using patient hiPSC-derived astrocytes[60,61] (Figure 1, Table 1).

Oligodendrocytes

Protocols to differentiate hiPSCs into pre-OL progenitors were first established in 2012[62]. Retinoic acid (RA) and purmorphamine, a small-molecule agonist of Sonic Hedgehog (Shh) signaling, were used to make pre-OL progenitors that express the markers oligodendrocyte transcription factor 2 (OLIG2) and NK2 homeobox 2. Pre-OL progenitors were then further differentiated into bipotential OL progenitor cells (OPCs) that expressed markers SOX10 and platelet-derived growth factor receptor alpha (PDGFRA) using PDGF-AA, triiodothyronine, and neurotrophin-3. The OPCs at this stage were further developed into either $O4^+$ and myelin basic protein-positive (MBP^+) human-induced OLs or $GFAP^+$ astrocytes. OPCs ameliorate neurological deterioration and support survival of *shiverer* mice after engraftment[62]. However, this protocol requires a lengthy 120-d culture period. Efficient and robust

generation of hiPSC-derived OPCs in 95 d has been achieved more recently[63,64]. Improved differentiation of myelinating OLs was obtained using brain extracellular matrix from decellularized human brain tissue[65]. Fast and efficient OL generation has additionally been achieved with SOX10 overexpression, either by introducing lentiviral vectors at the neuroepithelial stage or by direct transfection of hiPSCs prior to differentiation[66,67].

Shaker *et al*[68] published a 42-d protocol to derive organoids containing myelinating human OLs and astrocytes. Differentiated OLs that were produced using hiPSCs from primary progressive multiple sclerosis patients were found to be functional and supported *in vivo* myelination in *shiverer* mice[63]. Death of OLs is a hallmark of Pelizaeus-Merzbacher disease, an X-linked leukodystrophy caused by mutations in proteolipid protein 1 (PLP1)[63]. Human-induced OLs from individuals with PLP1 mutations have helped to identify important subgroups based on cell-intrinsic phenotypes and to elucidate the pathogenesis of various PLP1 mutations[15,68]. Involvement of OLs in neurodegenerative diseases, including AD and PD as well as multiple system atrophy[69], have also been studied using human-induced OLs.

OTHER CELL TYPES OF THE CENTRAL NERVOUS SYSTEM

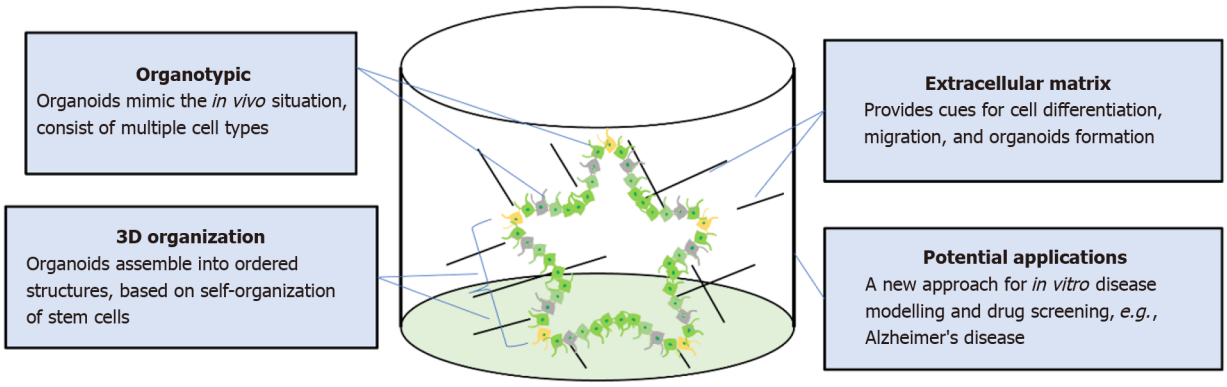
Microglia

Although murine models have been the main tool for studying the genetics and function of microglia, there are important distinctions between murine microglia and human microglia when it comes to aging and associated diseases[70,71]. Historically, viable microglia cells have been obtained by extracting them from brain tumors or epileptic foci removed from surgery, but this procedure is logistically very challenging. These hurdles were reduced when multiple methods to differentiate microglia from hPSCs were developed[70-75]. Muffat *et al*[73] published the first protocol by producing microglia-like cells from regular and patient hESCs and hiPSCs. This method used serum-free neuroglial differentiation media, which contained various components with concentrations adjusted to biologically match human cerebrospinal fluid. Abud *et al*[76] described a two-step method to successfully derive microglia-like cells (iMGLs) from 10 different hiPSC lines in 5 wk. The transcriptome profile of the derived iMGLs was strikingly similar to that of both adult human and fetal microglia[75]. Most microglial directed differentiation protocols involve hematopoiesis[73,75,76]. Some reported studies use chemically-defined protocols to generate human microglia through the formation of myeloid progenitors in 30 d[77]. Ionized calcium binding adapter molecule 1, a protein that belongs to the calcium-binding protein family, is one of the main markers of microglia[78]. It is primarily involved in rearranging cytoskeleton and has been used as a marker for the 3D reconstruction of microglial cells[79,80]. Other general markers used for microglial identification are CD45 and CX3C motif chemokine receptor 1. In a recent study, Dräger *et al*[81] described an effective 8-d protocol for generating induced transcription factor microglia-like cells (iTF-Microglia) based on the inducible expression of six transcription factors (human MAF BZIP transcription factor B, CCAAT enhancer-binding protein, interferon regulatory factor (IRF8) PU.1, and IRF5).

The risk of developing late-onset AD is linked to several genes, including triggering receptor expressed on myeloid cells 2 (*TREM2*) and *CD33* expressed by microglia. Microglia accumulate around amyloid plaques during AD and exacerbate pathophysiology by secreting cytokines and chemokines that induce inflammation. Microglia that have been generated using hiPSCs can be effectively used to model neurological diseases *in vitro*[80,81]. Alternatively, microglia derived from patient hiPSCs have also been used for modeling neurodegenerative diseases. Recently, patient hiPSCs expressing the AD-linked R47Hhet *TREM2* variant was used to elucidate the signal transduction deficit observed during AD progression[82]. Another study using microglia derived from AD patient hiPSCs reported that dysregulated peroxisome proliferator-activated receptor gamma (PPARγ)/p38 MAPK signaling causes the phenotypic deficits observed in *TREM2* variants. The results of this study concluded that the activation of PPARγ/p38MAPK signaling can ameliorate metabolic deficits within these cells and consequently rescue critical microglial cellular functions such as β-amyloid phagocytosis[83].

BRAIN ORGANOID DEVELOPMENT FROM HPSCS

Protocols for producing brain organoids were derived from the EB and SFEBq methods for neural induction. The formation of brain organoids is based on the self-organization and self-renewal of stem cells to generate a mixed cell population in 3D suspension culture (Figure 2). In 2013, the first study on brain organoids was reported to generate whole brain tissues with regional specific structures using an EB-based culture involving Matrigel support in a spinning bioreactor[24]. The organoids were used to model microcephaly, a neurodevelopmental disease whose pathologic features are difficult to recapitulate using animal models. This was the first work done to generate brain-like tissue *in vitro* and apply them to study human pathological disorders. In the following years, a simpler method was



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Figure 2 Self-organization of brain organoids. Human brain organoids are generated based on the self-organizing properties of stem cells. Organoids usually contain multiple cell types including mature neurons and immature neural progenitors. The key to organoid regeneration is the extracellular matrix that is used to support stem cell growth and differentiation. Brain organoids have been widely utilized to model neurological pathology in disease such as Alzheimer's disease and microcephaly.

developed to generate cortical spheroids in 3D static culture without Matrigel and agitation[25]. This method first derived neural progenitors using dual SMAD inhibition and then induced regional specific patterning by supplementing culture with the growth factors FGF2 and EGF. The last step of this protocol extended cultivation of brain aggregates and replaced the growth factors with the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) for up to 3 mo. The generated cortical spheroids exhibited a cortical layer-like structure and were developmentally comparable with the human fetal cortex. Brain spheroids and organoids have been widely used to model human brain development and neurological diseases *in vitro* and have provided a promising platform for drug screening[84] (Table 2, Figure 2).

Forebrain organoids

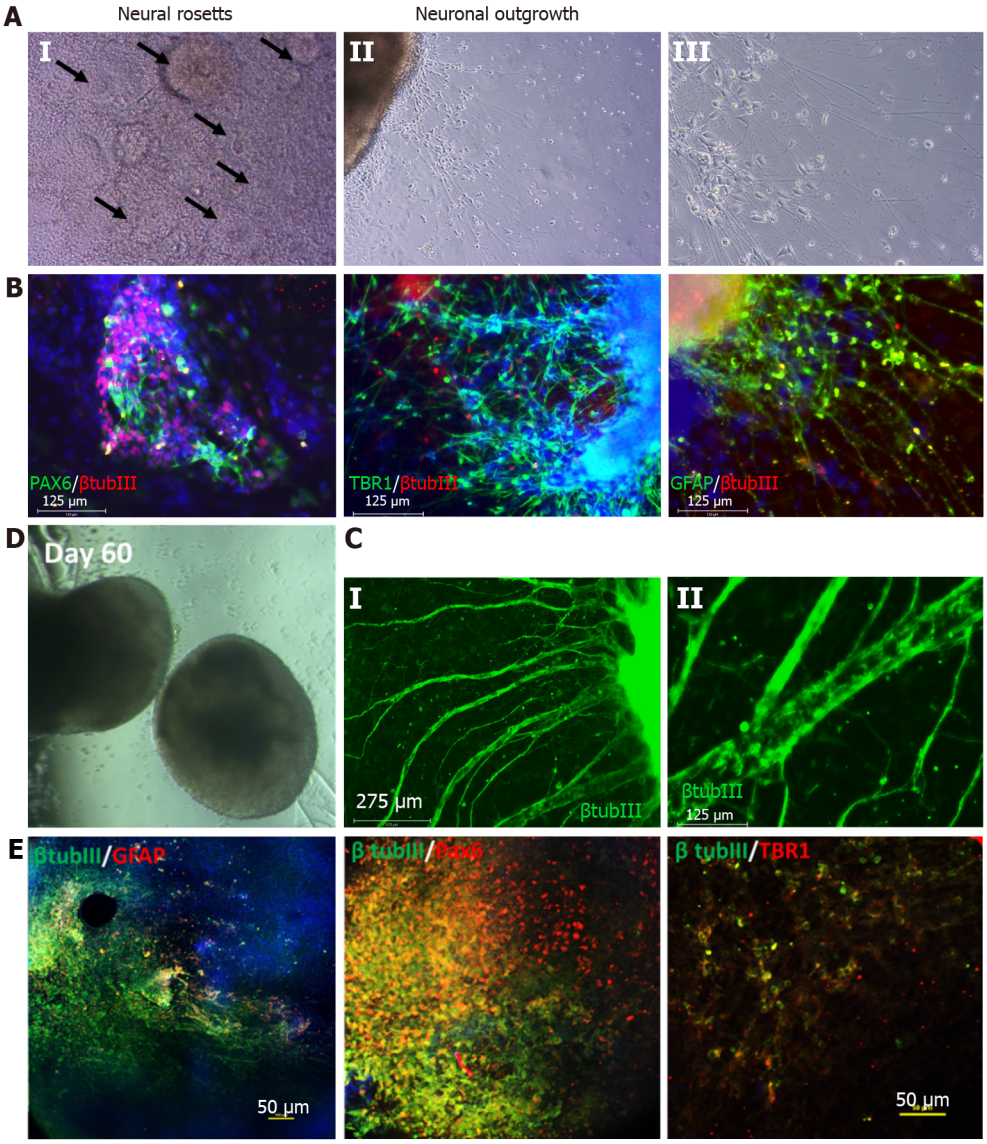
The areas of the brain originating from the telencephalon and diencephalon are referred to as the forebrain. The telencephalic region consists of the cerebral cortex and cerebellum, whereas the diencephalon includes the thalamus, hypothalamus, and pituitary glands. Self-organized cortical organoids or dorsal forebrain organoids were first reported using the SFEBq method. Multiple cortical layer tissues were generated through inhibition of TGF-β and Wnt signaling, resulting in dorsal-ventral patterning[22,85]. Lancaster *et al*[17] reported the first 3D culture system for deriving cortical organoids from hPSCs. Later, the Pasca lab generated more complex cortical spheroids and organoids from hPSCs, containing both neurons and astrocytes[25] (Figure 3). In following years, several groups attempted to develop protocols to derive cortical organoids from hPSCs. However, a common problem faced by many of these approaches was the presence of multiple ventricular subtypes within each organoid[26,86-91]. Cortical organoids derived from familial AD patient hiPSCs show increased levels of phosphorylated Tau and cytoplasmic neurofibrillary tangle-like deposits[92,93] (Table 1). Recent studies have shown that culture variations have an impact on the AD phenotypes seen in cerebral organoids and should be considered when using these models[94]. Cortical organoids were also used in stroke modeling to study the effects of oxygen-glucose deprivation (OGD), neuronal death that followed, and damaged neural networks[95]. These models use 2-8 h of OGD with O_2 (0.1%), CO_2 (5.0%), and N_2 (95.0%) gas levels and deoxygenated glucose-free medium to induce ischemia[95]. It has also been discovered that hypoxic conditions can reduce the number of progenitors and impair the differentiation of immature neurons during the development stage of brain organoids[96,97]. Ventral forebrain tissue-like medial ganglionic eminence (MGE) organoids are usually patterned by high Shh and low Wnt signals[85,98]. These MGE organoids contain diverse GABAergic interneurons subtypes including somatostatin, parvalbumin, calretinin, and calbindin. These MGE organoids were assembled to model the migration of human interneurons towards the cerebral cortex[98-100]. In 2020, the Pasca lab reported a method to generate striatal organoids expressing medium spiny neuron markers such as DARPP32 using activin A, IWP-2, and SR11237[101]. Brain organoid technology has also been utilized to generate organoids that can model other regions of forebrain tissue including thalamic organoids[102], hypothalamic organoids[103-105], and hippocampal organoids[106,107].

The hippocampus plays a significant role in learning, memory, and emotion. Hippocampal atrophy or hyperexcitability can cause neurological disorders such as schizophrenia and neurodegenerative diseases like AD. Hippocampal spheroids can be derived from hiPSCs using dual SMAD inhibition, Shh, and Wnt pathway inhibition followed with Wnt activation[107,108]. Commonly reported hippocampal markers include zinc finger and BTB domain-containing 20 and prospero homeobox 1. Hippocampal spheroids can be used to model AD pathology either by the exogenous addition of

Table 2 Comparison of methods for brain organoids generation from human pluripotent stem cells

Organoid type or brain region modeled	Method brief description	Model application	Ref.
EB-like aggregates; cerebral cortex	SFEBq, static suspension culture with cell sorting	Form self-organized structure mimicking the early cortiogenesis	Eiraku *et al*[23], 2008
Cerebral organoid; whole brain	Spinning bioreactor with Matrigel supporting	Form pyramidal identities with spatial separation mimicking the developing human brain at early stage; modeling microcephaly	Lancaster *et al*[17], 2013
Cortical neuroepithelium; cerebral cortex	Improved SFEBq, in 40% oxygen in Lumox plates	Inside-out layer pattern for human cortex	Kadoshima *et al*[86], 2013
Cortical spheroid; cerebral cortex	Static suspension culture with FGF-2 and EGF	Generated laminated cerebral cortex-like structure with some functions	Paşca *et al*[26], 2015
Cerebellar-plate-like neuroepithelium; cerebellum	Static suspension culture with FGF-19 and SDF-1	Mimicking the early development of human cerebellum	Muguruma *et al*[129], 2015
Telencephalic organoids; forebrain	Static suspension culture after neural rosettes isolation manually	Modeling autism spectrum disorder	Mariani *et al*[130], 2015
Dorsomedial telencephalic-like tissue; hippocampus	Improved SFEBq, in 40% oxygen	Modeling the development of human hippocampus	Sakaguchi *et al*[107], 2015
Forebrain organoids; cerebral cortex	Miniaturized spinning bioreactor	Zika virus exposure	Qian *et al*[27], 2016
Midbrain organoids; midbrain	Miniaturized spinning bioreactor	Midbrain organoids contained TH^+ cells	Qian *et al*[27], 2016
Hypothalamic organoids; hypothalamus	Miniaturized spinning bioreactor	Modeling early hypothalamus development	Qian *et al*[27], 2016
Midbrain organoids; midbrain	Static suspension culture on orbital shaker	Midbrain produced neuromelanin and dopamine	Jo *et al*[131], 2016
Pituitary organoid; anterior pituitary	Improved SFEBq	Formed pituitary placode with pituitary hormone-producing cells	Ozone *et al*[132], 2016
Cerebral organoid; cerebral cortex	Microfilament-engineered organoids under agitation	Formed polarized cortical plate and radial units	Lancaster *et al*[133], 2017
Cerebral organoid; whole brain	Spinning bioreactor with Matrigel supporting	Brain organoids formed spontaneously active neuronal networks	Quadrato *et al*[134], 2017
Brain assembloids; assembly dorsal and ventral forebrain organoids	Static suspension culture	Modelling migration of human interneurons and their functional integration into microcircuits using healthy and timothy syndrome cell line	Birey *et al*[99], 2017
Fused cerebral organoids; assembly dorsal and ventral forebrain organoids	Static suspension culture with Matrigel supporting on orbital shaker	Modelling migration of human interneurons in cerebral cortex	Birey *et al*[99], 2017
Fused cortical organoids and MGE organoids	Static suspension culture on orbital shaker	Modelling migration of human interneurons	Xiang *et al*[101], 2017
Neoplastic cerebral organoid	Static suspension culture with Matrigel supporting on orbital shaker	Modelling brain tumorigenesis	Bian *et al*[135], 2018
Granted brain organoids in mouse	Spinning bioreactor	Formed functional networks and blood vessels in the grafts	Mansour *et al*[136], 2018
Cortical spheroid	Static suspension culture	Modelling Alzheimer's disease	Yan *et al*[87], 2018
Cerebral organoids	Static suspension culture with Geltrex supporting on orbital shaker	Modelling Alzheimer's disease	Gonzalez *et al*[93], 2018
Neuromuscular organoid	Static suspension culture supporting on orbital shaker	Formed functional neuromuscular junctions and modelling myasthenia gravis	Faustion Martins *et al*[137], 2020
Section spherical organoid	Manually slicing forebrain organoids	Sliced organoids exhibited separated upper and deep cortical layer	Qian *et al*[90], 2020
Cortico-motor assembloids; assembly cortical spheroids, spinal spheroids, and skeletal muscle spheroids	Static suspension culture	Modeling cortical-motor circuits	Andersen *et al*[18], 2020
Cortico-striatal assembloids; assembly cortical spheroids and striatal spheroids	Static suspension culture	Modeling cortical-striatal circuits and 22q13.3 deletion syndrome	Miura *et al*[102], 2020
Air-liquid interface cerebral organoids	Slicing mature organoids and cultured in air-liquid interface not completely submerged in liquid	Formed network with functional output	Giandomenico *et al*[138], 2019

ASD: Autism spectrum disorder; EGF: Epidermal growth factor; FGF2: Fibroblast growth factor 2; hPSCs: Human pluripotent stem cells; SDF: Stromal cell-derived factor; SFEBq: Serum-free floating culture of EB-like aggregates with quick aggregation.



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Figure 3 Characterization of cortical organoids for neural and astrocyte marker expression. A: Brightfield images showing the neural rosettes and neuronal outgrowth from the organoids replated to an attachment plate at day 35 of differentiation; B: Resulting immunocytochemistry analysis of neural marker paired box 6 (PAX6), cortical deep layer VI marker T-box brain transcription factor 1 (TBR1), astrocyte marker glial fibrillary acidic protein (GFAP) co-stained with common neural marker β tubulin III, scale bar 125 μm; C: Immunostaining at later stage of the replating showing thick axon like extensions from the organoids, scale bar: 275 μm; D: Brightfield images of the day 60 cortical organoids; E: Confocal images of the day 60 organoids showing astrocyte marker GFAP, neural marker PAX6, cortical deep layer VI marker TBR1 co-stained with common neural marker β tubulin III, scale bar: 50 μm.

amyloid beta 42 oligomer[107,108] or by using amyloid precursor protein/PS1 variant hiPSCs. Current hippocampal organoids reflect the early stages of embryonic hippocampus development and successfully can create dentate gyrus granule and carbonic anhydrase 3 (CA3) pyramidal-like neurons, but are unable to produce CA1 pyramidal-like neurons.

Midbrain organoids

The protocol to differentiate human midbrain-like organoids (hMLOs) employs several molecules to mediate the differentiation of neuroepithelial cells. These factors include hBDNF, hGDNF, dibutyryl cyclic adenosine monophosphate, ascorbic acid, TGF-β3, and 1 purmorphamine[109,110]. The presence of dopamine transporter tyrosine hydroxylase as well as the expression of G-protein-regulated inward-rectifier potassium channel 2 are both characteristics of midbrain dopaminergic (mDA) neurons in hMLOs. Common midbrain genes including engrailed, nuclear receptor 4A2, LIM homeobox

transcription factor 1 beta (*LMX1B*), *LMX1A*, monoamine oxidase B, calbindin 1, tyrosine hydroxylase, catechol-O-methyltransferase, and dopa-decarboxylase have also been detected in these organoids. Additionally, neurons in hMLOs have been found to exhibit action potentials with large sag currents, indicating the existence of mDA neurons[111] (Figure 3).

According to single cell sequencing studies, hMLOs replicate early embryonic neurodevelopment and recapitulate disease characteristics[112,113]. However, the methods to generate midbrain organoids can take a significant amount of time, and can vary from batch to batch. To scale up the generation of midbrain organoids, Mohamed *et al*[114] recently published microfabricated disk technology using eNUVIO EB-Disks. Another study found that the use of recombinant spider-silk microfibers functionalized with full-length human laminin produced similar ventral midbrain organoids with lower inter-organoid variability[114,115]. Alternatively, an automated approach, termed automated midbrain organoids, was published by Renner *et al*[116] that produced high-throughput 3D midbrain organoids. The high-throughput production of hMLOs from hPSCs in spinner flasks was also reported using TH-TdTomato reporter hPSC lines as well[116].

As the second most prevalent neurodegenerative disease worldwide[117], PD is frequently studied using hPSC-derived hMLOs[87,111,112,115,118-120]. The disease is characterized by the loss of dopaminergic neurons in the substantia nigra and is mainly caused by mutations in glucocerebrosidase and LRRK2 genes in addition to α-synuclein (α-syn; *SNCA*) gene triplications[111,121,122]. The hMLOs generated from patients with these mutations display PD traits such as oligomeric and fibrillar α-syn aggregates, loss of mDA neurons, and Lewy body-like inclusions[111,118,119]. Since hPSCs can be edited using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 technology, *SNCA* gene genome correction has been demonstrated to revert PD patient hPSCs back to wild-type phenotypes[119,123]. These hMLOs have also been successfully generated from hiPSCs carrying the *LRRK2*-G2019S mutation[124]. Biallelic pathogenic variations in the phosphatase and tensin homolog-induced kinase 1 gene that controls mitochondrial function is also connected to the etiology of PD[125]. Human Parkinsonism can also have a more direct cause such as the toxicity of some drugs including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which has also been modeled using hMLOs[126-128] (Table 2)[129-138].

Hindbrain organoids

The medulla, pons, and cerebellum make up the hindbrain region, which is developed from the metencephalon and myelencephalon. Methods to generate hindbrain organoids commonly involve purmorphamine-mediated Shh signaling activation to convert neuroepithelial cells into ventral identity neurons. RA is used as a potent caudalizing agent to promote the fate of hindbrain cells instead of Wnt signaling activation, which is required for midbrain patterning[138]. Markers of hindbrain neurons include serotonergic neuron marker serotonin, human fifth Ewing variant, gastrulation brain homeobox 2, choline acetyltransferase (ChAT), and HB9. Due to their location, cerebellar neurons cannot be easily studied at the cellular or molecular level. Thus, using hiPSC-derived technologies is advantageous in this situation. The cerebellum can be divided into inhibitory GABAergic neurons known as Purkinje cells, which are derived from pancreas-specific transcription factor 1α progenitors, and excitatory glutamatergic neurons known as granule cells, which are descended from atonal homolog 1 (also known as MATH1) progenitors[139].

The first published granule cell differentiation protocol using hiPSCs involved several factors including FGF8B, Wnt proteins, BMPs, and RA. This method recapitulates anteroposterior and dorsoventral patterning and thereby induces *MATH1*-expressing mitotic neural progenitors, which can later be differentiated into cerebellar granule cells. Purkinje cells derived from hiPSCs initially had an immature phenotype, and thus needed to be co-cultured with mouse cerebellar granule cell precursors to allow for maturation[129,140]. However, cells made using this approach had substantial functional variability. Silva *et al*[141] recently published a protocol that generates mature cerebellar neurons without the need of such a co-culture system. This method involves stimulating the development of cerebellar precursors with FGF19, followed by self-organization and differentiation using stromal cell-derived factor 1 and BDNF/GDNF. Cerebellar neurons derived from hiPSCs are also helpful for modeling diseases, particularly cerebellar ataxia, a neurodegenerative disease that affects cerebellar neurons and eventually leads to motor incoordination. Cerebellar neurons derived from hiPSCs of either healthy human participants or ataxia patients were used in several recent studies to create an *in vitro* disease model. Spinocerebellar ataxia type 6 patient hiPSC-derived Purkinje cells have been used to model both thyroid hormone depletion-dependent degeneration and downregulation of the transcriptional targets TATA-Box Binding Protein Associated Factor 1 and BTG anti-proliferation factor 1, indicating their potential as a pathogenesis tool[141].

Recently, protocols for generating brain stem organoids have also been published, offering a new tool for evaluating the pathophysiology of disorders that impact the brainstem. Human brain stem organoids express the medullary marker ChAT, the pons marker hydroxylase, and the mature and functioning excitatory and inhibitory neuron markers vesicular glutamate transporter 1 and glutamic acid decarboxylase 67 in addition to various other relevant markers[142]. Both $OLIG2^+$ and MBP^+ OLs, as well as $S100^+$ astrocytes, are expressed in brain stem organoids[142].

Assembloids

Assembloids are systems that combine one type of spheroid or organoid with another type of spheroid or organoid. For example, assembloids can be produced by combining the dorsal and ventral forebrain, the cerebral cortex with the thalamus, or the cerebral cortex with any other non-neural cell type such as microglia, immunological cells, pericytes, and endothelial cells. The Pasca lab published the first assembloid study in 2017, in which human cortical spheroids were mixed with human subpallium spheroids[98]. The assembloids were created using human subpallium spheroids and cortical spheroids differentiated from hiPSCs from timothy syndrome (TS) patients with mutations in the α1c subunit of the L-type calcium channel (*CACNA1C*) gene. The two types of spheroids were combined in simple conical tubes and left undisturbed for 3 d to produce assembloids. The interneurons within the assembloids migrated, suggesting high potential for the study of certain aspects of migratory disorders such as TS. These patient-derived assembloids showed less effective interneuron movement, which was reversed by the administration of L-type calcium channel blockers.

Since then, many labs have sought to use assembloids to elucidate the interactions that occur between different physiological systems. The Knoblich lab reported the use of fused cerebral organoids that combined dorsal and ventral forebrain tissue cultures. They showed migration of C-X-C chemokine receptor type 4-dependent GABAergic interneurons from the ventral forebrain to the dorsal forebrain, which had a more MGE identity[143]. Assembloids of cortical organoids with integrated pericyte-like cells which express angiotensin-converting enzyme 2 have also been shown to enhance severe acute respiratory syndrome coronavirus 2 infection, suggesting the involvement of multiple cell types[100]. Another study employed cortico-striatal assembloids to recapitulate neurodevelopmental disorders that impair the cortico-striatal pathway, including schizophrenia, obsessive-compulsive disorder, and autism spectrum disorder[144-146]. These cortico-striatal assembloids were developed from patients with Phelan-McDermid Syndrome, a severe developmental disorder also known as 22q13.3DS. It is important to note that these patient-derived assembloids had a higher number of calcium spike events than striatal organoids, offering a better representation of altered neural activity. Interneuron migration has also been reported in a separate assembloids study that fused human MGE organoids with human cortical organoids[147].

CURRENT LIMITATIONS AND POTENTIAL ADVANCEMENTS

The extended culture times required by current methods to produce neural cell types as well as organoids restrict their application. Another consideration is the cell-line-to-cell-line and batch-to-batch variabilities of hiPSC differentiation. Therefore, accelerated protocols with less variable outcomes should be developed. For hiPSC-derived astrocytes, the major drawback is their lack of regional identity. Most protocols derive astrocytes with cortical identity, which may not be useful for modeling disease pathophysiology affecting the ventral part of the brain. Therefore, it is essential to employ experimental approaches that can produce astrocyte subtypes with the appropriate rostro-caudal and dorso-ventral identities. In the case of hiPSC-derived OLs, the lack of advanced OL disease models created using genetically-modified hiPSCs also limits their application. Finally, the lack of vascularization in current organoid and assembloid systems prevents the important study of cell-type crosstalk. Therefore, incorporating vasculature as well as reducing culture time would benefit multiple methods of neural lineage disease modeling.

CONCLUSION

Research in hPSCs has proven to be extremely helpful in creating disease models that can corroborate results gleaned from animal models and overcome their associated limitations. Distinct brain cell types can be produced using hPSCs including neurons, astrocytes, OLs, microglia, in addition to more advanced heterogeneous systems such as brain organoids. These systems have contributed to the development of models for neurological diseases such as AD, PD, and many others. Current models that employ hPSCs have certain shortcomings related to the absence of vasculature as well as microglia. However, developing research in the field of tissue engineering that use cocultures, organ-on-chip and assembloids may be able to get around these limitations in the years to come.

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FOOTNOTES

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Conflict-of-interest statement: Lauren E Woodard and Julie Bejoy have two patent applications submitted on "Accelerated protocol for deriving podocytes from hiPSCs" and "nephron progenitor exosomes" listed below; Inventors: Bejoy J and Woodard LE accelerated the protocol for the differentiation of podocytes from human pluripotent stem cells. Patent Application filed August 26, 2022. PCT/US2022/075447; Inventors: Bejoy J and Woodard LE Nephron progenitor exosomes, patent Application filed October 6, 2022. PCT/US2022/077692.

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REVIEW

Factors affecting osteogenesis and chondrogenic differentiation of mesenchymal stem cells in osteoarthritis

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Abstract

Osteoarthritis (OA) is a common degenerative joint disease that often involves progressive cartilage degeneration and bone destruction of subchondral bone. At present, clinical treatment is mainly for pain relief, and there are no effective methods to delay the progression of the disease. When this disease progresses to the advanced stage, the only treatment option for most patients is total knee replacement surgery, which causes patients great pain and anxiety. As a type of stem cell, mesenchymal stem cells (MSCs) have multidirectional differentiation potential. The osteogenic differentiation and chondrogenic differentiation of MSCs can play vital roles in the treatment of OA, as they can relieve pain in patients and improve joint function. The differentiation direction of MSCs is accurately controlled by a variety of signaling pathways, so there are many factors that can affect the differentiation direction of MSCs by acting on these signaling pathways. When MSCs are applied to OA treatment, the microenvironment of the joints, injected drugs, scaffold materials, source of MSCs and other factors exert specific impacts on the differentiation direction of MSCs. This review aims to summarize the mechanisms by which these factors influence MSC differentiation to produce better curative effects when MSCs are applied clinically in the future.

Key Words: Osteoarthritis; Mesenchymal stem cells; Differentiation; Hypoxia; Dexamethasone; Cell therapy

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Core Tip: Several reviews have summarized the current status of mesenchymal stem cells (MSCs) in the treatment of osteoarthritis (OA). These studies usually focus on the paracrine function of MSCs. However, the differentiation function of MSCs also plays an important role in the treatment of diseases. This is the first review to report the factors that may affect the differentiation direction of MSCs in the treatment of OA and aims to provide guidance for more accurate regulation when MSC therapy is applied in the future.

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INTRODUCTION

Osteoarthritis (OA) is one of the most common degenerative joint diseases, and its incidence increases with age[1]. With the rapid growth of the aging population, the prevalence of OA is increasing[2]. At present, there are more than 300 million OA patients worldwide[3]. The major symptoms of OA are pain and joint dysfunction, which seriously affect the quality of life of patients. Intra-articular microenvironment changes occur as OA develops. Due to tissue injury, severe hypoxia occurs in the joint cavity, and the expression level of hypoxia inducible factor 1 alpha increases significantly[4]. Many inflammatory cytokines infiltrate joints with OA, including interleukin-1 and tumour necrosis factor alpha (TNF-α). The expression of transforming growth factor-beta (TGF-β) in cartilage is significantly lower than that in healthy joints, which can lead to metabolic disorders of chondrocytes[5].

OA is an incurable disease at present, and cartilage degeneration and subchondral bone remodeling are considered the main pathogenic mechanisms of OA. There are no drugs that can delay the progression of OA[6]. The goal of clinical treatment is to relieve symptoms such as pain and loss of function[7]. The common treatments for OA include physiotherapy, pain relievers and nonsteroidal anti-inflammatory drug administration, intra-articular glucocorticoid injection and surgery. When OA progresses to the advanced stage, the only treatment option for most patients is total knee replacement surgery, which causes patients great pain and anxiety[8]. Some innovative new treatment options have been proposed, including mesenchymal stem cell (MSC) therapy.

Mesenchymal stem cells are a branch of stem cells, with the stem cell characteristics of self-renewal and differentiation potential[9]. MSCs can repair tissue damage after injury by differentiating into different tissue cells, so they can play an important role in disease treatment. In 1968, Professor Friedenstein[10] first discovered the existence of MSCs in bone marrow and established an adherent method to isolate and culture MSCs *in vitro*. Pittenger *et al*[11] proved for the first time that MSCs have multidirectional differentiation ability. Since then, MSCs have been widely studied and applied to the treatment of clinical diseases. In 2006, the International Society for Cell Therapy (ISCT)[12] established three minimal criteria for defining MSCs unequivocally: (1) The cells must have the ability to adhere to plastic surfaces when cultivated in standard conditions; (2) they must express CD105, CD73, and CD90, but not CD45, CD34, CD14/CD11b, CD79a/CD19, or HLA-D; and (3) they must have the ability to differentiate into at least the following cell types *in vitro*: Osteoblasts, adipocytes, and chondroblasts.

MSCs can be isolated from various tissues, such as bone marrow, adipose tissue, cord blood and placenta. MSCs from different sources have different characteristics[13]. At present, bone marrow MSCs (BMSCs) and adipose MSCs (ADSCs) are the most commonly used. Heo *et al*[14] found that only BMSCs and ADSCs have the ability to differentiate into three lineages, including osteoblasts, adipocytes and chondrocytes, to meet the minimum MSC standard proposed by the ISCT[15]. However, Beeravolu *et al*[16] believe that MSCs from the human umbilical cord and fetal placenta can also differentiate into three lineages.

At present, there are approximately 1519 studies on "mesenchymal stem cells" registered, according to clinicaltrials.gov (April 2023). MSCs have been used as cellular therapy for various degenerative, inflammatory and autoimmune diseases in a large number of clinical trials. These clinical trials include diseases of the musculoskeletal system, respiratory system, blood system and cardiovascular system and have already shown the effectiveness and safety of MSCs. These cells are most commonly used for the treatment of OA in the musculoskeletal system. At present, the pathogenesis of OA is not completely clear. A large number of studies have shown that subchondral bone destruction[17,18] and cartilage degeneration[19-21] participate in pathogenesis. Osteogenesis and chondrogenesis of MSCs play a key role in the treatment of OA.

Lamo-Espinosa *et al*[22] recruited 30 patients with OA and injected MSCs into the experimental group and hyaluronic acid (HA) into the control group. After 12 mo, magnetic resonance imaging (MRI) showed that the experimental group receiving the high dose of MSCs had a greater cartilage thickness, which is an indicator of the regeneration of cartilage in OA patients, than the control group. Tang *et al*

[23] injected MSCs into rabbit models of OA. Nine weeks later, the knee joints of the rabbits were collected and analyzed. They found that when MSCs were injected, the articular cartilage of the rabbit showed characteristics of good reconstruction, such as a regular surface, restored cartilage thickness, nearly normal chondrocyte morphology, and uniformly distributed red Safranin O staining in the articular cartilage. At present, no experiment has been performed on the differentiation of MSCs in isolated subchondral bone tissue. However, many similar studies that applied MSCs to bone defects have been performed and proved the feasibility of osteogenic differentiation of MSCs *in vivo*. For example, in the treatment of femoral head necrosis in the same hypoxic environment, after MSCs are implanted, the expression of bone-related genes is improved, and alkaline phosphatase and type I collagen are increased, which are indicators of bone formation[24]. MSCs are considered promising candidates for bone and cartilage repair and regeneration in OA. But the differentiation of transplanted MSCs is influenced by the microenvironment. Therefore, this article reviews the factors affecting the osteogenesis and chondrogenesis of MSCs (Figure 1).

MECHANISM OF OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION OF MSCS

The differentiation of MSCs depends on a number of factors, including chemical, physical and biological factors. These factors activate different signaling pathways and transcription factors that regulate MSC differentiation into different cells[25]. The differentiation of MSCs is precisely controlled by various signaling pathways[26]. These signaling pathways activate lineage-specific transcription factors[27].

Chondrogenesis and osteogenesis of MSCs are interrelated processes. There are two ways for MSCs to form bone: Endochondral or intramembranous ossification. In endochondral ossification, MSCs first differentiate into chondrocytes and secrete cartilage matrix, and then they are stimulated by osteoblasts to form bone. In contrast, in intramembranous ossification, MSCs differentiate into osteoblasts directly [28]. Therefore, there are some common signaling pathways and transcription factors involved in the osteogenic differentiation and chondrogenic differentiation of MSCs.

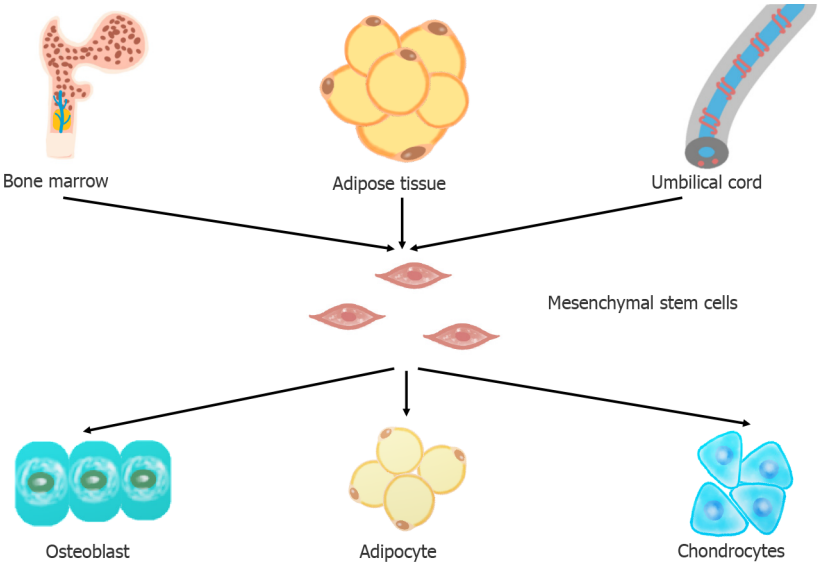
TGF-β signaling, Wnt/β-catenin signaling and Notch signaling are the key pathways involved in chondrogenic differentiation of MSCs. The key cytokines include Sox9, Runx2, TGF-β, FGF and others [29]. Sox9 plays an essential role during chondrogenic differentiation and is considered an early sign of chondrocyte formation. Complete deletion of Sox9 can prevent the formation of cartilage. When it is overexpressed, it significantly inhibits the proliferation of chondrocytes[30]. Sox9 is a regulator of the type II collagen (*ColII*) gene, which is a specific marker of cartilage formation. The expression of *ColII* in chondrocytes has been found to be in direct proportion to the concentration of Sox9[31]. TGF-β can promote the differentiation of MSCs into chondrocytes and inhibit the terminal differentiation of chondrocytes into mast cells[32]. The differentiation of chondrocytes induced by TGF-β is mainly mediated by the Smad signaling pathway[33], which can upregulate the expression of Sox9 transcription factors and promote the synthesis of collagen and proteoglycan.

The main paracrine signaling pathways involved in the osteogenic differentiation of MSCs include bone morphogenetic protein (BMP) signaling, Wnt signaling, and Notch signaling[28,34-36]. The key transcriptional regulatory factors include Runx2, β-catenin, and osterix[27]. Runx2 is indispensable for the osteogenic differentiation of MSCs because it is a common convergence point for many signaling pathways[25,37]. It leads to the differentiation of MSCs into osteoblasts and inhibits the differentiation of adipogenesis and chondrogenesis. Runx2 promotes the differentiation of MSCs into osteoblasts in the early stage and promotes the maturation and mineralization of osteoblasts in the later stage by regulating extracellular matrix proteins, such as ColI and alkaline phosphatase (ALP). When Runx2 is absent, neither periosteal nor endochondral ossification occurs[38]. BMP2 is also an effective osteogenic induction factor that promotes the expression of Runx2, thus promoting the differentiation and maturation of osteoblasts[28]. Osterix is an osteoblast-specific transcription factor that is only expressed in osseous tissue and plays a decisive role in the differentiation of MSCs into osteoblasts[39]. Activation of the Wnt signaling pathway induces osterix expression. Overexpression of osterix in MSCs leads to osteogenic differentiation and an enhanced bone regeneration ability of MSCs[40]. The activity of β-catenin is also regulated by Wnt signaling. β-catenin can facilitate the shift of MSC fate to osteoblasts and enhance endochondral ossification. Its deficiency hinders the osteogenesis of MSCs and promotes the formation of cartilage and fat[41,42] (Figure 2).

FACTORS AFFECTING THE OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION OF MSCS IN THE MICROENVIRONMENT OF OA

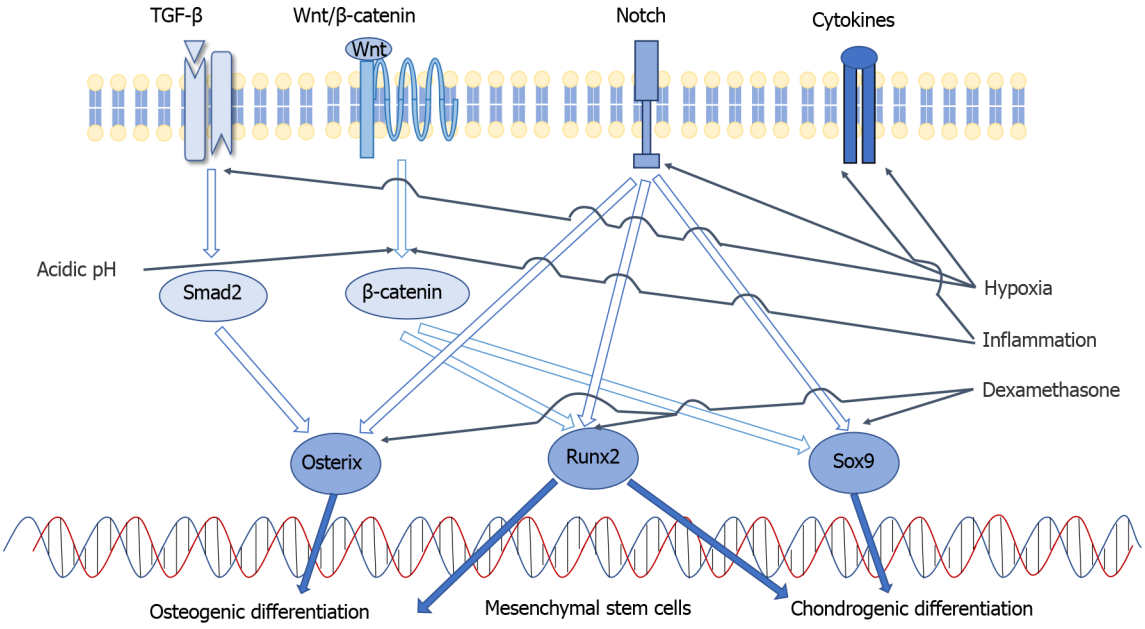
Oxygen concentration

The effect of oxygen concentration on MSCs has been studied for over twenty years (hypoxia promotes murine bone marrow-derived stromal cell migration and tube formation). Although there are still some controversies, a large number of studies have proven that low oxygen tension (hereinafter "hypoxia")



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Figure 1 Common sources and differentiation potential of mesenchymal stem cells.



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Figure 2 Signaling pathways involved in osteogenic and chondrogenic differentiation of mesenchymal stem cells and the mechanism of certain factors. TGF-β: Transforming growth factor-beta.

exerts a significant impact on the differentiation of MSCs. Hypoxia often occurs in the stem cell microenvironment, which induces beneficial signals, such as upregulation of pluripotency markers, for MSCs to maintain their functions[43]. In the general microenvironment *in vivo*, the oxygen concentration is usually low. For example, the oxygen concentration in healthy bone marrow is only 1.3% to 7.0%[44], and that in articular cartilage is only 2% to 5%[45]. In particular, OA often occurs in a hypoxic environment. Nitric oxide synthase and hypoxia-inducible factor-1 are often upregulated in OA and aggravate the hypoxic environment[46]. Although the oxygen concentration in the microenvironment of MSCs is low, a 21% O_2 concentration (hereinafter "normoxia") is routinely used in cell culture.

Ciapetti *et al*[22] isolated and cultured BM-MSCs under 2% O_2. They observed a higher tendency of osteogenic differentiation of these cells compared with the cells cultured in standard normoxia. Significant changes in the MSC immunophenotype, such as increased CD73 and CD90 expression, were observed, but CD105 expression was reduced. MSCs cultured under hypoxia have better mineralization, higher mineral density and higher calcium matrix deposition than those cultured under normoxia.

Alizarin red S staining is a convenient method for detecting calcium salt deposition. The positive area observed in hypoxic cultured MSCs was greater than that in cells cultured in normoxia, which means that osteoblast differentiation was enhanced[47,48]. Fennema *et al*[49] found that hypoxic culture increased the levels of osteogenic genes, such as osteopontin, osteocalcin, ALP and ColI[50]. Hypoxia activates the Notch signaling pathway, increases the expression of CBF-1α, and promotes the osteogenic differentiation of MSCs[51,52].

Similarly, hypoxic conditions increased the chondrogenic differentiation efficiency compared to normoxic conditions. Chondrogenic differentiation of MSCs can be quantitatively assessed by Safranin O staining[53,54], and the positive area increases after hypoxic culture. Immunohistochemistry demonstrated that the expression of *ColII* increased[50]. Hypoxia may enhance the chondrogenic differentiation capacity of MSCs by enhancing the expression of chondrogenic genes[55]. This may be manifested as increased mRNA expression of glycosaminoglycans, aggrecan, transcription factor and Sox9[56,57]. The level of cartilage oligomeric matrix protein is higher under hypoxic conditions[53]. These factors play crucial roles in chondrocyte differentiation. Hypoxia affects the overall cellular response through TGF-β, leading to upregulation of cartilage molecular markers, such as *ColII* and Sox9 [58]. However, some studies have shown that hypoxia can enhance chondrogenesis and inhibit osteogenesis of MSCs[59,60]. These differences between studies may be due to differences in culture conditions and sources of MSCs and therefore larger sample sizes and more precise experiments are required to fully elucidate the effect of hypoxia on MSC differentiation.

Inflammation

OA was once referred to as noninflammatory arthritis, but it is now considered a persistent low-grade inflammatory disease, and many inflammatory cells are involved[61]. Chronic inflammation activates the Wnt/β-catenin pathway, which leads to mitochondrial damage and further impairs the differentiation of MSCs[62]. Interferon-gamma (IFN-γ) and TNF-α are two important inflammatory cytokines involved in OA inflammation[63]. Li *et al*[64] pretreated MSCs with IFN-γ and TNF-α to simulate the inflammatory microenvironment. They found that the inflammatory microenvironment promoted chondrogenic differentiation of MSCs and inhibited their osteogenic differentiation.

Acidic pH

Inflammation decreases the extracellular pH and makes the OA joint cavity a weakly acidic microenvironment[65]. The pH of joints with OA (6.40 ± 0.08) was obviously lower than that of normal joints (7.01 ± 0.26)[66]. Decreasing pH was shown to inhibit the proliferation and metabolism of MSCs in culture. Furthermore, the activity of alkaline phosphatase was reduced, which means that the osteogenic differentiation of MSCs was decreased[67]. At physiologic pH (8.0), MSCs exhibit the strongest osteogenic differentiation potential[68]. A pH of 8.0 is recommended for a greater therapeutic effect of MSCs in OA.

Osmolar pressure

The osmolar pressure in the joint cavity of healthy adults is 404 mOsm/L ± 57 mOsm/L, while that in OA patients is 297.0 mOsm/L ± 16.9 mOsm/L. The joint cavity of OA patients is exposed to a hypoosmotic environment[69,70]. At present, there is little research on the effect of osmotic pressure on the osteogenic and chondrogenic differentiation of MSCs. Some studies have demonstrated that hyperosmolarity promotes the chondrogenic differentiation of MSCs and cartilage repair[71-73]. No study on the effect of hypo-osmotic stress has been conducted.

Cytokines

In contrast to normal bone, subchondral bone in advanced OA is characterized by osteosclerosis, including a higher bone volume fraction, a greater number of trabecular bones in the load-bearing area, and an increase in the thickness of the original trabeculae. This may be due to the overexpression of growth factors in the joints of patients with OA, such as insulin-like growth factor 1 and TGF-β[74]. Both of these cytokines have been shown to promote osteogenic and chondrogenic differentiation of MSCs [75,76]. The local expression of basic fibroblast growth factor (bFGF) in the joints of patients with OA is significantly higher than that in healthy people[77]. bFGF has been proven to be an important growth factor for maintaining the stemness of MSCs[78].

OTHER COMMON INFLUENCING FACTORS IN THE TREATMENT OF OA

Glucocorticoids

Dexamethasone is a member of the glucocorticoid class, and it is considered to be the mildest corticosteroid drug used for OA treatment[79]. Intra-articular injection of glucocorticoids is one of the treatment methods for OA[80], and dexamethasone can also be used as an immunosuppressive agent for MSC transplantation. Moreover, dexamethasone is generally considered one of the main components that induces MSCs to differentiate toward osteogenic, adipogenic and chondrogenic lineages[81]. The

osteogenic differentiation of MSCs mainly relies on osteogenic induction medium, which is usually composed of dexamethasone, ascorbic acid and β-sodium glycerophosphate. Dexamethasone induces increased expression of Runx2, osterix, and bone matrix proteins. Furthermore, it can induce osteogenic differentiation by inhibiting Sox9 expression[82]. Ascorbic acid and β-sodium glycerophosphate increase the content of ColI and stimulate the formation of a mineralized matrix[11]. Human MSCs need dexamethasone to produce ALP, a marker used to distinguish osteoblasts in culture[83,84]. The chondrogenic differentiation medium often includes dexamethasone, ascorbic acid and TGF-β3. Dexamethasone enhances the expression of the cartilage-specific gene Sox9[85]. The adipogenic differentiation medium often contains dexamethasone, ascorbic acid, 3-isobutyl-1-methylxanthine, insulin and other components[86]. Although the detailed mechanism of differentiation induced by dexamethasone is currently unknown, dexamethasone clearly affects the direction of MSC differentiation [87].

Doi *et al*[88] found that dexamethasone had certain effects on the osteogenic differentiation of MSCs. The effect of dexamethasone on inducing MSCs to differentiate into osteoblasts depends on the dosage and exposure time of the drugs[89]. It has been reported that short-term use of low-dose (10^{-8}, 10^{-7} mol/L) dexamethasone can stimulate the osteogenesis of MSCs and significantly increase the formation of mineralized nodules and the expression of osteogenic markers (BSPII and Runx-2) in cells[90,91]. It has also been reported that high concentrations of dexamethasone (10^{-6} mol/L) can inhibit the osteogenic differentiation of MSCs and induce them to differentiate into adipocytes. A high dose can reduce the osteogenic differentiation-related surface phenotype, as indicated, for example, by decreased surface expression of CD73. The higher concentration of dexamethasone resulted in enhanced lipid droplet formation and higher expression of lipid-forming markers (PPAR-γ and CEBP-α) in cells. In addition, a high concentration of dexamethasone exacerbates apoptosis of MSCs, inhibits MSC proliferation, and promotes senescence of MSCs[83]. A concentration of dexamethasone up to 10^{-6} mol/L imposes toxic effects on MSCs[89,92]. When the concentration of dexamethasone was lower than 10^{-8} mol/L, no differentiation of osteoblasts was detected[93,94]. Therefore, 10^{-7} mol/L is considered the most appropriate concentration for inducing MSCs to differentiate into bone[91].

Similarly, dexamethasone can also promote chondrogenic differentiation of MSCs[93]. Tangtrongsup *et al*[95] found that chondrogenic differentiation was suppressed in dexamethasone-free cultures. It can increase the proteoglycan content and collagen type II intracellular content. Dexamethasone may not function as a specific chondrogenic factor to directly promote cartilage differentiation, rather, it may promote it by inducing cells to upregulate cartilage factors, such as Runx2 and Noggin[96]. Its influence is mainly dependent on the context[97].

In addition to the dosage, the duration of dexamethasone treatment also affected the differentiation of MSCs. Dexamethasone is commonly used in bone trauma to relieve edema and pain, but long-term use may lead to osteoporosis through bone loss and bone marrow lipogenesis. Some studies suggest that long-term exposure of MSCs to dexamethasone may negatively impact their differentiation[91]. Others found that a lack of dexamethasone inhibits the differentiation of MSCs, so continuous delivery *in vivo* should be given priority[95]. Moreover, Song *et al*[98] found that within 4 wk, as exposure time increased, stimulation of osteogenic differentiation by dexamethasone strengthened, and calcium deposition increased. According to some authors, the sensitivity of MSCs to dexamethasone depends on the stage of cell maturation. Dexamethasone mainly acts on early stem cells, so it should be applied in the early stage[84]. It has also been suggested that early exposure to dexamethasone has little effect on MSC differentiation, and thus, continuous exposure for at least one week is required[89]. At present, there is little research on the effect of the duration of dexamethasone exposure on MSC differentiation, and more accurate experiments are needed to verify this hypothesis. As a glucocorticoid, dexamethasone is not suitable for systematic use to regenerate local tissue defects due to possible negative effects on healthy tissues and organs. Therefore, the application of local controlled release devices of dexamethasone in MSC therapy is reasonable.

Scaffolds

MSCs can be injected directly into the damaged site or differentiate into target cells together with the tissue engineering scaffold. Many tissue engineering experiments have proven that biological scaffolds can enhance the osteogenic and chondrogenic differentiation of MSCs. HA is often used to form a stable 3D environment for MSC chondrogenesis *in vitro*, which allows for better provision of oxygen and nutrients to MSCs. HA can promote the osteogenic process of endochondral ossification of MSCs[99]. Three-dimensional nanofibrous scaffolds, such as poly-((D,L)-lactide-ε-caprolactone)dimethacrylate scaffolds and poly(-caprolactone) nanofibrous scaffolds, have been shown to enhance chondrogenic differentiation of MSCs[100,101]. The combination of MSCs with biomaterials can improve the differentiation ability of MSCs. These studies have demonstrated that better efficacy can be achieved by injection of scaffolds loaded with MSCs.

Sources of MSCs

The source of MSCs also significantly impact their differentiation. By reviewing clinical trials, we found that bone marrow-derived MSCs (BM-MSCs), adipose tissue-derived MSCs (AD-MSCs) and umbilical cord-derived MSCs (UC-MSCs) are mainly used in OA research. MSCs from different sources have

different characteristics and differentiation potentials[102].

BM-MSCs offer the advantages of strong differentiation ability, mild implantation reaction and strong expansion ability *in vitro*. However, BM-MSCs need to be obtained from the patient's bone marrow, and bone marrow collection is a painful and invasive process. The number of BM-MSCs derived *in vivo* is quite low and requires *in vitro* amplification. Moreover, the differentiation potential and proliferative capacity of BM-MSCs decrease with the age of the donor[103]. Adipose tissue was first identified as an alternative source of MSCs in 2001[104,105]. AD-MSCs offer the advantage that they can be obtained in large quantities in a simple, minimally invasive manner. They can be extracted from excess adipose tissue that is discarded as waste during liposuction, avoiding immunogenicity and ethical concerns. The quantity and quality of MSCs from adipose tissues were significantly higher than those of other tissues [106]. Some studies have suggested that the differentiation potential of AD-MSCs depends on the source of adipose tissue[107]. MSCs from visceral adipose tissues have greater osteogenic differentiation capacity[108,109]. UC-MSCs exhibit superior clonogenic, proliferation and migration capacities[43,110]. They can secrete relevant chondrogenic factors[111,112]. Furthermore, UC-MSCs are less mature, which makes them a better choice for allogeneic therapy[113]. Wharton's jelly (WJ) is the most frequently used source of umbilical cord tissue[114]. WJ-MSCs are relatively novel for cell and tissue engineering therapy and are considered promising candidates for the development of cell-based therapies[113,115].

Many studies have shown that BM-MSCs have higher osteogenic and chondrogenic differentiation potential[116,117]. They exhibit a relatively high incidence of bone and cartilage formation[50], and they can generate more mature bone tissue and more compact cartilage pellets[118]. BM-MSCs express high levels of CD90, which means that they are more suitable for bone repair and regeneration[119].

AD-MSCs are more inclined to differentiate into adipocytes, and their potential for osteogenic differentiation and chondrogenic differentiation is relatively low[107]. Some studies hypothesize that the chondrogenic potential of MSCs derived from adipose tissue is higher than that of MSCs derived from UC sources[120]. However, other studies have suggested that AD-MSCs and UC-MSCs show similar chondrogenic potential[110].

Although BM-MSCs have stronger differentiation ability, AD-MSCs and UB-MSCs perform better for pain relief and functional improvement in OA[121]. AD-MSCs are considered the most effective MSCs in relieving pain, while UC-MSCs are considered the most effective MSCs in improving function in OA patients[122]. Therefore, AD-MSCs and UC-MSCs showed better anti-arthritis efficacy than BM-MSCs [123].

CONCLUSION

In this review, we summarize the common factors that affect the differentiation of MSCs in the OA microenvironment. MSCs can differentiate into different lineages, and these processes are precisely regulated by signaling pathways. Many factors can affect the differentiation direction of MSCs by acting on these signaling pathways. The multidirectional differentiation potential and tunability of MSCs make them a promising treatment for OA and other diseases. A large number of studies have confirmed their safety and effectiveness.

At present, a large number of studies focus on the paracrine effect of MSCs. However, the differentiation function of MSCs can also play an important role in disease treatment. Chemical, physical and biological factors can affect the differentiation of MSCs. Therefore, there are many conditions that can affect the efficacy of MSCs. To control the differentiation of MSCs more precisely to improve their efficacy in the treatment of diseases, it is necessary to understand how various influencing factors work. However, there are few studies on the factors that affect the differentiation direction of MSCs, and we are still at a preliminary stage in understanding how these factors determine the fate of MSCs. More research is needed on the differentiation of MSCs, which is of great value for developing novel therapies for diseases and applying MSCs to clinical practice.

FOOTNOTES

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MINIREVIEWS

Potential regulatory effects of stem cell exosomes on inflammatory response in ischemic stroke treatment

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Abstract

The high incidence and disability rates of stroke pose a heavy burden on society. Inflammation is a significant pathological reaction that occurs after an ischemic stroke. Currently, therapeutic methods, except for intravenous thrombolysis and vascular thrombectomy, have limited time windows. Mesenchymal stem cells (MSCs) can migrate, differentiate, and inhibit inflammatory immune responses. Exosomes (Exos), which are secretory vesicles, have the characteristics of the cells from which they are derived, making them attractive targets for research in recent years. MSC-derived exosomes can attenuate the inflammatory response caused by cerebral stroke by modulating damage-associated molecular patterns. In this review, research on the inflammatory response mechanisms associated with Exos therapy after an ischemic injury is discussed to provide a new approach to clinical treatment.

Key Words: Mesenchymal stem cell-derived exosome; MicroRNA; Inflammation; Ischemic stroke; Adipose-derived stem cell; Toll-like receptor

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Core Tip: Mesenchymal stem cell-derived exosome (MSC-Exos) transplantation is a novel treatment method for ischemic stroke that exhibits certain achievements in trials. Here, we review the strategies developed for MSC-Exos in the neuroinflammatory response of patients with stroke and provide potential therapeutic targets. These methods provide new insights for the future clinical application of MSC-Exos in the treatment of ischemic stroke.

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INTRODUCTION

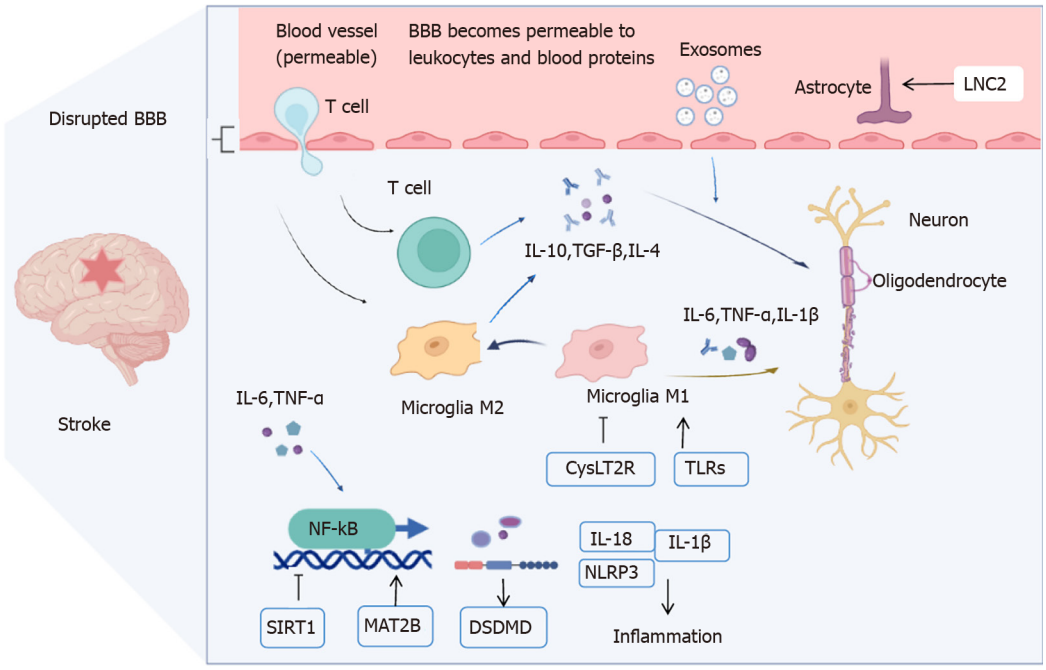
Stroke is a common clinical condition that frequently occurs in middle-aged and elderly people and is a global public health problem with high disability and mortality rates; it ranks third in the list of diseases affecting human lifespan[1]. The main goals of stroke treatment are vascular recanalization and reduction of cerebral ischemic injury. At present, the main recanalization methods are intravenous thrombolysis and endovascular mechanical thrombectomy; however, owing to the restricted time window and various comorbidities, few patients can benefit from these procedures[2,3]. Increasing evidence has suggested that inflammatory cytokines promote the migration of immune cells to damaged tissues through the blood-brain barrier (BBB) after stroke, aggravating the inflammatory response and leading to nerve cell injury[4,5]. However, the exact molecular mechanisms underlying the inflammatory response after stroke remain unclear, hindering the development of effective and specific treatments.

The effectiveness of stem cell transplantation, which can regulate the immune-inflammatory response and the permeability of the BBB, in the treatment of ischemic stroke (IS) has been verified[6,7]. However, pluripotent stem cells are obstructed by the BBB and cannot effectively enter the central nervous system, leading to risks, such as tumorigenicity, thrombosis, and pulmonary embolism, limiting their clinical application. It has been suggested that the therapeutic mechanism of mesenchymal stem cells (MSCs) may involve secreted exosomes (Exos) rather than the direct replacement of brain cells[8]. MSC-derived extracellular vesicles (MSC-EVs) possess the biological characteristics of cells and can penetrate the BBB, reduce the risk of tumors and pulmonary embolism, considerably improve therapeutic efficiency, and reduce complications, thereby having broad therapeutic prospects. MSC-derived Exos (MSC-Exos) reduce inflammatory responses after stroke[9-12]. However, the specific mechanism by which Exos alleviate the inflammatory response after stroke has not yet been explored.

Therefore, in this review, we present the current progress in research on the unique biological characteristics of MSC-Exos and the specific mechanism of action of MSC-Exos in the neuroinflammatory response after stroke. This review aims to explore the role of Exos in the neuroinflammatory response in stroke and provide potential therapeutic targets, with the expectation of offering a reference for future clinical treatments.

PATHOLOGICAL CHANGES AFTER CEREBRAL ISCHEMIA

Brain cell death after stroke can lead to a series of pathological processes including cell energy failure, neuronal apoptosis, leukocyte infiltration, inflammatory immune responses, tight junction (TJ) protein breakage and degradation, BBB destruction, and increased permeability[13,14]. The main goals of IS treatment are to restore blood flow and improve functional outcomes as soon as possible[15]. In addition, methods of regulating immune inflammation and oxidative stress responses, anti-apoptosis, and promotion of angiogenesis and neurogenesis are of great significance for the treatment of cerebral apoplexy in ischemic and hypoxic injured brain tissues[16]. The BBB controls the inflow and outflow of biological substances necessary for metabolic activity and neuronal function in the brain; therefore, its structural and functional integrity is essential for maintaining the brain microenvironment. The BBB is mainly comprised of vascular endothelial cells, pericytes, the basement membrane, astrocytes, neurons, and microglia, which exchange substances that connect the central and peripheral nervous systems. The mechanisms of BBB injury after stroke include modification of TJ proteins, regulation of transporter expression, and inflammatory damage[17]. The intravascular inflammatory response marks the beginning of BBB disruption and leukocyte infiltration in ischemic brain tissue[18]. The inflammatory response after stroke is an important factor in BBB disruption and nerve cell edema, leading to damage to mental function and even death (Figure 1).



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Figure 1 Pathological changes after stroke. Blood-brain barrier (BBB) disruption after stroke is permeable to leukocytes and blood proteins. Microglia are stimulated, and these activated microglia (M1), in turn, release tumor necrosis factor-α, interleukin (IL)-6, and IL-1β, which activate the nuclear factor kappa-B inflammatory response of reactive astrocytes (A1) and further amplify this effect. Exosomes can penetrate BBB, promote microglial M1 polarization to M2 and T cell activation, mediate lipocalin-2, sirtuin 1, methionine adenosyl transferase 2B, pyrin domain-containing protein, cysteinyl leukotriene receptor 2, and other signaling pathways to promote the release of anti-inflammatory cytokines IL-10, transforming growth factor-β, and IL-4. By BioRender.com. BBB: Blood-brain barrier; CysLT2: Cysteinyl leukotriene receptor 2; IL: Interleukin; LNC2: Lipocalin-2; MAT2B: Methionine adenosyl transferase 2B; NF-κB: Nuclear factor kappa-B; SIRT1: Sirtuin 1; TLRs: Pyrin domain-containing protein; TNF-α: Tumor necrosis factor-α.

Microglia are the resident immune cells of the brain that polarize into different phenotypes (M1 or M2)[19]. M1 inflamed microglia lead to BBB dysfunction and vascular 'leakage,' whereas M2 microglia have inflammation-inhibiting, immune-regulating, tissue-repair, and damage-eliminating functions; they also protect the BBB[20]. Activated M1 microglia release the pro-inflammatory factors tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, which activate the nuclear factor kappa-B (NF-κB)-mediated inflammatory response of reactive astrocytes (A1s) and further amplify this effect[21]. Owing to inflammatory response stimulation, the structure of the neurovascular unit (NVU) changes, which inhibits central nervous system restoration. This change in the microenvironment stimulates M2 microglia to initiate phagocytosis and secrete transforming growth factor-β (TGFβ), IL-4, and IL-10, and the engulfment of immune cells, indirectly protecting against inflammation-induced BBB disruption [22]. M2 polarization promotes the release of anti-inflammatory cytokines and tissue repair, including neurogenesis, axonal remodeling, angiogenesis, and oligodendrogenesis[21,23]. Activated matrix metalloproteinase (MMP)-2 and MMP-9 by microglia after stroke degrade the basement membrane and TJ proteins, resulting in BBB disruption, leukocyte infiltration, and angioedema, thus aggravating brain injury[17,24,25]. Pericytes also release cytokines that play vital roles in maintaining the structural integrity of the BBB. Under pathological conditions, dysfunctional pericytes can cause basement membrane degradation or alter NVU coordination, leading to BBB instability[17]. In addition, BBB injury along with the activation of TGFβ signaling in astrocytes may be a mechanism to disrupt NVU structure, as TGFβ overproduction affects the function of pericytes and vascular smooth muscle cells[26,27]. Microglial polarization is closely related to stroke progression; M1 microglia promote astrocyte differentiation to the A1 phenotype through a variety of signaling pathways, including the immune inflammatory response, angioedema, BBB disruption, neuronal apoptosis, and glutamate excitotoxicity, thereby exacerbating brain injury caused by IS[28,29]. The inflammatory reaction of the nervous system is closely related to the polarization of microglia, pericyte-secreted factors, astrocyte differentiation, and leukocyte species. However, the underlying mechanism of action of Exos in the treatment of neuroinflammation in stroke remains unclear.

BIOLOGICAL CHARACTERISTICS OF MESENCHYMAL STEM CELL EXOSOMES

MSCs are pluripotent stem cells with self-renewal, differentiation, secretion, and homing properties. They were first discovered in the bone marrow, where they are abundant and easily extractable, and are

also found in the dental pulp, umbilical cord, hemocytes, and adipose tissue (such as bone marrow MSCs, dental MSCs, umbilical cord-derived MSCs, adipose-derived MSCs, and hematopoietic stem cells)[30,31]. To overcome the problems with primary MSCs, human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and pluripotent stem cells (iPSCs), represent a promising solution[32]. MSCs can differentiate into lipogenic, chondrogenic, osteoblastic, endothelial, neural, and epithelial lineages, both *in vivo* and *in vitro*[33,34]. MSCs can reduce inflammatory responses, release trophic factors to promote therapeutic effects, induce angiogenesis, promote neurogenesis, reduce infarct volume, and replace damaged cells *via* immunomodulation[10,35,36]. Extracellular vesicles secreted by MSCs can be divided into three types based on their size and intracellular origin: Apoptotic bodies, microvesicles, and Exos. Apoptotic bodies are ≥ 1000 nm and microvesicles are 100–1000 nm in diameter. Exos (30–100 nm in diameter) originate from multivesicular bodies and are released by exocytosis, which is dependent on cytoskeletal reorganization, but independent of intracellular Ca^{2+} concentration[37,38]. When multivesicular bodies fuse with the cell membrane, Exos are released from the cells. Previously, these vesicles were considered waste products actively excreted by cells; however, studies have shown that Exos have key functions, such as transmitting information between cells, tissue regeneration, and immune regulation[39] (Figure 2).

Exos are released upon fusion with the cell membrane and trigger the release of different cellular substances. Exos can carry the same bioactive substances as their source cells and are vital for information transmission between cells, such as immune regulation and promotion of cell migration, proliferation, differentiation, and matrix synthesis[40]. Moreover, the exogenous Exos of stem cells express specific surface markers (CD9, CD63, CD81, and CD92), express specific phenotypes of stem cells (CD29, CD44, CD73, and CD90)[41], and carry heat shock proteins (HSP) proteins (HSP8, HSP60, HSP70, and HSP9), signal transduction proteins, and multivesicular production-related proteins. More importantly, they can directly transfer bioactive molecules, including non-coding regulatory microRNAs (miRNAs), messenger RNAs (mRNAs), and proteins from donor cells to recipient cells. MiRNAs are short (approximately 22 nucleotides), single-stranded, non-coding RNAs transcribed in the nucleus by RNA polymerase II from one gene or between two different genes to regulate different cellular processes such as differentiation, proliferation, metabolism, inflammation, stress response, angiogenesis, and signaling transduction[42]. miRNAs mainly affect gene expression by degrading the corresponding miRNAs or suppressing translation[43]. Alexander *et al*[44] showed that exosomal miRNAs participate in regulating inflammatory responses; miR-146a-containing Exos can inhibit inflammation, whereas miR-155-containing Exos promote inflammation following exposure to the same inflammatory stimulus.

Exos contain a variety of active substances that form the basis for disease treatment. These bioactive substances carried by Exos can target specific cells for information transmission and enter the cytoplasm by fusion with receptor cell membranes or endocytosis, thereby changing the target cell function by transmitting proteins, lipids, and nucleic acids[45]. Exos can be effectively isolated from donor cells and protect their contents from the external environment, ensuring the complete transmission of effective information[46]. Exos act as mediators that facilitate intercellular communication and influence the recipient cell activity by delivering content. DiR-labeled MSC-Exos were injected into a rat model of stroke *via* the caudal vein, and the *in vivo* tracer showed that Exos could penetrate the BBB and reach the brain tissue. The fluorescence signal peaked on the third day and then gradually decreased[47]. Matsumoto *et al*[48] also demonstrated that Exos can increase long-term neuroprotective effects after stroke, modulate peripheral immune responses, and increase angiogenesis and axonal dendritic remodeling. Therefore, the use of Exos for the treatment of neurological diseases has great potential[49, 50]. These results suggest that Exos is an important therapeutic target for the treatment of stroke (Figure 3).

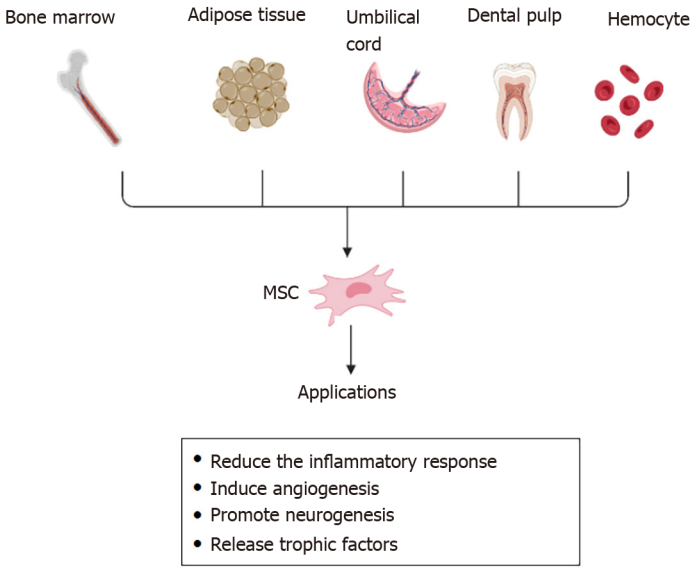
MSC-EVS REGULATE INFLAMMATORY RESPONSE IN IS TREATMENT

The potential therapeutic mechanisms of stem cell Exos involve promoting dendritic and axonal growth, repairing nerves, and promoting angiogenesis through direct actions[51,52]. Through indirect action, it can promote the secretion of inflammatory factors by cells by exogenously producing Exos that appear to interact with recipient brain cells, thereby stimulating them to release their own Exos and playing a role in anti-inflammation and neurological repair. Transplantation of MSC-Exos improves inflammatory responses in IS, maintaining BBB function, decreasing brain edema, regulating energy metabolism, and promoting antioxidant, anti-inflammatory, and anti-apoptotic effects[53]. In IS, miRNAs are involved in a variety of cellular functions, such as injured tissue repair and remodeling, and different neuronal activities. Their target genes play a crucial regulatory role in the inflammatory process of post-ischemic reperfusion injury, which explains their potential use as therapeutic targets in IS and is the focus of Exos research[54,55]. According to current research, the main signaling pathways mediated by Exos after cerebral ischemia are as follows (Table 1)[12,41,56-63].

Table 1 Application of stem cell exogenous exosomal miRNA in treatment of ischemic stroke

MicroRNA	Source	Model(s)	Functional effects	Pathway(s)	Ref.
miR-21a-5p	MSCs	OGD microglia	Induces microglial M2 polarization by targeting STAT3	STAT3	Xin *et al*[12], 2022
miR-138-5p	BMSCs	MCAO mouse, OGD astrocytes	Promotes astrocyte proliferation and inhibits inflammatory response	LCN2	Deng *et al*[41], 2019
miR-542-3p	MSCs	MCAO mice, OGD human glial cells	Suppresses inflammation and prevents cerebral infarction	TLR4	Cai *et al*[56], 2021
miR-146a-5p	HUMSCs	MCAO mouse, OGD microglia	Anti-inflammation	IRAK1/TRAF6	Zhang *et al*[57], 2021
miR-223-3p	MSCs	MCAO Rats, OGD microglia	Anti-inflammation	CysLT2R	Zhao *et al*[58,59], 2020
miR-21-3p	MSCs	MCAO rats	BBB protection, anti-inflammation, anti-apoptosis	MAT2B	Li *et al*[60], 2019
miR-26a-5p	HUMSCs	I/R mice, OGD microglia	Inhibits microglial M1 polarization	TLRs	Li *et al*[61], 2020
miR-150-5p	BMSCs	MCAO rats	Decelerates neuronal apoptosis, reduces inflammation	TLR5	Li *et al*[62], 2022
miR-30d-5p	ADSCs	Patients, MCAO rats, OGD microglia	Promotes M2 microglial/macrophage polarization	Inflammatory mediators	Jiang *et al*[63], 2018

ADSCs: Adipose-derived stem cells; BMSCs: Bone marrow mesenchymal stem cell; CysLT2R: Cysteinyl leukotriene receptor 2; HUMSCs: Human umbilical cord blood mesenchymal stem cells; I/R: Ischemia/reperfusion; IRAK1: Interleukin-1receptor-associated kinase 1; MCAO: Middle cerebral artery occlusion; MAT2B: Methionine adenosyl transferase 2B; MSCs: Mesenchymal stem cells; OGD: Oxygen-glucose deprivation; STAT3: Signal transducer and activator of transcription 3; TLRs: Toll-like receptors; TRAF6: Tumor necrosis factor receptor-associated factor 6; TLR4: Toll-like receptor 4; TLR5: Toll-like receptor 5.

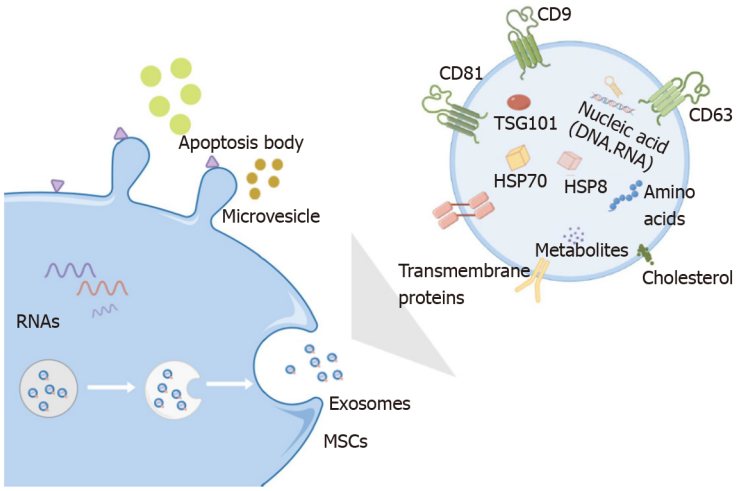


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Figure 2 Sources and potential function for stroke of mesenchymal stromal cells. These cells are found in bone marrow, dental pulp, umbilical cord, hemocyte, and adipose tissue. Mesenchymal stromal cells reduce the inflammatory response, release trophic factors, induce angiogenesis, and promote neurogenesis in stroke. By BioRender.com. MSC: Mesenchymal stem cell.

NF-kB signaling pathway

The transcription factor NF-κB regulates many aspects of innate and adaptive immunity and plays an important role in the inflammatory response. It is also involved in the migration of immune effector cells to the inflammatory system, thereby allowing the secretion of pro-inflammatory cytokines[64]. Han *et al*[65] showed that MSCs-Exos protect MCAO-injured rats, possibly by regulating the AMP-activated protein kinase (AMPK) and JAK2/STAT3/NF-κB signaling pathways. NF-κB is a central inflammatory mediator responding to many immune receptors. NF-κB mediates the induction of pro-inflammatory



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Figure 3 Exosomes. Extracellular vesicles are classified into three types: Apoptotic bodies, microvesicles, and exosomes (Exos). Apoptotic bodies are ≥ 1000 nm, microvesicles are 100–1000 nm, and Exos are 30–100 nm in diameter. The contents carried by the Exos are mainly DNA, mRNA, microRNA, functional proteins, and other active substances. The membrane structure can express a variety of antigens and antibody molecules. The surface markers of the Exos mainly include CD63, CD81, CD9, and heat shock protein 70. By Figdiaw. MSCs: Mesenchymal stem cells.

cytokines, such as TNF-α, IL-1, and IL-6, in monocytes/macrophages[66]. Fann *et al*[67] confirmed the involvement of NF-κB signaling in the activation of pyrin domain-containing inflammasomes following IS. Preconditioning MSCs with lithium modifies EV secretion patterns, enhancing the therapeutic potential of the derived EVs (Li-EVs) and significantly increasing the resistance of cultured astrocytes, microglia, and neurons to hypoxic injury compared with control and native EVs. Li-EVs reduce the abundance of post-hypoxic and post-ischemic TLR4 (leading to activation of the NF-κB signaling pathway) and decrease proteasomal activity, which together contribute to reduced levels of poststroke encephalitis[68]. The miRNAs carried by Exos play a significant physiological role. Cai *et al*[56] confirmed that MSC-derived exosomal microRNA-542-3p (miR-542-3p) prevented ischemia-induced glial cell inflammatory responses by inhibiting TLR4. Interleukin-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) may be parts of an NF-κB-induced negative feedback loop[69]. Zhang *et al*[57] found that injected Exos produced by human umbilical cord MSCs (HUMSC-Exos) enter the site of ischemic injury and be internalized by cells, both *in vivo* and *in vitro*. *In vitro*, HUMSC-Exos treatment attenuates microglial inflammation induced by oxygen-glucose deprivation (OGD). *In vivo*, HUMSC-Exos treatment significantly reduced infarct volume, alleviated behavioral deficits, and improved microglial activation 3 d after transient cerebral ischemia. MiR-146a-5p from HUMSC-Exos can attenuate microglial-mediated neuroinflammation through the IRAK1/TRAF6 pathway and ensuing neurological deficits after IS. NF-κB signaling pathway activation is a 'master regulator' of inflammation and is associated with the generation of free radicals and the activation of proteolytic enzymes and pro-inflammatory cytokines, playing an important role in regulating apoptosis after stroke[70,71]. Taken together, these results show that NF-κB signaling is essential for the regulation of brain tissue inflammasomes under ischemic conditions. In addition, MSC-Exos treatment decreased the activation of the NF-κB signaling pathway, thereby attenuating inflammasome expression and activation under ischemic conditions. These findings suggest that therapeutic interventions targeting neuroinflammasome activation may provide new opportunities for the treatment of IS.

NOD-like receptor family pyrin domain-containing 3 signaling pathway

NOD-like receptor family pyrin domain-containing 3 (NLRP3) plays an important role in mediating the inflammatory responses during cerebral IS[72]. The NLRP3 inflammasome is a multiprotein complex comprising NLRP3 and pyroptosis-related factors (ASC and caspase 1)[73]. The NF-κB and mitogen-activated protein kinase (MAPK) pathways play a major role in the expression and activation of NLRP1 and NLRP3 inflammasomes in primary cortical neurons[67]. Bone marrow MSC-Exos (BMSC-Exos) can reduce brain infarct area and cerebral edema, thus improving neurological function. MSC-Exos can downregulate the expression of NLRP3 inflammasome and pyroptosis-related proteins on the surface of neurons[74]. Moreover, it improved the transition from M1 to M2 phenotype both *in vivo* and *in vitro*. BMSC-Exos relieve cerebral ischemia/reperfusion (I/R) injury by suppressing NLRP3 inflammasome-mediated inflammation and pyroptosis *via* modulation of microglial polarization[74]. Sarmah *et al*[75] came to similar conclusions by treating a rat MCAO model with intra-arterial injections of MSCs; the levels of NLRP-1 and NLRP-3 inflammasomes and their related components IL-1β, caspase-1, and ASC were significantly reduced. NLRP3 apoptotic bodies are involved in astrocyte and microglial

polarization and are closely related to the development of the inflammatory cascade after stroke, and BMSC-Exos reduce the inflammatory response after stroke by inhibiting NLRP3 activation.

Sirtuin signaling pathway

Sirtuins (SIRTs) are NAD+-dependent deacylases with multiple roles in energy metabolism regulation, cell survival, transcriptional regulation, inflammation, circadian regulation, and DNA repair[76]. SIRT-1 and SIRT-3 are both associated with the inflammatory response in stroke patients. Xin *et al*[12] used an *in vivo* neonatal male mouse model of hypoxic-ischemic (HI) injury and induced *in vitro* hypoxia-glucose deficiency, thus simulating microglial BV-2 cells to deliver miR-21a-5p (miR-21a-5p) as a therapeutic intervention through MSC-Exos. Treatment of BV-2 cells with MSC-EVs increased cell viability and miR-21a-5p levels, which were decreased after glucose-oxygen deprivation. In both *in vitro* and *in vivo* models of HI injury, the effects on microglial polarization and STAT3 phosphorylation decreased when miR-21a-5p levels were reduced in MSC-Exos. These results suggest that MSC-Exos attenuate HI brain injury in neonatal mice by delivering miR-21a-5p, which induces microglial M2 polarization by targeting STAT3. Adipose-derived MSC-derived miR-22-3p reduces infarct volume and apoptosis in a stroke model[77]. Sarmah *et al*[75] demonstrated that intraarterial MSCs increase SIRT-1 to inhibit the NF-κB pathway, reducing inflammasome signaling and apoptosis, thereby exerting a neuroprotective effect. SIRT1 may be an independent risk factor for cerebral infarction, and a high concentration of SIRT1 in cerebral infarction may be associated with disruption of the BBB[78].

Cysteinyl leukotriene signaling pathway

Cysteinyl leukotrienes (CysLTs), including leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4), are derived from 5-lipoxygenase metabolites of arachidonic acid after cell necrosis and are effective mediators of inflammation[79]. The effects of CysLTs are mainly mediated by the CysLT1 and CysLT2 receptors (CysLT1R and CysLT2R), which are active in various cell types during pathological brain injury. CysLT2 is expressed in the cerebral cortex, hippocampus, substantia nigra, and lateral ventricle[80]. Zhao *et al*[58,59] showed that the overexpression of miR-223-3p (miR-223-3p) in MSC-Exos can reduce MCAO-induced infarction, improve neurological deficits, and promote learning and memorization. MiR-223-3p inhibits the expression of pro-inflammatory factors and promotes the secretion of anti-inflammatory factors in the ischemic cortex and hippocampus. Western blot and quantitative real-time PCR analyses also showed that exosomal miR-223-3p reduced the mRNA and protein expression of CysLT2R *in vitro* and *in vivo*. Exosomal miR-223-3p from MSCs alleviated cerebral I/R injury by inhibiting the pro-inflammatory response mediated by M1 polarization of microglia, which may be related to the inhibition of CysLT2R by exosomal miR-223-3p.

Lipocalin-2

Lipocalin-2 (LCN2), a 25 kDa protein, is a neutrophil gelatinase-associated protein that affects different cellular processes during stroke. The pro-inflammatory mediator LCN2 plays a key role in I/R injury [81]. Genetic or pharmacological inhibition of these pro-inflammatory mediators (iNOS, IL-6, CCL2, and CCL9) provides neuroprotection against stroke and reduces the expression of inflammatory factors by down-regulating LCN2[41]. Deng *et al*[41] used a mouse MCAO model to explore the effects of BMSC-derived exosomal miR-138-5p in IS *in vivo*. Overexpression of miR-138-5p promoted cell proliferation and inhibited apoptosis of OGD-damaged astrocytes, accompanied by decreased expression of inflammatory factors. This was achieved by downregulating LCN2, and the expression of LCN2 protein was subsequently detected by Western blot analysis. More importantly, BMSCs attenuated neuronal injury in IS mice by delivering miR-138-5p to astrocyte Exos. Therefore, the exogenous exosomal miR-138-5p from BMSCs promotes astrocyte proliferation and inhibits the inflammatory response after IS by targeting LCN2, thereby reducing neurological impairment, which may provide a new target for IS treatment.

Methionine adenosyl transferase 2B

Methionine adenosyl transferase (MAT) is an enzyme involved in cell cycle regulation. Mammals have three major MAT genes: MAT1A, MAT2A, and MAT2B[60]. TNF-induced activation of MAT2B promotes tumor growth through the NF-κB pathway in hepatoma cells[81]. MiR-21-3p antagomir can control the inflammatory response by inhibiting NF-κB signaling; these functions of miR-21-3p are exerted by directly targeting MAT2B[82]. This interaction forms the basis of the function of miR-21-3p/MAT2B in regulating inflammation. Li *et al*[60] found that miR-21-3p expression was elevated in the MCAO model, and the inhibition of exogenous adipose-derived stem cell Exos miR-21-3p could inhibit the expression of MAT2B in neural cells, thereby improving the BBB status and inhibiting apoptosis and inflammatory responses. MiR-21-3p antagomir could inhibit the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL6) and promote the expression of anti-inflammatory cytokines (IL-10). Thus, miR-21-3p can protect neural cells by inhibiting the expression of MAT2B and thus inhibiting apoptosis and inflammatory responses.

Toll-like receptors (TLRS)

Evidence suggests that Toll-like receptors (TLRs) play important roles in the development of ischemic brain injury in adults[83]. TLRs, comprising 13 members (TLR-1 to TLR-13), are type 1 integral membrane proteins responsible for detecting invading pathogens and initiating immune responses[84, 85]. The microglial TLR pathway is activated following cerebral ischemia and inhibition of TLR signaling by exosomal miR-26a-5p decreases cholesterol 25-hydroxylase protein expression, which in turn inhibits microglial M1 polarization and relieves nerve injury after brain injury[61]. The gene expression of many inflammatory mediators, such as TNF-α, IL-1, and IL-6, can reduce the development of nervous system inflammation by inhibiting TLR4 transduction pathway downregulation[86]. Upregulation of miR-326 attenuates IL-10, IL-1β, and TNF-α pro-inflammatory cytokine expression in response to lipopolysaccharide stimulation by targeting TLR4[87]. TLR5 activates NF-kB and MAPK pathways that regulate the transcription of genes encoding immune mediators[88]. Qiao *et al*[89] elucidated that TLR5 downregulation is accompanied by alleviated neurological deficits, reduced infarct volume, and reduced edema after IS[90]. Li *et al*[62] validated that BMSC-Exos can improve neurological function and pathological changes, decelerate neuronal apoptosis, and reduce inflammatory factors in MCAO rats. Exosomal miR-150-5p from BMSCs mitigates cerebral I/R injury by inhibiting TLR5 expression. These studies showed that TLRs and their related miRNAs are associated with inflammation after IS.

Inflammatory mediators

Dabrowska *et al*[91] transplanted human bone marrow stem cells and their secreted Exos into a rat model of local brain injury. The results showed that monocyte chemoattractant protein-1 (MCP-1) expression increased locally after brain injury, whereas MCP-1 expression decreased in the transplanted HUMSCs and Exos groups. In addition, they observed that the infusion of pro-inflammatory cytokines and chemokines with HUMSCs or EVs in rats with untreated focal brain injury was associated with reduced microglial/macrophage and astrocyte activation. MSC-Exos therapy can reduce the expression of the inflammatory cytokines TNF-α and IL-6, increase the expression of the cytokines IL-4 and IL-10, and reduce brain injury. Exos from stem cells can enhance the activation of CD4+ and CD8+ lymphocytes, decrease the number of dendritic cells, regulate peripheral immunosuppression caused by stroke[9], and pass antigens to the immune system through the BBB[92]. IL-4, CD206, and IL-10 are markers of M2 microglial secretion, whereas TNF-α, IL-6, and iNOS are markers of M1 microglial secretion[93]. Yang *et al*[94] found that the MCAO model also verified that Exos intervention reduced the infarct volume and promoted the polarization of microglia to M2 phenotype. These results demonstrate that adipose-derived stem cell Exos can prevent stroke by shifting microglia from an M1 to M2 phenotype in the hippocampus[94].

Microglial polarization

Microglia are macrophages of the central nervous system and are important components of innate and adaptive immune responses[95]. The microglial M1 type can secrete pro-inflammatory factors, whereas the M2 type can secrete anti-inflammatory factors; therefore, the fact that MSCs and Exos can promote the polarization of microglia to M2 is notable for the treatment of IS. The M2 type protects nerve cells mainly by engulfing debris and promoting the repair and regeneration of brain tissue. In contrast, inflammatory factors of the M1 phenotype aggravate post-stroke symptoms. Therefore, the microglial response after stroke is an important prognostic factor[96]. Different miRNAs transported by Exos contribute to the differentiation of microglia into distinct phenotypes. Increased levels of miR-124-3p in microglial Exos promote M2 microglial polarization, reduce brain damage, and improve stroke outcomes[97]. Adipose-derived MSCs (ADMSCs) participate in the repair process of tissues through paracrine effects after relieving nerve injury; ADMSCs have similar biological characteristics to MSCs. Stimulation of AMSC-derived Exos with inflammatory factors was found to convert M1 microglia into M2 microglia, suggesting that AMSC-derived Exos promote microglial polarization by activating pro-inflammatory microenvironment signals[98]. miRNAs are critical regulators of genes involved in various biological processes; miR-146a-5p-enriched BMSC-Exos directly target IRAK1 and nuclear factor-activated T cell 5 (NFAT5), which contributes to inflammatory responses and polarize M1 microglia/macrophages[99]. Exos containing miR-216a-5p, miR-124, miR-155, miR-182, miR-17-5p, miR-30d-5p, and miR-223-3p were found to promote microglial M2 polarization[63,100-103]. BMSC-Exos promote microglial polarization from M1 to M2, inhibit inflammation-related signaling pathways, and reduce endothelial cell injury and neurological impairment caused by IS[104-106]. Although astrocytes may play a role in brain inflammation, little is known about their role in stroke pathology[107].

Activated regulatory T cells

Changes in regulatory T cell (Treg) numbers and function after stroke are accompanied by a decrease in immunosuppressive function, which affects stroke prognosis[108]. The immunosuppressive function of Tregs is largely impaired during stroke and Treg-derived anti-inflammatory factors, including transforming growth factor-beta (TGF-b) and IL-10, are reduced[109,110]. MiRNAs delivered by stem cell Exos can induce anti-inflammatory polarization as important regulators of Treg homeostasis and

function[111,112]. MSC-Exos induce anti-inflammatory IL-10 and TGF-β transcription, attenuate pro-inflammatory factors IL-1β, IL-6, and TNF-β, and inhibit the differentiation and activation of Tregs[113, 114]. Furthermore, MSC-Exos are absorbed by endothelial cells, impair T-cell function by inhibiting T-cell proliferation *in vitro*, and increase endothelial cell proliferation, migration, and capillary formation in a dose-dependent manner[115]. Wang *et al*[116] showed that the intravenous injection of MSC-EVs reduced neurological deficits, cerebral infarct volume, brain edema, and neuronal injury in both young and old mice. The neuroprotective and anti-inflammatory effects of MSC-EVs were demonstrated through a decrease in leukocyte infiltration and, specifically, polymorphonuclear neutrophil, monocyte, and macrophage infiltration, in the cerebral ischemic areas of aged mice. In addition, MSC-EVs significantly decreased the number of monocytes and activated Tregs. The expression and phosphorylation of signal transducer and activator 1 (STAT1) are increased in mice with miR-146a deficient Tregs, and miR-125a is involved in the differentiation of Tregs[117]. Exosomal miR-16 and miR-21, derived from bone marrow stem cells, can increase the production of Tregs and exert anti-inflammatory effects[118]. Although breakthroughs have been made in elucidating the working mechanism of Tregs over the past decade, the mechanism by which this minor population of peripheral immune cells has a significant beneficial effect after stroke injury remains largely unknown[108].

CONCLUSION

Brain injury after stroke is a complex pathological process. This review summarizes the recent studies on the mechanism of action of MSC-Exos in regulating inflammatory responses during IS treatment. MSC-Exos regulate microglial polarization through various pathways such as NF-κB, NLRP3, and STATs, indicating that microglial M1 to M2 phenotype polarization is closely related to the inflammatory response after IS.

However, some essential questions remain unanswered. Stroke-induced brain injury involves multiple mechanisms that cannot be explained by a single one. Immune inflammation plays a crucial role in this process, especially the NF-κB, NLRP3, and other signaling pathways. After immune inflammation, microglia, leukocytes, and other inflammatory cells are activated and release many pro-inflammatory factors. Additionally, nerve cells are affected by varying degrees of damage caused by ischemia and hypoxia after stroke.

However, these studies have some limitations. MSC-Exos can mediate different signaling pathways to reduce inflammatory responses after stroke in animal models. However, these results have not been translated into clinical practice. Most studies have focused on exosomal miRNAs, indicating that they play an important role in regulating cellular functions. However, research on other bioactive molecules contained in Exos, such as miRNAs, mRNAs, and proteins, is limited. This does not mean that this mechanism of action of miRNAs can explain how Exos attenuate post-stroke inflammation. The dosage, mode of administration, and duration of action of the Exos should be elucidated. Exos are considered ideal biomarkers and drug delivery vehicles, with great potential for overcoming the limitations of stem cell therapy[119]. The use of Exos as drug-loaded systems will facilitate breakthroughs in the research and development of targeted drugs for clinical treatment. Moreover, new directions and methods will be provided for stroke treatment.

FOOTNOTES

Author contributions: Chen N drafted the manuscript; Wang YL, Sun HF, Wang ZY, and Zhang Q reviewed the literature, and designed and revised the manuscript; Fan FY, Liu FX, and Ma YC generated the graphs; Zhang YK guided the construction of the manuscript; all the authors have read and approved the manuscript.

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Clinical relevance of stem cells in lung cancer

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Abstract

Lung cancer is the major cause of cancer-related deaths worldwide, it has one of the lowest 5-year survival rate, mainly because it is diagnosed in the late stage of the disease. Lung cancer is classified into two groups, small cell lung cancer (SCLC) and non-SCLC (NSCLC). In turn, NSCLC is categorized into three distinct cell subtypes: Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC is the most common lung cancer, accounting for 85% of all lung cancers. Treatment for lung cancer is linked to the cell type and stage of the disease, involving chemotherapy, radiation therapy, and surgery. Despite improvements in therapeutic treatments, lung cancer patients show high rates of recurrence, metastasis, and resistance to chemotherapy. Lung stem cells (SCs) are undifferentiated cells capable of self-renewal and proliferation, are resistant to chemotherapy and radiotherapy and, due to their properties, could be involved in the development and progression of lung cancer. The presence of SCs in the lung tissue could be the reason why lung cancer is difficult to treat. The identification of lung cancer stem cells biomarkers is of interest for precision medicine using new therapeutic agents directed against these cell populations. In this review, we present the current knowledge on lung SCs and discuss their functional role in the initiation and progression of lung cancer, as well as their role in tumor resistance to chemotherapy.

Key Words: Lung cancer; Cancer stem cells; Biomarkers; Chemoresistance

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Core Tip: Lung cancer stem cells (CSCs) could have a functional role in primary tumor initiation, invasion and metastasis, resistance to chemotherapeutic drugs, and recurrence in lung cancer. To improve lung cancer treatments, it is necessary to identify and characterize CSCs populations in lung tissue and develop targeted therapies against these cell types. This review discusses the current knowledge on CSCs in lung tissue and future perspectives in lung cancer treatment.

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INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide, in both men and women. Lung cancer is classified into two histological types, small cell lung cancer (SCLC) derived from cells of the neuroendocrine lineage and non-SCLC (NSCLC) derived from epithelial cells. The latter is the most frequent type of lung cancer in the population, since represents approximately 85% of all lung tumors, while SCLC represents the remaining 15%[1].

Based on the morphology of the transformed cells, NSCLC is classified into three subtypes: Squamous cell carcinoma (SCC), adenocarcinoma (AD), and large cell carcinoma (LCC). AD arises in peripheral lung tissue from type II alveolar epithelial cells that line the small alveoli, while SCC arises in the central lung region from cells of the stratified squamous epithelium that line the airways from the trachea to the main bronchi. LCC is a heterogeneous group of tumors that lack the morphological characteristics of AD and SCC sub-types or SCLC and arise from epithelial cells that line the lungs[1,2].

SCLC arises from lung epithelial cells from the main bronchi to the terminal alveoli. Their histologic features correlate with the site of origin and reflect epithelial variations of the airways[2]. The classification and main characteristics of lung cancer are shown in Figure 1.

All forms of lung cancer have a poor prognosis, mainly SCLC and SCC, which are typically seen in smokers. SCLC is the most aggressive lung cancer because it grows quickly and spreads to other tissues, generating metastases in the liver, brain, bones, and adrenal glands[3]. LCC has a better prognosis, which depends on the cell subtype. The poor prognosis is generally associated with the ability of the cells to metastasize and the late diagnosis of the disease, since the symptoms and signs do not usually appear in the early stages[4]. People with lung cancer may have chest pain, frequent coughing, blood in the mucus, trouble breathing, swallowing or speaking, loss of appetite and weight, and tiredness[5].

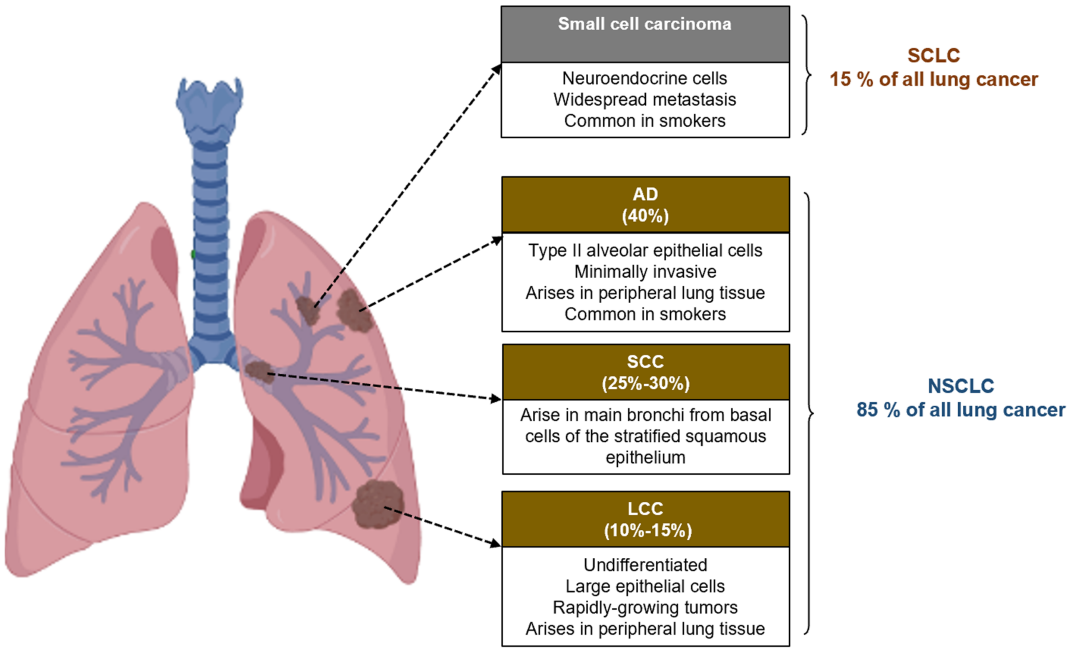
The prevalence of lung cancer worldwide is 13%, with an incidence of 1.8 million new cases per year. Lung cancer is responsible for about 20% of all cancer-related deaths worldwide and has one of the lowest 5-year survival rates (7% in SCLC and 11-17% in NSCLC, depending on the subtype and stage of the cancer[6].

The main cause of lung cancer is long-term smoking, with a 25-fold increase in the risk of developing the disease. However, there are other risk factors such as genetic factors, long-term menopausal hormone replacement therapy, exposure to radon, asbestos, metals (arsenic, cadmium, and chromium), organic chemicals found in smoke coal, and a history of lung disease such as tuberculosis, emphysema, or chronic bronchitis[7]. Smoking can cause all types of lung cancer, but is most strongly linked to SCLC and SCC, while AD is the most common type of lung cancer in patients who have never smoked. In the first two, cancer development frequently occurs in people 60 to 70 years of age with a long-term history of tobacco smoking, while AD occurs in younger people[7].

In this review, we update knowledge about the properties of SCs and their participation in the initiation and progression of lung cancer, pointing out the biomarkers that may be useful in therapeutic strategies or as a prognosis of the disease. In addition, we discuss the role of SCs in the resistance to therapeutic treatments.

SOMATIC MUTATIONS IN LUNG CANCER

Lung carcinogenesis is a chronic process that involves multiple genetic, cellular, and tissue alterations as a consequence of mutations in genes that regulate growth, differentiation, and apoptosis. Cancers are caused by the accumulation of mutations in critical genes, specifically those that control cell growth and division or the repair of damaged DNA. These changes allow cells to grow and divide uncontrollably to form a tumor. In almost all cases of lung cancer, these genetic changes, which are present only in certain lung cells, are acquired during life as a result of exposure to carcinogens, long-term hormone therapy, or chronic lung disease[8].



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Figure 1 Classification and main characteristics of lung cancer. Lung cancer is classified into two histological types, small cell lung cancer (SCLC) derived from cells of the neuroendocrine lineage and non-SCLC (NSCLC) derived from epithelial cells. NSCLC is classified into three subtypes: Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. SCLC: Small cell lung cancer; NSCLC: Non-SCLC; AD: Adenocarcinoma; SCC: Squamous cell carcinoma; LCC: Large cell carcinoma.

The most frequently mutated genes in lung cancer are *epidermal growth factor receptor* (*EGFR*), *Kirsten rat sarcoma virus* (*KRAS*) and *tumor protein 53* (*TP53*)[9,10]. The proteins encoded by these genes participate in signaling pathways that contribute to lung tumorigenesis. Mutations in the *EGFR* gene cause the expression of receptors on the cell membrane that are constitutively activated, triggering signaling pathways within cells involved in lung tumorigenesis. Binding of epidermal growth factor (EGF) to EGFR induces receptor phosphorylation at tyrosine residues and activates multiple downstream signaling pathways, such as the Ras-Raf-MAPK, PI3K, and STAT pathways, which induce proliferation and cell invasion, angiogenesis, inhibition of apoptosis and metastasis. The constitutive activity of EGFR has been observed in more than 60% of patients with NSCLC and is due to different mutations present in the receptor[11]. *KRAS* was first identified as a viral oncogene in the Kirsten RAt Sarcoma virus. The *KRAS* gene encodes the Ras proteins, low molecular weight enzymes with guanosine triphosphate hydrolase (GTPase) activity located on the inside of the plasma membrane. Ras GTPases are activated in response to the binding of ligands to several receptors such as EGF, platelet-derived growth factor, integrin, and cytokine receptors. Mutations in the *KRAS* gene cause loss of the ability of Ras to hydrolyze GTP to guanosine diphosphate leading to constant activation of Ras[12]. Similar to constitutive activation of EGFR, mutated Ras stimulates multiple signaling pathways relevant to tumor development. Mutations in the *KRAS* gene are of high frequency in NSCLC but are also present in other types of cancer such as colorectal and pancreatic cancer[13]. Another protein associated with the onset of lung cancer is the p53 tumor suppressor protein encoded by the *TP53* gene. This tumor suppressor responds to several cellular stresses to regulate expression of target genes, thereby inducing DNA repair or cell cycle arrest and apoptosis[14]. Mutations in the *TP53* gene synthesize a non-functional p53 protein, causing accumulation of damaged DNA in cells and dysregulation of the cell cycle. Mutations in the *KRAS* and *TP53* genes have been found in up to 30% of patients with lung cancer and have been considered as predictors of poor prognosis[9]. Mutations in other genes such as *CDKN2A*, *STK11*, *BRAF*, *PIK3CA*, *RB1*, *PTEN* or *MET* have been associated with the onset of lung cancer, although they showed a lower mutation rate than the *EGFR*, *KRAS* or *TP53* genes. Most of them are involved in the regulation of cell proliferation, differentiation, or apoptosis[9].

Lung tumor tissue is characterized by a heterogeneity of cell types with different phenotypic characteristics and properties as a result of the accumulation of gene mutations and differential cell signaling in the tumor microenvironment. However, it has been shown that only a small fraction of cells in tumor tissue have the ability to form tumor spheroids *in vitro* and develop tumors when they are transplanted into immunodeficient animals. These observations raise the need to study which are the cell populations in the tumor with tumorigenic potential.

ROLE OF STEM CELLS IN TUMORIGENESIS

Cancer stem cells (CSCs) are defined as a subpopulation of tumor cells residing in the tumor with SCs characteristics. CSCs have the ability to self-renew, generate identical daughter cells with stem cell properties, differentiate into multiple cancer cell lineages, facilitate tumor growth and survival, and metastasize to distal sites. In addition, they could be the cause of evasion of the immune system, resistance to chemotherapy and radiation therapy, and cancer relapse[15]. CSCs differ from normal tissue-specific SCs only in their uncontrolled growth and altered genotypes[16].

The seminal proposition that CSCs could be responsible for tumor initiation was introduced by the end of XIX century[17], however the first conclusive evidence for CSCs in acute myeloid leukemia was demonstrated in 1997 by Bonnet and Dick[18]. These authors isolated a subpopulation of CD34+/CD38- leukemic cells capable of initiating tumors in non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice histologically similar to the donor. In recent years, CSCs have been identified in several tumors, including breast, brain, colon, and lung cancer[19].

CSCs are a small proportion of the cell population of a tumor (less than 1%) that exhibit high tumorigenic potential. This is supported by experiments where thousands of tumor cells need to be inoculated into syngeneic or immunodeficient animals for a solid tumor to develop. Some years ago, this requirement was thought to be due to the loss of viability of tumor cells during transfer or the absence of a suitable niche for tumor growth. The new paradigm indicates that only CSCs (a small fraction of the transplanted cells) has the potential to generate a tumor[20]. This hypothesis can be reinforced by the fact that metastases are histologically heterogeneous and similar to the primary tumor, which implies that the cell that gave rise to it has the potential to differentiate into multiple cell types, such as a SCs[19].

Several questions have been raised about the origin of CSCs in tumor tissue. The first hypothesis is that CSCs could originate from tissue-specific normal SCs that have dysregulated cell growth. In this sense, it has been shown that all tissues harbor SCs that play an essential role in tissue repair[16]. These normal SCs could be transformed into CSCs due to the accumulation of gene mutations or the action of chemical mediators released under conditions of tissue damage[21]. This hypothesis is accepted for tissues with a high degree of cell renewal, such as intestinal epithelium or skin, but it is controversial for tissues that are quiescent or that renew slowly, such as lung epithelium. In this sense, some authors have identified a fraction of cells in active division (less than 1.3%) located in specific niches of the tracheal and bronchiolar epithelium with characteristics of SCs that could become CSCs[22,23]. The second hypothesis is that CSCs could originate from more differentiated progenitor cells that have acquired the capacity for self-renewal or differentiated tumor cells (called CSCs-like cells) due to mutations in genes that regulate the cell cycle, hypoxia, or chemical mediators of the tumor microenvironment such as nitric oxide or certain interleukin[24].

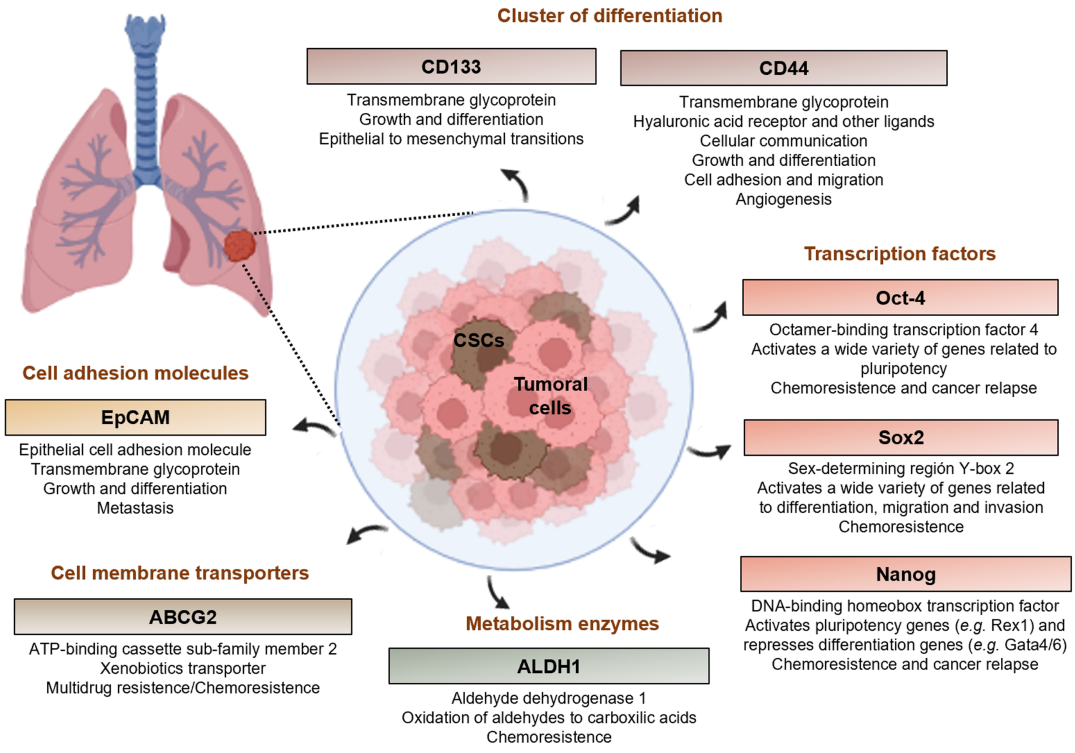
STEM CELL MARKERS IN LUNG CANCER

Tumor tissue is heterogeneous, showing different cell populations that have their own signaling pathways, leading to more complex therapeutic strategies. Factors contributing to intratumoral heterogeneity include genetic mutations, epigenetic changes, interactions with the microenvironment and CSCs[25]. CSCs represent only a small group of cells within the heterogeneity of tumor tissue. SCs trigger specific signaling pathways, such as Notch, Hedgehog, and Wnt, that allow them to self-renew and proliferate in tissue repair processes. These pathways are tightly controlled, but their aberrant activation in CSCs can induce the expression of tumor molecular markers, leading to tumorigenicity and chemoresistance[26].

CSCs have been identified in several types of cancer through proteins that are differentially expressed in these cells. These proteins include cell membrane receptors, cell adhesion molecules, cell membrane transporters, enzymes involved in metabolism, and transcription factors. Its are used as biomarkers to predict the prognosis of the disease, evaluate the most appropriate chemotherapy treatments and the efficacy of the therapies in tumor remission[15].

Biomarkers that have been identified in lung cancer include cluster of differentiation-133 (CD133), cluster of differentiation-44 (CD44), epithelial cell adhesion molecule (EpCAM), aldehyde dehydrogenase 1A1 (ALDH1A1), ATP-binding cassette sub-family G member 2 (ABCG2) and the transcription factors octamer-binding transcription factor 4 (Oct-4), sex-determining region Y-box 2 (Sox2), and Nanog[15,26]. Although these biomarkers have different structures and functional roles, its are linked to the SCs properties and uncontrolled proliferation of tumor cells. The biomarkers identified in lung CSCs are shown in Figure 2.

Biomarkers are not specific for lung CSCs and may be expressed in other types of cancer, such as breast, brain, colon or liver cancer. Since a single marker is not sufficient to accurately identify CSCs, combinations of several markers are used to identify and isolate CSCs in tumor tissue[27].



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Figure 2 Stem cell biomarkers in lung cancer. Biomarkers identified in cancer stem cells in lung tissue include clusters of differentiation, cell adhesion molecules, cell membrane transporters, enzymes of cell metabolism, and transcription factors. CSCs: Cancer stem cells; ABCG2: ATP-binding cassette subfamily G member 2; ALDH: Aldehyde dehydrogenase.

Cluster of differentiation

CD133: CD133, also known as prominin-1, is a transmembrane glycoprotein involved in cell growth and differentiation through its involvement in multiple signaling pathways[27,28]. It has been identified as the main biomarker of SCs in normal tissue and of stemness in tumor tissue from patients with NSCLC or SCLC.CD133 expression is essential to maintain CSCs characteristics such as tumor cell proliferation, migration, and invasion, and the ability to resist chemotherapy[27].

In lung cancer, the high level of expression of CD133 has been correlated with epithelial to mesenchymal transitions and the formation of metastases in lymph nodes and other tissues, which reveals that CD133 is a biomarker of tumor aggressiveness and poor prognosis of the pathology[29-31]. The molecular mechanisms involved in CD133-mediated cell growth and resistance to chemotherapy are still under study, but appear to be associated with the Wnt/catenin, PI3K-AKT and SRC-FAK signaling pathways[32,33]. Furthermore, CD133 has been shown to directly interact with vascular endothelial growth factor (VEGF), stimulating angiogenesis and leading to tumor growth[34]. CD133 is also used as diagnostic, predictive, or therapeutic biomarkers in other types of cancer including breast, stomach, liver, prostate, colorectal, pancreatic, and renal[28].

CD44: CD44 is a transmembrane glycoprotein of high structural and functional diversity due to alternative splicing processes and post-translational modifications. It can bind to a wide variety of ligands on the cell surface, including hyaluronic acid (HA), and trigger multiple cellular processes such as cell-cell signaling, cell growth and differentiation, cell adhesion and migration, angiogenesis, or cell survival[26].

CD44 regulates several signaling pathways to promote cancer progression, including Notch, Hedgehog, and Wnt pathways[26]. In addition, it can act as a co-receptor and heterodimerizes with growth factor receptors [EGFR, fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor, VEGF receptor, transforming growth factorβ receptor] and leads to activation of the PI3K-AKT and MAPK pathways[15]. It is hypothesized that CD44 could play an important role in tumorigenesis mediated by the constitutive activation of EGFR, whose expression is frequently mutated in patients with NSCLC. In this last sense, CD44 could be an important marker to predict the efficacy of chemotherapy using EGFR-specific tyrosine kinase inhibitors in patients with lung cancer[15].

CD44 is expressed in almost all tumor types, but it has been identified as the main CSCs biomarker in lung ADs and in SCC. Its expression is associated with a more aggressive tumor phenotype, with the ability to metastasize and resist chemotherapy[35].

Cell adhesion molecules

EpCAM: EpCAM is a transmembrane protein and acts as an adhesion molecule on the lung epithelium. It is a potential biomarker for lung tumors of epithelial origin[36]. EpCAM expression in lung tumor epithelium is upregulated by metastasis-associated protein 1 and leads to increased metastatic capacity of tumor cells[36]. The co-expression of EpCAM with CD44 and CD166 in NSCLC indicates a greater self-renewal capacity, clonal heterogeneity and stemness. These biomarkers in lung tumor tissue indicate a poor prognosis for the disease[37].

Cell membrane transporters

ABCG2: ABCG2 is a transporter of xenobiotic compounds into the extracellular space, which has been implicated in the development of multidrug resistance (MDR) in cancer. The wide variety of ABCG2 substrates includes several antitumoral drugs such as paclitaxel, doxorubicin, cisplatin, topotecan, mitoxantrone and irinotecan. ABCG2 also expels the fluorescent dye Hoechst 33342 out of the cell, a property that is unique to SCs and is used for identification of these cells[38]. The expression of ABCG2 in SCs has been conserved in many tissues, such as the pancreas, lung, heart, testis, liver, brain, prostate, and embryonic tissue, demonstrating the importance of this transporter for cell survival[39]. In lung cancer, increased expression of ABCG2 has been found in SCs, associated with the upregulation and activity of the transcription factors Sp1, Sp3, YAP1 and Nrf2[40-42]. Its expression in lung CSCs is associated with cancer relapse and poor prognosis[42].

Enzymes involved in metabolic pathways

ALDH1: ALDH1 is an enzyme that participates in cellular metabolism through the oxidation of aldehydes to carboxylic acids and is a marker of SCs of normal and tumor tissue[43]. It has three main isotypes, ALDH1A1, ALDH1A2 and ALDH1A3, but its activity is mainly attributed to the ALDH1A1 isotype[43].

Increased expression of ALDH has been found in NSCLC overexpressing a subpopulation of CD44+/EpCAM+ cells[44]. Overexpression of the ALDH1 and CD133 markers was found exclusively in SCC and AD[45]. ALDH1A1 overexpression is associated with an aggressive chemotherapy-resistant tumor phenotype. Despite this, ALDH1 could be a useful therapeutic target for tumors growing in tissues that do not normally express high levels of ALDH1A1, such as the lung, breast, colon, and stomach[43].

Transcription factors

Otherwise, the transcription factors involved in the normal activity of SCs could be deregulated in cancer, and activate cellular pluripotency genes and suppress differentiation genes, triggering signaling pathways responsible for the characteristics of CSCs. Transcription factors that regulate the functions of lung CSCs, such as Oct-4, Sox2, and Nanog, have been identified.

Oct-4: Oct-4 also known as POU5F1, is a member of the POU transcription factor family that contains a binding domain to the ATGCAAAT sequence in target genes[15]. The *Oct-4* gene encodes four protein isoforms called Oct4A, Oct4B-190, Oct4B-265, and Oct4B-164. Of these, Oct4A is known to transcribe a wide variety of genes that regulate SCs stemming. Several Oct-4A target genes have been identified in CSCs of lung cancer, including Fgf4, Utf1, Opn, Rex1/Zfp42, and Fbx15[15].

Oct-4A transcriptional activity is upregulated by post-translational modifications, through phosphorylation at residues Ser229, Ser236 and tyr327 or sumoylation at residue Lys118. Its expression is high in SCs, however Oct-4 is ubiquitinated and degraded by the proteasome in the cell differentiation process[15].

In lung cancer, Oct4 activity is associated with chemotherapy resistance, cancer relapse, and worse outcomes[15].

Sox2: Sox2 is a member of the high mobility group (HMG) box gene family encoded by the sex-determining region Y-box gene. HMG is a sequence of 80 amino acids that acts as a DNA-binding domain in several target genes related to the maintenance of pluripotency in embryonic SCs and CSCs [46]. Sox2 is closely associated with early embryonic development, neuronal differentiation, bronchial morphogenesis, and airway epithelial maturation[46].

In lung cancer, Sox2 can regulate the transcription of the c-MYC, Wnt and Notch oncogenes and increase metastatic capacity through the FGFR-ERK1/2-SOX2 signaling pathway[47]. In SCC, Sox2 can induce expression of tumor-related factors p63 and keratin 6 and lead to cancer differentiation, migration, and invasion. In SCLC, Sox2 is crucial in the PI3K-Akt-Sox2 signaling pathway and may mediate chemoresistance[47]. In addition, Sox2 together with Oct-4 increase its expression under hypoxia in tumor tissue, which induces the expression of the CD133 marker and the self-renewal and maintenance of lung CSCs[48].

Sox2 transcriptional activity can be increased by phosphorylation at amino acid residue Thr118 or methylation at Arg113, which inhibits its proteasome degradation or promotes its homodimerization, respectively[49].

Sox2 and Oct4 have been located in the cell nucleus of SCLC and lung AD, but not in their paracancerous tissues or benign tumor tissues, pointing to the importance of Sox2 and Oct4 as potential markers for cancer therapies[50].

Nanog: Nanog, a DNA-binding homeobox transcription factor, may promote cell proliferation, renewal, and stem properties. Nanog can regulate cell pluripotency through two mechanisms of action. Thus, Nanog can repress the transcription of genes essential for cell differentiation, such as Gata4 and Gata6, or it can activate the transcription of genes necessary for self-renewal, such as Rex1[15].

Nanog expression is upregulated by Nr5a2 promoting CSCs properties and tumorigenesis in NSCLC [51]. Nanog is highly expressed in pluripotent cells and its expression is downregulated during differentiation. Its transcriptional activity is increased by phosphorylation at amino acid residues Ser52, 65 and 71 and Thr287, which abolishes its ubiquitination and degradation by the proteasome[52].

Nanog is increased in many types of carcinomas, including lung cancers, and is associated with chemoresistance, cancer relapse, and poor prognosis[15,52].

STEM CELL MARKERS AS THERAPEUTIC TARGETS FOR THE TREATMENT OF LUNG CANCER

Most clinical trials involve the use of drugs whose targets of action are protein tyrosine kinases, regulators of the cell cycle or cell signaling pathways. Several chemotherapy drugs are not effective in controlling tumor growth and metastases. Thus, novel therapeutic agents directed against CSCs are a hope for patients who do not respond to conventional therapies or who relapse after cancer treatment. These therapies include the use of synthetic or natural inhibitors, monoclonal or bispecific antibodies (BsAb), antibodies-drug conjugates, aptamer-drug conjugates, or chimeric antigen receptor T (CAR-T) therapies. Novel therapies using stem cell biomarkers as pharmacological targets are summarized in Table 1.

Inhibitory drugs

Salazosulfapyridine in combination with cisplatin and pemetrexed is under phase I study for advanced NSCLC (Trial registration-UMIN 000017854). This drug inhibits the intracellular uptake of cysteine by the cysteine-glutamate antiporter in CSCs that overexpress CD44 marker, preventing the synthesis of the antioxidant GSH, essential for cellular redox homeostasis, and leading to the inhibition of tumor growth[53].

Phase II trial using disulfiram in combination with cisplatin plus vinorelbine have shown to be effective for the treatment of metastatic NSCLC (Trial registration-NCT 00312819)[54]. *In vitro* studies in NSCLC cell lines treated with cisplatin have shown the growth of chemoresistant ALDH1+ cell subpopulations. These cells were sensitive to the cytotoxic effects of cisplatin after treatment with diethylaminobenzaldehyde and disulfiram, inhibitors of ALDH1 activity[55]. In xenograft models in NOD/SCID mice, it has been shown that disulfaran inhibits the activity of ALDH1A1 and the expression of Sox2, Oct-4 and Nanog, reducing the size of tumors derived from ALDH+ CSCs and cancer recurrence [56].

ABCG2 transporter is one of the main CSCs markers under study due to its role in MDR. It has been found that the secalonic acid D, a metabolite of marine-derived mangrove endophytic fungus, can down-regulate the expression of ABCG2 by activation of Calpaina 1, a protease that shortens the half-life of the transporter[57]. In addition, Verteporfin, an inhibitor of the YAP1 transcription factor, was found to down-regulate the expression of the ABCG2 transporter in lung CSCs[41].

A-803467, a tetrodotoxin-resistant sodium channel blocker, inhibits ABCG2 transporter activity, increasing the sensitivity to chemotherapeutic drugs such as topotecan, doxorubicin and mitoxantrone in multidrug resistant cells. A combination of A-803467 and ABCG2 substrates may potentially be a novel therapeutic treatment in tumors overexpressing the ABCG2 protein[58].

FL118, a topoisomerase 1 inhibitor similar to camptothecin analogues (such as irinotecan or topotecan) used in the clinic, selectively inhibits the expression of several members of the proapoptotic protein family such as Survivin, Xiap, CIAP2 and BCL-2. It has also been found that FL118 down-regulate the expression of ABCG2, ALDH1A1 and Oct-4. FL118 improves sensitivity to chemotherapy and inhibits the growth of CSCs and metastases. Moreover, FL118 is effective for human tumors that acquire irinotecan, topotecan and cisplatino resistance due to its ability to bypass the drug resistance induced by ABC transporters[59].

Monospecific and Biespecific antibodies

mAbMEM-85 is a monoclonal antibody of therapeutic interest since it inhibits the growth of lung cancer cells in murine models. It has been shown that mAbMEM-85 recognizes the hyaluronate binding site in the C-terminal region of CD44 in lung cancer SCs[60].

Axitinib is a monoclonal antibody that inhibits the activity of the ABCG2 transporter in CSCs, and reverses MDR. Studies in A549 human lung cancer cells and nude mice bearing S1-M1-80 xenografts

Table 1 Potential therapeutic strategies targeting lung cancer stem cell markers

Stem cell marker-target	Therapeutic strategies in clinical trials and preclinical studies
Differentiation cluster	
CD133	Docetaxel-loaded liposomes conjugated to anti-CD133 aptamers
	Gefitinib-loaded nanomicelles conjugated to anti-CD133 aptamers
	Salinomycin sodium-loaded nanoparticles conjugated to anti-CD133 antibody
	CD133-specific CAR-T cells plus anti-PD-1 antibody and a CD73 inhibitor
CD44	Salazosulfapyridine plus cisplatin and pemetrexed (Phase I clinical trial for the treatment of advanced non-squamous NSCLC)
	mAb MEM-85 (monoclonal antibody)
	HA-Cisplatin conjugated
	HA-Irinotecan conjugated (Phase II study for treatment of SCLC)
	HA-Apoferritin conjugated
	HA-conjugated cisplatin-loaded nanoparticles
Cell adhesion molecules	
EpCAM	Doxorubicin-loaded nanoparticles conjugated to EpCAM aptamer
	Catumaxomab BsAb anti-human EpCAM/CD3 T-cell antigen (Phase I study for treatment of NSCLC)
	MT110 BsAb anti-EpCAM/CD3 T-cell antigen (Phase I clinical trial for treatment of lung adenocarcinoma)
	MuS110 BsAb anti-human EpCAM/CD3 T-cell antigen
	2C11x4-7 BsAb anti-murine EpCAM/CD3 T-cell antigen
Cell membrane transporters	
ABCG2	Secalonic acid D
	Axitinib in combination with topotecan or mitoxantrone
	FL118 (topoisomerase 1 inhibitor) in combination with irinotecan, topotecan or cisplatino
	A-803467 (tetrodotoxin-resistant sodium channel blocker) in combination with topotecan, doxorubicin or mitoxantrone
	Verteporfin (YAP1 inhibitor)
Metabolic enzymes	
ALDH	Disulfiram in combination with cisplatin plus vinorelbine (Phase II clinical trial for the treatment of NSCLC)
	Disulfiram alone or in combination with diethylaminobenzaldehyde and cisplatin
	FL118 in combination withirinotecan, topotecan or cisplatino
Transcription factors and signaling pathway	
Oct-4	FL118 in combination with irinotecan, topotecan or cisplatino
Notch-signaling pathway	BsAb-5 directed against c-MET and CTLA-4 in CD166+ NSCLC

mAb: Monoclonal antibody; HA: Hyaluronic acid; BsAb: Bispecific antibody; CAR-T: Chimeric antigen receptor T; c-MET: Cellular mesenchymal-to-epithelial transition factor; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; NSCLC: Non-small cell lung cancer; ABCG2: ATP-binding cassette subfamily G member 2; CD133: Cluster of differentiation-133; CD44: Cluster of differentiation-44; EpCAM: Epithelial cell adhesion molecule; ALDH: Aldehyde dehydrogenase; Oct-4: Octamer-binding transcription factor 4.

that overexpress ABCG2 have shown that axitinib increases tumor cell apoptosis induced by chemotherapeutic drugs such as topotecan and mitoxantrone without causing additional toxicity[61].

BsAb target two different epitopes or antigens simultaneously. One of the Fab fragments recognizes epitopes on cytotoxic drugs or CD8+ T or NK immune cells, while the other Fab fragment can bind epitopes on CSCs. Then, BsAbs can selectively direct effector cells or chemotherapeutic drugs towards CSCs, promoting their destruction. BsAb have been extensively explored in translational and clinical studies in lung cancer.

Catumaxomab is a BsAb with binding sites directed to human EpCAM and CD3 T-cell antigen. A phase I study has shown that catumaxomab is safe and tolerable when administered intravenously in patients with NSCLC[62]. Other anti-CD3-EpCAM BsAbs have been studied in immunocompetent mice bearing lung tumors, showing potent inhibition of local and disseminated tumor growth[63]. MT110 BsAb targeting EpCAM/CD3 T-cell antigen has been tested in patients with colorectal, gastric, and lung cancer[64]. MuS110 BsAb was found to have similar *in vitro* characteristics and *in vivo* antitumor activity as MT110[65].

BsAb-5 target cellular mesenchymal-to-epithelial transition factor (c-MET) and cytotoxic T-lymphocyte-associated protein 4 in CD166+ lung CSCs with high affinity and specificity. BsAb-5 has been shown to reduce tumor size in mouse models by a mechanism that involves inhibition of the c-MET-Notch pathway in CSCs and the up-regulating effector T cells. BsAb could be a possible drug for the treatment of human NSCLC[66].

Drugs conjugated to specific ligands of CSCs

The extracellular domain of CD44 contains a HA binding site, a property that is used for the development of HA-conjugated antitumor drugs. Preclinical studies have shown that HA can be effectively used to deliver chemotherapy and selectively decrease CD44+ lung CSCs. Thus, a phase II clinical trial (ACTRN 12611000520932) using HA-irinotecan has shown to be effective in the treatment of SCLC[67]. Other studies show that cisplatin or apoferritin conjugated to HA are effective in eliminating lung CSCs[68,69].

Drugs-loaded nanoparticles

In recent years, nanoparticulate systems have been developed to encapsulate antitumor drugs. The efficacy of these systems in the treatment of cancer has been improved by the conjugation of the nanoparticles to specific ligands, antibodies or aptamers directed against SC markers. In this sense, it has been shown that the intratracheal administration of HA-conjugated cisplatin-loaded nanoparticles attenuated lung cancer growth in mice[70]. Other systems such as salinomycin sodium-loaded nanoparticles conjugated to anti-CD133 antibody, doxorubicin-loaded nanoparticles conjugated to EpCAM aptamer and gefitinib or docetaxel-loaded liposomes conjugated to anti-CD133 aptamers have been shown to be effective in inhibiting tumor growth[71-74].

CAR-T therapies

CAR-T cell is a new therapeutic approach that involves the development of a receptor expressed in T cells that recognizes certain specific antigens on the membrane of cancer cells, triggering an antitumor immune response. The efficacy of specific CAR-T therapy against the AC133 epitope of CD133 has been studied in a mouse model with orthotopic xenograft. AC133-specific CAR-T cells reduced tumor size and prolonged survival in the humanized orthotopic SCLC model but were unable to eliminate tumors completely. However, the combination of AC133-specific CAR-T cells with an anti-PD-1 antibody and a CD73 inhibitor was able to eliminate chemoresistant CSCs[75].

CONCLUSION

Current chemotherapies for lung cancer involve the use of drugs whose targets of action are protein tyrosine kinases, regulators of the cell cycle or cell signaling pathways. Despite improvements in treatments, some patients do not respond to therapies or have cancer relapses months or years after treatment. It is hypothesized that one of the main causes of cancer relapse is the ineffectiveness of anticancer drugs to eliminate CSCs in tumor tissue. Chemotherapy and radiotherapy induce senescence of tumor cells. Factors released by senescent cells into the tumor microenvironment could activate signaling pathways that induce phenotypic and functional changes in SCs, increasing their plasticity and uncontrolled growth. In addition, CSCs could be chemoresistant due to the expression of transporters in cell membranes that expel xenobiotic compounds into the extracellular space.

In lung cancer, several biomarkers have been identified in CSCs associated with the maintenance of tumorigenicity, metastasis and chemoresistance. These biomarkers could be useful as targets for the effective treatment of lung cancer. Thus, new drugs directed against CSCs include the use of inhibitors, monoclonal and BsAb, antibody-drug conjugates or aptamer-drug conjugates, and CAR-T therapies. These therapies are a hope for patients who do not respond to conventional treatments or relapse in lung cancer.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Single cell RNA sequencing reveals mesenchymal heterogeneity and critical functions of *Cd271* in tooth development

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Abstract

BACKGROUND

Accumulating evidence suggests that the maxillary process, to which cranial crest cells migrate, is essential to tooth development. Emerging studies indicate that *Cd271* plays an essential role in odontogenesis. However, the underlying mechanisms have yet to be elucidated.

AIM

To establish the functionally heterogeneous population in the maxillary process, elucidate the effects of *Cd271* deficiency on gene expression differences.

METHODS

p75NTR knockout (*Cd271*$^{-/-}$) mice (from American Jackson laboratory) were used to collect the maxillofacial process tissue of p75NTR knockout mice, and the wild-type maxillofacial process of the same pregnant mouse wild was used as control. After single cell suspension, the cDNA was prepared by loading the single cell suspension into the 10x Genomics Chromium system to be sequenced by NovaSeq6000 sequencing system. Finally, the sequencing data in Fastq format were obtained. The FastQC software is used to evaluate the quality of data and CellRanger analyzed the data. The gene expression matrix is read by R software, and Seurat is used to control and standardize the data, reduce the dimension and cluster. We search for marker genes for subgroup annotation by consulting literature and database; explore the effect of p75NTR knockout on mesenchymal stem cells (MSCs) gene expression and cell proportion by cell subgrouping, differential gene analysis, enrichment analysis and protein-protein interaction network analysis; understand the interaction between MSCs cells and the differentiation trajectory and gene change characteristics of p75NTR knockout MSCs by cell communication analysis and pseudo-time analysis. Last we verified the findings

single cell sequencing *in vitro*.

RESULTS

We identified 21 cell clusters, and we re-clustered these into three subclusters. Importantly, we revealed the cell–cell communication networks between clusters. We clarified that *Cd271* was significantly associated with the regulation of mineralization.

CONCLUSION

This study provides comprehensive mechanistic insights into the maxillary- process-derived MSCs and demonstrates that *Cd271* is significantly associated with the odontogenesis in mesenchymal populations.

Key Words: *Cd271*; Mesenchymal stem cells; Single cell RNA sequencing; Osteogenesis; Mineralization; Tooth development

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Core Tip: Our study reveals the following findings: (1) High cellular heterogeneity and molecular details; (2) Significant functional and signaling differences between cell types; (3) Novel subclusters of mesenchymal stem cells; and (4) Crucial cell-cell interactions of mesenchymal subpopulations. We provided new insights into the biological features of mesenchymal stem cells at the single cell level. Our findings contribute to thorough exploration of the mechanism of *Cd271* in regulating odontogenesis and osteogenesis which add to the theory of tooth development.

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INTRODUCTION

During the early stage of embryogenesis, cranial neural crest cells migrate throughout the maxillary and mandibular processes, which are defined as ecto-mesenchymal stem cells (MSCs)[1,2]. Ecto-MSCs were regarded as the primitive dental cells in the classical theory of tooth development. To date, in-depth studies of the odontogenesis and osteogenesis of MSCs are still lacking.

Cd271 (low-affinity nerve growth factor receptor, p75 neurotrophic receptor) is a member of the tumor necrosis factor receptor superfamily, and is implicated in various biological functions[3]. It is used as a specific cell surface marker for purifying and identifying MSCs[4]. Previous studies focused on cellular physiological functions in processes, such as migration, proliferation, differentiation, survival and apoptosis[5-8]. *Cd271* is involved in the regulation of morphogenesis and the development of various tissues, including nerves, fat, liver and teeth[9-13]. In addition to these features, emerging studies indicate that *Cd271* plays a critical role in initiating tooth development, differentiation and mineralization of odontogenic stem cells. Mitsiadis *et al*[14] indicated that *Cd271* surrounded the developing tooth germ within nerve fibers and was highly expressed in the epithelium and mesenchyme in the early stages of odontogenesis within the cells of origin[14]. The spatial–temporal expression of *Cd271* was similar to the mineralization factor *Runx2* during the early development of tooth germ[15], which was supported by other studies[16,17]. These studies indicate that *Cd271* is involved in the physiological processes of tooth development and biomineralization. However, the molecular mechanisms of *Cd271*, as a transmembrane signaling molecule, in the morphogenesis and development of teeth remains largely unknown.

Conventionally, MSCs were considered a unified group of fusiform cells. Nevertheless, accumulating evidence suggests that MSCs are functionally and morphologically heterogeneous in essence[18,19]. High-throughput single cell RNA sequencing (scRNA-Seq) is powerful for disclosing the complexity and diversity of cells and relationships among genes involved in tissues, and offers an opportunity to explore unbiased gene expression profiling of cells[20]. scRNA-Seq also provides insights into specific changes in cell lineages, trajectory inference, and the identification of biomarkers[21,22]. scRNA-seq has been used to study MSCs derived from adipose tissue, bone marrow, endometrium, placenta and dental pulp[23-27]. However, application of scRNA-seq in maxillary-process-derived MSCs is still absent.

Herein, we used the maxillary process from mouse embryos as a model to understand the development of maxillary-process-derived MSCs. We applied scRNA-Seq analysis to elucidate the cellular heterogeneity and explore molecular details better compared with conventional methods. Our study provides novel insights into the biological features of MSCs at the single cell level and the mechanism of *Cd271* in regulating odontogenesis and osteogenesis.

MATERIALS AND METHODS

Animal preparation and tissues dissection

Cd271 knockout (*Cd271*$^{-/-}$) and wild-type (*Cd271* $^{+/+}$) mice were used in this study. The *Cd271*$^{-/-}$ mice were gifts from The Jackson Laboratory (Bar Harbor, ME, United States). These mutant mice that exhibit the targeted deletion of exon III of the *Cd271* locus do not express functional full-length *Cd271*. All animal experiments were performed according to the protocols approved by the Medical Ethics Committee of Wenzhou Medical University (No. wydw2019-0224). We completed experimental steps under ethical guidelines. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were housed to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) prior to experimentation. The homozygotes of *Cd271* $^{+/+}$ and *Cd271*$^{-/-}$ mice were mated to produce heterozygous offspring. The heterozygous mice were mated to generate three types of genotype embryos, *Cd271*$^{+/+}$ and *Cd271*$^{-/-}$and *Cd271*$^{+/-}$. We obtained embryos through abdominal surgery, and chose homozygous embryos from embryonic day 16.5. The embryos were placed in a 6-cm Petri dish and washed with Phosphate buffer saline (PBS). We cut the amniotic membrane with ophthalmic scissors to separate the fetal mice and washed them with PBS again. After cutting the head of the fetus, we cut the maxillary process tissue under a stereomicroscope. After the removal of nonpurpose tissues like blood stains and fatty layers, we rinsed the tissue twice in PBS. The genotype of wild-type and *Cd271* knockout mice was confirmed with a one-step genotyping kit (Vazyme, Nanjing, China).

Single cell isolation and library preparation

Maxillary processes were dissociated into single cells in dissociation solution (0.35% collagenase IV5, 2 mg/mL papain, and 120 U/mL DNase I) in 37 °C water bath with shaking for 20 min at 100 rpm. Single cell suspensions were washed and resuspended to load to the 10X Chromium platform. More than 25000 single cells were captured and subjected to 10X Chromium Controller machine to generate gel beads-in-emulsion (GEMs). mRNA was prepared using the 10X Genomics Chromium Single Cell 3′ Reagent Kit V2 (10X Genomics, Pleasanton, CA, United States). Cells were divided by partitions into the GEMs along with GelBeads coated with oligos in this step. These oligos utilized poly-dT sequences to capture mRNAs and cell-specific and transcript-specific barcodes. The following cDNA amplification generated adequate quantities for library construction according to the standard protocol. Libraries were sequenced on an Illumina NovaSeq 6000 sequencing system (paired-end multiplexing run, 150 bp) by the LC-Bio Technology (Hangzhou, China) at a minimum depth of 20000 reads per cell.

scRNA-Seq data processing

We processed sequencing reads using CellRanger (version 6.0.1). Then we created objects by Seurat (version 4.0.5) R package, and merged data using the function of Merge[28]. The ratio of mitochondrial genes to all genetic material was used to judge whether a cell was in a steady state. Generally, it was thought that a cell might be in a state of stress when it had a higher proportion of mitochondrial genes. Therefore, we filtered cells with > 10% mitochondrial gene content. Considering potential cellular diploidy, we filtered cells with < 1000 or > 6000 genes. After the above steps, we finally obtained 17426 cells.

Data were normalized using the log-normalization method. After controlling for the relationship between mean expression and dispersion, we identified highly variable genes in individual cells. We input variable genes to perform principal component analysis (PCA) and identified significant principal components based on the function of JackStraw[29]. A total of 20 principal components were selected as statistically significant inputs to the Uniform Manifold Approximation and Projection (UMAP). We examined the distribution of UMAP and PCA between these samples. The data showed correlations. We compared the mean expression of genes between samples and found an excellent Pearson correlation between them. We divided the cells clustered by the FindCluster into 21 clusters.

Cell annotation

We identified cell types based on specific maker genes. The MSCs lineage was identified by *Col1a1* and *Col3a1*. *Lgals7* and *Krtdap* marked the epithelial cell lineage; muscle cell marker genes included *Actc1* and *Tnnt1*; macrophage marker genes included *Pf4* and *C1qb*; glial marker genes included *Dct* and *Ptgds*; T cell marker genes included *Cma1* and *Cpa3*; endothelial cell marker genes included *Egfl7* and *Cdh5*; and perivascular cell marker genes included *Rgs5* and *Ndufa4 L2*. The FindAllMarkers function was

used to find differentially expressed genes between each cell type.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) can identify whether predefined gene sets show significant differences between biological processes by a computational method. Typically, GSEA is used to estimate expression in dataset samples. To study differences in the biological processes between the two groups, we downloaded the reference gene set c2.cp.kegg.v7.4.entrez.gmt from the MSigDB database on the basis of the gene expression profiling dataset[30]. We used GSEA included in the R package ClusterProfiler to perform enrichment analysis and visualization of the dataset. The cut-off standards were set as nominal $P < 0.05$, FDR q < 0.25 and normalized enrichment score (NES) > 0.6.

MSCs reclustering

On account of our main focus on MSCs, we extracted a subpopulation of MSCs. Based on hierarchical clustering and defined marker genes, we reclustered the MSCs using the FindClusters function and assigned the MSCs to 13 cell subpopulations. The progenitor subpopulation was identified by *Cdk1* and *Dkk1*, the osteoblasts subpopulation was identified by *Runx2* and *Sp7*, and the fibroblasts subpopulation was determined by maker genes *Dlk1* and *Shox2*. We next counted the proportions and number of these cell subpopulations in wild-type or *Cd271* knockout mice.

Gene ontology functional enrichment analysis and protein–protein interaction network

Gene Ontology (GO) is an approach for functional enrichment analysis of genes in diverse levels and dimensions. GO analysis comprises three levels: Biological process, molecular function and cellular components. The Wilcoxon rank-sum test was used to identify genes differentially expressed in MSCs between wild-type and *Cd271* knockout samples (logFC > 0.25, $P < 0.05$). Differentially expressed genes that were identified between wild-type and *Cd271* knockout samples were subjected to GO functional annotation by the ClusterProfiler (version 4.2.0) R package to identify the significantly enriched biological processes[31].The enrichment results were visualized in the form of a lollipop plot, and the significance threshold for the enrichment analysis was set at a corrected $P < 0.05$.

The STRING online database (https://string-db.org/) analyzed the interaction between the marker genes. We constructed a protein–protein interaction (PPI) network for the results obtained by Cytoscape (3.9.0). The functional interactions between the proteins expressed by the genes were mapped, including direct physical interactions and indirect functional correlations.

Pseudotemporal analysis

Cell differentiation of MSCs (progenitors, osteoblasts and fibroblasts) was inferred by the Monocle (2.22.0) R package and default parameters recommended by the developers[32]. The integrated gene expression profiling of each cell type was exported from Seurat into Monocle to establish the cellular gene dataset. The variable genes were defined by the process of DispersionTable, and the cells were sorted with the function of setOrderingFilter. The DDRTree method was used to reduce the dimension, and the orderCells function was used to estimate the cell arrangement along the trajectory. Based on clustering characteristics and marker gene analysis, we obtained the trajectory map of the differentiation time of MSCs. The study of each trajectory used a standard protocol with default parameters.

Cell–cell communication analysis

We identified the underlying interactions between MSCs and other cell populations by CellChat (version 1.1.3) (http://www.cellchat.org/) R package, which is commonly used to analyze cell–cell communication networks from single cell transcriptome sequencing profiling[33]. Taking advantage of CellChat, we inferred the scRNA-Seq data quantitatively and researched cell–cell communication networks. We predicted the main ingoing and outgoing signal patterns by network analysis and pattern recognition methods, as well as the coordination function between cells and signals. We counted all the important receptor–ligand pairs in the intercellular signal transmission by bubble chart, and we selected the signaling with a higher contribution to the cell for network centrality analysis.

Cell culture and mineralization induction

MSCs were isolated from embryonic maxillary processes. After washing with high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, United States) three times, the cells were digested with trypsin–EDTA (Gibco) at 37 °C for 10 min, and centrifuged at 1000 rpm for 5 min. The cells were resuspended in a growth medium with DMEM, 10% fetal bovine serum (FBS) (Gibco), and 1% antibiotics (100 mg/mL penicillin and 100 μg/mL streptomycin) to generate primary MSCs. We maintained the cells at 37 °C in a 5% CO_2 humidified incubator and replaced the medium every 3 d.

For mineralization induction, cells were seeded in DMEM supplemented with 10% FBS at 4×10^4 cells per well in 24-well plates. At 75%–80% confluence, we changed the medium to mineralization induction medium, which consisted of DMEM with 10% FBS, 100 IU/mL penicillin, 100 IU/mL streptomycin, 0.1 μM dexamethasone, 10 mmol/L β-glycerol phosphate (Sigma–Aldrich, St. Louis, MO, United States) and 50 μM ascorbic acid (Sigma–Aldrich). We cultured the cells for up to 7 d, and replaced the medium

every 3 d.

Alkaline phosphatase activity and staining

The alkaline phosphatase (ALP) activity kit (Nanjing Jiancheng Biotech, China) was used to detect intracellular ALP activity. Cells were lysed in RIPA lysis buffer (Beyotime, China) without protease and phosphatase inhibitors to induce mineralization for 7 d. We centrifuged the lysate at 12000 rpm and 4 °C for 30 min, and incubated the supernatant with reaction buffer at 37 °C for 15 min. We stopped the color development and measured the absorbance at 520 nm. We measured the protein concentration of the lysate with a Bicinchoninic Acid Assay (BCA) protein assay kit (Beyotime). The ALP staining assay was performed with a Beyotime kit. On day 7 of induction, the cells were fixed for 30 min and stained with Alkaline Phosphatase Assay Kit (Beyotime, China) in the dark for 30 min. The cells were observed and imaged for histochemical detection of ALP with a Nikon microscope.

Alizarin Red staining and quantification

After osteogenic inducting for 14 d, the cells were fixed with 4% paraformaldehyde for 30 min. They were stained with 1% Alizarin Red (pH 4.3) (Beyotime) for 20 min at room temperature and washed three times with deionized water. We observed and imaged the calcium deposits under a Nikon microscope. To quantify calcium deposits, we destained the stained cells with 10% cetylpyridinium chloride monohydrate (Sigma–Aldrich) in 10 mmol/L sodium phosphate (pH 7.0) for 30 min. We transferred a 200-μL aliquot to a 96-well plate to measure the absorbance at 550 nm by a Varioskan Flash Spectral Scanning Multimode Reader (ThermoFisher Scientific, United States).

Quantitative real-time polymerase chain reaction

Total RNA was isolated from the cells by an RNA prep pure Cell Kit (TIANGEN, Beijing, China). cDNA was synthesized following the instructions for the HiScript® III RT Super Mix for quantitative real time polymerase chain reaction (qPCR) kit (Vazyme, Nanjing, China). Quantitative real-time polymerase chain reaction (PCR) was performed with ChamQ Universal SYBR qPCR Master Mix kit (Vazyme) and Real-Time PCR Detection System (Quantstudio5, United States). Gene-specific primer pairs are show in Supplementary Table 1.

Cell Counting Kit-8 proliferation

Cell Counting Kit-8 (CCK-8; Dojindo Kagaku, Japan) was used to investigate the proliferation rate of E16.5d *Cd271* knockout and wild-type MSCs. The cells were seeded at 2 × 10^3 cells/well in a 96-well plate. After mixing the CCK-8 solution, the cells were cultured at 37 °C for 2 h in the dark. Absorbance was detected for 7 d continuously by a microplate reader at 450 nm.

Statistical analysis

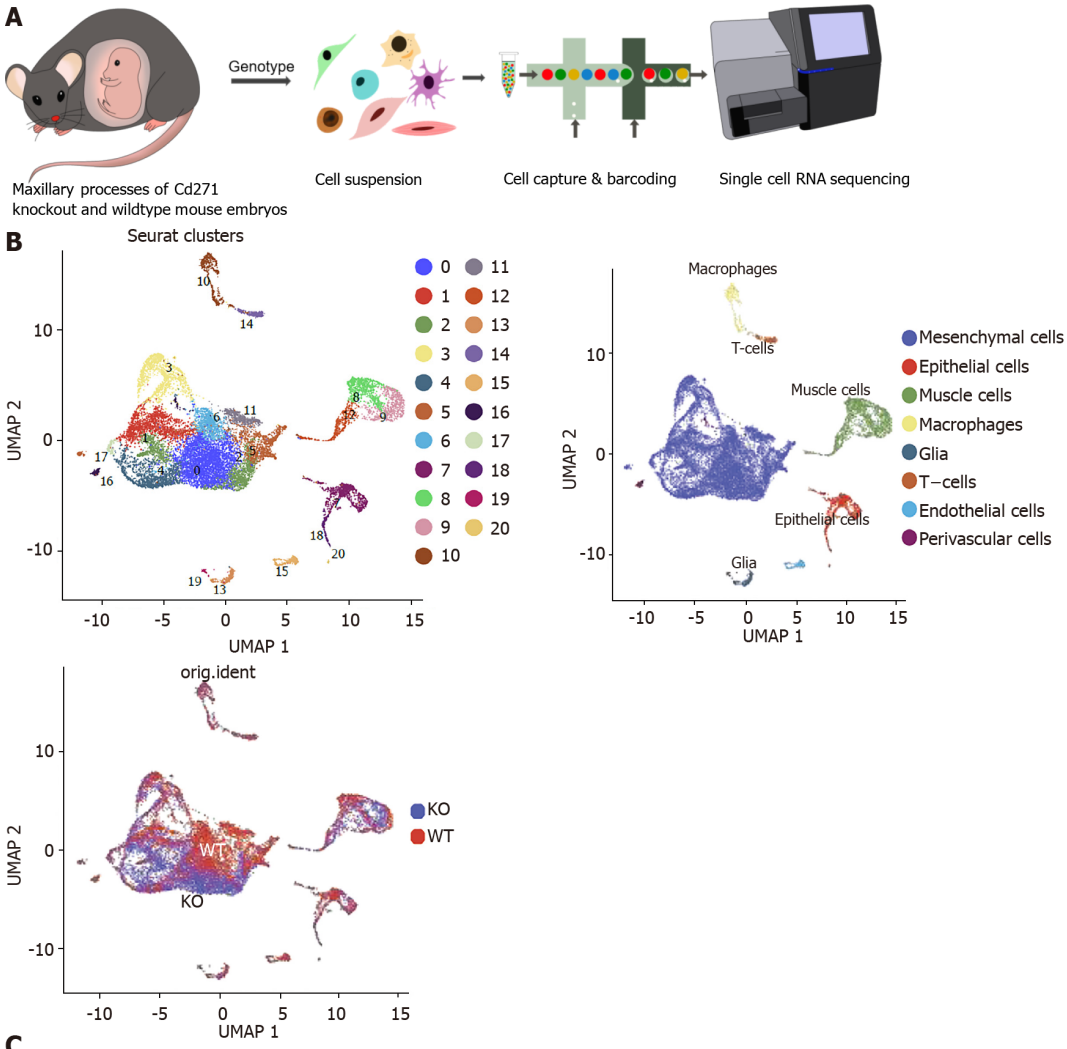
Except the scRNA Seq, all the experiments were repeated more than three times. A one-way analysis of variance (ANOVA) or t test (GraphPad Prism 9.0 software, La Jolla, CA, United States) was used to identify significant differences. $P < 0.05$ was considered statistically significant.

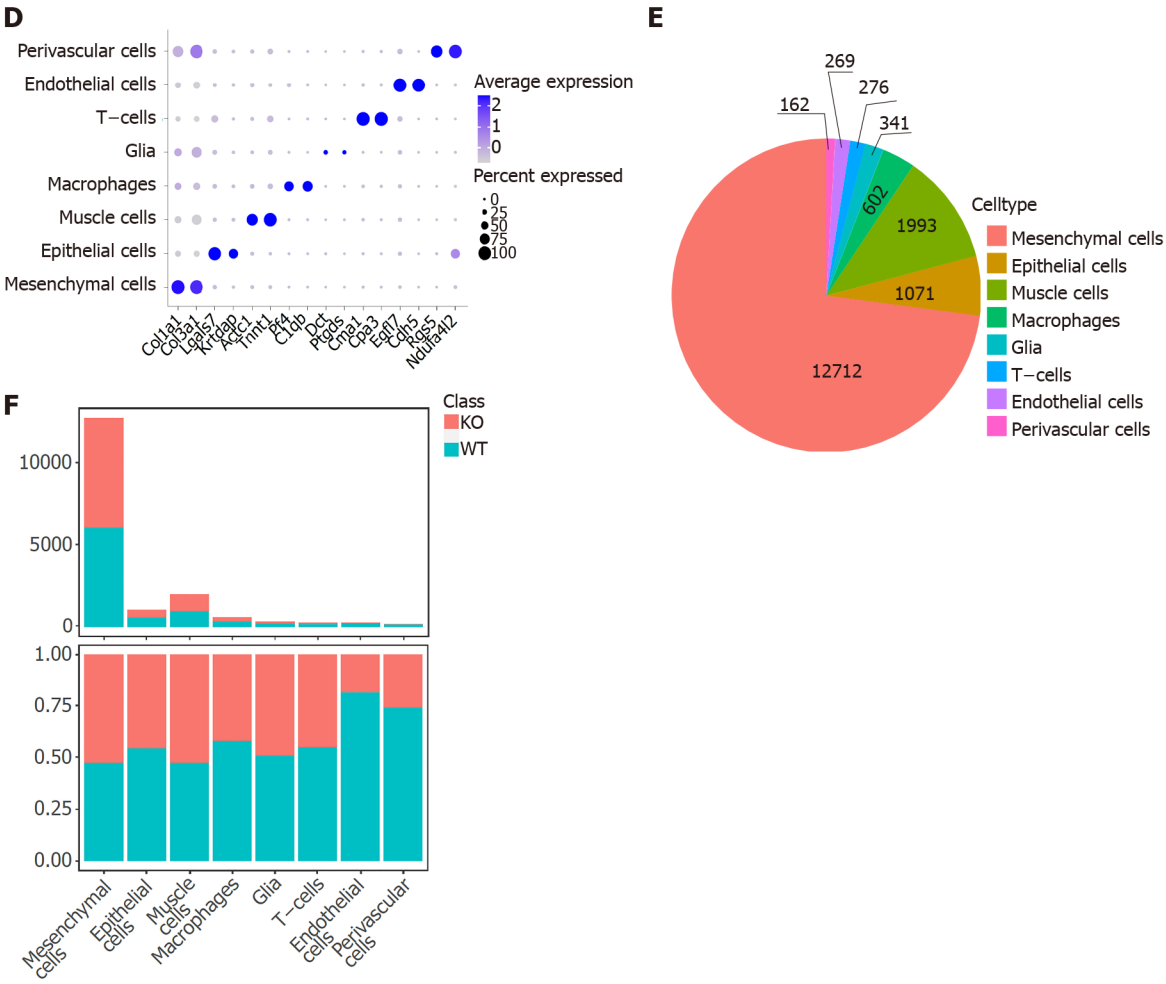
RESULTS

scRNA-Seq analysis showed high cellular heterogeneity of murine maxillary process

To reveal cellular heterogeneity, we performed scRNA-Seq with murine maxillary processes from wild-type and *Cd271* knockout embryo tissues (Figure 1A and Supplementary Figure 1). After quality controlling and normalizing scRNA-Seq data, we obtained transcriptomes of 17426 cells (Supplementary Figure 2). UMAP clustering divided the cells into 21 independent clusters. Based on established lineage-specific marker genes, these 21 clusters were assigned to eight cell lineages (Figure 1B). We identified differentially expressed genes by comparing the gene expression profiles of one cluster with the others (Figure 1C). The MSC lineage was identified by *Col1a1* and *Col3a1*, and epithelial cells were marked by *Lgals7*. Based on these established lineage-specific maker genes, we assigned these clusters to eight cell types and plotted them (Figure 1D). We identified nine mesenchymal clusters (0–6, 11 and 17), a total of 12712 cells, which constituted the majority of sequenced cells by > 72.94%. Clusters 7, 18 and 20 were annotated as epithelial cells with a relative proportion of 6.14%. Muscle cells consisted of clusters 8, 9 and 12 with a relative proportion of 11.43%, while cluster 10 accounted for 3.11% as macrophages. Clusters 13 and 19 were annotated as glial cells (341, 1.95%). Clusters 14–16 were annotated as T cells (276, 1.58%), endothelial cells (269, 1.54%), and perivascular cells (162, 0.92%), respectively (Figure 1E).

We also analyzed the single cell sequence profiling from wild-type and *Cd271* knockout mice to establish the distribution of different types of cells and the source of samples. We counted the number and proportion of each cell type using histograms (Figure 1F). MSCs occupied most of the cell transcriptomes from the maxillary process, and the proportion of these cells between *Cd271* knockout


A
Genotype
Maxillary processes of Cd271 knockout and wildtype mouse embryos
Cell suspension
Cell capture & barcoding
Single cell RNA sequencing
B
Seurat clusters
UMAP 1
UMAP 2
Macrophages
T-cells
Muscle cells
Epithelial cells
Glia
Mesenchymal cells
Epithelial cells
Muscle cells
Macrophages
Glia
T−cells
Endothelial cells
Perivascular cells
orig.ident
KO
WT
C
Col1a1
Dcn
Agtr2
Col3a1
Col1a2
Smoc2
Ptn
Lum
Aldoa
Bsg
Ap2s1
Fis1
Timm13
Eif3l
Drg1
Itga1
Myh9
Nid1
Sntb1
Cald1
Tns1
Egfl7
Esam
Fabp4
Emcn
Ecscr
Plvap
Pf4
C1qc
Selenop
Fcrls
Ctsb
Fcer1g
Cluster 0
Cluster 6
Cluster 11
Cluster 1
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Figure 1 Single cell RNA sequencing analysis of maxillary processes from wild-type and *Cd271* knockout mice. We identified 21 clusters of cells, and the single cell RNA sequencing data were characterized. A: Schematic representation of the experimental workflow. The *Cd271* knockout and wild-type maxillary processes were collected from mouse embryos at 16.5 d. After genotyping, cells were suspended as single cells, and the cDNA library was constructed and sequenced; B: Cells extracted from the samples were subjected to Uniform Manifold Approximation and Projection hierarchical clustering and color-coded by cluster grouping, predominant cell type, and sample origin; C: Heatmap showing expression levels of differentially expressed genes in each cluster; D: Dot plot showing prominent marker genes for each cell type (mesenchymal cells, epithelial, muscle cell, macrophage, glial, T cell, endothelial, and perivascular); E: Pie chart showing the number of each cell type; F: Histogram showing the number and proportion of cells from wild-type and *Cd271* knockout mice. UMAP: Uniform Manifold Approximation and Projection; KO: Knockout; WT: Wild-type.

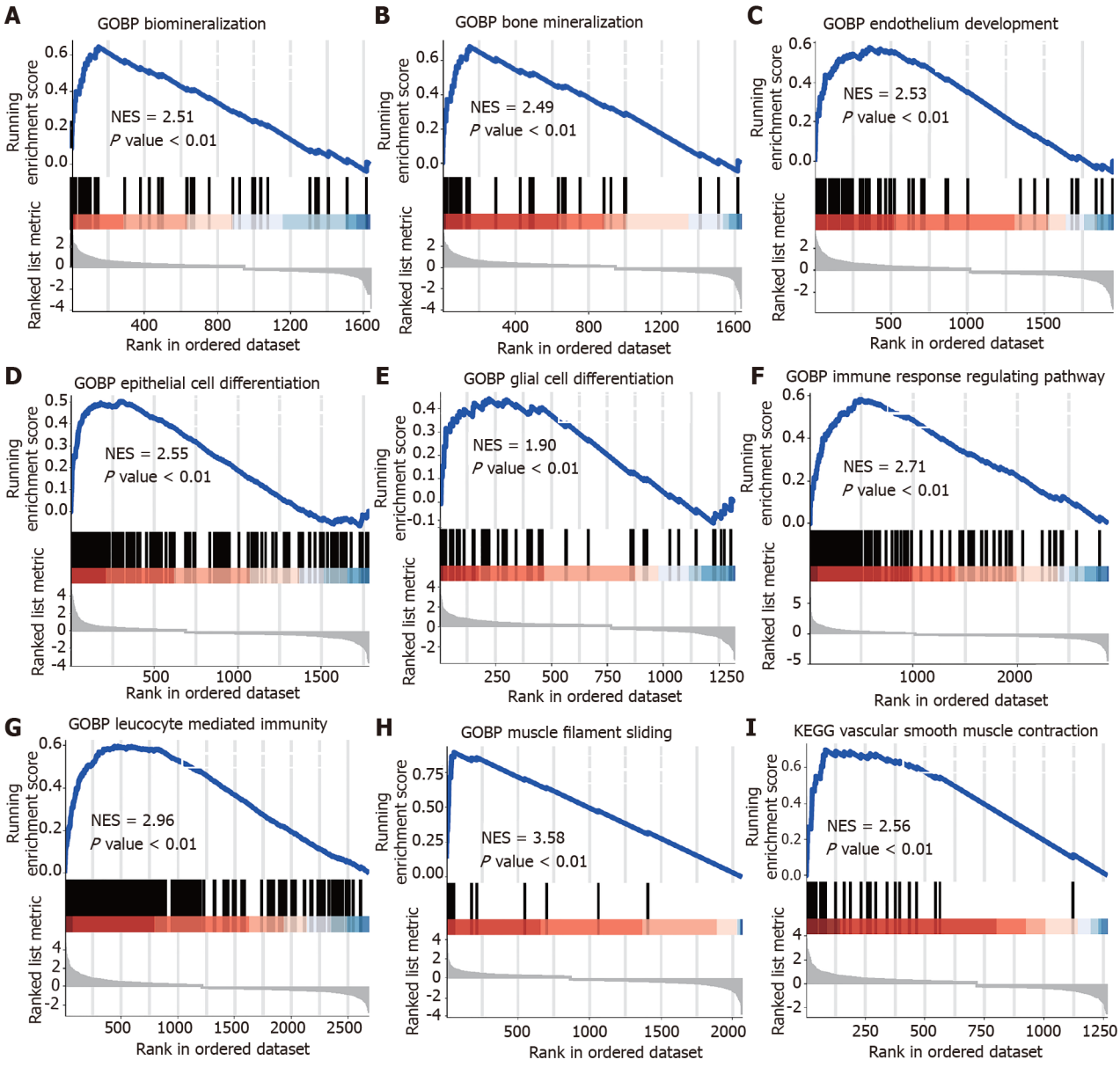
and wild-type was similar.

GSEA showed significant functional and signaling differences between cell types

To identify the relevant molecular mechanisms and possible pathways, we subjected all expressions to GSEA. The results showed a high correlation with MSCs and a close relationship with biomineralization. The results suggested that biomineralization (NES = 2.51, $P < 0.01$) and bone mineralization (NES = 2.49, $P < 0.001$) associated pathways enriched in MSCs (Figure 2A and B). GSEA also revealed that the differentially expressed genes enriched endothelial development, epithelial and glial cell differentiation, immune response, myofilament sliding and vascular smooth muscle contraction (Figure 2C–I).

Unsupervised clustering revealed novel subclusters of MSCs

As MSCs contribute much to embryogenesis and are essential in odontogenesis and osteogenesis, we performed unsupervised reclustering of MSCs. We performed the Subcluster analysis to investigate the heterogeneity within the mesenchymal populations. We observed further heterogeneity in subclusters 0–12 (Figure 3A). We used the published markers to identify the subclusters in MSCs. For instance, *Cdk1* and *Dkk1* marked progenitors, *Runx2* and *sp7* marked osteoblasts, and *Dlk1* and *Shox2* marked fibroblasts. We scored these marker genes in MSC clusters (Figure 3B). In this way, MSC clusters could also be identified into three subpopulations: Progenitors, fibroblasts and osteoblasts. We counted the cell proportions of the MSCs subclusters from wild-type and *Cd271* knockout maxillary processes. The



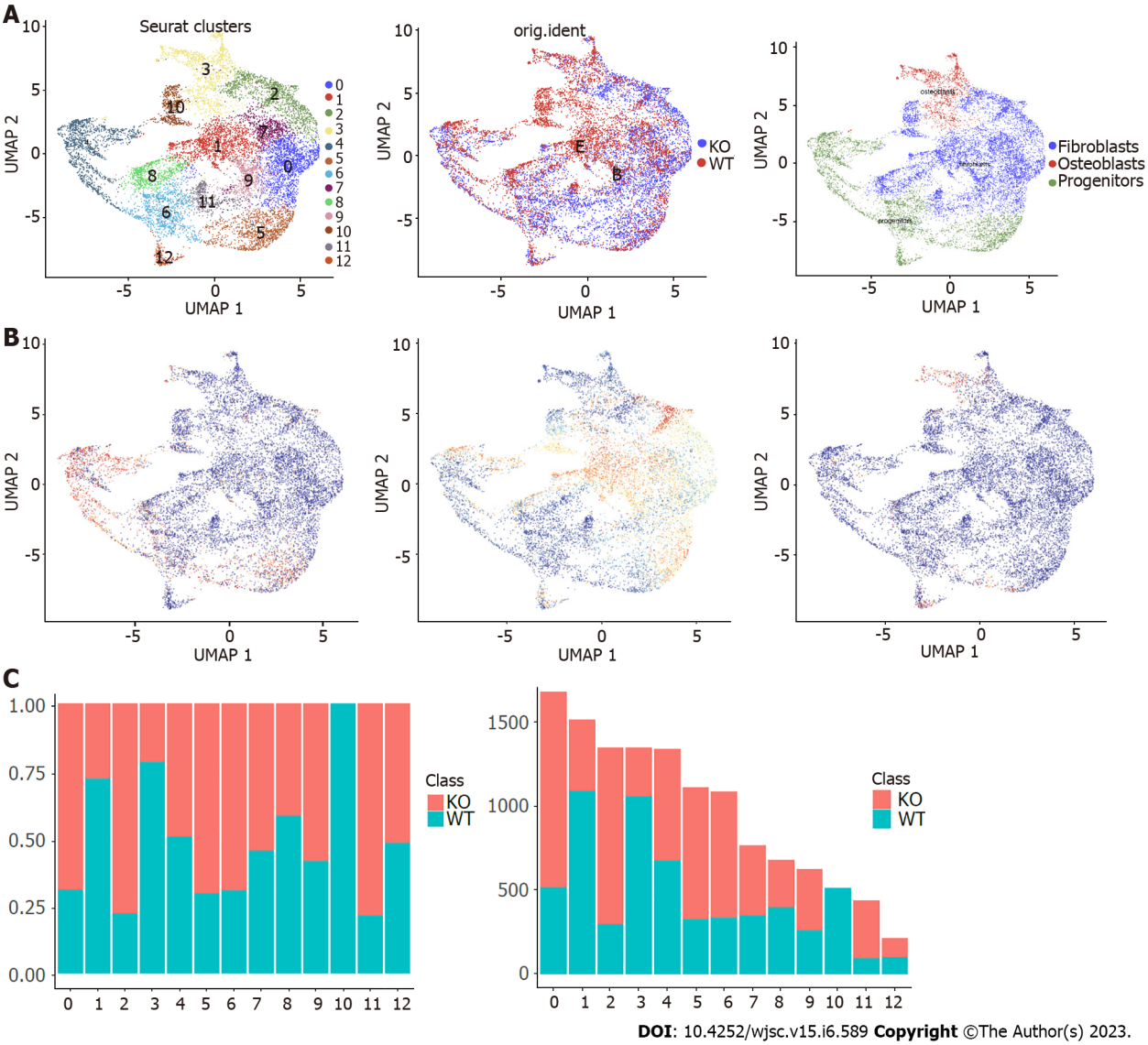
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Figure 2 Gene set enrichment analysis plots for representative signaling pathways upregulated in maxillary process. Gene set enrichment analysis enriched differentially expressed genes into multifunctional gene networks. A and B: Representative gene set enrichment analysis maps for mesenchymal stem cells; C: Endothelial cells; D: Epithelial cells; E: Glia; F: T cells; G: Macrophages; H: Muscle cells; I: Perivascular cells. Normalized enrichment score normalized enrichment score, corrected for multiple comparisons using false discovery rate method, *P* values shown in plots. NES: Normalized enrichment score.

results showed that the numbers of subclusters 1, 3 and 10 were lower, while the other subclusters were higher in *Cd271* knockout compared with wild-type mice (Figure 3C).

Cd271 knockout MSCs exhibited weaker proliferation and osteogenic differentiation potential

We compared MSCs between wild-type and *Cd271* knockout maxillary processes. GO functional enrichment analysis revealed that differentially expressed genes were enriched in ossification, myeloid cell differentiation and biomineralization cell proliferation-related signaling (Figure 4A). To compare the differences in proliferation and osteogenic differentiation between wild-type and *Cd271* knockout MSCs, we investigated the potential for MSC proliferation. As the CCK-8 assay showed, the *Cd271* knockout MSCs exhibited weaker cell proliferation (Figure 4B). We induced the cells with an osteogenic induction medium and performed the mineralization assay. ALP staining and a quantitative assay revealed a lower and lighter mineralization level of *Cd271* knockout than wild-type MSCs. Lighter and fewer mineralized nodules were observed by Alizarin Red staining in *Cd271* knock out MSCs (Figure 4C). These results indicated more inadequate osteogenic differentiation potential in *Cd271* knockout MSCs. We selected several differentially expressed genes with considerable interaction by



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Figure 3 Further cluster analysis of mesenchymal stem cells. A: Mesenchymal stem cells were reclustered and color-coded according to cluster, sample origin and cell type; B: Feature scores of progenitors, fibroblasts and osteoblasts marker genes were displayed in the Uniform Manifold Approximation and Projection plot; C: Histogram showing the proportion and the number of cells in each cluster of wild-type and *Cd271* knockout mice. UMAP: Uniform Manifold Approximation and Projection; KO: Knockout; WT: Wild-type.

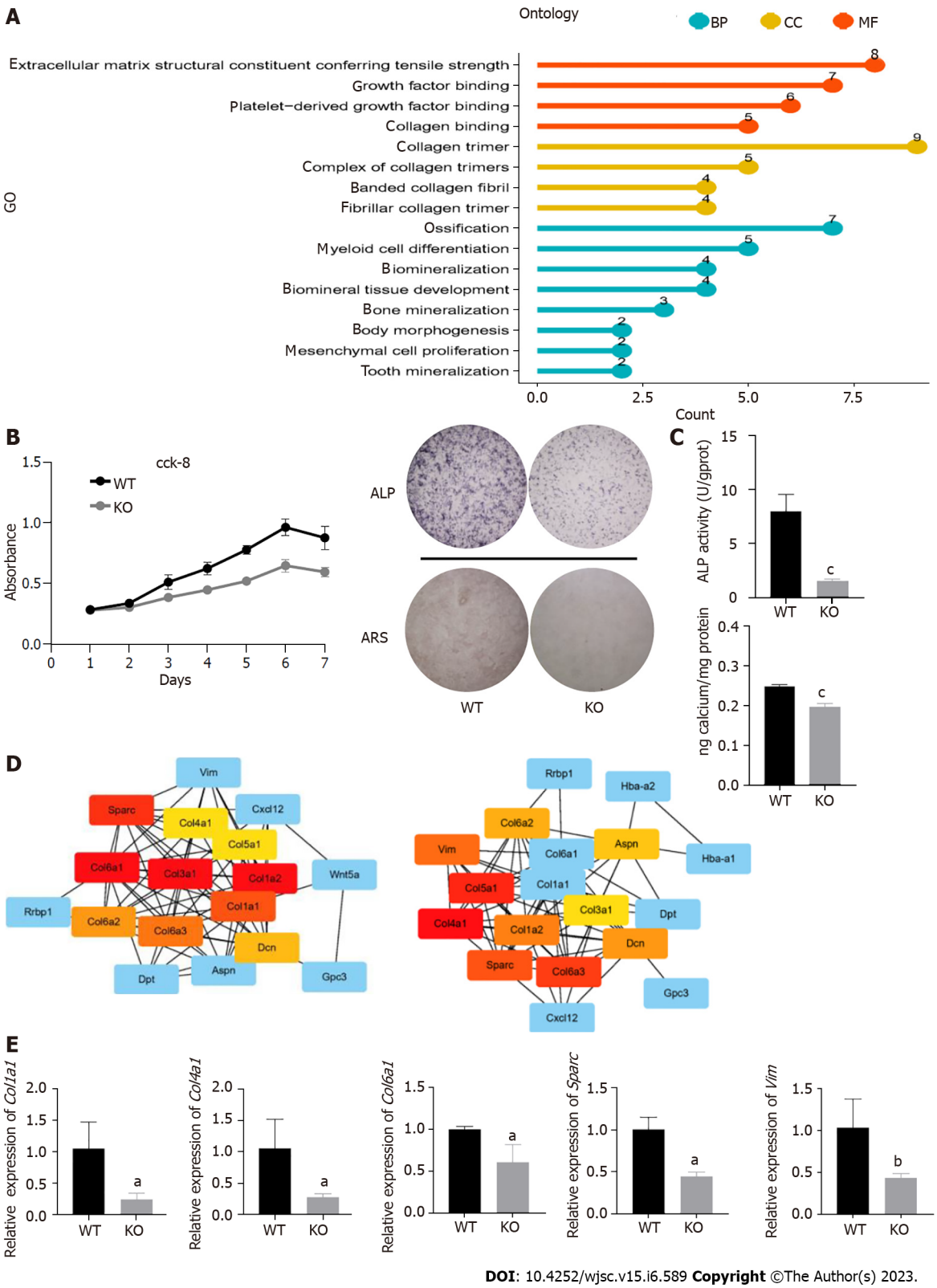
Cytoscape, and measured the gene expression by real-time quantitative PCR (Figure 4D). Proliferation, ossification and osteoblast differentiation-related genes, such as *Col4a1*, *Col6a1*, *Sparc*, *Vim*, *Col1a1* and *Col3a1*, were significantly decreased in *Cd271* knockout MSCs (Figure 4E). *Cd271* was involved in regulating osteogenic differentiation in MSCs, and our results implied a weaker potential of proliferation and osteogenic differentiation in *Cd271* knockout MSCs.

Different sets of genes appeared to shift along the differentiation trajectory

We selected MSCs to institute a pseudotemporal trajectory map that contained terminals consistent with distinct cell fates. The progenitor cells aggregated at the root and branches, indicating a good cell cycle of the progenitor cells. Osteoblasts and fibroblasts were scattered in various branches, showing high invasive potential (Figure 5A–C). We extracted differentially expressed genes over pseudotemporal changes and plotted heatmaps. Different sets of genes were also found to be shifted during the progression of MSCs, stressing the actional change of the progenitor cells, which managed the cell fate transition in the mesenchyme. We clustered the differentially expressed genes into three and performed GO analysis. Clusters 1 and 2 were significantly enriched in biological processes of osteoblast differentiation, biomineralization and odontogenesis, while cluster 3 was mainly enriched in cell metabolism (Figure 5D).

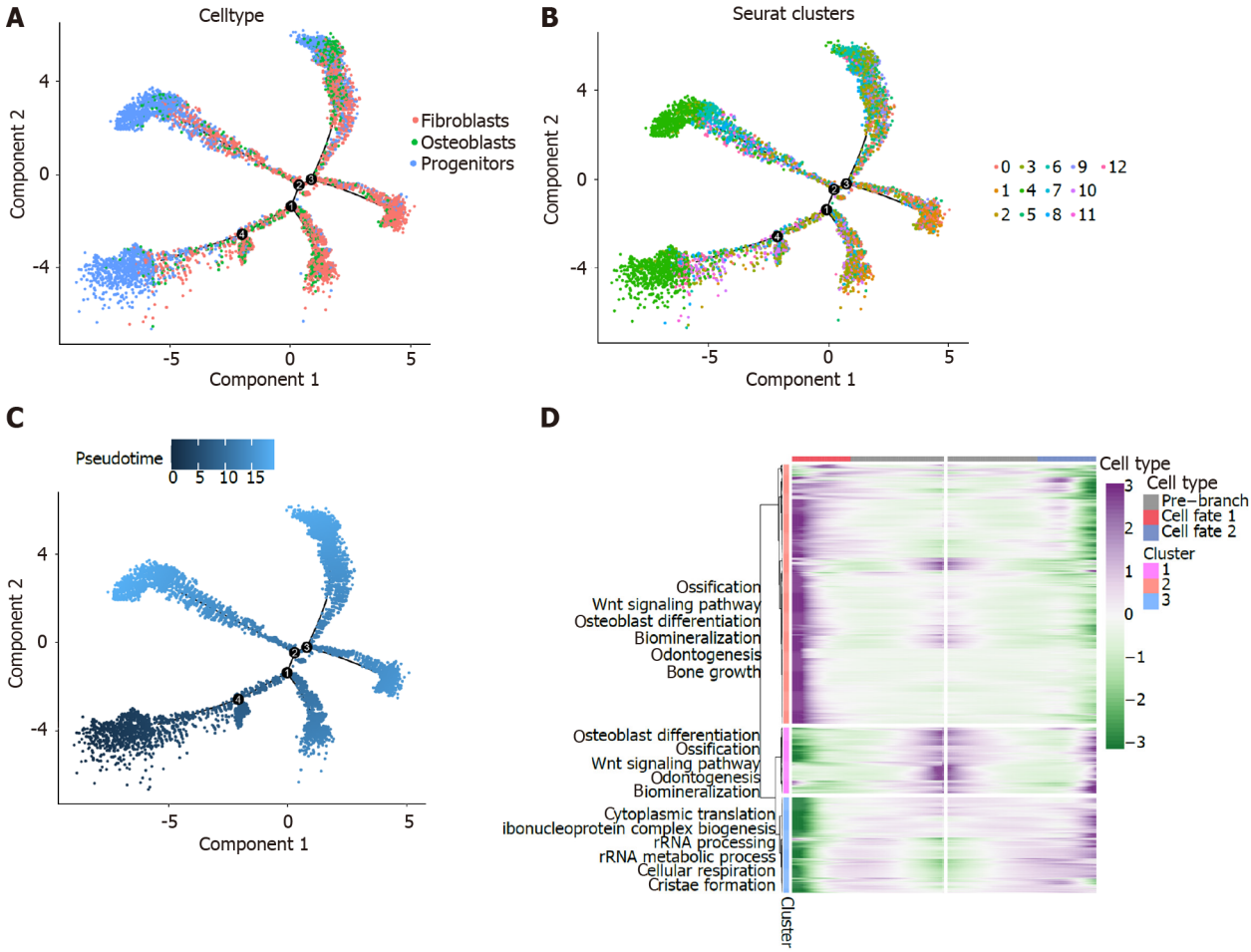
Cell–cell interactions of mesenchymal subpopulations were crucial in the maxillary process

The dataset of scRNA-Seq provided an opportunity to analyze intercellular communication mediated by



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Figure 4 *Cd271* knockout mesenchymal stem cells showed weaker proliferation and osteogenic differentiation potential. A: Gene Ontology enrichment analysis of differentially expressed genes between *Cd271* knockout and wild-type mesenchymal stem cells (MSCs); B: Growth curves of *Cd271* knockout and wild-type MSCs; C: Mineralization assay of *Cd271* knockout and wild-type MSCs. Alkaline phosphatase (ALP) and Alizarin red staining of *Cd271* knockout MSCs was significantly lighter. ALP activity and calcium quantification were significantly lower in *Cd271* knockout compared with wild-type MSCs; D: Protein–protein interaction network interaction analysis of wildtype *vs* *Cd271* knockout differentially expressed genes; E: Quantitative real-time polymerase chain reaction assay of some differentially expressed genes in *Cd271* knockout and wild-type MSCs. Data are presented as mean values ± SD (n = 3 biologically independent experiments. Two-sided unpaired t-test, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$) BP: Biological Process; CC: Cell component; MF: Molecular function; ARS: Alizarin red S; ALP: Alkaline phosphatase; KO: Knockout; WT: Wild-type.



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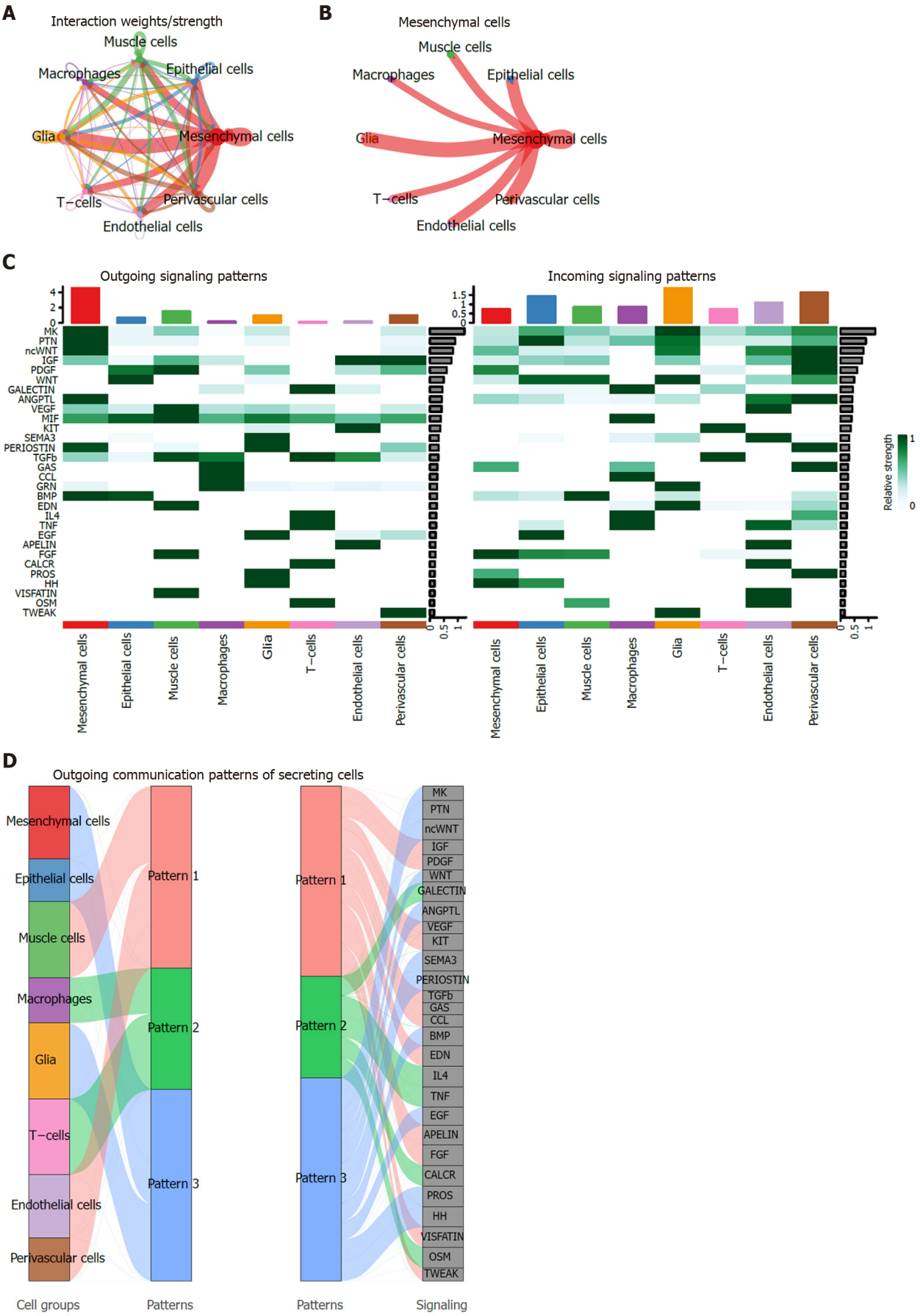
Figure 5 The potential developmental trajectories of mesenchymal stem cells by Monocle. A: Pseudo-sequential diagram based on the differentiation process of mesenchymal stem cell (MSC) subpopulations; B: Pseudo-sequence plot of MSCs colored by cluster classification; C: Pseudo-sequential diagram of MSCs drawn with timeline coloring; D: Heatmap of differentially expressed genes in pseudotemporal analysis. The differentially expressed genes were divided into three gene clusters and gene ontology biological process enrichment analysis was performed.

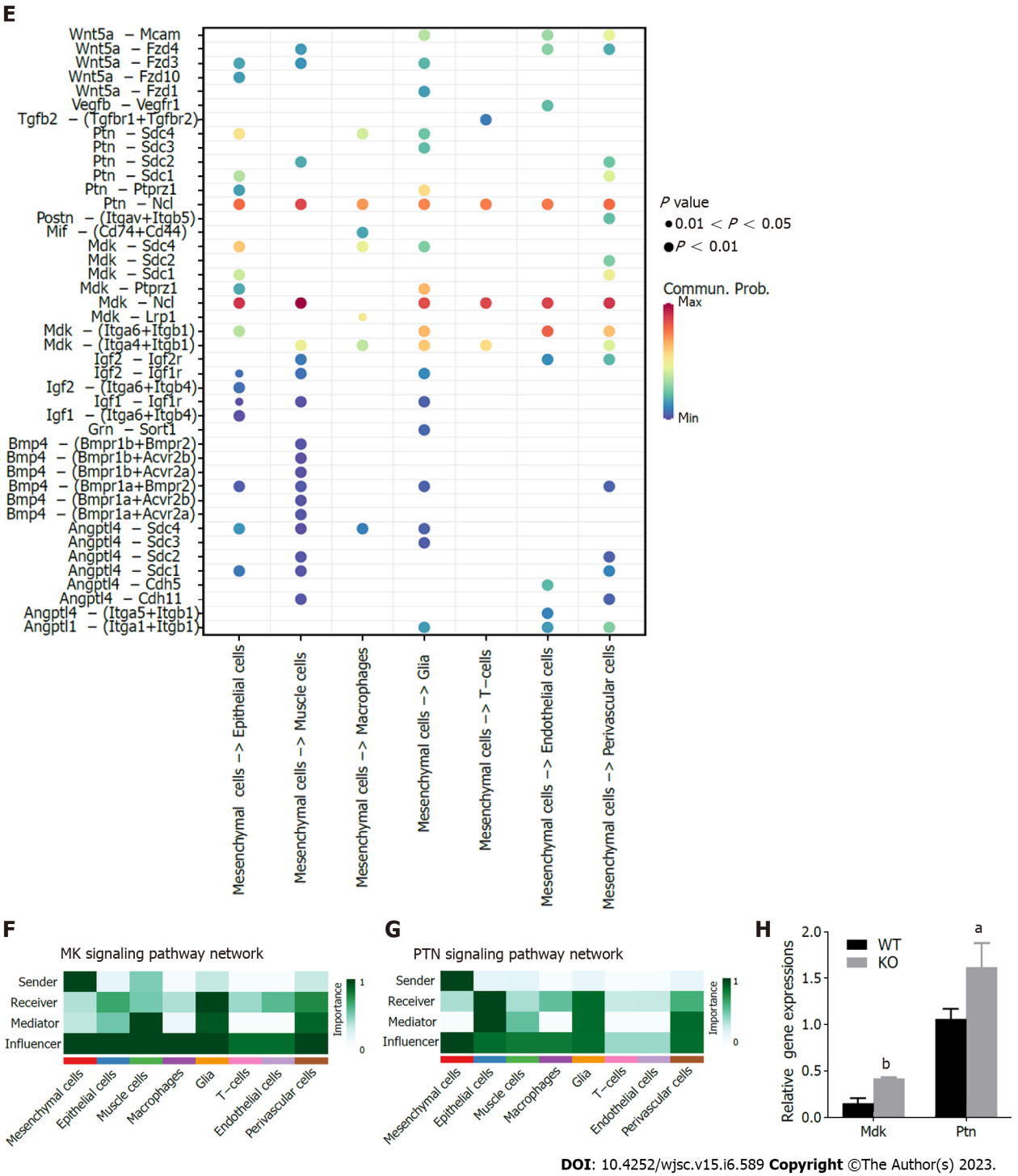
ligand–receptor interactions. To gain insights into potential signaling interactions between different cellular subpopulations, we interrogated our data with CellChat (version 1.1.3) (http://www.cellchat.org/) R package, which could predict the ligand and receptor interactions at single cell resolution[33]. We found dense communication between MSCs and other cells, and the most concentrated interactions occurred in the mesenchymal subpopulation (Figure 6A and B). It revealed the importance of mesenchymal interaction signaling. We detected 30 signaling pathways in eight cell groups, including MK, PTN, ncWNT, IGF, PDGF, WNT and VEGF signaling pathways. We compared the contribution of efferent (or afferent) signaling between cells, confirming that MSCs had higher interaction than other cell types while acting as outgoing signaling pathways (Figure 6C). We analyzed the potential signaling of MSCs, revealing a series of signaling pathways, such as odontogenesis-related signaling such as WNT, BMP and HH signaling (Figure 6D).

We explored ligand–receptor signals broadcast by MSCs, which revealed that MSCs might affect other cells in the ligand–receptor interaction of MK and PTN signaling (Figure 6E). We performed network centrality analysis on MK and PTN signaling, which confirmed that MSCs were important signal senders in intercellular communication (Figure 6F and G). The expression of *Mdk* and *Ptn* in wild-type and *Cd271*$^{-/-}$ MSCs exhibited significant differences by quantitative real-time PCR assay. The expression of *Mdk* and *Ptn* was higher in the *Cd271* knockout MSCs (Figure 6H). These results disclosed underlying ligand–receptor interactions and suggested that MK and PTN signaling may be influenced by *Cd271* in mesenchymal subpopulations.

DISCUSSION

Although MSCs have been extensively studied, there is still a lack of knowledge about the key


A
Interaction weights/strength
Muscle cells
Macrophages
Epithelial cells
Glia
Mesenchymal cells
T−cells
Perivascular cells
Endothelial cells
B
Mesenchymal cells
Muscle cells
Macrophages
Epithelial cells
Glia
Mesenchymal cells
T−cells
Perivascular cells
Endothelial cells
C
Outgoing signaling patterns
Incoming signaling patterns
MK
PTN
ncWNT
IGF
PDGF
WNT
GALECTIN
ANGPTL
VEGF
MIF
KIT
SEMA3
PERIOSTIN
TGFb
GAS
CCL
GRN
BMP
EDN
IL4
TNF
EGF
APELIN
FGF
CALCR
PROS
HH
VISFATIN
OSM
TWEAK
Relative strength
Mesenchymal cells
Epithelial cells
Muscle cells
Macrophages
Glia
T−cells
Endothelial cells
Perivascular cells
D
Outgoing communication patterns of secreting cells
Mesenchymal cells
Epithelial cells
Muscle cells
Macrophages
Glia
T−cells
Endothelial cells
Perivascular cells
Pattern 1
Pattern 2
Pattern 3
Cell groups
Patterns
Signaling



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Figure 6 Inference, analysis and visualization of cellular communication networks from a single dataset *via* CellChat. A: Interaction intensity plot between cells in single cell sequence profiling; B: Interaction intensity map between mesenchymal stem cells (MSCs) and other cell types; C: Contribution map of 30 signaling pathways detected by CellChat to intercellular efferent (or afferent) signaling; D: Alluvial plot of cell outgoing signaling patterns, showing the correspondence between cell populations and signaling pathways; E: Important ligand–receptor pairs for MSCs sending signals to other cell types. F and G: Contribution of efferent (or afferent) signals between cells, the MK signaling pathway (F) and PTN signaling pathway (G) were selected for visualization of network centrality scores; H: Expression of *Mdk* and *Ptn* in *Cd271* knockout and wild-type MSCs was analyzed by quantitative real-time polymerase chain reaction. Data are presented as mean values ± SD (n = 3 biologically independent experiments. Two-sided unpaired t-test, $^{a}P < 0.05$, $^{b}P < 0.01$). KO: Knockout; WT: Wild-type.

mechanisms in tooth development. Researchers have found that the maxillary process included various cell subpopulations with diverse phenotypic and functional characteristics[34]. How this heterogeneity appears in osteogenesis and odontogenesis remains unclear. *Cd271* used to be a marker of the MSCs, and it had been reported that *Cd271* promotes differentiation in MSCs[35-37]. Hence, we used *Cd271* knockout mouse embryos to investigate what happens to MSCs in their initial developmental stage. We constructed a single cell profiling of representative mouse embryo maxillary processes and selected MSCs to explore their characteristics and critical regulatory mechanism *Cd271* related.

Maxillary-process-derived MSCs are recognized as primitive odontogenic stem cells. For instance, researchers obtained the MSCs from the first branchial arch of mice and the maxillary process tissue of rats in succession, and revealed the pluripotency of MSCs[38,39]. Wen *et al*[40] found that $p75^{+/+}$ MSCs have good multidirectional differentiation potential[40]. We identified nine MSC clusters among 21 clusters identified using scRNA-Seq in mouse embryo maxillary processes. MSC clusters accounted for the majority, suggesting that MSCs contribute much to embryogenesis. Emerging studies have revealed that the marker genes of MSCs, *Col1a1* and *Col3a1*, promote collagen production and influence odontogenesis or osteogenesis[41-43]. The decreased expression of these two genes in *Cd271* knockout MSCs compared to wild-type, suggested that *Cd271* was related to MSC regulation of odontogenesis or osteogenesis.

In the past, several studies concentrated on the whole population of MSCs rather than the relationships among the subclusters. Our subcluster analysis disclosed that these MSC clusters could be separated into three subpopulations: Fibroblast, progenitors and osteoblast, which had not been separated before. One of the characteristics of MSC clusters is the expression of osteoblast differentiation-associated genes such as *Runx2*, *Sox9*, *Msx1* and *Omd*[44-47]. Previous studies showed that proteins such as Runx2 and Sox9 were positively regulated by *Cd271* in MSCs[48-50]. However, these studies did not show the heterogeneity of MSCs, or which subpopulations of MSCs these proteins derived from. Our results revealed that these proteins were mainly expressed in the osteoblast subpopulation. The number of osteoblast subpopulations decreased while progenitor subpopulations increased. This suggested that some progenitors were hindered from differentiating into osteoblasts in the *Cd271* knockout MSC clusters. Consistently, GO enrichment analysis and GSEA verified significant enrichment of the ossification, biomineralization and bone mineralization pathways in the *Cd271* knockout and wild-type cells. Based on our scRNA-Seq data, we gained differentially expressed genes between two groups for the first time. We picked out several differentially expressed genes with extensive interactions; for instance, *Sparc*, *Vim*, *Col4a1*, *Col6a1*, *Col1a1* and *Col3a1*. These genes are considered to relate to cell proliferation, bone mineralization and multipotent differentiation[41-43,51-54]. In *Cd271* knockout MSCs, we verified by quantitative real-time PCR that these differentially expressed gene clusters had lower expression. Functional experiments also demonstrated a weaker odontogenic and osteogenic differentiation and proliferation in the *Cd271* knockout MSCs. Consistent with previous studies, our results confirmed that the MSC clusters had an essential role in odontogenesis and osteogenesis and *Cd271* possibly regulated it.

Pseudotemporal analysis uncovered a developmental trajectory among these three subpopulations. Significantly, the progenitor subpopulation was found to develop into the osteoblast and fibroblast subpopulations. We concluded that the progenitors were the base subpopulation and increasingly transformed into the osteoblast and fibroblast subpopulations in MSCs. Consistent with our GO and GSEA findings, the differentially expressed genes that emerged in pseudotemporal analysis showed a significant relation with ossification and osteoblast differentiation in MSCs. Collectively, our results revealed that MSCs were progressively diversified and determined to the odontogenic fate. Thus, the mesenchyme in the maxillary process provides a remarkable model to study the development of maxillary-process-derived MSCs.

It is known that the interactions between dental mesenchyme and epithelium play a crucial part in the integral tooth developmental process. Nevertheless, the cell–cell interactions within the maxillary process have not been extensively studied[55]. In our study, scRNA-Seq offered the opportunity to identify communicating pairs on the basis of the expression of their cell-surface receptors and ligands. CellChat showed that the most concentrated interactions occurred between mesenchymal and other cells. It indicated that MSCs play a vital role in cell communication. It is worth noting that MSCs mainly participated in MK, PTN, ncWNT, WNT and BMP signaling. Previous studies reported that *Cd271* might regulate the odontogenic differentiation of MSCs through the BMP, WNT and PI3K pathways[17,49,56]. However, there are no reports that MDK signaling participates in regulating MSCs. In the CellChat, the receptor interactions of MDK and PTN appeared to be obvious. It reminded us that the *Mdk* and *Ptn* might play a primary role in the developmental process of MSCs. *Mdk* and *Ptn* expression notably increased in *Cd271* knockout cells during induction of mineralization for 7 d. Our subsequent study will explore the molecular mechanism of how *Cd271* regulates *Mdk* to influence osteogenesis.

Although our studies revealed several significant discoveries, there were some limitations. First, different clustering can generate different results; thus, further groupings are needed to perform future analysis. Second, the current study was based on scRNA-Seq. Therefore, we did some biological observation but did not illustrate the direct mechanisms of *Cd271* involved in tooth development. Therefore, further studies about direct mechanisms and *in vivo* studies are needed.

CONCLUSION

Our study reveals high cellular heterogeneity, molecular details and cell-cell interactions in MSCs. It provides a valuable resource for understanding the development of maxillary-process-derived MSCs. And it enables the maxillary process to serve as an excellent model to explore tooth development and

cell fate determinations. Furthermore, we have found significant functional and signaling differences between *Cd271* knockout and wildtype MSCs. We have clarified that *Cd271* is significantly associated with the regulation of mineralization. We tentatively propose that *Mdk* signaling is involved in the regulatory mechanism of mineralization. These findings contribute to thorough exploration of the mechanism of *Cd271* in regulating odontogenesis and osteogenesis which add to the theory of tooth development.

ARTICLE HIGHLIGHTS

Research background

Tooth loss has become a common problem in human life. Compared with traditional denture restoration, dental tissue engineering has become the most ideal means to solve this problem, and it is also one of the most active research fields of stomatology in recent years. The tooth development involves complex signal pathways. Ecto-mesenchymal stem cells (MSCs) were regarded as the primitive dental cells in the classical theory of tooth development. To date, in-depth studies of the odontogenesis and osteogenesis of MSCs are still lacking.

Research motivation

We contribute to thorough exploration of the mechanism of odontogenesis and osteogenesis to add to the theory of tooth development.

Research objectives

Our study provides novel insights into the biological features of MSCs at the single cell level and the mechanism of *Cd271* in regulating odontogenesis and osteogenesis.

Research methods

We used the maxillary process from mouse embryos as a model to understand the development of maxillary-process-derived MSCs. We applied single cell RNA sequence analysis to elucidate the cellular heterogeneity and explore molecular details. And we verified the findings from single cell sequencing *in vitro* by lab experience such as cell staining, cell counting and quantitative real time polymerase chain reaction.

Research results

Our study reveals: (1) High cellular heterogeneity and molecular details; (2) Significant functional and signaling differences between cell types; (3) Novel subclusters of mesenchymal stem cells; and (4) Crucial cell-cell interactions of mesenchymal subpopulations. Besides, we contribute to thorough exploration of the mechanism of *Cd271* in regulating odontogenesis and osteogenesis.

Research conclusions

Our study reveals high cellular heterogeneity, molecular details and cell-cell interactions in MSCs. We found significant functional and signaling differences between *Cd271* knockout and wildtype MSCs. We clarified that *Cd271* is significantly associated with the regulation of mineralization.

Research perspectives

We need illustrate that *Mdk* signaling is involved in the regulatory mechanism of mineralization in future research. And direct mechanisms of *Cd271* involved in tooth development are needed in further studies.

FOOTNOTES

Author contributions: Zhang YY conceived experiments, interpreted data and wrote a manuscript; Li F, Zeng XK and Zou YH performed the experiment; Zhu BB, Ye JJ, Zhang YX, and Jin Q provided a critical insight into this study; Nie X supervised this study; All authors read and approved the final version for submission.

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Culture and identification of neonatal rat brain-derived neural stem cells

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Abstract

BACKGROUND

Timing of passaging, passage number, passaging approaches and methods for cell identification are critical factors influencing the quality of neural stem cells (NSCs) culture. How to effectively culture and identify NSCs is a continuous interest in NSCs study while these factors are comprehensively considered.

AIM

To establish a simplified and efficient method for culture and identification of neonatal rat brain-derived NSCs.

METHODS

First, curved tip operating scissors were used to dissect brain tissues from new born rats (2 to 3 d) and the brain tissues were cut into approximately 1 mm^3

sections. Filter the single cell suspension through a nylon mesh (200-mesh) and culture the sections in suspensions. Passaging was conducted with TrypL™ Express combined with mechanical tapping and pipetting techniques. Second, identify the 5th generation of passaged NSCs as well as the revived NSCs from cryopreservation. BrdU incorporation method was used to detect self-renew and proliferation capabilities of cells. Different NSCs specific antibodies (anti-nestin, NF200, NSE and GFAP antibodies) were used to identify NSCs specific surface markers and muti-differentiation capabilities by immunofluorescence staining.

RESULTS

Brain derived cells from newborn rats (2 to 3 d) proliferate and aggregate into spherical-shaped clusters with sustained continuous and stable passaging. When BrdU was incorporated into the 5th generation of passaged cells, positive BrdU cells and nestin cells were observed by immunofluorescence staining. After induction of dissociation using 5% fetal bovine serum, positive NF200, NSE and GFAP cells were observed by immunofluorescence staining.

CONCLUSION

This is a simplified and efficient method for neonatal rat brain-derived neural stem cell culture and identification.

Key Words: Neonatal rats; Brain-derived neural stem cells; Culture; Identification

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Core Tip: How to harvest sufficient neural stem cells (NSCs) is a basic requirement for the study and clinical application of NSCs. This study describes a simplified and efficient method for neonatal rat brain-derived NSC culture and identification comprehensively considering the influencing factors including timing of passaging, passage number, passaging approaches and methods for cell identification. It demonstrates that combination of TrypL™ Express and mechanical tapping and pipetting techniques makes a more efficient way of passaging. The optimal timing for NSC passage is on the fourth to fifth day of primary or passage NSC culture.

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INTRODUCTION

Spinal cord injuries (SCIs) are associated with high morbidity, disability and medical costs around the world[1-3]. Prevention, treatment and rehabilitation of SCIs remains one of the global health issues. Pathophysiological basis for sensory, motor and sphincter dysfunction below the neurological level of injury is the substantial variations, necrosis and loss of neurons along with ruptured tracts of spinal cord as well as demyelination induced by primary and/or secondary SCIs[4,5]. Therefore, the research fucus on the key to promoting recovery of function following SCIs falls on how to replenish the lost neurons and boost the regeneration of axis cylinder and myelin sheath. It should be noted that neural stem cells (NSCs) have the capacity to self-renew and proliferate, and differentiate into nerve cells of central nervous system including neuron, astrocyte and oligodendrocyte[6]. Hence, by replenishing neurons, forming axis cylinder, increasing oligodendrocyte and reconstructing medullary sheath and then remolding the injured structure and function of spinal cord, NSCs replacement as a possible therapeutic approach could bring hope to SCI patients. In view that rapid and simplified accesses to a large number of pure NSCs for research are needed, it is necessary to develop a method for NSC amplification, culture, purification and identification *in vitro* to provide sufficient-necessary condition for further investigation of NSCs.

The present study cultured, passaged and harvested large amounts of high pure NSCs with growth factor containing serum-free medium using TrypLE™ Express digestion method combined with mechanical disassociation method[4,7,8], which provide the platform for the further study of NSCs.

MATERIALS AND METHODS

Rats

In this study, Brain-derived NSCs were abstracted from several two-to-three-day old newborn SD rats provided by Southwest Medical University Animal Resource Center.

Reagents

Hyclone DEME/F12 (1:1); Hyclone standard fetal bovine serum; Peprotech EGF (20 μg); Peprotech bFGF (10 μg); Gibco B27 Plus Supplement (50×); Gibco TrypL™ Express (1×); rabbit anti-nestin polyclonal antibody (Wuhan Boster Biological Technology, Ltd.); mouse anti-NSE monoclonal antibody and mouse anti-GFAP monoclonal antibody (Wuhan Boster Biological Technology, Ltd.); RBITC-labeled goat anti-mouse IgG (Beijing Biosynthesis Biotechnology Co., Ltd.); RBITC-labeled goat anti-rabbit IgG (Beijing Biosynthesis Biotechnology Co., Ltd.).

Preparation of serum free medium for NSC culture

Take 100 mL of DMEM/F12 (1:1) and add EGF (20 ng/mL), bFGF (20 ng/mL), B27 Plus Supplement (2%), glutamine (2 mmol/L) and 1 mL of penicillin-streptomycin solution (100 U/mL). NaOH filtered with 0.22 μm microporous membrane filter were used to adjust PH of the solution between 7.2 and 7.4. After preparation, the medium was stored at 4°C. Serum free medium (SFM) should be kept fresh. A weekly dosage of 100 mL for on preparation is generally recommended.

Primary culture, passage, cryopreservation, resuscitation and identification of NCSs

Preparation of extracts and primary NSC culture: Newborn SD rats (2–3 d old) were selected and immersed in 75% alcohol for 10 min for disinfection. In supine position, the rats were fixed to foam board and placed on the clean bench with cranial cavity exposed. Brain tissues were isolated and placed in sterilization incubators containing phosphate-buffered saline (PBS) (0.01 M, PH 7.2, pre-warmed to 37°C, 10 min, the same below) for washing to remove blood stains on the surface. Then pia mater as well as choroid plexus tissues were removed under a dissecting microscope. The dissected brain tissues were washed and transferred to a new sterilization incubator. Using curved tip operating scissors, dissected brain tissues were cut into approximately 1 mm^3 sections and were transferred to centrifuge tubes (10 mL, the same below) using sterile pipettes. Centrifuge the sections at 1000 rpm for 1 min and remove the supernatant. Save the cerebral cortex in three separate tubes, each containing 1 mL of mixed enzyme, shake them gently for 5 min in a 37°C water-bath and centrifuge them at 1000 rpm for 5 min, and then remove the supernatant. Add 5% fetal bovine serum, pipette and tap gently 10 times using pipettes, shake gently in a 37°C water-bath, centrifuge for 5 min at 1800 rpm, and remove the supernatant. Then add DMEM/F12 (1:1), pipette and tap gently 20 times using pipettes, filter using stainless-steel cell strainers (200-mesh), centrifuge for 5 min at 1000 rpm and remove the supernatant. Use 0.5 mL of SFM to resuspend cells. Pipette and tap the cells gently and form single-cell suspensions. Seed the cells at a rate of 1 × 10^6/mL (cell density determined by trypan blue method excluding dead cells) in 25 mL glass culture flasks and cultured in carbon dioxide constant temperature incubators (stable temperatures of 37°C, saturated humidity, a CO_2 level of 5%). Centrifuge the cells for 5 min at 1000 rpm every other day or every two days depending on how the cells developed and refresh half of the media with fresh growth media. Approximately, four or five days later, initiate passaging. Observe and record cell growth status under inverted phase contrast microscope every day including medium color, impurities in medium, cell refraction, density, size of neurosphere formation and neurosphere refraction.

Passaging of NSCs: After four to five days of primary culture, which is dependent on the growth process of cell and neurospheres, gather all neurospheres and culture solution and place in a 15 mL cone shaped tube, centrifuge for 5 min at 1000 rpm and remove the supernatant. Add 1 mL of TrypLE™, pipette cells gently using pipettes, shake them gently for 5 min in a 37°C water-bath and centrifuge them at 1800 rpm for 5 min, and then remove the supernatant. Add 0.5 mL of SFM to resuspend cells. Using 1000 μL pipettes, tap the cells gently 10 times (for 200 μL pipettes, approximately 20 times). Seed the cells at a rate of 1 × 10^6/mL (cell density determined by trypan blue method excluding dead cells) in a 25 mL glass culture flask and cultured in the above described constant temperature incubators (stable temperatures of 37°C, saturated humidity, a CO_2 level of 5%).

Cryopreservation and resuscitation of NSCs: Add 1 mL of freshly prepared cell freezing medium (DMEM/F12:fetal bovine serum:DMSO = 7:2:1) to the single-cell suspensions. Adjust the cell density to 5×10^6/mL, and preserve the suspension in a 4°C refrigerator for three hours. Place the cryovials in a small plastic storage box and keep the box immediately in a −80°C gas-phase liquid nitrogen freezer overnight and finally cryopreserve the cryovials in a liquid-phase liquid nitrogen freezer. For cell resuscitation, remove cryovials from the liquid-phase liquid nitrogen freezer and place the cryovials in a 37°C water-bath for rapid melting. Transfer the cells to centrifuge tubes and add 4 mL of basal media. Centrifuge the cells for 5 min at 1000 rpm and remove the supernatant. Add SFM and resuspend cells. Seed the cells at a rate of 1 × 10^6/mL (cell density determined by trypan blue method excluding dead cells) in 25 mL glass culture flasks and cultured in the above described constant temperature incubators.

Identification of NCSs: Identify the 5th generation of passaged NSCs and the revived NSCs from cryopreservation. Use bromodeoxyuridine (BrdU) incorporation method to detect the self-renew and proliferation abilities of cells. Use diverse neurocyte-specific antibodies such as anti-nestin, anti-NF200, anti-NSE and anti-GFAP antibodies to examine NSC specific surface markers and muti-differentiation capabilities by immunofluorescence staining.

BrdU incorporation method for the detection of the proliferation of NSCs: (1) At 24 h after the culture of 5th generation of passaged NSCs, add BrdU to SFM to make BrdU reach a concentration of 10 μmol/L. Continue to culture the cells for five days and seed the NSCs cultured in suspensions at a rate of 1 × 10^6/mL (500 μL) in a carbon dioxide constant temperature incubator containing 24-well culture plates with polylysine coated coverslips (stable temperatures of 37°C, moderate saturated humidity, a CO_2 level of 5%) for four hours; (2) Draw the solution and gently wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (3) Add 4% paraformaldehyde for a fixation for 15 min at room temperatures; (4) Draw the stationary fluids and wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (5) Use 37°C blocking buffer (10% sheep serum and 0.3% Triton) for 1 h closed incubation (150 μL per well, almost spread the coverslips) and then draw the stationary fluids. Add 150 μL of mouse anti-BrdU primary monoclonal antibody (dilute the fluids with PBS to adjust the concentration to 1:200) and cover coverslips. Use PBS (0.01mol/L, PH7.2) as positive control to replace mouse anti-BrdU primary monoclonal antibody. Continue to culture the cells in a 37°C constant temperature incubator for two hours; (6) Blot fluids in the last step and wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (7) Add FITC labeled goat anti-mouse IgG second antibody (dilute the fluids with PBS to adjust the concentration to 1:40) (cells should be protected against exposure to light for each of the following steps). Continue to culture the cells in a 37°C constant temperature incubator for one hour; (8) Blot the second antibody and wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (9) Add 20 μL of anti-fluorescence quencher over the slide; and (10) Lift the coverslip using a self-made right-angled tip needle and then press it with the side containing the cells down avoiding creating air bubbles. View the cells under a fluorescence microscope, take photos and make records.

Nestin immunofluorescence staining: (1) Take a small amount of 5th generation of cultured neurosphere containing NSCs and seed them in a carbon dioxide constant temperature incubator containing 24-well culture plates with polylysine coated coverslips (stable temperatures of 37°C, moderate saturated humidity, a CO_2 level of 5%) for four hours; (2) Discard the solution and wash gently 3 × 5 min with PBS; (3) Add 4% paraformaldehyde for a stationary phase for 15 min at room temperatures; (4) Discard the stationary liquid and wash gently 3 × 5 min with PBS; (5) Use 37°C blocking buffer (10% sheep serum and 0.3% Triton) for 1 h closed incubation (150 μL per well, almost spread the coverslips) and then blot the stationary fluids. Add rabbit anti-nestin monoclonal primary antibody (dilute the fluids with PBS solution to adjust the concentration to 1:200). The primary antibody was substituted for PBS solution as a negative control. Continue to culture the cells in a 37°C constant temperature incubator for two hours; (6) Discard the liquid used in the last step and gently wash 3 × 5 min with PBS (0.01 M, PH7.2); (7) Add RBITC labeled goat anti-rabbit IgG second antibody (dilute the fluids with PBS solution to adjust the concentration to 1:40) (avoid light exposure for each of the following steps). Then culture the cells in a 37°C constant temperature incubator for one hour; (8) Blot the second antibody. Wash 3 × 5 min with PBS (0.01 mol/L, PH7.2); (9) Add DAPI staining solution (150 μL per well, almost spread the coverslips) for 3 min at room temperatures and then blot the DAPI staining solution. Wash 3 × 5 min with PBS (0.01mol/L, PH7.2). Add 20 μL of anti-fluorescence quencher over the slide; and (10) Lift the coverslip and then press it with the side containing the cells down on the slide containing 20 μL of anti-fluorescence quencher (Be careful to avoid creating air bubbles).

Observe the cells under a fluorescence microscope, take photos and make records. Differentiation of NSCs and immunofluorescence staining of GFAP, NF200 and NSE. (1) Using SFM containing 5% fetal bovine serum, seed the 5th generation of NSCs in 24-well culture plates with polylysine coated coverslips and culture the cells for 5 d; Repeat steps (2), (3) and (4) in the section of BrdU incorporation method for the detection of the proliferation of NSCs or in nestin immunofluorescence staining; (5) Use 37°C blocking buffer (0.3% Triton X-100 and 10% sheep serum) for 1 h closed incubation (150 μL per well, almost spread the coverslips, similarly hereinafter) and then discard the stationary fluids. Add mouse anti-GFAP monoclonal antibody, mouse anti-NSE monoclonal antibody and mouse anti- NF200 monoclonal antibody, respectively (all as primary antibody, dilute the fluids with PBS to adjust the concentration to 1:200), and the primary antibody was substituted for prepared PBS solution as a negative control and then cultured in the above described constant temperature incubators for two hours; (6) Discard the liquid in the last step, and wash 3 × 5 min with prepared PBS; (7) Add RBITC-labeled goat anti-mouse IgG secondary anti-body (dilute the fluids with PBS to adjust the concentration to 1:40), and incubate the cell in a above mentioned incubator for 1 h; Repeat steps (8), (9) and (10) in the section of BrdU incorporation method for the detection of the proliferation of NSCs or in nestin immunofluorescence staining.

RESULTS

Development and identification of NSCs

The first-generation cells derived from newborn SD rats appear small, equal sized, glossy spheres. At 24 h after the primary culture, larger amounts of cells aggregate in the middle of potion of the growth medium. These single floating cells can differentiate and renew (Figure 1A). There are also plenty of glossy, spherical-shaped, adherent single cells at the bottom of the culture bottle. Moreover, many structural looseness, highly refractile, spherical-shaped clusters and membraniform flocculation also exist in the culture medium, which are considered as clumps of dead cells and uneliminated tissues such as blood vessels and pia mater, respectively. At 2 to 3 d after the primary culture, several and even dozens of translucent, highly refractile, spherical-shaped or thyrsiform cell clusters are discovered (Figure 1B). What's more, only a small amount dead cell clusters were left in the culture medium. At four to five days after the primary culture (Figure 1C), the cell mass bulks up to a spherical shape when large numbers of cell clusters aggregate and form translucent slippery spheres under an inverted phase-contrast microscope. However, refractivity at the center of the sphere decreases and few aggregated dead cells are observed. Compared with that of neurospheres at five days after the primary culture, the volume of neurospheres at eight days after the primary culture is obviously big and the refractivity is poor and it shows dark brown in most parts of the sphere center (Figure 1D). Passaging conducted at that time only dissociates neurospheres into smaller cell clumps and few single floating cells rather than single-cell suspensions. It is speculated that this is associated with tight cell-cell junction around the neurospheres. Moreover, many small sized adherent cells are observed at two days after the passaging with small quantity of low density single floating cells. Adherent differentiation occurs in plenty of large volume neurospheres during the continuous culture.

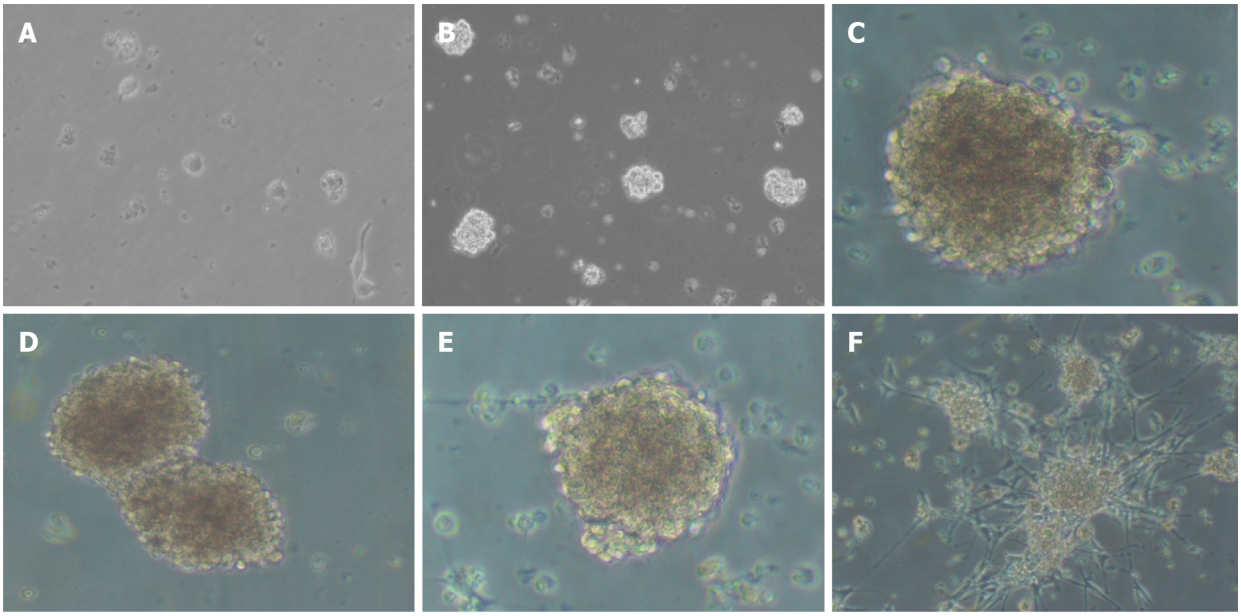
Generally, there are two methods of cell dissociation: mechanical and enzymatic. It is discovered that it is hard to control enzyme concentration and time, which easily lead to cell death. Comparatively, TrypLE™ Express digestion method combined with mechanical disassociation method can harvest sufficient normomorph, uniform size single floating cells with glossy cytoplasm and there are relatively few cell debris and impurities in the culture medium. In addition, medium-sized cloned neurospheres in good shape can be observed in the passaged NSC culturation (Figure 1E). At four hours after the induction of dissociation of 5th generation of passaged NSCs using 5% fetal bovine serum, adherent neurospheres and gradual migration of cells away from neurospheres are observed. At approximately five days after the induction of dissociation, spheres become flattened and form various shaped cells surrounding the neurospheres. At approximately five days after the induction of dissociation, the spheres flatten and make up lots of various shapes of cells. These cells connect to each other into a mesh-structure (Figure 1F).

Identification of NCSs

The incorporation of BrdU into the 5th generation of passaged cells showed positive after BrdU immunofluorescence staining (green fluorescence, Figure 2A) and nestin immunofluorescence staining (red fluorescence, Figure 2B). At five days after the induction of dissociation of 5th generation of passaged cells using 5% fetal bovine serum, NF200 (green fluorescence, DAPI nucleic acid stain revealed the blue fluorescent cell nucleus, Figure 2C), NSE (red fluorescence, blue fluorescent obtained with DAPI nucleic acid stain, Figure 2D) and GFAP (red fluorescence, DAPI nucleic acid stain revealed the blue fluorescent cell nucleus, Figure 2E) positive immunoreactivities were found.

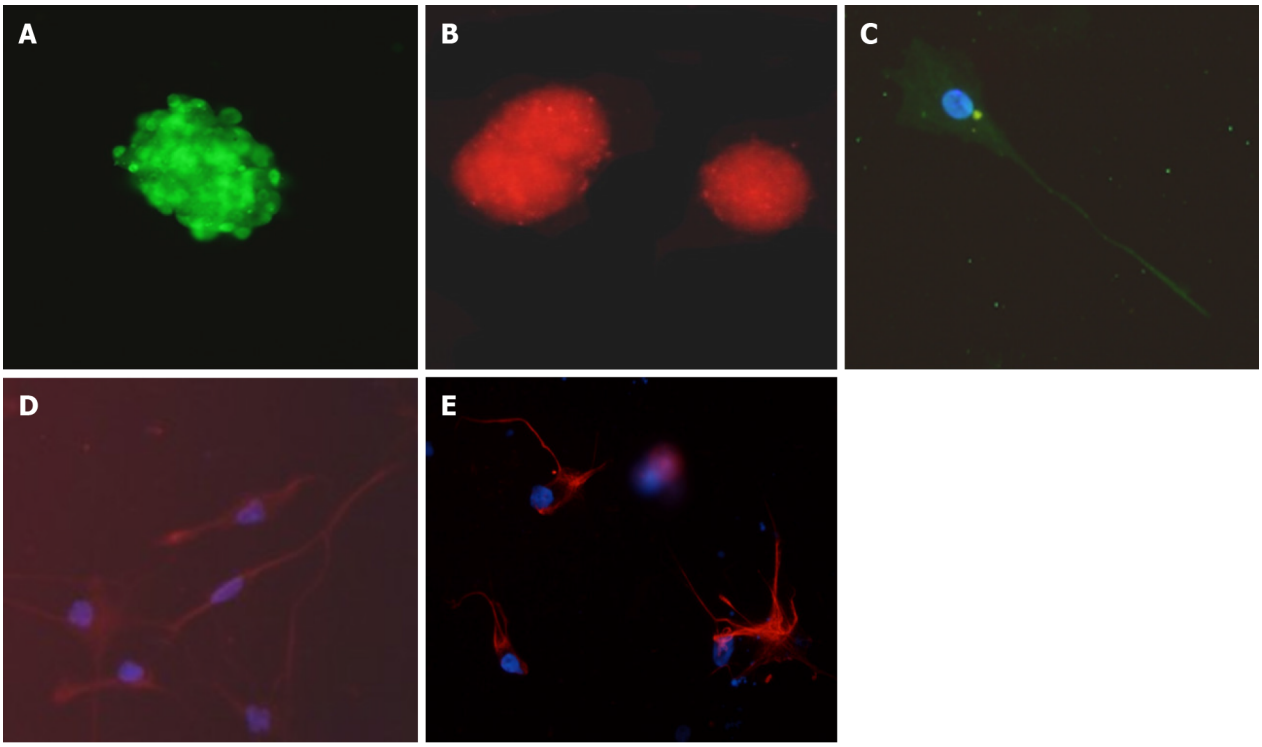
DISCUSSION

Since 1990s, many researchers have demonstrated that NSCs are widely present in central nervous system (CNS) including spinal cord[6,9-17]. NSCs not only have the probabilities of division, regeneration proliferation and multidirectional differentiation (differentiate into nearly all types of CNS nerve cells such as neuron and glial cell), but also exhibit low immunogenicity and good histocompatibility[6,9-17]. This refutes the theory that the damaged cells are unable to be regenerated and repaired following the injuries to the nerve and the spinal cord, and provides new hope to the reconstruction of structure and function in CNS disorders such as SCIs and cerebral injuries[6,18]. NSCs, as the seed cells enhancing CNS regeneration, spontaneously become cores and foundation of neurological research. Efficient approaches for enhancing pure NSC yield in the research thus becomes crucial. The present study established a method for NSC amplification, culture, purification and identification *in vitro* to provide sufficient-necessary condition for further investigation of NSCs. Cell culture technology is the basic premise for the investigation of NSCs. Mature techniques for the isolation and culture of NSCs have been developed. In addition to proving the presence of NSCs and its potential of self-renewal and multidirectional differentiation, Reynolds and Weiss *et al*[9] also demonstrated that NSCs could proliferate massively *in vitro*, which establish crucial conditions for future investigation of NSCs. However, application of methods for *in vitro* amplification of stem cells is currently restricted to CNS. By contrast, cloning of monolayer cells[6,19] offers an alternative approach for NSC culture. This



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Figure 1 Development of neural stem cells derived from newborn rats (inverted phase-contrast microscope). A: At 24 h after the primary culture (100×); B: 3 d after the primary culture (100×); C: 5 d after the primary culture (100×); D: 8 d after the primary culture (100×); E: 4 d after 5 passages (×200); F: 5 d after induction of dissociation (×200).



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Figure 2 Immunofluorescence staining. A: BrdU immunofluorescence staining (green fluorescence, ×100); B: Nestin immunofluorescence staining (red fluorescence, ×100); C: NF200 + DAPI immunofluorescence staining (green fluorescence refers to NF200, blue fluorescence refers to DAPI, ×200); D: NSE + DAPI immunofluorescence staining (green fluorescence refers to NSE, blue fluorescence refers to DAPI, ×200); E: GFAP + DAPI immunofluorescence staining (green fluorescence refers to GFAP, blue fluorescence refers to DAPI, ×200).

approach is widely used because using this approach we can not only derive an abundant amount of pure NSCs in a short time but also determine the differentiation capacity for a single NSC or its precursor cells.

For the timing of NSC passaging, the present study found that although passaging at two-to-three-day primary culture could facilitate the dissociation of neurospheres and formation of single-cell suspensions, the number of single cells derived was unsatisfactory at low densities which was not conductive to amplification and proliferation of NSCs after passage. Passaging performed when primary NSCs are cultured for seven to eight days could only dissociate neurospheres into small clusters of cells with a small amount of single floating cells instead of single-cell suspensions, which was presumably related to the tight junctions between peripheral neurosphere cells[20]. Moreover, small clusters of adherent cells with small portion of low density single floating cells were discovered after only two days of passage. Differentiation of adherent large neurospheres occurred after a continued cultivation. The observation of nine-to-ten-day primary NSC culture revealed that differentiation occurred in plenty of adherent large neurospheres with lots of dead cells, which also inhibits passaging. Comparatively, when passaging was performed after four to five days of primary culture, it not only promotes easy generation of single-cell suspensions dissociated from neurospheres but also harvests sufficient single floating cells with fewer dead cells and improved amplification and proliferation capabilities of NSCs. It follows that the optimal timing for NSC passage is after four-to-five-day primary or passage NSC culture. During the progress of *in vitro* culture, junctions can be formed between NSCs, which assemble to form neurospheres, through cytoskeletal protein and calnexin induced cell-cell adhesion mediation[20]. For neurospheres formed through primary culture or passage culture carried out for two to three days, the junction between NSCs is relatively loose making dissociation and formation for single floating cells easy. However, an analysis of the grow curve over this period displayed that passaging which is carried out when NSCs entered the logarithmic growth phase from the incubation phase might destroy the rapid amplification of NSCs resulting in passaged NSCs' repeated incubating and missing the optimal timing for amplification and at last failure to yielding sufficient NSCs in a short time. On the fourth to fifth day of primary culture or subculture, the number of NSCs increased significantly, the volume of neurospheres grew bigger and cell-cell junctions became tight when the junctions were still able to be dismantled by some means to obtain single cells comfortably. Notably, cell culture gradually reached a plateau period with anchoring cell-cell junctions over time. Oppositely, when the primary or passage culture last for seven to eight days, cell-cell junctions would become so tight that it was difficult to cleave or dissociate. Forced disconnection might end up with NSC damage, dissolution, death, adherence and differentiation. Furthermore, it was observed that the number of neurospheres decreased with their increasing volume suggesting the bulked volume of neurospheres might arise from neurosphere fusion instead of large amplification of NSCs. This can be interpreted as with the increasing volume of neurospheres, it is difficult for the cells within the spheres to contact the culture medium, which then prevents cells within the spheres from absorbing nutrition and affecting the process of metabolism, causing decline in the proliferation abilities of cells within the neurospheres, death of a large number of cells and generation of necrotic cores[21]. From this view, balance should be reached between easy dissociation of neurospheres into single cells and rapid amplification of NSCs without damaging the proliferation abilities of cells within spheres by the excessive volume of neurospheres or causing death during the choice of timing for NSC passage. After summarizing the results of experiments, it is believed that the optimal timing for NSC passage is on the fourth to fifth day of primary or passage NSC culture.

Currently, the commonly used methods for passaging are enzymatic digestion method and mechanical method. After analyzing the investigations[4,7,8], the present study discovered that: (1) Although mechanical method alone is easy to operate and needs a very few of centrifugation, the force and times of tapping and pipetting is difficult to control and it increases the probability of mechanical damage to cells, which is not conducive to the experimental operation; (2) for enzymatic digestion method, trypsin is mainly used. However, the effect of trypsin in digestion is likely to cause chemical damage to NSCs. Moreover, usage of serum or enzyme inhibitor to counteract the digestive effect of trypsin may reduce the vitality and proliferation rate of NSCs and then influence the experimental results; (3) concerning the combination of enzymatic digestion method and mechanical tapping and pipetting method, in spite of a very few of blasting is needed and small scale of blasting can dissociate neurospheres into single-cell suspensions, it does not elevate the number of single cells while the amplification capability of NSCs and number of neurospheres decrease significantly; and (4) in terms of TrypL™ Express digestion method combined with mechanical tapping and pipetting method, TrypL™ Express, a high purity recombinant enzyme, has trypsin-like property, which cleaves peptide bonds on the C-terminal sides of lysine and arginine. TrypL™ Express has high thermostability compared with trypsin. There is no need to use serum or enzyme inhibitor to compromise its digestive effect. Instead, the effect of TrypL™ Express is mild with less toxicity. However, the action of TrypL™ Express should not last long. This study used TrypL™ Express combined with mechanical tapping and pipetting methods as the passaging method. Using this passaging method, sufficient regular shaped, even sized sheen floating single cells were harvested. What's more, cell culture medium is clear with few fragments and elemental impurities, and NSCs holds vigorous growth and proliferation abilities. After observing the process of NSC culture post passaging, it discovered proper sized and good shaped colony-like neurospheres. Based on this, this study recommend using TrypL™ Express combined with mechanical tapping and pipetting method as the method for NSCs passaging.

Additionally, immunofluorescence staining was used to identify whether the cultured cells derived from newborn rats' brains were NSCs. The 5th generation of passaged cells showed positive after both BrdU immunofluorescence staining and nestin immunofluorescence staining. BrdU, a thymidine analog, can incorporate into newly synthesized DNA during the S-phase of the cell cycle[22]. Its property of continuous self-replication and self-reproduction by stem cells makes it take in labeled Brdu during replication. Thus, when immunocytochemical detection is carried out, BrdU positive suggests the cells are in the division and proliferation phrase. Accordingly, NSCs' potential of division and proliferation can be identified by Brdu detention. Nestin, also called NES, is a major cytoskeletal protein in stem cells in the mammalian CNS and its expression is inversely associated with cell differentiation[23-25]. Nestin is a specific biomarker for NSCs and functions as one of factors to identify NSCs. However, nestin is also expressed in pancreatic progenitor cells and endodermal cells. Therefore, nestin positive cells are not necessarily NSCs. The identification of NSCs cannot only based on a certain feature. Apart from nestin immunofluorescence staining, specific biomarkers for NSC differentiation should be considered. The present study selected neuron-specific biomarkers NF200 and NSE and astrocyte-specific biomarker GFAP respectively for the identification of muti-differentiation of NSCs. This study used immunofluorescence staining for the detection of cultured cells derived from newborn rat brains in respect of the above described some characteristics of NSCs. It was observed that the 5th generation of passaged culture cells showed BrdU positive after BrdU immunofluorescence staining and nestin positive after nestin immunofluorescence staining. NF-200, NSE, GFAP positive cells were discovered following immunofluorescence staining at five days after induction of dissociation was performed in 5% fetal bovine serum. This implied that the cultured cells fulfil the criteria for NSC identification - expression of nestin, abilities of differentiation, renewal and proliferation and moreover the capabilities of dissociation. The composition of all these factors can demonstrate the cultured cells are NSCs.

There still are limitations on the development of methodologies to efficiently isolate, culture, and identify NSCs *in vitro*, and evaluating these obtained NSCs is the precondition for its clinical application. Further studies should try to find relevant influencing factors behind these issues.

CONCLUSION

Above all, the approaches used in this study yields sufficient purified NSCs, which then are employed for the further study of NSCs.

ARTICLE HIGHLIGHTS

Research background

The present study explores to establish a simplified and efficient method for culture and identification of neural stem cells (NSCs) derived from newborn rats in the aspects of timing of passaging, passage number, passaging approaches and methods for cell identification.

Research motivation

The study of NSCs is crucial for it will provide important insight into understanding and treating brain disorders. Efficient way to harvest sufficient neural stem cells will facilitate the use of NSCs in clinical scenarios.

Research objectives

This study aimed to explore a more effective protocol for culturing and identifying NSCs derived from newborn rats.

Research methods

Passaging of brain tissues sections dissected from new born rats (2 to 3 d) was conducted using TrypL™ Express combined with mechanical tapping and pipetting techniques. After five passages, NSCs as well as the revived NSCs from cryopreservation were identified. BrdU incorporation method was used to detect self-renew and proliferation capabilities of cells. Different NSCs specific antibodies (anti-nestin, NF200, NSE and GFAP antibodies) were used to identify NSCs specific surface markers and muti-differentiation capabilities with immunofluorescence staining.

Research results

With TrypL™ Express combined with mechanical tapping and pipetting techniques, spherical-shaped cell clusters were successfully obtained. When BrdU was incorporated into the 5th generation of passaged cells, positive BrdU cells and nestin cells were observed by immunofluorescence staining. After induction of dissociation using 5% fetal bovine serum, positive NF200, NSE and GFAP cells were

observed by immunofluorescence staining.

Research conclusions

The present study developed a simplified and efficient method for neonatal rat brain-derived neural stem cell culture and identification.

Research perspectives

First, there still are limitations on the development of methodologies to efficiently isolate, culture, and identify NSCs *in vitro*. Second, evaluating these obtained NSCs is the precondition for its clinical application. Further studies should try to find relevant influencing factors behind these issues.

FOOTNOTES

Author contributions: Zhou QZ designed the study; all authors contributed to the data collect and manuscript writing; all authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Synergism of calycosin and bone marrow-derived mesenchymal stem cells to combat podocyte apoptosis to alleviate adriamycin-induced focal segmental glomerulosclerosis

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Abstract

BACKGROUND

Bone marrow-derived mesenchymal stem cells (MSCs) show podocyte-protective effects in chronic kidney disease. Calycosin (CA), a phytoestrogen, is isolated from *Astragalus membranaceus* with a kidney-tonifying effect. CA preconditioning enhances the protective effect of MSCs against renal fibrosis in mice with unilateral ureteral occlusion. However, the protective effect and underlying mechanism of CA-pretreated MSCs ($MSCs^{CA}$) on podocytes in adriamycin (ADR)-induced focal segmental glomerulosclerosis (FSGS) mice remain unclear.

AIM

To investigate whether CA enhances the role of MSCs in protecting against podocyte injury induced by ADR and the possible mechanism involved.

METHODS

ADR was used to induce FSGS in mice, and MSCs, CA, or MSCs^{CA} were administered to mice. Their protective effect and possible mechanism of action on podocytes were observed by Western blot, immunohistochemistry, immunofluorescence, and real-time polymerase chain reaction. *In vitro*, ADR was used to stimulate mouse podocytes (MPC5) to induce injury, and the supernatants from MSC-, CA-, or MSCs^{CA}-treated cells were collected to observe their protective effects on podocytes. Subsequently, the apoptosis of podocytes was detected *in vivo* and *in vitro* by Western blot, TUNEL assay, and immunofluorescence. Overexpression of Smad3, which is involved in apoptosis, was then induced to evaluate whether the MSCs^{CA}-mediated podocyte protective effect is associated with Smad3 inhibition in MPC5 cells.

RESULTS

CA-pretreated MSCs enhanced the protective effect of MSCs against podocyte injury and the ability to inhibit podocyte apoptosis in ADR-induced FSGS mice and MPC5 cells. Expression of p-Smad3 was upregulated in mice with ADR-induced FSGS and MPC5 cells, which was reversed by MSC^{CA} treatment more significantly than by MSCs or CA alone. When Smad3 was overexpressed in MPC5 cells, MSCs^{CA} could not fulfill their potential to inhibit podocyte apoptosis.

CONCLUSION

MSCs^{CA} enhance the protection of MSCs against ADR-induced podocyte apoptosis. The underlying mechanism may be related to MSCs^{CA}-targeted inhibition of p-Smad3 in podocytes.

Key Words: Calycosin; Mesenchymal stem cells; Focal segmental glomerulosclerosis; Apoptosis; Smad3

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Core Tip: Calycosin (CA)-pretreated mesenchymal stem cells (MSCs^{CA}) enhanced the protective effect of MSCs against adriamycin (ADR)-induced podocyte injury *in vitro* and *in vivo* by inhibiting apoptosis, accompanied by more reversal of the upregulated expression of p-Smad3 after ADR induction. Smad3 overexpression eliminated the inhibitory effect of MSCs^{CA} on podocyte apoptosis, suggesting that MSCs^{CA} inhibit podocyte apoptosis by targeting p-Smad3. These results broaden our understanding of the potential of MSCs pretreated with herbal extract and provide new theories for possible therapeutic mechanisms for ADR-induced focal segmental glomerulosclerosis.

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INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is the most common primary glomerulopathy and the dominant pathological type of chronic kidney disease (CKD)[1,2], associated with high albuminuria and end-stage renal disease (ESRD) with a poor prognosis[3,4]. FSGS is linked with injury or even depletion of podocytes, manifested by the gradual disappearance of podocyte-specific markers such as podocin[5, 6]. As podocyte injury plays a critical role in FSGS progression, protecting podocytes is promising to prevent ESRD in patients with FSGS[7].

Apoptosis of podocytes has been widely studied in previous studies[8-10], and inhibition of podocyte apoptosis has been reported to delay FSGS progression[11]. Podocyte apoptosis is characterized by the loss of Bcl-2 protein and the increase of Bax protein[12,13]. Recently, Smad3-related pathways have been reported to be involved in podocyte apoptosis[14]. However, the underlying mechanism remains unclear, and no specific effective treatment can prevent podocyte apoptosis.

Mesenchymal stem cells (MSCs) are multipotent stem cells that exhibit varying potential for multilineage cell differentiation as well as the capacity for self-renewal[15]. Therefore, using MSCs to treat various diseases is worth exploring[16-18]. MSCs treat diabetic nephropathy by protecting podocytes[19-21], and bone marrow-derived MSC (BMSC) transplantation can attenuate FSGS progression in a rat model of FSGS[22,23]. In addition, the protective effects of MSC derivatives or exosomes on podocytes have also been reported[24,25]. However, the application of MSCs is also

limited. For instance, MSCs may be losing their biological function after being isolated and cultured for a long time. After infusion, MSCs must face harsh environments with various stressors such as inflammation, hypoxia, high acidity, or reduced energy reserve. On this account, preconditioning, genetic modification, and delivering MSCs with biomaterials have been developed[26]. Thus, it is important to explore how MSCs can overcome adverse microenvironments to enhance their therapeutic benefits.

Calycosin (CA), a phytoestrogen with a kidney-tonifying effect, is isolated from *Astragalus membranaceus*. It has been reported that CA is the top component of potentially active compounds for the treatment of nephrotic syndrome[27]. Moreover, CA has also been found to be an active ingredient in the treatment of adriamycin (ADR) nephropathy using network pharmacology combined with transcriptomics[28]. Our research group used Ca-pretreated MSCs to treat mice with unilateral ureteral occlusion (UUO) and found that they improved renal fibrosis and inhibited necrosis of renal tubular epithelial cells more than normal MSCs did[29]. However, the protective effect on podocytes and their mechanism of action remain unknown.

In rodents, ADR can induce rapid podocyte injury characterized by massive foot process effacement and glomerulosclerosis, which serves as a model of FSGS[30,31]. In the present study, we compared the antiapoptotic efficacy of CA-pretreated MSCs ($MSCs^{CA}$) to that of MSCs or CA in a mouse model of FSGS induced with ADR and *in vitro*, as well as the possible mechanisms of action involved.

MATERIALS AND METHODS

Animal experiments

The C57BL/6 mice utilized in this investigation were bought from Chengdu Dashuo Biotechnology Co., LTD. in China. They were male, 8 wk old, and weighed 22-25 g. All the mice were kept in a specific disease-free space with 12 h of light and dark cycles and had free access to water and food. The mice were randomly divided into the following six groups: Normal control group; ADR injection group; ADR with Dulbecco's modified Eagle's medium (DMEM; 200 μL) injection (ADR + DMEM); ADR with 200 μL MSCs (10^6 cells/mL) (ADR + MSCs); ADR with 200 μg/mL CA (10^6 cells/mL) (ADR + CA); and ADR with MSCs preconditioned with 200 μg/mL CA (10^6 cells/mL) (ADR + $MSCs^{CA}$). For ADR-induced FSGS, the mice were injected with 10 mg/kg ADR (Shenzhen Main Luck Pharmaceuticals Inc.) *via* the tail vein. The normal control mice were injected with vehicle (saline). MSCs, CA dissolved in DMEM, and $MSCs^{CA}$ were injected *via* the tail vein 4 wk after ADR injection once weekly. Since both MSCs and CA are soluble in DMEM, mice in the ADR + DMEM group were given an equal volume of normal DMEM as the solvent control. All mice were killed at 8 wk after ADR injection. All animal experiments were carried out in accordance with the recommendations of the Institute of Nutrition and Health's Animal Care and Utilization Committee, and were approved by the Southwest Medical University's Animal Ethics Committee (No. 20210223-024).

Isolation of MSCs

As previously described, MSCs were isolated from the leg bone marrow of male C57BL/6 mice aged 6-8 wk[32]. Briefly, cells were grown at 37 °C and 5% CO_2 in DMEM Petri plates with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, United States), 1 g/L glucose, and 1% penicillin-streptomycin (Beyotime, Shanghai, China). At 24 h, the medium was changed to remove the non-adherent cells. The MSCs were passed once 90% confluence was reached. As described previously[26], anti-CD29 (102205; Biolegend), anti-CD90 (ab24904; Abcam, Cambridge, MA, United States), and anti-CD11b (101205; Biolegend, San Diego, CA, United States) antibodies were used to label MSCs, and the purity of the MSCs was analyzed using a BD FACSVerse (Becton, Dickinson and Company, Franklin Lakes, NJ, United States).

CA pretreatment of MSCs

CA (≥ 94% purity) was purchased from Cayman Chemical Company (Ann Arbor, MI, United States). The stoste used for MSC pretreatment included full medium and CA (200 g/mL) dissolved in DMEM as previously described[29]. After incubation for 72 h, the MSCs and $MSCs^{CA}$ were injected into mice, and the supernatants were used to treat mouse podocytes (MPC5) for 48 h.

Urine albumin-creatinine ratio

Random urine samples were collected, followed by determining the albumin concentration with a mouse albumin ELISA kit (Sangon Biotech, China) and creatinine with a creatinine assay kit (Nanjing Jiancheng, Jiangsu Province, China). The urine albumin-creatinine ratio was calculated by dividing the urine albumin concentration by the creatinine concentration.

Hematoxylin-eosin staining

Mouse kidneys were fixed in 4% neutral formaldehyde followed by paraffin embedding. The paraffin sections were rehydrated in a graded ethanol series and subjected to hematoxylin-eosin (HE) staining

(Beyotime, Shanghai, China) as previously described[33].

Immunohistochemistry

The sections underwent antigen retrieval in 0.01 M citric acid solution (pH 6.0) in a microwave oven for 10 min after deparaffinization and rehydration. To inhibit endogenous peroxidase, the slices were incubated with 5% H_2O_2 for 15 min. The sections were then further blocked for 30 min at room temperature with 5% bovine serum albumin (BSA), and then incubated overnight at 4 °C with anti-p-Smad3 antibody (C25A9; Cell Signaling Technology, Danvers, MA, United States). The slices were treated with secondary antibodies for 1 h at room temperature following PBS washing. Images were recorded with a light microscope (Eclipse 80i; Nikon, Japan).

TUNEL assay

TUNEL assay was used to evaluate podocyte apoptosis in the kidneys after ADR induction, as previously described[34]. After the mouse kidneys were fixed in 4% neutral formaldehyde followed by paraffin embedding, the paraffin sections were used for staining. Podocyte apoptosis was measured through the utilization of a One-step TUNEL In Situ Apoptosis Assay Kit (AF488; Green) (E-CK-A321; Elabscience, China). The images were captured with an orthotopic fluorescence microscope (DM4B; Leica, Germany).

Cell culture and treatment

Prof. San-Tao Ou (Department of Nephrology, Southwest Medical University) kindly donated the conditionally immortalized MPC5 cell line. The cells were grown at 33 °C in RPMI-1640 medium supplemented with 10 IU/mL recombinant interferon and 10% FBS. After the MPC5 cells were cultured at 37 °C for 14 d to induce differentiation, the differentiated cells were treated with different concentrations of ADR for 24 h. A Smad3 overexpressing MPC5 cell line was established with the Smad3 overexpression plasmid pcDNA3.1-Smad3 which was described previously[35].

Immunofluorescence

After treatment, the MPC5 cells or frozen sections were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 (in PBS), and blocked with 5% BSA for immunofluorescence. After that, the frozen sections or MPC5 cells were incubated with anti-podocin (BA0290; Boster, Wuhan, China), anti-Bax (AF0120; Affinity, United States), and anti-Bcl-2 (AF6139; Affinity) antibodies at 4 °C overnight. After washing with PBS, the frozen sections or MPC5 cells were incubated with Alexa Fluor 594 Donkey anti-mouse/rabbit secondary antibodies (Thermo Fisher Scientific, Waltham, MA, United States) for 1 h at room temperature. The nuclei were stained with 4¢,6-diamidino-2-phenylindole (Sangon Biotech). Images were captured with a fluorescence microscope (EVOS FL Auto, Thermo Fisher Scientific, United States).

Real-time quantitative polymerase chain reaction

TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to separate total RNA from cells or kidneys, and a Reverse Transcription Kit (Promega, Madison, WI, United States) was used to obtain cDNA. Using Master Mixture (TaKaRa, Dalian, China) and LightCycler 480 equipment (Roche, Germany), the podocin mRNA expression levels were assessed. The internal control used was GAPDH. Using $2^{-\Delta\Delta Ct}$ analysis, the relative expression of the target gene was standardized to GAPDH expression. The primer sequences used are shown in Supplementary Table 1.

Western blot analysis

Using RIPA lysis buffer (Beyotime), total proteins were extracted from kidneys or cells. The protein concentrations were determined with a BCA protein assay kit (Beyotime). Proteins were transferred onto polyvinylidene difluoride membranes after being separated by 12% SDS-PAGE. Then the membranes were incubated with anti-podocin (BA0290; Boster), anti-p-Smad3 (C25A9; Cell Signaling Technology), anti-Smad3 (C67H9; Cell Signaling Technology), anti-Bax (AF0120; Affinity), anti-Bcl-2 (AF6139; Affinity), and anti-GAPDH (AB0037; Abways, China) antibodies at 4°C overnight. The membranes were treated with the relevant secondary antibody at room temperature for 1 h after being rinsed with Tris-buffered saline with Tween (TBST). The protein bands were depicted with an enhanced ECL kit (Boster) and a chemiluminescence imaging system (ChemiScope 6200; Clinx, China). ImageJ software (NIH, Bethesda, MD, United States) was used to calculate the band gray intensity.

Apoptosis detection by flow cytometry

The cells were digested with trypsin-EDTA solution (C0201; Beyotime), collected in a centrifuge tube, and centrifuged for 5 min at 1800 rpm, and the supernatant was discarded. The cells were resuspended with 1 mL precooled PBS. According to the Annexin V-FITC/PI Apoptosis Detection kit's instructions (Vazyme, Nanjing, China), the prepared propyl iodide staining solution was added to the cells and incubated at 37 °C for 10 min without light. Red fluorescence was detected at an excitation wavelength of 488 mm and light scattering was detected with a BD FACSVerse (Becton, Dickinson).

Statistical analysis

The mean and standard deviation of the data are displayed. Using SPSS 21.0 software (IBM Corp., Chicago, IL, United States), one-way analysis of variance was used to compare the data. $P < 0.05$ was considered statistically significant.

RESULTS

MSCs^{CA} enhance the protective effect of MSCs on podocyte injury in ADR-induced FSGS

To investigate whether CA pretreatment enhances the protective effect of MSCs on podocyte injury in ADR-induced FSGS mice, we treated mice with MSCs, CA, or MSCs^{CA}. Due to MSCs, CA, and MSCs^{CA} being dissolved in DMEM, a DMEM group was separately designed as the solvent control group to exclude the protective effect of DMEM-containing nutrients on podocytes (Figure 1A). The identification of MSCs, the chemical formula of CA, and its appropriate concentration can be found in our previous research[26]. Eight weeks after ADR injection, increased urinary albumin excretion was detected in ADR-treated mice, and MSCs^{CA} reversed this more significantly than MSCs or CA alone. However, there was no difference between the DMEM group and the model group (Figure 1B). HE staining showed that glomerular atrophy and FSGS were prominent in the ADR and DMEM groups, but MSCs^{CA} treatment reversed this change and was superior to MSCs and CA treatment (Figure 1C). Immunostaining, real-time quantitative polymerase chain reaction (RT-PCR), and Western blot analysis showed that the expression of podocin, a podocyte-specific marker, was significantly reduced in the ADR and DMEM groups; however, MSCs^{CA} treatment best restored its expression (Figure 1D-G). The above evidence indicated that MSCs^{CA} treatment better protected podocytes from ADR injury in FSGS mice.

The capacity of MSCs to prevent apoptosis in ADR-induced FSGS is improved by CA pretreatment

To determine the effect of MSCs^{CA} on renal cell apoptosis, Western blot and TUNEL assay were performed. Expression of Bax protein as an apoptosis marker was significantly increased in the ADR and DMEM groups compared with the normal group, and their levels were reduced after MSC or CA treatment (Figure 2A and B). MSCs^{CA} reduced ADR-induced Bax protein expression more significantly than MSCs or CA. The changing trend in Bcl-2 protein expression was opposite to that of Bax in each group (Figure 2A and C). TUNEL assay showed an obvious increase in the brightness and range of green fluorescence in the ADR group, which was weakened by MSCs^{CA} treatment (Figure 2D). The above data indicated that MSCs^{CA} enhanced the antiapoptotic effect of MSCs on kidney cells of ADR-induced FSGS mice.

P-Smad3 is upregulated in podocytes of ADR-induced FSGS mice and reversed after MSCs^{CA} treatment

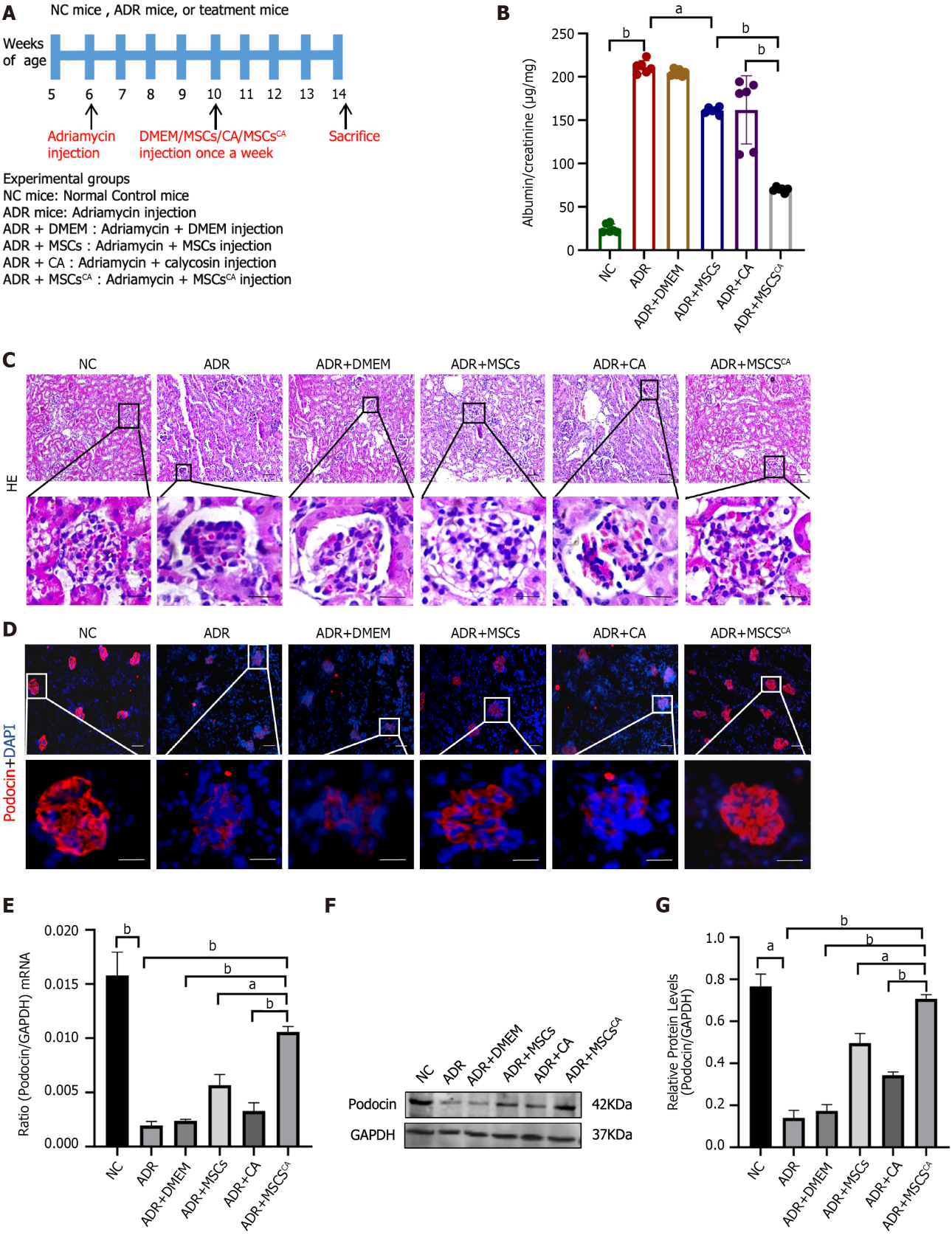
It has been reported that the Smad3 protein is involved in podocyte apoptosis[13], so we examined the effect of MSCs^{CA} on the expression of Smad3 and p-Smad3 proteins. As expected, we found by Western blot and immunohistochemistry that MSCs^{CA} treatment significantly reversed the upregulation of p-Smad3 in ADR-treated mouse renal podocytes, and the effect was superior to that of MSC and CA treatment (Figure 3). The above evidence suggested that p-Smad3 was involved in the ADR-induced injury of podocytes and the recovery after MSCs^{CA} treatment.

The capacity of MSCs to reduce the injury caused by ADR-stimulated MPC5 cells in vitro is improved by CA pretreatment

To further demonstrate the enhanced potential of MSCs^{CA} to protect podocytes from ADR injury, we cultured and treated MPC5 cells. Immunofluorescence staining, RT-PCR, and Western blot showed that 1.2 μM/mL ADR decreased the expression of podocin mRNA and protein, while the expression was significantly promoted by treatment with conditioned medium from MSCs, or CA. Importantly, conditioned medium from MSCs^{CA} further elevated the expression of podocin mRNA and protein compared with the ADR group (Figure 4). Therefore, MSCs^{CA} protected podocytes from ADR injury better than MSCs or CA alone.

CA pretreatment enhances the ability of MSCs to inhibit apoptosis in ADR-stimulated MPC5 cells

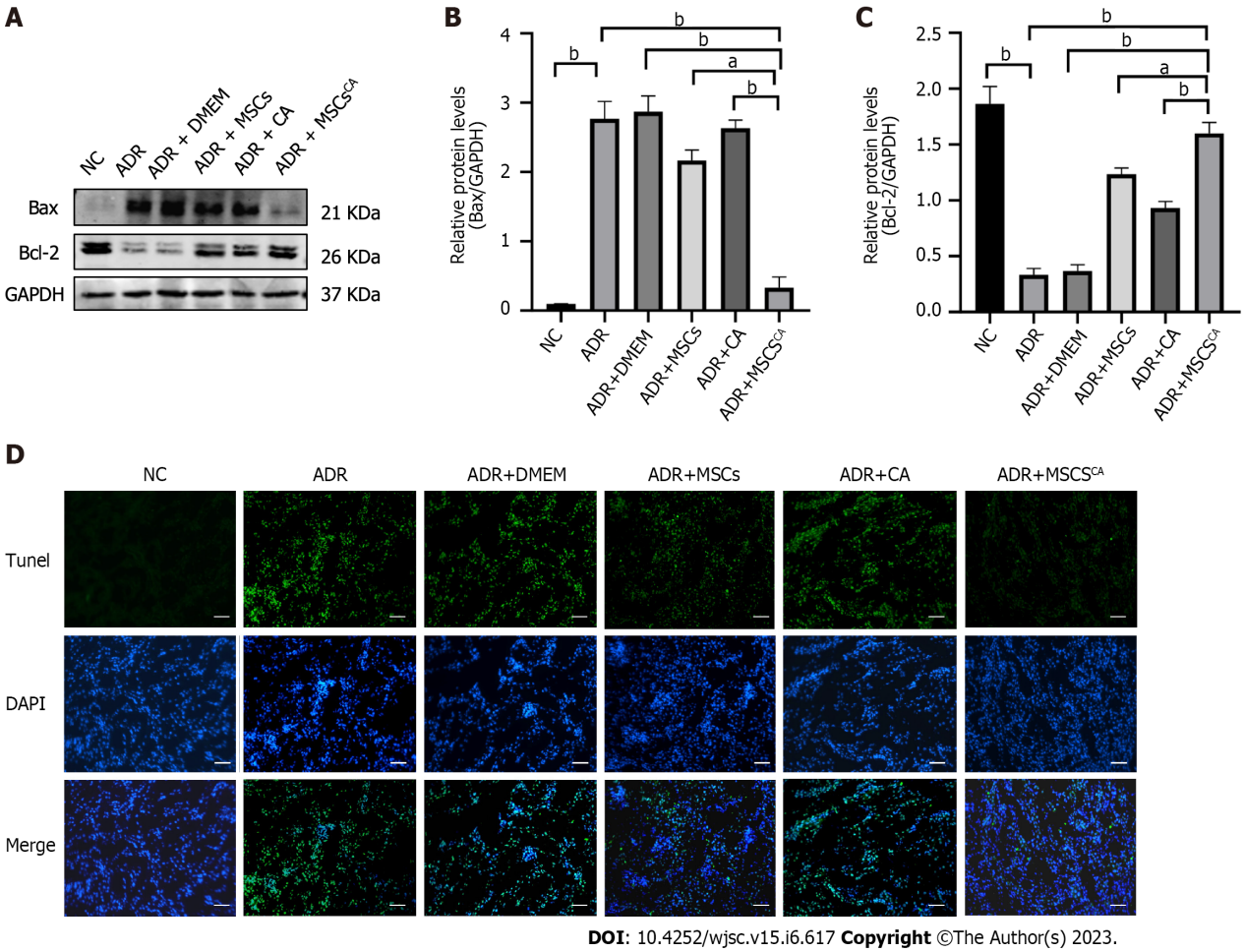
Immunofluorescence staining and Western blot demonstrated that the protein level of Bax was upregulated in MPC5 cells treated with ADR, but its expression was significantly inhibited by treatment with a conditioned medium from MSCs, or CA alone (Figure 5). The effects of the conditioned medium from MSCs^{CA} were more pronounced. The trend for Bcl-2 protein expression was the opposite. These findings revealed that Ca-pretreated MSCs enhanced the inhibitory effect of MSCs on podocyte apoptosis.



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Figure 1 Mesenchymal stem cells pretreated with calycosin enhance the protective effect of mesenchymal stem cells on podocyte injury in adriamycin-induced focal segmental glomerulosclerosis mice. A: Mice received adriamycin injections through the tail vein at week 6, were injected with Dulbecco's modified eagle medium, mesenchymal stem cells (MSCs), calycosin (CA), and MSCs pretreated with CA (MSCs^{CA}) at week 10, respectively, and were sacrificed at week 14; B: Levels of albumin/creatinine ratio in urine (n = 6), $^{a}P < 0.05$, $^{b}P < 0.001$; C: Pathological changes in the kidneys of mice examined by hematoxylin-eosin staining. Typical glomeruli are indicated by black boxes and enlarged to the next row. Bar = 50 μm; D: Changes in the kidneys of mice examined by

podocin immunofluorescence staining. Glomeruli are indicated by white boxes and enlarged to the next row. Bar = 50 μm; E: Relative (podocin/GAPDH) mRNA expression analyzed by real-time quantitative polymerase chain reaction. Data are expressed as the mean ± SD (n = 6). $^{a}P < 0.05$, $^{b}P < 0.001$; F and G: Relative protein levels (podocin/GAPDH) detected by Western blot. Data are expressed as the mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.001$. NC: Normal control; ADR: Adriamycin; DMEM: Dulbecco's modified eagle medium; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

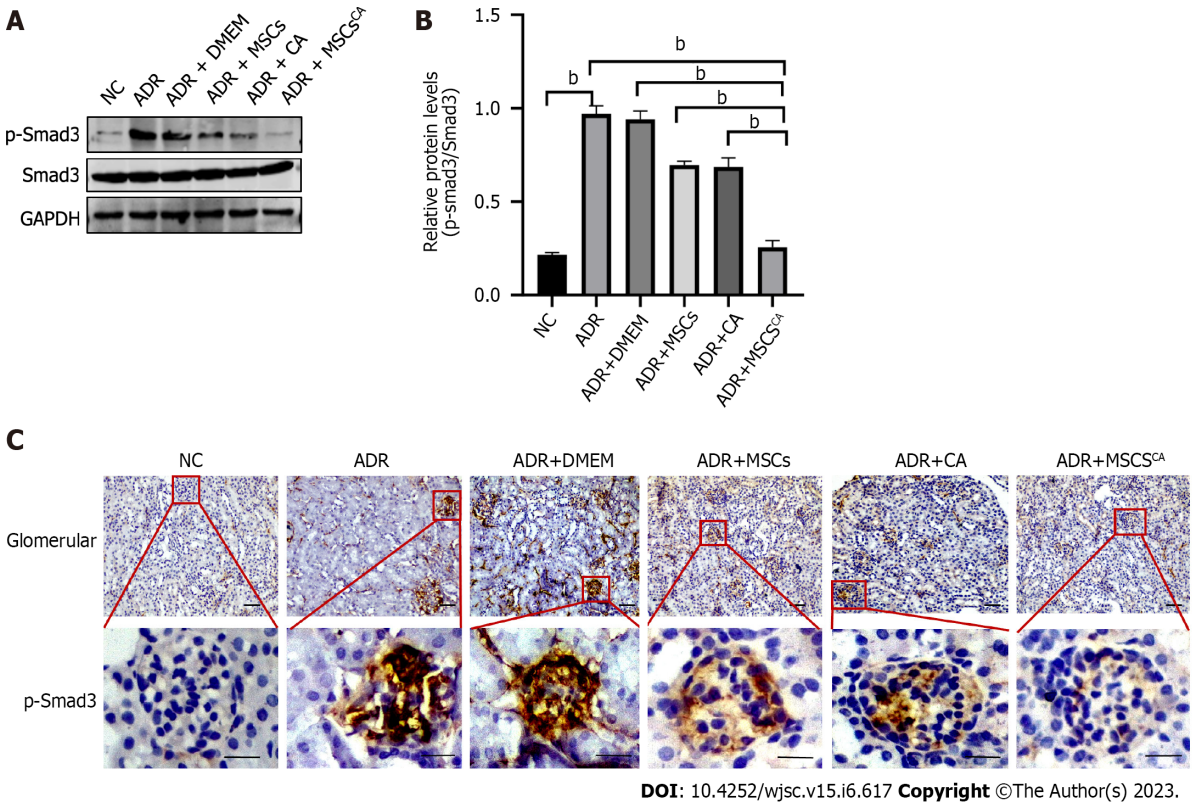


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Figure 2 Calycosin pretreatment enhances the ability of mesenchymal stem cells to inhibit apoptosis in adriamycin-induced focal segmental glomerulosclerosis mice. A-C: Protein expression levels of Bax and Bcl-2 in the kidneys measured by Western blot and normalized to control. Data are expressed as the mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.001$; D: Apoptosis in each group as determined by TUNEL assay. Bar = 50 μm. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

MSCs^{CA} improve ADR-induced podocyte apoptosis by targeting p-Smad3 expression

As described previously, p-Smad3 is involved in ADR-induced FSGS mice. Further experiments were conducted to explore whether MSCs^{CA} inhibit podocyte apoptosis by targeting p-Smad3. Expression of p-Smad3 in MPC5 cells was markedly elevated by ADR stimulation and subsequently significantly downregulated by MSCs^{CA} treatment. The upregulated expression of p-Smad3 was also reversed by MSCs or CA, but to a lesser extent (Figure 6A and B). When Smad3 accompanied by p-Smad3 in MPC5 cells was overexpressed, Bax protein expression was upregulated but Bcl-2 protein expression was downregulated. Meanwhile, MSCs^{CA} treatment no longer showed a protective effect against ADR-induced podocyte apoptosis compared with the group without Smad3 overexpression (Figure 6C-G). Flow cytometry was used to detect apoptosis, and it was found that the apoptosis rate of MPC5 cells was significantly increased after ADR induction compared with the normal group, and MSCs^{CA} reversed this increase. However, after overexpression of Smad3, the apoptosis rate was increased compared with the normal group and the model group regardless of whether MSCs^{CA} were administered. This means that treatment with MSCs^{CA} did not improve the apoptosis of podocytes with Smad3 overexpression (Figure 6H-L). The graphical abstract (created in BioRender.com) is shown in Figure 6M. The above evidence suggested that MSCs^{CA} improved podocyte apoptosis through targeted inhibition of p-Smad3.



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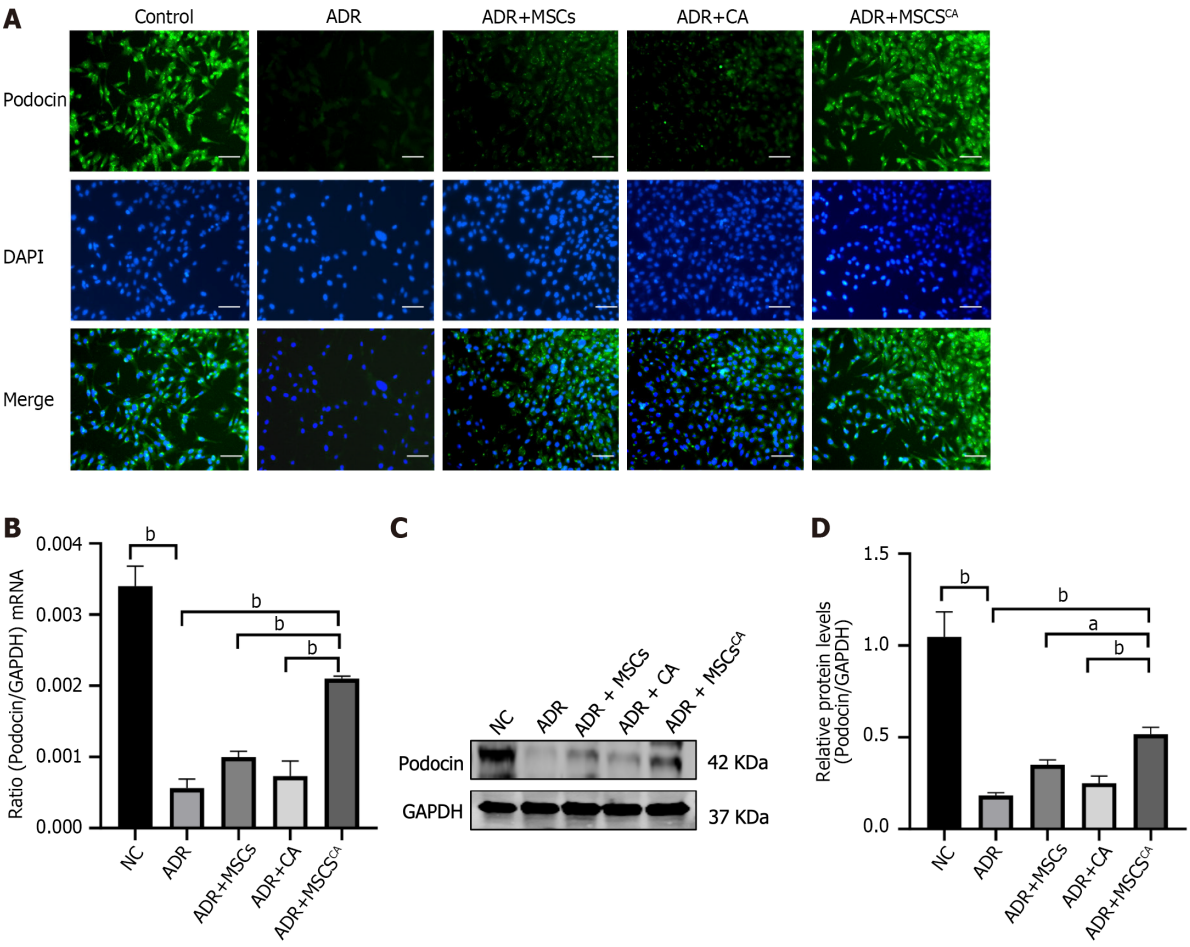
Figure 3 P-Smad3 is upregulated in podocytes of adriamycin-induced focal segmental glomerulosclerosis mice and reversed after treatment with mesenchymal stem cells pretreated with calycosin. A and B: Protein expression levels of p-Smad3 and Smad3 detected using Western blot and normalized to control. Data are expressed as the mean ± SD (n = 3). $^{b}P < 0.001$; C: Immunohistochemistry staining for p-Smad3 in mouse glomeruli, which are indicated by red boxes and enlarged to the next row. Bar = 50 μm. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

DISCUSSION

Increasing evidence has shown that MSCs and derived extracellular vesicles can ameliorate renal deterioration in CKD[36,37]. However, because of a hostile environment with several stresses such as inflammation, high acidity, hypoxia, and depleted energy reserve, few MSCs survive *in vivo* after intravenous or direct local injection[38-40]. The question of whether preconditioning BMSCs can shield them from the damaging environment at the injury site and enhance their functionality has drawn more attention in research. These pretreatments involve the application of supportive materials, cytokines, and natural or synthetic chemicals[41-44]. Researchers have been investigating the preconditioning of MSCs using Chinese herbal medicine or its primary monomer components. There is evidence that resveratrol-pretreated adipose-derived stem cells show increased regenerative capacity in a rat model of diabetes-induced cardiomyopathy[45]. Further research has shown that preconditioning MSCs obtained from umbilical cords with the active ingredient of a Chinese herb, triptolide, primed MSCs to be activated and inhibited the immune response before being delivered[46]. Previous results from our group have also shown that CA-pretreated BMSCs show enhanced antifibrotic activity in UUO mice and inhibit tubular epithelial cell necrosis[29]. Therefore, we investigated whether MSCs^{CA} enhance podocyte protection. Similar to previous studies, MSCs^{CA} protected podocytes from ADR-induced apoptosis, both *in vivo* and *in vitro*, which means that they may be a potential therapy for FSGS.

CA is the top ingredient in *Astragalus*, which is one of the most widely used herbs in Chinese medicine to treat kidney disease[47-49]. The effectiveness of CA in CKD has been confirmed in recent years[50-52]. However, whether its combination with MSCs can enhance their efficacy in treating CKD remains to be seen. It has been shown that human MSCs are stimulated to enhance osteogenesis and mineralization by CA-7-O-glucoside obtained from *Astragalus membranaceus*[53]. This result gave us confidence and we also identified the advantages and potential of MSCs^{CA} in the treatment of FSGS, which extends the application of CA and MSCs in FSGS.

The main pathological manifestations of FSGS are podocyte injury and the therapeutic options for FSGS are limited, requiring further research and exploration. Therefore, we explored the mechanism of podocyte injury. Podocyte apoptosis is the main type of podocyte injury, which includes podocyte dedifferentiation, autophagy, and epithelial-mesenchymal transformation[54]. Podocyte apoptosis is caused by many factors, including drugs, infection, and immune disorders[55-57]. ADR is one of the



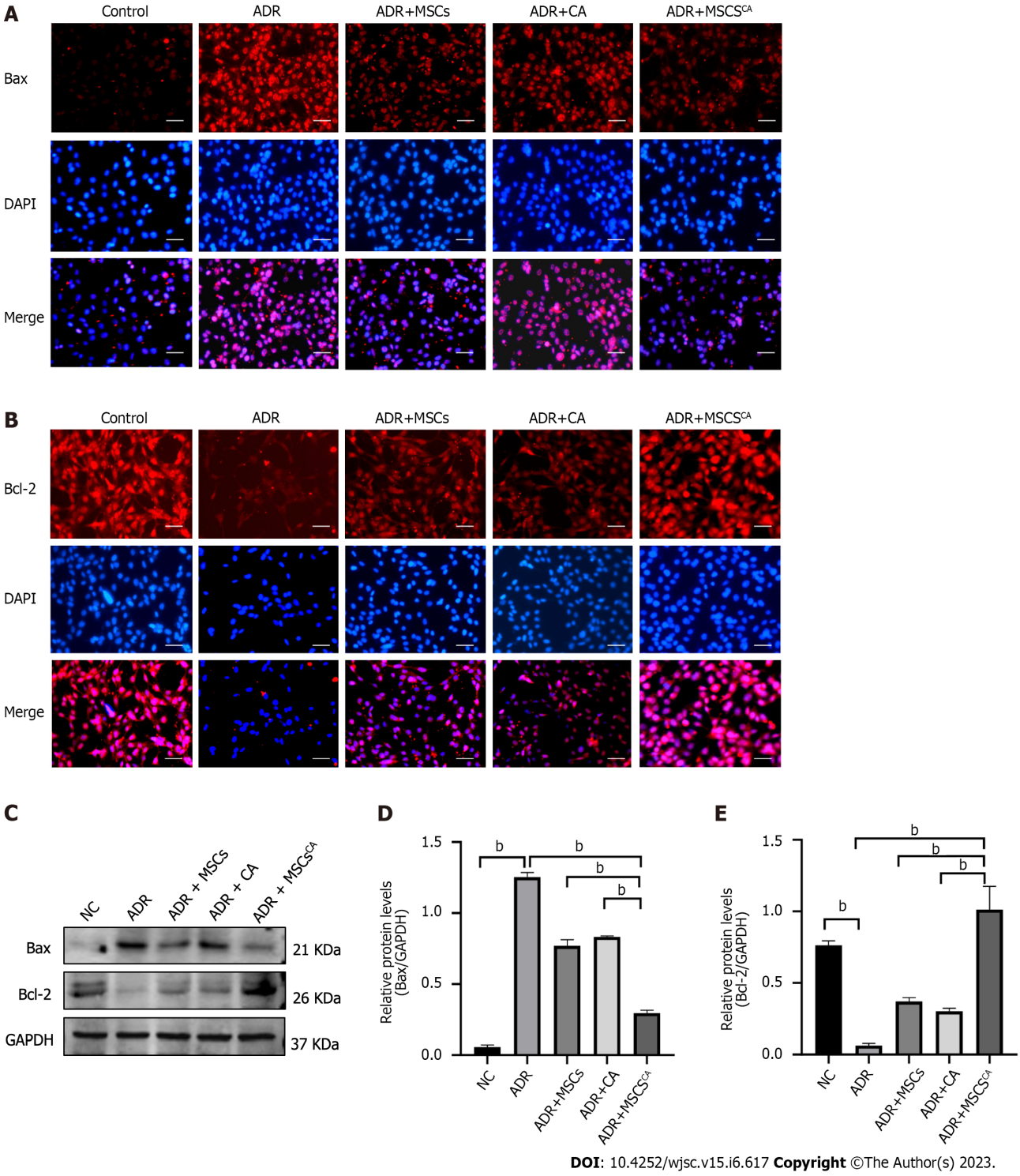
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Figure 4 Calycosin pretreatment enhances the ability of mesenchymal stem cells to ameliorate the injury of adriamycin-stimulated mouse podocyte cells *in vitro*. A: Podocin expression in each group as determined by immunofluorescence staining. Bar = 50 μm; B: Analysis of relative (podocin/GAPDH) mRNA expression by real-time quantitative polymerase chain reaction. Data are expressed as the mean ± SD (n = 3). $^{b}P < 0.001$; C and D: Protein expression levels of podocin detected by Western blot and normalized to control. Data are expressed as the mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.001$. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

drugs that causes podocyte apoptosis due to its pharmacological action and distribution[58]. However, how to protect podocytes from ADR needs further research to find more effective targeted drugs.

Smad3 is involved in apoptosis, and podocytes are no exception[59,60]. Activation of Smad3 and its related pathway proteins induces podocyte apoptosis[14,61]. The canonical Smad pathway is a crucial regulatory route in the etiology of renal inflammation and fibrosis, according to earlier research. Major receptor-associated Smads include Smad2 and Smad3. Mad-homology 2 domain is located at the C-terminus of Smad3, which has unique phosphorylation sites and sequences triggered by transforming growth factor (TGF)-β1. The binding of phosphorylated Smad3 to TGF-β1 signaling receptors promotes fibrosis[62]. Our study showed that MSCs^{CA} significantly downregulated the expression of p-Smad3 in the kidneys of ADR-induced FSGS mice and ADR-induced MPC5 cells. Subsequently, we overexpressed Smad3 in MPC5 cells and confirmed that MSCs^{CA} targeted inhibition of p-Smad3 to improve podocyte apoptosis using rescue experiments. This provides a new possible mechanism and target for preventing podocyte apoptosis by MSCs^{CA}.

There were some limitations to this study. Although we have revealed that MSCs^{CA} improve podocytes apoptosis by inhibiting Smad3 signaling, this study still has certain limitations and the underlying mechanism deserves further exploration. First, how does MSCs^{CA} intervene in the Smad3 signal, directly or indirectly? We speculate that CA may activate the anti-apoptotic activity of MSCs or affect the differentiation, mobilization, and homing of BMSCs as well as the abundance of beneficial exosomes, but the main mechanism and responsible factors are still unknown. Second, it is still unclear which molecules in podocytes respond to the activity of MSCs and what are their potential relationship with Smad3. Understanding these mechanisms is conducive in expanding the application of MSCs^{CA}, and we will answer each question one by one in future research.

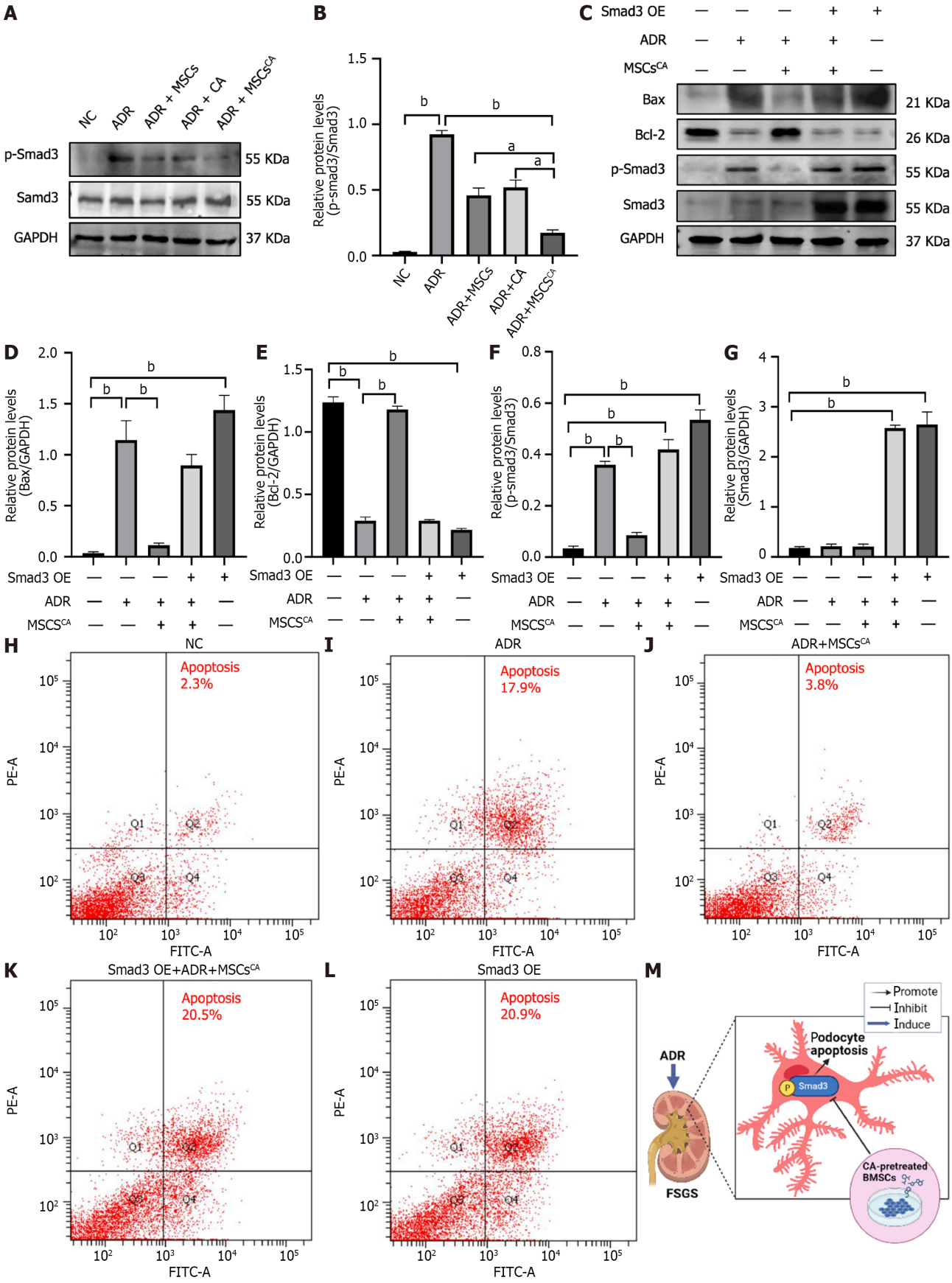


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Figure 5 Calycosin pretreatment enhances the ability of mesenchymal stem cells to inhibit apoptosis in adriamycin-stimulated mouse podocyte cells. A and B: Expression of Bax and Bcl-2 in each group as determined by immunofluorescence staining. Bar = 50 μm; C-E: Protein expression levels of Bax and Bcl-2 detected by Western blot and normalized to control. Data are expressed as the mean ± SD (n = 3). $^{b}P < 0.001$. NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin.

CONCLUSION

This study showed that MSCs^{CA} improve ADR-induced podocyte apoptosis by targeting Smad3 inhibition, and are superior to MSCs or CA. Thus, our study provides a new perspective on the synergistic application of MSCs and a new theory for the mechanism of improvement of podocyte apoptosis.



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Figure 6 Calycosin-pretreated mesenchymal stem cells improve adriamycin-induced podocyte apoptosis by targeting p-Smad3 expression. A and B: Protein expression levels of p-Smad3 and Smad3 in mouse podocyte cells (MPC5) detected by Western blot and normalized to control. Data are expressed as the mean ± SD (n = 3). $^{a}P < 0.05$, $^{b}P < 0.001$; C-G: Protein expression levels of Bax, Bcl-2, p-Smad3, and Smad3 in Smad3-overexpressing MPC5

cells detected by Western blot and normalized to control. Data are expressed as the mean ± SD (n = 3). $^{b}P < 0.001$; H-L: Cell apoptosis detected by flow cytometry; M: Graphical abstract (created in BioRender.com). NC: Normal control; ADR: Adriamycin; MSCs: Mesenchymal stem cells; CA: Calycosin; MSCs^{CA}: Mesenchymal stem cells pretreated with calycosin; FSGS: Focal segmental glomerulosclerosis.

ARTICLE HIGHLIGHTS

Research background

Focal segmental glomerulosclerosis (FSGS) has become a global public health problem due to its high incidence and lack of treatment. Prevention of podocyte apoptosis is essential in the treatment of FSGS. Bone marrow-derived mesenchymal stem cells (BMSCs) have been found to protect podocytes, but have some limitations, such as low survival rate *in vivo* and poor homing function. In our previous study, calycosin (CA)-pretreated BMSCs enhanced the antifibrotic activity in kidneys compared with BMSCs. Therefore, CA-pretreated MSCs are expected to be a new method to protect podocytes in the treatment of FSGS.

Research motivation

Although MSCs have been confirmed to improve podocyte apoptosis in mice, their availability and effectiveness *in vivo* are limited. Currently, there is still a lack of effective therapeutic methods for FSGS, and their mechanism of action is not clear.

Research objectives

To evaluate the therapeutic effect of CA-pretreated BMSCs in a mouse model of adriamycin (ADR)-induced FSGS *in vivo* and MPC5 cells *in vivo*.

Research methods

MSCs^{CA} were compared with MSCs or CA to observe their inhibitory effects on podocyte apoptosis in mice with ADR-induced FSGS *in vivo* and ADR-treated MPC5 cells *in vitro*, to explore the possible mechanism by which MSCs^{CA} improves podocyte apoptosis.

Research results

In vivo results showed that MSCs^{CA} reduced podocyte apoptosis, improved podocyte injury and depletion, alleviated glomerulosclerosis and albuminuria, and downregulated p-Smad3 expression in ADR-induced FSGS mice, which were superior to MSCs and CA. Similar to *in vivo* studies, MSCs^{CA} alleviated ADR-induced apoptosis of MPC5 cells more significantly than MSCs and CA. Through rescue experiments, we found that the potential of MSCs^{CA} to protect podocytes may be realized through targeted inhibition of p-Smad3 expression.

Research conclusions

MSCs^{CA} improve ADR-induced podocyte apoptosis by targeting Smad3 inhibition, which are superior to MSCs or CA.

Research perspectives

Our findings provide a new potential strategy for the treatment of FSGS.

FOOTNOTES

Author contributions: Wang L and Fahsai K contributed equally to this article as co-corresponding authors; Wang L and Fahsai K designed the study and supervised the laboratory experiments; Hu QD conducted the experiments and drafted the manuscript; Tan RZ assisted with the experiments; Zou YX and Li JC collected the samples; Fan JM and Wang L contributed new reagents and analytic tools; and all authors read and approved the final manuscript.

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SYSTEMATIC REVIEWS

Current overview of induced pluripotent stem cell-based blood-brain barrier-on-a-chip

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Abstract

BACKGROUND

Induced pluripotent stem cells (iPSCs) show great ability to differentiate into any tissue, making them attractive candidates for pathophysiological investigations. The rise of organ-on-a-chip technology in the past century has introduced a novel way to make *in vitro* cell cultures that more closely resemble their *in vivo* environments, both structural and functionally. The literature still lacks consensus on the best conditions to mimic the blood-brain barrier (BBB) for drug screening and other personalized therapies. The development of models based on BBB-on-a-chip using iPSCs is promising and is a potential alternative to the use of animals in research.

AIM

To analyze the literature for BBB models on-a-chip involving iPSCs, describe the microdevices, the BBB *in vitro* construction, and applications.

METHODS

We searched for original articles indexed in PubMed and Scopus that used iPSCs to mimic the BBB and its microenvironment in microfluidic devices. Thirty articles were identified, wherein only 14 articles were finally selected according to the inclusion and exclusion criteria. Data compiled from the selected articles were organized into four topics: (1) Microfluidic devices design and fabrication; (2) characteristics of the iPSCs used in the BBB model and their differentiation conditions; (3) BBB-on-a-chip reconstruction process; and (4) applications of BBB

microfluidic three-dimensional models using iPSCs.

RESULTS

This study showed that BBB models with iPSCs in microdevices are quite novel in scientific research. Important technological advances in this area regarding the use of commercial BBB-on-a-chip were identified in the most recent articles by different research groups. Conventional polydimethylsiloxane was the most used material to fabricate in-house chips (57%), whereas few studies (14.3%) adopted polymethylmethacrylate. Half the models were constructed using a porous membrane made of diverse materials to separate the channels. iPSC sources were divergent among the studies, but the main line used was IMR90-C4 from human fetal lung fibroblast (41.2%). The cells were differentiated through diverse and complex processes either to endothelial or neural cells, wherein only one study promoted differentiation inside the chip. The construction process of the BBB-on-a-chip involved previous coating mostly with fibronectin/collagen IV (39.3%), followed by cell seeding in single cultures (36%) or co-cultures (64%) under controlled conditions, aimed at developing an *in vitro* BBB that mimics the human BBB for future applications.

CONCLUSION

This review evidenced technological advances in the construction of BBB models using iPSCs. Nonetheless, a definitive BBB-on-a-chip has not yet been achieved, hindering the applicability of the models.

Key Words: Induced pluripotent stem cells; Cell differentiation; Blood-brain barrier; Neurovascular unit; Organ-on-a-chip; Microfluidic device

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Core Tip: This systematic review provided a current perspective on the applicability of induced pluripotent stem cells within blood-brain barrier (BBB)-on-a-chip with high technology advances in commercial chips and promotion of an efficient human neurovascular unit, able to screen for drugs, mimic brain dysfunctions, such as stroke and Huntington's disease, and suitable for future personalized therapeutic approaches. However, the composition and construction of the BBB models lack consensus in the literature.

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INTRODUCTION

Induced pluripotent stem cells (iPSCs) have been largely studied for their numerous applications in drug screening, toxicological studies, cell therapy, and disease modeling[1]. Their relevance may be justified by their resemblance to embryonic stem cells, given their ability to differentiate into any one of the three germ layers: ectoderm, mesoderm, and endoderm[1,2], making them attractive candidates for cell therapy-based regenerative medicine[3].

Different from primary cells, iPSCs are easily attainable and able to mature into almost any desired cell type. In general, they can be formed by reprogramming cells obtained from a tissue biopsy or from more accessible sources such as peripheral blood, renal epithelial cells, or dental pulp[4,5]. These characteristics, as opposed to primary cells, have promoted more reliable models for complex human structures such as the blood-brain barrier (BBB)[6].

The BBB secures the brain with a homeostatic environment, controlling the interaction, communication, and molecular and ion exchange between the central nervous system (CNS) and the peripheral blood[7]. The neurovascular unit (NVU) is generally composed of brain microvascular endothelial cells (BMECs), astrocytes (ACs), and pericytes (PCs), being directly involved in regulating CNS blood flow, and consequently, neuronal activity[8].

Recreating the BBB microenvironment *in vitro* allows for the investigation of barrier dysfunction in neurodegenerative diseases, and drug delivery to the CNS, in addition to the evaluation and screening

of the permeability of substances across the BBB[9,10]. In this regard, classic BBB *in vitro* models have traditionally utilized Transwell® technology, which enables the construction of multicellular models with paracrine interactions between the co-cultures of ECs and ACs or PCs. Notwithstanding its usefulness, Transwell® technology lacks a key element for the *in vivo*-like functioning of ECs: Laminar flow[6,9]. Moreover, many of the existing BBB models employ primary BMECs, immortalized BMECs, or human umbilical vein ECs (HUVECs) as EC-layer cultures. Even though they can constitute fair BBB constructs, access to primary cell sources can be difficult for ethical purposes and immortalized lines often fail to mimic the actual BBB function found *in vivo*[6,9].

In the building of *in vitro* BBB mimics, iPSC-derived BMECs (iBMECs) present improved barrier properties compared to primary BMECs, allowing for the modeling of genetic diseases and personalized therapy approaches[2]. These properties become more apparent when iBMECs are cultured in a complex environment. The traditional *in vitro* culture model, despite being simple and reproducible, fails to mimic cell-cell and cell-extracellular matrix (ECM) interactions[11]. Animal models, which present ideal barrier permeability, transport mechanisms, and morphological characteristics, present low reliability given that 80% of clinical trials fail in drug-delivery tests[12]. These limitations may be overcome with the development of organs-on-a-chip that simulate complex morphology in a three-dimensional (3D) culture[13].

The construction and applications of BBB-on-a-chip using microfluidic devices provide a lower-cost alternative with an impact on ethical issues, allowing for the reduced use of animals, reagents, and sample volume, and also permitting real-time microscopic analysis, with shortened reaction and analysis times, high throughput, automation, and portability[14,15]. Furthermore, from a morphological point of view, the microdevices assure a compatible reproduction of the BBB model due to the presence of laminar flow and shear stress, which convey better maintenance of barrier functions, homeostasis, and transport. This model has already been applied to several studies of neurological disorders such as Alzheimer's disease, stroke, infectious diseases, and brain cancer[15]. Despite being the best alternative for simulation and in-depth study of the BBB, studies involving microfluidic devices lack clear protocols for large-scale manufacturing and adequate liquid perfusion, making the process imprecise and hard to operate, in addition to the difficulty in collecting channel material for detailed assessments[16].

Regarding the construction of BBB *in vitro* models on-a-chip using iPSCs, the literature still lacks a consensus on the best conditions to mimic BBB dysfunction upon CNS disorders and to screen for drugs and other therapeutic approaches. In view of these gaps, this systematic review searched the current literature for examples that meet the abovementioned criteria, that is, iPSCs, BBB models, and microfluidic devices.

MATERIALS AND METHODS

Search strategy

The articles used in this review were identified through searches performed in the PubMed and Scopus databases following the PRISMA guidelines[17]. The following selected criteria of interest, keyword strings ((Induced Pluripotent Stem Cell) AND (Microfluidic Device) AND (Blood-Brain Barrier)) and Boolean operators (DecS/MeSH) were used:

PubMed: (((((((“organs-on-chips”[Title/Abstract]) OR (“organs-on-a-chip”[Title/Abstract])) OR (“microfluidic device”[Title/Abstract])) OR (“lab-on-chips”[Title/Abstract])) OR (microfluidics[Title/Abstract])) AND ((((“blood-brain barrier”[Title/Abstract]) OR (“Brain Blood Barrier”[Title/Abstract])) OR (“Blood Brain Barrier”[Title/Abstract])))) AND ((((“Induced Pluripotent Stem Cell”[Title/Abstract]) OR (“Induced pluripotent stem cells”[Title/Abstract])) OR (iPSC[Title/Abstract])))) NOT (review[Publication Type]);

Scopus: ((TITLE-ABS-KEY (ipsc) OR TITLE-ABS-KEY (“induced pluripotent stem cell”) OR TITLE-ABS-KEY (“induced pluripotent stem cells”))) AND (((TITLE-ABS-KEY (“organs-on-chips”) OR TITLE-ABS-KEY (“organs-on-a-chip”) OR TITLE-ABS-KEY (“microfluidic device”) OR TITLE-ABS-KEY (“lab-on-chips”) OR TITLE-ABS-KEY (microfluidics) OR TITLE-ABS-KEY (“lab-on-chip”))) AND ((TITLE-ABS-KEY (“blood brain barrier”) OR TITLE-ABS-KEY (“brain blood barrier”) OR TITLE-ABS-KEY (“blood-brain barrier”)))) AND (LIMIT-TO (LANGUAGE, “English”)) AND (LIMIT-TO (DOCTYPE, “ar”)).

Inclusion and exclusion criteria

Only original full-text articles written in English have been included, without publication year limit, containing: (1) iPSCs; (2) microfluidic devices; and (3) simulation of the BBB by specialized cells. From the perspective of the Problem, Intervention, Comparison, and Outcome criterion, the addressed Problem was unclear literature on the best conditions to mimic the BBB using microfluidic device technology (BBB-on-a-chip) and IPSCs; the Intervention was to analyze the conditions for BBB-on-a-chip manufacture regarding the design and material for an adequate construction of the NVU with a functional environment for testing; the Comparison was related to conventional analyses; and the Outcome was a microdevice that mimics the BBB applied in neurological conditions or not. The

exclusion of articles followed the following criteria: (1) Book chapters; (2) reviews; (3) duplicate articles in the databases; and (4) a study that did not report the use of iPSCs in the BBB-on-a-chip.

Data extraction

The selected articles under evaluation were analyzed under four topics, which were represented in three tables and one figure that addressed the following characteristics: (1) Microfluidic devices design and fabrication; (2) characteristics of the iPSCs used in the BBB models and their cultivation conditions; (3) iPSC differentiation process into iBMECs for reconstruction of the BBB-on-a-chip model; and (4) applications of BBB microfluidic 3D models using iPSCs.

Data compilation and review

In this systematic review, the preselection of titles was performed by authors Alves ADH and Gamarra LF from the defined search strategies. The ten authors (Alves ADH, Nucci MP, Ennes do Valle NM, Missina JM, Rego GNA, Mamani JB, Dias OFM, Garrigós MM, de Oliveira FA, Gamarra LF), in pairs, independently, and randomly reviewed and analyzed the eligibility of the articles according to the selection criteria mentioned above. In case of discrepancy in study selection between two authors, the criteria were discussed with a third reviewer and resolved.

Ennes do Valle NM and Mamani JB searched for the fabrication and characteristics of microfluidic devices; Alves ADH, Nucci MP, Ennes do Valle NM, Missina JM, and Rego GNA searched for differentiation and characterization protocols for iPSCs; Alves ADH, Nucci MP, Ennes do Valle NM, Missina JM, and Gamarra LF searched for the insertion and manipulation of cells in the microfluidic device; de Oliveira FA, Mamani JB, Dias OFM, and Garrigós MM searched for the objectives and applications of the selected studies. Analyses of data extracted from tables and flowcharts were performed by full peer consensus, respecting the above distribution. In this review, all authors wrote the entire text.

Risk of bias assessment

The selection of articles was performed in pairs and a third independent author decided if the articles should be included. The data selected in the tables were divided by the authors into the groups already described above, and data verification was carried out by the following group. The final inclusion of studies into the systematic review was by agreement of all reviewers.

Data analysis

The variables grouped in the tables and charts were distributed in percentages or range of distribution and used to characterize and illustrate the most frequently used results in this review.

RESULTS

Overview of the reviewed literature

A search was performed based on the abovementioned selection strategy and keywords, resulting in 30 original articles in English: 11 from PubMed and 19 from Scopus. Following the exclusion criteria, 5 of the 11 articles from PubMed were excluded: 4 under the "review article" criterion and 1 under the "book chapter" criterion. Regarding the studies found in Scopus, 10 were manuscripts duplicated by the other database. After the eligibility analysis, only two articles were withdrawn: One for not having reported a study using iPSCs, and the other for not involving BBB functionality. Finally, this systematic review included 14 articles[18-31] that met all selection and inclusion criteria established by the authors (Figure 1).

Microfluidic device design and fabrication

The main theme of this review has been studied in current years (2017-2022), as shown in Table 1. The latest studies were performed in commercial chips[18-21], whereas older studies were performed using chips manufactured in-house[22-31]. In general, the studies with commercial chips did not detail the type of polymer[18-20] or the technology or mold used[18-21]. Only one of them specified the use of polydimethylsiloxane (PDMS), a well-known polymer for manufacturing microfluidic chips[21]. The majority of studies that manufactured the chip in-house used PDMS (57%)[22,25-29,31], but two studies adopted the less than usual materials polymethylmethacrylate (PMMA)[23] and Objet VeroClear® photopolymer[30]. Most of the chips fabricated in-house were molded by soft lithography[24-29,31], except the study by Choi *et al*[23], which employed computer numerical control; and the study by Wang *et al*[30], which used a 3D object printer. Details about mold material and fabrication were seldom explored in soft lithography applications; however, the mainly reported material was SU-8 made by photolithography[25-27].

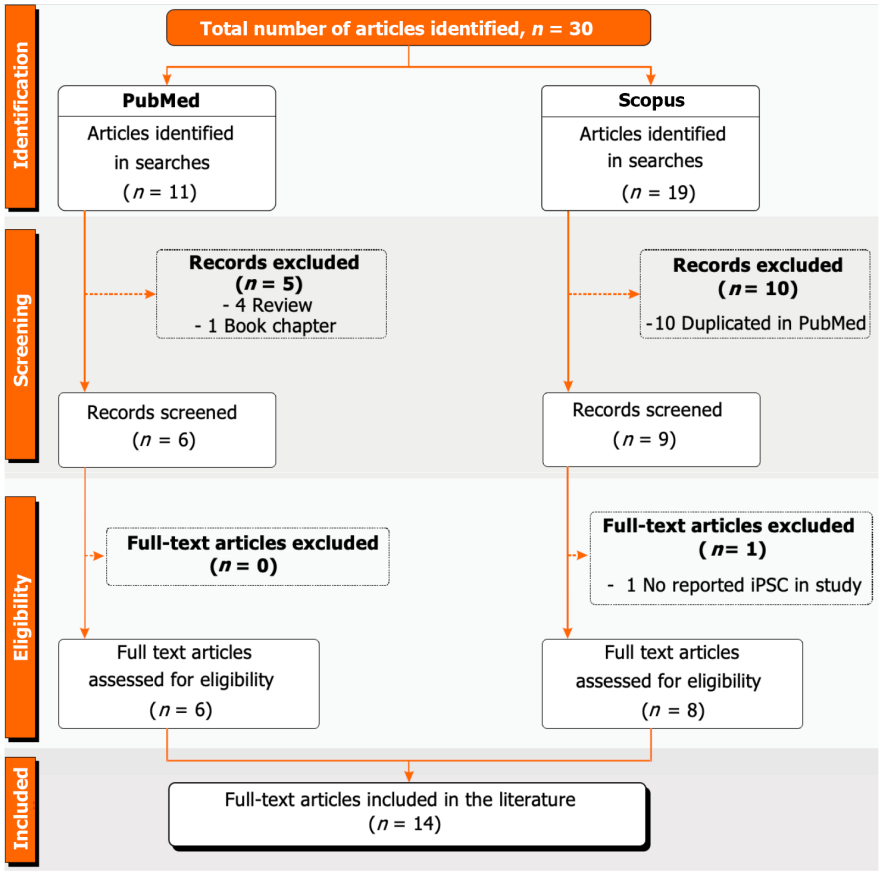
Half of the articles evaluated displayed membrane-free microfluidic chips[18-20,24,25,29,31], as shown in Table 1. The remaining studies reported the presence of a membrane dividing the channels within the chip: Three (21%) made of PDMS[21,26,27], one made of polyethylene terephthalate (PE)[28],

Table 1 Microfluidic device design and fabrication

Ref.	Manufacturing					Characteristics of microdevices
	Fabrication	Main material of device	Technology used	Mold details	Membranes	Dimensions as width × height
Kurosawa *et al* [18], 2022	MIMETAS® (OrganoPlate® 3-lane plate)	Unspecified polymer	NR	NR	Membrane-free	Top and bottom channels: 320 μm × 220 μm. Gel channels: 360 μm × 220 μm. PhaseGuides®: 100 μm × 55 μm
Fengler *et al* [19], 2022	MIMETAS® (OrganoPlate® 3-lane plate)	Unspecified polymer	NR	NR	Membrane-free	Top and bottom channels: 320 μm × 220 μm. Gel channels: 360 μm × 220 μm. PhaseGuides®: 100 μm × 55 μm (width × height)
Wevers *et al* [20], 2021	MIMETAS® (OrganoPlate® 3-lane plate)	Unspecified polymer	NR	NR	Membrane-free	Top and bottom channels: 320 μm × 220 μm. Gel channels: 360 μm × 220 μm. PhaseGuides®: 100 μm × 55 μm
Noorani *et al* [21], 2021	Emulate® (brain on-a-chip)	PDMS	NR	NR	PDMS membrane	Brain channel: 1 mm × 1 mm. Blood channel: 1 mm × 0.2 mm
Middelkamp *et al*[22], 2021	In house	PDMS	Soft lithography	Material: PMMA. Fabrication: Microm-milling	PETE membrane (5 μm thick)	Straight bottom channel: 500 μm × 500 μm. Open-top compartment: 500 μm × 1500 μm
Choi *et al*[23], 2021	In house	PMMA	CNC	NA	PETE membrane	HUVEC microchannels: 800 μm × 200 μm. iBMECs microchannels: 800 μm × 500 μm
Motallebnejad *et al*[24], 2020	In house	NR	Soft lithography	NR	Membrane-free	800 μm × 100 μm
Lee *et al*[25], 2020	In house	PDMS	Soft lithography	Material: SU-8. Fabrication: Photolithography	Membrane-free	Fluidic channel: 1340 μm × 150 μm. Main channel: 2200 μm × 150 μm
Jagadeesan *et al* [26], 2020	In house	PDMS	Soft lithography	Material: SU-8. Fabrication: Photolithography	PDMS membrane (50 μm thick)	Top microchannel: 1 mm × 1 mm. Bottom microchannel: 1 mm × 0.2 mm
Vatine *et al*[27], 2019	In house	PDMS	Soft lithography	Material: SU-8. Fabrication: Photolithography	PDMS membrane (50 μm thick)	Top microchannel: 1 mm × 1 mm. Bottom microchannel: 1 mm × 0.2 mm
Park *et al*[28], 2019	In house	PDMS	Soft lithography	Material: Prototherm. Fabrication: 3D printed (Proto labs)	PE membrane (20 μm thick)	Hollow microchannels: 1 mm × 1 mm. Top channel: 1 mm × 1 mm. Bottom channel: 1 mm × 0.2 mm
Campisi *et al* [29], 2018	In house	PDMS	Soft lithography	Material: Silicon Wafer. Fabrication: NR	Membrane-free	Fluidic channel: 1000 μm × 150 μm. Main channel: 1300 μm × 150 μm. Distance between posts: 200 μm
Wang *et al*[30], 2017	In house	Objet VeroClear photopolymer	3D object printer (Objet 30Pro, Stratasys Ltd., Rehovot, Israel)	NA	PC membrane (0.4 μm pore size)	Main channel: 300 μm × 160 μm
DeStefano *et al* [31], 2017	In house	PDMS	Soft lithography	Material: Aluminum mold. Fabrication: NR	Membrane-free	390 μm, 450 μm, 550 μm, and 770 μm (different width)

3D: Three-dimensional; CNC: Computer numerical control; hBMECs: Human brain microvascular endothelial cells; HUVEC: Human umbilical vein endothelial cells; NA: Not applied; NR: Not reported; PDMS: Polydimethylsiloxane; PC: Porous polycarbonate; PE: Polyethylene terephthalate; PETE: Polyester track-etched; PMMA: Poly(methyl methacrylate); SU-8: Negative photoresist.

two made of polyester (PETE)[22,23], and one of porous polycarbonate (PC)[30]. Almost all microfluidic chips with membranes were designed to comprise a top and a bottom channel[21-23,26-28]. An exception was found in the study by Wang *et al*[30], whose chip design was more complex, containing four parts that were stacked to compose the BBB model. Five studies (36%) presented three-channel microfluidic chips: Two side channels that were generally used to perfuse medium, and one middle channel where the cells were generally cultured[18-20,25,29]. The remaining studies employed simpler chip designs that comprised a single[24] or multiple channels[31] parallel to each other. Channel



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Figure 1 PRISMA flow chart of the study selection process applied in this systematic review. iPSC: Induced pluripotent stem cell.

dimensions were largely variable (from 160 μm to 2200 μm) among the selected articles, defined according to the desired use of the chip.

Characteristics of the iPSCs used in the BBB models and their cultivation and differentiation conditions

To build the BBB, as shown in Table 2 and Figure 2A, IMR90-C4 from human fetal lung fibroblasts was the main iPSC line used (41.1%)[18,19,21,23,24,28,30], commonly cultivated in Matrigel-coated flasks. Few studies (17.6%) have reported the use of different iPSC lines[20,26,27], one of which used nine different types of iPSCs[27] that were extracted from skin fibroblasts or the peripheral blood of healthy (5) or unhealthy donors (2). Two of these skin fibroblast lines from unhealthy donors were modified by clustered regularly interspaced short palindromic repeat (CRISPR)[27]. These diverse cell lines were useful in modeling BBB dysfunctions and their corrections[27].

Concerning the coating applied for culturing the iPSC lines, fibronectin was selected for cultivation of human iPSC (hiPSC)-ECs (11.8%)[25,29]. Other particular iPSC lines, such as Ax0018 (neural stem cells)[20] and BC1-hiPSC (bone marrow)[31], were cultured on Matrigel, whereas GM25256 (skin fibroblast)[22], and ACS-1024 (bone marrow)[24] were cultured on laminin. The ECM on which EZ-Spheres (neural progenitors) were grown was not defined[26]. Interestingly, the study by Motallebnejad *et al*[24] compared cultivation of the ACS-1024 line on different laminin isoforms (LN511 and LN411), or on collagen IV associated with fibronectin[24]. Overall, Matrigel was the coating of choice in the majority of the selected studies (47.4%)[18-20,23,24,27,28,30,31]. The least common coatings, such as basement membrane matrix coating[26] and geltrex[20], appeared in 5.3% of the analyzed articles (Figure 2A and Table 2).

The iPSCs generally go through a complex differentiation process, involving multiple medium replacements with the addition of supplements and composition changes. Most iPSC cultivation protocols were undertaken in mTeSR1 medium (53.3%) associated[23] or not[18,19,24,26-28,30,31] with essential medium 8 (E8, 6.7%) for about 3 d, mainly for culturing the IMR90-C4 cell line. Part of these studies (35.7%)[18,19,21,24,31] added the Rho-associated, coiled-coil containing protein kinase inhibitor (Y27632) to the cultures overnight to inhibit cell proliferation before differentiation[32,33]. Unconditioned medium (UM), normally composed of Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with knockout serum replacement + non-essential amino acids (NEAA) or minimum essential medium (MEM) with NEAA (MEM-NEAA) + glutamax or L-glutamine + β-mercaptoethanol, was used

Table 2 Characteristics of the induced pluripotent stem cells used in the blood-brain barrier model, their cultivation and differentiation conditions

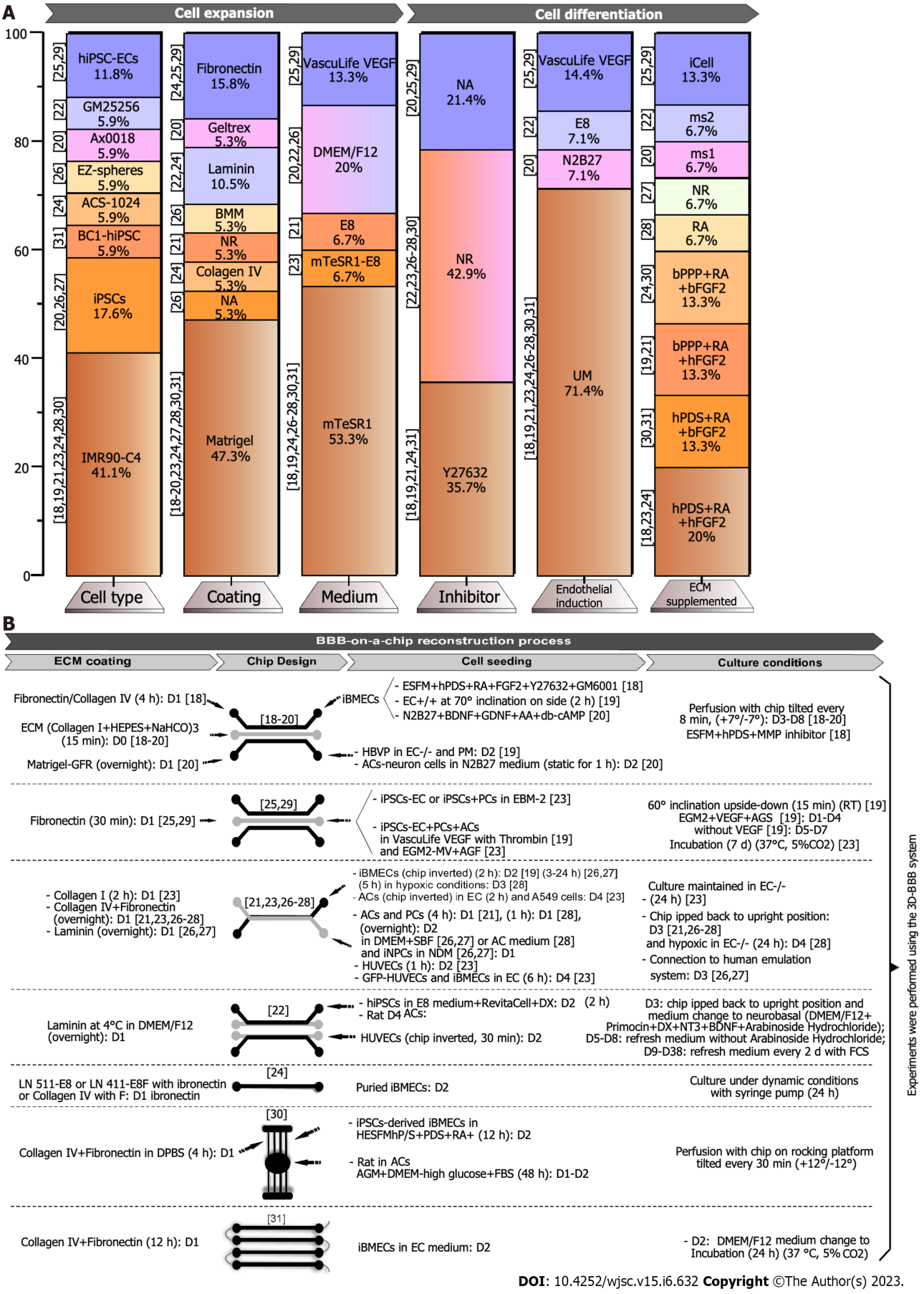
Ref.	Cell origin	Cell differentiation				BBB components model	
		iPSCs line	Flask coating	Medium	Supplement	Differentiated cell/medium	Co-culture/medium
Kurosawa *et al* [18]	Human fetal lung fibroblast	IMR90-C4	Matrigel	Day 3-2: mTeSR1-cGMP	Day 3: With Y27632 Day 2: Without Y27632	iBMECs (10^7) in ESFM	NA
				Day 0-5: UM	KOSR (20%) + glutamax (0.5%) + NEAA (1%) + β-mercaptoethanol (0.0007%)		
				Day 6-8: $EC^{+/+}$ (HESFM)	hPDS (1%) + RA (10 μM) + hFGF2 (20 ng/mL)		
Fengler *et al*[19]	Human fetal lung fibroblast	IMR90-C4	Matrigel	Day 3-1: mTeSR1	Y27632 (10 μM)	iBMECs purified[1] (10^6) in HBVP conditioned	NA
				Day 0-5: UM (DMEM/F12-HEPES)	Glutamax + KOSR + NEAA + β-Mercapto-ethanol		
				Day 6: $EC^{+/+}$ (HESFM)	bPPP (1%) + RA (10 μM) + bFGF (20 ng/mL)		
Wevers *et al*[20]	Human astrocytes	iPSCs	Geltrex	DMEM	FBS (10%) + N2 (1 ×) + P/S (1%)	iBMECs (10^4) in NR medium	Astrocyte-neuron cells (1:4) (1.5×10^4 cells/μL) in N2B27
	Human neural stem cells	Ax0018	Matrigel-GFR	Day 0-21: N2B27	BDNF (20 ng/mL) + GDNF (10 ng/mL) + AAc (100 μM) + db-cAMP1 (10 μM)		
Noorani *et al*[21]	Human fetal lung fibroblast	IMR90-C4	NR	Day 1: E8	Y27632 (10 μm)	iBMECs (1.5×10^7 cells/mL) in NR medium	Primary ACs (10^6 cells/mL) and PCs (3.5×10^5 cells/mL) (3:1) in NR medium
				Day 0-6: UM	KOSR (20%) + NEAA (1%) + Glutamax (0.5%) + β-Mercapto-ethanol (0.1 mM)		
				Day 7-8: $EC^{+/+}$ (HESFM)	bPPP (1%) + bFGF (20 ng/mL) + RA (10 μm)		
				Day 9: $EC^{-/-}$ (HESFM)	Without bFGF and RA		
Middelkamp *et al*[22]	Adult skin fibroblasts	GM25256	Laminin	Day 1: DMEM/F12	Primocin (0.1 mg/mL) + DX (4 μg/mL) + N2 (1 ×) + MEM-NEAA (1 ×) + NT3-RHP (10 ng/mL) + BDNF-RHP (10 ng/mL)	iNeurons (10^4) in E8	Rat ACs (1:1) and HUVECs (4×10^4) in ECGM-2
				Day 1 (after two hours): E8	RevitaCell (1 ×) + DX (4 μg/mL)		
				Day 3-38: Neurobasal	Day 3: Primocin (0.1 mg/mL) + B-27 (1 ×) serum free + glutamax (1 ×) + DX (4 μg/mL) + NT-3 (10 ng/mL) + BDNF (10 ng/mL) + arabinoside hydrochloride (2 μm) Day 5: Refresh medium without arabinoside hydrochloride (2 μm)		

					Day 9-38: Refresh medium with 2.5% fetal calf serum		
Choi *et al*[23]	Human fetal lung fibroblast	IMR90-C4	Matrigel	Day 3: mTeSR1™-E8™	NR	iBMECs purified[1] (1.2×10^7 cells/mL) in HESFM	AC (10^6 cells/mL) in $EC^{-/-}$
				Day 0-5: UM (DMEM/F12)	KOSR (20%) + NEAA (100x) + glutamax (0.5%) + β-Mercapto-ethanol (0.007%) (5% O_2)		
				Day 6-8: $EC^{+/+}$ (HESFM)	Human serum (1%) + bFGF (20 ng/mL) + RA (10 μm) (5% O_2)		
				Day 9: $EC^{-/-}$ (HESFM)	Without bFGF and RA		
Motallebnejad *et al*[24]	Human fetal lung fibroblast	IMR90-C4	Matrigel	Days 3-1: mTeSR1-cGMP	Day 3: Y27632 (10 μm)	iBMECs purified[1] in NR medium	NA
					Day 2: Without Y27632		
				Days 0-5: UM	NR		
				Days 6-8: $EC^{+/+}$ (HESFM)	Days 6-7: hPDS (1%) + RA (10 μM) + FGF2 (20 ng/mL)		
					Day 8: bPPP (1%) or FBS (2%) + FGF (20 ng/mL) + RA (10 μM)		
				Day 9: $EC^{-/-}$ (HESFM)	Without bFGF and RA		
	Healthy human African American male from the bone marrow CD34+ cells	ACS-1024	LN 511-E8 or LN 411-E8 or collagen IV + fibronectin	HESFM	Day 8: bPPP (1%) or FBS (2%) + FGF (20 ng/mL) + RA (10 μM)		
					Day 9: Without bFGF and RA		
Lee *et al*[25]	Endothelial cells	hiPSC-ECs	Human fibronectin	VascuLife VEGF	iCell media supplement	hiPSC-ECs (6×10^6 cells/mL) in VascuLife VEGF with thrombin	PCs and ACs (10^6 cells/mL) in VascuLife VEGF with thrombin
Jagadeesan *et al*[26]	Neural progenitors	EZ-Spheres	NA	Day 1: EZ-sphere medium (DMEM/F12)	bFGF (100 ng/mL) + EGF (100 ng/mL) + heparin (5 μg/mL) + B27 (2%)	iNPCs (10^6 cells/mL) in NDM	ACs (9×10^5 cells/mL) and PCs (3×10^5 cells/mL) in in DMEM
					B27 (2%) + vitamin A + N2 (1%) + hBDNF, (20 ng/mL)		
	NR	hiPSCs	Basement membrane matrix-coated	Day 1: mTeSR1	NR	iBMECs [(14-20) × 10^6 cells/mL] in $EC^{-/-}$ (HESFM)	
				Day 2-8: UM (DMEM/F12)	KOSR (10%) + NEAA (1%) + glutamine (0.5%) + β-Mercapto-ethanol (100 μm)		
				Day 9-10: $EC^{+/+}$ (HESFM)	bPPP (1%) + bFGF (20 ng/mL) + RA (10 μM)		
				Day 11: $EC^{-/-}$ (HESFM)	Without bFGF and RA		
Vatine *et al*[27]	Adult skin fibroblasts	CS03iCTR CS83iCTR CS03iCTR$^{mut, 2}$ CS01iMCT8	Matrigel	Day 3: mTeSR1	NR	iNPCs (10^6 cells/mL) in NDM and iBMECs (1.4×10^4 cells/mL)	ACs (9×10^5 cells/mL) and PCs (3×10^5 cells/mL) in DMEM

		CS01iMCT-8^{cor,2}					
	Peripheral blood	CS0172iCTR					
		CS0188iCTR		Day 0: UM	Without bFGF		
		CS0617iCTR					
	Huntington's disease	CS81iHD					
Park *et al*[28]	Human fetal lung fibroblast	IMR90-C4	Matrigel	Day 3: mTeSR1	NR (5% O_2)	iBMECs purified[1] (2.3×10^7 cells/mL) in EC	ACs (7×10^5 cells/mL) and PCs (3×10^5 cells/mL) in ACM
				Day 0-6: UM (DMEM/F12)	KOSR (100 mL) + NEAA (5 mL) + glutamax (2.5 mL) + β-Mercaptoethanol (3.5 μL) (5% O_2)		
				Day 7-9: $EC^{+/+}$	RA (5% O_2)		
Campisi *et al*[29]	Blood from 30-39-year-old healthy females	hiPSC-ECs	Human fibronectin	VascuLife VEGF	iCell media supplement + VEGF (50 ng/mL)	hiPSC-ECs (2×10^6 cells/mL) in EBM-2	Monoculture hiPSC-ECs (6×10^6 cells/mL) in EBM-2
							PCs (2×10^6 cells/mL) in EGM-2 MV
							PCs and ACs (2×10^6 cells/mL) in EGM-2 MV
Wang *et al*[30]	Human fetal lung fibroblast	IMR90-C4	Matrigel	Day 3: mTeSR1	NR	iBMECs in HESFM	Rat primary ACs in AGM
				Day 0-5: UM (DMEM/F12)	HEPES + KOSR (20%) + MEM-NEAA (1 ×) + L-glutamine (1 mM) + β-Mercaptoethanol (0.1 mM)		
				Day 6-8: $EC^{+/+}$ (HESFM)	hPDS (1%) + bFGF (20 ng/mL) + RA (10 μM)		
DeStefano *et al*[31]	CD34 positive bone marrow	BC1-hiPSCs	Matrigel	Day 4-3: mTeSR1-E8	NR Day 3: Y27632 (10 μm)	iBMECs (10^5) in EC	NA
				Day 0-5: UM (DMEM/F12)	KOSR (20%) + NEAA (1%) + L-Glutamine (0.5%) + β-Mercaptoethanol (0.84 μm)		
				Day 6-7: $EC^{+/+}$ (HESFM)	hPDS (1%) + bFGF (20 ng/mL) + RA (10 μm)		
				Day 8: EC (HESFM)	DB-cAMP (400 μm) or Y27632 (10 μm)		

[1]Induced pluripotent stem cell-derived brain microvascular endothelial cells were selectively expanded before to seed in the chip.

[2]Modified by clustered regularly interspaced short palindromic repeats. For human induced pluripotent stem cells-brain microvascular endothelial cells purification, day 8: ECM [1 μg/cm^2 LN 511-E8 or LN 411-E8 (iMatrix, iWAi), 1 μg/cm^2 full length laminin 511 (Biolamina), 100 μg/mL fibronectin (Millipore Sigma), or a mixture of 400 μg/mL collagen IV (Millipore Sigma) and 100 μg/mL of fibronectin] protein-coated ThinCert cell culture inserts (Greiner Bio-One), well plates or ibidi μ-slides. AT: Adipose tissue; BDNF-RHP: Brain-derived neurotrophic factor recombinant human protein; EGF: Epidermal growth factor; BM: Bone marrow; bPPP: Platelet-poor plasma derived bovine serum; CXCR4: C-X-C chemokine receptor type 4; db-cAMP1: 2′-O-Dibutyryladenosine-3′,5′-cyclic monophosphate; DMEM: Dulbecco's modified eagle medium; E8 medium: Essential 8 medium; EGF: Epidermic growth factor; FDF2: Fibroblast growth factor 2; FL: Fetal lung; GFP: Green fluorescent protein; HESFM: Human endothelial serum free medium; hFGF2: Human fibroblast growth factor 2; hiPSC: Human induced pluripotent stem cell; HOXB4: Homeobox B4; hPDS: Human serum from platelet-poor human plasma; IB: Intrabone; IP: Intraperitoneal; IV: Intravenous; KOSR: Knockout serum replacement; Matrigel-GFR: Growth factor reduced matrigel; MEC: Brain microvascular endothelial cell; MEM: Minimum essential medium; NA: Not applied; NEAA: Non-essential amino acids; $Nos2^{-/-}$: Deficient in type 2 nitric oxide; NR: Not reported; NT3-RHP: Neurotrophin-3 recombinant human protein; P/S: Penicillin/streptomycin; PDGFB: Platelet-derived growth factor subunit B; PPDS: Platelet poor derived serum; AAc: Ascorbic acid; SDF-1: Stromal cell-derived factor 1; solG-CSFR: Soluble granulocyte colony-stimulating factor decoy receptor; TPO: Thrombopoietin; UCB: Umbilical cord blood; MMP3: Matrix metallopeptidase 3.



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Figure 2 Induced pluripotent stem cell differentiation process into induced pluripotent stem cell-derived brain microvascular endothelial-like cells for reconstruction of the blood-brain barrier-on-a-chip model. A: Percent analysis of cells and culture media used in induced pluripotent stem

cell expansion and differentiation before blood-brain barrier (BBB)-on-a-chip reconstruction; B: Schematic summary of the main findings in this systematic review on the cells and culture conditions applied in the BBB-on-a-chip reconstruction process. Studies were grouped by similar device designs. AAc: Ascorbic acid; AC: Astrocyte; ACS-1024: Bone-induced pluripotent stem cell line; AGF: Astrocyte growth factor; AGM: Astrocyte growth medium; BC1: Lymphoma cell line; BDNF: Brain-derived neurotrophic factor; bFGF: Bovine fibroblast growth factor; BMM: Basement membrane matrix; bPPP: Basic platelet-poor plasma; D1: Day 1; D2: Day 2; D3: Day 3; D38: Day 38; D4: Day 4; D5: Day 5; D6: Day 6; D8: Day 8; D9: Day 9; db-cAMP1: 2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate; DMEM/F12: Dulbecco's modified Eagle medium with F12; DPBS: Dulbecco's phosphate-buffered saline; DX: Doxycycline; E8: Essential 8 medium; EC-/-: Human endothelial serum-free medium without retinoic acid + basic fibroblast growth factor; EC: Endothelial cell; EC+/+: Human endothelial serum-free medium with retinoic acid + basic fibroblast growth factor; ECM: Extracellular matrix; EGM-2MV: Microvascular endothelial cell growth medium-2; ESFM: Endothelial serum-free medium; FBS: Fetal bovine serum; FCS: Fetal calf serum; FGF2: Fibroblast growth factor 2; GDNF: Glial cell line-derived neurotrophic factor; GFP: Green fluorescent protein; GFR: Growth factor reduced; GM25256: Cell line of induced pluripotent stem cell derived from adult skin fibroblasts; GM6001: Broad spectrum MMP inhibitor; HBVP: Human brain vascular pericytes; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HESFM: Human endothelial serum-free medium; hFGF: Human fibroblast growth factor; hiPSC-EC: Human-induced pluripotent stem cell-derived endothelial cell; hPDS: Human serum from platelet-poor human plasma; hPDS: Platelet-poor plasma-derived human serum; HUVEC: Human umbilical vein endothelial cell; iBMECs: Induced pluripotent stem cell-derived brain microvascular endothelial cells; IMR90-C4: Induced pluripotent stem cell line; iPSCs: Induced pluripotent stem cells; LN: Laminin; ms1: Brain-derived neurotrophic factor + glial cell line-derived neurotrophic factor + ascorbic acid + 2'-O-Dibutyryladenosine-3',5'-cyclic monophosphate; ms2: Primocin + glutamax + doxycycline + neurotrophin-3 + brain-derived neurotrophic factor + fetal calf serum; mTeSR1: Basal medium type for induced pluripotent stem cells; N2B27: Culture medium; NA: Not applied; NR: Not reported; NT3: Neurotrophin-3; P/S: Penicillin/streptomycin; PCs: Pluripotent cells; PM: Pericyte medium; RA: Retinoic acid; RA: Retinoic acid; RT: Room temperature; SFB: Serum-free medium; UM: Unconditioned medium; VEGF: Vascular endothelial growth factor; Y27632: Dihydrochloride inhibitor.

in 71.4% of the studies[18,19,21,23,24,26-28,30,31] to promote differentiation to ECs (for 6 d on average), followed by a change to human endothelial serum-free medium containing diverse substances, such as retinoic acid (RA), platelet-poor-derived bovine serum (bPPP) or human serum from platelet-poor human plasma (hPDS), and basic fibroblast growth factor, to induce BMEC formation (Figure 2A and Table 2).

Few studies have described different processes to generate iBMECs. Lee *et al*[25] used solely vasculife vascular endothelial growth factor medium with iCell media supplement[29]. The study by Wevers *et al* [20] used DMEM/F12 and N2B27 supplemented with brain-derived neurotrophic factor (BDNF) + glial cell line-derived neurotrophic factor + ascorbic acid + 2′-O-Dibutyryladenosine-3′,5′-cyclic monophosphate. Finally, the study by Middelkamp *et al*[22] utilized DMEM/F12 supplemented with primocin + doxycycline (DX) + N2 + MEM-NEAA + neurotrophin-3 (NT3) recombinant human protein + BDNF recombinant human protein, followed by a change to E8 supplemented with revitacell + DX, and a later change to neurobasal medium supplemented first with primocin + B27SF + glutamax + DX + NT3 + BDNF, in addition to arabinoside hydrochloride for 24 h, followed by the addition of fetal calf serum. DX induced the overexpression of neurogenin *2* in GM25256 iPSCs, being one of the key factors for the differentiation of GM25256 into iNeurons (Table 2).

All of the abovementioned processes were described for iPSC differentiation on a plate, with the exception of the study by Campisi *et al*[29], whose cell differentiation step occurred inside the microfluidic chips. Furthermore, two studies used hypoxic conditions during the cell differentiation process[23,28].

BBB-on-a-chip reconstruction process

To build the BBB-on-a-chip model, the microdevices must first receive a coating layer to sustain 3D cell growth, as depicted in Figure 2B. From this perspective, diverse coating substances and different cell-seeding strategies have been described, depending on the chip design. As seen in Figure 2B, previously to cell culture on-a-chip, fibronectin was the principal ECM component coating the microfluidic channels (39.3%)[18,21,23-26,28-31], among which 28.6% corresponded to a mixture of fibronectin + collagen type IV. More recent studies have applied an ECM composed of collagen type I + 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid + sodium bicarbonate (10.7%)[18-20]. Laminin coating was used in 14.3% of the analyzed studies[22,26,27], wherein a mixture of different types of laminin + fibronectin was employed in the study by Motallebnejad *et al*[24]. Additionally, one study utilized only Matrigel[20], and another utilized collagen type I[23] as channel coating. The mentioned coatings were carefully selected for each channel, depending on the desired environment and cultured cell type.

Regarding cell seeding, five studies have described an iBMEC single culture[18,19,24,29,31], in which two studies applied a selective cell expansion step before seeding[19,24]. A co-culture of iBMEC + ACs was reported in three studies[20,23,30], one of which used PCs in the co-culture[29]. Yet, most studies have built their BBB models based on an approach involving more than two cell types in co-culture, normally associating ACs and PCs with other ECs such as HUVECs[22], iBMECs[21,28], and iPSC-ECs [25], with the exception of Jagadeesan *et al*[26] and Vatine *et al*[27], who associated neural progenitor cells with ACs and PCs in the brain-side, and iBMECs in the blood-side of their BBB model (Figure 2B).

The particular aspects of cell culture inside the chips are displayed in Figure 2B. Among the observed aspects, we highlight the inversion or inclination of the chip to seed the cells[21-23,25-28], maintenance of the chip under hypoxic conditions[28], and tilting of the chips for gravity-dependent bidirectional medium flow[18-20,30].

Applications of BBB microfluidic 3D models using iPSCs

Regarding the general objective of the studies (Table 3), the great majority of the articles (roughly 80%) [18-21,23,24,26-30] were aimed at developing a microfluidic BBB model, as well as assessing the convenience of the manufactured model by quantifying common molecular markers that are normally expressed in cells that make up the BBB *in vivo*. Five of these studies (35.7%) analyzed drug transport for BBB characterization purposes[18,19,27,28,30]. Only two studies described actual applications for their models: Wevers *et al*[20] used their developed chip to model BBB disruption upon ischemic stroke, and Lee *et al*[25] tested the permeability of their model to commercial and synthesized polymer nanoparticles (NPs). Middelkamp *et al*[22] compared a 2D Transwell BBB model to one built on-a-chip, whereas DeStefano *et al*[31] evaluated the effect of shear stress on their BBB microfluidic chips.

The characterization of the BBB model was performed by means of structural and functional analyses. Concerning structural analyses, the expression of tight junction proteins was evaluated in 85.7% of the studies using immunocytochemistry (85.7%)[18-21,23-31]. Part of these studies also performed quantitative polymerase chain reaction (qPCR) (42.9%)[23-25,29,31]. Less frequently used techniques were fluorescence (F-actin staining)[24], transcriptional analysis[27], mass spectrometry (MS) (proteomics)[28], and western blot analysis[31]. Transport protein and receptor expression was evaluated in 57.1% of the cases[18,20,21,23,26-28,31] by immunocytochemistry (42.9%)[18,21,23,26-28], qPCR (21.4%)[18,20,31], fluorescence (permeability glycoprotein inhibition, 14.3%)[20,21], transcriptional analysis (7.1%)[27], and MS (7.1%)[28]. Two studies (14.3%) characterized neuronal differentiation by immunocytochemistry[22,26], one of which also applied RNA sequencing and transcriptomic analysis to further characterize the HUVECs[22]. Structural characterization of ACs and PCs was performed by immunocytochemistry by Campisi *et al*[29]. Lastly, DeStefano *et al*[31] used microscopic techniques to structurally and functionally characterize ECs seeded on-a-chip[31] (Table 3).

Functional characterization of the BBB model was more varied, with the use of diverse techniques. Tight junction functionality (71.4%) was mostly analyzed by fluorescence (50%)[18-20,22,24,26,30] using diverse fluorescent markers[18,23,24,26,28-30] such as zonula occludens-1 (ZO-1), claudin-5[21,22,26,28], occludin (OCLN)[21,22], glucose transporter type 1[21,24,26], platelet/EC adhesion molecule-1 (PECAM-1)[22,24,26,27], and vascular endothelial (VE)-cadherin[22]. Other techniques used to evaluate tight junction functionality were transepithelial electrical resistance (TEER) measurements (42.9%)[19,20,24,27,28,30], enzyme-linked immunosorbent assay (21.4%)[19,27,28], (ultra or high performance) liquid chromatography with tandem MS (LC-MS/MS) (21.4%)[18,27,30], immunocytochemistry[23,27] (14.3%), transendothelial migration of cancer cells[23], electron transmission microscopy (7.1%)[28], and transmitted light microscopy (7.1%)[27]. Microvessel permeability (57.1%)[19-21,25,27-30] and microvessel integrity (7.1%)[19] were assessed by fluorescence (57.1%)[19-21,25,27-30]. Transport protein function was evaluated by 21.4% of the studies[18,27,28] by fluorescence (14.3%)[27,28] and high-performance LC-MS (7.1%)[18]. Neuronal functionality was evaluated by calcium fluorescence imaging (14.3%)[20,27] and immunocytochemistry (7.1%)[27]. Mitochondrial membrane potential was analyzed by luminescence and ATP quantification (7.1%)[20]. In a diverse approach, the study by Vatine *et al*[27], among other evaluations, perfused whole human blood through the chip channels to perform a whole-blood neuronal toxicity assay using a colorimetric technique, focusing on the quantification of lactic dehydrogenase[27] (Table 3).

Overall, the analyzed studies were successful in building structural and functional BBB models on-a-chip using iPSCs. Most of them highlighted the future use of their models for drug screening tests, in addition to mimicking physiological conditions found in healthy and unhealthy individuals, wherein one of the studies reported the applicability of their device for modeling cancer cell invasion and migration through the BBB. The studies also focused on the potential for the development of personalized therapies, mainly in conditions of disease. Some interesting outcomes have reported long-lasting BBB function for the developed chips, with an average BBB function duration of 7 d[21,28] to 10 d[30] (Table 3).

DISCUSSION

This systematic review showed that this theme, BBB models with iPSCs in microdevices, is quite novel in the scientific literature; without setting a time limit, only articles from 2017 to 2022 were found. Furthermore, the articles published in the last 2 years have already shown great technological advances in BBB-on-a-chip in commercial devices[18-21]. Curiously, the same chip by MIMETAS® was used in three studies by different labs around the world (Japan[18], the Netherlands[19], and Germany[20]), and another commercial BBB-on-a-chip was also used by an American lab[21]. These advances in technological on-chip manufacturing allow the scientific community to direct further efforts to apply the developed BBB-on-a-chip to model pathological conditions, such as stroke[21], and design personalized therapeutic approaches.

Among the developed BBB models on a microdevice, some showed distinctive features, mainly for the chips fabricated in house. For example, the study by Choi *et al*[23] described a BBB microchip whose functionality was based on a track-etched 10 μm pore polyester membrane covered with a collagen type

Table 3 Applications of blood-brain barrier microfluidic three-dimensional models using induced pluripotent stem cells

Ref.	Application	Characterization	Evaluation technique	Outcomes
Kurosawa *et al*[18]	Build and evaluate a BBB 3D *in vitro* model	Capillary structure formation and tight junction proteins expression	Immunocytochemistry	Formation of the capillary structure, functional tight proteins; lower expression of ABC transporters than levels found *in vivo*, except for BCRP; expression of functional SLC transporters
		Transport proteins and receptors expression	Immunocytochemistry	
			qPCR	
		Tight junction functionality	Fluorescence (lucifer yellow and antipyrine)	
			HPLC-MS/MS (test-drug transport)	
		Transport proteins function	HPLC-MS/MS (test-drug transport)	
Fengler *et al*[19]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	Capillary diameter CA. 40 times larger than *in vivo* brain vessels; physiologically relevant TEER values; physiologically similar localization of BCRP and GLUT-1 proteins. Promising BBB model for future drug screening tests
		Microvessel integrity	Fluorescence (DEX-A647 and sodium fluorescein)	
		Microvessel permeability	Diazepam, Emricasan, Ac-YVAD-CMK, Z-DEVD-FMK, ZVAD (OH)-FMK, Staurosporine, and IL-1β	
		Tight junction functionality	ELISA (Diazepam)	
			TEER measurements	
Wevers *et al*[20]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	Barrier functionality similar to that found *in vivo*; microfluidic model suitable for evaluating disruption of the BBB; successful ischemic stroke modeling. Potential use for modeling the BBB under sub-optimal conditions (disease) and for evaluating potential therapies
		Tight junction functionality	TEER measurements	
		Microvessel permeability	Fluorescence (sodium fluorescein)	
		Transport proteins expression	Fluorescence: P-gp inhibition	
			qPCR	
		Neuronal functionality	Calcium fluorescence imaging	
	Ischemic stroke modeling	Microvessel permeability	Fluorescence (FITC-dextran)	
		Mitochondrial membrane potential	Luminescence (CellTiter-GLO)	
			ATP quantification	
Noorani *et al*[21]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	BBB functionality remains intact for up to 7 d and is similar to that found *in vivo*; a more physiologically relevant BBB model; shear stress contributes positively to BBB tightness
		Microvessel permeability	UPLC-MS/MS: [$^{13}C_{12}$] sucrose and [$^{13}C_6$] mannitol	
		Transport proteins expression	Immunocytochemistry	
			Fluorescence: P-gp inhibition	
Middelkamp *et al*[22]	Compare 2D cultures to microfluidic chip cultures	Neuronal differentiation and characterization of HUVECs	Immunocytochemistry	Culture in microfluidic chips promotes gene expression that more closely resembles that found *in vivo*
			RNA sequencing	
			Transcriptomic analysis	
Choi *et al*[23]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	cECMTE membrane with 10 m pores in microfluidic device were successful in mimicking the *in vivo* BBB, also allowing for cancer cell tissue migration. Promising BBB model for studying cancer metastasis, cell communication, and migration
			qPCR	
		Tight junction functionality	Fluorescence (lucifer yellow)	
			Transendothelial migration of cancer cells (CellMask)	
			Immunocytochemistry	
		Transport proteins expression	Immunocytochemistry	

Motallebnejad *et al*[24]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	LM511-E8 ECM contributes to long-lasting endothelial cell and BBB function, in addition to promoting better shear stress responses. Authors recommend the use of LM511-E8 ECM for future studies involving BBB function
			Fluorescence (F-actin staining)	
			qPCR	
		Tight junction functionality	TEER measurements	
			Fluorescence (rhodamine B-labeled neutral dextran)	
Lee *et al*[25]	BBB permeability to polymer nanoparticles	Tight junction and transport proteins expression	qPCR	Fast analysis of polymer nanoparticles permeability; physiologically reliable BBB model
		Permeability to polymer nanoparticles	Fluorescence (polymer nanoparticles and FITC-dextran)	
			3D fluorescence intensity maps	
Jagadeesan *et al*[26]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	Successful fabrication of BBB model personalized for different human individuals; BBB models were able to mimic physiological differences between healthy and ill individuals
		Tight junction functionality and microvessel permeability	Fluorescence: FITC-dextran	
		Transport proteins expression	Immunocytochemistry	
		Neuronal differentiation		
Vatine *et al*[27]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	Successful fabrication of BBB model personalized for different human individuals; BBB models were able to mimic physiological differences between healthy and ill individuals
			Transcriptional analysis	
		Microvessel permeability and tight junction functionality	Fluorescence (FITC-dextran and 2NDBG)	
			ELISA (human albumin, IgG and transferrin)	
			LC-MS/MS (T_3, colchicine, levetiracetam and retigabine)	
			Transmission light microscopy	
			TEER measurements	
			Immunocytochemistry	
		Transport proteins expression	Immunocytochemistry	
			Transcriptional analysis	
		Transport protein function	Fluorescence (rhodamine-123)	
		Whole-blood neuronal toxicity	Colorimetric assay (quantification of lactic dehydrogenase)	
		Neuronal functionality	Immunocytochemistry	
			Calcium fluorescence imaging	
Park *et al*[28]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	BBB functionality remains intact for up to 7 d. Promising BBB model for future drug and antibody transport studies
			Multiplex qPCR	
			MS (proteomics)	
		Tight junction functionality and microvessel permeability	Electron transmission microscopy	
			TEER measurements	
			Fluorescence (dextrans, cetuximab, angiopep-2, MEM75, 13E4)	
			ELISA (dextrans, cetuximab)	
		Transport proteins expression	Immunocytochemistry	
			MS	
		Transport proteins function	Fluorescence (rhodamine-123 and doxorubicin)	

Campisi *et al*[29]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	Tri-culture of human iPSC-derived endothelial cells, astrocytes and pericytes spontaneously arranged into a BBB-like model. Promising BBB model for future preclinical experiments
			qPCR	
		Tight junction functionality and microvessel permeability	Fluorescence (FITC-dextran)	
		Characterization of astrocytes and pericytes	Immunocytochemistry	
Wang *et al*[30]	Build and evaluate a BBB 3D *in vitro* model	Tight junction proteins expression	Immunocytochemistry	Pumpless media perfusion system that resembles the blood residence time within brain tissues; physiologically relevant TEER values maintained for up to 10 d. Promising BBB model for future drug permeability studies
		Tight junction functionality and microvessel permeability	TEER measurements	
			Fluorescence: FITC-dextran and doxorubicin	
			LC-MS/MS (caffeine and cimetidine)	
DeStefano *et al*[31]	Evaluate BBB upon shear stress	Characterization of iPSC-derived endothelial cells morphology and function	Microscopy (time-lapse imaging analysis using ImageJ)	BBB endothelial cells display unique features that differ from endothelial cells from other tissues; shear stress plays a key role in BBB-like function in microfluidic models
		Tight junction proteins expression	Immunocytochemistry	
			Western blot	
			qPCR	
		Transport proteins expression	qPCR	

2NDBG: 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]-D-glucose; 3D: Three-dimensional; Ac-YVAD-CMK: Caspase-1 inhibitor; ATP: Adenosine triphosphate; UPLC-MS/MS: Ultra-performance liquid chromatography-mass spectrometry (tandem); BBB: Blood-brain barrier; BCRP: Breast cancer resistance protein; cECMTE: Condensed extracellular matrix track-etched; CellTiter-GLO: Luminescent cell viability assay; DEX-A647: Dextran conjugated to Alexa 647; ECM: Extracellular matrix; ELISA: Enzyme-linked immunoassay; FITC-dextran: Fluorescein isothiocyanate dextran; GLUT-1: Glucose transporter 1; hBMEC: Human brain microvascular endothelial cells; HPLC-MS/MS: High-performance liquid chromatography-mass spectrometry (tandem); HUVEC: Human umbilical vein endothelial cell; iBMEC: Induced pluripotent stem cell-derived brain microvascular endothelial-like cell; IgG: Immunoglobulin G; IL-1β: Interleukin 1 beta; iPSC: Induced pluripotent stem cell; LC-MS/MS: Liquid chromatography-mass spectrometry (tandem); LM511-E8: Fragment E8 of laminin 511; MS: Mass spectrometry; P-gp: Permeability glycoprotein; qPCR: Quantitative polymerase chain reaction; RNA: Ribonucleic acid; SLC: Solute carrier protein; TEER: Trans-epithelial electrical resistance; Z-DEVD-FMK: Caspase-3 inhibitor; ZVAD (OH)-FMK: Pan-caspase inhibitor.

I ECM. This study reported that the ECM-covered membrane with a larger pore size allowed for better modeling of the communications between neural cells and ECs, in addition to enabling the evaluation of cancer cell metastasis through the BBB[23]. Other articles also based their 3D BBB models on microfluidic channels separated by porous membranes of various materials - PDMS[21,26,27], PC[30], PE[28], PETE[22,23] - and various pore sizes that ranged from 0.4 mm to 7 mm. Nevertheless, none of these studies focused on the importance of the membrane material or pore size to the envisaged BBB function.

However, half of the selected articles, and curiously, the most recent ones, showed similar geometries with straight membrane-free channels. The advantage of the presence of a membrane is controversial in the literature regarding its role in the BBB model. Due to the relatively greater thickness of the artificial membrane compared to the basal lamina *in vivo*, cell-cell interactions are limited[34,35]. More recent articles use the diffusible factors produced by the cells in culture themselves for indirect communication between ACs and the BBB endothelium, without the physical restriction imposed by the artificial membrane[35].

Organs-on-chips are a currently sought alternative due to the possibility of physiologically relevant 3D *in vitro* single- or pluricellular culture and, more importantly, due to the possibility of laminar medium flow, mimicking the environment and shear stress found in capillary vessels. Also, the microchip culture of neural cells has shown more *in vivo*-like results, with more mature neurons and ACs[22]. However, the establishment of laminar flow might be cumbersome, given the need for apparatuses such as syringes, capillary tubes, and peristaltic pumps adapted for the sterile environment required for cell culture. Some studies have attempted to overcome this problem with creative alternatives, such as rocking platforms that allow the bidirectional flow of media within the microchips[18-20,30]. Regardless, the cerebral tissue is rather complex and involves a multitude of interacting cell types and ECM proteins. *In vitro* modeling at such a level is already challenging *per se*, whether or not the laminar flow is involved.

Regarding the material used for chip fabrication, conventional PDMS was used in most cases (57%)[22,25-29,31]. One of the studies adopted PMMA[23], which consists of the least hydrophobic material

utilized in microfabrication. In addition, this material is ideal for mass production, and it is stiffer than PDMS. However, rapid prototyping is somewhat complicated by the fact that PMMA bonding is more difficult than the straightforward bonding of PDMS[36,37]. Conversely, PDMS may nonselectively adsorb proteins and hydrophobic molecules, which could possibly interfere with the characterization process of the BBB-on-a-chip and future drug screening applications[38]. Hence, some studies have presented alternatives to tackle this issue, such as covering the microchip surface with bovine serum albumin[39], grafting with anti-fouling molecules[40], or silanization[41].

For construction of the *in vitro* NVU, the cell cultures have been classically established in Transwell plates, mainly using primary brain ECs isolated from animal and human sources, which retain very tight barriers *in vitro* and may be useful tools for studying paracellular permeability[42,43]. Immortalized EC lines, such as HUVECs, have also been used in these BBB models, although they do not provide the permeability profile or the protein expression typically present in the human BBB[44]. Moreover, there is great difficulty in obtaining primary human brain ECs from healthy individuals[45].

The aforementioned drawbacks have been recently addressed with the use of iPSCs. After going through a complex differentiation protocol, these stem cells have shown *in vivo*-like barrier function [27]. Their BBB phenotype can be further enhanced by co-cultivation with other cells that exert barrier function *in vivo*, such as ACs and PCs[24,27]. In view of this aspect, we found in this systematic review a great diversity of iPSC commercially available cell lines from human sources, such as the IPS cell line IMR90-C4 from human fetal lung fibroblasts[18,19,21,23,24,28,30]. We further verified that one study used an iPSC line from unhealthy donors, with the advanced purpose of verifying BBB dysfunction in a pathological model and an attempt to correct this dysfunction through gene editing (CRISPR)[27]. In addition, building BBB models from human-derived iPSCs may be a valuable tool for personalized medicine, wherein a patient's CNS disease could be precisely modeled *in vitro*, enabling the evaluation of personalized treatment.

On the other hand, iPSC-based BBB models have shown short-lived BBB function, which is generally maintained for up to 2 d[21]. Current efforts have been aimed at extending the BBB profile of these cultures[21,28,30]. 3D BBB on-a-chip devices form more physical and physiologically accurate morphologies. The cell-cell and cell-matrix interactions produced in microfluidic models generally provide a robust BBB function, with permeability to different substances (drugs, antibodies, proteins) that resemble that observed *in vivo*[18,19,27,28].

Most of the studies featured in this review (86%) differentiated their iPSCs into BMECs. This is expected since BMECs are the specialized cells in the CNS vasculature that show barrier properties. On the other hand, four studies differentiated the iPSCs into neural cells (neurons and ACs)[20,22,26,27]. The NVU, which makes up the BBB, is mainly composed of strict interactions among BMECs, the ECM, basal lamina, PCs, ACs, and adjacent neurons[11,46]. Even though neurons are indeed considered to be part of the BBB, they are physiologically positioned farther from the microvessels that make up the NVU (10 μm to 20 μm) than ACs or PCs[47]. The studies that reported co-culture of ECs with neurons mentioned in the current review did not clearly state the reasons for using neurons in their BBB models, but some studies stated that co-culture of iBMECs with neurons induces the upregulation of membrane transporters typical of the BBB, promoting a more robust function[29,48], as reported in a study by Wevers *et al*[20]. Microfluidic chips co-cultured both with iBMECs and neural cells showed a more robust phenotype regarding BMECs and strong indicators of epithelial cell barrier integrity and permeability (TEER from 1000 to 4000 Ω × cm^2)[18,19,21,27,30].

Although studies have pointed out drawbacks using primary BMECs or iBMECs in the EC monolayer in BBB-on-a-chip, they still constitute a more reliable model. One of the biggest challenges in using iBMECs in BBB research is the difficulty in producing an *in vivo*-like BMEC phenotype *in vitro* following the currently available protocols, which generally result in more epithelial-like phenotypes with the suboptimal expression of membrane transporters[6,20,26,27,49]. A recent meta-analysis study showed that iPSCs could only be reliably differentiated into BMECs through exposition to endothelial ETS transcription factors ETS variant transcription factor 2, Friend leukemia integration 1 transcription factor, and Ets-related gene[49]. Nevertheless, the differentiation of iPSCs into iBMECs can further benefit from co-culture with neural cells, such as ACs and neurons[27,50]. While a more robust differentiation protocol is not established, some studies make use of alternative ways to make an EC layer, such as the use of HUVECs[22], primary BMECs[20], or even immortalized lines[10]. On the other hand, the isolation of human primary BMECs is a challenge, since they comprise less than 0.1% of the cells in the CNS and tend to de-differentiate in culture[27,51]. Studies with HUVECs generally point out some limitations, such as the formation of an incomplete EC monolayer[22]. Immortalized cells, despite being easily commercialized, generally constitute BBB models with poor barrier properties[27,52].

Fully iPSC-based chips were useful in modeling BBB disruptions from diseased patients, such as the alterations found in Huntington's disease and in monocarboxylate transporter 8 deficiency[26,27]. Wevers *et al*[20] and Middelkamp *et al*[22] also differentiated the iPSCs into neurons, albeit not providing further justifications for doing so. With the exception of Vatine *et al*[27], who observed improved barrier functions with a fully iPSC-derived BBB chip, the other studies considered in this review have not versed on whether their obtained outcomes were positive or negatively dependent on the use of iPSCs.

Normally the microfluidic chips receive a coating made of collagen type IV/fibronectin before cell seeding[21,23,24,26-28,30,31]. These coating agents improve cell adhesion to the chip substrate through micropatterning, offering a fibrous base to guide cell differentiation and cell-to-cell contacts[16]. Motallebnejad *et al*[24] considered that the *in vivo* brain endothelial basement membrane is made up of laminins. Keeping this in mind, they compared the characteristics of the iPSC-derived ECs grown on an ECM based on E8 fragments of laminin 411 or laminin 511 (laminin511-E8) to ECs grown on the common collagen IV/fibronectin ECM. The authors found that the ECM made of laminin511-E8 presented more significant results, with the iPSC-ECs grown on this ECM exhibiting a longer-lasting EC phenotype and improved barrier stability compared to cells grown on the collagen IV/fibronectin ECM. iPSC-ECs grown on laminin511-E8 presented higher expression levels of junctional proteins such as claudin-5, VE-cadherin, and PECAM-1. Curiously, three other articles used a laminin ECM coating[22,26,27], but on the "brain side" channel of their microfluidic devices. One of the aforementioned studies also used the laminin coating on the "blood side" channel for culturing HUVECs, but without mentioning the importance of the laminin ECM[22].

Another important aspect of microfluidic device analyses in many studies is perfusion, whether by culture media or other biologically-relevant liquids, due to the shear stress and their important advantage in *in vitro* studies[10,16,53]. For BBB models, the role of shear stress on BBB function remains inconclusive. Among the studies selected for this review, eight (57%) considered shear stress[19-22,24,27,30,31], whereas two[22,30] designed their BBB mimics to minimize shear stress. Vatine *et al*[27] reported an increase in the expression of tight junction genes, such as tight junction protein 1/*ZO-1*, *OCLN*, *PECAM-1,* and cadherin 5/VE-cadherin, even under shear stress as low as 0.01 or 0.5 dyne/cm^2. A study by Noorani *et al*[21] found ambiguous results for cultures under shear stress; perfusion did not induce changes in tight junction gene expression but decreased the permeability levels of sucrose and mannitol. In turn, the work by DeStefano *et al*[31] focused solely on the effect of increasing shear stress (0, 4, or 12 dyne/cm^2) on the genotype and phenotype of iPSC-derived ECs. The authors found no correlation between the presence of shear stress and the expression of a more robust EC phenotype, since tight junctions are already formed under static conditions. Some authors considered that perfusion makes a bigger difference in BBB models made of non-iPSC-derived EC cultures such as HUVECs or primary ECs[31,54], whereas iPSC-derived ECs do not experience drastic changes in BBB markers upon shear stress[21,31]. Motallebnejad *et al*[24] gathered evidence that culturing iPSC-derived ECs on laminin511-E8 ECM improves the response of ECs to shear stress, wherein the cells exhibit changes in morphology, surface area, and the upregulation of tight junction proteins.

Regarding BBB applications, the specialized literature has not yet been able to establish a consistent microfluidic BBB model that can cover a broad range of applications, from modeling CNS disorders, including cancer metastasis, to screening for drugs able to permeate the BBB to treat such maladies. This is reflected in the goals of most studies selected to be part of this review. The great majority of the articles (roughly 80%) were aimed at developing a microfluidic BBB model, as well as assessing the convenience of the manufactured model by quantifying common molecular markers that are normally expressed in cells that make up the BBB *in vivo*[18-21,23,24,26-30]. Only one of the studies focused not on the BBB model itself but on the permeation potential of either commercial or synthesized polymer NPs through the *in vitro* 3D BBB mimic[25]. The BBB is a significant obstacle to the effective transport of large molecules for the treatment of brain disorders, and a study by Lee *et al*[25] showed that iPSC-ECs are appropriate for generating the 3D *in vitro* BBB model[25]. Surface modification of NPs with ligands for specific binding to BBB cells enhances NP accumulation in the brain through receptor-mediated endocytosis, providing good conditions to explore personalized therapies[25].

Another different application for the 3D BBB model was reported in a study by Wevers *et al*[20], which employed hypoxia together with hypoglycemia and lack of perfusion to successfully mimic ischemic stroke on-a-chip[20]. This hypoxic condition was also used to improve the iPSC differentiation to BMECs inside the microfluidic device by Park *et al*[28]. Based on the hypoxic environment found during the embryological development of the BBB *in vivo*, the whole differentiation step of iPSCs into ECs, both on a plate and in the microfluidic chip, was performed under low oxygen (5% O_2). For the cells cultured under hypoxia, the authors observed the upregulation of diverse tight junction proteins and membrane transporters, contributing to a more robust BBB function. Moreover, they found that the differentiated ECs produce hypoxia-inducible factor 1α, a molecular marker whose presence was found to improve the function and durability of the BBB model[28].

Concerning the characterization techniques, the structural evaluation of the BBB model was mainly targeted by immunocytochemistry, whereas functional aspects have been mainly assessed using fluorescence techniques. Immunocytochemistry assays are the golden standard for identifying proteins related to the BBB, as well as for identifying different stages of BBB maturation (early, intermediate, and late phases of BBB development)[55]. Fluorescence assays are easily accessible and generally offer a vast array of markers that can be used for various purposes, from evaluating BBB permeability and integrity to neuronal functionality. Many studies mentioned TEER measurements as an important method to characterize BBB function[20,21,24,27,28,30,56]. However, they pose a number of disadvantages. The TEER values across BBB culture models are frequently influenced by disregarded physical and technical factors such as temperature, viscosity, and current density, generated by various electrode types, surface size, circumference, and porosity of the insert membrane, leading to severalfold differences within the

same biological model[57]. Additionally, these TEER values can vary according to cell type (primary, immortalized, iPSC-derived), cell source (animal or human), and culture type (2D, 3D culture, co-culture)[58]. Therefore, the comparison of TEER values among different studies is highly discouraged in the relevant literature.

This systematic review highlights the main aspects of the current literature on BBB models on-a-chip, from iPSC differentiation to the NVU formation and characterization inside the microfluidic devices, also considering the functional duration of the proposed models by diverse approaches, focusing on future applications. However, the lack of uniform nomenclature and protocols made it a challenge to deeply analyze and comprehend all the steps in the processes involved.

The construction of BBB-on-a-chip still faces numerous challenges in producing feasible, reproducible, and reliable models. This becomes apparent in that, among the 14 articles studied in this review, 12 focused on the development of a robust *in vitro* model[18,19,21-24,26-31], and only 2 focused on the application of a model to study BBB disruption following ischemic stroke[20] and study the BBB permeability to polymer NPs[25]. Even though the use of iPSCs in microfluidic systems poses an advancement in these BBB constructs, there remain obstacles in the use of this type of cells, especially related to differentiation protocols, which need to be further developed in order to result in more dependable cell phenotypes. Moreover, on-chip technology remains a costly *in vitro* alternative, and the in-house manufacture of microfluidic devices is hampered by the need for a clean room and specialized equipment and personnel.

The integration of sensors to monitor barrier permeability and neuronal response to drug exposure is an important aspect scarcely addressed. The addition of such sensors could enable more precise and real-time measurements of drug transport across the BBB and its effects in brain cells. In terms of potential impacts, this technology can accelerate the development of new drugs by allowing for faster and more accurate screening of compounds that cross the BBB for therapeutic effects on the brain. One important step towards this scenario was already taken in the study by Vatine *et al*[27], who were successful in perfusing whole human blood through their microfluidic device, creating promising conditions to evaluate disease[27]. Moreover, more reliable BBB-on-a-chip constructs may help to better understand the *in vivo* functioning of the BBB across different individuals, representing a significant impact on personalized medicine.

CONCLUSION

Despite the well-known literature on iPSCs, microfluidic devices, and BBB *in vitro* models in isolation, the combination of the three subjects is a currently relevant theme, already presenting with advanced technology in the use of commercial microdevices for modeling the BBB. The BBB-on-a-chip models, in spite of their particularities in each study, have shown to be efficient for reconstructing the NVU *in vitro*, being able to reproduce the characteristics found *in vivo* in terms of structure and function. They have displayed promising qualities for investigating barrier disorders, drug delivery, mimicking disease, and personalized medicine.

ARTICLE HIGHLIGHTS

Research background

Induced pluripotent stem cells (iPSCs) offer a potential alternative to building blood-brain barrier (BBB)-on-a-chip models that more closely resemble the structure and functions found *in vivo*.

Research motivation

iPSC-derived BBB models on-a-chip are a promising field that still lacks improvements and uniformity within the specialized literature.

Research objectives

To search the literature and analyze the selected data on the cultivation of iPSCs within microfluidic environments to mimic the human BBB.

Research methods

A literature search using the PRISMA approach using the following terms: “iPSC,” “BBB,” and “microfluidic device,” wherein 14 studies were selected based on the inclusion and exclusion criteria, and data were organized into three tables and one flow chart.

Research results

Studies have been found from 2017 to 2022, wherein the microdevices were either commercially

available or manufactured in-house using soft-lithography. iPSCs were differentiated into endothelial or neural cells and seeded in the chips individually or in co-culture onto an extracellular-matrix layer mainly made of collagen IV/fibronectin. The selected studies focused principally on the structural and functional design of the human BBB model on-a-chip, displaying future application potential for drug screening and disease modeling.

Research conclusions

Despite the lack of consensus in protocols, the studies analyzed herein were able to efficiently reproduce a human microfluidic BBB *in vitro* making use of iPSCs.

Research perspectives

The developed BBB models on-a-chip have shown great potential to study physiopathological conditions related to the central nervous system, to apply advanced technology approaches for novel therapies (drug delivery through nanoparticles), and to develop genetic modification therapies for BBB dysfunctions through clustered regularly interspaced short palindromic repeats methodologies.

FOOTNOTES

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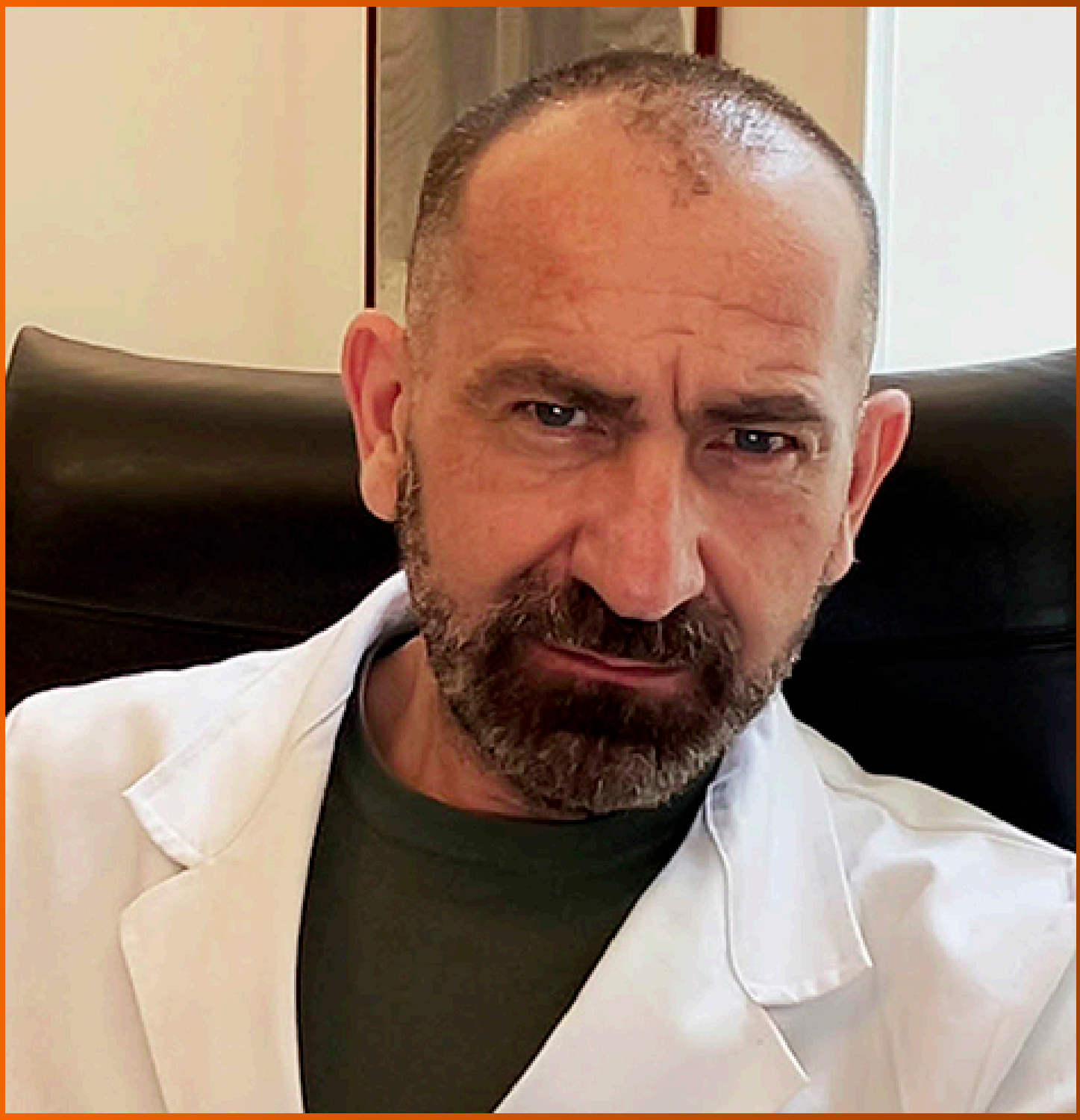


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OPINION REVIEW

Could extracellular vesicles derived from mesenchymal stem cells be a potential therapy for acute pancreatitis-induced cardiac injury?

Long-Fei Pan, Ze-Qun Niu, Song Ren, Hong-Hong Pei, Yan-Xia Gao, Hui Feng, Jiang-Li Sun, Zheng-Liang Zhang

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Abstract

Acute pancreatitis (AP) often leads to a high incidence of cardiac injury, posing significant challenges in the treatment of severe AP and contributing to increased mortality rates. Mesenchymal stem cells (MSCs) release bioactive molecules that participate in various inflammatory diseases. Similarly, extracellular vesicles (EVs) secreted by MSCs have garnered extensive attention due to their comparable anti-inflammatory effects to MSCs and their potential to avoid risks associated with cell transplantation. Recently, the therapeutic potential of MSCs-EVs in various inflammatory diseases, including sepsis and AP, has gained increasing recognition. Although preclinical research on the utilization of MSCs-EVs in AP-induced cardiac injury is limited, several studies have demonstrated the positive effects of MSCs-EVs in regulating inflammation and immunity in sepsis-induced cardiac injury and cardiovascular diseases. Furthermore, clinical studies have been conducted on the therapeutic application of MSCs-EVs for some other diseases, wherein the contents of these EVs could be deliberately modified through prior modulation of MSCs. Consequently, we hypothesize that MSCs-EVs hold promise as a potential therapy for AP-induced cardiac injury. This paper aims to discuss this topic. However, additional research is essential to comprehensively elucidate the underlying mechanisms of MSCs-EVs in treating AP-induced cardiac injury, as well as to ascertain their safety and efficacy.

Key Words: Acute pancreatitis; Cardiac injury; Mesenchymal stem cells; Extracellular vesicles; Inflammation; Therapeutic strategies

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Core Tip: Acute pancreatitis (AP) often causes cardiac injury, leading to poor prognosis. Mesenchymal stem cells (MSCs) and their extracellular vesicles (EVs) possess anti-inflammatory properties and have been studied as a potential therapy for inflammatory diseases. Although preclinical studies on the use of MSCs-EVs for AP-induced cardiac injury are lacking, research has demonstrated their positive effects in various inflammatory diseases such as sepsis-induced cardiac injury and cardiovascular diseases. Therefore, MSCs-EVs may represent a promising strategy for treating AP-induced cardiac injury.

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INTRODUCTION

Acute pancreatitis (AP) often results in important extra-pancreatic organ damage, including the lungs, kidneys, heart, liver, and intestines[1-3]. Cardiac injury associated with AP frequently manifests with non-specific symptoms, or is overshadowed by symptoms caused by AP itself, making it easy to be overlooked and leading to the misconception that cardiac injury is uncommon in AP[4]. However, studies have reported a 100% incidence rate of cardiac injury in all cases of AP, with cardiac injury occurring earlier than damage to organs such as the lungs and kidneys[5,6]. The severity of cardiac injury is closely related to the severity of AP, with severe acute pancreatitis (SAP) having the highest occurrence rate of severe cardiac injury, which can reach up to 60.5%, and in some severe cases, SAP can even lead to cardiac dysfunction[7,8]. Retrospective analysis has also confirmed significantly elevated serum cardiac injury markers in SAP patients upon admission[6]. Among SAP patients who succumbed to multiple organ dysfunction syndrome, approximately 86.4% experienced cardiovascular failure[9]. Cardiac injury is a significant contributing factor to the challenges in curing and increased mortality rates of SAP, with approximately 10%-30% of SAP fatalities attributed to SAP-induced cardiac injury[7]. Therefore, it is crucial to give adequate clinical attention to AP-induced cardiac injury. However, the exact mechanisms underlying AP-induced cardiac injury are complex, involving multiple factors, and the precise mechanism remains unclear, posing a significant challenge in the development of effective treatments.

Mesenchymal stem cells (MSCs) are among the most readily accessible types of stem cells, which can be extracted from sources such as bone marrow, adipose tissue, umbilical cord, and dental pulp. They possess unique characteristics of self-renewal and multi-directional differentiation, allowing them to differentiate into various cell types. MSCs participate in various inflammatory diseases through the paracrine secretion of bioactive molecules and can also promote tissue repair [10,11]. Moreover, MSCs can regulate the phenotype of immune cells and alleviate inflammatory responses[12]. However, the long preparation period required for MSCs transplantation makes it unsuitable for emergency situations. Additionally, MSCs carry the risks of immunogenicity and tumorigenicity, which to some extent limits their application [13].

The various biological functions of MSCs are primarily carried out through the secretion of bioactive molecules, encompassing a diverse range of chemical factors, cytokines, immunomodulatory factors, extracellular matrix components, as well as several other proteins, nucleic acids, and lipids[14]. MSCs also release extracellular vesicles (EVs) that serve as the principal mediators for MSCs to exert their immune and inflammatory regulatory effects. MSCs-EVs act as carriers, transporting bioactive molecules such as growth factors, cytokines, chemokines, mRNA, miRNA, lncRNA, *etc.*, which they transmit through paracrine or endocrine secretion[15]. EVs have been identified as effective therapeutic vehicles capable of delivering various proteins and regulatory genes to target organs. MSCs-EVs are believed to possess similar anti-inflammatory and other biological effects as MSCs, while offering advantages over MSCs such as smaller size, targeted transport, and low immunogenicity[16]. Therefore, MSCs-EVs hold the potential to replace MSCs in exerting therapeutic effects on certain diseases.

MSCS-EVS HAVE EMERGED AS A PROMISING TREATMENT OPTION FOR VARIOUS INFLAMMATORY DISEASES

With the increasing focus on the immunomodulatory effects of MSCs, extensive research has been conducted to investigate their mechanisms in inflammatory diseases. MSCs-EVs, which possess similar anti-inflammatory effects as MSCs and the ability to avoid potential risks associated with cell transplantation, have been widely studied in the context of inflammatory diseases. Multiple studies have demonstrated the efficacy of MSCs-EVs in improving inflammatory responses in animal models of various diseases, including brain ischemia-reperfusion injury, acute lung injury/acute respiratory distress syndrome (ARDS), inflammatory bowel disease, acute liver injury, acute kidney injury, sepsis, AP, myocardial ischemia-reperfusion injury, and acute myocardial infarction[17]. For instance, MSCs-EVs can regulate inflammatory and immune responses following brain ischemia by modulating the central nervous system, peripheral immune system, and immune regulatory molecules, thereby promoting neurological function recovery[18]. By transmitting miRNA, MSCs-EVs can reduce the secretion of pro-inflammatory cytokines, oxidative stress, and prevent

lung tissue infiltration by inflammatory cells, thereby alleviating ventilator-associated lung injury[19]. Moreover, MSCs-EVs can regulate gene expression and inhibit the production of inflammatory cytokines by transmitting miRNA or other bioactive molecules, thereby reducing neutrophil infiltration, improving lung inflammation and oxidative damage, and promoting the survival, proliferation, and differentiation of alveolar epithelial cells and endothelial cells. This, in turn, facilitates lung tissue repair, regeneration, and improvement of lung function[19-22]. In acute liver failure, MSCs-EVs can inhibit inflammasomes, reduce levels of inflammatory factors, and alleviate inflammatory response, thus improving acute liver injury[23].

MSCs-EVs for the treatment of sepsis and sepsis-induced cardiac injury

MSCs-EVs have shown promise in treating sepsis by modulating the immune response and mitigating inflammatory damage through various mechanisms. These mechanisms include regulating cytokine production, reducing oxidative stress, and promoting immune cell proliferation and differentiation[24]. In septic mice, MSCs-EVs have demonstrated the ability to decrease pro-inflammatory cytokine levels while promoting the production of anti-inflammatory cytokines, thus improving survival rates[25]. Furthermore, MSCs-EVs have been discovered to alleviate sepsis-induced acute lung injury by suppressing the mitogen-activated protein kinase (MAPK)/nuclear factor-κB (NF-κB) pathway. This regulation leads to the modulation of immune cell activity, reduction of oxidative stress levels, and promotion of cell survival and regeneration[26]. Additionally, MSCs-EVs can also improve the severity of sepsis-induced renal injury by directly delivering biologically active molecules, such as circRNA[27]. Moreover, through the delivery of miRNA and other biologically active molecules, MSCs-EVs can inhibit macrophage apoptosis, regulate macrophage polarization, and induce macrophage M2 polarization. These actions help to modulate immune responses and inflammation, promote tissue repair, and ultimately alleviate sepsis-induced acute lung and kidney injury[28-31].

Between 40% and 60% of sepsis patients experience cardiac injury[32]. Numerous studies have demonstrated the potential of MSCs-EVs in alleviating sepsis-induced cardiac injury. For example, MSCs-EVs carrying miR-223 can prevent myocardial cell apoptosis and suppress the inflammatory response by inhibiting the NF-κB signaling pathway, which in turn can help to prevent sepsis-induced cardiac injury[33]. MSCs-EVs enriched with miR-146a-5p can promote cell proliferation and survival by regulating Myb-like protein 1 expression, thus protecting septic myocardial cells or tissue[34]. In addition, the delivery of miR-223-3p by MSCs-EVs can suppress sepsis-induced cardiac inflammation, pyroptosis, and dysfunction[35]. Moreover, MSCs-EVs containing miR-141 can activate β-catenin by targeting phosphatase and tensin homolog deleted on chromosome 10 (PTEN), thereby mitigating septic mouse myocardial injury[36]. Furthermore, MSCs-EVs can also deliver circRTN4 to inhibit cardiac fibrosis and inflammation through the miR-497-5p/mitsugumin 53 signaling axis, thus preventing sepsis-induced cardiac injury[37]. Finally, the presence of PTEN-induced putative kinase 1 in MSCs-EVs can restore mitochondrial $_{m}Ca^{2+}$ efflux, thereby preventing septic myocardial cell mitochondrial calcium overload[38].

MSCs-EVs can be used for the treatment of AP

Limited studies have been conducted on the role of MSCs-EVs in AP. Some studies have demonstrated the potential of human umbilical cord MSCs-EVs in reducing the severity of traumatic pancreatitis by colonizing injured pancreatic tissue, which in turn regulates inflammatory cytokine levels and inhibits acinar cell apoptosis[39]. Furthermore, human umbilical cord MSCs-EVs have shown promise in improving the inflammatory response in mild to moderate traumatic pancreatitis by suppressing inflammation, increasing cell proliferation, and inhibiting pancreatic acinar cell apoptosis. These effects promote the repair of pancreatic tissue, leading to effective relief of traumatic pancreatitis[40]. In addition, MSCs-EVs derived from hair follicles have also been found to promote pancreatic tissue repair and enhance pancreatic function by reducing inflammation in pancreatic cells and inhibiting cell pyroptosis-related signaling pathways[41]. These findings suggest that MSCs-EVs have therapeutic potential in treating AP and may offer a promising alternative to traditional treatments.

Table 1 presents a summary of the factors delivered by MSCs-EVs in inflammatory diseases, the signaling pathways that these factors mediate, and the ultimate effects of MSCs-EVs on inflammation or immunity.

RESEARCH PROGRESS OF USING MSCS-EVS FOR TREATING AP-INDUCED CARDIAC INJURY

In recent years, there has been growing recognition of the therapeutic potential of MSCs-EVs in the treatment of inflammatory diseases. Sepsis and AP, which are closely associated with inflammatory reactions, can result in cardiac and other organ injuries due to the amplification of the inflammatory response during their course. MSCs-EVs, on the other hand, have been shown to have potential therapeutic effects for both conditions.

Although research on the use of MSCs-EVs for AP-induced cardiac injury is limited, several studies have confirmed their potential in treating sepsis-induced cardiac injury, as well as their potential as a therapeutic strategy for various cardiovascular diseases. For instance, MSCs-EVs have been demonstrated their ability to improve myocardial inflammation, reduce cell apoptosis, and promote cardiac remodeling and function following acute myocardial infarction. This is achieved by inhibiting the activation of the NF-κB signaling pathway through the transmission of miR-302d-3p[42]. Additionally, MSCs-EVs have been found to alleviate myocardial ischemia-reperfusion injury by suppressing M1 polarization of macrophages through the inhibition of Toll-like receptor 4 (TLR4) *via* the transmission of miR-182[43]. Based on these findings, it is reasonable to speculate that MSCs-EVs may also exert therapeutic effects on AP-induced cardiac injury.

Table 1 Therapeutic potential of MSCs-EVs in inflammation disease

Cell model/animal model	Target cells in tissue	Source of MSCs	Cargo of MSCs-EVs	Factors or pathways involved	Therapeutic effects and mechanisms	Ref.
ALI						
Mouse BMDMs stimulated with LPS/C57BL/6 mouse with LPS-induced ALI	Alveolar macrophages	Human AT-MSCs	miR-27a-3p	NF-κB1	*In vitro*: MSCs-EVs facilitated M2 polarization of BMDMs through the inhibition of NF-κB1 expression; *in vivo*: Systemic or intratracheal administration of MSCs-EVs reduced NF-κB1 expression in alveolar macrophages *via* miR-27a-3p delivery, promoting macrophage M2 polarization and alleviating LPS-induced ALI	[20]
Mouse MLE-12 cells (lung epithelial cells) barrier model/ICR mouse with sulfur mustard-induced ALI	Lung epithelial cells	Mouse BM-MSCs	Not detected	GPRC5A/YAP axis	*In vitro*: MSCs-EVs dose-dependently inhibited sulfur mustard-induced lung epithelial cell apoptosis and promoted the repair of adherens and tight junction integrity through the regulation of the GPRC5A/YAP axis, ultimately facilitating the recovery of epithelial barrier function; *in vivo*: Administration of MSCs-EVs protected lung epithelial cells from apoptosis and epithelial barrier damage by regulating the GPRC5A/YAP axis, promoting the restoration of barrier function and exerting a protective effect against pulmonary edema in ALI	[21]
HLMVECs injured by a mixture of IL-1β, TNF-α, and interferon-γ which were often used as a surrogate for ALI pulmonary edema fluid/-	-	Human BM-MSCs	Ang1 mRNA	Not detected	*In vitro*: MSCs-EVs partially increased Ang1 secretion in injured HLMVECs through the transfer of Ang1 mRNA, subsequently promoting the secretion of anti-permeability factors, restoring intercellular junctions, and preventing the formation of "actin stress fiber", thereby dose-dependently restoring protein permeability across HLMVECs during ALI; *in vivo*: -	[22]
Sepsis						
-/BALB/C mouse with LPS-induced sepsis	Not detected	Human UC-MSCs	Not detected	Not detected	*In vitro*: -; *in vivo*: Administration of MSCs-EVs effectively mitigated the destructive effects of inflammation caused by sepsis by reducing inflammatory factors, thereby alleviating tissue damage	[25]
-/C57BL/6 mouse with CLP-induced sepsis-induced ALI	Not detected	Human UC-MSCs	Not detected	MAPK/NF-κB pathway	*In vitro*: -; *in vivo*: MSCs-EVs can inhibit the phosphorylation and activation of the MAPK/NF-κB pathway, increase heme oxygenase 1 expression, enhance nuclear factor erythroid 2-related factor 2 expression, and upregulate antioxidant enzyme levels, thereby suppressing the infiltration of polymorphonuclear neutrophils to alleviate lung inflammation, improving pulmonary microvascular permeability to mitigate pulmonary edema, ultimately enhancing the survival rate of mice with sepsis-induced ALI	[26]

-/C57BL/6N mouse with CLP-induced sepsis-induced renal injury	Not detected	Hypoxia pretreated mouse AT-MSCs	mmu_circ_0001295	Not detected	*In vitro*: -; *in vivo*: EVs secreted by hypoxia-pretreated MSCs can mitigate the elevated levels of plasma chemokines and cytokines induced by sepsis through the delivery of mmu_circ_0001295, thereby improving renal microvascular dysfunction, suppressing renal vascular leakage, and ultimately mitigating sepsis-induced renal dysfunction to enhance the survival rate of septic mice	[27]
Mouse RAW264.7 cells (monocytes/macrophages) stimulated with LPS/C57 mouse with LPS-induced sepsis	BMDMs	Mouse BM-MSCs	miR-17	BRD4/EZH2/TRAIL axis	*In vitro*: MSCs-EVs suppressed the inflammation caused by RAW264.7 cells under LPS stimulation by delivering miR-17 to regulate the BRD4/EZH2/TRAIL axis; *in vivo*: MSCs-EVs, through the delivery of miR-17 to regulate the BRD4/EZH2/TRAIL axis, decreased serum levels of pro-inflammatory cytokines and suppressed their expression in BMDMs, ultimately improving LPS-induced sepsis in mice and enhancing survival rates	[28]
Mouse BMDMs stimulated with LPS/C57BL/6 mouse with CLP-induced sepsis	Liver macrophages	IL-1β pretreated mouse MSCs (source not mentioned)	miR-21	PDCD4	*In vitro*: MSCs-EVs induced M2-like polarization of macrophages, and IL-1β-pretreated MSCs-derived EVs exhibited an enhanced capacity to promote macrophage polarization towards an M2-like phenotype; *in vivo*: MSCs-EVs, by delivering miR-21, suppressed the effects of PDCD4 and induced M2-like polarization of macrophages, resulting in reduced inflammation, alleviated symptoms, prevented the progression of sepsis, and ultimately improved the survival rate	[29]
-/C57BL/6 mouse with CLP-induced sepsis-induced ALI or LPS-induced ALI	Alveolar macrophages	Mouse BM-MSCs	SAA1	LPS	*In vitro*: -; *in vivo*: MSCs-EVs delivering SAA1 induced LPS internalization by mouse alveolar macrophages, leading to a decrease in inflammatory cytokine levels and ultimately alleviating sepsis-induced ALI	[30]
Mouse MH-S cells (alveolar macrophages) stimulated with LPS/C57BL/6 mouse with LPS-induced ARDS	Alveolar macrophages	Mouse BM-MSCs	Not detected	HIF-1α/glycolysis-related protein	*In vitro*: MSCs-EVs suppressed M1 polarization and promoted M2 polarization of alveolar macrophages by inhibiting cellular glycolysis, thereby exerting anti-inflammatory effects; *in vivo*: Intratracheal administration of MSCs-EVs attenuated the LPS-induced inflammatory response by suppressing glycolysis in alveolar macrophages *via* regulation of HIF-1α, leading to improved lung pathology, reduced lung edema, increased PaO_2/FiO_2 ratio, and therefore enhancing survival rate	[31]
Mouse RAW264.7 cells (monocytes/macrophages) or primary cardiomyocytes stimulated with LPS respectively/C57BL/6 mouse with LPS-induced sepsis-induced cardiac injury	Cardiomyocytes	Mouse BM-MSCs	miR-223, STAT3 and Sema3A proteins	STAT3, Sema3A	*In vitro*: MSCs-EVs suppressed the release of inflammatory cytokines in LPS-induced macrophages through the delivery of miR-223 and reduced LPS-induced cardiomyocyte apoptosis and cell death; *in vivo*: MSCs-EVs	[33]

					carrying miR-223 suppressed the expression of STAT3 and Sema3A, resulting in reduced serum levels of TNF-α, IL-1β, and IL-6, which in turn decreased cardiomyocyte apoptosis, improved cardiac function, and conferred cardio-protection in sepsis, ultimately reducing mortality. Additionally, by inhibiting miR-223 to pre-treat MSCs, the protein cargo within the secreted EVs can be reprogrammed, leading to an increased delivery of Sema3A and STAT3 proteins that exert detrimental effects on recipient cells	
Rat H9c2 cells (cardiomyocytes) stimulated with LPS/C57BL/6 mouse with LPS-induced sepsis-induced cardiac injury	Myocardium	Rat BM-MSCs	miR-146a-5p	MYBL1	*In vitro*: MSCs-EVs, by delivering miR-146a-5p, suppressed MYBL1 to inhibit the progression of LPS-induced cardiomyocyte inflammation, promoting cell proliferation, and inhibiting cell apoptosis; *in vivo*: MSCs-EVs administration can ameliorate cardiac injury and improve survival rates in septic mice	[34]
Human HL-1 cells (cardiomyocytes) model of cardiac dysfunction induced by LPS/C57BL/6 mouse with LPS-induced myocarditis	Cardiomyocytes	Mouse BM-MSCs	miR-223-3p	FOXO3/NLRP3 axis	*In vitro*: MSCs-EVs inhibited LPS-induced inflammation and pyroptosis in cardiomyocytes by delivering miR-223-3p, which targeted FOXO3 to suppress NLRP3 expression; *in vivo*: MSCs-EVs restricted myocardial tissue infiltration of inflammatory cells and inflammatory response, decreased cardiomyocyte pyroptosis, thus improving cardiac dysfunction by shuttling miR-223-3p, which targeted the FOXO3/NLRP3 axis	[35]
-/KM mouse with CLP-induced sepsis-induced cardiac injury	Cardiomyocytes	Mouse BM-MSCs	miR-141	PTEN/β-catenin axis	*In vitro*: -; *in vivo*: MSCs-EVs ameliorated myocardial impairment and improved cardiac function by attenuating myocardial inflammatory infiltration and cell apoptosis in septic mouse myocardial tissues through the delivery of miR-141 and regulation of the PTEN/β-catenin axis	[36]
RAT H9c2 cells or human AC16 cells (cardiomyocytes) stimulated with LPS respectively/wistar rat with CLP-induced sepsis-induced cardiac injury	Cardiomyocytes	Human MSCs (source not mentioned)	circRTN4	miR-497-5p/MG53 axis	*In vitro*: MSCs-derived exosomal circRTN4 improved cell survival and suppressed apoptosis in LPS-stimulated cardiomyocytes by targeting the miR-497-5p/MG53 axis; *in vivo*: MSCs-EVs, administered through injection into three different sites around renal tissue for three consecutive days after CLP, delivered circRTN4 to suppress oxidative stress, reduce inflammation factors, and alleviate apoptosis, resulting in the mitigation of cardiac injury	[37]
Human AC16 cells (cardiomyocytes) stimulated with LPS/C57BL/6 mouse with CLP-induced sepsis-induced cardiac injury	Cardiomyocytes	Human UC-MSCs	PINK1 mRNA	PKA/NCLX axis	*In vitro*: MSCs-EVs mediated the delivery of PINK1 mRNA to regulate cardiomyocyte $_{m}Ca^{2+}$ efflux through the PKA/NCLX axis; *in vivo*: MSCs-EVs mediated the transfer of PINK1	[38]

					mRNA, leading to the maintenance of normal $_{m}Ca^{2+}$ efflux, alleviation of mitochondrial calcium overload, and subsequent mitigation of cardiomyocyte injury caused by mitochondrial damage, resulting in improved cardiac function and increased survival rate	
AP						
-/SD rat with impactor-induced traumatic AP	Pancreatic tissue	Human UC-MSCs	Not detected	Not detected	*In vitro*: -; *in vivo*: MSCs-EVs inhibited the apoptosis of pancreatic acinar cells, controlled the systemic inflammatory response, and thereby attenuated pancreatic tissue injury and facilitated the repair of pancreatic tissue	[39, 40]
Mouse MPC-83 cells (pancreatic acinar cells) stimulated with caerulein/C57BL/6J mouse with caerulein-induced AP	Pancreatic acinar cells	Mouse HF-MSCs	Not detected	Pyroptosis-related protein	*In vitro*: MSCs-EVs enhanced cell viability, mitigated inflammation, and attenuated the expression of pyroptosis-related proteins in caerulein-stimulated pancreatic acinar cells; *in vivo*: Intraperitoneal or intravenous administration, especially intravenous injection, of MSCs-EVs, can mitigate pancreatic acinar cell pyroptosis, alleviate the inflammatory response and oxidative stress in AP, thus reducing the severity of pancreatic injury	[41]
Cardiovascular diseases						
Mouse HL-1 cells (cardiomyocytes) hypoxia model/C57BL/6J mouse with LAD ligation-induced AMI	Cardiomyocytes	Mouse BM-MSCs	miR-302d-3p	BCL6/MD2/NF-κB axis	*In vitro*: MSCs-EVs carrying miR-302d-3p improved the viability of hypoxic cardiomyocytes, suppressed inflammation, and inhibited apoptosis by targeting the BCL6/MD2/NF-κB axis; *in vivo*: Intramyocardial injection of MSCs-EVs carrying miR-302d-3p near the infarcted area attenuated cardiomyocyte apoptosis and cardiac inflammation by targeting the BCL6/MD2/NF-κB axis, leading to reduced infarct size and myocardial fibrosis, thereby suppressing post-AMI cardiac remodeling and improving cardiac dysfunction	[42]
Mouse RAW264.7 cells (monocytes/macrophages) stimulated with LPS/C57BL/6J mouse with LAD ligation-induced ischemia-reperfusion injury	Cardiac macrophages	Mouse BM-MSCs	miR-182	TLR4/NF-κB/PI3K/Akt signalling cascades	*In vitro*: MSCs-EVs carrying miR-182 facilitated the polarization of macrophages from an M1 to M2 phenotype in an inflammatory environment by inhibiting the TLR4/NF-κB signaling pathway and activating the PI3K/Akt signaling pathway through cross-talk between them; *in vivo*: MSCs-EVs carrying miR-182 regulated myocardial inflammation and reduced infarct size, thereby attenuating myocardial ischemia-reperfusion injury and improving cardiac function in mice through the promotion of macrophage M2 polarization *via* targeting the TLR4/NF-κB/PI3K/Akt signaling cascades	[43]

MSCs: Mesenchymal stem cells; EVs: Extracellular vesicles; BM-MSCs: Bone marrow-derived MSCs; AT-MSCs: Adipose tissue-derived MSCs; UC-MSCs: Umbilical cord-derived MSCs; HF-MSCs: Hair follicle-derived MSCs; Akt: Protein kinase B; ALI: Acute lung injury; AMI: Acute myocardial infarction; Ang1: Angiopoietin-1; AP: Acute pancreatitis; BCL6: B-cell leukemia/lymphoma 6; BMDMs: Bone marrow-derived macrophages; BRD4: Bromodomain-containing protein 4; CLP: Cecal ligation and puncture; EZH2: Enhancer of zeste homolog 2; FOXO3: Forkhead box protein O3; GPRC5A: G protein-coupled receptor family C group 5 member A; HIF-1α: Hypoxia-inducible factor-1α; HLMVECs: Human lung microvascular endothelial cells; IL-1β: Interleukin-1β; LAD: Left anterior descending coronary artery; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MD2: Myeloid differentiation protein 2; MG53: Mitsugumin 53; MYBL1: Myb-like protein 1; NCLX: Mitochondrial Na^+/Ca^{2+} exchanger; NF-κB: Nuclear factor-κB; NF-κB1: Nuclear factor-κB subunit 1; NLRP3: NOD-like receptor thermal protein domain associated protein 3; PDCD4: Programmed cell death 4; PI3K: Phosphatidylinositol 3-kinases; PINK1: PTEN-induced putative kinase 1; PKA: Protein kinase A; PTEN: Phosphatase and tensin homolog deleted on chromosome 10; SAA1: Serum amyloid A1; Sema3A: Semaphorin 3A; STAT3: Signal transducers and activators of transcription 3; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; YAP: Yes-associated protein.

In a study conducted by Chen *et al*[44], MSCs-EVs were found to be effective in reducing oxidative stress and inflammatory damage in cardiac tissue, decreasing cell apoptosis, improving cardiac function, and ultimately increasing the survival rate of rats with SAP. These beneficial effects were attributed to the activation of the protein kinase B (Akt)/nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) signaling pathway. In our own research[45], we delved into the potential protective effects and mechanisms of MSCs-EVs in SAP-induced cardiac injury. Our findings suggest that MSCs-EVs can downregulate the high mobility group box-1/TLR4 signaling axis and activate the Akt signaling pathway by delivering miR-29a-3p. This, in turn, helps improve myocardial inflammation, enhance myocardial cell vitality, reduce cell apoptosis, and ultimately alleviate myocardial damage while preserving cardiac function. Therefore, we hypothesize that MSCs-EVs may play a crucial role in the onset and progression of SAP-induced cardiac injury.

IS THERE POTENTIAL FOR USING MSCS-EVS IN THE TREATMENT OF AP-INDUCED CARDIAC INJURY?

Studies have demonstrated that injecting EVs derived from MSCs overexpressing macrophage migration inhibitory factor into the infarcted area of the heart can enhance myocardial angiogenesis, reduce cell apoptosis, decrease cardiac remodeling, and thereby improve cardiac function[46]. Pre-treatment of bone marrow-derived MSCs with fibronectin type III domain-containing protein 5 can promote the secretion of more EVs, which can inhibit the secretion of pro-inflammatory cytokines, increase anti-inflammatory cytokine levels, and promote M2 polarization of macrophages *via* NF-κB signaling pathway and Nrf2/HO-1 axis[47]. Furthermore, when bone marrow-derived MSCs are pre-treated with lipopolysaccharide, the EVs secreted by these MSCs can inhibit M1 polarization of macrophages, promote M2 polarization, alleviate inflammation and cell apoptosis, and thereby facilitate tissue repair in various inflammatory diseases[48]. These studies highlight the potential of MSCs-EVs for targeted interventions, as the cargo and level of bioactive molecules loaded in EVs can be modulated according to the specific therapeutic goals. The *In vitro* plasticity of MSCs-EVs suggests a promising avenue for their utilization in the treatment of specific diseases.

The potential of using MSCs-EVs for clinical therapy is being explored in the initial stages. A Phase I clinical trial has been conducted on MSCs-EVs containing KrasG12D siRNA for the treatment of metastatic pancreatic cancer, and Phase I and II clinical trials on MSCs-EVs transfected with miR-124 for the treatment of stroke have been initiated[49]. Encouraging results have been reported in some clinical studies. A Phase I clinical trial confirmed the safety and efficacy of intravenous injection of placenta-derived MSCs-EVs for treating complex anal fistula in non-Crohn's disease patients [50]. In another Pilot Randomized Clinical Trial, placenta-derived MSCs-EVs were injected intraparenchymally to patients who underwent decompressive craniectomy after malignant middle cerebral artery infarction, and no significant adverse events were observed[51].

Due to the membrane of MSCs-EVs can be modified with specific ligands or peptides, they can be engineered to effectively target specific tissues or cells[52]. Additionally, MSCs-EVs can be stored for extended periods of time. Therefore, when compared to cell-based therapies, MSCs-EVs offer several advantages, including lower risks of immune rejection and tumorigenesis, lower costs, and on-demand availability. As a result, MSCs-EVs may present a promising potential as a viable strategy for treating AP-induced cardiac injury. However, there are still challenges to overcome in optimizing the separation and characterization of EVs, ensuring their purity and potency, and determining appropriate doses and delivery routes for MSCs-EVs in the treatment of AP-induced cardiac injury. Furthermore, potential safety concerns associated with the use of MSCs-EVs, such as the risk of thrombosis formation, immunogenicity, and potential tumorigenicity due to targeted delivery failure, must be carefully evaluated in clinical trials.

CONCLUSION

In conclusion, AP poses a significant global health threat, particularly when accompanied by cardiac injury, as it can complicate the treatment of SAP and worsen prognosis. Although there is currently limited preclinical research on the effectiveness of MSCs-EVs in treating AP-induced cardiac injury, multiple studies have demonstrated their ability to mitigate inflammation in various inflammatory diseases, including AP, regulate the immune response, promote tissue

regeneration, and improve sepsis-induced cardiac injury and various cardiovascular diseases. Therefore, we postulate that MSCs-EVs may hold promise as a potential treatment for AP-induced cardiac injury. However, further experimental research is necessary to explore their mechanisms, clarify treatment targets, and identify intervention pathways.

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FOOTNOTES

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REVIEW

Human retinal secretome: A cross-link between mesenchymal and retinal cells

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Abstract

In recent years, mesenchymal stem cells (MSC) have been considered the most effective source for regenerative medicine, especially due to released soluble paracrine bioactive components and extracellular vesicles. These factors, collectively called the secretome, play crucial roles in immunomodulation and in improving survival and regeneration capabilities of injured tissue. Recently, there has been a growing interest in the secretome released by retinal cytotypes, especially retinal pigment epithelium and Müller glia cells. The latter trophic factors represent the key to preserving morphofunctional integrity of the retina, regulating biological pathways involved in survival, function and responding to injury. Furthermore, these factors can play a pivotal role in onset and progression of retinal diseases after damage of cell secretory function. In this review, we delineated the importance of cross-talk between MSCs and retinal cells, focusing on common/induced secreted factors, during experimental therapy for retinal diseases. The cross-link between the MSC and retinal cell secretomes suggests that the MSC secretome can modulate the retinal cell secretome and vice versa. For example, the MSC secretome can protect retinal cells from degeneration by reducing oxidative stress, autophagy and programmed cell death. Conversely, the retinal cell secretome can influence the MSC secretome by inducing changes in

MSC gene expression and phenotype.

Key Words: Secretome; Mesenchymal stem cells; Retinal cells; Extracellular vesicles; Retinal diseases

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Core Tip: Recently, the mesenchymal stem cell secretome, a solution rich with paracrine bioactive factors and extracellular vesicles, acquired a significant role in immunomodulation and survival induction of damaged tissues. A secretome is also released by retinal cells, physiologically or following pathological stimuli. One of the most promising therapeutic frontiers is represented by a possible "cross-talk" between mesenchymal stem cells and retinal cells through the secretomes in order to improve the knowledge on released factors mechanisms of action during their potentially beneficial role.

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INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have been indicated as the most effective source for cell-based therapy, particularly in regenerative medicine. In particular, MSCs produce major therapeutic effects releasing soluble paracrine bioactive components and extracellular vesicles (EVs) constituting the so called secretome. These secreted factors play crucial roles in modulating immunity and improving survival and regeneration capabilities of injured tissue[1].

Secreted trophic factors are also key to preserving the morphofunctional integrity of the retina, regulating biological pathways involved in survival, function and response to injury[2]. Additionally, these factors can play a fundamental role in onset and progression of retinal diseases after damage of cell secretory functions[3]. In this review, we discussed the link between the secretome of MSCs to the retinal cell secretome, in order to highlight the current knowledge of secreted factor involvement in retinal diseases.

Main features of MSCS

One of the most recent fields of therapy research concerns MSCs, multipotent non-hematopoietic stem cells that originate from the mesoderm. They can reach a pathological site following the release of different biologically active immunomodulatory and regenerative factors related to different diseases[4]. There are multiple sources of MSCs, including umbilical cord blood, placenta, adipose tissue, skin and bone marrow tissue, with the latter representing the most widely used source[5]. Isolation of different types of MSCs, such as adipose tissue-derived mesenchymal stromal/stem cells (ASCs), is a non-invasive process, and this represents a fundamental advantage from an ethical and/or legal point of view[6]. The key point for MSC use is their low immunogenicity, permitting allogeneic trans-plantation in the medical setting[7].

Moreover, recent studies have shown that MSCs can produce an immune response, mediated by T cells regulated by IFN-γ[8]. When activated, MSCs can reach the correct pathological site to exert reparative functions, triggered by a huge number of secreted factors from the injured cells, such as cytokines, chemokines and growth factors[9]. Among the latter, placental growth factor plays a pivotal role, along with VEGF, EPO, SDF-1, ANG2, G-CSF, stem cell factor, PDGF, EGF, HGF and IGF-1[10]. Regarding cytokines and chemokines, the former include TNF-α and interleukins such as IL-1b, IL-2, IL-3, IL-6 and IL-8, and the latter includes, among others, CCL5 and CCL22[11].

Various studies have confirmed that human MSCs evade allorecognition, affect T lymphocytes and dendritic cell activities and produce a local immunosuppressant microenvironment by releasing the already cited cytokines[12]. Moreover, MSCs can be easily genetically manipulated, with elevated metabolic activity and low mutation rate, and can efficiently secrete a wide number of proteins[13]. Today, preclinical and clinical trials using MSCs, especially human bone marrow-derived MSCs (hBMMSCs) and human adipose mesenchymal stem cells (hADSCs), have been performed in different kinds of pathologies with promising results, such as autoimmune disease, joint reconstruction, vascular disease, nerve injury, organ transplantation, degenerative disease and severe infection[14].

In particular, one of the fields with the highest number of ongoing clinical trials is represented by eye diseases (Table 1). The protective activity of MSCs was initially linked to their direct differentiation and replacement of injured tissues, as evidenced by human MSCs becoming hepatocyte-like cells or rat MSCs turning into neuron-like cells[15]. However, today the protective action of MSCs is well known to be primarily mediated through paracrine properties, exerted by what is defined as the MSC secretome.

MSC secretome

The secretome released by MSCs consists of a conditioned medium (CM) made up of soluble elements (cytokines and growth factors) and a vesicular part made up of exosomes and microvesicles, which are fundamental for protein and genetic material transfer towards other cells[16]. The most recent *in vitro* and *in vivo* studies on the features of the MSC

Table 1 Ongoing clinical trials on eye diseases based on the use of mesenchymal stem cells

Title	Sponsor/collaborators	URL
Safety and Efficacy of Pluripotent Stem Cell-derived Mesenchymal Stem Cell Exosome (PSC-MSC-Exo) Eye Drops Treatment for Dry Eye Diseases Post Refractive Surgery and Associated With Blepharospasm	Second Affiliated Hospital, School of Medicine, Zhejiang University | Zhejiang University | Hangzhou yuansheng biotechnology Co., Ltd	https://ClinicalTrials.gov/show/NCT05738629
Therapeutic Effect of Stem Cell Eye Drops on Dry Eye Disease	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	https://ClinicalTrials.gov/show/NCT05784519
Safety of Cultured Allogeneic Adult Umbilical Cord Derived Mesenchymal Stem Cells for Eye Diseases	The Foundation for Orthopaedics and Regenerative Medicine	https://ClinicalTrials.gov/show/NCT05147701
The Role of Transscleral Cyclophotocoagulation in Patients Undergoing a Boston Keratoprosthesis	Centre hospitalier de l'Université de Montreal (CHUM) | Fonds de recherche en ophtalmologie de l'Université de Montreal	https://ClinicalTrials.gov/show/NCT04232982
Characterization of Potential Biomarkers of Eye Disease and Vision	Association for Innovation and Biomedical Research on Light and Image	https://ClinicalTrials.gov/show/NCT02500862
Long-term Safety of UC-MSC Transplantation in Patients With Retinitis Pigmentosa	PT. Prodia Stem Cell Indonesia	https://ClinicalTrials.gov/show/NCT05786287
Clinical Evaluation of Two Daily Disposable Lenses in Sphere Design	Coopervision, Inc.	https://ClinicalTrials.gov/show/NCT05516082
Efficacy of Locally Delivered Allogeneic Mesenchymal Stromal Cells	University of Illinois at Chicago | United States Department of Defense	https://ClinicalTrials.gov/show/NCT05705024
Diquafosol *vs* Hyaluronic Acid for Diabetic Dry Eye	He Eye Hospital	https://ClinicalTrials.gov/show/NCT05682547
Eye Length Signal With Myopia Control	Brien Holden Vision Institute	https://ClinicalTrials.gov/show/NCT04813640
Patient Acceptability of Autonomous Telemedicine	Ufonia | Buckinghamshire Healthcare NHS Trust | Innovate UK	https://ClinicalTrials.gov/show/NCT04885868
Phase 2b Pivotal Study of Izokibep in Non-infectious, Intermediate-, Posterior- or Pan-uveitis	ACELYRIN Inc.	https://ClinicalTrials.gov/show/NCT05384249
Dose Optimization for Safe and Efficient Fluorescein Angiography (DOSE Study)	Seoul National University Bundang Hospital	https://ClinicalTrials.gov/show/NCT05664555
Analysis of the Results of Intense Pulsed Light Treatment Previously to Laser Refractive Surgery	Vissum, Instituto Oftalmologico de Alicante	https://ClinicalTrials.gov/show/NCT05139511
Study of the Association Between Digital Eye Syndrome With Binocular Vision and the Ocular Surface in Higher Education Students in the Area of Health Technologies	Universidade Nova de Lisboa | NOVA Medical School Faculdade de Cinecias Medicas, Universidade Nova de Lisboa | Escola Superior de Tecnologia da Salude de Lisboa (ESTeSL) | University of lâvora | CINTESIS@RISE, NOVA Medical School Faculdade de Ciencias Medicas, Universidade Nova de Lisboa | Comprehensive Health Research Center (CHRC), Universidade Nova de Lisboa	https://ClinicalTrials.gov/show/NCT05675475
Caffeine Consumption and Cataract Prevention	Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT05194696
Rotational Stability of the TECNIS Eyhance Toric	Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT05126368
Reading Performance in Patients With Acrysof IQ Vivity Versus Acrysof IQ	Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT05194657
Performance of Two Intraocular Lenses With Extended Depth of Vision	Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT05194150
Assessment of Retinal Vascular Changes With and Without ILM Peeling in Diabetic Vitrectomy Using OCT-A	Kasr El Aini Hospital	https://ClinicalTrials.gov/show/NCT05739539
Primary Vitrectomy With Silicone Oil or SF6 for Rhegmatogenous Retinal	Cairo University	https://ClinicalTrials.gov/show/NCT05377606

Detachment		
Intravitreal Sirolimus as Therapeutic Approach to Uveitis	Stanford University | Santen Inc.	https://ClinicalTrials.gov/show/NCT01280669
Clinical Trial With Artiflex Presbyopic	Ophtec BV	https://ClinicalTrials.gov/show/NCT04632784
Post-Market Evaluation of the EVO ICL	Staar Surgical Company	https://ClinicalTrials.gov/show/NCT05538754
Clinical Evaluation of Two Multifocal Contact Lenses	Coopervision, Inc.	https://ClinicalTrials.gov/show/NCT05457608
Ologen Collagen Matrix Versus Mitomycin-C in Patients With Juvenile-onset Open Angle Glaucoma	L.V. Prasad Eye Institute	https://ClinicalTrials.gov/show/NCT03548805
A Comparative Study of Visual Outcome of Two Extended Depth of Focus Intraocular Lenses After Cataract Surgery	Cairo University	https://ClinicalTrials.gov/show/NCT05647421
Keratometric Change After XEN, Trabeculectomy and Tube Shunts	Centre hospitalier de l'Universitè de Montreal (CHUM) | Allergan	https://ClinicalTrials.gov/show/NCT04602923
Post-market Follow Up Study on Paragon CRT 100 (Paflufocon D)	Coopervision, Inc. | TigerMed	https://ClinicalTrials.gov/show/NCT04187599
Evaluating Two Multifocal Daily Disposable Contact Lenses	Coopervision, Inc.	https://ClinicalTrials.gov/show/NCT05579886
A Clinical Comparison of Two Soft Multifocal Contact Lenses	Coopervision, Inc.	https://ClinicalTrials.gov/show/NCT05794126
Ocular Surface Disease and IOP Monitoring With Travoprost Without Conservatives	Democritus University of Thrace	https://ClinicalTrials.gov/show/NCT05319470
Performance of Two Hydrophobic IOLs	Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT05639049
RayOne EMV Mini-monovision - Efficiency and Safety of 3 Grades of Mini-monovision	Somich, s.r.o.	https://ClinicalTrials.gov/show/NCT05417633
A Clinical Study to Evaluate the Potential Role of ACTH Gel in Patients With Scleritis	Metropolitan Eye Research & Surgery Institute | Mallinckrodt | Stanford University | Ocular Imaging Research and Reading Center | Foresight Studies, LLC	https://ClinicalTrials.gov/show/NCT03465111
Zimbabwe Eyecare And Learning (ZEAL): Formative Research on Hyperopia and Educational Outcomes in Primary School Children	Queen's University, Belfast | L.V. Prasad Eye Institute | University of Zimbabwe | University of Ulster | New England College of Optometry | Peek Vision | Zimbabwe Optometric Association | Clearly | Christian Blind Mission	https://ClinicalTrials.gov/show/NCT05538182
Vision and Balance Changes After Bilateral Implantation of Toric IOLs	University of Plymouth | Carl Zeiss Meditec AG | Glasgow Caledonian University | University of St Mark and St John Plymouth | University Hospital Plymouth NHS Trust	https://ClinicalTrials.gov/show/NCT05629078
Multicenter Study on the Efficacy and Safety of OCS-01 in Subjects With Uveitis Related and Post Surgical Macular Edema	Quan Dong Nguyen | Global Ophthalmic Research Center (GORC) | Oculis | Stanford University	https://ClinicalTrials.gov/show/NCT05608837
Inflammatory Biomarkers in Ocular Surface in Primary Open Angle Glaucoma or Ocular Hypertension Under Topical Prostaglandins	Instituto Universitario de Oftalmobiologia Aplicada (Institute of Applied Ophthalmobiology) - IOBA | Hospital Clvinico Universitario de Valladolid	https://ClinicalTrials.gov/show/NCT05039684
Ranibizumab *vs* Bevacizumab for Type 1 Retinopathy of Prematurity	Zagazig University | Cairo University	https://ClinicalTrials.gov/show/NCT05033106
Dresden Corneal Disease and Treatment Study	Technische Universitut Dresden	https://ClinicalTrials.gov/show/NCT04251143
Reliability, Validity of the Turkish Version of the Primary Sjogren Syndrome Quality of Life (PSS-QoL) Questionnaire	Gazi University	https://ClinicalTrials.gov/show/NCT04858464
Mean Visual Acuity Changes Following Five Injections of Aflibercept	McMaster University	https://ClinicalTrials.gov/show/NCT02645266

Targeted Fluorescence Imaging in AMD	University Medical Center Groningen	https://ClinicalTrials.gov/show/NCT05262244
Treatment of Ligneous Conjunctivitis in Children With Plasminogen Deficiency	University of Saskatchewan | Canadian Blood Services	https://ClinicalTrials.gov/show/NCT05404932
Can the Risk for AMD be Modulated?	Association for Innovation and Biomedical Research on Light and Image	https://ClinicalTrials.gov/show/NCT05735730
Study to Evaluate the Response to Supplementation With Postbiotics in Patients With Macular Degeneration	Institut de la Macula y la Retina | Igen BioLab SLU	https://ClinicalTrials.gov/show/NCT05056025
Development of a Tele-Physiotherapy Tool for the Early Management of Muskuloskeletal Pain in People With Visual Impairement (TeleEDxPhysio)	Escuela Universitaria de Fisioterapia de la Once | Universidad de Zaragoza	https://ClinicalTrials.gov/show/NCT05478200
Clinical Trial to Evaluate Safety and Efficacy of Cell Therapy in Patients With Cicatricial Conjuntivitis	Instituto de Investigacion Sanitaria de la Fundacion Jimenez Diaz | Effice Servicios Para la Investigacion S.L	https://ClinicalTrials.gov/show/NCT05520086
Advanced Glaucoma Progression Study	University of California, Los Angeles | National Eye Institute (NEI)	https://ClinicalTrials.gov/show/NCT01742819
Retrobulbar Methylprednisolone as Adjunctive Treatment in Optic Neuritis Trial	Asociacion para Evitar la Ceguera en Mexico	https://ClinicalTrials.gov/show/NCT04942002
Nystagmus Assessment for Patients Consulting in the Emergency Department for Acute Vertigo	CHU de Quebec-Universite Laval	https://ClinicalTrials.gov/show/NCT05176015
Effectiveness of Periocular Drug Injection in CATaract Surgery	Luigi Rondas | European Society of Cataract and Refractive Surgeons | Academisch Ziekenhuis Maastricht	https://ClinicalTrials.gov/show/NCT05158699
Clinical Trial Comparing Two Non-Surgical Treatments for Severe Blepharoptosis	Massachusetts Eye and Ear Infirmary | National Eye Institute (NEI)	https://ClinicalTrials.gov/show/NCT04678115
Stem Cell Ophthalmology Treatment Study II	MD Stem Cells	https://ClinicalTrials.gov/show/NCT03011541
Effect of Intravenous Methylprednisolone and Intravenous Erythropoietin in Toxic Optic Neuropathies: Randomized Clinical Trial	Asociacion para Evitar la Ceguera en Mexico	https://ClinicalTrials.gov/show/NCT05748561
SPT Screening in Irradiated Hereditary Retinoblastoma Survivors	Amsterdam UMC, location VUmc | ODAS	https://ClinicalTrials.gov/show/NCT02329002
Community Access Through Remote Eyesight (CARE) Study	New England College of Optometry | National Institute on Disability, Independent Living, and Rehabilitation Research | University of California, Los Angeles	https://ClinicalTrials.gov/show/NCT04926974
Electro-acupuncture and Transcorneal Electrical Stimulation (TES) for Retinitis Pigmentosa	Nova Southeastern University | National Eye Institute (NEI)	https://ClinicalTrials.gov/show/NCT02086890
Evaluation of NeoRetina Artificial Intelligence Algorithm for the Screening of Diabetic Retinopathy at the CHUM	Centre hospitalier de l'Universitè de Montreal (CHUM) | DIAGNOS Inc.	https://ClinicalTrials.gov/show/NCT04699864
Clemastine Fumarate as Remyelinating Treatment in Internuclear Ophthalmoparesis and Multiple Sclerosis	Amsterdam UMC, location VUmc	https://ClinicalTrials.gov/show/NCT05338450
The K-Map Study, Global Prevalence of KC	University Hospital, Geneva | ELZA Institute	https://ClinicalTrials.gov/show/NCT03115710
Methotrexate For The Prevention and Treatment of Proliferative Vitreoretinopathy in Pediatric Patients	Stanford University	https://ClinicalTrials.gov/show/NCT04830878
A Collaborative Resource of Heidelberg Multimodal Imaging of Intermediate and Early Atrophic AMD Cases to Study Prediction of Disease Progression	Association for Innovation and Biomedical Research on Light and Image | European Vision Institute Clinical Research Network	https://ClinicalTrials.gov/show/NCT05698316

Clinical Trial of Multi-Periscopic Prism Glasses for Hemianopia	Massachusetts Eye and Ear Infirmary | National Eye Institute (NEI)	https://ClinicalTrials.gov/show/NCT04827147
Feasibility Tests for Various Prism Configurations for Visual Field Loss	Massachusetts Eye and Ear Infirmary | National Eye Institute (NEI)	https://ClinicalTrials.gov/show/NCT04424979
Quality Assurance Via Telephone Interviews After Cataract Surgery	Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT05215002
0.01% Hypochlorous Acid in the Treatment of Blepharitis	Eye & ENT Hospital of Fudan University | ShuGuang Hospital	https://ClinicalTrials.gov/show/NCT05608980
Birdshot Chorioretinopathy: Prospective Follow-up and Immuno-genetic Studies(CO-BIRD)	Assistance Publique - Hopitaux de Paris	https://ClinicalTrials.gov/show/NCT05153057
Characterization of Retinal Disease Progression in Eyes With Non Prolif-erative Diabetic Retinopathy in Diabetes Type 2 Using Non-invasive Procedures (CHART)	Association for Innovation and Biomedical Research on Light and Image | European Vision Institute Clinical Research Network	https://ClinicalTrials.gov/show/NCT04636307
Swiss Pediatric Inflammatory Brain Disease Registry (Swiss-Ped-IBrainD)	University of Bern | Schweizerische Multiple Sklerose Gesell-schaft | University Hospital Inselspital, Berne | Roche Pharma (Switzerland) Ltd | Novartis	https://ClinicalTrials.gov/show/NCT05017142
Topotecan Episcleral Plaque for Treatment of Retinoblastoma	Targeted Therapy Technologies, LLC	https://ClinicalTrials.gov/show/NCT04156347
Comparison of Phacoemulsification and Corneal Damage Between FLACS and Standard Phaco With Two Handpieces	Centre hospitalier de l'Universitè de Montreal (CHUM)	https://ClinicalTrials.gov/show/NCT05119270
Computer-based Tutorial and Automated Speech Recognition for Intravitreal Drug Injections	Prim. Prof. Dr. Oliver Findl, MBA | Vienna Institute for Research in Ocular Surgery	https://ClinicalTrials.gov/show/NCT04142164
OCS-05 in Patients With Acute Optic Neuritis	Oculis | Neurotrials	https://ClinicalTrials.gov/show/NCT04762017
PROgressive Supranuclear Palsy CorTico-Basal Syndrome Multiple System Atrophy Longitudinal Study UK	University College, London | University of Cambridge | University of Oxford | University of Manchester | Newcastle University | University of Sussex | Royal Gwent Hospital	https://ClinicalTrials.gov/show/NCT02778607
Therapeutic Recommendations For The Treatment Of Children With A Retinoblastoma	French Africa Pediatric Oncology Group	https://ClinicalTrials.gov/show/NCT04425434
Temperature on Evaporative Dry Eye	He Eye Hospital	https://ClinicalTrials.gov/show/NCT05720754
Prevalence of Visual Dysfunction in Neurological Disorders	University of Florida	https://ClinicalTrials.gov/show/NCT04836715
Intravitreal Infliximab for Proliferative Vitreoretinopathy	Cairo University	https://ClinicalTrials.gov/show/NCT04891991
A Patch Free Treatment for Young Children With Amblyopia	University of Waterloo | Retina Foundation of the Southwest | McGill University | Queensland University of Technology	https://ClinicalTrials.gov/show/NCT04086524
Effectiveness of the Serious Game 'Broodles' for Siblings of Children With Visual Impairment and/or Intellectual Disability	VU University of Amsterdam	https://ClinicalTrials.gov/show/NCT05376007
Screening for Oculocerebral Lymphoma With the Phenotype of NK Cells in Patients With Uveitis	Hospices Civils de Lyon	https://ClinicalTrials.gov/show/NCT05388838
10-year Progression of Diabetic Retinopathy: Identification of Signs and Surrogate Outcomes	Association for Innovation and Biomedical Research on Light and Image	https://ClinicalTrials.gov/show/NCT04650165
Management of DE With IPL in Combination With DQS	He Eye Hospital	https://ClinicalTrials.gov/show/NCT05694026
Endophthalmitis Post Intravitreal Injections	Rajeev Muni | Unity Health Toronto	https://ClinicalTrials.gov/show/NCT04035369
Discovering Early Biomarkers in Circulating Endothelial Cells for	Aarhus University Hospital | University of Aarhus	https://ClinicalTrials.gov/show/NCT05169502

Diabetes Complications by Single Cell RNA Sequencing		
Diabetic Retinopathy Classification: ETDRS 7-fields *vs* Widefield Imaging (ClarusDR)	Association for Innovation and Biomedical Research on Light and Image	https://ClinicalTrials.gov/show/NCT05746975
Evaluation of Desensitization Therapy and Re-treatment of Eye Movement Information [EMDR] in Patients With Post-traumatic Stress Disorder [PTSD]	Centre hospitalier de Ville-Evrard, France	https://ClinicalTrials.gov/show/NCT04431765
Prediction of Progression of Retinal Ischemia in Diabetes	Association for Innovation and Biomedical Research on Light and Image	https://ClinicalTrials.gov/show/NCT05581225
Personalized Parkinson Project PSP Cohort	Radboud University Medical Center | UCB Pharma | Verily Life Sciences LLC	https://ClinicalTrials.gov/show/NCT05501431
Enriched Eggs for Retina Health in Type 2 Diabetes	University of Manitoba | Egg Farmers of Canada	https://ClinicalTrials.gov/show/NCT04496817
Pneumatic Retinopexy Versus Vitrectomy for Retinal Detachment in Patients With Extended Criteria	Unity Health Toronto	https://ClinicalTrials.gov/show/NCT02871531
Adherence to Lifestyle Changes for Age-related Macular Degeneration	Erasmus Medical Center | CORR foundation	https://ClinicalTrials.gov/show/NCT05667441
Timing of Glaucoma Drainage Device With Boston Keratoprosthesis	Centre hospitalier de l'Universitè de Montreal (CHUM)	https://ClinicalTrials.gov/show/NCT02084745
Patient Satisfaction and Visual Function Following Implantation of Trifocals or Extended Range of Vision Intraocular Lenses	Queen's University | University of Toronto	https://ClinicalTrials.gov/show/NCT04900662
A Computerized, Adaptive Therapeutic Gaming Approach Training Visual Perceptual Skills in Children With CVI	Universitaire Ziekenhuizen KU Leuven | Vrije Universiteit Brussel | Fund for Scientific Research, Flanders, Belgium	https://ClinicalTrials.gov/show/NCT05014503
Re-Orchestration of Interregional Oscillatory Activity to Promote Visual Recovery	Ecole Polytechnique Federale de Lausanne	https://ClinicalTrials.gov/show/NCT05220449
Effect of Type of Head Positioning on Retinal Displacement in Vitrectomy for Retinal Detachment	Unity Health Toronto	https://ClinicalTrials.gov/show/NCT04035343
Macular Perfusion Changes After Anti-VEGF Versus Targeted Retinal Photocoagulation in Proliferative Diabetic Retinopathy	Cairo University	https://ClinicalTrials.gov/show/NCT04674254
Metabo-lipidomics of the Ocular Surface for Cataract Surgery	University Hospital, Tours	https://ClinicalTrials.gov/show/NCT05802550
Macular Involvement in Diabetic Retinopathy Evaluated With Swept-Source OCT	University of British Columbia	https://ClinicalTrials.gov/show/NCT03765112
EyeConic: Qualification for Cone-Optogenetics	University Hospital, Basel, Switzerland | Institute of Molecular and Clinical Ophthalmology Basel	https://ClinicalTrials.gov/show/NCT05294978
Suprachoroidal Visco-buckling for the Treatment of Rhegmatogenous Retinal Detachment	King's College Hospital NHS Trust | Norfolk and Norwich University Trust Foundation | St Thomas' Hospital, London | University of Sunderland | Moorfields Eye Hospital NHS Foundation Trust | Mid and South Essex NHS Foundation Trust | Sheffield Teaching Hospitals NHS Foundation Trust	https://ClinicalTrials.gov/show/NCT04557527
S.T.O.P. Technology Contact Lenses Versus Dual-focus Contact Lenses for Slowing Down Myopia Progression in Children	nthalmic Pty Ltd | Brighten Optix Corporation	https://ClinicalTrials.gov/show/NCT05243836
PMCF Study on EDOF (Isopure) *vs* Monofocal (Micropure) IOL	Beaver-Visitec International, Inc. | targomedGmbH	https://ClinicalTrials.gov/show/NCT04249492
ARTFL LEFFTDS Longitudinal Fronto-temporal Lobar Degeneration (ALLFTD)	Mayo Clinic | University of California, San Francisco | National Institute on Aging (NIA) | National Institute of Neurological Disorders and Stroke (NINDS)	https://ClinicalTrials.gov/show/NCT04363684
Systematic Assessment of Laryngo-	Kliniken Beelitz GmbH | University Hospital	https://ClinicalTrials.gov/show/NCT04706234

pharyngeal Function in Patients With MSA, PD, and 4 repeat Tauopathies	Muenster | Medical University of Warsaw | University Hospital Carl Gustav Carus | University of Ulm | Medical University Innsbruck | Hannover Medical School | University of Barcelona

secretome have highlighted its role in facilitating cell survival, proliferation, differentiation and physiological processes [17]. A huge number of secreted growth factors are well known today, including VEGF, SDF-1, TGF-β, IGF-1, fibroblast growth factor (FGF), nerve growth factor-beta (NGF-β), HGF, G-CSF and EGF[18]. With regard to MSC secreted cytokines and chemokines, the most investigated are CCL2, CCL5 and CXCL12 (SDF-1)[19].

One of the most useful aspects of the MSC secretome is the possibility to tailor or modify its composition depending on the desired cell-specific therapeutic effects. This promising possibility depends on MSC tissue sources or on the number of passages, allowing the creation of distinct secretory profiles and exosomal compositions[20]. However, several controversial studies have already been published. It was shown, for example, that the impact of MSCs extracted from adipose tissue was more noticeable on axonal growth than MSCs coming from bone marrow, while cell passaging did not influence the secretome content/activities supporting postnatal neuronal survival and axonal growth[21]. During the last few years, it has been revealed that MSCs are able to modify the microenvironment by releasing EVs, primarily distinct into apoptotic bodies, microvesicles and exosomes[22] (Table 2). The latter subtype consists of a bilayered lipid film of 30-120 nm, originating from convex membranes in late endosomes, determining the production of multi-alveolar bodies[23].

Different and various proteins are typical markers of exosomes, such as tetraspanin (CD9, CD63, CD81), annexin, heat shock proteins, caveolin and clarins as well as protein characteristics of source cells[24]. Furthermore, exosomes present specific lipids, comprising lipid raft portions, ceramides, sphingomyelin, cholesterol, GM1 ganglioside and phosphatidylserine. Additionally, they can contain nucleic acids, mRNA and ncRNA[25]. MSC-derived exosome biosynthesis and secretion are complex pathways that differ in microenvironmental stimuli, like inflammation or hypoxia[26]. The mTOR and Wnt pathways seem to play a pivotal role in exosome release[27]. Interestingly, recent studies have shown that MSC-derived exosomes may be involved in antigen presentation and immunologic response, coagulation, angiogenesis and apoptosis, as confirmed by the expression of antigens such as CD9, CD44 and CD89 on their surface [28]. Thus, the secretome obtained from the culture of MSCs would appear to promote tissue repair and modulate immune response *in vitro* and *in vivo*, showing a translational impact on regenerative medicine[29]. The use of CM could present diverse advantages if compared to the original MSC implantation, such as: (1) Removal of the inherent risks of cell transplantation; (2) Simpler storage, transport and conservation requirements; and (3) Possible application as a ready-to-go biologic product[30].

Secretome preconditioning modulated by the MSC cultural microenvironment

In recent years, it has been shown how preconditioning approaches for improving paracrine secretion, such as hypoxia, biochemical stimuli and 3D microenvironment, can increase the viability, proliferation and paracrine features of MSCs, thus expanding the therapeutic potential of these cells and their derived products[31]. In detail, dynamic culture conditions, such as 3D aggregate culture and fluid flow, could noticeably impact cellular behavior[32]. Boosted levels of growth factors and cytokines were detected in 3D MSC cultures grown on rotatory orbital or shaking platforms, in stirred systems, such as stirred tank reactors or spinner flasks, and in microgravity bioreactors[33]. Nevertheless, little is still known about the dynamic culture conditions and procedures for 3D aggregate MSC cultures as a scalable and reproducible plan for secretome production. However, the possibility of culturing cells under 3D conditions in a way to better mimic the *in vivo* environment has emerged[34].

A dynamic cross-talk between the cells could permit them to constantly modify their secretome following received stimuli, generating a microenvironment able to promote secretome enrichment for specific applications. Additionally, enhancing the manufacturing process allows MSC cell populations to be obtained that can be cryopreserved for clinical applications to expand clinical efficacy[35]. Recently, the use of matrix-conjugated hydrogel cell culture materials normalized a culture of induced pluripotent stem cell-derived MSCs (iPSC-MSCs), leading to a well-defined secretory profile able to promote enhanced neovascularization both *in vitro* and *in vivo*[36]. Using such innovative biomaterials, it was possible to stimulate reproducible secretion of proangiogenic and immunomodulatory cytokines from iPSC-MSCs that improved tubulogenesis of endothelial cells in Geltrex and neovascularization in chick chorioallantoic membranes [37].

Treatment with both IFN-γ and TNF-α permitted optimization of the MSC secretome. Recently, a unique supernatant of MSCs from human umbilical cord-derived MSCs, pretreated with TNF-α, was discovered to be more powerful in promoting macrophage migration, M2 polarization and pha-gocytosis, due to the induced high levels of CCL2 and IL-6 [38]. Another way to overcome the expansion limitation of MSCs is to work with MSCs derived from human-induced pluripotent MSCs, serving as a reproducible and sustainable cell source. In a way similar to hBMMSCs, human-induced pluripotent MSCs can release EVs with *in vitro* immunomodulatory properties, with an increased expression of well-known immunomodulatory genes, such as *HLA-DRA*, *IDO1* and *CXCL8/IL8* and at least another 100 regulated by NFKB signaling, known to play a pivotal role in immune response[39].

Interestingly, it has recently been shown that hypoxic preconditioning appears to induce the ASC secretome to release a secretome with enhanced anti-apoptotic effects by promoting the autophagic process of ASCs[40]. Furthermore, the specific content of EVs can be modulated by hypoxia, with their source cell responding by triggering HIF at low oxygen levels. The pleiotropic effects of HIF regulate the expression of many genes involved in pathways such as inflammation, angiogenesis, migration, differentiation, metabolism, proliferation and apoptosis. Expression of these genes is reflected in the interior of secreted EVs, which showed a greater regenerative ability than those achieved under normal oxygen conditions[41]. Moreover, the preconditioning of MSCs in an oxidative stress (OS) environment provides the release of

Table 2 Secretome and extracellular vesicle features

Type	Dimension	Origin	Collection	Content
Medium-size EVs (microvesicles)	200-1000 nm	Plasma membrane shedding	Filtration, ultracentrifugation, chromatography, precipitation, immunoaffinity	Similar to exosomes + cytosolic/plasma membrane/post-translational modified proteins
Small-size EVs (exosomes)	Up to 200 nm	Multivesicular bodies pathway	Filtration, ultracentrifugation, chromatography, precipitation, immunoaffinity	Receptors, transcription factors, enzymes, proteins, lipids, nucleic acids (DNA, mRNA and miRNA)
Soluble factors	Up to 5 nm	Protein synthesis	Protein extraction methods	Proteins, growth factors, chemokines, cytokines, enzymes
Secretome	From protein size to 1000 nm	Cell secretion/shedding	Cell culture media (concentration is possible)	The combination of the other components

EV: Extracellular vesicle; miRNA: MicroRNA.

many proteins, growth factors, cytokines and exosomes that could increase the antioxidant ability of MSCs against OS, enforcing the secretome as an encouraging, novel, cell-free tissue regeneration approach[42].

Characterization of MSC secretome EVs

A detailed analysis of the secretome structure might contribute to the improvement of secretome application for regenerative purposes and allow the discovery of novel biomarkers circulating in patient blood, improving pathology diagnosis and discovering new therapeutics targets[43]. As already anticipated, secretome fractions consist of lipids, proteins and non-coding RNAs able to impact the physiology of target cells. MSC-EVs were shown to contain a significant number of microRNAs (miRNAs), such as miR-210, miR-200b-3p and miR-4732-3p, involved in improving myocardial function[44]. BM-MSC-EVs, PD-L1-MSC-EVs and human umbilical cord-derived MSC-EVs also exhibited a healing role in autoimmune conditions[45]. miR-146a and miR-27a/b, upregulated in ASC-derived EVs, were able to induce neoangiogenesis pathways, while miR-122-5p, miR-27a, miR-206 and lncRNA MALAT1 played a relevant role in osteogenic regenerative processes[46].

Other studies identified specific factors from the secretome released by tumor cells that might be actively involved in cancer progression, thus representing optimal biomarkers[47]. Additionally, a customized secretome could be rich in proapoptotic factors that are helpful against cancer or higher levels of proangiogenic and pro-osteogenic factors suitable for regenerative applications. An interesting case is represented by human fetal MSCs, producing a secretome rich in anti-apoptotic factors as well as proangiogenic and antiangiogenic and osteogenic differentiative proteins[48]. On the contrary, the multipotent fetal dermal cell secretome is enriched in upregulated proteins involved in wound healing processes, angiogenesis and cellular metabolism[49]. Such data underlined that the fetal MSC secretome could be more beneficial for regenerative purposes if compared to the adult MSC secretome. In agreement with reports on the secretome derived from 3D cultured cells, fetal cells cultured under 3D conditions might further improve the therapeutic abilities of their secretome[50].

Unique immunomodulatory properties emerged for amniotic MSCs. Their secretome was able to reduce the polarization of T cells toward inflammatory helper T cell subgroups, inducing regulatory T cells, to decrease the proliferation of activated peripheral blood mononuclear cells, to affect monocyte polarization to antigen-presenting cells stimulating the synthesis of anti-inflammatory macrophage (M2) markers and to reduce the activation of B lymphocytes into plasma cells[51]. The most intriguing aspect of secretome EV fractions is the functional mitochondria release from human mesenchymal stromal cells[52]. Recent studies in non-orthopedic tissues proposed that MSCs can rescue damaged cells by donating mitochondria, repairing mitochondrial activity in target cells, preserving cell viability and stimulating tissue repair. To obtain this goal, MSCs might be able to package mitochondria for export into EVs, and these "mitoEVs" could provide a delivery approach for cell-free mitochondria-targeted therapy[53].

Therapeutic role of the secretome in central nervous system pathologies

The MSC secretome is a significant element of the paracrine and autocrine cell signaling mechanism, playing a crucial role in the regulation of many physiological and pathological processes. In particular, its effects on immunomodulation, neuronal survival and regeneration, due to the action of soluble and vesicular factors, are pivotal in reducing or even arresting neuronal disease evolution and in promoting repair[54]. Thus, the various MSC secreted factors and vesicles seem to be an effective tool for the protection and survival of neuronal and glial cells[55]. Traumatic brain injury (TBI) is determined by external mechanical forces able to cause physical, cognitive and emotional impairments[56]. In this case, the MSC-derived secretome may be used to control the secondary injury mechanisms of TBI, modulate the abnormal inflammatory cascade, reduce proinflammatory cytokines and stimulate neural stem cell proliferation and differentiation [57]. Moreover, EVs released by MSCs reduced neuroinflammation and supported neurogenesis and angiogenesis, rescuing spatial learning and motor damage in TBI animal models[58].

Spinal cord injury is characterized by long-term functional deficits following the loss of neurons and glial cells, inflammation and demyelination[59]. The paracrine factors secreted into the lesion site by MSCs, such as HGF, BDNF and NGF could promote immunomodulation, glial scar reduction, axonal regeneration and neurite outgrowth. Additionally, the

ASC-derived secretome reduced the production of TNF-α by M1 macrophages while it improved TGF-β1 and IL-10 production by M2 macrophages[60]. MSC exosomes could stimulate anti-inflammatory and proangiogenic effects and axonal regeneration and suppress glial scar formation and cell apoptosis, reducing lesion size and improving functional recovery after traumatic spinal cord injury[61].

Ischemic stroke is a cerebrovascular pathology induced by blood vessel occlusion or injury, leading to a blood supply defect, determining focal tissue loss and endothelial and neuronal cell death[62]. The use of MSC secreted factors such as IGF-1 and BDNF could induce neuroprotection by impeding neuronal damage and tissue loss and reduce astrocyte injury by GFAP downregulation[63].

Parkinson's disease (PD) is a neurodegenerative pathology characterized by the progressive degeneration of dopaminergic neurons. In PD, it has already been seen that the addition of the MSC secretome can promote a partial reversion of PD histological impairments and gains in animal motor ability by the secretion of immunomodulatory, anti-inflammatory, neurogenic, neurodevelopmental, neurorescuing or antiapoptotic factors[64]. Recent evidence highlighted the particular ability of the MSC secretome to reduce one of the hallmarks of the disease, the alpha-synuclein aggregates, through an MMP-2-based mechanism[65].

MAIN FEATURES OF RETINAL CELLS

Even if peripherally localized, the retina represents an important part of the central nervous system. Though it presents the same types of functional elements and neurotransmitters sited in other portions of the central nervous system, the retina includes five classes of neurons: photoreceptors (rods and cones); bipolar cells; amacrine cells; horizontal cells; and ganglion cells. Light absorption by the photopigment in the outer segment of rods and cones, the two photoreceptors, starts a cascade of events that changes the receptor membrane potential and the quantity of neurotransmitter released by the rod and cone synapses onto the adjacent bipolar cells, in the outer plexiform layer. Then, in the inner plexiform layer, the short axonal processes of bipolar cells realize a synapse with the dendritic processes of ganglion cells whose axons form the optic nerve. Horizontal and amacrine cells, instead, present their cell bodies within the inner nuclear layer and are mainly involved in lateral interactions between already described retinal cells, impacting the sensitivity of the visual system to light contrast over a wide range of intensities. The amacrine cell processes, which ramify laterally in the inner plexiform layer, are postsynaptic to bipolar cells and presynaptic to ganglion cells, while the processes of horizontal cells instead extend in the outer plexiform layer. The existence of different subgroups of amacrine cells that play a distinct role within visual pathways is relevant. Furthermore, the neural retina and the choroid are connected by a monolayer of cells constituting the retinal pigmented epithelium (RPE). Light absorption, epithelial transport, spatial buffering of ions, visual cycle regulation and phagocytosis of rod and cone outer segment membranes represent the main functions exerted by the RPE[66].

RPE cell secretome and retinal diseases

The RPE is characterized by a polarized nature, with molecules expressed by these cells either secreted to the apical or basolateral membrane by the Na^+/K^+-ATPase associated channel or by the anion channel, respectively. These products, mainly growth, anti/proangiogenic and neurotrophic factors, are critical for the correct functioning of the neuroretina and choroid. Among them, the most characterized are VEGF[67,68], TGF-β[67,69], PEDF[70], MMPs[71], NGF[72], FGF-1, FGF-2, and FGF-5[73], IGF-1[74], BDNF[75], PDGF[76], CTGF[77], LEDGF[78], interleukins[79], tissue inhibitor of matrix metalloproteases[80], PIGF[81], angiogenin[82], EPO[83], somatostatin[84] and apolipoprotein A1[85]. These factors could also play a fundamental role in the etiology of several retinal diseases such as diabetic retinopathy (DR), age-related macular degeneration (AMD) and retinopathy of prematurity[86].

Deep proteomic analyses of RPE cells cultivated in these pathological condition microenvironments suggested that previously described molecules could be involved in membrane and cytoskeleton dynamics, mitochondrial trafficking, protection/induction of cellular stress, apoptosis, differential modulation of multidrug resistance-associated proteins and in other metabolic events already during the first stages of the diseases[87]. In physiological conditions, RPE cells release EVs characterized by proteins associated with biological pathways involved in AMD etiology, including drusen composition. Recently, it was shown that drusen-associated proteins are secreted as cargo of EVs produced by RPE cells in a polarized apical to basal way. Remarkably, drusen-associated proteins revealed differential regulation of polarized secretion in homeostatic conditions and in response to AMD stressors[88]. Findings suggested that a finely-tuned mechanism is pivotal to regulate directional sorting and secretion of drusen-associated proteins *via* RPE secretome EVs, supporting the influential role of vesicles as a strategic source of drusen proteins and critical elements to drusen development[89].

OS changed the release of several factors implicated in neovascularization and AMD, stimulating a proangiogenic microenvironment by increasing the secretion of VEGF, PTN and CRYAB and reducing the production of anti-PEDF and CFH. Apical secretion was influenced more than basolateral for PEDF, CRYAB and CFH, while directional secretion was impacted more for VEGF, which may have implications for choroidal neovascularization[90]. VEGF-A is an important proangiogenic factor released by different retinal cytotypes (endothelial cells, Müller cells, ganglion cells and pericytes) but primarily by the basolateral side of RPE in homeostatic conditions, shifting to apical during pathological conditions [91].

In particular, VEGF overexpression was highlighted in hypoxic and hyperglycemic conditions, by both *in vitro* and *in vivo* studies, demonstrating that in pathological conditions VEGF causes alteration of tight junction proteins and transepithelial resistance[92]. It has been confirmed that VEGF R2, placed in the apical side of RPE cells, can induce

disruption of the RPE barrier by promoting VEGF signaling[93]. Considered together, such findings led to the development of anti-VEGF therapies to treat retinal neovascularization in patients with DR and other related diseases. However, many associated complications are still present, such as repeated injection requirements, increased ocular pressure, macular edema, subconjunctival hemorrhage, pain, uveitis and the compromised viability of RPE, photoreceptors, choriocapillaris and Müller glia[94].

One of the most interesting relates to splice variants of VEGF, such as VEGF165b, expressed by RPE cells. It can appear to act as a powerful antiangiogenic isoform of VEGF with significant results in treating induced choroidal neovascularization and was decreased in DR[95]. Nevertheless, while inner retinal barrier and Müller cell association with VEGF is well known, the outer retinal barrier properties of RPE in relation to VEGF in diabetes and other ocular neovascularization-related diseases should be better investigated. One of the most significant glycoproteins of the RPE secretome is PEDF, a serine protease inhibitor with neuroprotective, antiangiogenic and anti-inflammatory features. In homeostatic conditions, PEDF is apically released from the RPE and preserves retinal and choriocapillaris integrity by preventing endothelial cell proliferation[96]. It was seen that PEDF was downregulated in human hyperglycemic RPE cells as well as in patients affected by proliferative DR (PDR), diabetic macular edema (DME), retinopathy of prematurity, retinitis pigmentosa and leber congenital amaurosis[97]. Thus, PEDF is primarily considered for its therapeutic potential, showing positive effects in photoreceptor survival, morphology and function while reducing vascular permeability in correlation with reduced levels of angiogenic factors (VEGF, VEGFR-2), cytokines and chemokines[98]. Additionally, recent animal studies have proven that in an oxygen-induced retinopathy model and in a rat model of choroidal neovascularization, PEDF upregulation blocked retinal neovascularization and inflammation[99].

Another prosurvival cytokine able to stimulate fibroblast chemotaxis/proliferation and preserve pericyte viability and physiological vascularization of the retina is PDGF. Similar to VEGF, it can promote pathologic neovascularization in PDR and DR and in a hypoxia-regulated microenvironment[100]. PDGF receptor activation suggests an autocrine mechanism in epiretinal membrane development and retinal wound repair[101]. Today, one of the most promising research fields is understanding the cross-talk of PDGF with other signaling pathways in order to identify the best molecular targets for combinatorial therapies. This idea arose from several animal studies that established that antagonism to PDGF-BB (a homodimeric form of the PDGF family), together with anti-VEGF, enhanced the arrest of retinal neovascularization[102].

An important cofactor of VEGF is PlGF. It can alter retinal fibrovascular integrity and RPE permeability by interaction with VEGF and activation of Akt and HIF-1 pathways[103]. Thus, it was found at high levels in AMD and PDR patients [104]. The use of anti-PlGF monoclonal antibody in different animal models revealed reduced inflammation and vascular leakage with no adverse effects in retinal ganglion cell (RGC) viability[105]. However, novel strategies that avoid the weaknesses observed in repeated intraocular injections should consider PlGF as a valid therapeutic target. RPE cells cultured in high glucose medium also showed an elevated expression of CTGF, one of the main fibrogenic factors involved in fibroblast proliferation and extracellular matrix synthesis, which could control the microenvironment around the distal retinal/RPE/Bruch's membrane complex and protect against neurodegenerative diseases[106]. Increased retinal CTGF levels might play an essential role in DR, probably by reducing VEGF levels[107]. Thus, the combined use of anti-CTGF and anti-VEGF in treating complications of DR could exert more beneficial effects than a monotherapy drug[108].

CTGF is corroborated in its activity by the more well-known FGF, which plays a crucial role in stimulating vascularization, angiogenesis and cell survival and acting as autocrine factors. FGF1, FGF2 and FGF5 are principally released in the RPE, reaching their highest levels in non-proliferative retinopathy, PDR with active proliferative retinopathy and diabetic conditions, respectively[109]. Recently, targeting of retinal FGFs exhibited worthy results in improving visual acuity of DME and exudative AMD patients, even if further studies are mandatory to determine long-term effects[110]. The secretome produced by human RPE cells also contained IGF-1 and IGF-2, natural proteins promoting growth and insulin-like metabolic effects, together with their receptor (IGF-R) and binding protein (IGFBP-2)[111]. Both growth factors seem to play a pivotal role for RPE autocrine/paracrine-mediated modulation of proliferation[112]. Recent evidence showed that another IGFBP family member, IGFBP-3, was able to reduce DR by considerably decreasing TNF-α levels and proapoptotic markers[113].

Among secreted factors, TGF-β represents one of the main elements that can modulate main cellular physiological processes, like growth, differentiation, proliferation and apoptosis[114]. However, there is scant information on its efficacy and potential mechanisms in relation to retinal homeostasis or pathology. In detail, comparable secretion levels of TGF-β from polarized RPE, differentiated from human embryonic stem cells and human RPE, promoting retinal homeostasis and sustaining the potential of human embryonic stem cell-RPE in replacement therapies, have recently been highlighted[115]. Human stem cell-derived RPE treated with reactive oxygen species for 1 wk or 3 wk released more than 1000 proteins, many of which showed relevant changes due to induced stress.

In particular, secreted APOE and TGF-β were decreased 4-fold, and urotensin-II, one of the most effective vasoconstrictors, doubled, similar to BMP1[116]. The glycoprotein EPO represents one of the most promising molecules found in the RPE secretome. It acts as an erythropoiesis regulator with different additive features such as vessel integrity, recruitment of endothelial progenitor cells, neuroprotection and antioxidative properties[117]. High levels of EPO were recently found in DR, PDR and DME patients[118]. Especially in hyperglycemic conditions, EPO seems to protect the RPE barrier, reducing retinal vasculogenesis, downregulating VEGF and VEGFR expression and protecting tight junctions by increasing the flow of Ca^{2+} ions in blood-brain barrier animal models[119]. However, the administration of EPO in the late stage of a hypoxia-induced murine retinopathy model worsened retinal neovascularization, suggesting that EPO might play a protective role in early DR and a pathologic one in late DR[120]. This dual nature of EPO could be related to its action mechanism, whose first step is its hypoxia-modulated binding to cell surface receptor EPOR. Thus, it can be predicted that in the first stages of DR, EPO exerts neuroprotective functions, while in the advanced stage of DR EPO acts as a neovasculogenesis inducing molecule that is regulated by hypoxia[121].

MMPs, apically secreted by RPE, are calcium-dependent endopeptidases involved in angiogenesis and are fundamental for ocular extracellular matrix and photoreceptor outer segment homeostasis[122]. Recently, it has been seen that basolateral secretion of MMP is related to AMD, and increased levels of MMP-2 and MMP-9 were also observed in the Bruch's membrane of AMD and DR patient eyes[123]. Thus, inhibitors of both cited MMPs might also exert an advantageous role by blocking capillary cell apoptosis, growth of vessels and reduce inflammatory-mediated permeability[124].

In addition to angiogenic and antiangiogenic factors, numerous inflammatory chemokines and cytokines were elevated in retinal diseases, such as PD and PDR. Among them, the most investigated were MCP-1, IL-6 and IL-8. It was seen that MCP-1 and IL-8 secretion levels are directly correlated to blood glucose levels, suggesting a crucial role in altered blood retinal barrier (BRB) activities of DR affected patients[125]. MCP-1 carries out chemoattractant activity for monocytes and lymphocytes to promote endothelial proliferation and may limit the impairment of neurosensory retina[126]. IL-6 and IL-8, were overexpressed in cultured RPE cells stimulated with IL-1b or TNF-β, suggesting that polarized release of growth factors/cytokines is favored in retinal diseases[127].

Additionally, it is interesting to cite the recently discussed role of somatostatin as a neuromodulator of retinal homeostasis, as hypothesized by its downregulation in the RPE of diabetic eyes[128]. Finally, several substrates of the serine protease HTRA1 were found in the RPE secretome, proposing a link between it and complement modulation and amyloid deposition in AMD etiopathogenesis. In detail, a cleavage of fibromodulin (90%), CLU (50%) and vitronectin (54%) involved in regulation of the complement pathway was seen, along with a cleavage of 2-macroglobulin (55%) and ADAM9 (54%) related to amyloid deposition as well as some cell surface protein cleavages including talin-1 (21%), fascin (40%) and chloride intracellular channel protein 1 (51%)[129].

Müller glia cell secretome and retinal diseases

Regarding the RPE, Müller cells can modulate trophic secretion depending on the healthy or pathological status of the retina[130]. The Müller cell physiological secretome mainly contains molecules that are crucial to increase BRB tightness, like thrombospondin-1 and PEDF[131]. In pathological circumstances, factor synthesis and secretion both shift towards an inflammatory environment. Under hyperglycemic conditions, IL-1b release by Müller cells is increased, leading to vascular impairment and cell death *via* a paracrine mechanism. Thus, by inhibiting IL-1b or knocking down its receptor, it was possible to decrease inflammation and photoreceptor/retinal vessel disruption in murine models, exerting a possible therapeutic role for ocular dystrophies related to chemokine expression and/or diabetes[132]. Furthermore, the proinflammatory IL-6 and TNF-α can be secreted by Müller cells, determining a possible promotion of both vascular dysfunction and angiogenesis, even if IL-6 may exert protective effects toward photoreceptor cells[133].

Stimulation of porcine and human Müller cells with IL-4, IL-6, IL-10, VEGF, INF-γ, TGF-β1, TGF-β2, TGF-β3 and TNF-α resulted in a primarily proinflammatory phenotype with release of cytokines and factors of the complement system[134]. Additionally, Müller cells expressed proteins linked to biosynthesis and maturation of phagosomes. These findings underline the relevance of Müller cell signaling in chronic retinal inflammation[135]. Additionally, under hyperglycemia and hypoxic conditions, Müller cells shift PEDF secretion to VEGF, contributing to ocular vascular diseases[136]. Therefore, inhibition or knockdown of Müller cell-derived VEGF could reduce ischemia-induced impairment of the BRB, prevent ischemia-induced retinal neovascularization and decrease vascular leakage[137].

Recent evidence highlighted that in the diabetic retina expression of VEGF could be regulated by increasing the activity of the receptor for retinoic acid alpha, which also stimulates the expression of glial cell line-derived neurotrophic factor, with a final significant decrease of vascular leakage[138]. Nevertheless, in recent years, the neuroprotective effects of VEGFR-2 in Müller glia have also been described, suggesting its significance for cell survival and consequential viability of neuronal cells in the diabetic retina[139]. Interestingly, the secretome of Müller cells also contains increased levels of MMP-2 and MMP-9 in patients with PDR and AMD, respectively[140]. It was proposed that the stabilization of HIF-1a could raise the level of VEGF, inducing MMP-2 expression in neighboring endothelial cells, with consequent retinal neovascularization[141]. As MMPs regulate crucial cellular pathways through angiogenesis and apoptosis, their targeting could represent an important therapeutic strategy for ocular diseases. Moreover, new evidence has proven that Müller glia release neurotrophic factors, such as CLU, osteopontin and basigin, that support RGC survival. The latter two significantly enhance RGC survival *in vitro*, suggesting that the survival-promoting activity of the Müller cell secretome is multifactorial[142].

Recently, it was shown that human iPSC-derived multinucleated giant cells (hiMGCs) could represent an alternative to primary MGCs in understanding glial cell involvement in retinal disorders, including DR. Under culture with palmitate, a major free fatty acid with elevated plasma levels in diabetic patients, hiMGCs and primary MGCs expressed low transcript levels of *AQP4*, *RLPB1*, *SLC1A3*, *KCNJ1* and *KCJN10*. Furthermore, the analysis of the palmitate-treated hiMGC secretome evidenced an upregulation of proangiogenic factors powerfully related to DR, including ANG2, endoglin, IL-1b, CXCL8, MMP-9, PDGF-AA and VEGF[143]. One of the most interesting pieces of evidence regarding the Müller cell secretome was linked to the production of different EVs from endfeet and microvilli of retinal Müller cells in adult mice. In particular, VAMP5 was identified as a Müller cell-specific snap receptor member that is part of EVs and responsive to ischemia, with relevant changes between the secretomes of Müller cells and neurons *in vitro*[144].

Other glial cell secretome and retinal diseases

Undifferentiated rat RGC line RGC-5 can secrete numerous protein markers of RGCs, even if they are unable to react to glutamate or N-methyl-D aspartate. Furthermore, it has recently been highlighted that human nonpigmented ciliary epithelial (HNPE) cells could release several neuroproteins located in the aqueous humor, many of which can influence the activity of neuronal cells. Recent works identified about 130 unique proteins from the HNPE cell-conditioned SF-medium, most of which are involved in cell differentiation. These results led to the hypothesis that a differentiation

system of HNPE cell-conditioned SF-medium with RGC-5 cells can promote a differentiated phenotype in RGC-5 cells, functionally close to primary cultures of rat RGCs[145].

The secretome of retinoblastoma, the solid malignancy of the developing retina, is immunosuppressive and induces a protumoral phenotype. This conclusion was the result of complex analyses that identified the cytokine extracellular matrix metalloproteinase inducer and macrophage migration inhibitory factor, both characterized by detected immunosuppressive activity and secreted at high levels in retinoblastoma primary cell cultures. In addition, macrophages derived from peripheral blood mononuclear cells increased the expression of M2-like polarization markers following exposure to retinoblastoma-conditioned medium or recombinant migration inhibitory factor[146].

CROSS-LINK BETWEEN RETINAL AND MESENCHYMAL SECRETOMES: DIFFERENT MSC-DERIVED SECRETOMES AS INNOVATIVE APPROACHES FOR RETINAL DISEASES

The MSC secretome is currently studied extensively for the treatment of several retinal diseases. Its therapeutic potential lies in its richness of immunomodulatory, antiangiogenic and neurotrophic factors, preventing retinal degeneration and improving retinal morphology and function. Additionally, exosomes secreted by MSCs showed anti-inflammatory and antiapoptotic effects (Figure 1 and Table 3). Based on MSC origins and their particular secreted factors, several promising preclinical and clinical studies were initiated to explore the potential advantages of MSC secretome for the treatment of retinal diseases.

Novel evidence on the role of MSC secreted factors in retinal disease etiopathogenetic pathways

Novel evidence showed that MSC conditioned media inhibits abnormal neovascularization and decreases vaso-obliteration (promoting revascularization) in retinopathies by restoring neuronal Sema3E levels, which reduce pathological concentrations of IL-17A (and associated proinflammatory factors, such as IL-1b) in myeloid cells[147]. Among MSC released factors, PDGF secretion may play a crucial role in MSC-mediated RGC neuroprotection. These results were obtained from the arrest of PDGF signaling by small molecule PDGF inhibitors, neutralizing antibody or downstream phosphatidylinositol 3 kinase, which blocked RGC neuroprotection conferred by MSC co-culture. Furthermore, intravitreal injection of PDGF led to relevant optic nerve neuroprotection *in vivo* after experimental induction of high intraocular pressure[148]. Application of conditioned media obtained from MSCs protected against Aβ 1-42 oligomer-induced retinal pathology in RGCs of both rat and ARPE-19 cells, due to proteins associated with SIRT1/pAKT/pGSK3β/β-catenin, tight junction proteins and the apoptosis pathway[149]. Furthermore, in recent years, the administration of EVs in models of neurological disorders has highlighted a relevant improvement of neurological dysfunction. In particular, miRNAs from MSC-EVs, as one of the central mediators that control various genes and decrease neuropathological change, have been identified in various neurological pathologies[150].

Bone marrow MSC-derived secretome regulates retinal cell neuroprotection

The BMMSC secretome protects retinal morphology, regulates autophagy-, proapoptotic– and pronecroptotic–related gene and protein expression and promotes the activation of antioxidant machinery, exerting a neuroprotective ability during retinal degeneration[151]. A recent expression analysis of about 1000 proteins exhibited high levels of paracrine factors secreted by hBMMSCs that might be fundamental in the neuroprotective effect of the stem cell secretome over *in vitro* retinal degeneration. These results support the hypothesis that the paracrine effect of hBMMSCs may slow photoreceptor death and be a therapeutic possibility in retinal photoreceptor degenerative diseases[152]. Additionally, rat BMMSCs cultured with the secretome from neonatal rat retinal cells were able to differentiate into RGC-like cells, exhibiting protein expression patterns similar to those of isolated RGCs such as Map2, nestin and Thy1.1[153].

Adipose MSC-derived secretome regulates retinal cell regeneration

Recent evidence showed an important therapeutic effect of hADSCs and its secretome on an *in vivo* model of sodium iodate retinal neurodegeneration. The studies highlighted that the hADSC secretome effects were particularly striking, especially in terms of photoreceptor regeneration and retinal function, as underlined by increased expression of retinal regeneration markers such as Pax6, Chx10, S-Opsin (Opn1sw), Nrl, Crx and GFAP[154]. Oxidatively stressed ARPE-19 cells treated with adipose MSC conditioned media and/or combined with nicotinamide, vasoactive intestinal peptide or both factors showed an improved recovery from the damaged status. Additionally, the same treatment could determine better protection of the neuroretinal architecture, mainly rods and cones, and a lower degree of glial cell activation[155].

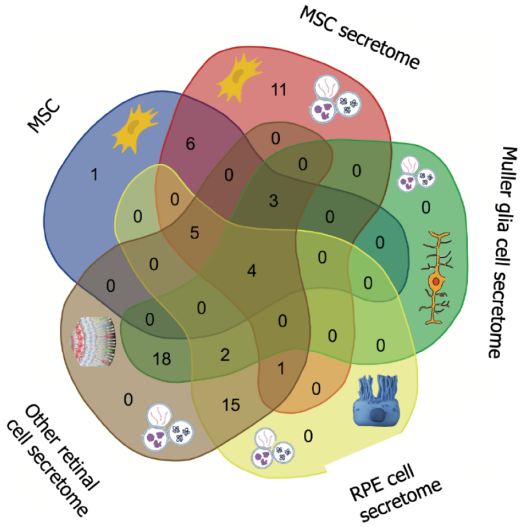
The preclinical efficacy of adipose-derived stem cell concentrated conditioned medium (ASC-CCM) was recently tested in repetitive ocular blast injury mice, highlighting a significant rescue from retinal injury and a significant restoration of visual function, also associated with a significant reduction of neuroinflammation markers, retinal GFAP and OS. Furthermore, *in vitro*, oxidatively stressed Müller cells pre-incubated with ASC-CCM exhibited normalized levels of GFAP, viability and catalase activity[156]. Intravitreal injection of ASC-CCM was safe and efficient against the visual impairments of mild TBI. Blast mice treated with ASC-CCM exhibited improved vision at 5 mo but minimal effects at 10 mo, associated with alterations of GFAP and proinflammatory gene expression in retina. Thus, the unchanged glial response and the risk of retinal injury with live cells suggested that ASC-CCM might have better safety and efficacy than live cells for visual dysfunction therapy[157].

Table 3 Key molecules of the mesenchymal secretome derived from different cell types in the treatment and regeneration of retinal cells

MSC	MSC secretome	Müller glia cell secretome	RPE cell secretome	Other retinal cell secretome
IFN-γ[8]	IFN-γ[8]	TSP-1[131]	VEGF[68]	TSP-1[131]
VEGF[10]	VEGF[10]	PEDF[70]	TGF-β[69]	PEDF[70]
PIGF[81]	PIGF[81]	IL-1b[11]	PEDF[70]	IL-1b[11]
SDF-1[10]	SDF-1[10]	IL-1R[143]	MMPs[71]	IL-1R[143]
ANG2[10]	ANG2[10]	TNF-α[11]	NGF[72]	IL-6[11]
G-CSF[10]	G-CSF[10]	IL-4[134]	FGF-1[73]	TNF-α[11]
SCF[10]	SCF[10]	IL-6[134]	FGF-2[73]	IL-4[134]
PDGF[10]	PDGF[10]	IL-10[134]	FGF-5[73]	IL-10[134]
EGF[10]	EGF[10]	VEGF[10]	IGF-1[74]	VEGF[10]
HGF[10]	HGF[10]	IFN-γ[8]	BDNF[75]	IFN-γ[8]
IGF-1[10]	IGF-1[10]	TGF-β1[34]	PDGF[76]	TGF-β1[34]
TNF-α[11]	TNF-α[11]	TGF-β2[34]	CTGF[77]	TGF-β2[34]
IL-1b[11]	IL-1b[11]	TGF-β3[34]	LEDGF[78]	TGF-β3[34]
IL-2[11]	IL-2[11]	MMPs[71]	IL-1b[79]	MMPs[71]
IL-3[11]	IL-3[11]	CLU[121]	IL-2[79]	CLU[121]
IL-6[11]	IL-6[11]	SPP1[142]	IL-3[79]	SPP1[142]
IL-8[11]	IL-8[11]	BSG[142]	IL-6[79]	BSG[142]
CCL5[11]	CCL5[11]	AQP4[143]	IL-8[79]	AQP4[143]
CCL2[11]	CCL2[11]	RLBP1[143]	TIMP[80]	RLBP1[143]
	TGF-β[18]	SLC1A3[143]	PIGF[81]	SLC1A3[143]
	FGF[18]	KCNJ1[143]	ANGIOGENIN[82]	KCNJ1[143]
	NGF-β[18]	KCNJ10[143]	EPO[83]	KCNJ10[143]
	CXCL12[19]	ANG2[143]	SOMATOSTATIN[84]	ANG2[143]
	miR-210[44]	ENDOGLIN[143]	APOA1[85]	ENDOGLIN[143]
	miR-200b-3p[44]	CXCL8[39]	APOE[116]	CXCL8[143]
	miR146a[46]	PDGF[10]	TGF-β[69]	PDGF[76]
	miR27a/b[46]	VAMP5[144]	BMP1[116]	VAMP5[144]
	miR-122-5p[46]		HTRA1[129]	TGF-β[69]
	miR-206[46]			NGF[72]
	MALAT[46] (lncRNA)			FGF-1[73]
				FGF-2[73]
				FGF-5[73]
				IGF-1[74]
				BDNF[75]
				PDGF[76]
				CTGF[77]
				LEDGF[78]
				IL-2[79]
				IL-3[79]
				IL-8[79]

	TIMP[80]
	PIGF[81]
	ANGIOGENIN[82]
	EPO[83]
	SOMATOSTATIN[84]
	APOA1[85]
	APOE[116]
	BMP1[116]
	HTRA1[129]

MSC: Mesenchymal stem cell; RPE: Retinal pigment epithelium; FGF: Fibroblast growth factor; NGF: Nerve growth factor.



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Figure 1 Common factors released by retinal and mesenchymal cell secretomes. Both groups of cells can release different clusters of factors that are able to influence the microenvironment by paracrine mechanisms. The most common factors are highlighted in the cytotypes. More details are available in Table 3. MSC: Mesenchymal stem cell; RPE: Retinal pigment epithelium.

Human uterine cervical and Wharton's jelly MSC-derived secretomes regulate retinal cell immunomodulation

The treatment of oxidatively stressed ARPE-19 cells with human uterine cervical stem cells-conditioned medium evidenced a significant increase of *VEGFA*, *HO-1*, *HSPB1*, *GCLC*, *PDGFA* and *PDGFB* mRNA expression, highlighting a potential stimulation of detoxifying genes, protection from damage by OS and better vascularization[158]. Recent studies demonstrated that RPE cell viability and the expression of anti-apoptotic Bcl2 were reduced significantly in conditioned media secreted by human Wharton's jelly MSC (WJMSCs)-treated RPE cells, while expression of proapoptotic biomarkers Bax and IL-1b was not significantly changed. WJMSCs are a subgroup of MSCs isolated from the Wharton jelly of the umbilical cord characterized by a high potential of proliferation and a secretome rich in trophic factors and immunomodulatory cytokines. Moreover, previously described experiments showed that the WJMSC secretome could induce apoptosis in RPE cells through activating apoptosis pathways, being a potential therapeutic target for pathologies like proliferative vitreoretinopathy[159].

CONCLUSION

The crosslink between mesenchymal cell and retinal cell secretomes is a topic of interest for regenerative medicine, as both types of cells secrete trophic factors that can modulate cellular pathways involved in survival, function and response to injury. The mesenchymal cell secretome is a collection of molecules secreted by MSCs and have a positive effect on re-establishing the intra-articular homeostasis and stimulating regeneration by different growth factors, cytokines and miRNA that are contained within the EVs of the secretome. The retinal cell secretome is mainly composed of the secreted factors from the RPE and Müller cells, which are key to maintain the structural and functional integrity of the retina. The crosslink between these two types of secretomes could potentially enhance the neuroprotective effect of the MSC

secretome on retinal degeneration, by modulating OS, autophagy and programmed cell death. This scenario could be of particular interest especially for MSC secretome-only factors, such as FGF, CXCL12, CCL511, NGF-, described miRNAs and the lncRNA MALAT, whose complementary action might play a functional compensation role towards retinal cell alterations.

However, many challenges still exist, including the specific characterization of secretome released factors to further target therapy to the pathology profile, better manipulation of the retinal secretome or from other cell sources for noteworthy therapeutic effect, improving methods for intraocular administration of secretome factors and developing personalized combinations of trophic factors involved in different pathological pathways (inflammation, reactive oxygen species, angiogenesis, proliferation) to evaluate the collective therapeutic potential. Nevertheless, the possible improvement of new efficient pharmaceutical formulations related to the secretome of MSCs and retinal cells, with the addition of exogenous factors or drugs without the necessity to deliver cells into the eye may represent a novel milestone towards a personalized approach to retinal disease.

FOOTNOTES

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MINIREVIEWS

Neural stem cells for Parkinson's disease management: Challenges, nanobased support, and prospects

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Abstract

Parkinson's disease (PD), characterized by loss of nigrostriatal dopaminergic neurons, is one of the most predominant neurodegenerative diseases affecting the elderly population worldwide. The concept of stem cell therapy in managing neurodegenerative diseases has evolved over the years and has recently rapidly progressed. Neural stem cells (NSCs) have a few key features, including self-renewal, proliferation, and multipotency, which make them a promising agent targeting neurodegeneration. It is generally agreed that challenges for NSC-based therapy are present at every stage of the transplantation process, including preoperative cell preparation and quality control, perioperative procedures, and postoperative graft preservation, adherence, and overall therapy success. In this review, we provided a comprehensive, careful, and critical discussion of experimental and clinical data alongside the pros and cons of NSC-based therapy in PD. Given the state-of-the-art accomplishments of stem cell therapy, gene therapy, and nanotechnology, we shed light on the perspective of complementing the advantages of each process by developing nano-stem cell therapy, which is currently a research hotspot. Although various obstacles and challenges remain, nano-stem cell therapy holds promise to cure PD, however, continuous improvement and development from the stage of laboratory experiments to the clinical application are necessary.

Key Words: Parkinson's disease; Synuclein; Neural stem cells; Nanomaterials; Nano-stem cell therapy

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Core tip: Neural stem cell (NSC) transplantation is a groundbreaking therapy with therapeutic effects for many neurodegenerative diseases. Parkinson's disease (PD) is the second most common neurodegenerative movement disorder. The ability of NSCs for neural differentiation has been suggested as an effective mechanism in PD subjected to cell transplantation. Here, the potential therapeutic mechanisms, challenges, nanobased support, and manifestations of NSCs in PD are discussed, along with examples of preclinical and clinical studies.

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INTRODUCTION

Parkinson's disease (PD), with a global prevalence of > 6 million, is the second most common neurodegenerative movement disorder. Currently, no disease-modifying cure is available, and occurrence of PD has been projected to double over the next generation[1]. PD is an age-related disorder affecting about 5% of the population by the age of over 85 years.

The primary pathological property of PD is the presence of abnormal α-synuclein (ASN) in the form of neural inclusions called Lewy bodies, whose toxic effect is hypothesized to contribute to progressive degeneration and cell loss [2,3]. Dopaminergic (DAergic) neurons in the substantia nigra pars compacta specifically undergo degeneration leading to dopamine (DA) and several other biochemical deficits in the nigrostriatal system, resulting in the development of motor symptoms, including bradykinesia, resting tremor, muscular rigidity, and postural instability[4]. Moreover, loss of other types of neurons and the presence of Lewy pathology in many parts of the central, enteric, and autonomic nervous systems have been demonstrated that probably contribute to non-motor manifestations, such as autonomic dysfunction, olfactory impairment, and mood, cognitive or sleep disturbances present in PD patients[5,6].

Dopamine replacement therapy (DRT) is the gold standard treatment for alleviating PD symptoms. However, as the disease progresses, compensatory mechanisms for denervation affect the duration and delay the alleviating effect of DRT in a nonlinear manner[7]. Surgical procedures are also used, including stereotactic ablations or deep brain stimulation. Nevertheless, these approaches only partially manage the severe motor symptoms and alleviate DAergic desensitization supporting DRT in advanced PD patients[8,9]. Aggregation of ASN, besides the gain-of-toxic function, can also result in loss of function, thereby decreasing its functions[10]. Since ASN exerts physiological activity towards newly formed neurons by promoting the development and maturation of dendrites and the spine, depending on the expression level [11], the intraneuronal inclusions of ASN contribute to the PD pathogenesis by also affecting neurogenesis[12]. Therefore, stimulation of endogenous neurogenesis or cell-replacement therapy is considered a therapeutical option against PD neurodegeneration[13]. Accordingly, strategies aiming at rebuilding the pathway and reshaping the brain have been developing[14].

Endogenous neural stem cells (NSCs) exist throughout life and are found in specific areas of the human brain. NSCs exhibiting abilities to self-renew and differentiate into neurons, astrocytes, or oligodendroglia are responsible for restoring brain function under normal circumstances. The regeneration of DAergic neurons from stem cells is considered an alternative treatment for PD[15].

In the early phase of preclinical and clinical studies, foetal ventral midbrain tissues were grafted into the striatum of rats[16,17] and PD patients[18-23]. The foetal DAergic neuron transplants survived, released DA, and improved behavioural deficits in animal models of PD[16,17] and individuals[20,21]. However, apart from these benefits, in some patients, dyskinesia developed[22,23]. Moreover, independent postmortem examinations revealed that the healthy neurons grafted into the brains of PD patients acquired Lewy bodies several years after transplantation[18,19] due to a prion-like mechanism with permissive templating of synucleinopathy progression[1,6]. In this context, DA neuron-based transplant approaches are not curative; nevertheless, they offer a competitive therapeutic effect[5]. The rapid development of cell- and molecular-based research opens new perspectives for cell therapy approaches in managing PD [5]. During the last decade, cumulative research on using different types of stem cells as starting material supports the potential of stem-cell-based DAergic neuron replacement therapy for PD[5]. The considered candidates are NSCs, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and mesenchymal stem cells (MSCs)[24]. Importantly, translation into the clinic of the stem cells transplantation approach requires avoidance of adverse immunological response and proliferation and related risk of tumorigenesis[5].

This review focuses on the therapeutic potential of NSCs in PD. Accordingly, we give an overview of their use in experimental studies and clinical trials and discuss challenges related to their application, alongside the pros and cons of NSC-based therapy in PD. Given the state-of-the-art accomplishments of stem cell therapy, gene therapy, and nanotechnology, we shed light on the perspective of complementing the advantages of each process by developing nano-stem cell therapy, also using genetically engineered NSCs.

EMERGENCE OF NSCS IN ADULT BRAIN AND ALTERNATIVE SOURCES OF NSCS

NSCs are an important central nervous system (CNS) element. NSCs exist in the embryonic brain and adult nervous system during human life and are found in two specific brain regions (Figure 1A). The subgranular region of the dentate gyrus and the lateral wall of the subventricular zone both contain a significant number of adult endogenous NSCs[25]. These cells have a few key features, including self-renewal, proliferation, and multipotency, which make them a major component in the CNS, maintaining the cell pool of nervous tissues[15]. NSCs can differentiate into neurons, astrocytes, or oligodendrocytes and may replace the loss of DAergic neurons in PD[13]. Moreover, NSCs can encourage endogenous repair mechanisms as they can migrate near the site of damage to increase neuroblasts and promote tissue repair. NSCs can have immunomodulatory effects such as cytokine, chemokines, and chemokine receptors secretion and T cell proliferation inhibition[26,27] (Figure 1B). Neurotransmitters, neuropeptides, cytokines, metabolites, extracellular matrix proteins, and accessory cells control NSCs proliferation[13].

The alternative sources of NSCs are foetal and adult nervous system tissues, umbilical cord blood, bone marrow, peripheral blood, amniotic fluid, Wharton jelly, skin- or adipose-derived MSCs, or iPSCs[28] (Figure 1A). In the transplantation procedure, these stem cells are either *in vitro* pre-differentiated or, according to the concept that signals in the injured tissue will recruit the stem cells and induce their differentiation *in situ*, NSCs are applied directly in the undifferentiated state[28]. Recently, trans-differentiation, especially chemically induced, whereby somatic cells are reprogrammed into patient-specific neuronal cells by skipping an intermediate proliferative pluripotent stem cell stage, offers perspectives on alternative autologous cell therapeutic strategies for PD[29]. By producing the desired type of CNS cells, these sources have all been used to demonstrate their potential for treating various neurodegenerative diseases[30].

EXPERIMENTAL APPROACHES TO NSC TRANSPLANTATION

Several preclinical studies have shown that different types of human NSCs, after their transplantation, could migrate, survive, and proliferate in specific brain sites. Immunohistochemical examinations support evidence that the transplanted human NSCs could transform into functional neurons and integrate into neural circuits[31]. In *in vivo* studies, it has been demonstrated that NSCs transplantation was an efficient therapeutic method for PD (Table 1). Yang *et al*[32] have shown that the clonal line C17.2 of NSCs grown *in vitro* under control conditions and transplanted into intact or 6-hydroxydopamine (6-OHDA)-lesioned rats spontaneously developed into neuronal-like cells, expressed traits of a DA phenotype, and integrated into the host brain[32]. Svendsen *et al*[33] have demonstrated that expanded populations of human CNS progenitor cells maintained in a proliferative state in culture and can migrate and differentiate into both neurons and astrocytes following intracerebral grafting into the striatum of adult rats with unilateral DAergic lesions[33]. NSCs, secreting *in vitro* nerve growth factor and neurotrophin-3, engrafted into the 6-OHDA lesioned striatum of rats, have been reported to survive and partially differentiate into tyrosine hydroxylase (TH)-positive cells. A significant rotational behaviour improvement accompanied these effects[33].

The ability of NSCs for neural differentiation and to exert a trophic effect by secreting nerve growth factor and neurotrophin-3 on dying DA neurons in the striatum has been suggested as an effective mechanism against behavioural deficits in PD rats subjected to cell transplantation[34]. A high-throughput quantitative proteomic study revealed that the protein profile of the subventricular zone severely distorted in mice by 6-OHDA injection was normalized and striatal astrocytes were activated after human NSC transplantation accompanied by an increase in neurotrophic factors. In addition, the grafted mice exhibited improved behavioural performance. The changes in the proteome have been suggested to result from the stimulation of host nigral DAergic neurons and their nigrostriatal projections rather than their replacement[35]. To increase trophic factor secretion, genetically engineered human NSCs were developed[36]. Stereotaxic transplantation of human NSCs (HB1.F3 clone) secreting stem cell factor into the 6-OHDA-lesioned striatum of rats has been demonstrated to result in functional improvements and ameliorated Parkinsonian behavioural symptoms. It was accompanied by the activation of endogenous neurogenesis in the subventricular zone, alongside the preservation of TH-positive cells of the nigrostriatal pathway[37]. Similarly, human olfactory bulb NSCs genetically engineered to express human nerve growth factor ameliorated the cognitive deficits associated with 6-OHDA-induced lesions in PD model rats. Transplanted cells exhibiting enhanced survival and differentiation rate migrated to damaged areas to promote repair or neuroprotection through cell replacement, integration, and/or neuroprotection[38]. Foetus-derived human NSCs (hVM1 clone 32) transplanted in their undifferentiated state protected against motor and nonmotor deficits in middle-aged parkinsonian mice, although degeneration of the nigrostriatal pathway was not significantly improved. Thus, the multifactoriality of NSCs was suggested[39]. Yoon *et al*[40] have reported that although the human-brain-derived NSCs injected into the striatum of rats with the 6-OHDA-induced lesion of the nigrostriatal pathway differentiated into DAergic neurons, no functional or behavioural improvements were observed[40]. Transplantation of human-brain-derived NSCs in the form of cell aggregates with a pre-established cell network into the striatum of 6-OHDA-injected rats appeared to be more effective than single-cell transplantations. The transplanted cells not only survived but also exerted a therapeutic effect attributed to secreting DA[41].

Nonhuman primate models of PD represent the highest clinical translational value due to close-to-human similarities in physiology, anatomy, and immunology and are used to validate therapeutical strategies at preclinical stages[42]. Successful transplantation of undifferentiated human NSCs into the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-challenged primates promoted homeostatic adjustments attributed to normalization of endogenous neuronal number-to-size ratios and protection of host nigrostriatal circuitry that resulted in behavioural improvement. In addition, the percentage of cells containing ASN inclusion dropped from 80% to 20% upon human NSC transplantation.

Table 1 Experimental studies on the therapeutic role of neural stem cells in Parkinson's disease models

Cells type	Model	Effects	Ref.
Undifferentiated NSCs line C17.2	6-OHDA rat model	Striatum deposition ↑ Expression of β-tubulin III, NSE, NeuN ↑ TH- and AADC-positive cells ↓ Motor behaviour deficits	Yang *et al*[32]
hCNSPCs	6-OHDA rat model	Striatum deposition ↑ Expression of β-tubulin III ↑ TH- and hGFAP-positive cells ↓ Motor behavior deficits	Svendsen *et al*[33]
hNSCs	6-OHDA mice model	Striatum deposition ↓ IL-1 β, IL-2, and TNF-a ↑ IL-10 ↓ Motor behavior deficits	Zuo *et al*[35]
hfNSC line (HB1.F3)	6-OHDA rat model	Striatum deposition ↑ Expression of nestin, HuD, β-tubulin III-positive, MAP2-positive, and NeuN positive and HuC negative ↑ TH-positive cells ↓ Motor behavior deficits	Yasuhara *et al*[37]
hNFG-GFP-OBNSCs	6-OHDA rat model	Striatum deposition normal histoarchitecture of the striatum ↑ Striatal neurons ↓ Necrotic cells ↓ Motor behavior deficits	Marei *et al*[38]
human dental papilla-derived stem cells and hbNSCs	6-OHDA rat model	Striatum deposition ↑ MAP2- positive cells ↑ TH- and GIRK2-positive cells ↔ No differences behavioral amelioration	Yoon *et al*[40]
hbNSCs	6-OHDA rat model	Striatum deposition ↑ TH- and human β2 microglobulin-positive cells ↔ No differences behavioral amelioration	Shin *et al*[41]
hNSCs	MPTP-induced monkeys	Striatum deposition ↑ TH- and DAT- positive cells ↑ DA level ↓ Motor behavior deficits	Redmond *et al*[43]
hpNSCs	MPTP-induced monkeys	Striatum deposition ↑ Striatal DA concentration, TH- positive cells, TH fiber innervation ↓ Behavior deficit	Gonzalez *et al*[44]

6-OHDA: 6-hydroxydopamine; AADC: Aromatic L-amino decarboxylase; DA: Dopamine; DAergic: Dopaminergic; DAT: Dopamine transporter; GIRK2: G Protein-activated inward rectifier potassium channel 2; hNSCs: Human NSCs; hbNSCs: Human brain-derived NSCs; hfNSCs: Human fetal-derived NSCs; hGFAP: Human glial fibrillary acidic protein; hNFG-GFP-OBNSCs: Olfactory bulb neural stem cells (OBNSCs) genetically engineered to express hNGF and GFP; hpNSCs: Human parthenogenetic stem cell-derived NSCs; HuC: Very early neuronal marker; HuD: Postmitotic neuronal marker; MAP2:

Microtubule-associated protein 2; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NSE: Neuron-specific enolase; NeuN: Matured neuronal marker; NPCs: Neural progenitor cells; TH: Tyrosine hydroxylase; TNF-α: Tumor necrosis factor- α.

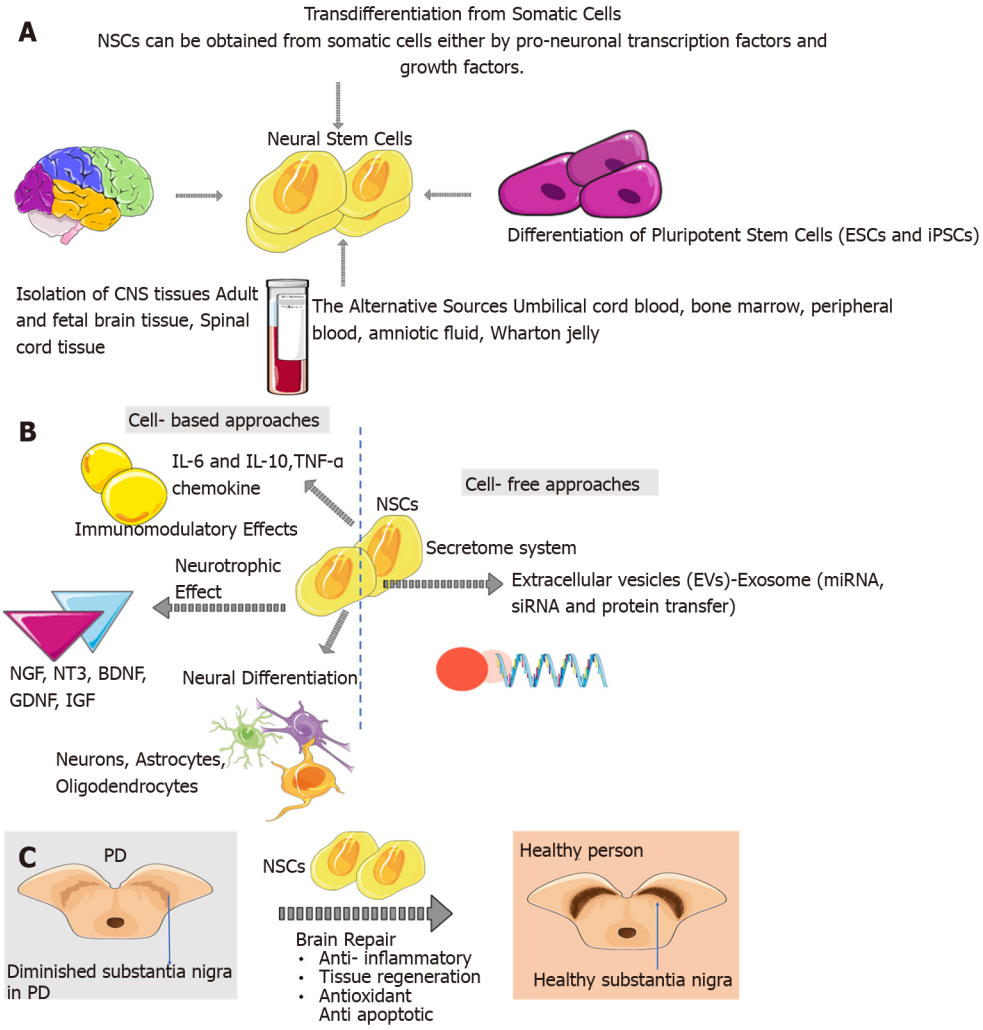


Figure 1 Emergence of neural stem cells in the adult brain and alternative sources of neural stem cells. A: The sources of neural stem cells (NSCs) are embryonic stem cells and induced pluripotent stem cells, adult/foetal brain tissue and spinal cord tissue. Also, NSCs can be obtained from somatic cells either by transcription factors and growth factors. The alternative sources of NSCs are foetal and adult nervous systems, umbilical cord blood, bone marrow, peripheral blood, amniotic fluid, Wharton jelly; B: NSCs can have immunomodulatory effects such as cytokine, chemokines, and chemokine receptors secretion and T cell proliferation inhibition. NSCs can have neurotrophic effects such as nerve growth factor, neurotrophin-3, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor. NSCs can target secretome and paracrine effects *via* extracellular vesicles like exosomes. Also, NSCs play a role in neural differentiation; C: NSCs after their transplantation, could migrate, survive, and proliferate in specific brain sites. And NSCs play a role in brain repair mechanisms such as antiapoptotic, anti-inflammatory, and antioxidant. CNS: Central nervous system; BDNF: Brain-derived neurotrophic factor; ESCs: Embryonic stem cells; EVs: Extracellular vesicles; GDNF: Glial cell line-derived neurotrophic factor; iPSCs: Induced pluripotent stem cells; NGF: Nerve growth factor; NT-3: Neurotrophin-3; NSCs: Neural stem cells; TFs: Transcription factors; PD: Parkinson's disease; TGF: Transforming growth factor. Citation: The parts of the figures were drawn using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/Licenses/by/3.0/).

Importantly, no tumours, overgrowth, or inappropriate stem cell migration in the transplanted primates were observed [43]. Gonzalez *et al*[44] evaluated the safety and efficacy of two doses of human parthenogenetic stem-cell-derived NSCs injected in the striatum and substantia nigra of MPTP-lesioned monkeys with moderate to severe PD symptoms. They found that human parthenogenetic NSCs engrafts promoted behavioural recovery accompanied by raised striatal DA concentration, fibre innervation, and several DA neurons. Increased expression of genes and pathways downregulated in PD has been observed. Importantly, the 12 mo post-transplantation of human parthenogenetic NSCs was safe and well tolerated by the animals with no serious adverse events[44]. This safety aspect is significant as the potential for inappropriate stem cell migration and tumour formation must be minimized before cell transplantation[5].

Stem cell therapeutic strategies for PD not only deal with the direct replacement of damaged or lost cells but also target secretome and paracrine effects on neurotrophic and growth factors and endogenous neural precursor cell activity[45,46] (Figure 1B). The heterogeneous population of membrane-bound exosomes and extracellular vesicles (EVs) is a part of the

secretome system[45]. Nanosized EVs derived from human iPSC (hiPSC)-NSCs have been suggested as a safer alternative to NSCs. Upadhya *et al*[47] demonstrated that EVs exhibited similar neuroreparative properties as NSCs, and they could be administered noninvasively as an autologous or allogeneic off-the-shelf product. EVs comprising miRNAs and/or proteins promoting synaptogenesis, synaptic plasticity, and better cognitive function have been demonstrated to incorporate well into neurons, microglia, and astrocytes of rodents after intranasal treatment, resulting in enhancement of hippocampal neurogenesis and an anti-inflammatory effect[47]. The results hold the promise of using stem-/progenitor-cell-derived EVs, or EVs loaded with bioactive molecules such as DA, catalase, curcumin, and siRNAs, to alleviate parkinsonian symptoms[48]. Specifically, exosomes biologically active EVs, with high miR-133b, which are lacking in PD patients, can promote the growth of neurites[49]. Lee *et al*[50] have demonstrated that NSC-derived EVs *via* a paracrine signalling-based mechanism exerted antioxidant, antiapoptotic, and anti-inflammatory effects, thereby protecting against loss of neuronal population and function of target cells, both *in vitro* and *in vivo* PD models[50]. Nevertheless, isolation, characterization, and testing of the biological properties of EVs retain critical issues for translation[47]. An emerging alternative to the cell-based approach is the nanotechnology approach for the targeted delivery of growth factors aiming to support and expand resident CNS stem cells for endogenous repair[51].

PROS AND CONS OF THE USE OF NSCs IN PD

Injected neural progenitor cells (NPCs) accumulate in the brain and integrate with the injured zone; however, in only a small percentage, most of the cells remained undifferentiated. The promotion of tissue survival is associated with anti-inflammatory and antiapoptotic effects at both mRNA and protein levels and increased secretion of growth factors[52] (Figure 1C). Additionally, NPCs cooperate with nearby immune and resident cells of the CNS to modulate the focal release of stem cell regulators and aid in the functional recovery from CNS injuries[53,54]. As Marsh and Blurton-Jones [55] have reviewed, there is strong evidence that NSC-mediated changes in neurotrophies resulting in increased synaptic plasticity, long-term potentiation, and neuronal survival contribute to the improvement of cognitive and motor performance observed *in vivo*. Thus, NSCs providing neurotrophic support to affected neuronal populations and synapses is a promising approach for treating neurodegenerative diseases such as PD[55]. Safety, including a potential tumorigenic effect, raised concerns. Amariglio *et al*[56] (2009) have reported a donor-derived brain tumour in a boy with ataxia telangiectasia following intracerebellar and intrathecal injection of human foetal NSCs[56]. Moreover, the complex issue of the immune rejection of transplanted cells in the brain has been demonstrated[5]. Therefore, the development of stem-cell-derived neuronal transplant therapies in PD requires consideration of this safety aspect alongside the effectiveness ones, which can be managed by addressing issues related to type and source of cells, their reproducible generation, accurate zone of transplantation and maintenance of function and viability of these grafted cells should be considered[5].

Considering transplantation safety, protocol standardisation, and the ethical aspect, approaches with the use of ESCs and IPSCs for making DAergic neurons are being developed[5,14].

ESCs, undifferentiated pluripotent cells derived from mammalian blastocysts, can make all types of cells in the body. Also, iPSCs, laboratory-generated counterparts of ESCs, that are generated from adult somatic cells, possess similar properties and can differentiate into any other cell. ESCs and iPSCs are used in many clinical trials are going on globally and have wide applications in regenerative medicine for specific diseases and conditions. The results of these trials do not, however, reach the general population because of the long and complex time frame needed to complete the clinical trials and publish the data obtained[57].

iPSCs have some advantages, such as obtaining the reprogrammed cells directly from the patients, thus potentially reducing the risk of transmissible infections and immune reactions. Additionally, they can change into any type of cell approach to treating PD[58]. Using iPSCs for treating PD is ethically permissible because patients' somatic cells can be differentiated into a pluripotent state to produce DAergic neurons implanted into the brain. Several investigations elucidated the promising role of iPSCs in regenerating DAergic neurons for treating PD. Transplantation of iPSCs ameliorated PD symptoms[58]. iPSCs have some disadvantages, such as powerful pluripotency; hence the risk of tumour development of iPSCs may be greater than other stem cells. The heterogeneity of iPSCs for clinical therapy could lead to potential tumorigenicity risks *in vivo*. Additionally, iPSCs made from autologous PD patients may contain pathogenic gene mutations that impact the outcome of cell replacement therapy[59]. Although grafted autologous iPSCs cells are considered immune-privileged, they can still be recognized as foreign (allogeneic) by the patient's immune system in some situations. Cell preparation protocol and transplantation site have been suggested to influence iPSC-derived cell immunogenicity[60]. Hence, decreasing the heterogeneity and increasing the controllability of iPSCs and cell preparation through isolation, purification, amplification, and modification of stem cells is necessary to decrease the tumorigenicity and immunogenicity potentials[61].

Although these scalable and traceable sources with well-established protocols and the efficacy in preclinical PD models have made ESCs and iPSCs promising candidates for cell-based therapy (Table 2), the risks of tumour development and immunosuppression have not been eliminated. These sources are still not ethically neutral, especially ESCs, as the embryo is used in the persevering protocol[5,14].

Despite these great efforts, there is still a gap between experimental therapeutic approaches and their translation into clinical practice. Although several clinical trials (Table 3) have been studied using cell-based treatments for PD (Clinical Trials. gov; NCT03119636[62], NCT05635409[63], NCT02452723[64], NCT03815071[65], UMIN000033564[66], NCT-03128450[67], NCT03309514[68], NCT01898390[69], NCT02795052[70], NCT00976430[71], NCT03724136[72], NCT03684122[73]) these days, the main obstacles restricting the clinical use of stem cells refer mainly to ethical concerns,

Table 2 Experimental studies on the therapeutic role of alternative stem cell sources in Parkinson's disease models

Stem cells source	Model	Effects	Ref.
hPESC line	MPTP-induced monkeys	Striatum deposition	Wang *et al*[93]
Q-CTS-hESC-1		↑ DA level	
		↑ Expression of TH-, hNCAM and GIRK2-positive cells	
		↑ Behavioral improvement	
NPCs	6-OHDA rat model	↑ DA level	Song *et al*[94]
		↑ Nurr1 and Foxa2 transcription factors	
		Improvement in behavior is not mentioned	
hiPSC lines	6-OHDA rat model	Striatum deposition	Song *et al*[95]
		↑ DAergic neurons density	
		↑ Behavioral amelioration	
MSCs	6-OHDA rat model	↑ TH-positive fibers in the striatum and TH-positive neurons in the SNpc	Wang *et al*[96]
		↑ Therapeutic effects of SDF-1α	
		↑ Behavioral amelioration	
hMSCs	6-OHDA rat model	striatum deposition	Cova *et al*[97]
		↑ DAergic neuron density	
		↑ Ki67/ PCNA – positive cells level	
		↑ Dcx expression	
		None of the grafted cells were TH- or DAT-positive	
		Improvement in behavior is not mentioned	

6-OHDA: 6-hydroxydopamine; DA: Dopamine; DAergic: Dopaminergic; DAT: Dopamine transporter; Dcx: Double cortin; GIRK2: G protein-activated inward rectifier potassium channel 2; hMSCs: Human adult MSCs; hiPSC: Human iPSC; hPESC: Human parthenogenetic ESCs; hNCAM: Human specific neural cell adhesion molecule; PCNA: Proliferating cell nuclear antigen; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NPCs: Neural progenitor cells; Ki67: Proliferating cell nuclear antigen, SDF-1α: Stromal cell-derived factor 1α; SNpc: Substantia nigra pars compacta; TH: Tyrosine hydroxylase.

immune response, tumorigenesis, and toxicity[31].

PROSPECTS FOR NSC-BASED THERAPY

From the short review above, several challenges for NSC-based therapy emerge, which can be identified at every stage of the neurosurgical procedure, including preoperative cell preparation and quality control, perioperative procedures, and postoperative graft preservation, adherence, and overall success of the therapy[74].

Strict quality control and safety assessments of the relevant cells must be in place at the preoperative stage. To achieve the optimal number of DAergic neurons, the required number of implanted cells in the final graft should be achieved [74]. The DA neurons can be derived from different stem cells or directly from NSCs. The use of foetus-derived NSCs has shown outstanding potential in rodent models of PD, and the possibility for tumorigenicity is minimal[75]. Safety issues related to the risk of tumorigenesis by grafted stem cells need to be evaluated for the long-term survival and phenotype stability of stem-cell-derived neurons in the graft following transplantation to identify the appropriate stem cell type. In addition, cell dose, case selection, and delivery route are also necessary as a precondition for the clinical use of cell-based therapies[76]. Patients receiving treatment with NSCs products are at risk for safety issues related to delivery methods, immune and allergic reactions to the medication, tumour development, heterotopic neuronal differentiation with functional disruption, and viral and microbial contamination[26]. Therefore, the fundamental concern of cell-based therapies is that before entering the clinic, transplantable cell populations must be differentiated and characterized according to good manufacturing practice regulations both *in vitro* and *in vivo*[77]. This, in turn, requires access to a large pool of differentiated cells stored in a tissue bank. However, providing sufficient *in vitro* differentiation of neurons from NSCs is challenging[78]. Cell origin is a significant problem that must be considered in treating PD by NSC transplantation as the high concentration of NSCs is needed to treat diseases[79].

Table 3 Clinical trials for cell-based therapy in Parkinson's disease

Study identifier	Cell type	Aim of the study
NCT03119636[62]	hESC-NPCs	Safety and efficacy evaluation of intracerebral transplantation
NCT05635409[63]	hESC-DA neurons	Safety and tolerability evaluation of intraputamenal transplantation
NCT02452723[64]	ISC-hpNSC®	Safety evaluation of intracerebral transplantation
NCT03815071[65]	iPS-NSCs	Safety and efficacy evaluation
UMIN000033564[66]	iPSC-derived dopaminergic progenitors	Safety and efficacy of transplantation into the corpus striatum
NCT03128450[67]	hNSCs	Safety and efficacy evaluation of nasal delivery
NCT03309514[68]	NSC-derived neurons	Safety and efficacy evaluation of intracerebral injection
NCT01898390[69]	hVMT	Safety and efficacy evaluation
NCT02795052[70]	BMSC	Efficacy evaluation of intravenous and intranasal delivery
NCT00976430[71]	BMSC	Safety and efficacy evaluation
NCT03724136[72]	BMSC	Efficacy evaluation of intravenous and intranasal delivery on cognitive impairment
NCT03684122[73]	Umbilical cord derived MSCs differentiated into NSCs	Safety evaluation of intrathecal and intravenous injections

BMSC: Bone-marrow-derived stem cell; iPS-NSC cells: Pluripotent stem-cell-derived neural stem cells; ISC-hpNSC®: Human parthenogenetic stem-cell-derived neural stem cells developed by international stem cell corporation; hNSCs: Human neural stem cells; hVMT: Human ventral mesencephalon tissue; hESC-NPCs: Human embryonic stem cells-derived neural precursor cells.

For the promotion of the behaviour of NSCs, the fabrication of culture surfaces is developed. In this context, nanomaterials, with their nano size, unique physicochemical properties, and capability for surface functionalization, offer the potential for designed manipulation of cell behaviour. Fabricating the extracellular matrix with biocompatible nanomaterial-based scaffolds with topology simulating the microenvironment *in vivo* seems to be a promising strategy. Pandanaboina *et al*[78] demonstrated successful differentiation of embryonic rat NSCs on the nanocellulose coupled with lysine scaffold as 87% of the cells attached to the surface, and more than half of the NSCs had differentiated into functional neurons[78]. Previously they found that plasmonic gold nanorods integrated into growth surfaces tuneable stimulated and modulated embryonic rat NSCs[80]. A robust scaffold for culturing NSCs *in vitro* is three-dimensional graphene (3DG) foam, which behind supporting their growth, maintains cells in an active proliferative state through the modulation of metabolism and several metabolic processes. 3DG-stimulated pathways are involved in amino acid incorporation and glucose metabolism[81] (Figure 2).

As NSCs are regulated by a specialized vasculature during adult neurogenesis, transplanted, they may be importantly affected by the adjoining vasculature and endothelial cells[76]. Chou *et al*[82] have shown the importance of establishing an appropriate neurovascular microenvironment to deliver NSCs to replace lost DA neurons. This study indicated the molecular and cellular signalling involved in forming vasculature-like structures and increased neuronal differentiation through endothelial signalling[82]. The ability to properly locate and track transplanted NSCs is necessary to develop the efficacy of NSC therapies and ensure the treatment's safety[25].

A critical step for efficient NSCs therapy is the survival and integration of the grafted DAergic neurons, due to the lack of both stromal and growth factor support of the cells and the risk of ischaemic stress and an innate immune response [51]. Prion-like transmission synucleinopathy to the healthy neurons grafted into the brains of PD patients should be considered[18,19,21]. Nanomaterials capable of suppressing oxidative stress[83], neuroinflammation[84], or even toxic protein aggregation[85] can act as bioactive nanomedicines addressing the limitations of cell-based therapy for PD. The strategy of combining nano-enabled therapy with stem-cell-based therapy shows potential for the treatment of PD[46]. Accordingly, NSCs isolated from human olfactory bulb human coengrafted with carbon nanotubes (CNTs) restored cognitive deficits and neurodegenerative changes in the trimethyl-induced rat model of neurodegeneration. Moreover, the CNTs supported engrafted NSCs by stimulating differentiation into neurons rather than glial cells[86]. To support individual cells over the first few weeks at the postgrafting stage, attached growth-factor-loaded nanoparticles (NPs) are directly to the cell before grafting has been developed[51]. Nanomaterial carriers can be designed into "intelligent" nanodrug delivery systems functionalised with targeting ligands with the controlled release of loaded drugs/genes/cells for effective transport of them to inaccessible and specific brain areas[46]. Preclinical data have supported the safety and efficacy of the surrogate NPs stroma as no adverse effects after injection of stroma/cell constructs into the brain were evident, and human foetal ventral mesencephalic cell survival *in vivo* was increased fourfold[87]. At the postoperative stage, monitoring the injected/transplanted cells is critical for the optimisation of the neurosurgical procedure. Nanomaterials, with their unique optical properties, cellular uptake, surface functionalisation, and good biocompatibility, are

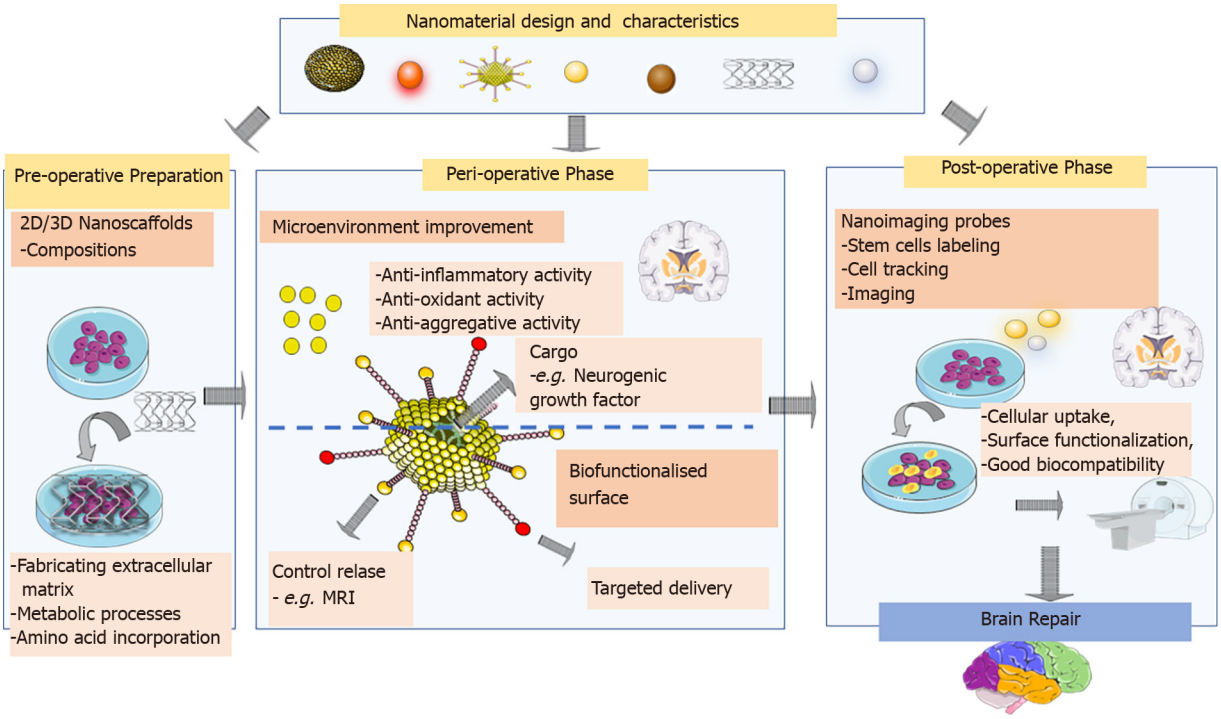


Figure 2 Application of nanomaterials in stem cell therapy of Parkinson's disease. Preoperative preparation: Nanomaterials, with their small size, unique physicochemical properties, and capability for surface functionalisation, offer the potential for designed manipulating cell behaviour. A promising strategy is that fabricating the extracellular matrix with biocompatible nanomaterial-based scaffolds with topology simulating the microenvironment. Preoperative phase: nanomaterials capable of suppressing oxidative stress, neuroinflammation, and toxic protein aggregation can act as bioactive nanomedicines addressing the limitations of cell-based therapy for Parkinson's disease. Moreover, "intelligent" nano-drug delivery systems functionalised by targeting ligands with the controlled release of loaded molecules like growth factor aims to support and expand stem cells for brain repair. At the postoperative stage, nanomaterials, with their unique optical properties, cellular uptake, surface functionalisation, and good biocompatibility, are promising candidates for cell tracking and imaging. MRI: Magnetic resonance imaging. Citation: The parts of the figures were drawn using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/Licenses/by/3.0/).

promising candidates for cell tracking and imaging[46] (Figure 2).

Advances in the development of stem cell and gene therapies create new opportunities. The combination of stem cell and gene therapy could be a technical breakthrough that increases the therapeutic effectiveness of stem cells. Engineered cells overexpressing genes involved in DA synthesis or neurotrophic factors might increase their functional capability and solve differentiation and survival issues, thus improving gene therapy's efficacy[88]. Taking into account the safety of cell-based therapy the cells can be reprogrammed to avoid as many adverse effects as possible, including immune reactions and tumours[89].

CHALLENGES FOR NANO-STEM CELL THERAPY

Nano-stem cell therapy can face many challenges, mainly related to the safety profile of NPs, including potential cytotoxicity and unknown impact on stem cell differentiation and biodegradability. There is ample scope for further research for understanding mechanisms by which cells interact with nanomaterials, the biotransformation of NPs, and their influence on cell functions, making it challenging to control the safety and effectiveness of nano-cell therapy[46,90]. Since the activity of NPs strongly depends on their sizes, structures, shapes, and surface chemistry and is cell type dependent[91], several problems related to methods for the synthesis, bio-functionalisation, characterisation, and tailoring of 3D nanostructures in tissue engineering should be addressed[46,90].

Given the challenges and state-of-art accomplishments of nanotechnology, it can be expected to lead to the development of nano-stem cell therapy for PD. It is supported by recently merged multifunctional nanotheragnostic approaches for effective stem cell therapy in managing brain cancers[92].

CONCLUSION

PD significantly affects society because it carries a mounting socioeconomic burden, while no cure is currently available. The studies discussed in this review have shown the potential of NSC-based therapy for PD (Figure 1C). Although NSCs present major promise in treating this neurodegenerative disease, they raise significant concerns. They may form a

tumour, which is a more devastating condition than PD. Moreover, immunological rejection, ethical issues, limited cell sources, and complications related to skill to maturation into desired neuronal cell types and proper differentiation are important reasons for further research to facilitate NSC therapy in PD.

Currently, combining stem cell therapy, including genetically engineered cells, with nanotechnology approaches to complement each other's advantages provides new insights into the improving the therapy protocol and efficacy. The safety profile and the efficacy of nano-stem cell therapy in treating neurodegenerative diseases is a research hotspot. It is believed that future advancements in new nanomaterials will surely benefit NSCs-based therapy from this technology.

In conclusion, although various obstacles and challenges remain, nano-stem cell therapy holds promise to cure PD. Clearly, its continuous improvement and development from the stage of laboratory experiments to the clinical application are necessary.

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ORIGINAL ARTICLE

Basic Study

Commitment of human mesenchymal stromal cells to skeletal lineages is independent of their morphogenetic capacity

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Abstract

BACKGROUND

Mesenchymal stromal cells (MSCs) are multipotent cell populations obtained from fetal and adult tissues. They share some characteristics with limb bud mesodermal cells such as differentiation potential into osteogenic, chondrogenic, and tenogenic lineages and an embryonic mesodermal origin. Although MSCs differentiate into skeletal-related lineages *in vitro*, they have not been shown to self-organize into complex skeletal structures or connective tissues, as in the limb. In this work, we demonstrate that the expression of molecular markers to commit MSCs to skeletal lineages is not sufficient to generate skeletal elements *in vivo*.

AIM

To evaluate the potential of MSCs to differentiate into skeletal lineages and generate complex skeletal structures using the recombinant limb (RL) system.

METHODS

We used the experimental system of RLs from dissociated-reaggregated human placenta (PL) and umbilical cord blood (UCB) MSCs. After being harvested and reaggregated in a pellet, cultured cells were introduced into an ectodermal cover

obtained from an early chicken limb bud. Next, this filled ectoderm was grafted into the back of a donor chick embryo. Under these conditions, the cells received and responded to the ectoderm's embryonic signals in a spatiotemporal manner to differentiate and pattern into skeletal elements. Their response to differentiation and morphogenetic signals was evaluated by quantitative poly-merase chain reaction, histology, immunofluorescence, scanning electron microscopy, and *in situ* hybridization.

RESULTS

We found that human PL-MSCs and UCB-MSCs constituting the RLs expressed chondrogenic, osteogenic, and tenogenic molecular markers while differentially committing into limb lineages but could not generate complex structures *in vivo*. MSCs-RL from PL or UCB were committed early to chondrogenic lineage. Nevertheless, the UCB-RL osteogenic commitment was favored, although preferentially to a tenogenic cell fate. These findings suggest that the commitment of MSCs to differentiate into skeletal lineages differs according to the source and is independent of their capacity to generate skeletal elements or connective tissue *in vivo*. Our results suggest that the failure to form skeletal structures may be due to the intrinsic characteristics of MSCs. Thus, it is necessary to thoroughly evaluate the biological aspects of MSCs and how they respond to morphogenetic signals in an *in vivo* context.

CONCLUSION

PL-MSCs and UCB-MSCs express molecular markers of differentiation into skeletal lineages, but they are not sufficient to generate complex skeletal structures *in vivo*.

Key Words: Human mesenchymal stromal cells; Recombinant limbs; Mesenchymal stromal cell morphogene-sis; Mesenchymal stromal cell *in vivo* differentiation; Skeletal tissues

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Core Tip: Human mesenchymal stromal cells (MSCs) from umbilical cord blood or placenta can differentiate into osteogenic and chondrogenic lineages in culture systems and have been used in regenerative medicine. Here, we used the recombinant limb (RL) model to provide evidence that MSCs do not have the ability to generate skeletal structures *in vivo*. MSCs received and responded to the ectoderm's embryonic spatiotemporal signals in this RL system. However, the expression of differentiation markers of skeletal lineages was not sufficient to generate skeletal structures *in vivo*.

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INTRODUCTION

The limbs have an intricate anatomy that results from highly coordinated morphogenesis and cellular differentiation processes, which establish the correct position and shape of muscles, bones, cartilage, tendons, skin, and nerves[1]. During embryonic development, limb bud mesodermal cells (LBMCs) form a bud covered by a layer of ectodermal cells. The morphogenesis and differentiation of limb tissues are controlled by the response to signals that emanate from three signaling centers: The apical ecto-dermal ridge (AER), which controls the proximodistal axis; the zone of polarizing activity, which directs the anteroposterior axis; and the ventral and dorsal LB ectoderms, which control the dorsal-ventral axis[1,2]. Fibroblast growth factor 8 (FGF8) and Wnt family member 3a signals emanate from the AER and maintain the MCs in an undifferentiated and proliferative state. Once cells stop receiving AER signals, they begin the differentiation process toward the chondrogenic and tenogenic lineages, followed by endochondral ossification to form the skeletal elements[1,2]. As development progresses, the coordination between signals is essential for patterning and differentiation to fully develop the skeletal elements of a functional limb[1,2].

SRY-box transcription factor 9 (SOX9) is the earliest molecular marker of chondrogenic commitment[3]. Besides, SOX9 induces the expression of extracellular matrix proteins such as collagen type 2 (Col2α) and aggrecan (ACAN)[4]. On the other hand, growth differentiation factor 5 (GDF5) promotes cell condensa-tion and cell adhesion during the initial stages of chondrogenesis[5,6]. By contrast, hypoxia-inducible factor alpha (HIF-1α) is critical in maintaining the chondrogenic environment[7]. Once cartilage anlagen is formed, ossification begins by expressing Runt-related transcription factor 2 (RUNX2)[8-10]. At the same time, the dorsal and ventral margins of early embryonic LB initiate tendon specification by express-ing the earliest known marker of tenocyte progenitors, scleraxis (SCX)[11,12]. SCX induces the transcription of major structural collagens and the glycoprotein tenomodulin[13]. Mohawk (MKX) is involved in tendon maturation by

maintaining SCX expression, which restricts cells to the tenogenic lineage[13,14].

Regenerative medicine has emerged as an alternative treatment for degenerative diseases or an adjuvant therapy[15,16]. To this end, the potential use of mesenchymal stromal cells (MSCs) has garnered great interest. Because MSCs share characteristics with LBMCs and have the potential to differentiate into osteogenic or chondrogenic lineages[17-21], in this work, we used the recombinant limb (RL) model to evaluate the capacity of human MSCs to form complex skeletal structures[22]. The RL model recapitulates the differentiation, morphogenesis, and patterning programs that occur during normal limb development[23-25]. RLs consist of an ectodermal cover obtained from an early chicken LB (CK-LB) filled with dissociated-reaggregated cells grafted into the back of a donor chick embryo. Because of the high capacity of human MSCs from umbilical cord blood (UCB-MSCs) or placenta (PL-MSCs) to differentiate into osteogenic and chondrogenic lineages, we evaluated their ability to generate skeletal structures *in vivo*. Our results demonstrate that although MSCs receive and respond to the ectoderm's embryonic spatiotemporal signals, they do not generate complex skeletal structures, possibly due to the intrinsic characteristics of MSCs.

MATERIALS AND METHODS

Isolation and culture of MSCs

MSCs were collected from Villa Coapa Hospital, Mexican Social Security Institute (IMSS; Mexico City, Mexico) according to their ethical guidelines, including informed consent. MSCs derived from bone marrow (BM; n = 3) were used to validate the specificity of all primers used in this study. BM cells were obtained from hematologically normal BM transplantation child donors at the Bernardo Sepulveda Hospital (National Medical Center, IMSS). A cell population enriched for MSCs was isolated according to Montesinos *et al*[25] using a negative selection procedure [RosetteSep™ system; STEMCELL Technologies Inc. (STI), Vancouver, Canada] and a Ficoll gradient. The cells were resuspended in low-glucose Dulbecco's modified Eagle medium (Lg-DMEM; Gibco BRL, Rockville, MD, United States) supplemented with 10% fetal bovine serum (FBS) and seeded at a density of 0.2 × 10^6 cells/cm^2 into T25 cell culture flasks (Corning Inc., Costar, New York, NY, United States). After 4 d, the nonadherent cells were removed by pipetting, and fresh medium was added. Every 5 d, a medium change was performed. When the cultures reached 80% confluence, they were trypsinized (0.05% trypsin, 0.53 mmol/L EDTA; Gibco BRL, New York, NY, United States) and subcultured at a density of 0.01 × 10^6 cells/cm^2 into T75 flasks (Corning). In the second passage, cells were harvested and analyzed.

UCB-MSC and PL-MSC samples were obtained from two volunteer donors from normal full-term deliveries according to the institutional guidelines of Troncoso Hospital, IMSS, as previously described by Montesinos *et al*[25]. UCB-derived MSCs were obtained using a negative selection procedure (RosetteSep™ system; STI), as described for BM. Cells were resuspended in Lg-DMEM (Gibco) supplemented with 10% FBS (STI) and seeded at a density of 0.2 × 10^6 cells/cm^2 in T25 culture flasks (corning), and subsequent cultures were manipulated as described for BM. PL-derived MSCs were obtained using an enzymatic digestion procedure. The internal area (about 2 cm^3) of the central PL lobules was washed with phosphate-buffered saline (PBS) and dissected into small pieces with sterilized scissors and forceps. The chopped tissues were digested with trypsin-EDTA (Gibco) at 37 °C for 10 min. A single-cell suspension was collected by flushing the tissue parts through a 100-μm nylon filter (Falcon; Becton, Dickinson and Company, San Jose, CA, United States) with Lg-DMEM containing 10% FBS. The cell suspension was centrifuged for 10 min at 400 g to collect the cell pellet. Then, the cells were resu-spended in Lg-DMEM supplemented with 10% FBS. The total number of nucleated and viable cells was determined, seeded, and manipulated as described for BM. After the third passage, the cells were analyzed and cryopreserved. For all experiments, MSCs were used in passages 4-5.

RLs

RLs were established according to the protocol by Marín-Llera *et al*[24]. PL-MSCs, UCB-MSCs, or 22 Hamburger-Hamilton (HH) CK-LBMCs were collected to stuff 22 HH CK limb ectoderms, according to Hamburger and Hamilton [26]. After obtaining PL-MSCs, UCB-MSCs, or CK-LBMCs, they were centrifuged at 3000 rpm and incubated at 37 °C between 1.5 h and 2 h to form a compact pellet. Separately, to obtain ectoderms, LBs from about 10 of the 22 HH embryos were dissected in PBS, transferred to a tube, and digested in 0.5% trypsin in PBS for 30 min at 37 °C. Then, the LBs were transferred to PBS supplemented with 10% FBS, and the ectoderm was peeled off. Next, the pellet of PL-MSCs, UCB-MSCs, or CK-LBMCs was detached from the bottom of the tube and transferred to a small petri dish containing the ectoderms. Later, a pellet fragment was stuffed into each ectoderm. Ectoderms were individually transferred into a previously windowed 22 HH chick embryo, positioned between somites 15-20, and fixed with palladium wires over a previously scratched wound. Manipulated embryos were incubated for 24 h, 48 h, or 72 h at 37.5 °C until their collection and processing. The leftover pellet from three sources was independently frozen to evaluate its basal expression compared to RLs by quantitative polymerase chain reaction (qPCR). All RL experiments were performed in triplicate.

qPCR

RNA from RLs was extracted with NucleoSpin RNA (Cat. No. 740955; Macherey-Nagel, Düren, Ger-many) according to the manufacturer's instructions. Retrotranscription of total RNA was achieved using the RevertAid RT Kit (Cat. K1691; Thermo Fisher Scientific, Waltham, MA, United States). Gene expression levels were quantified using SYBR green (Cat. No. 4309155; Thermo Fisher Scientific), normalizing to the transcript level of ribosomal protein L13. The expression level was evaluated relative to a calibrator according to the $2^{-\Delta\Delta Ct}$ equation. Each plotted value represents the mean ± SD of at least three independent experiments. For each independent qPCR replicate, RNA was obtained from 25 to 30 RL pools for each condition. In addition, leftover pellets of MSCs were collected and processed for RNA extraction. The student's t-test

was performed to compare the expression levels between pellets and RLs and to determine the statistical significance among RL sources. Statistical significance was set at $P < 0.05$. To analyze the specificity of the amplification, a melting curve was achieved for each primer. The specificity of all primers was validated with human MSCs-BM before amplification in the RL. The primer sequences used in this study are included in Supplementary Table 1.

Alcian blue and hematoxylin staining

For Alcian blue staining of RLs, samples were fixed in 5% trichloroacetic acid (Cat. No. A-5268; Sigma-Aldrich, St. Louis, MO, United States) for 24 h and stained with 1% Alcian blue in ethanol-hydrogen chloride for 24 h. Then, the RLs were transferred to 100% ethanol for 24 h and cleared with methyl salicylate (Cat. No. M-2047; Sigma-Aldrich) until the skeleton was observed. RL images were acquired with the AxioZoom v16 microscope (Carl Zeiss, Oberkochen, Germany) using ZEN lite software (Carl Zeiss). After image acquisition, Alcian blue-stained RLs were dehydrated in ethanol and xylol before embedding in paraffin. Ten-micrometer sections were obtained with a microtome (RM2125 RTS; Leica, Wetzlar, Germany). For hematoxylin and eosin (H&E) staining, samples were rehydrated with an ascending gradient of xylol-ethanol, followed by incubation with H&E dyes. Next, slides were dehydrated with ethanol-xylol and mounted with DPX medium (Cat. 44581; Sigma-Aldrich). Images were acquired with the Olympus BX51-WI microscope equipped with a fluorescence and gyratory disc unit (Tokyo, Japan) using Stereo Investigator 9 software (MicroBrightField Inc., Colchester, VT, United States).

Detection of cell death with LysoTracker staining

RLs were incubated in 1 μM LysoTracker Red DND-99 (Cat. L7528; Molecular Probes, Eugene, OR, United States) at 37 °C for 15 min. Then, the samples were rinsed with PBS and fixed in 4% paraformaldehyde overnight at 4 °C. Next, the RLs were dehydrated in an increasing methanol-PBS-Tween series and cleared with 2:1 benzylic alcohol-benzyl benzoate solution for 1 h (following the 1999 recommendations by Parish). Images were acquired with an Olympus BX51-WI microscope equipped with a fluorescence and gyratory disc unit using Stereo Investigator 9 software (MicroBrightField).

Scanning electron microscopy

RLs were washed twice with PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2) for 2 h. The fixation solution was washed three times with 0.1 M sodium cacodylate at 4 °C. For postfixation, samples were immersed in 1% osmium tetroxide in cacodylate solution for 2 h before dehydration in graded concentrations of ethanol. The samples were placed in microporous capsules submerged in pure ethanol and placed in the Critical Point drying equipment (SPI) for desiccation. Ethanol was replaced with liquid carbon dioxide until its critical constants (31.3 °C and 1072 PSI) were met. After drying, the sample was mounted on an aluminum specimen holder with a silver-based adhesive. The coating was performed by ionizing the sample with a layer of gold in a low vacuum ionizer. Images were acquired using the DSM-950 Carl Zeiss microscope.

In situ hybridization

RNA antisense probes were labeled with UTP-digoxigenin (Cat. No. 11209256910; Roche Applied Science, Indianapolis, IN, United States) and used for whole-mount *in situ* hybridization as previously described[6]. Samples were treated with 15 μg/mL proteinase K for 20 min at 21 °C, and the hybridization temperature was 68 °C. The *Fgf8* signal was visualized with BM Purple substrate for alkaline phosphatase (Roche). Images were acquired with the AxioZoom v16 microscope (Carl Zeiss).

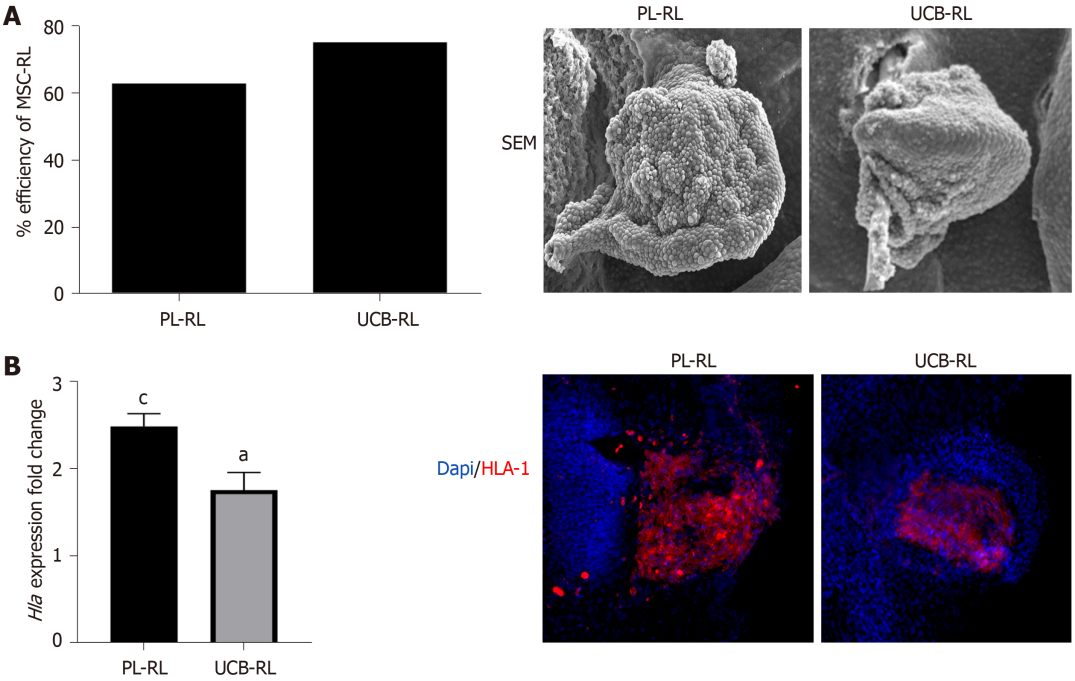
RESULTS

RLs serve as a model to evaluate the morphogenesis and differentiation processes of human MSCs

We used RLs as an experimental model to evaluate the capacity of PL-MSCs and UCB-MSCs to differentiate into limb tissues and to determine their capacity to generate well-organized tissues in an *in vivo* context. In the first instance, we evaluated the competence of human MSCs to respond to embryonic signals present in the limb ectoderm (Figure 1). The results showed that 24 h postimplantation (hpi), the PL-MSCs and UCB-MSCs were successfully integrated and formed RLs, with an efficiency of 62.95% and 76.87%, respectively (Figure 1A). Next, we measured the expression and distribution of major histocompatibility complex 1 (HLA1) to determine whether the RLs were exclusively formed by MSCs. We observed that both MSCs-RLs expressed significantly higher levels of HLA1 compared with their own pellet before transplantation. In addition, the cells in the center of the RLs were HLA1$^+$ (Figure 1B). These results demonstrated the successful establishment of RLs composed of human MSCs for the evaluation of MSC behavior *in vivo*.

Morphology of MSCs changes as RL develops

To assess the capacity of human MSCs to form well-organized tissues, the phenotype of MSC-RLs and their cell organization were evaluated at 24 hpi, 48 hpi, and 72 hpi and compared with RLs formed with CK-LBMCs as a reference of the morphogenetic behavior under RL conditions. In contrast to CK-RL, no evidence of central skeletal elements was observed in RLs from either MSC source (Figure 2). Histolo-gical analyses revealed that the ectodermal cover in all RL conditions was maintained as a monostrati-fied epithelium, while cellular condensation in the center of the MSCs-RL was observed at all time points (Figure 2). Interestingly, the morphology of cell condensations in MSCs-RLs from both sources changed as the development of the RLs progressed, but UCB-RLs showed more evident progressive elongation of a



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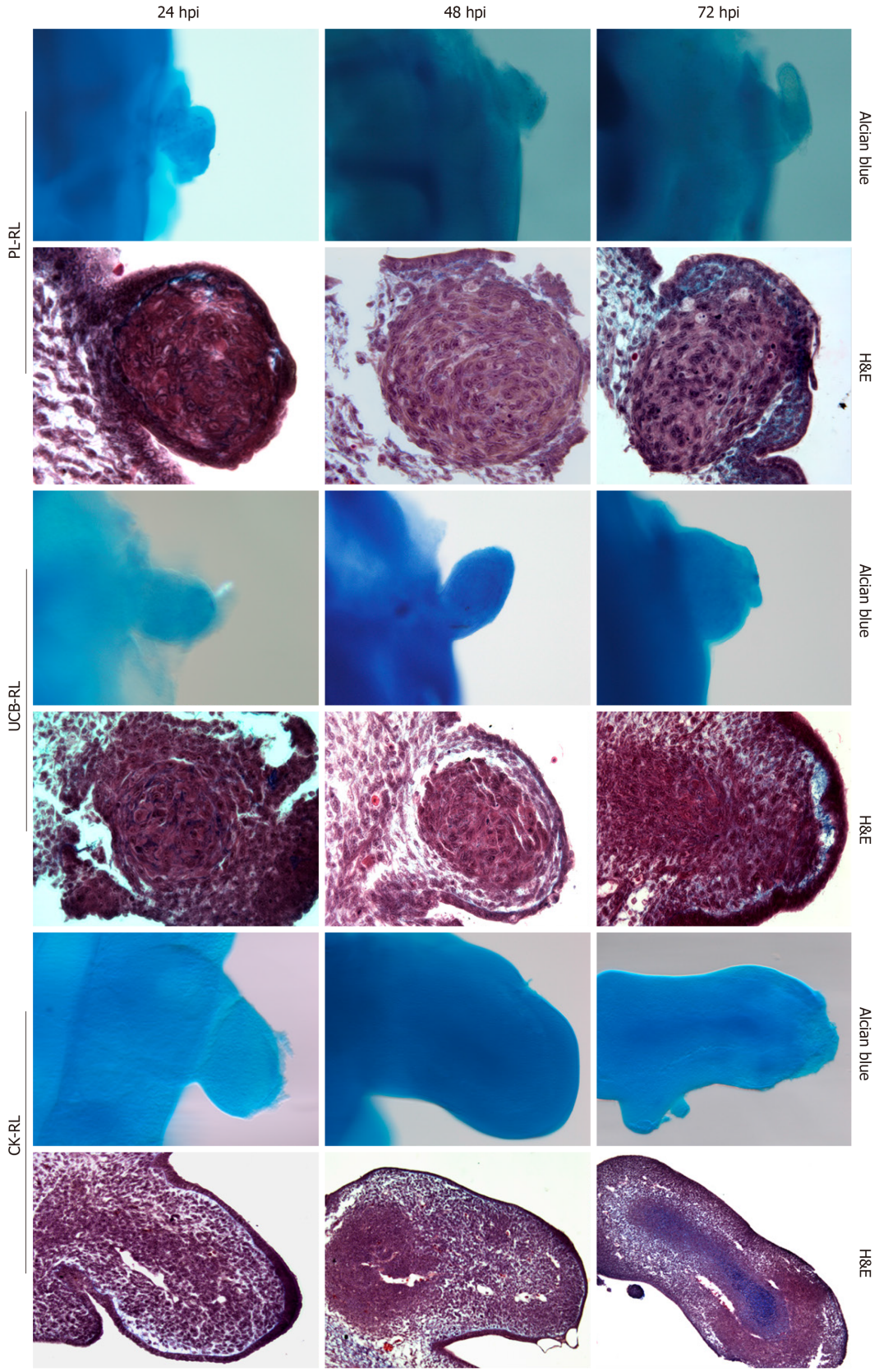
Figure 1 Validation of the recombinant limb system using human mesenchymal stromal cells. A: Implantation efficiency of umbilical cord blood (UCB)-recombinant limb (RLs) and placenta (PL)-RLs in chicken embryos after 24 h. Scanning electron microscopy of PL-RLs (n = 3) and UCB-RLs (n = 2) at 24 h postimplantation (hpi). The embryonic ectoderm and apical ectodermal ridge were conserved in RLs from both sources; B: Levels of human leukocyte antigen (*Hla*) detected by quantitative polymerase chain reaction at 24 hpi RLs from PL and UCB relative to its own pellet. Data represent three independent experiments. The major histocompatibility complex (HLA) expression pattern is shown in RLs from PL (n = 3) and UCB (n = 2). HLA expression was observed in the center of the RLs, indicating that mesenchymal stromal cells constituted the RLs. Statistical significance was set as follows: $^{a}P < 0.05$; $^{c}P < 0.0005$. UCB: Umbilical cord blood; PL: Placenta; RL: Recombinant limb; HLA: Human leukocyte antigen.

central structure than PL-RLs. Although Alcian blue staining around the condensed cells was observed in MSCs-RLs from both sources, cell morphology analyses showed no cartilage anlage formation after 72 hpi as observed in CK-RLs (Figure 2). The lack of well-formed skeletal elements and cartilage morphology in MSCs-RLs suggests that MSCs cannot form complex skeletal structures because they can remain undifferentiated or in an early committed state. These results also suggest that PL-MSCs and UCB-MSCs respond differently to embryonic ectoderm signals.

Molecular and in situ characterization of cell differentiation in MSC-RLs

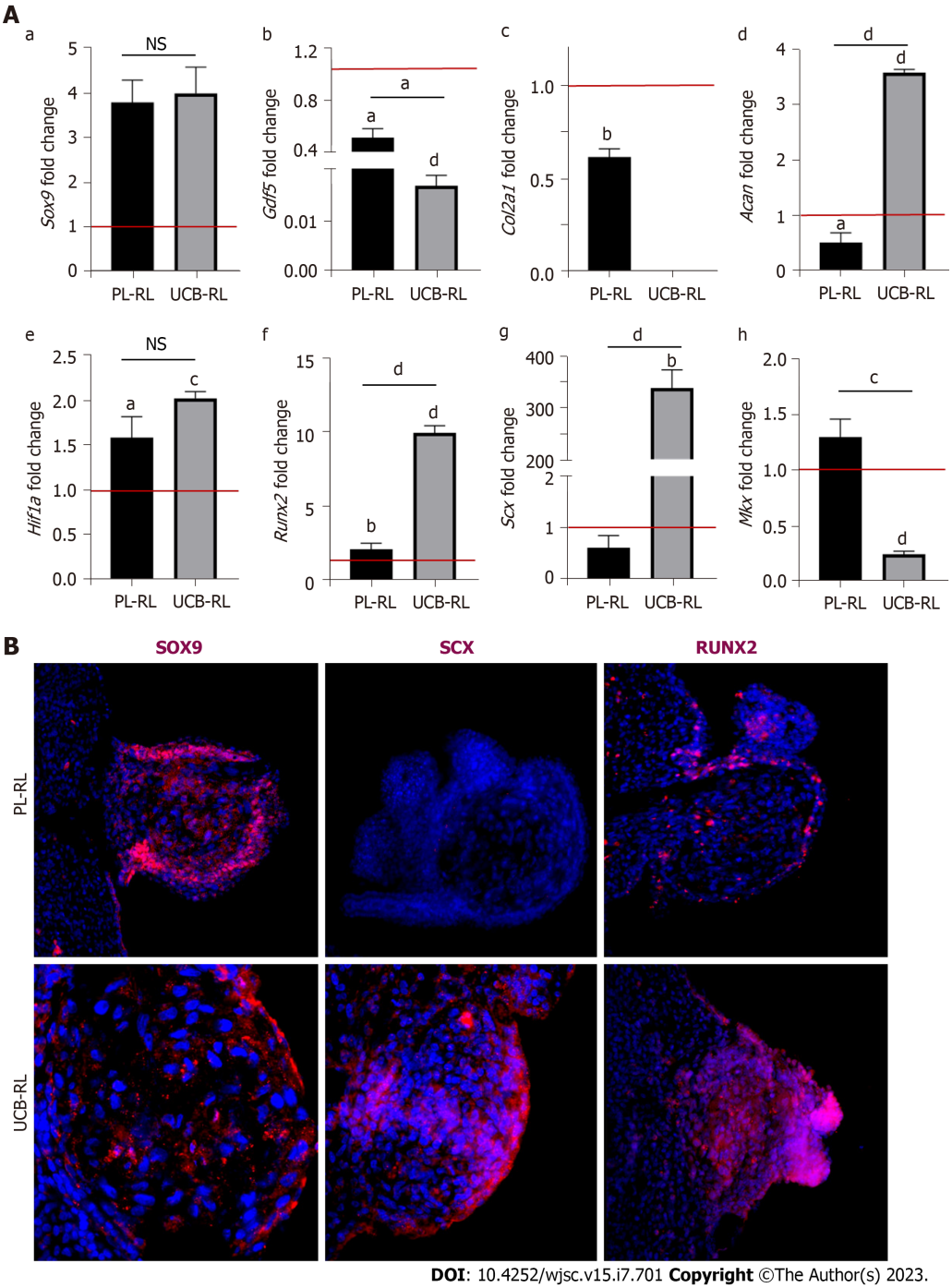
In contrast to CK-RL, histological analyses of MSCs-RLs did not indicate specific tissue formation at any evaluated time. Thus, to determine if the PL-MSCs and UCB-MSCs committed to limb cellular lineages during MSCs-RL formation, we evaluated the expression profile of chondrogenic (*Sox9, Gdf5*, *Col2a1*, *Acan,* and *Hif1a*), osteogenic (*Runx2*), and tenogenic (*Scx* and *Mkx*) differentiation genes at 24 hpi in MSCs-RLs from both sources (Figure 3A). The results confirmed that MSCs commit to limb lineages but respond differentially to embryonic ectodermal signals as the expression of molecular markers of chondrogenic, osteogenic, and tenogenic lineages was induced at different levels in MSCs-RLs from both sources compared with its initial pellet (Table 1).

Comparative analyses of *Sox9* expression levels between MSCs-RLs showed no significant differences ($P < 0.8421$), but remarkably, it was highly induced in both MSCs after receiving ectodermal signals (Figure 3A, Table 1). By contrast, low *Gdf5* expression was observed in both sources compared with its initial levels. In addition, it was significatively reduced in UCB-RLs compared with PL-RLs ($P < 0.0205$; Figure 3A, Table 1). Regarding later chondrogenic-associated genes, expression of *Col2a1* was not detected in UCB-RLs, whereas its expression was significatively reduced in PL-RLs (Figure 3A, Table 1). On the other hand, *Acan* was overexpressed only in UCB-RLs and was significatively reduced in PL-RLs ($P < 0.0001$; Figure 3A, Table 1). Interestingly, the levels of *Hif1a* observed in both MSCs-RLs suggest that a hypoxic environment is favored under these conditions (Figure 3A, Table 1). Regarding osteo-genic commitment, *Runx2* was positively regulated in both PL-RLs and UCB-RLs; however, the expression levels of this gene in UCB-RLs were significatively higher than those observed in PL-RLs ($P < 0.0001$; Figure 3A, Table 1). The tenogenic marker *Scx* was markedly induced in UCB-RLs compared with PL-RLs ($P < 0.0004$), in which expression was inhibited (Figure 3A, Table 1). By contrast, *Mkx* expression was significantly downregulated in UCB-RLs compared with PL-RLs ($P < 0.0002$; Figure 3A, Table 1). To evaluate the spatial organization of committed cells, the protein distribution of SOX9, SCX, and RUNX2 was evaluated in MSC-RLs (Figure 3B). Importantly, the high protein expression was correlated with gene expression in all RLs, as assessed by qPCR (Figure 3B, Table 1). However, these results demonstrated that the localization of SOX9, SCX, and RUNX2 was not restricted to the center of the MSCs-RLs (Figure 3B). Together, these results suggest that at 24 hpi, cells in MSCs-RLs are committed early to the chondrogenic lineage. UCB-RLs can also commit to osteogenic lineage,



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Figure 2 Phenotypic and histological characterization of the mesenchymal stromal cells-recombinant limbs. Alcian blue and hematoxylin and eosin staining of recombinant limbs from the placenta (n = 8), umbilical cord blood (n = 7), and chicken (n = 4) at 24, 48, and 72 h postimplantation. hpi: Hour postimplantation; UCB: Umbilical cord blood; PL: Placenta; RL: Recombinant limb; CK: Chicken; H&E: Hematoxylin and eosin.



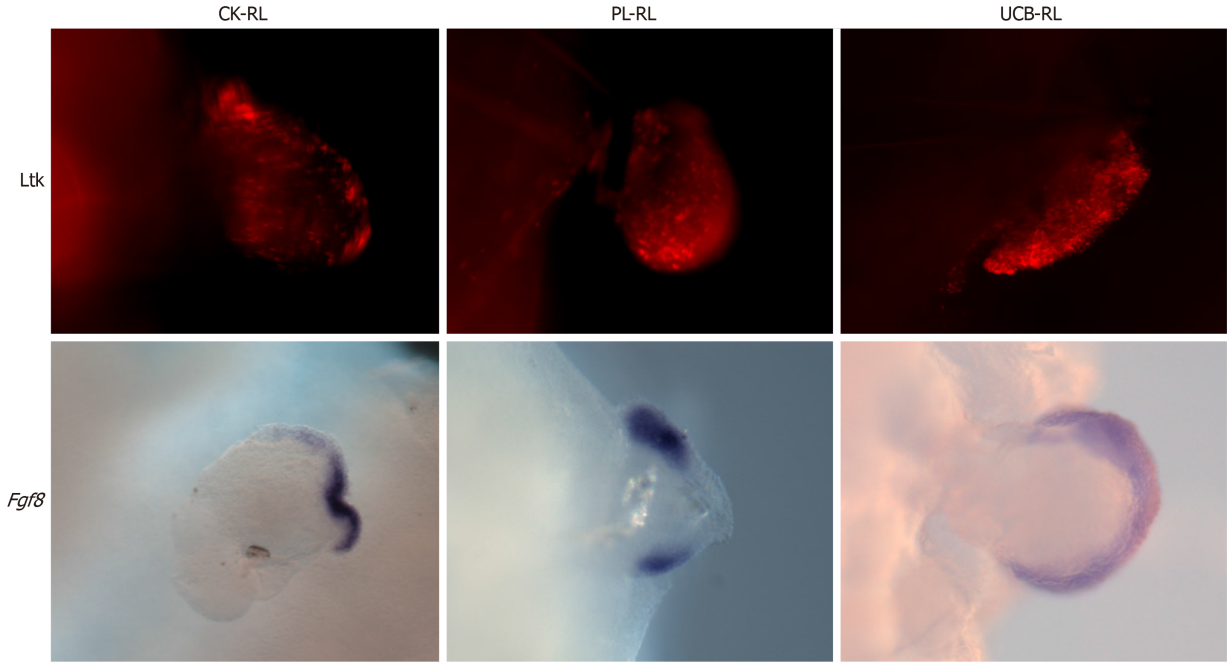
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Figure 3 Mesenchymal stromal cells differentially commit to chondrogenic, osteogenic, and tenogenic lineages in the recombinant limb system. A: Quantitative polymerase chain reaction analysis of cartilage (a-e), bone (f), and tendon (g and h) cell fate-regulating genes of the mesenchymal stromal cells-recombinant limbs (MSCs-RLs) at 24 h postimplantation (hpi) relative to its own pellet set to 1.0. Statistical significance comparing MSCs-RLs from the two sources is shown; B: Protein expression pattern of master genes of the three differentiation lineages in MSCs-RLs at 24 hpi. Data represent three independent experiments. Statistical significance was set as follows: $^{a}P < 0.05$; $^{b}P < 0.005$; $^{c}P < 0.0005$; $^{d}P < 0.0001$. For more details see Table 1. Sox9: SRY-box transcription factor 9; Gdf5: Growth differentiation 5; Col2α1: Collagen type 2 alpha 1; Acan: Aggecan; Hif1α: Hypoxia-inducible factor 1 alpha; Runx2: Runt-related transcription factor 2; Scx: Scleraxis; Mkx: Mohawk; UCB: Umbilical cord blood; RL: Recombinant limb; PL: Placenta; NS: Not significant.

although preferentially to a tenogenic cell fate, but this cell fate is not promoted in PL-RLs.

Evaluation of Fgf-8 expression and cell death in MSCs-RLs

Signals from the ectoderm and AER are indispensable for maintaining cell survival and proliferation during limb development and RL formation[23]. During limb development, the undifferentiated cellular state and cell commitment balance depend on signals from the AER[2]. To determine whether the lack of ectodermal signals or massive cell death was the mechanism underlying the inability of MSC to form skeletal structures, ectoderm integrity by *Fgf8* expression and lysotracker staining were evaluated in RLs (Figure 4). The results showed that the cell death process was present in



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Figure 4 Cell death and fibroblast growth factor 8 expression pattern in mesenchymal stromal cells and chicken recombinant limbs at 24 h postimplantation. Lysotracker staining (Ltk) to evaluate cell death in recombinant limbs (RLs) from the placenta (PL-RLs; *n* = 6), umbilical cord blood (UCB-RLs; *n* = 6), and chicken (CK-RLs; *n* = 4) is shown. *In situ* hybridization of fibroblast growth factor 8 (*Fgf8*) in PL-RLs showed disrupted pattern of this gene in 2/4 RLs; *Fgf8* expression in UCB-RLs was detected in 3/6 evaluated samples, while 5/5 CK-RLs continued to express *Fgf-8* at 24 h postimplantation. UCB: Umbilical cord blood; PL: Placenta; RL: Recombinant limb; CK: Chicken; Ltk: Lysotracker staining; Fgf8: Fibroblast growth factor 8.

the ectoderm of all evaluated RLs; UCB-RLs showed a higher number of dead cells than PL-RLs and CK-RLs. Interestingly, in PL-RLs and CK-RLs, dead cells were concentrated in the apical area of the ectoderm, whereas in UCB-RLs, dead cells were distributed homogeneously on the ectoderm of all samples evaluated (Figure 4). Despite the death of ectodermal cells, *Fgf8* expression was maintained in some MSCs-RLs (see details in the figure legend) or its expression in PL-RLs was disrupted in the center of the ectoderm (Figure 4). These results suggest that at 24 hpi, signals continue to emanate from the AER, which are received by the MSCs or CK-LBMCs beneath the AER.

DISCUSSION

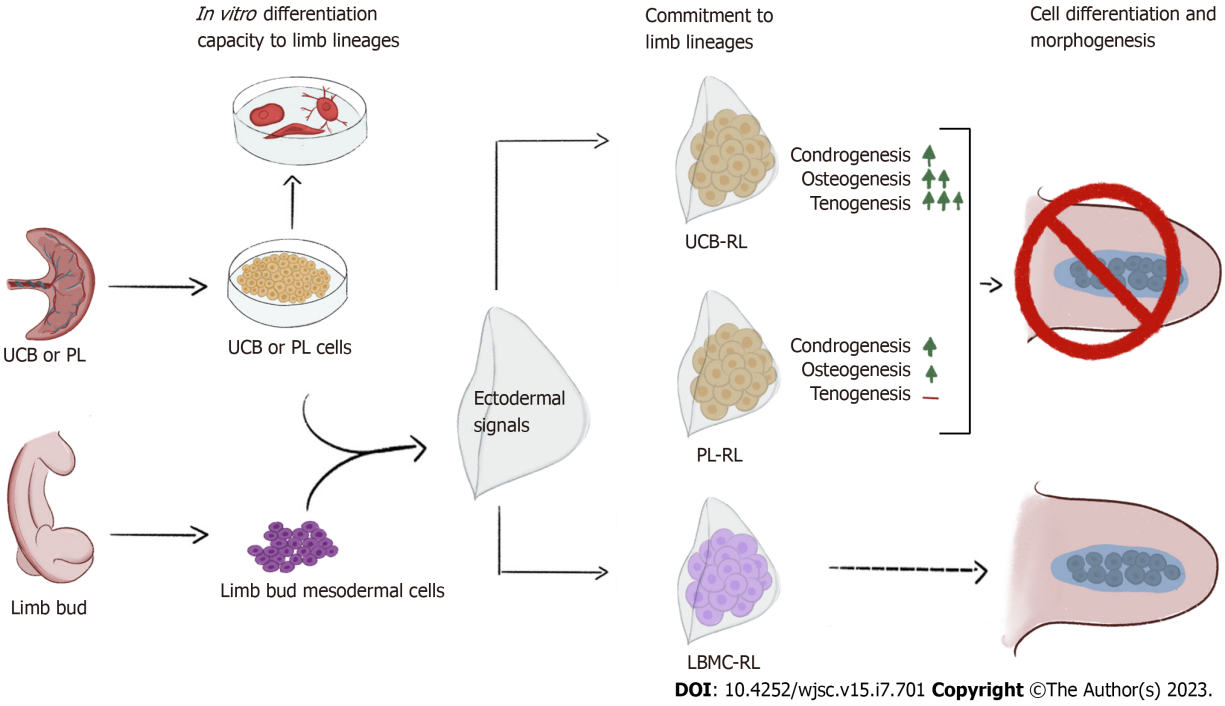
Cells commit to specific lineages once the expression of master genes occurs in response to inducing factors. However, the ability to interpret signals and acquire a particular fate and behavior depend on the developmental history of the cells. The capacity to organize complex structures depends on cell differentiation, cell recruitment, cell movement, differential cell proliferation, and cell death, which occur during the morphogenetic process of tissues and organs.

In this study, we used the RL model to evaluate the morphogenetic abilities of MSCs other than those observed in cell culture. Because RLs provide limb spatial-temporal signals, we evaluated the ability of MSCs to generate complex skeletal structures[23-25]. We found that human MSCs can integrate into an RL implanted in the CK embryo. PL- or UCB-MSCs from RLs respond differently to ectodermal signals, suggesting that the source of MSCs might be important to delineate a particular lineage *in vivo* (Figure 5). Although MSCs from both sources start the chondrogenic program with high levels of SOX9 expression, they do not necessarily equally follow and complete the cell differentiation process (Figure 5). *In vitro* studies have shown that UCB-MSCs preferentially differentiate into osteogenic lineages[17] and present a higher *in vivo* osteogenic and chondrogenic differentiation potential than BM-MSCs when grafted subcutaneously with a scaffold of hydroxyapatite/tricalcium phosphate[19]. By contrast, PL-MSCs preferentially differentiate into an osteogenic lineage when seeded into microcarriers or nanofiber scaffolds[20,21]. These findings suggest that depending on the tissue source, MSCs can represent a cell population with a different number of osteogenic or chondrogenic progenitors or both. Similarly, the molecular markers from chondrogenic and osteogenic lineages were induced at higher levels in UCB-RLs than in PL-RLs. However, the high expression levels of *Runx2* and *Scx* genes did not lead to the formation of well-defined bone tissue or tendons. In addition, the gene expression pattern of *Sox9, Scx,* and *Rux2* did not associate with a specific tissue pattern or arrangement inside the RLs. The expression of molecular markers was not sufficient to promote the formation of well-defined structures, likely because the morphogenetic program was not triggered. Interestingly, PL-RLs expressed high levels of *Mkx* while UCB-RL expressed high levels of *Scx*. It is unknown if in this system both genes are needed to form well-structured tendons. Nevertheless, the expression of *Mkx* or *Scx* can drive the *in vitro* differentiation of BM-MSCs to a tenocyte fate[13,27].

Table 1 Analysis of gene expression fold-changes of differentiation genes between pellets and their corresponding recombinant limbs of different mesenchymal stromal cell sources at 24 h postimplantation

Source	*Sox9*	*Col2a1*	*Acan*	*Gdf5*	*Hif1α*	*Runx2*	*Scx*	*Mkx*
UCB-RL *vs* UCB-pellet	3.99 ± 0.4, $P <$ 0.0004	ND	3.58 ± 0.10, $P <$ 0.0001	0.01 ± 00.003, $P <$ 0.0001	1.58 ± 0.79, $P <$ 0.0002	9.96 ± 0.65 $P <$ 0.0001	377.61 ± 78.24, $P <$ 0.002	0.24 ± 0.03, $P <$ 0.0001
PL-RL *vs* PL-pellet	3.79 ± 0.4, $P <$ 0.0007	0.61 ± 0.12, $P <$ 0.002	0.55 ± 0.21, $P <$ 0.052	0.56 ± 0.12, $P <$ 0.01	1.61 ± 0.28, $P <$ 0.03	2.18 ± 0.59, $P <$ 0.03	0.61 ± 0.33, $P <$ 0.14	1.29 ± 0.22, $P <$ 0.21

Gene expression fold-change was evaluated comparing the pellet of mesenchymal stromal cells (MSCs) after forming the recombinant limb (RL) with MSCs-RLs from both sources. Data from the pellet were set to 1.0. Statistical significance comparing placenta-RL *vs* umbilical cord blood-RL is shown in Figure 3A. All data are presented as mean ± SD. Statistical significance was set at $P < 0.05$. Acan: Aggecan; Col2α1: Collagen type 2 alpha 1; Gdf5: Growth differentiation 5; Hif1α: Hypoxia-inducible factor 1 alpha; Mkx: Mohawk; ND: Not detected; Scx: Scleraxis; Sox9: SRY-box transcription factor 9; Runx2: Runt-related transcription factor 2; UCB: Umbilical cord blood; RL: Recombinant limb; PL: Placenta.



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Figure 5 Mesenchymal stromal cells commit to limb mesodermal lineages but are not capable of forming skeletal structures. Mesenchymal stromal cells (MSCs) from umbilical cord blood (UCB) and placenta (PL) differentiated into chondrogenic, osteogenic, and tenogenic lineages *in vitro*. However, under the influence of differentiation and morphogenetic signals from limb ectoderm in the recombinant limb (RL) system, MSCs committed to limb lineages but failed to organize into tridimensional structures and form skeletal elements as observed with limb bud mesodermal cells under the same conditions. Similarly, the response to ectodermal signals depended on the source of MSCs: UCB-RLs had higher expression of osteogenic and tenogenic markers than PL-RLs, whereas in PL-RLs, tenogenic markers were downregulated after receiving ectodermal signals. UCB: Umbilical cord blood; PL: Placenta; LBMC: Limb bud mesodermal cell; RL: Recombinant limb.

The inability of MSCs to generate complex structures suggests that MSC populations, in addition to being composed of a wide variety of cell types, are unable to initiate or follow a morphogenetic program *in vivo*. Although MSCs can differentiate into skeletal lineages, it does not guarantee that they will organize into skeletal elements. It is possible that the RL model lacks signals to support the formation of skeletal elements. However, CK-LBMCs respond to differentiation signals, resulting in the generation of skeletal elements. Thus, the RL system provides signals that instruct cells to form skeletal elements.

We did not observe exacerbated cell death in the MSCs-RLs, but in some MSCs-RLs the loss of *Fgf8* expression in the AER was evident. Additional studies are needed to determine if this cell death results from the loss of FGF8 loop signaling between the AER and MSCs, or if MSCs cannot recover and maintain the loop to maintain FGF8 expression. Our data suggest that the differentiation process in MSCs is detached from the process of morphogenesis and patterning, although it is possible that addi-tional signals may be needed to promote the integration of morphogenetics with differentiation signals in MSCs. Accordingly, other studies have shown that after implantation in the kidney subcapsular region or nude mice[28-31], MSCs organize better than *in vitro* but never originate complex structures such as skeletal elements or other limb musculoskeletal tissues.

Although MSCs are considered relevant in regenerative medicine, their intrinsic cellular properties may explain why the reported therapeutic effects of MSCs are mostly indirect through immunomodulation or paracrine mechanisms rather than reliable integration into adult tissues or *de novo* tissue forma-tion[32-39].

CONCLUSION

This study demonstrates that the expression of differentiation markers of skeletal lineages in MSCs is not sufficient to generate skeletal structures *in vivo*, possibly due to the intrinsic characteristics of MSCs. In regenerative medicine, cells must be incorporated into well-defined tissues or generate new complex structures with a well-defined patterning. Thus, further application of MSCs in regenerative medicine needs to focus on understanding their biological characteristics to gain insights into how MSCs can integrate into adult tissues or properly rebuild tissues and organs.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) differentiate *in vitro* to different skeletal lineages; however, it is un-known if they have the ability to form complex skeletal structures.

Research motivation

Although MSCs are considered relevant in regenerative medicine, reliable integration into adult tissues or *de novo* tissue formation has not been demonstrated. The application of MSCs in regenerative medicine needs to focus on understanding their biological characteristics to gain insights into how MSCs can integrate into adult tissues or properly rebuild tissues and organs.

Research objectives

To evaluate the ability of MSCs to organize and form complex skeletal structures *in vivo* under the influ-ence of embryonic signals.

Research methods

The recombinant limb (RL) is an experimental system that recapitulates the embryonic environment and its influence on cells to generate skeletal structures. Here, umbilical cord blood (UCB)-MSCs or placenta (PL)-MSCs were placed in an RL to assess their ability to form skeletal structures. The evaluation was conducted by Alcian blue staining, immunofluor-escence, and quantitative polymerase chain reaction of molecular markers of skeletal lineages.

Research results

MSCs expressed molecular markers of skeletal lineages but were unable to generate complex skeletal structures. PL-MSCs or UCB-MSCs integrated into an RL implanted in a chicken embryo. They res-ponded differently to ectodermal signals starting the chondrogenic, osteogenic, or tenogenic program with high *SRY-box transcription factor 9*, *Runt-related transcription factor 2*, and *scleraxis* gene expression levels. However, PL-MSCs or UCB-MSCs did not complete the cell differentiation and morphogenetic processes, likely due to the intrinsic characteristics of MSCs.

Research conclusions

PL-MSCs or UCB-MSCs express molecular markers of skeletal lineages but do not organize into com-plex skeletal structures. The use of RLs is an excellent model for determining the ability of cells from other origins than limb bud mesodermal cells to generate skeletal structures. The inability of MSCs to form skeletal structures might be due to their intrinsic characteristics. Additional studies are needed to understand the properties of MSCs and whether they can integrate into adult tissues or properly rebuild tissues and organs.

Research perspectives

The application of MSCs to regenerative medicine needs to focus on understanding their biological characteristics to gain insights into how MSCs can integrate into adult tissues or properly rebuild tissues and organs.

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FOOTNOTES

Author contributions: Marín-Llera JC and Chimal-Monroy J conceptualized the research and wrote the manuscript; Marín-Llera JC, Montesinos-Montesinos JJ, and Chimal-Monroy J discussed the data; Marín-Llera JC, García-García RD, and Garay-Pacheco E performed the experiments; Adrian Cortes-Morales V maintained and prepared the mesenchymal stromal cells; and all authors approved the final version of the manuscript.

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ORIGINAL ARTICLE

Basic Study

Transplantation of human induced pluripotent stem cell derived keratinocytes accelerates deep second-degree burn wound healing

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Abstract

BACKGROUND

Current evidence shows that human induced pluripotent stem cells (hiPSCs) can effectively differentiate into keratinocytes (KCs), but its effect on skin burn healing has not been reported.

AIM

To observe the effects of hiPSCs-derived KCs transplantation on skin burn healing in mice and to preliminarily reveal the underlying mechanisms.

METHODS

An analysis of differentially expressed genes in burn wounds based on GEO datasets GSE140926, and GSE27186 was established. A differentiation medium containing retinoic acid and bone morphogenetic protein 4 was applied to induce hiPSCs to differentiate into KCs. The expression of KCs marker proteins was detected using immunofluorescence staining. A model of a C57BL/6 mouse with deep cutaneous second-degree burn was created, and then phosphate buffered saline (PBS), hiPSCs-KCs, or hiPSCs-KCs with knockdown of *COL7A1* were injected around the wound surface. The wound healing, re-epithelialization, engraftment of hiPSCs-KCs into wounds, proinflammatory factor level, and the NF-κB pathway proteins were assessed by hematoxylin-eosin staining, carboxi-fluorescein diacetate succinimidyl ester (CFSE) fluorescence staining, enzyme linked immunosorbent assay, and Western blotting on days 3, 7, and 14 after the injection, respectively. Moreover, the effects of *COL7A1* knockdown on the proliferation and migration of hiPSCs-KCs were confirmed by immunohistochemistry, EdU, Transwell, and damage repair assays.

RESULTS
HiPSCs-KCs could express the hallmark proteins of KCs. *COL7A1* was down-regulated in burn wound tissues and highly expressed in hiPSCs-KCs. Transplantation of hiPSCs-KCs into mice with burn wounds resulted in a significant decrease in wound area, an increase in wound re-epithelialization, a decrease in proinflammatory factors content, and an inhibition of NF-κB pathway activation compared to the PBS group. The in vitro assay showed that *COL7A1* knockdown could rescue the inhibition of hiPSCs-KCs proliferation and migration, providing further evidence that *COL7A1* speeds up burn wound healing by limiting cell proliferation and migration.

CONCLUSION
In deep, second-degree burn wounds, *COL7A1* can promote KC proliferation and migration while also suppressing the inflammatory response.

Key Words: Induced pluripotent stem cell; Keratinocytes; Cell transplantation; Burn wound healing; COL7A1

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Core Tip: Current evidence shows that human induced pluripotent stem cells (hiPSCs) can effectively differentiate into keratinocytes (KCs), but its effect on skin burn healing has not been reported. Therefore, this study was intended to observe the effects of hiPSCs-derived KCs transplantation on skin burn healing in mice and to preliminarily reveal the underlying mechanisms. Transplantation of hiPSCs-KCs into mice with burn wounds resulted in a significant decrease in wound area, an increase in wound re-epithelialization, a decrease in proinflammatory factors content, and an inhibition of NF-κB pathway activation, which rescued by *COL7A1* knockdown.

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INTRODUCTION

The skin acts as an environmentally protective barrier and has vital physiological functions such as sensation, absorption, secretion, excretion, and protection, as well as being involved in a variety of metabolic processes in the body and maintaining the stability of the internal environment[1]. Therefore, skin integrity is crucial to maintain its function. However, due to extensive skin integrity defects caused by different pathological conditions (including burns, scalds, vasculopathy-triggered skin ulcers, pressure ulcers, diabetic ulcerated feet, and others), which are caused by the action of internal and external factors, the underlying wounds develop pathological inflammatory reactions, which cannot adequately heal through normal repair procedures. Subsequently, such poorly-healing wounds will cause severe disability or patient death, together with adversely impacting the economic status of the patient's family and healthcare system[2-5]. Thus, developing novel therapeutics for chronic wounds is crucial to enhance the management practices and subsequent outcomes.

In August 2006, Takahashi and Yamanaka[6] at Kyoto University, Japan, first transfected four pluripotency related genes, *Oct4, Sox, c-myc,* and *KLF4*, into mouse fibroblasts using retroviruses as gene introduction vectors, which led to the creation of pluripotent stem cells with similar characteristics to ESCs and named them induced pluripotent stem cells (iPSCs)[6]. Today, iPSC technology has revolutionized basic research and clinical disease treatment in biology[7,8]. For instance, valproic acid strongly promotes the differentiation of human pluripotent stem cells into spermatogonial stem cell-like cells and provides a reliable tool and model for studying human germ cell development and male infertility[9]. Another study discovered that transplanting retinal pigment epithelial cells derived from human iPSCs (hiPSCs) is promising as a new treatment modality for age-related macular degeneration and Stargardt disease[10]. Moreover, the transplantation of hiPSC-derived neural stem/progenitor cells (hiPSC-NS/PCs) is a promising therapeutic approach for various neuropathological conditions[11,12].

For burn trauma, inducing re-epithelialization has received much attention as a therapeutic focus and a key indicator to evaluate the efficacy of clinical interventions[13]. To complete re-epithelialization, keratinocytes (KCs) migrate, proliferate, and differentiate, with migration being the most important[14]. In this context, KC initiation and migration are the initiating events and rate-limiting steps in the re-epithelialization process because impaired migration causes non-healing wounds and chronic wound formation, while the inhibition of their proliferation and differentiation functions does not[15,16]. A study showed that KC abnormalities were the main drivers that triggered psoriasis pathology, and the ability of iPSCs to efficiently differentiate into mature KCs played a crucial role in psoriasis treatment[17]. Replacement cells derived from hiPSCs have the potential for sufficient scalability, and selecting hiPSCs as the human pluripotent stem cells (hPSC) of choice ensures immunocompatibility. Ibrahim *et al*[18] showed that hiPSC-derived KCs would lose CD200 expression and express keratin 14 (*K14*), indicating the emergence of more mature terminally differentiated cells that

could be transplanted to treat large burns or ulcers. Accordingly, while the ability to obtain sufficient KCs from hiPSCs for transplantation repair is promising, further research is needed before this technology can be applied to skin wound repair. Moreover, the specific mechanism of hiPSCs involvement in burn repair has not been fully revealed.

In this study, we used the cell induced differentiation technique of hiPSCs to successfully differentiate hiPSCs into KCs. Subsequently, to study the effects of transplanting differentiated KCs on murine skin wound healing, a skin-deep second-degree burn model was created and successfully differentiated KCs were injected around the wound. In addition, a bioinformatics approach was taken to further explore the possible mechanism of hiPSCs-KCs transplantation role in wound healing.

MATERIALS AND METHODS

Bioinformatics analysis

GSE140926 and GSE27186 were found in the GEO database (https://www.ncbi.nlm.nih.gov/gds) for burn trauma-related mRNA expression profile datasets. The GSE140926 dataset was derived from the GPL15433 platform and included 3 burned eschars and 3 normal skin tissue samples. On the other hand, the GSE27186 dataset was derived from the GPL570 (Affymetrix Human Genome U133 Plus 2.0 Array) platform and included two iPSC and two KC samples. The raw data of the chip, including data matrix files in txt document format and corresponding platform annotation files, were subsequently downloaded. The difference analysis was performed *via* the limma package on R software, which employed the classical bayson's *t*-test analysis method with $|\log \text{Fold Change}| \geq 0.5$, and $P < 0.05$ as the filtering criteria. Visualization Differentially expressed genes (DEGs) were used to generate a volcano plot. There were three types of annotations present in gene ontology (GO) functional annotations: Molecular function, biological pathway (BP), and cellular component (CC). To perform GO functional annotation of DEGs, we used the online tool David (https://david.ncifcrf.gov/). GO pathway enrichment analysis was performed with the cluster profiler package on R software with the following filtering criteria: False discovery rate < 0.05, and adjusted $P < 0.05$. The enrichment results were visualized using R, as well.

Induced differentiation of hiPSCs into KCs

HiPSCs used in this study were purchased from Beijing Saibe Biotechnology Co., Ltd (Beijing, China). HiPSCs in the logarithmic growth period were digested and inoculated into the culture plate. The cells were grown in a cell incubator with 5% CO_2 at 37 °C for two days in DMEM culture medium containing 10% fetal bovine serum. Cells were cultured in a cell culture chamber with 5% CO_2 saturated humidity at 37 °C, and 0.1 mol/L retinoic acid (RA) and 10 mol/L bone morphogenetic protein 4 (BMP-4) (Thermofisher, United States) were added to the differentiated medium on days 3, 5, and 7. On the 14th day of differentiation induction, 0.1 mol/L Ca^{2+} was added to the differentiation medium, and the morphological changes of cells were observed.

Immunofluorescence staining

A total of 300 μL of each of primary antibody *K14* (1:200), *involucrin* (1:200), *loricrin* (1:200), and *COL7A1* (1:200) (Abcam, United States) dilutions diluted in phosphate buffered saline (PBS) were added to hiPSCs-KCs and incubated overnight according to the manufacturer's instructions. On the following day, after washing the cells using PBS, the corresponding fluorescent secondary antibodies Goat anti-mouse (1:200), and Goat anti-rabbit (1:200) were added and incubated in the dark at 37 °C for 1 h. After 15 min in the dark at room temperature, 300 μL of DAPI (1:1000 dilution) was added, and the images were acquired using an inverted fluorescence microscope.

Cell transfection

The shRNA sequences against *COL7A1* were purchased from Shanghai SANGON company (SANGON, Shanghai, China). *COL7A1* shRNA and its negative control NC shRNA were transfected into hiPSCs-KCs using Lipofectamine®3000 transfection reagent according to the manufacturer's protocol. hiPSCs-KCs were employed for the following experiments two days after transfection.

Clinical specimens

Skin samples were obtained from the remaining burned and normal skin tissue samples of 26 male patients, aged around 30 years old, who underwent autologous skin grafting in the Burn Department of the Second Affiliated Hospital of Soochow University from June 2019 to October 2021. All data extraction processes were reviewed and approved by the medical ethics committee of the First Affiliated Hospital of Soochow University while also obtaining informed patient consent.

Mouse burn model

Forty C57BL/6 male mice were purchased from Soochow University. The feeding formula was corn 35%, soy cake 15%, raw yellow beans 5%, wheat bran 15%, flour 15%, yeast meal 2%, bone meal 2%, sesame cake 5%, maltodextrin 2%, fish meal 2%, salt 0.5%, canola 1%, trace elements 0.2%, vitamins 0.1%, and choline chloride 0.2%. The experimental manipulation process strictly adhered to the relevant regulations and requirements of medical ethics. The mice were intraperitoneally injected with 40 mg/kg pentobarbital sodium. After shaving the dorsal hair of mice using a small animal shaver and smearing on the dorsal shaved area with 10% sodium sulfide, the back was wiped with water after 30 s to remove

any residuals. The mice were returned to the animal housing room for a further 24 h. Subsequently, after anesthetizing the mice by isoflurane inhalation, a 1.5 cm diameter circular mold was covered on the area that had been depilated on the back of the mice, and 92 °C hot steam was used to scald each back of the mice for 6 s. Deep second-degree burns were diagnosed when hematoxylin-eosin (HE) staining revealed scald tissue that was the full thickness of the epidermis and below the papillary layer of the dermis, the full thickness of unwounded skin, and still had hair follicles, sebaceous glands, and other skin appendages. After modeling, mice were randomly divided into four groups, including PBS, hiPSCs-KCs, hiPSCs-KCs + NC shRNA, and hiPSCs-KCs + *COL7A1* shRNA groups, which depicted the injection of 25 mL of 0.1 M PBS, 25 mL (2.0×10^7 cells) of hiPSCs-KCs, 25 mL of hiPSCs-KCs transfected with NC shRNA, and 25 mL of hiPSCs-KCs transfected with *COL7A1* shRNA around the postoperative wounds, respectively. On days 0, 3, 7, and 14 after injection, the wound healing area of each group was measured by a ruler, and photographed and recorded with a digital camera. To prevent the development of immune rejection, mice were injected with FTY720 (3 mg/kg/d), an immunosuppressive agent, to reduce any adverse reactions against hiPSCs. All animal experiments followed the Institutional Animal Care and Use Committee protocols.

HE staining

The skin 1 cm around the wound was cut and fixed using 4% paraformaldehyde at 4 °C for one week. After tissue paraffin embedding, and wax block sectioning, HE staining (Thermofisher, United States) was used according to the manufacturer's requirements. Finally, the slides were blocked after dehydration using ethanol, and observed under a microscope.

Immunohistochemical staining

Deparaffinization, hydration, antigen retrieval, serum blocking, primary antibody (*COL7A1* and *Ki67*, Abcam, United States) and secondary antibody incubation, washing, DAB color development, hematoxylin counterstaining, and mounting of burn wound tissue sections were performed sequentially. The number of cells positive for *COL7A1* and *Ki67* expression was counted in three randomly selected high-power fields per section under the microscope.

Cell proliferation assay

hiPSCs-KCs, hiPSCs-KCs + NC shRNA, and hiPSCs-KCs + *COL7A1* shRNA cells were harvested from three different treatments. The hiPSCs-KCs (20×10^5/well) were seeded in culture dishes. The EdU solution was diluted using DMEM medium at a 5000:1 ratio to make an appropriate amount of 50 μmol/L EdU medium. Subsequently, 200 μl of EdU medium was added to the culture dish and incubated for 2 h. After discarding the medium, cells were sequentially incubated with fixative solution (PBS containing 4% paraformaldehyde), glycine, and Permeabilizer. After 30 min incubation in the dark at room temperature with DAPI reaction solution, an anti-fluorescent quencher blocking reagent was added, and images were observed and acquired under a fluorescence microscope for analysis.

Carboxifluorescein diacetate succinimidyl ester fluorescent labelling

After dilution in PBS with xylene sulfoxide solution containing 10 mmol/L carboxifluorescein diacetate succinimidyl ester (CFSE) (Millipore, United States), a 5 μmol/L working solution was obtained. CFSE working solution at a final concentration of 5 μmol/L was added to the hiPSCs-KCs and incubated at 37 °C for 30 min. hiPSCs-KCs suspension was injected around the mouse skin burn wounds. The fluorescence intensity was observed under an inverted fluorescence microscope on the 14th postoperative day.

Cell migration assay

HiPSC-KCs from all three treatment groups were gathered. In the upper chamber of the Transwell, 1×10^5 diluted hiPSCs-KCs were added per well, and in the lower chamber, the DMEM medium was placed. Following a 24-h incubation in 4% paraformaldehyde, the cells were fixed in blotting fixative, washed three times with PBS, and incubated with 0.01% crystal violet before being randomly selected for observation and counting in five different fields. For damage repair experiments, cells were streaked in the central area of cell growth with a sterile pipette tip and then rinsed with PBS to remove dead cells. The scratch width was observed under a microscope at 24 h after scratching and was photographed and recorded.

Western blotting

Total cell and tissue protein was extracted with RIPA lysate, and the protein concentration was determined by a BCA protein assay kit (Thermofisher, United States) in a microplate reader. After denaturation for 10 min with the addition of loading buffer, 50 μg of protein samples were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membrane was blocked with a blocking solution (5% nonfat dry milk) for 2 h and subsequently washed three times using TBST. Then, primary and secondary antibodies were added separately and shaken. Image J was used to analyze membrane protein band gray values.

RT-qPCR

The RNA concentration of 26 pairs of burned and normal skin tissue samples was determined, and the RNA was reverse transcribed into cDNA according to the instructions included in a reverse transcription kit. This cDNA was then used as a template for *COL7A1* amplification on Bio-Rad CFX90 RT-PCR. RT-PCR reaction conditions included pre-denaturation at 95 °C for 30 s, 39 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 5 s, and extension at 65 °C for 5 s. *GAPDH* was used as an internal reference and the relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data was processed *via* SPSS 22.0 statistical software and presented as the mean ± SEM of the results from at least three independent experiments. Kruskal-Walli's test was used for the non-parametric statistical analysis. Differences among more than two groups in the above assays were estimated using one-way ANOVA, with $P < 0.05$ considered significant.

RESULTS

GEO datasets screening identified high COL7A1 expression in hiPSCs-KCs and low expression in burn wounds

Data from the GSE140926 (containing 3 normal skin and 3 burn eschar tissue samples) and GSE27186 (2 hiPSCs and 2 hiPSCs-KCs samples) datasets were first screened for DEGs. A total of 3575 DEGs were found in hiPSCs-KCs samples compared to the hiPSCs ones, which included 1732 and 1843 downregulated and upregulated DEGs (Figure 1A). Furthermore, a total of 1634 DEGs were found in the burn eschar tissue samples compared to the human normal skin ones, which included 867 and 767 downregulated and upregulated DEGs (Figure 1B). Since KCs were damaged at the wound site, we selected DEGs downregulated at the wound site and upregulated in hiPSCs-KCs and intersected them to get 98 DEGs (Figure 1C). Using R software's cluster profiler package, 98 DEGs' GO pathway enrichment was analyzed (Figure 1D). Neuroinflammatory response, microglial activation, and leukocyte activation induced in inflammatory response were significantly enriched GO-BP pathways, and the main enriched genes were *AKR1C1*, *DKK3*, *COL7A1*, *HTRA1*, *ITGB4*, and others (Figure 1E). The most enriched GO-CC pathways were endocytic vesicle membrane and collagen-containing extracellular matrix, and the most enriched genes were *TLR3*, *RAB29*, *CTSC*, *COL7A1*, *LTBP2*, and others (Figure 1F). Furthermore, the significantly enriched GO-MF pathways included growth factor and steroid binding, while the main enriched genes included *PTPN3*, *FOXL2*, *CTSC*, *COL7A1*, *ITGB4* and others (Figure 1G). We additionally intersected the three groups of the significantly enriched DEGs and obtained four DEGs, including *COL7A1*, *HTRA1*, *CAV1* and *CTSC* (Figure 1H and I). Since *COL7A1* showed the greatest degree of differential expression in the burned tissue samples, we decided to investigate it further.

hiPSCs successfully differentiated into keratinocyte

We used RA and BMP-4 to induce differentiation in hiPSCs before plating them to differentiate. The ability of hiPSCs to differentiate into KCs was then evaluated by analyzing the expression and cellular distribution of several KC-expressed proteins using immunofluorescence. Results showed that after 14 d of differentiation induction, the hiPSCs significantly expressed KCs marker proteins (*K14*, *involucrin*, and *loricrin*), as evidenced by increased fluorescence intensity (Figure 2). Afterward, at day 14, hiPSCs-KCs were implanted into mice.

COL7A1 was significantly under-expressed in second-degree burn tissues

To validate *COL7A1* mRNA expression in human deep second-degree burn wound and normal skin tissues, RT-qPCR and Western blotting were applied and the results showed that *COL7A1* expression was downregulated in burn wound tissue (Figure 3A and B). Representative photomicrographs of HE stained mouse burned skin 24 h after the injury showed a damaged structure throughout the epidermis and deep dermis (Figure 3C). This might suggest that the mouse deep second-degree burn model was successfully constructed. Immunohistochemical detection of *COL7A1* expression levels in mouse burn wounds and the surrounding normal skin showed that *COL7A1* expression remarkably diminished in burn wounds (Figure 3D). We also compared fibroblast, fibroblast-like, and hiPSC-KC *COL7A1* expression levels. Immunofluorescence analysis revealed that *COL7A1* was only strongly expressed in hiPSCs-KCs cells, as indicated by increased green fluorescence (Figure 3E), while it was hardly expressed in hiPSCs and fibroblasts. Our findings further confirmed the results obtained by bioinformatics analysis.

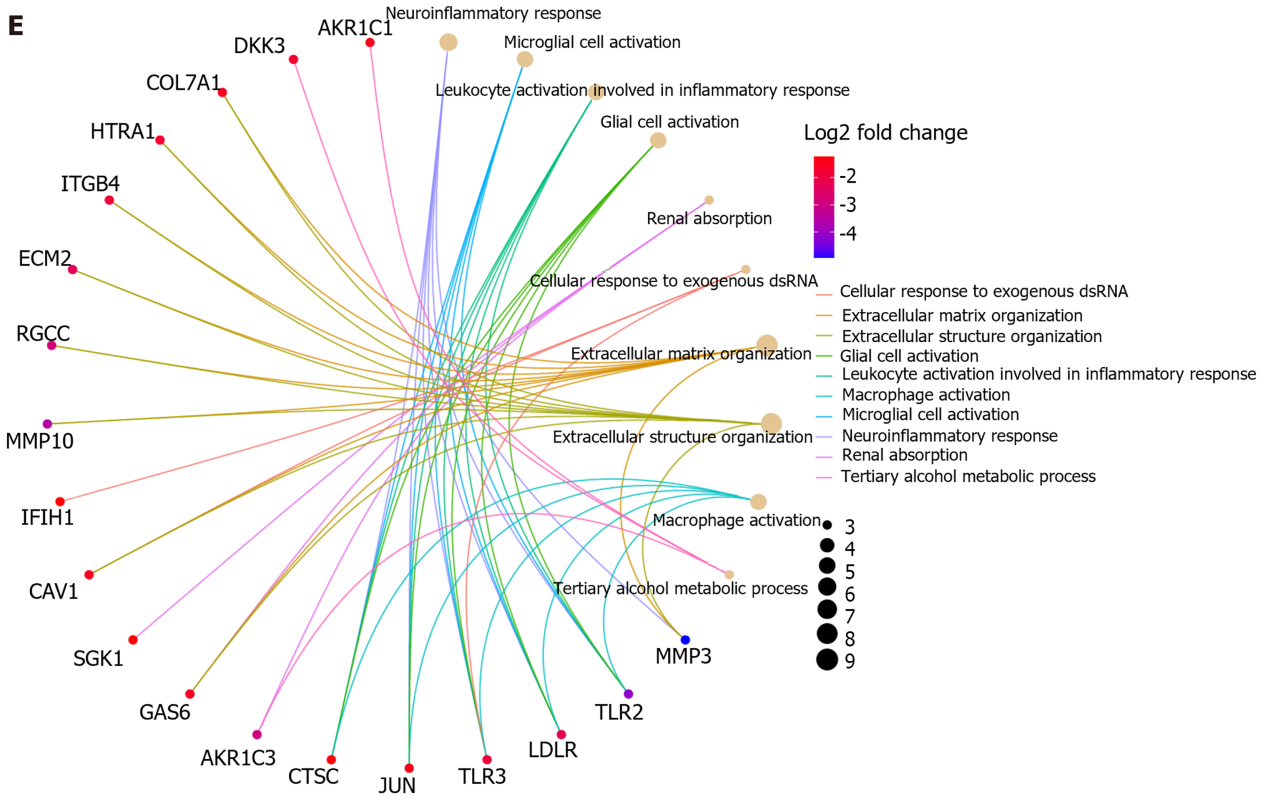
COL7A1 inhibition abolished the promoting effects of hiPSCs-KCs on burn healing and re epithelialization in mice

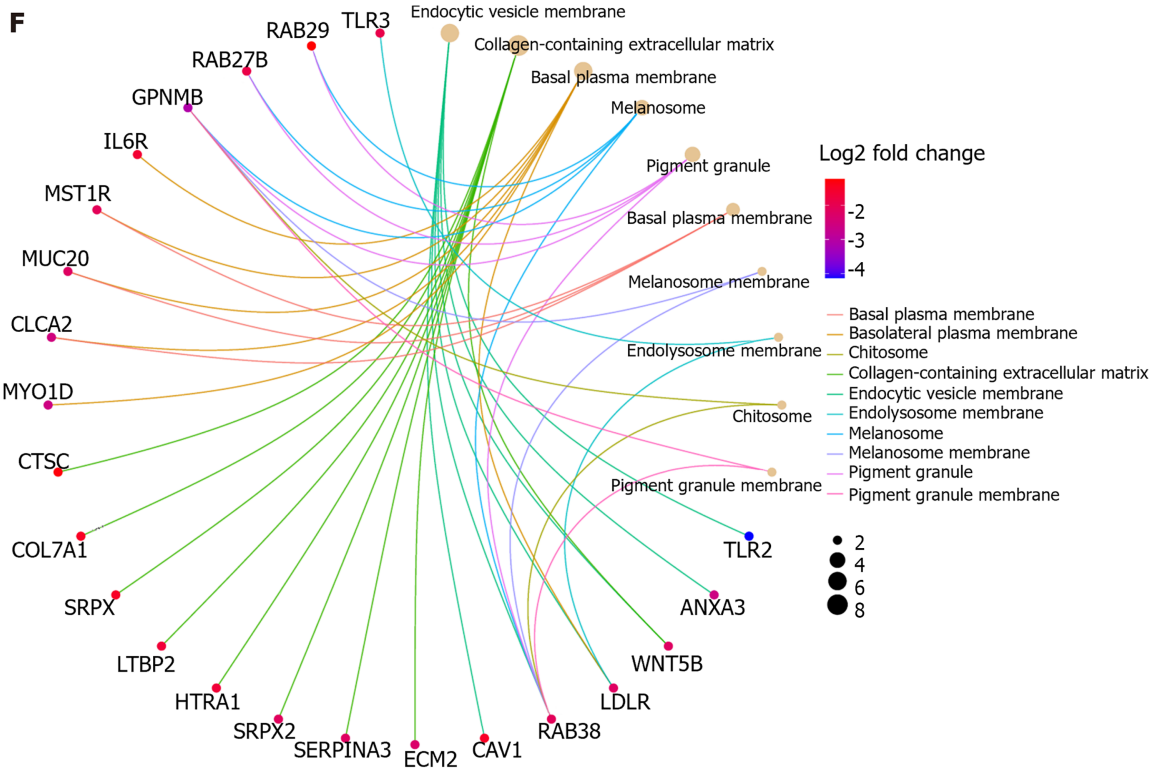
We evaluated the transfection efficiency of *COL7A1* shRNA before and after hiPSCs-KCs transplantation. The mRNA expression level of *COL7A1* decreased nearly five folds after transfection of hiPSCs-KCs with *COL7A1* shRNA (Figure 4A). Interestingly, as a point of reference for our experimental manipulation, we also built the *COL7A1* overexpression vector to demonstrate the efficiency of *COL7A1* overexpression. The data indicated that *COL7A1* overexpression was just as effective. Furthermore, *COL7A1* expression was significantly upregulated after transplantation of hiPSCs-KCs around mouse wounds, while *COL7A1* knockdown was associated with a decrease in *COL7A1* expression after transplantation of hiPSCs-KCs. All of this suggests that knocking down *COL7A1* is effective and can be used in future experiments. At 3, 7, and 14 d after hiPSCs-KCs transplantation, the healing of burn wounds in mice was recorded by taking photographs with a digital camera. We found that burn wound edges significantly contracted at 7 d after hiPSCs-KCs transplantation with obvious epithelialization and newly regenerated epidermal tissue that partially covers the wound bed. On the other hand, the normal tissue at the wound periphery contracted mildly after *COL7A1* knockdown in hiPSCs-KCs with very little newly regenerated epidermis at the wound edge.

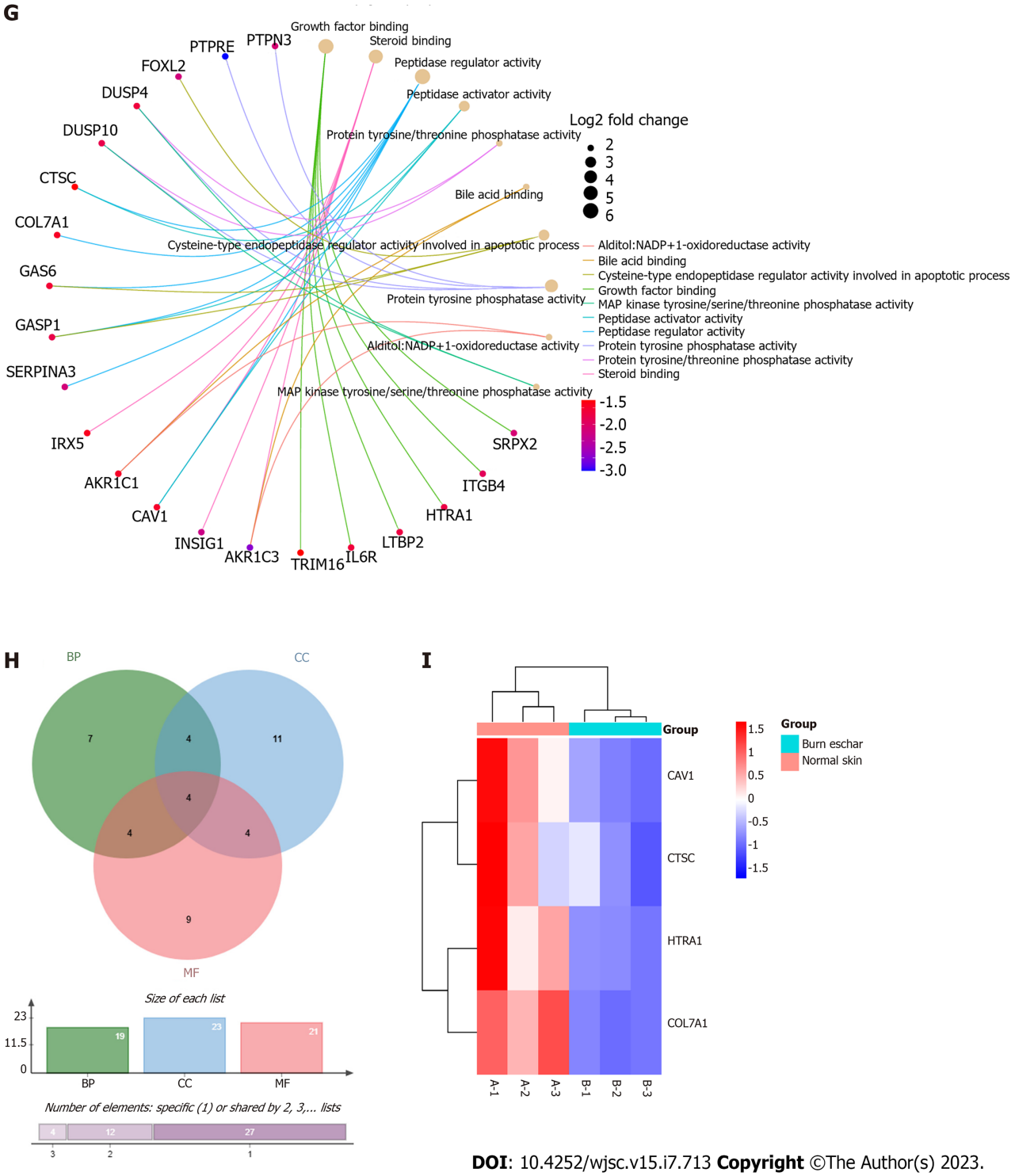
Moreover, by day 14, most of the hiPSCs-KCs transplanted group's wounds had been covered by newly formed epidermal tissue, and the wound margins had shrunk significantly. However, in the *COL7A1* knockdown group, wound size was reduced to a lesser extent and an exact re-epithelialization phenomenon was observed at the wound margin, but the majority of the wounds did not heal (Figure 4A and B).

Paraffin sections of the burned tissues were stained with HE at 7 and 14 d after hiPSCs-KCs transplantation to observe the pathological injury changes and regenerative epithelialization in each group. In the PBS group, the results showed


A
Down regulated (867)
Not changed
Up regulated (767)
GSE140926
B
Down regulated (1732)
Not changed
Up regulated (1843)
GSE27186
C
GSE27186
GSE140936
1634
98
769
Size of each list
1732
866
0
1732
867
GSE27186
GSE140926
Number of elements: specific (1) or shared by 2, 3,... lists
2403
2 (98)
1
D
GO results of three ontologies
neuroinflammatory response
microglial cell activation
leukocyte activation involved in inflammatory response
glial cell activation
renal absorption
cellular response to exogenous dsRNA
extracellular matrix organization
extracellular structure organization
macrophage activation
tertiary alcohol metabolic process
endocytic vesicle membrane
collagen−containing extracellular matrix
basolateral plasma membrane
melanosome
pigment granule
basal plasma membrane
melanosome membrane
endolysosome membrane
chitosome
pigment granule membrane
growth factor binding
steroid binding
peptidase regulator activity
peptidase activator activity
protein tyrosine/threonine phosphatase activity
bile acid binding
cysteine−type endopeptidase regulator activity involved in apoptotic process
protein tyrosine phosphatase activity
alditol:NADP+ 1−oxidoreductase activity
MAP kinase tyrosine/serine/threonine phosphatase activity
BP
CC
MF
Enrichment score


E
Neuroinflammatory response
AKR1C1
DKK3
Microglial cell activation
COL7A1
Leukocyte activation involved in inflammatory response
HTRA1
Glial cell activation
ITGB4
Renal absorption
ECM2
Cellular response to exogenous dsRNA
RGCC
Extracellular matrix organization
MMP10
Extracellular structure organization
IFIH1
Macrophage activation
CAV1
Tertiary alcohol metabolic process
SGK1
MMP3
GAS6
TLR2
AKR1C3
LDLR
CTSC
JUN
TLR3
Log2 fold change
-2
-3
-4
Cellular response to exogenous dsRNA
Extracellular matrix organization
Extracellular structure organization
Glial cell activation
Leukocyte activation involved in inflammatory response
Macrophage activation
Microglial cell activation
Neuroinflammatory response
Renal absorption
Tertiary alcohol metabolic process
3
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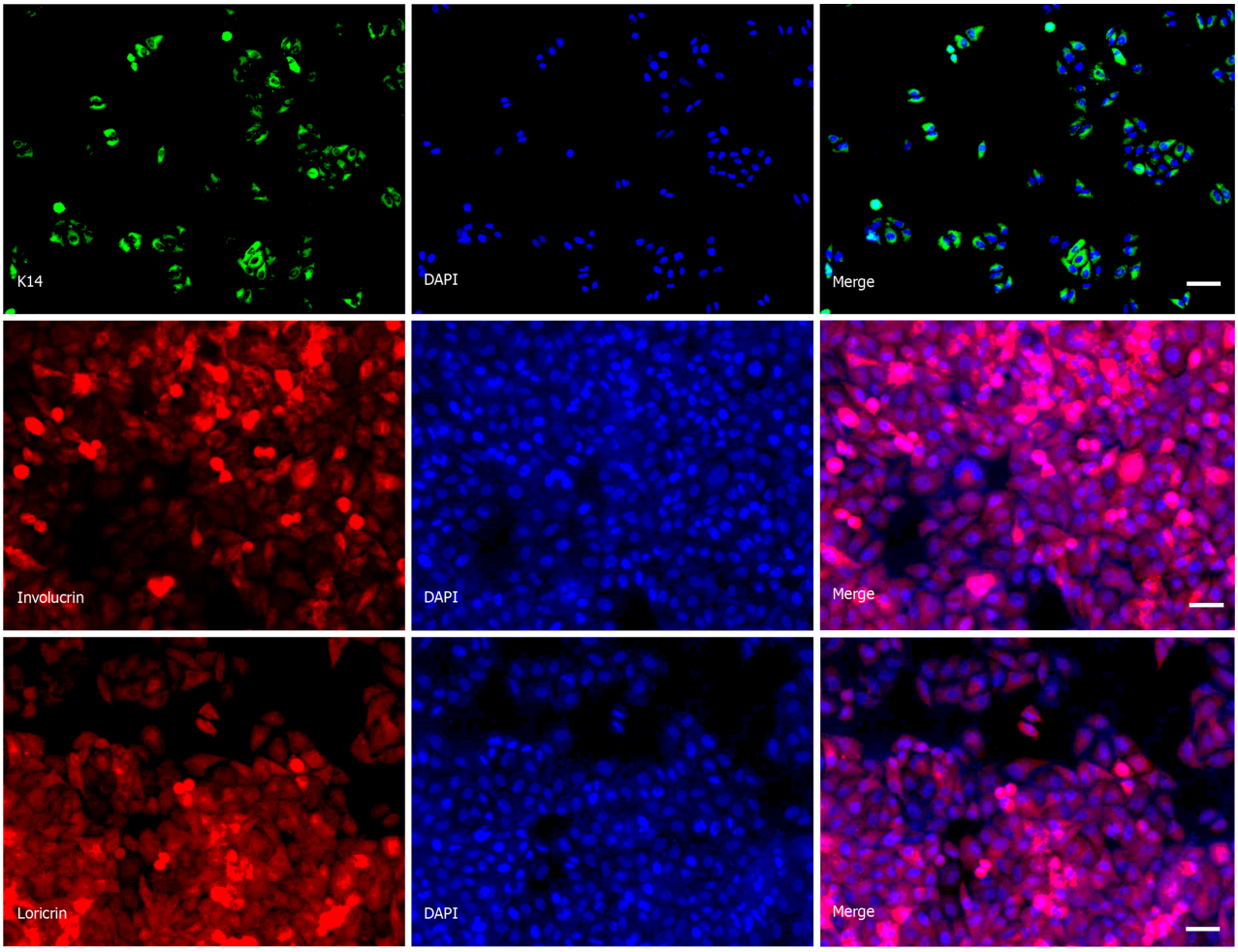

F
RAB29
TLR3
Endocytic vesicle membrane
RAB27B
Collagen-containing extracellular matrix
GPNMB
Basal plasma membrane
IL6R
Melanosome
MST1R
Pigment granule
MUC20
Basal plasma membrane
CLCA2
Melanosome membrane
MYO1D
Endolysosome membrane
CTSC
Chitosome
COL7A1
Pigment granule membrane
SRPX
TLR2
LTBP2
ANXA3
HTRA1
WNT5B
SRPX2
LDLR
SERPINA3
RAB38
ECM2
CAV1
Log2 fold change
-2
-3
-4
Basal plasma membrane
Basolateral plasma membrane
Chitosome
Collagen-containing extracellular matrix
Endocytic vesicle membrane
Endolysosome membrane
Melanosome
Melanosome membrane
Pigment granule
Pigment granule membrane
2
4
6
8



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Figure 1 Screening based on GEO datasets identified high expression of *COL7A1* in human induced pluripotent stem cells derived keratinocytes and low expression in burn wounds. A: Volcano plot of differentially expressed genes (DEGs) in GSE140926 dataset, which included 3 normal skin tissue samples and 3 burn eschar tissue samples. Abscissa axis indicates log2 (Fold change); B: Volcano plot of DEGs in GSE27186 dataset, which included 2 human induced pluripotent stem cells (hiPSCs) samples and 2 hiPSCs- keratinocytes samples. Abscissa axis indicates log2 (Fold change); C: The venn diagram exhibited the intersection of the downregulated DEGs in the GSE140926 dataset and the upregulated DEGs in the GSE27186 dataset; D: Gene ontology (GO) entry enrichment bar plot. Abscissa is the proportion of genes identified and ordinate is the GO entry name; E: Visualization of significant DEGs enriched in GO-biological pathway (BP) entries; F: Visualization of significant DEGs enriched in GO-cellular component entries. G: Visualization of significant DEGs enriched in GO-MF entries; H: The Venn diagram exhibited the intersection of GO-BP, GO-CC, and GO-MF; I: Heatmap of the 4 DEGs in GSE140926.

that inflammatory cells had clearly infiltrated the skin, the epidermal nuclei were deeply stained and pyknotic, the dermis was severely damaged, and the skin structure had been destroyed. In the hiPSCs-KCs group, the inflammatory cell infiltration reduced, the nucleus staining became pale, the skin structural damage improved, and the re-epithelialization of the wounds on the postoperative day 14 was complete. However, the improvement in skin injury was prevented after *COL7A1* knockdown in hiPSCs-KCs, and the re-epithelialization capacity of the wound was diminished (Figure 4C and



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Figure 2 Human induced pluripotent stem cells successfully differentiated into keratinocyte. Retinoic acid (RA) and BMP-4 were used to induce differentiation of hiPSCs into keratinocyte. The images as immunofluorescence staining of keratinocyte marker proteins K14, involucrin and loricrin at 14 d of induced differentiation, scale bar: 25 μm.

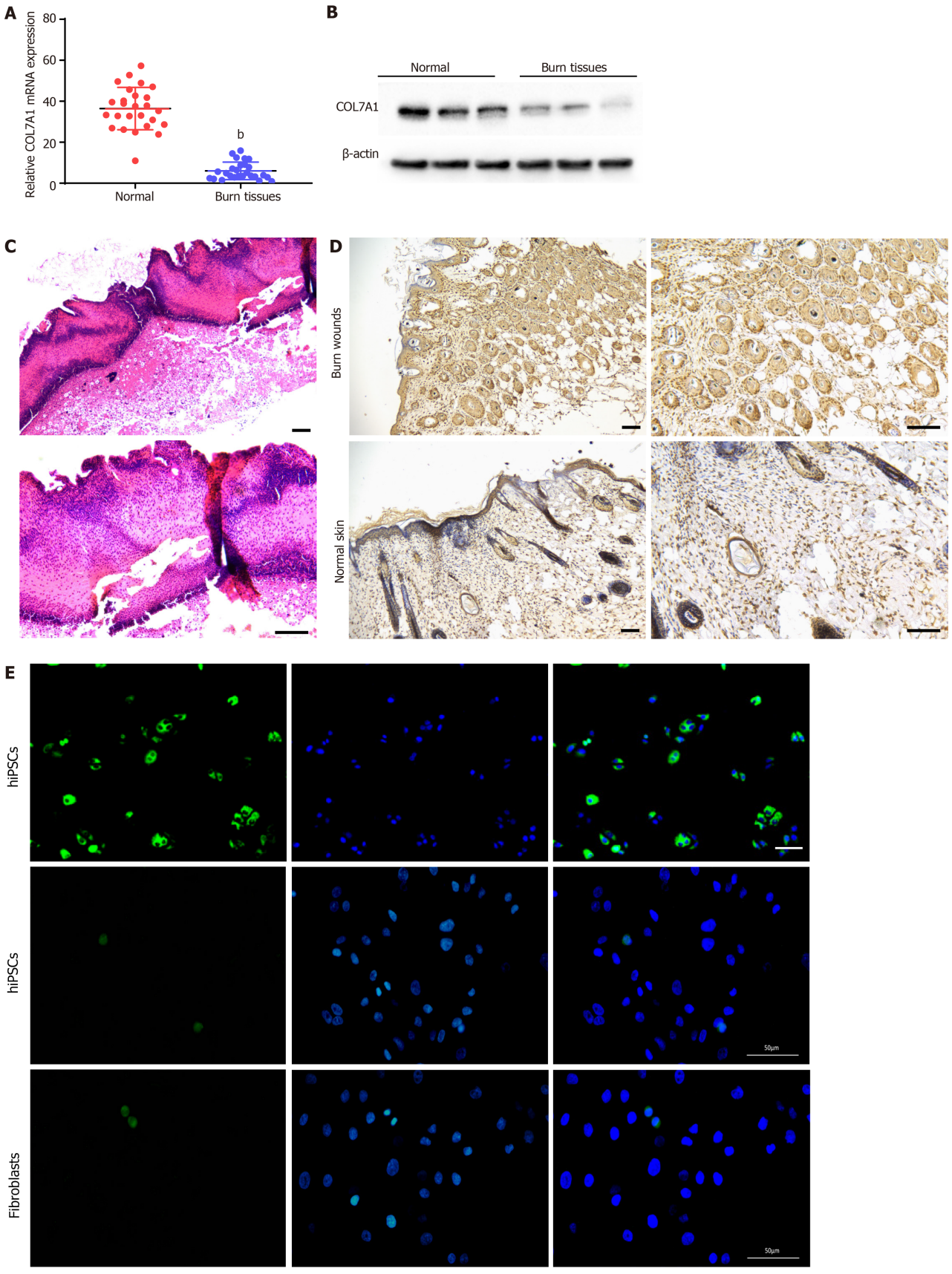
D, Supplementary Figure 1). Accordingly, it can be suggested that hiPSCs-KCs transplantation promoted skin burn healing and wound re-epithelialization in mice, which was partially counteracted by *COL7A1* knockdown.

COL7A1 knockdown abolishes the facilitative effect of hiPSCs-KCs on keratinocyte proliferation in mouse burn wounds

We further explored whether the accelerating effect of hiPSCs-KCs on wound healing was associated with hiPSCs-KC proliferation. Immunohistochemical staining was used to evaluate *Ki67* positive cells around the wounds in each group at 14 d after hiPSCs-KCs transplantation. The results demonstrated that the rate of *Ki67* positive cells in burn wound tissues significantly increased after hiPSCs-KCs transplantation, whereas it decreased after *COL7A1* knockdown in hiPSCs-KCs (Figure 5A and B). Furthermore, the results were consistent with cell experiments, and *COL7A1* knockdown significantly inhibited the EdU fluorescence intensity in hiPSCs-KCs (Figure 5C and D). Figure 5E displays the results of double immunofluorescence staining with EdU and the human marker human leukocyte antigen (HLA) to identify the proliferating cell type. Transplantation of hiPSCs-KCs led to a dramatic increase in the number of proliferating cells, and analysis showed that most of the proliferating cells showed overlap with the human marker HLA, suggesting that most of the proliferating cells were hiPSCs-KCs. Additionally, a small number of active proliferating cells that did not coincide with HLA was noted, indicating that these cells were natural mouse cells involved in tissue repair. Therefore, it can be suggested that hiPSCs-KCs proliferates actively after human hiPSCs-KCs transplantation to the burn site in mice skin tissues and plays a major role in tissue repair, while a small number of mouse natural cells are activated and assist in tissue repair.

COL7A1 knockdown abolishes the positive impact of hiPSCs-KCs on keratinocyte migration in mouse burn wounds

We evaluated whether *COL7A1* accelerates wound healing by encouraging hiPSCs-KC migration. At day 14, frozen section results showed green fluorescence expression in the epithelium of skin wound healing in mice transplanted with CFSE-labeled hiPSCs-KCs, while the PBS group showed no fluorescence. This showed that hiPSCs-KCs could migrate into mouse skin wounds and participate in the epithelialization process. *COL7A1* knockdown also decreased green



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Figure 3 *COL7A1* was significantly under-expressed in deep II burn tissues. A: RT-qPCR analysis of *COL7A1* mRNA expression in 26 humans deep second degree burn wound tissues and 26 normal skin tissues; B: Western blotting analysis of *COL7A1* protein expression in 3 human deep second degree burn wound tissues and 3 normal skin tissues; C: Representative photomicrographs of hematoxylin-eosin stained burned skin of mice at 24 h post burn, scale bar: 100 μm; D: Immunohistochemical detection of *COL7A1* expression levels in burn wounds and surrounding normal skin of mice, scale bar: 100 μm; E:

Immunofluorescence detection of *COL7A1* expression levels in keratinocytes, hiPSCs and fibroblasts, scale bar: 50 μm. Data: Mean ± SEM. $^{b}P < 0.01$ *vs* Normal group.

fluorescence expression at wound healing in hiPSCs-KCs, suggesting that it prevented migration toward wounds (Figure 6A). Besides, Transwell assay and injury repair results indicated that *COL7A1* inhibition reduced the migration ability of hiPSCs-KCs, as demonstrated by the decreased cell number in the lower chamber of Transwell and the decreased scratch healing rate (Figure 6B and C). Consequently, hiPSCs-KC transplantation promoted the proliferation and migration of KCs towards the wound site around the mic cutaneous wounds, which in turn accelerated the epithelialization process and wound healing.

COL7A1 inhibition counteracted the inhibitory effects of hiPSCs-KCs on the inflammatory response to burn injury in mice

The effects of hiPSCs-KCs transplantation on the production of plasma inflammatory mediators in mice were investigated. Enzyme linked immunosorbent assay (ELISA) results showed that the levels of multiple inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, IL-10, and IL-12, remarkably decreased in the plasma of mice after hiPSCs-KCs transplantation (Figure 7). Furthermore, the production of plasma inflammatory mediators significantly increased after *COL7A1* knockdown in hiPSCs-KCs.

COL7A1 inhibition reversed the inhibitory effect of hiPSCs-KCs on the NF-κB pathway in mouse burn wounds

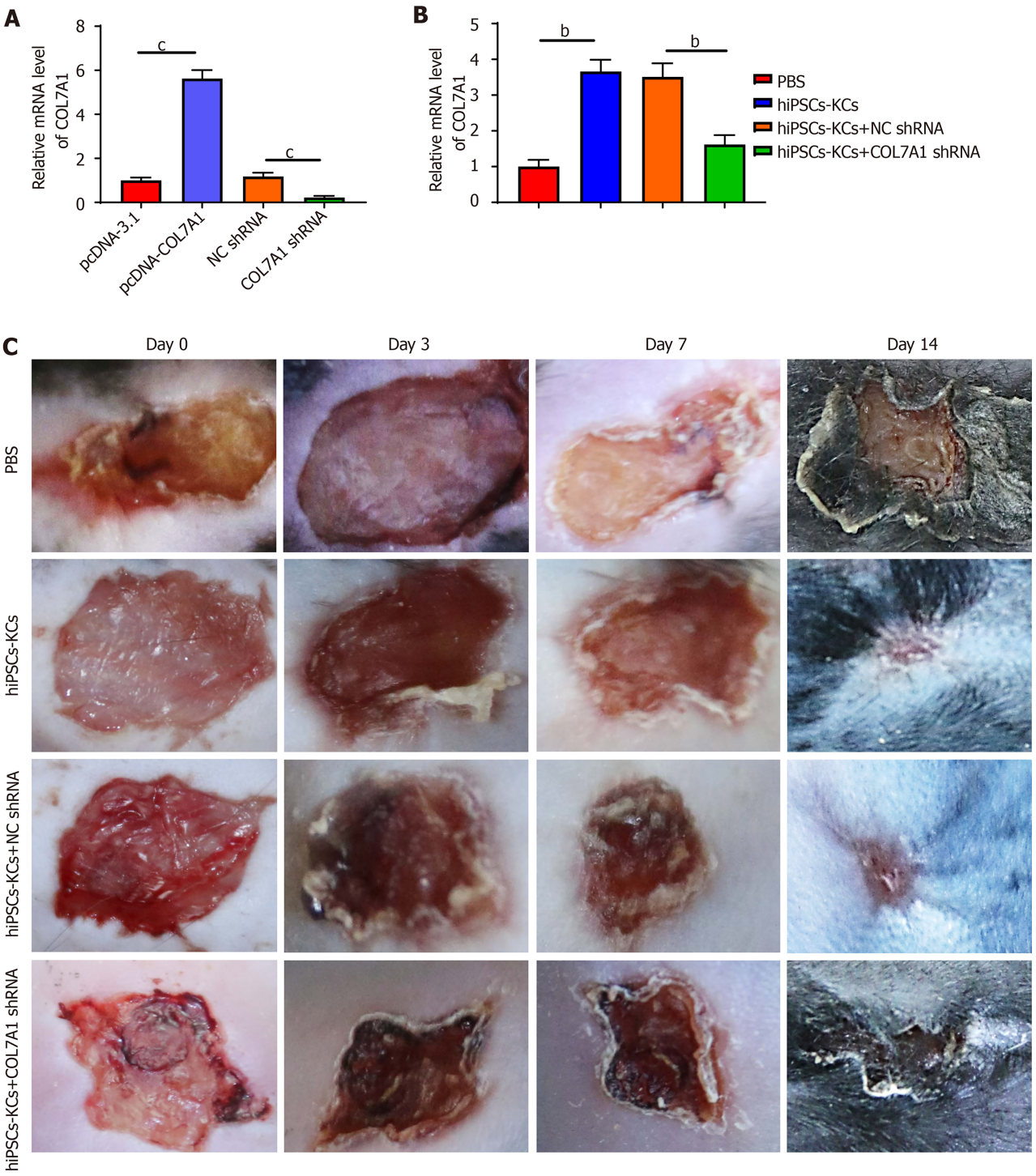
The NF-κB inflammasome pathway is a major player in the wound inflammatory response. Upon damage by stimuli, IκB α is degraded by phosphorylation, which dissociates NF-κB to form a complex with IκBα and translocate to the nucleus, causing auto activation, therefore activated NF-κB has an important role in mediating inflammatory activity. We examined the effect of *COL7A1* inhibition on NF-κB pathway protein expression after hiPSCs-KCs transplantation. Western blotting results confirmed that the protein level of p-*p65* considerably increased (Figure 8A) and that of IκBα decreased (Figure 8B) in mice wound tissues. In addition, p-*p65* protein expression decreased while IκBα protein expression was upregulated in wound tissues after hiPSCs-KCs transplantation, which was partially counteracted by *COL7A1* knockdown.

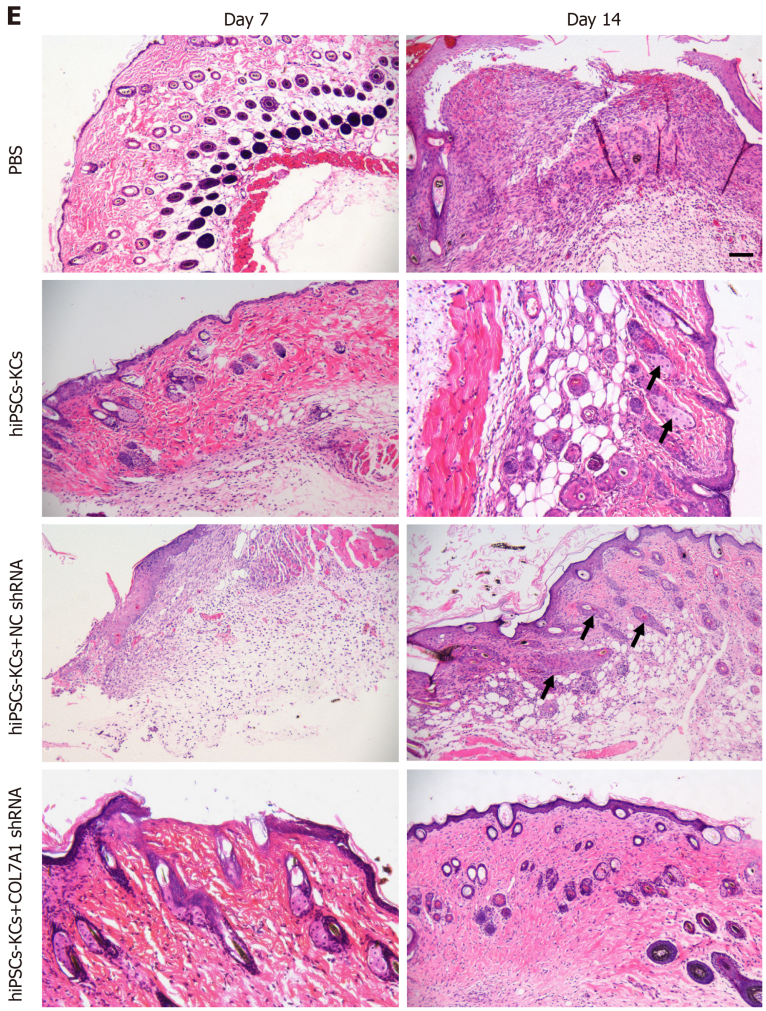
DISCUSSION

Burn injury usually refers to heat-induced tissue damage with skin tissue damage as the first manifestation[19]. Skin and mucosal tissues are commonly involved at the site of burn injury, and deep tissues, such as muscle, bone, and internal organs, might also be affected in severe cases[20]. The skin is the largest organ and the first barrier of the human body, which has many important functions such as blocking pathogen invasion, preventing dehydration, regulating body temperature, and providing sensation[21]. Besides, the skin is also an active immune organ, maintaining the homeostasis of relevant intracellular environments for both specific and nonspecific immunity[22,23]. If severe and extensive skin burns occur, the regenerative capacity of the skin can be disrupted, leading various pathological problems. Therefore, timely repair and closure of wounds can effectively prevent these issues, including infections, delay and prevent progressive damage to the underlying organs, prevent scar and contracture, and others.

Wound repair involves many physiological processes, including inflammation, KC migration, proliferation, granulation tissue formation, and matrix remodeling[24]. KCs proliferation is relatively slow in the normal skin basal layer, but rapid in the damaged skin[25]. In addition, they can secrete specific keratins, and various cytokines and signaling molecules, which work together to promote the healing of skin wounds[26]. One of the current therapies for treating skin wounds is cell therapy, which entails the transplantation of KCs at the wound's periphery so that they can proliferate and migrate within the wound as it heals[27].

In theory, hiPSCs can differentiate to various skin cell types. Moreover, they have become the most ideal and abundant seed cell source for skin tissue engineering due to their characteristics, such as simple expansion in vitro and simple gene interference or overexpression. This is because hiPSCs have solved the problems limiting the research and development of other types of stem cells, such as material source, immune rejection, ethics, and others[28]. Recently, researchers have focused on finding effective means to induce the differentiation of pluripotent stem cells into a single KC population. Using the ability of KCs to rapidly attach to collagen IV, Bilousova *et al*[29] induced pluripotent stem cell differentiation in mice with RA and BMP4, and ultimately obtained 80%-90% *K14* + KCs[29]. Further evidence that RA alone can induce efficient differentiation of hiPSCs into *K14*+/*p63*+ KCs was published in 2013 by Selekman *et al*[30]. This not only widened the use of KCs in tissue engineering, but also demonstrated the promise of hiPSCs for use in skin regeneration and repair[30]. Moreover, researchers found that miR-762-mediated promotion of keratinocyte and endothelial cell migration by exosomes from hiPSCs-KCs aided in the healing of deep second-degree burn wounds in mice[31]. In the current investigation, we utilized RA and BMP-4 to induce hiPSCs differentiation into KCs, which has been demonstrated by immunofluorescence. Subsequent injection of hiPSCs-KCs around the mice skin wounds was associated with significant improvement in skin burn wound healing rate and re-epithelialization in mice, which may be attributed to the ability of hiPSCs-KCs to promote the proliferation and migration of KCs around the wounds.


A
Relative mRNA level of COL7A1
8
6
4
2
0
c
c
pcDNA-3.1
pcDNA-COL7A1
NC shRNA
COL7A1 shRNA
B
Relative mRNA level of COL7A1
5
4
3
2
1
0
b
b
PBS
hiPSCs-KCs
hiPSCs-KCs+NC shRNA
hiPSCs-KCs+COL7A1 shRNA
C
Day 0
Day 3
Day 7
Day 14
PBS
hiPSCs-KCs
hiPSCs-KCs+NC shRNA
hiPSCs-KCs+COL7A1 shRNA
D
PBS
hiPSCs-KCs
hiPSCs-KCs+NC shRNA
hiPSCs-KCs+COL7A1 shRNA
Relative wound area (%)
150
100
50
0
NS
NS
a
a
b
b
0
3
7
14
Days
F
PBS
hiPSCs-KCs
hiPSCs-KCs+NC shRNA
hiPSCs-KCs+COL7A1 shRNA
%Re-epithelialization
150
100
50
0
b
a
b
a
7
14
Days

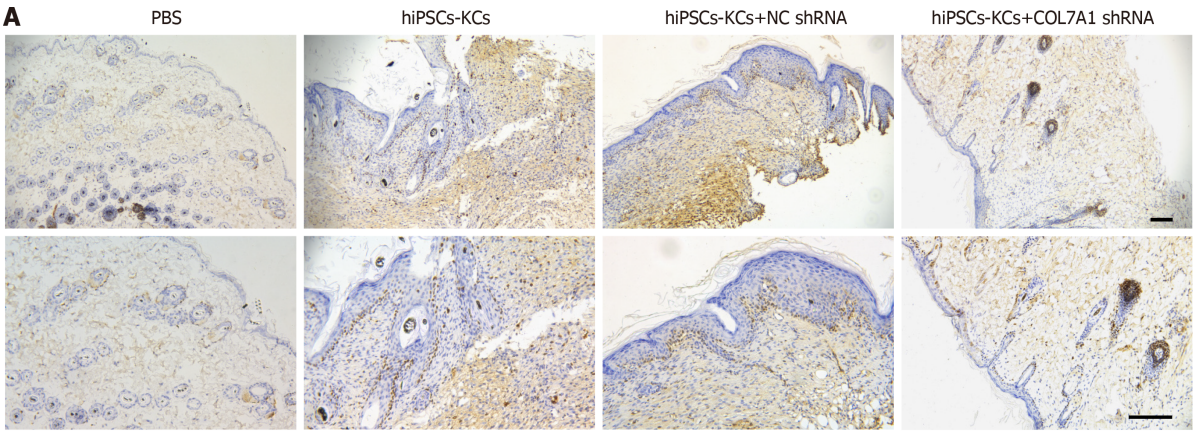


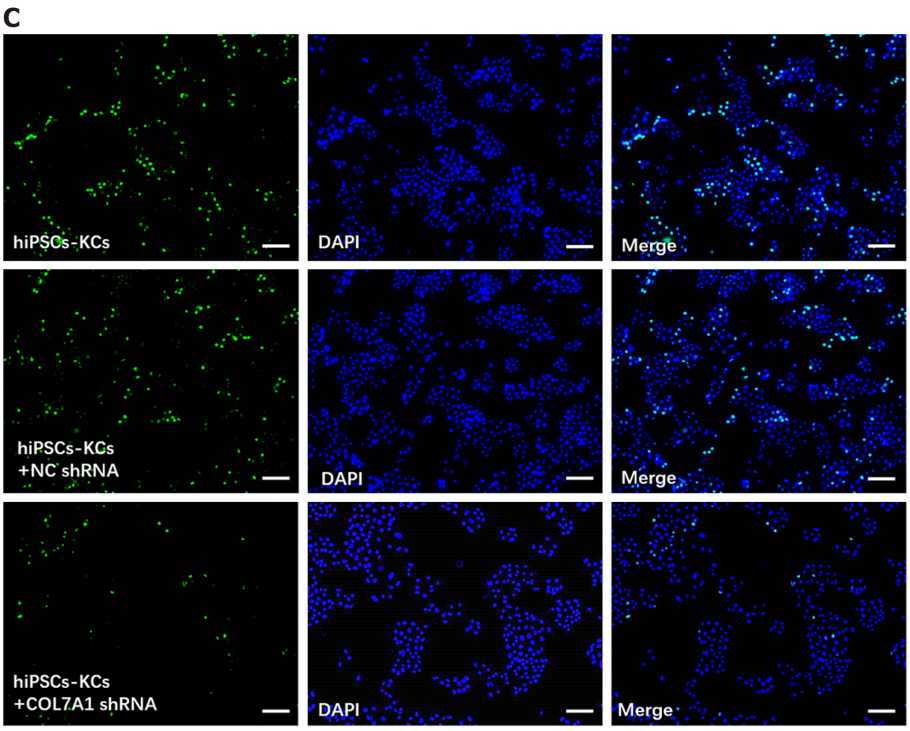
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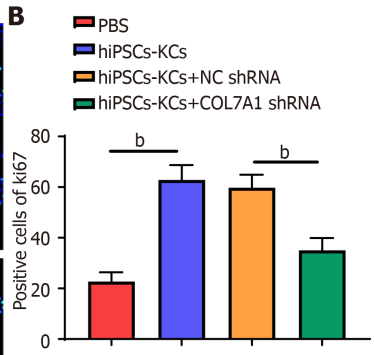
Figure 4 *COL7A1* inhibition abolished the promoting effects of human induced pluripotent stem cells derived keratinocytes on burn healing and re epithelialization in mice. A: RT-qPCR detection of the *COL7A1* mRNA levels in human induced pluripotent stem cells derived keratinocytes (hiPSCs-KCs) after transfection of pcDNA- *COL7A1* or *COL7A1* shRNA; B: RT-qPCR detection of the *COL7A1* mRNA levels in burned skin of mice after transplantation of hiPSCs-KCs or *COL7A1* knock-downed hiPSCs-KCs; C: At 3, 7, 14 d after hiPSCs-KCs transplantation, the healing of burn wounds in mice was recorded by taking photographs with a digital camera; D: Statistical analysis of relative size of burn wounds in each group of mice; E: Hematoxylin-eosin staining was performed after burn tissues were paraffin sectioned at 7 and 14 d after hiPSCs-KCs transplantation, and then wound healing as well as re-epithelialization were observed by histological sections in each group, scale bar: 200 μm; F: Re-epithelialization ratio of the burned window in mice of each group. Data: Mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. $n = 10$. NS: Not significant; PBS: Phosphate buffered saline; hiPSCs: Human induced pluripotent stem cells; KCs: Keratinocytes.

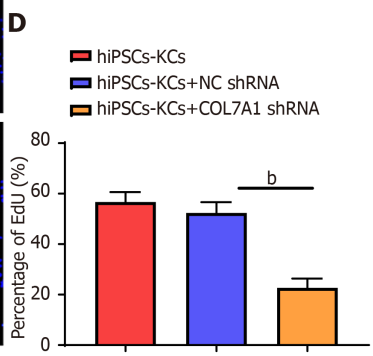
Through interactions with extracellular matrix (ECM) proteins like type IV collagen[32], the type VII collagen fibril (*COL7A1*), which is composed of three identical collagen chains, acts as an anchoring fibril between the outer epithelium and the underlying matrix to promote clustering and adhesion of the epithelial basement membrane. The skin, endometrium, and prostate are the most common normal human tissue types in which *COL7A1* is expressed[33]. KCs have been shown to have high levels of *COL7A1*[34]. By retroviral transduction, Pourreyron *et al*[35] increased KC adhesion, migration, and invasion in patients with recessive dystrophic epidermolysis bullosa (RDEB) by introducing type VII collagen into KCs. Mutations in the *COL7A1* gene lead to a lack of type VII collagen, which leads to RDEB. In addition, *COL7A1* was found to be an important factor in the physiological healing of wounds in humans and mice, which could aid in the development of therapeutic strategies for RDEB and other chronic wounds[36]. However, the function of *COL7A1* in the recovery from burns is not known currently. In the current investigtion, screening the GEO database revealed that *COL7A1* was highly expressed in hiPSCs-KCs, whereas it was poorly expressed in burn wounds. Besides, hiPSCs-KCs transplantation promoted cutaneous burn healing and wound re-epithelialization in mice, which was partially counteracted by *COL7A1* knockdown. Moreover, *COL7A1* positively contributes to the migratory ability of KCs. Subsequently, we also found that *COL7A1* knockdown partially abolished the promotion of KC migration towards the epithelium in wound healing by hiPSCs-KCs transplantation. According to the above findings, *COL7A1* is found mostly in KCs and promotes burn wound healing by enhancing KC migration.

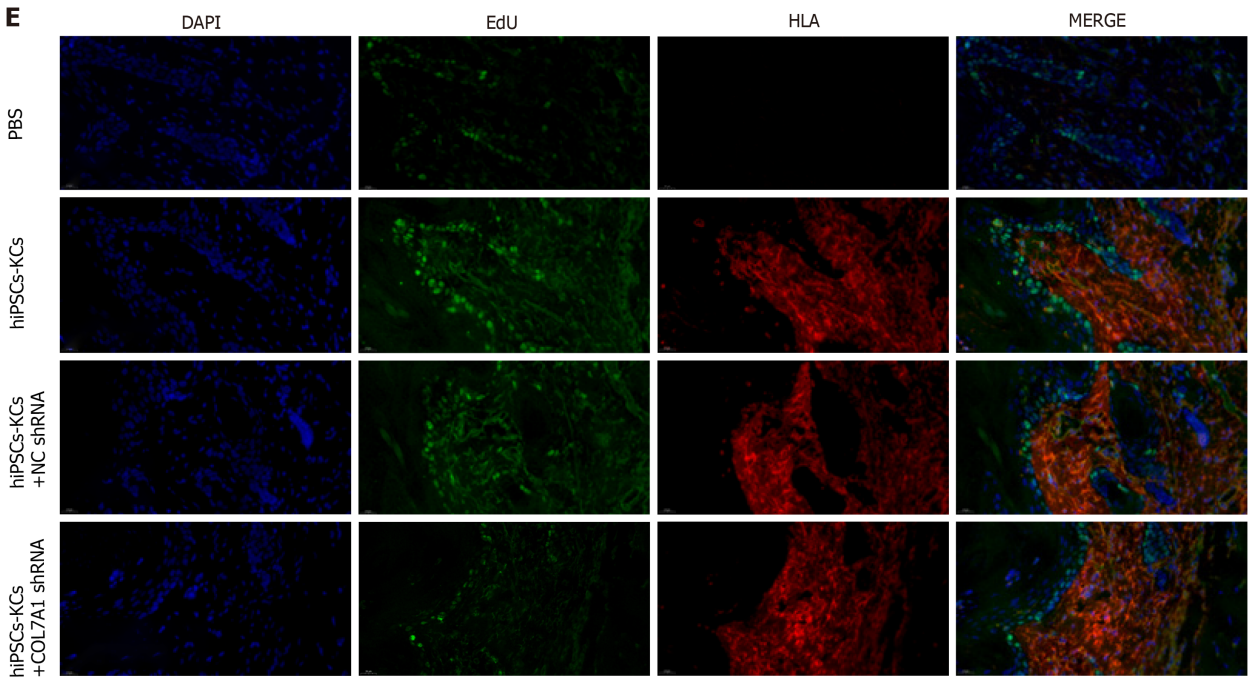
Studies have shown that direct burn injury to the skin mucosa disrupts the important barrier against the outside environment and tissue organ system function, breaks the relative balance between the body and the outside environment, and causes various degrees of systemic inflammatory responses[37]. A variety of cellular immune (macrophages, neutrophils, endothelial cells, *etc*.) and immune molecules mediate tissue cellular immune defense





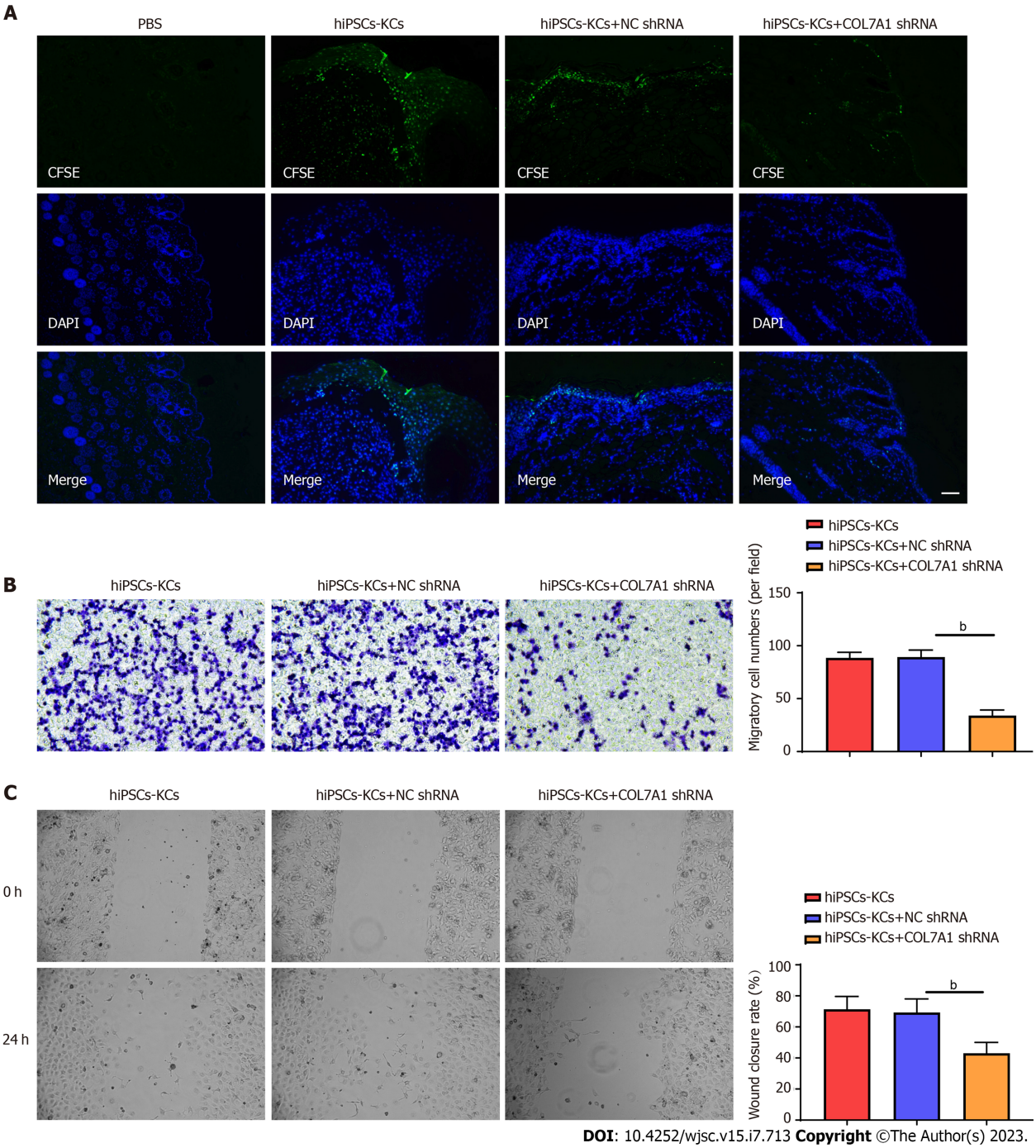






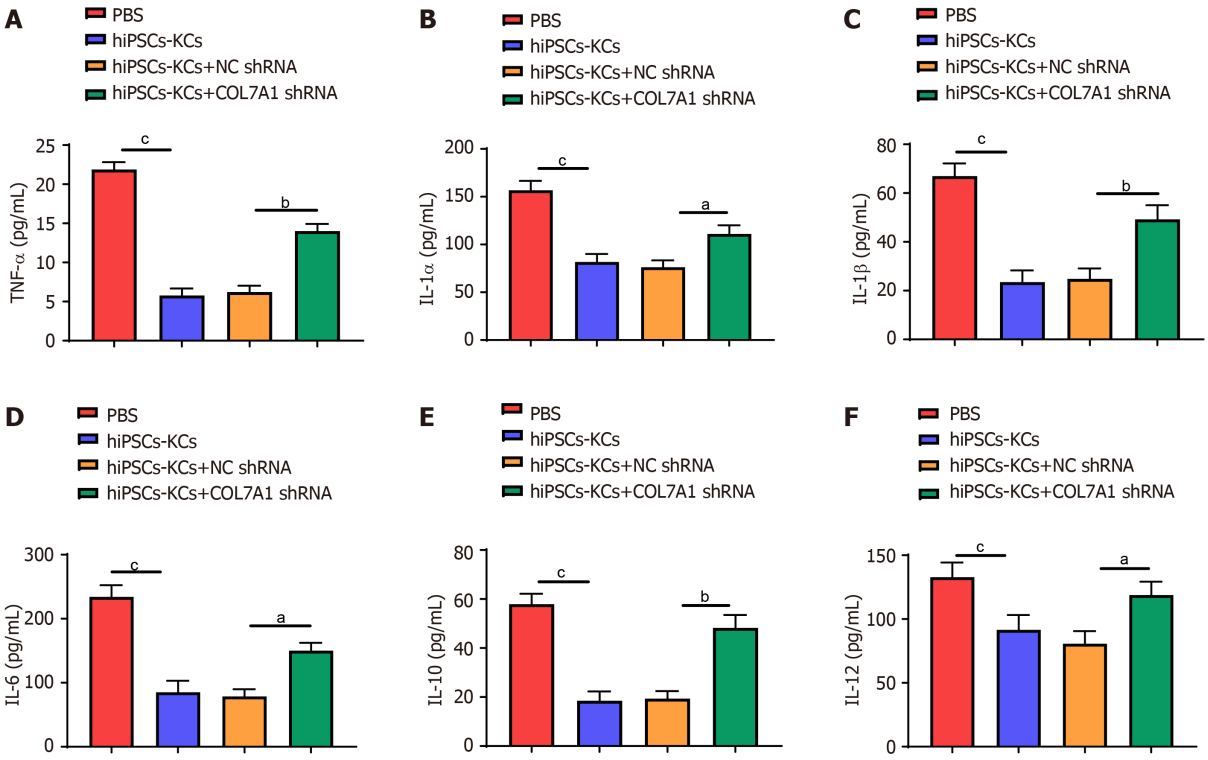
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Figure 5 *COL7A1* knockdown abolishes the facilitative effect of human induced pluripotent stem cells derived keratinocytes on keratinocyte proliferation in mouse burn wounds. A: The Ki67 positive cells in burn wound tissues of each group were detected by immunostaining on the 14th postoperative day. scale bar: 200 μm; B: The statistical graph of the number of Ki67 positive cells; C: The proliferation ability of keratinocytes in each group was detected by EdU staining, scale bar: 100 μm; D: The proliferation ability of keratinocytes in each group was detected by EdU staining; E: Immunofluorescence estimate of the EdU and HLA levels in burn tissue of mice in each group. $^{a}P < 0.05$, $^{b}P < 0.01$. $n = 10$. PBS: Phosphate buffered saline; hiPSCs: Human induced pluripotent stem cells; KCs: Keratinocytes; HLA: Human leukocyte antigen.



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Figure 6 *COL7A1* knockdown abolishes the facilitative effect of human induced pluripotent stem cells derived keratinocytes on keratinocyte migration in mouse burn wounds. A: On the 14th day after operation, frozen sections showed that carboxifluorescein diacetate succinimidyl ester labeled human induced pluripotent stem cells derived keratinocytes (hiPSCs-KCs) migrated to the epithelium of the wound healing site scale bar: 100 μm; B: Transwell assay was employed to determine the migration capability of hiPSCs-KCs transfected with *COL7A1* shRNA, scale bar: 100 μm; C: Damage repair assay was applied to test the migration capability of hiPSCs-KCs transfected with *COL7A1* shRNA. Data: Mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. $n = 10$. PBS: Phosphate buffered saline; hiPSCs: Human induced pluripotent stem cells; KCs: Keratinocytes; CFSE: Carboxifluorescein diacetate succinimidyl ester.



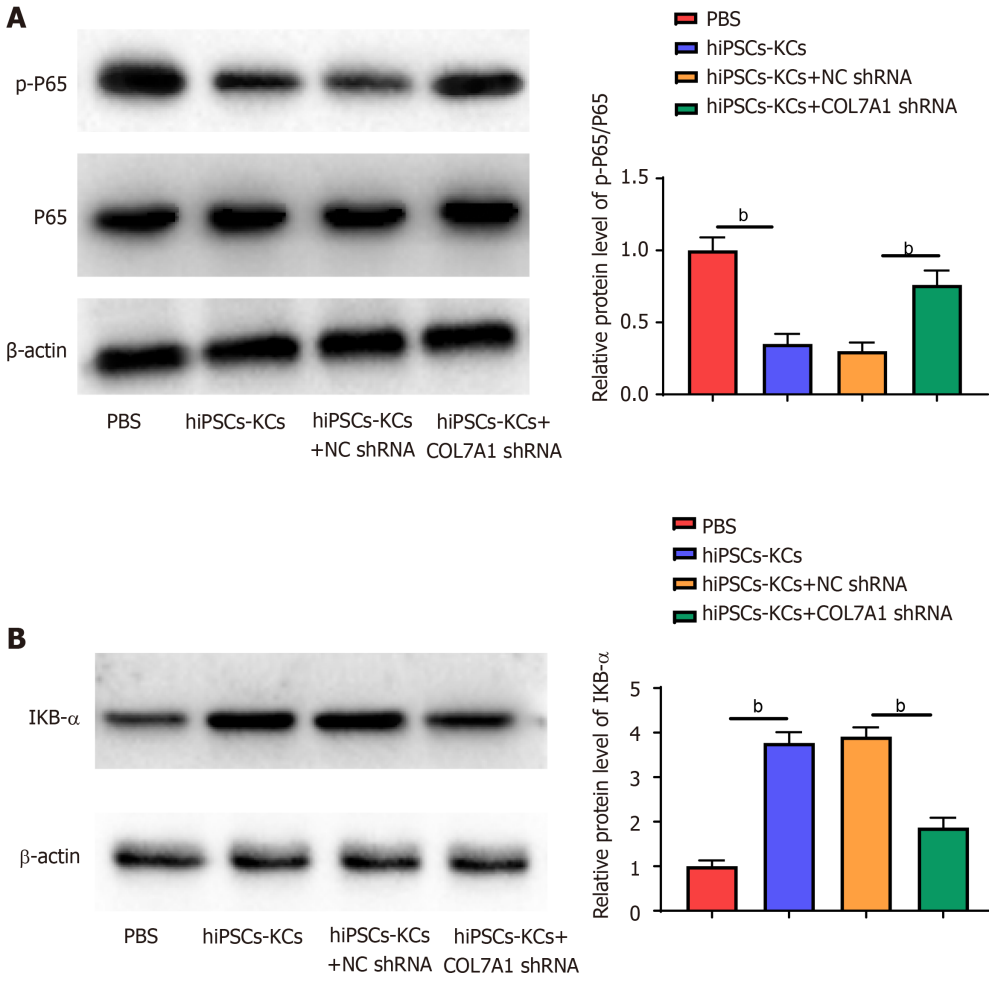
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Figure 7 *COL7A1* inhibition counteracted the inhibitory effects of human induced pluripotent stem cells derived keratinocytes on the inflammatory response to burn injury in mice. A: Enzyme linked immunosorbent assay (ELISA) detection of the tumor necrosis factor α contents in the plasma of mice in each group; B: ELISA detection of the interleukin (IL)-1α contents in the plasma of mice in each group; C: ELISA detection of the IL-1β contents in the plasma of mice in each group; D: ELISA detection of the IL-6 contents in the plasma of mice in each group; E: ELISA detection of the IL-10 contents in the plasma of mice in each group; F: ELISA detection of the IL-12 contents in the plasma of mice in each group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. $n = 10$. PBS: Phosphate buffered saline; hiPSCs: Human induced pluripotent stem cells; KCs: Keratinocytes.

functions to collectively clear the source of infection, promoting wound repair and healing[38]. After burn injury, the levels of TNF-α, IL-1α, IL-1β, IL-6, IL-10, and IL-12 change significantly[39]. This has been indicated in this investigation as the ELISA results on wound tissues showed that the levels of multiple inflammatory mediators, including TNF-α, IL-1α, IL-1β, IL-6, IL-10 and IL-12 significantly decreased in the plasma of mice after hiPSCs-KCs transplantation, which were furtherly reversed by *COL7A1* knockdown in hiPSCs-KCs. Accordingly, it can be suggested that *COL7A1* might accelerate wound epithelialization by inhibiting wound inflammatory response and promoting KC migration and proliferation. However, the molecular mechanism is still unknown.

NF-κB is one of the major regulatory factors in the pathways related to inflammatory mediators regulation[40,41]. Recently, more and more attention has been paid to the role of NF-κB in infections, autoimmune diseases, tumors, and other conditions. For instance, Wang *et al*[42] discovered that foamy macrophages may inhibit fibroblasts functions and accelerate wound healing by inhibiting the activation of NF-κB signaling pathway in tuberculous wounds. Moreover, in a study on mouse back burn skin tissues, Jiang *et al*[43] found that MDL-800, a highly efficient and selective *SIRT6* activator, significantly reduced the expression levels of proinflammatory factors TNF-α and IL-6 by inhibiting the NF-κB pathway, thereby demonstrating anti-inflammatory and pro-healing effects. It has also been shown by Chen *et al*[44] that CFTR promotes skin wound healing by inhibiting the NF-κB inflammatory response, decreasing KC proliferation, and encouraging its differentiation. Normally, NF-κB exists in a "silent" state from which it can be triggered by proinflammatory factors. When activated, it promotes the production of additional factors—including proinflammatory factors, adhesion factors, chemokines, and others—that can either prolong the acute phase of an inflammatory response or cause it to transition into chronic inflammation[45]. These findings indicate the importance of NF-κB in wound healing. Additionally, it has been demonstrated that when the NF-κB pathway is activated leading to the phosphorylation and degradation of IκBα proteins, *p65* is translocated from the cytosol to the nucleus to further regulate downstream inflammatory cytokine signals[46]. This is consistent with the findings of our investigation. NF-κB expression in burn wounds of mice was detected by Western blotting, and the results indicated that the protein level of p-*p65* strikingly increased and that of IκBα decreased in the wound tissues of the included mice. In addition, after hiPSCs-KCs transplantation, p-*p65* protein expression remarkably decreased in the wound tissues, while IκBα protein expression was upregulated, which was partially counteracted by *COL7A1* knockdown.

The above results indicate that *COL7A1* plays a role in accelerating wound healing by inhibiting the inflammatory response and promoting keratinocyte proliferation and migration in deep second-degree burn wounds. Therefore, it can be suggested that *COL7A1* is vital for encouraging wound healing and providing a new target for deep second-degree



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Figure 8 *COL7A1* inhibition reversed the inhibitory effect of human induced pluripotent stem cells derived keratinocytes on the NF-κB pathway in mouse burn wounds. A: The protein level of p-p65 in mouse wound tissues treated with hiPSCs-KCs, with or without *COL7A1* shRNA transfection, was employed by Western blotting; B: The protein level of IκBα in mouse wound tissues treated with hiPSCs-KCs, with or without *COL7A1* shRNA transfection, was employed by Western blotting. $^{b}P < 0.01$. $n = 10$. PBS: Phosphate buffered saline; hiPSCs: Human induced pluripotent stem cells; KCs: Keratinocytes.

burn wound treatment. However, there are a few limitations to our findings. For instance, we used FTY720 injection alone to suppress immune rejection in mice, based on previous experiments in the literature. FTY720 is a newly developed immunosuppressant that selectively reduces peripheral circulating lymphocytes and significantly prolongs the survival of transplanted organs in experimental animals. Ryu *et al*[47] reported an increase in the number of T lymphocytes in mesenteric lymph nodes and a reduction within the spleen among FTY720-treated mice. Besides, FTY720 has been shown to promote local wound healing *via* an immunomodulatory mechanism after being delivered using a biomaterial[48]. We hypothesized that local delivery of FTY720 can promote local wound healing through immunomodulatory mechanisms, which may also prevent delayed tissue repair after injury. Unfortunately, a control group without FTY720 injection was not considered in this investigation, so it was not possible to determine whether FTY720 could have an impact over tissue repair. Nevertheless, according to our findings, no immune rejection events were observed in our included mice, and the wound healing rate was within the normal range. Besides, future studies from our team will concentrate on modifying the experiment to account for FTY720's impact on tissue repair delay.

CONCLUSION

The above results indicate that *COL7A1* can play a role in accelerating wound healing by inhibiting the inflammatory response and promoting KC proliferation and migration in deep second degree burn wounds, revealing the mechanism of *COL7A1* for encouraging wound healing and providing a new target for deep second degree burn wound treatment.

ARTICLE HIGHLIGHTS

Research background

Based on the extensive skin integrity defects caused by burns, scalds, vasculopathy triggered skin ulcers, pressure ulcers, diabetic ulcerated feet, and so on, which are caused by the action of internal and external factors, the wounds develop pathological inflammatory reactions, which cannot achieve the healing through normal repair procedures, and then lead to slow or non-healing, causing severe disability or death to the patient, and at the same time, bringing a heavy economic burden to the patient's family. Studies have shown that impaired keratinocyte (KC) migration causes non healing wounds and is an important cause of chronic wound formation, whereas inhibition of their proliferation and differentiation functions does not result in the formation of non-healing wounds. Deriving the replacement cells from human-induced pluripotent stem cells (hiPSCs) allows for sufficient scale up and by using hiPSCs as the choice of human pluripotent stem cells (hPSC) will ensure immunocompatibility.

Research motivation

The specific mechanism of hiPSCs involvement in burn repair has not been fully revealed.

Research objectives

The current study showed that hiPSCs can effectively differentiate into KCs under the effect of inducers, but its effect on skin burn healing has not been reported. Therefore, this study was intended to observe the effects of hiPSCs-derived KCs transplantation on skin burn healing in mice, and to preliminarily reveal the related mechanisms by which it exerts its effects.

Research methods

In this study, we used the cell induced differentiation technique of hiPSCs to successfully differentiate hiPSCs cells into KCs (hiPSCs-KCs) using inducing factors. Subsequently, a murine skin-deep degree II burn model was constructed, and KCs induced to differentiate successfully were injected around the mouse skin wound, to deeply investigate the effect of transplantation of KCs induced to differentiate formed on the healing of murine skin wounds. In addition, a bioinformatics approach was taken to further explore the possible mechanism of hiPSCs-KCs transplantation involvement in wound healing.

Research results

The above results indicate that COL7A1 plays a role in accelerating wound healing by inhibiting the inflammatory response and promoting KC proliferation and migration in deep second-degree burn wounds. Therefore, it can be suggested that COL7A1 is vital for encouraging wound healing and providing a new target for deep second-degree burn wound treatment. However, there are a few limitations to our findings. For instance, we used FTY720 injection alone to suppress immune rejection in mice, based on previous experiments in the literature. FTY720 is a newly developed immunosuppressant that selectively reduces peripheral circulating lymphocytes and significantly prolongs the survival of transplanted organs in experimental animals. A study reported an increase in the number of T lymphocytes in mesenteric lymph nodes and a reduction within the spleen among FTY720-treated mice. Besides, FTY720 has been shown to promote local wound healing *via* an immunomodulatory mechanism after being delivered using a biomaterial. We hypothesized that local delivery of FTY720 can promote local wound healing through immunomodulatory mechanisms, which may also prevent delayed tissue repair after injury. Unfortunately, a control group without FTY720 injection was not considered in this investigation, so it was not possible to determine whether FTY720 could have an impact over tissue repair. Nevertheless, according to our findings, no immune rejection events were observed in our included mice, and the wound healing rate was within the normal range. Besides, future studies from our team will concentrate on modifying the experiment to account for FTY720's impact on tissue repair delay.

Research conclusions

The above results indicate that COL7A1 can play a role in accelerating wound healing by inhibiting the inflammatory response and promoting KC proliferation and migration in deep second degree burn wounds, revealing the mechanism of COL7A1 for encouraging wound healing and providing a new target for deep second degree burn wound treatment.

Research perspectives

This study will focus on the aspects of autologous transplantation of human iPSCs in the future.

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FOOTNOTES

Author contributions: Wu LJ contributed to methodology, investigation, data curation, original draft; Lin W contributed to review and editing; Liu JJ contribute to methodology, investigation, data curation, original draft; Chen WX contribute to writing, review & editing; He WJ contribute to investigation; Shi Y contribute to writing; Liu X contribute to review & editing; Li K contribute to idea, supervision, review & editing.

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ORIGINAL ARTICLE

Basic Study

Generation of a human haploid neural stem cell line for genome-wide genetic screening

Hai-Song Wang, Xin-Rui Ma, Wen-Bin Niu, Hao Shi, Yi-Dong Liu, Ning-Zhao Ma, Nan Zhang, Zi-Wei Jiang, Ying-Pu Sun

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Abstract

BACKGROUND

Haploid embryonic stem cells (haESCs) have been established in many species. Differentiated haploid cell line types in mammals are lacking due to spontaneous diploidization during differentiation that compromises lineage-specific screens.

AIM

To derive human haploid neural stem cells (haNSCs) to carry out lineage-specific screens.

METHODS

Human haNSCs were differentiated from human extended haESCs with the help of Y27632 (ROCK signaling pathway inhibitor) and a series of cytokines to reduce diploidization. Neuronal differentiation of haNSCs was performed to examine their neural differentiation potency. Global gene expression analysis was conducted to compare haNSCs with diploid NSCs and haESCs. Fluorescence activated cell sorting was performed to assess the diploidization rate of extended haESCs and haNSCs. Genetic manipulation and screening were utilized to evaluate the significance of human haNSCs as genetic screening tools.

RESULTS

Human haESCs in extended pluripotent culture medium showed more compact and smaller colonies, a higher efficiency in neural differentiation, a higher cell survival ratio and higher stability in haploidy maintenance. These characteristics effectively facilitated the derivation of human haNSCs. These human haNSCs can

be generated by differentiation and maintain haploidy and multipotency to neurons and glia in the long term *in vitro*. After PiggyBac transfection, there were multiple insertion sites in the human haNSCs' genome, and the insertion sites were evenly spread across all chromosomes. In addition, after the cells were treated with manganese, we were able to generate a list of manganese-induced toxicity genes, demonstrating their utility as genetic screening tools.

CONCLUSION

This is the first report of a generated human haploid somatic cell line with a complete genome, proliferative ability and neural differentiation potential that provides cell resources for recessive inheritance and drug targeted screening.

Key Words: Haploid; Neural stem cells; Extended pluripotency; Genetic screening; Cell therapy

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Core Tip: Human embryonic stem cells are widely used in preclinical and genetic screening studies. Haploid cells are ideal tools to perform genetic screening. To date, no human haploid somatic cell lines have been successfully created. We converted human haploid embryonic stem cells to an extended pluripotency state by optimizing the culture medium. The derived haploid neural stem cells can proliferate as a haploid genome and maintain multipotency to generate functional neurons and glia. The haploid neural stem cells can also be easily used for gene editing to generate numerous homozygous mutations for lineage-specific screens.

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INTRODUCTION

Haploid cells have one set of chromosomes that can be genetically analyzed. Compared to diploid cells, haploid cells are more prone to homozygous mutation. This characteristic is important for gene screening[1-5], especially for recessive gene screening, and the study of cellular and chromosomal fusion[6-8]. Although haploid genetics has been mostly restricted to unicellular organisms, recent reports of haploid embryonic stem cells (ESCs) have extended the research into animal species, including mice[9-13], rats[14] and monkeys[15]. More excitingly, haploid ESCs (haESCs) have been successfully derived from human[16-18] blastocysts. The success in producing human haESCs raises a particularly challenging question of whether these cells can be further differentiated while maintaining haploidy. Medaka (fish) haESCs can maintain haploidy without the need for cell sorting[19], whereas mammalian haESCs tend to diploidize in cell culture, which limits their potential for use in genetic screening. Almost all differentiated cells derived from mammalian haESCs are diploid cells.

Neural stem cells (NSCs) are somatic stem cells that can differentiate into both neuronal and glial progeny. Although we can obtain haploid neural precursors and neurons by differentiating mouse and monkey haESCs[20,21], the pathogenesis of many diseases differs between humans and animals. For instance, the α7-nicotinic acetylcholine receptor is widely studied as a target for treating the cognitive symptoms of Alzheimer's disease, attention deficit–hyperactivity disorder and schizophrenia[22]. However, the distribution of this receptor differs across species[23]. In general, there are many differences in gene expression among species[24].

This issue raises the question of how long human haESCs can maintain pluripotency in the stable haploid state. It also remains undetermined whether neural differentiation can be stably induced in haploid human cells, which would be useful to help clarify the specific pathogenesis of human neurological diseases. Previous studies have shown that haploid cells have poor differentiation potency. Yang *et al*[25] and Liu *et al*[26] indicated that by introducing a variety of small molecule compounds into the stem cell culture medium, traditional stem cells can be transformed into extended pluripotent stem (EPS) cells with higher pluripotency. Since their establishment, EPS cells have been widely used in different fields of research[27-32]. Recently, Zhang *et al*[33] demonstrated that inhibition of apoptosis reduced the diploidization of mouse haESCs during differentiation.

In this study, we introduced a small-molecule inhibitor during haESC culture and differentiation to restrain diploidization and enhance differential multipotency. Protocols were established for the derivation of human haploid neural stem cells (haNSCs) from human haESCs. These cells displayed neural differentiation potential *in vitro*. In addition, haNSCs can be maintained as predominantly haploid cells for more than 90 d without fluorescence activated cell sorting (FACS) enrichment. More importantly, we provided evidence that human haNSCs can be easily manipulated. These haNSCs are functional haploid adult stem cells and can be used for high-throughput genetic screening, providing a valuable resource for neural recessive gene studies.

MATERIALS AND METHODS

Reagents and media

The reagents and media used in the experiments were purchased from Life Technologies (Carlsbad, CA, United States) or Stem Cell Technologies, Inc. (Vancouver, Canada), unless otherwise indicated. Mouse embryonic fibroblasts were purchased from the Institute of Biochemistry and Cell Biology Shanghai Institutes for Biological Sciences Chinese Academy of Sciences.

Cell culture

Human haESCs were a kind gift from Dr. Jinsong Li of the State Key Laboratory of Cell Biology, Shanghai Key Laboratory of Molecular Andrology, Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of Chinese Academy of Sciences. The haESCs were cultured as previously reported[15,25,34]. Mouse embryonic fibroblasts were treated with 10 μg/mL mitomycin C for 3 h to generate a feeder layer and cultured in 10% fetal bovine serum (FBS) (BI) plus 1% penicillin-streptomycin Dulbecco's modified Eagle medium (DMEM). The ESC medium before optimization contained DMEM/F12 + 20% KnockOut Serum Replacement (Gibco, Billings, MT, United States) + 0.1 mmol/L non-essential amino acids, 100 U/mL penicillin-0.1 mg/mL streptomycin, 0.1 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine and 10 ng/mL basic fibroblast growth factor (bFGF). Human EPS cells were cultured in N2B27-LCDM (consisting of hLIF, CHIR99021, (S)-(+)-dimethindene maleate, minocycline hydrochloride) medium under 20% O_2 and 5% CO_2 at 37 °C. Then, 50 mL of N2B27 medium was prepared and included the following: 24 mL of DMEM/F12 (Gibco); 24 mL of Neurobasal (Gibco); 0.25 mL of N2 supplement (Gibco); 0.50 mL of B27 supplement (Gibco); 1% GlutaMAX (Sigma-Aldrich, St Louis, MO, United States); 1% non-essential amino acids (Gibco); 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich), penicillin-streptomycin (Gibco); and 5% KnockOut Serum Replacement (Gibco). The optimized medium was named N2B27-LCDM. Y-27632 (2 mmol/L; Peprotech, Rocky Hill, NJ, United States) was included in some cases. ESCs were passaged routinely using 1 mg/mL type IV collagenase (Sigma-Aldrich) (traditional culture ESCs) and 0.05 trypsin (optimized culture ESCs) every 3-4 d. The haNSCs were passaged every 4 d with 0.05% trypsin. The haNSC medium contained D/F12 + 1% N2 supplement + 20 ng/mL bFGF (Peprotech) + 20 ng/mL epidermal growth factor (EGF) (Peprotech). All cells were cultured in a 5% CO_2 incubator at 37 °C.

Neural differentiation of EhPGES1

Well-conditioned EhPGES1 were selected for differentiation, as the state of haESCs plays a decisive role in neural differentiation. EhPGES1 were digested to single cells with 1 mg/mL 0.05% trypsin. Then, these single cells were placed into gelatin-coated dishes for 30 min to allow the feeder cells to attach to the bottom of the dish. Single cells were suspended in embryoid body (EB) medium in a suspension petri dish for 4 d to form EBs. During the 1st d of differentiation, we added 10 μM ROCKi (Peprotech) to the EB medium. On day 4, the EB medium was replaced by neural induction medium (NIM) for another 2 d. On day 6, we transferred the EB from the suspension petri dishes to laminin-coated dishes and incubated overnight. The next day, EB paste was added to the bottom of the dish. We changed the NIM every other day.

The growth of flat cells and an increasing number of slender small cells were observed in the center of the implanted EB. After approximately 10 d in NIM, the central, small, lanky cells generated rosettes. Clusters were picked and expanded as described by Zhang *et al*[37] with slight optimizations[35-37]. In short, rosette clusters were attached to laminin-coated petri dishes in N2 medium containing human bFGF and EGF. Then, haNSCs were derived from haploid rosette clusters with continuous culture on polyornithine and laminin-coated dishes in N2 medium with 20 ng/mL bFGF and 20 ng/mL EGF for 3 mo. The differentiation efficiency from haploid rosette clusters to haNSCs under this condition was nearly 100% according to cell morphology evaluation.

Neuronal differentiation of haNSCs

haNSCs were examined for their neural differentiation potency. For differentiation into neurons, D/F12 medium was added as basal medium and supplemented with N2, B27 (Gibco), BDNF (20 ng/mL), glial-derived neurotrophic factor (20 ng/mL), dibutyryl-cAMP (1 mmol/L) and ascorbic acid (200 nM). Then, the haNSCs were replated on laminin-coated petri dishes at a density of 1 × 10^4 cells/cm^2. The addition of 1% FBS (BI) to the primary medium was used to differentiate haNSCs into astrocytes and oligodendrocytes[36].

DNA content analysis of cells

Human ESCs and NSCs were dissociated into single cells by treatment with 0.05% trypsin/EDTA at 37 °C for approximately 3 min. Cells were fixed in 75% ethyl alcohol at 4 °C for at least 12 h and stained with propidium iodide plus RNase A. Flow cytometry data were recorded by a BD Aria II system (BD Biosciences, Franklin Lakes, NJ, United States).

FACS

For sorting of haploid cells, haESCs and haNSCs were disaggregated by 0.05% trypsin/EDTA, washed with Dulbecco's phosphate-buffered saline (PBS) and incubated with 4 μg/mL Hoechst 33342 in a 37 °C water bath for approximately 15 min. Subsequently, the haploid cells were collected using a BD FACSAria III (BD Biosciences) for further culture. ROCKi (10 μM Y27632) was added before and after sorting.

RNA isolation and real-time PCR

Total RNA from all cells was extracted with TRIzol reagent (Invitrogen, Waltham, MA, United States) and treated with DNase I (Promega, Madison, WI, United States). cDNA was obtained by first-strand synthesis using 2 μg of RNA and the ReverTra Ace system for reverse transcriptase-PCR (Toyobo, Osaka, Japan). Real-time PCR was performed as described in our previous report[21]. All reactions were repeated three times. Data are presented as the mean ± SD.

Immunocytochemistry

Immunostaining and alkaline phosphatase staining of ESCs and NSCs were performed as described previously[38]. In short, cells were fixed in formaldehyde, rinsed with PBS and permeabilized with Triton X-100 (Sigma-Aldrich). Then, the cells were incubated with blocking buffer, rinsed with PBS and incubated with primary and secondary antibodies (Abcam, Cambridge, and United Kingdom). After being rinsed with PBS, the cells were examined under a fluorescence microscope. The data represent three independent experiments.

Karyotype (G-banding) analysis

Cells were incubated with 0.2 μg/mL colchicine for 1 h (ESCs) or 2 h (NSCs). Then, the cells were disaggregated with 0.05% trypsin/EDTA and resuspended in 5 mL of culture medium. The cells were then harvested from the 5 mL suspension in an automatic cell harvesting apparatus (Chromprep II; Sinochrome/Cytogenetics, Shanghai, China) according to the set procedure. Briefly, 4.5 mL of preheated potassium chloride (0.075 mmol/L) was added at 37 °C for 30 min. Hypotonic solution-treated cells were fixed in methanol:acetic acid (3:1 volume:volume) for 20 min at 37 °C, and the fixation step was repeated three times. Then, they were dropped onto precleaned charged slides by an automatic production machine (Chromprep AS; Sinochrome/Cytogenetics). After air drying, the cells were stained with Metaphase Auto-Stainer (Chromprep G; Sinochrome/Cytogenetics). More than 20 metaphase spread G-bands were analyzed by Ikaros karyotyping software with the MetaClient dashboard (MetaSystems, Altlussheim, Germany).

Growth curve detection

The Cell Counting Kit (CCK)-8 assays (Beyotime, Jiangsu Province, China) were performed as previously reported[15]. In short, cells were plated onto 96-well plates at a density of 1000 cells per well. Ten microliters of CCK-8 medium were added to each well on days 1, 2, 3, 4, and 5. The cells were incubated in a 37 °C 5% CO_2 incubator for 1-4 h. Optical density values of each well at 450 nm wavelength were detected by an enzyme labeling instrument.

Global gene expression analysis

The RNA-seq data were sequenced by Novogene (Beijing, China). Total RNA was extracted using TRIzol Reagent (Invitrogen). Following the manufacturer's instructions, raw data were processed with the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html) to remove noise and adaptors. The R heatmap function was used to perform hierarchical clustering.

PiggyBac transfection and splinkerette PCR

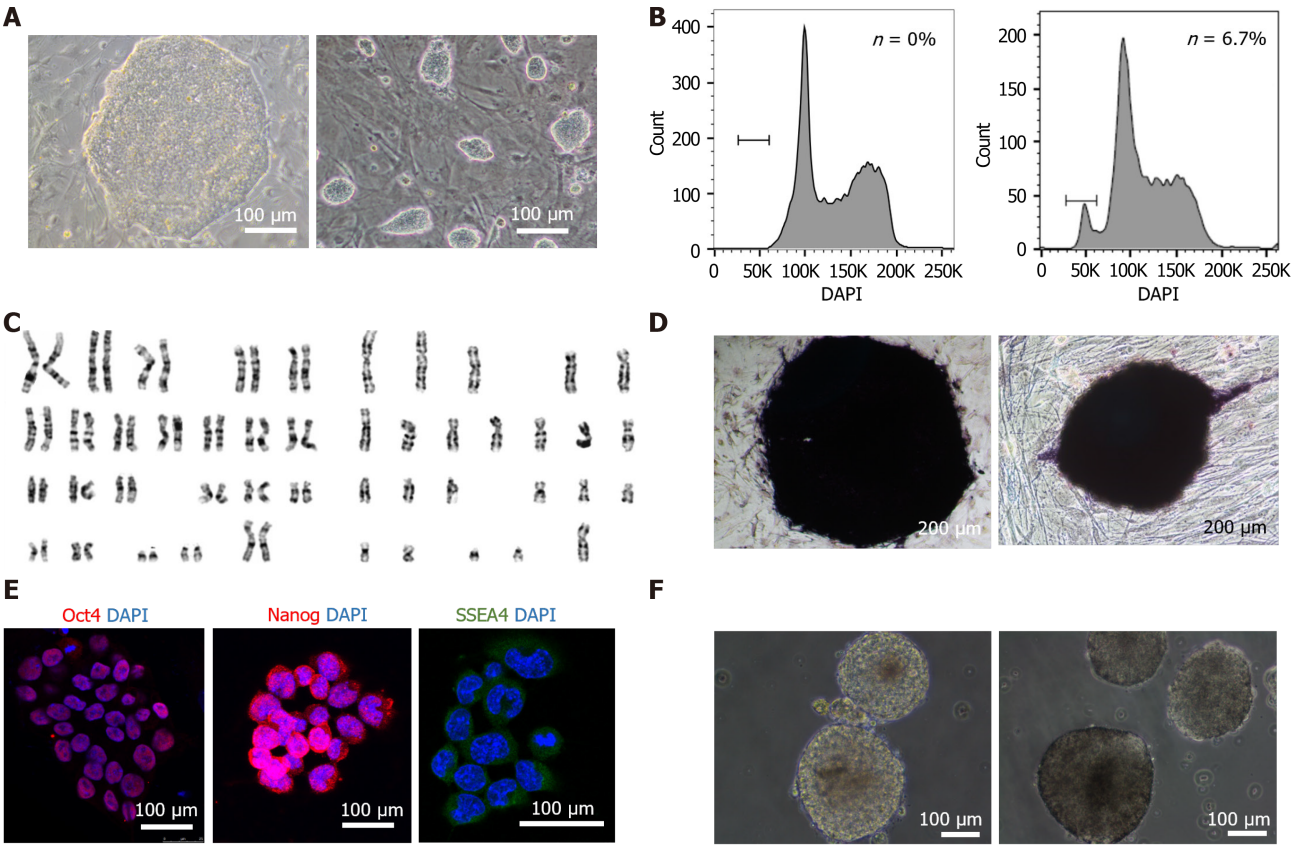
PiggyBac [PB Dual promoter PB513B-1; Applied Biosystems Inc (ABI), Waltham, MA, United States] and PBase (Super PiggyBac Transposase PB200PA-1; ABI) were cotransfected into cells with Lipofectamine 3000 (Invitrogen). Puromycin (250 μg/mL) was used to culture and screen the transfected cells. To characterize the sites of the PB element insertion, genomic DNA was digested with *BstYI* (Thermo Fisher Scientific, Waltham, MA, United States) at 37 °C for 16 h, and the products were self-ligated. Then, with the linked DNA as a template, PCR was performed with the primers PB-RF and PB-RR to detect the previously reported insertion sites. PCR was performed as previously reported[39]. The first round was performed as follows: 98 °C for 75 s; 2 cycles of 98 °C for 20 s and 64 °C for 20 s; 30 cycles of 98 °C for 20 s, 58 °C for 15 s and 72 °C for 2 min; 72 °C for 7 min; and a final hold at 4 °C. The second round was conducted as follows: 98 °C for 75 s; 30 cycles of 98 °C for 20 s, 58 °C for 15 s and 72 °C for 90 s; 72 °C for 7 min; and a final hold at 4 °C. Splinkerette PCR and deep sequencing were also performed to identify the integration sites. All primers used are listed in Supplementary Table 1.

High-throughput sequencing analysis

After sequencing of the PB mutation library, adapters and PB tags were removed from the read data before mapping to the genome using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html). HaSAPPy (https://github.com/gdiminin/HaSAPPy) was utilized to align the trimmed reads to the genome assembly from UCSC (mm10) (http://genome.ucsc.edu/cgi-bin/hgGateway? db = mm10)[40]. We analyzed the insertions for each gene with GENCODE[41] using the default parameters.

Statistical analysis

Student's *t*-test was used to evaluate the differences in gene expression levels among groups. All statistical analyses were performed using SPSS software 13.0 (SPSS Inc., Chicago, IL, United States) and Image-Pro Plus (Media Cybernetics, Rockville, MD, United States).



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Figure 1 Characterization of the pluripotency and haploidy of extended pluripotent human haploid embryonic stem cells. A: The human diploid embryonic stem cell (ESC) line H9 (left) was used as a control for extended human haploid ESCs (haESC) (right). Scale bar, 100 μm; B: Fluorescence activated cell sorting analysis of the DNA content of the human haESC line hPGES1 after approximately 6 wk of culture, without sorting. Left, diploid ESC line as a control; Right, haploid cell line EhPGES1. The "n" peak represents haploid cells; C: G-band analysis of the EhPGES1 cell line with a haploid set of 23 chromosomes (right) and diploid human ESCs as a control (46 chromosomes, left); D: Alkaline phosphatase staining of the human haESC line EhPGES1 (right) and diploid human ESCs as a control (left). Scale bar, 200 μm; E: Immunofluorescence analysis of the primate ESC markers Oct4 (red), Nanog (green) and SSEA4 (green) in the human haESC line EhPGES1. DAPI (blue) was used to stain the nuclei. Scale bar, 100 μm; F: Morphology of embryoid bodies from haploid-enriched cells (right) and diploid human ESCs as a control (left). Scale bar, 100 μm.

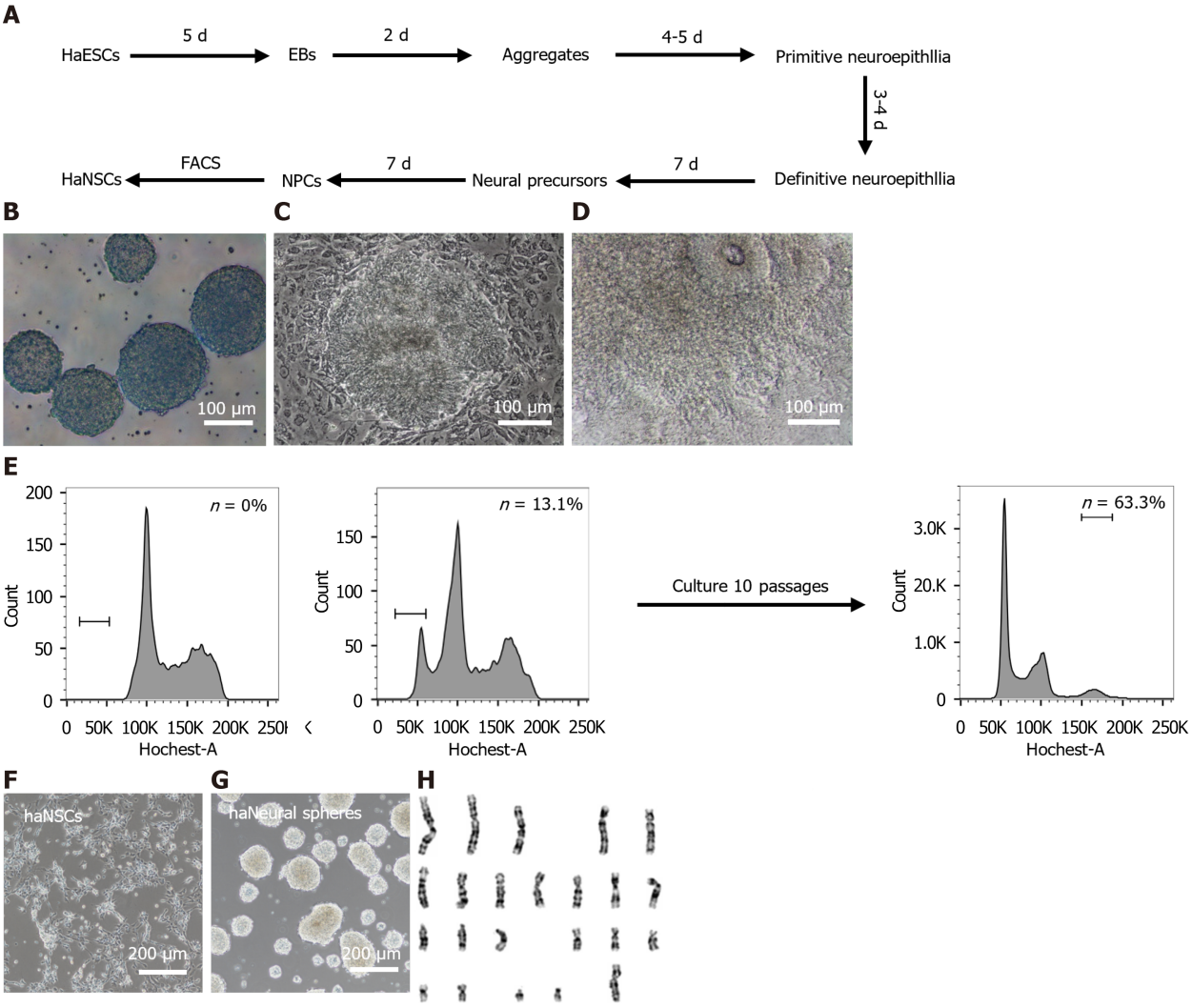
RESULTS

Characterization of the pluripotency and haploidy of extended pluripotent human haESCs

To determine whether traditional and extended pluripotent human haESCs exhibited pluripotency and intact haploid genomes, we compared them (cell line: hPGES1) with diploid human ESCs from zygotes (H9) in different respects. Traditional hPGES1 cells appeared as flat, compact colonies, a standard morphology for primed pluripotent cells (Supplementary Figure 1A) resembling that of diploid ESC H9 (Figure 1A), whereas extended pluripotent hPGES1 cells appeared as domed compact and small colonies, a standard morphology for naïve pluripotent cells resembling that of mouse ESCs (Figure 1A). After FACS of haploid cells, traditional and extended pluripotent hPGES1 cells maintained an acceptable percentage of haploidy for more than 6 wk without further sorting according to DNA content analysis (Figure 1B, Supplementary Figure 1B). We further assessed the chromosome numbers and morphology in traditional and extended pluripotent hPGES1 cells by G-band analysis and found that most cells were haploid (23 chromosomes) cells. A few cells were found to be haploid and aneuploid (Figure 1C, Supplementary Figure 1C and D). Next, we assessed the pluripotency of traditional and extended pluripotent hPGES1 cells from different aspects *in vitro*. We observed (by immunostaining) strong alkaline phosphatase activity (Figure 1D, Supplementary Figure 1D) and strong expression of pluripotent markers, including Oct4, Nanog, Sox2 and SSEA-4 (Figure 1E, Supplementary Figure 1E). After withdrawal of bFGF and feeder cells, traditional and extended pluripotent hPGES1 cells formed typical EBs, guaranteeing their utility for subsequent differentiation experiments (Figure 1F, Supplementary Figure 1F).

Human haNSCs were derived from extended pluripotent haESCs

Suspension aggregation and posterior adherent culture are the most common methods for neural differentiation of human ESCs[37,42]. We thus developed a scheme to derive haNSCs based on a previously reported protocol with slight modifications (Figure 2A). During the first 5 d, well-formed human haESCs (Supplementary Figure 2A) were suspended and aggregated in EBs. EBs with better morphology (Supplementary Figure 2B) were cultured in suspension in NIM for 2



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Figure 2 Derivation of human haploid neural stem cells *via* differentiation *in vitro*. A: Schematic overview of the strategy to efficiently derive haploid neural stem cell (haNSC) lines through embryoid body-mediated differentiation and fluorescence activated cell sorting; B: Morphology of 7-d aggregates. Scale bar, 100 μm; C: Morphology of primitive neuroepithelia. Dashed lines indicate the morphology of primitive neuroepithelia. Scale bar, 100 μm; D: Morphology of definitive neuroepithelia. Dashed lines indicate the morphology of definitive neuroepithelia. Scale bar, 100 μm; E: Fluorescence activated cell sorting of passage 4 human haNSCs and derived haNSCs maintained a haploid DNA content over 10 passages. Left, a diploid cell line as a control; middle, passage 4 haNSCs; right, derived haNSCs after 10; F: Morphology of sorted haNSCs. Scale bar, 200 μm; G: Morphology of haploid neural spheres from sorted haNSCs. Scale bar, 200 μm; H: G-band analysis of haNSCs with a haploid set of 23 chromosomes. haNSCs: Haploid neural stem cell; haESCs: Haploid embryonic stem cells; FACS: Fluorescence activated cell sorting; EB: Embryoid body; NPC: Neural progenitor cells.

d. Neuroectodermal structures were observed in the middle part of the EB on day 7 (Figure 2B). All aggregates were seeded in laminin-coated dishes for adherence-induced culture. Aggregates adhering to laminin began to form primitive neuroepithelium after 4-5 d (Figure 2C). After approximately 15 d of differentiation, a large number of neuroepithelial cells had accumulated, and rosette structures were formed in the centers of the adherent cells (Figure 2D). The rosettes were manually selected and expanded in NSC medium for 14 d (Supplementary Figure 2C) before FACS. Haploid haNSCs in G0/G1 phase were predominant at the first round of sorting (Figure 2E). Interestingly, purified haNSCs could be amplified in NSC medium for more than 10 generations without sorting, and the proportion of haploid cells was still very high. haNSCs retained the standard NSC morphology (microscopic and bipolar) before and after sorting (Supplementary Figure 2D, Figure 2F) and were able to aggregate into typical neurosphere structures in suspension in petri dishes (Figure 2G). We also examined the chromosomes of haNSCs by G-banding analysis and found that haNSCs maintained intact haploid genomes without any aneuploid mutations (Figure 2H, Supplementary Figure 2E). We compared the cell growth rates of haNSCs and diploid NSCs using the CCK-8 method. The results showed no significant difference between the two cell types (Supplementary Figure 2F).

Derived haNSCs showed multipotency for neural subtypes in vitro

NSCs are proliferative adult stem cells that have the potential to differentiate into functional neurons or neuroglial cells, making them useful resources for cell replacement therapy and screening research[43]. To determine whether the haNSCs that we had generated possessed neural lineage properties, we assessed NSC-specific markers by immunostaining. The

results showed that haNSCs expressed NSC markers (including Nestin, Sox1, and Pax6) in both monolayer cells (Figure 3A) and neural spheres (Figure 3B), similar to that of the diploid NSCs used as a control (Supplementary Figure 3A). Additionally, the gene expression level of the haNSCs was very similar to that of the diploid NSCs (Supplementary Figure 3B). Furthermore, there was a significant difference in gene expression between haESCs and human fibroblasts (Figure 3C). According to real-time PCR analysis, haNSCs expressed only NSC marker genes (Nestin, *PAX6* and *SOX1*) and resembled diploid NSCs (Figure 3D).

Next, to evaluate the differentiation potential of haNSCs, we induced them to differentiate into astrocytes, oligodendrocytes and neurons by applying specific growth factors for approximately 3 wk and then sorted out the haploid cells by FACS (Figure 3E). Immunostaining results demonstrated that astrocytes (GFAP) and oligodendrocytes (O4) could be obtained from haNSCs and maintained as haploid cells (Figure 3F). In a parallel experiment, diverse neurons (Tuj1- and Map2-positive) were also generated from haNSCs (Figure 3G). To evaluate the purity of the haNSCs, we randomly selected haNSC spheres for differentiation induction (Supplementary Table 2). The results showed that each haNSC sphere could differentiate into neurons, astrocytes and oligodendrocytes. Therefore, the haNSCs were deemed to have good homogeneity.

Better pluripotency yielded a higher efficiency of neural differentiation in human haESCs

Recently, primate ESCs, including human ESCs, were converted to a better state by introducing small molecule compounds[25,44] or regulating transcription[45], with morphology and molecular levels similar to those of mouse ESCs. Whether extended haESCs in primate species show better pluripotency than traditional primed state ESCs has not been clarified. Herein, we optimized the culture system of human haESCs on the basis of previous research[25] with slight improvements (Supplementary Figure 4A). After culture in the optimized extended medium for approximately three passages, dome-shaped human pluripotent stem cell colonies formed, which is a morphological feature characteristic of mouse ESCs (Figure 4A). Optimized hPGES1 could be digested into single cells by 0.05% trypsin-EDTA in the absence of Y27632 (Figure 4B). The purified haESCs were divided into two populations and cultured in traditional and optimized media. FACS analysis showed that the proportion of haploid cells was higher in the optimized medium, indicating that the optimized medium could inhibit diploidy to some extent (Figure 4C, Supplementary Table 3).

To compare the EB formation ability between the optimized extended haESCs and traditional primed haESCs, we suspended approximately 10^6 cells from each group and allowed them to aggregate in untreated dishes. The number of EBs generated from optimized haESCs was more than two-fold that generated from the traditional group (Figure 4D). Moreover, the aggregate-generating neural rosettes from the optimized group were larger and more abundant than those from the traditional group (Figure 4E). When first sorting the haNSCs from optimized and traditional haESCs separately, the number of surviving cells from the optimized group was greater than that from the traditional group, according to the analysis of the side scatter and forward scatter plots (Figure 4F), indicating that haESCs in optimized medium could produce more NSCs with higher survival rates. A similar trend was found in harvested cells; we found that sorted cells from the traditional group had lower survival rates (Supplementary Figure 4B). Therefore, sorted haNSCs from the traditional group were limited in number and survival rate and readily became diploid NSCs. Overall, we generated two haNSC lines from four parallel differentiation lines established from the optimized groups. No haNSC lines were obtained from six parallel differentiation lines established from the traditional groups (Supplementary Figure 4C).

Genetic manipulation and screening in human haNSCs

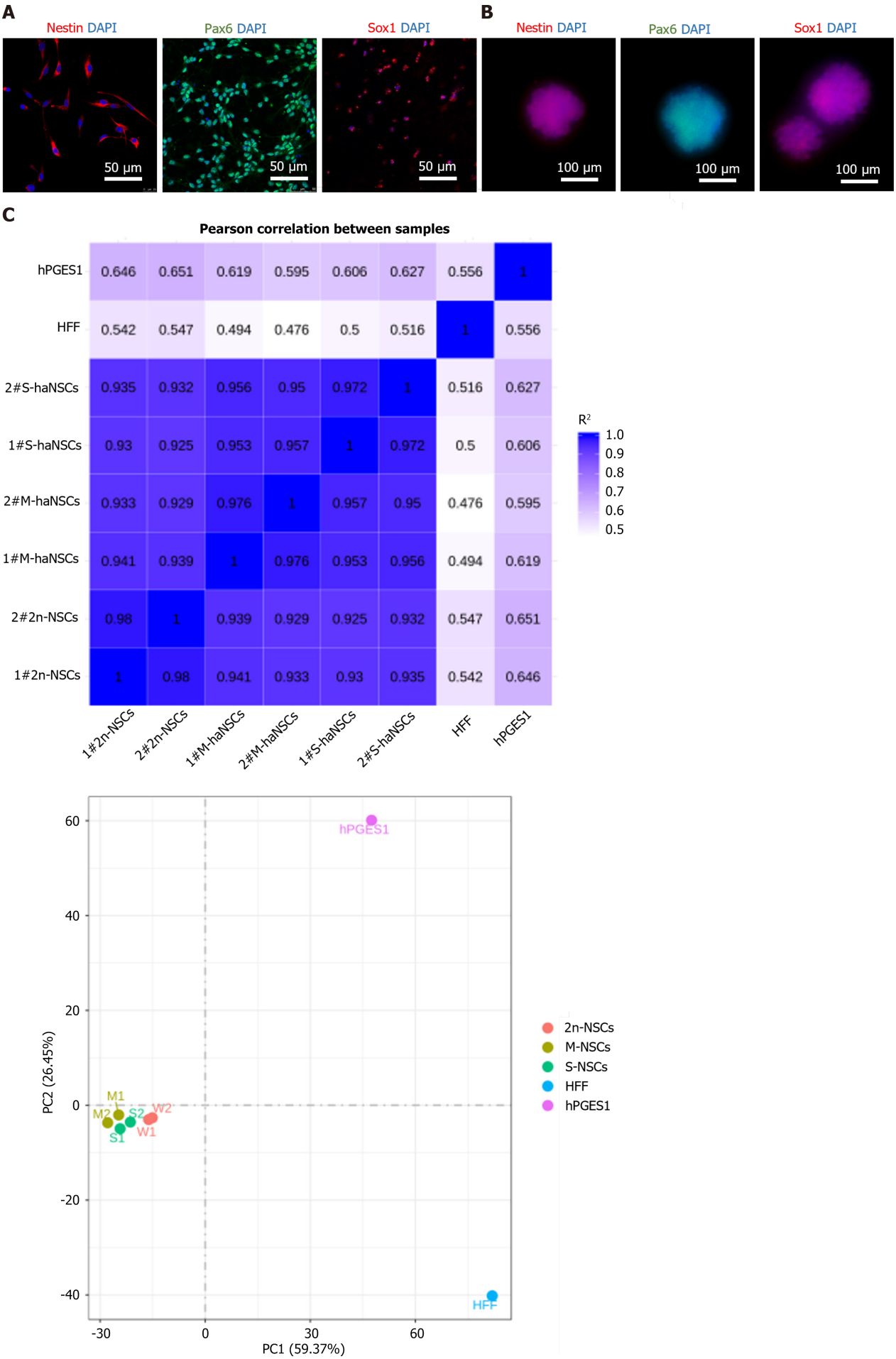
Next, we studied the feasibility of gene manipulation in human haNSCs. We initially applied transposable element-based strategies to establish transgenic haNSCs (Figure 5A). First, we employed the transposon and splinkerette PCR system, which has been applied for a variety of genetic manipulations in cells from mice[46,47], nonhuman primates[21] and humans[48]. We performed gene trapping in haNSCs to generate mutation libraries, which might be useful for neural lineage genetic screening.

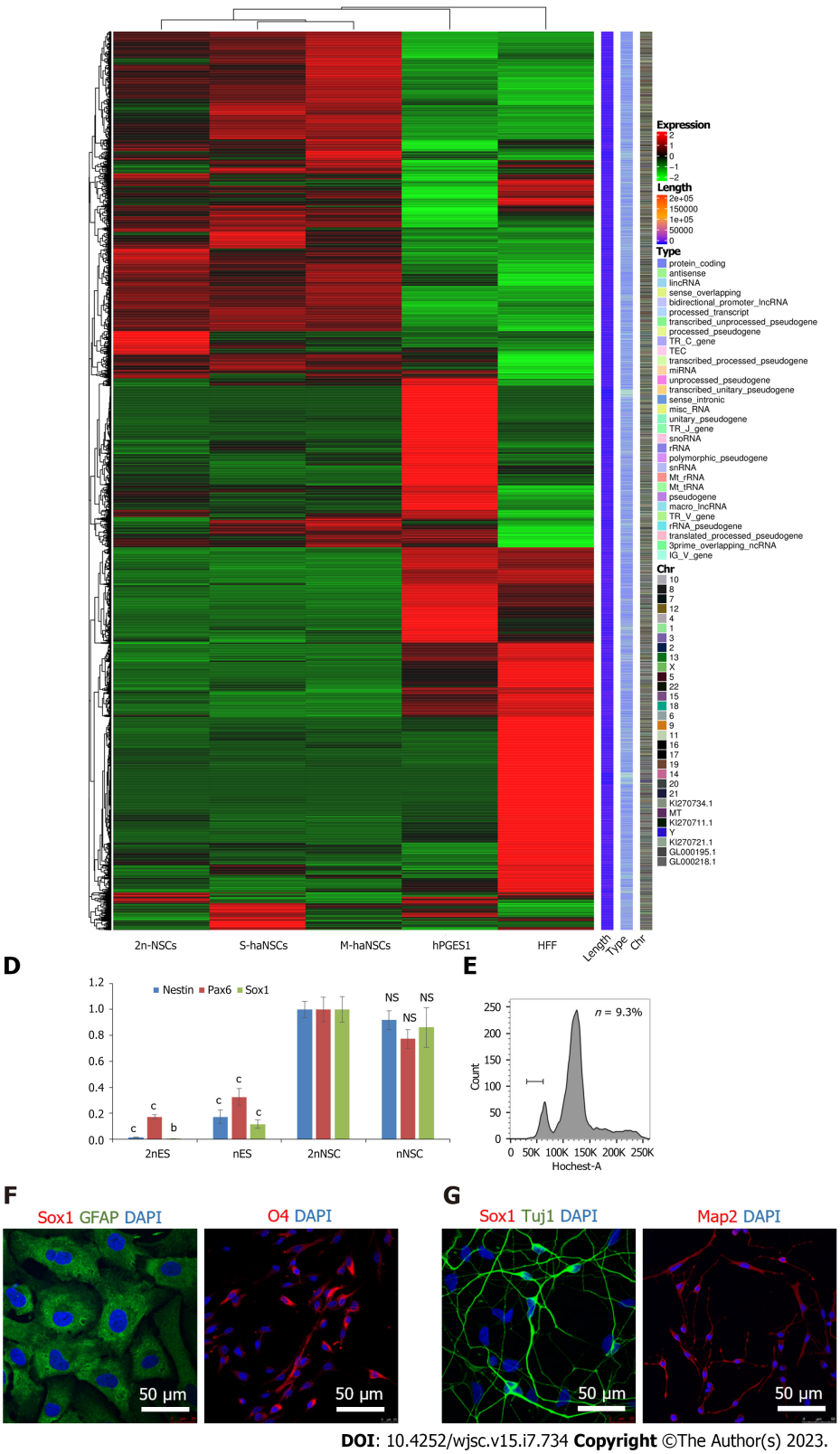
Specifically, haNSCs were transfected with PB transposon-based gene-trap vectors[46] carrying the enhanced green fluorescent protein (eGFP) gene. After transfection, haNSCs exhibited a PB insertion and expressed eGFP (Figure 5B). We then added the nerve agent manganese chloride to haNSCs carrying the mutant library. Five days later, all cells in the control group had died. We harvested the surviving haNSCs and amplified the integration sites by performing splinkerette PCR (Figure 5C). The PCR products were analyzed by agarose gel electrophoresis, and the 5′ inverted terminal repeat PCR results showed that the bands were relatively diffuse, indicating that there were multiple insertion sites in the haNSC genome (Figure 5D).

Deep sequencing analysis identified approximately 4 million independent insertions across more than 20000 genes, of which 51.8% were in the sense orientation (Figure 5E). We analyzed the hits across the whole genome, and the results showed that the insertion sites were evenly spread across all chromosomes, indicating genome-wide coverage of mutations (Supplementary Figure 5A). In addition, 93.00% of the insertions were located in intragenic regions (exon + intron + promoter + 5′/3′ untranslated region), whereas 6.24% of insertions occurred in intergenic regions (Figure 5F).

Gene Ontology enrichment analysis showed that insertions occurred preferentially in genes with specific functions in neural activity-related pathways, such as neuron projection guidance, neuron projection guidance and regulation of transmembrane ion transporters (Figure 5G). Ten genes with especially frequent insertions were identified by PB screening (Figure 5H). To comprehensively assess the inserted genes, we analyzed them for roles in previously reported neurotoxicity-related pathways (MAPK, Notch, Wnt and other pathways) (Supplementary Figure 5B). In summary, the derived haNSCs were found to be beneficial for genome-wide genetic screening.

Collectively, these results indicated that the optimized conditions supported the generation of human haNSCs, which can act as an ideal tool for screening key genes during neurogenesis.


A
Nestin DAPI
Pax6 DAPI
Sox1 DAPI
50 μm
B
Nestin DAPI
Pax6 DAPI
Sox1 DAPI
100 μm
C
Pearson correlation between samples
hPGES1 0.646 0.651 0.619 0.595 0.606 0.627 0.556 1
HFF 0.542 0.547 0.494 0.476 0.5 0.516 1 0.556
2#S-haNSCs 0.935 0.932 0.956 0.95 0.972 1 0.516 0.627
1#S-haNSCs 0.93 0.925 0.953 0.957 1 0.972 0.5 0.606
2#M-haNSCs 0.933 0.929 0.976 1 0.957 0.95 0.476 0.595
1#M-haNSCs 0.941 0.939 1 0.976 0.953 0.956 0.494 0.619
2#2n-NSCs 0.98 1 0.939 0.929 0.925 0.932 0.547 0.651
1#2n-NSCs 1 0.98 0.941 0.933 0.93 0.935 0.542 0.646
1#2n-NSCs 2#2n-NSCs 1#M-haNSCs 2#M-haNSCs 1#S-haNSCs 2#S-haNSCs HFF hPGES1
R²
1.0
0.9
0.8
0.7
0.6
0.5
60
40
20
0
-20
-40
PC2 (26.45%)
-30
0
30
60
PC1 (59.37%)
hPGES1
HFF
M1
M2
S2
S1
W2
W1
2n-NSCs
M-NSCs
S-NSCs
HFF
hPGES1



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Figure 3 Identification and differentiation of haploid neural stem cells. A: Immunofluorescence analysis of the neural stem cell (NSC)-specific markers Nestin (red), PAX6 (green), and SOX1 (red) in derived haploid NSCs (haNSCs). DAPI (blue) was used to stain the nuclei. Scale bar, 50 μm; B: Immunofluorescence analysis showed that haploid neural spheres expressed NSC-specific proteins Nestin (red), PAX6 (green) and SOX1 (red). Scale bar, 100 μm; C: Transcript levels of diploid NSCs, monolayer cultured haNSCs, haploid neural spheres, human haploid embryonic stem cells (hPGES1) and human fibroblasts; D: Real time PCR analysis of NSC marker genes (*SOX1*, *PAX6*, and Nestin); E: Fluorescence activated cell sorting of derived astrocytes, oligodendrocytes, and neurons by DNA content; F: Immunofluorescence analysis of astrocytes (GFAP, green) and oligodendroglia (O4, red). DAPI (blue) was used to stain the nuclei. Scale bar, 50 μm; G: Immunofluorescence analysis of Tuj1 (green) and Map2 (red) in neurons derived from haNSCs. DAPI (blue) was used to stain the nuclei. Scale bar, 50 μm. $^{b}P < 0.01$; $^{c}P < 0.001$.

DISCUSSION

Researchers have obtained haESCs from a variety of species, including mammals[9-13], rhesus monkeys[15] and even humans[21]. Because there are obvious differences in the pathogenesis of many diseases between humans and other species[23,24], we urgently need to increase the number of available human cell models to more directly and efficiently explain the pathogenesis of human diseases. In this study, we demonstrated that tissue-specific adult cells (haNSCs) can be generated *in vitro* by the conversion of haESCs without chromosome doubling. Although severe diploidization was observed during ESC-NSC conversion, it was possible to optimize the differentiation system without gene modification through the addition of a number of small molecule inhibitors to the culture medium. With these modifications, we were able to establish haNSC lines relatively easily.

As the derived haNSCs were generated from haESCs *in vitro*, their purity and NSC properties needed to be confirmed. Nestin, SOX1 and PAX6 are NSC-specific markers that have been widely used in research related to neural cell fate[37]. Our results showed that these haNSCs express NSC markers and are similar to brain-derived NSCs in morphology. After functional verification, we found that these cells can differentiate into neurons and glial cells *in vitro*. In this regard, haNSCs have differentiation potential resembling that of brain-derived NSCs.

HaESCs are an important tool for mammalian genetic research. Human haESCs can differentiate into haploid somatic cells of the ectoderm, mesoderm and endoderm[16]. This result suggests that primate species may have a potential superiority for a haploidy maintenance mechanism. Our experiments with human haNSCs confirmed this finding, as this cell group maintained a stable haploid state during the culture process. These advantages guaranteed their utility in a subsequent gene trapping procedure.

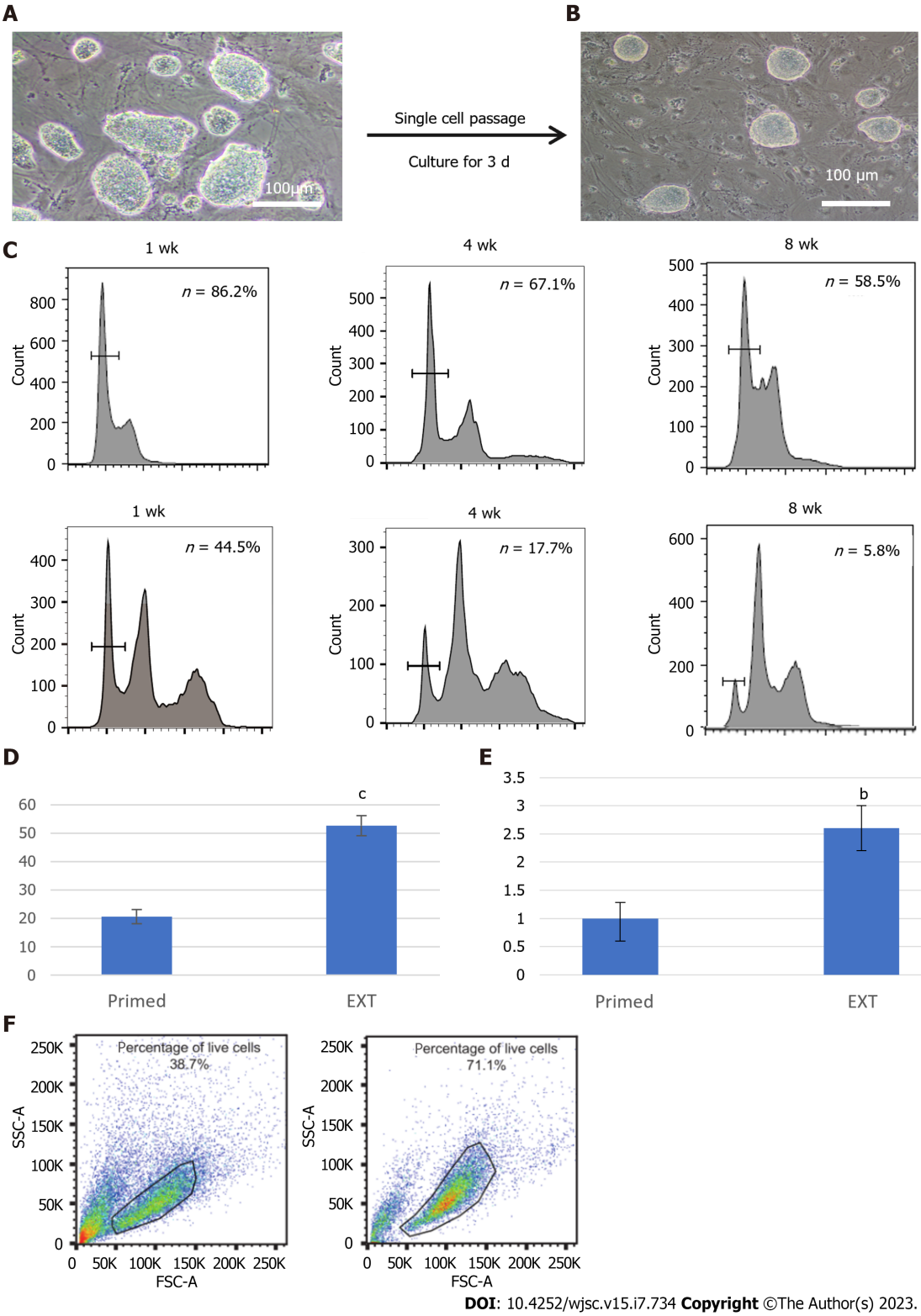
The PB transposon can insert a large number of random mutations in the genome[21,48]. Therefore, we combined the PB transposon and haNSCs to conduct genetic screening. Previous work demonstrated that neural cells are more susceptible to manganese-induced toxicity[49]. We performed genetic screening to uncover the target genes of manganese in haNSCs. The results of the gene trapping using the PB transposon system showed that random insertions could be generated efficiently in these haploid somatic cells. This method could be used in combination with high-throughput sequencing analysis to easily identify the gene insertion sites and insertion frequencies in all of the 23 chromosomes.

As described previously, human haESCs could differentiate into Tuj1-positive cells with a haploid genome. However, the cells lacked stability and proliferation capacity to act as an ideal tool for screening key genes.

There were limitations of this study. The diploidization of the haploid cells was not completely terminated; it was only slowed down. The underlying mechanism remains to be further studied and explored.

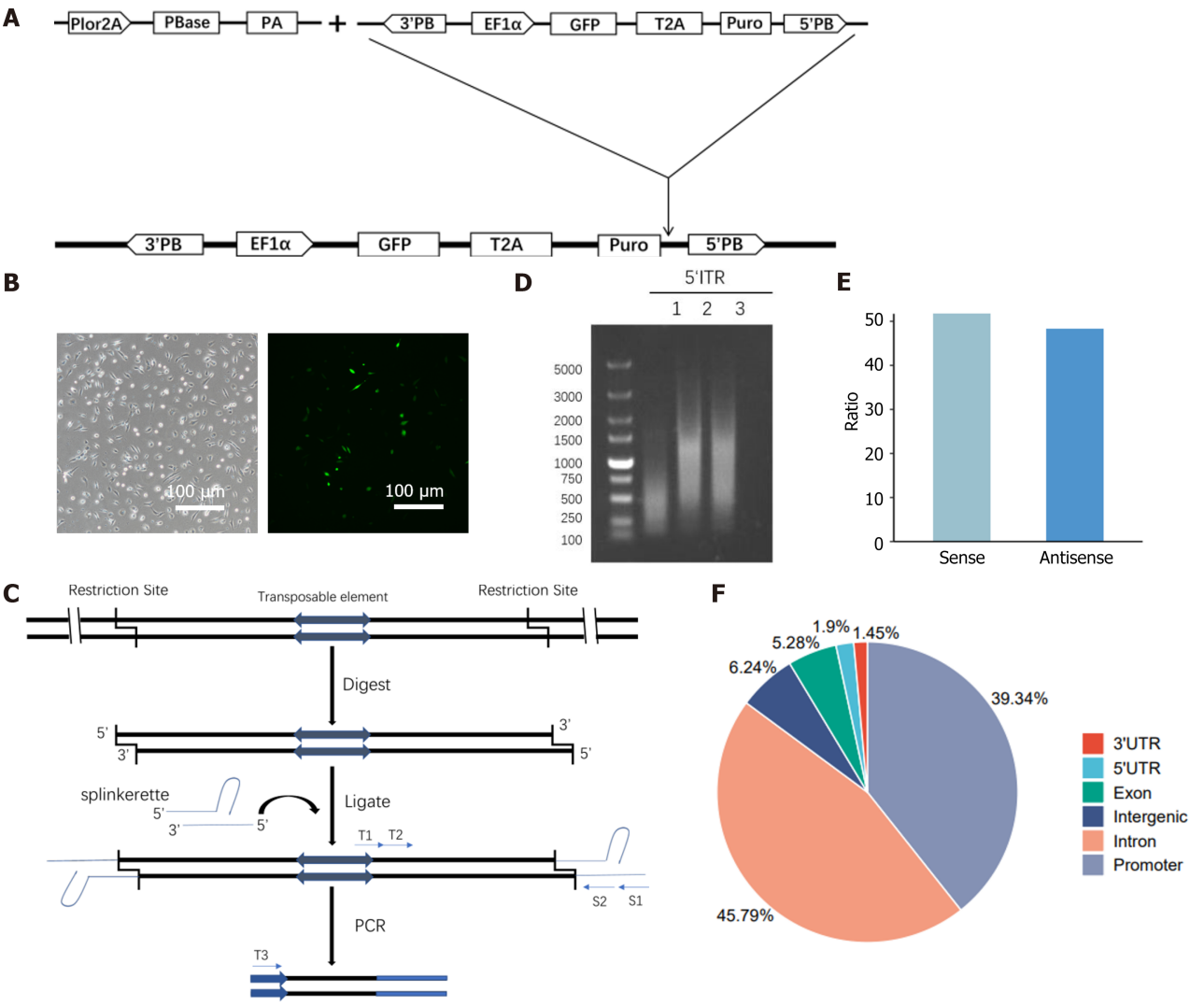
CONCLUSION

In conclusion, we optimized a culture system for human haESCs and achieved the derivation of human haNSCs (Figure 6). This is the first human haploid somatic stem cell line reported with good proliferative ability and pluripotency toward neural subtypes. Moreover, through chromosome G-band karyotype analysis, we confirmed for the first time that the differentiated haNSCs maintained 23 chromosomes while not exhibiting fragmentation, deletion, duplication, translocation, inversion or other balanced variations. Meanwhile, we identified manganese-induced toxicity genes with the help of this haploid cell line. This cell line will be valuable for genetic screening relevant to human nervous system function and drug targeting research.



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Figure 4 Comparison of pluripotency and survival of haploid embryonic stem cells in different culture media. A: EhPGES1 formed dome-shaped colonies after culture in optimized extended medium for approximately three passages. Scale bar, 100 μm; B: Morphology of day 3 optimized hPGES1 after single cells were passaged by 0.05% trypsin-EDTA in the absence of Y27632. Scale bar, 100 μm; C: Fluorescence activated cell sorting analysis of haploid embryonic stem cells after culture for 1 wk, 4 wk, and 8 wk in different media. Haploidy maintenance capacity between the traditional (bottom) and optimized (top) culture media; D: Statistical analysis of embryoid bodies (differentiation in 5 d) under traditional culture conditions and optimized culture conditions. *t*-test, $^{c}P < 0.001$; E: Statistical analysis of rosette areas in traditional culture conditions and optimized culture conditions. *t*-test, $^{b}P < 0.01$; F: Fluorescence activated cell sorting analysis of the proportion of live cells among haploid neural stem cells in different culture media as evaluated by forward scatter plot and side scatter plot. Traditional culture system (left) and optimized culture system (right).

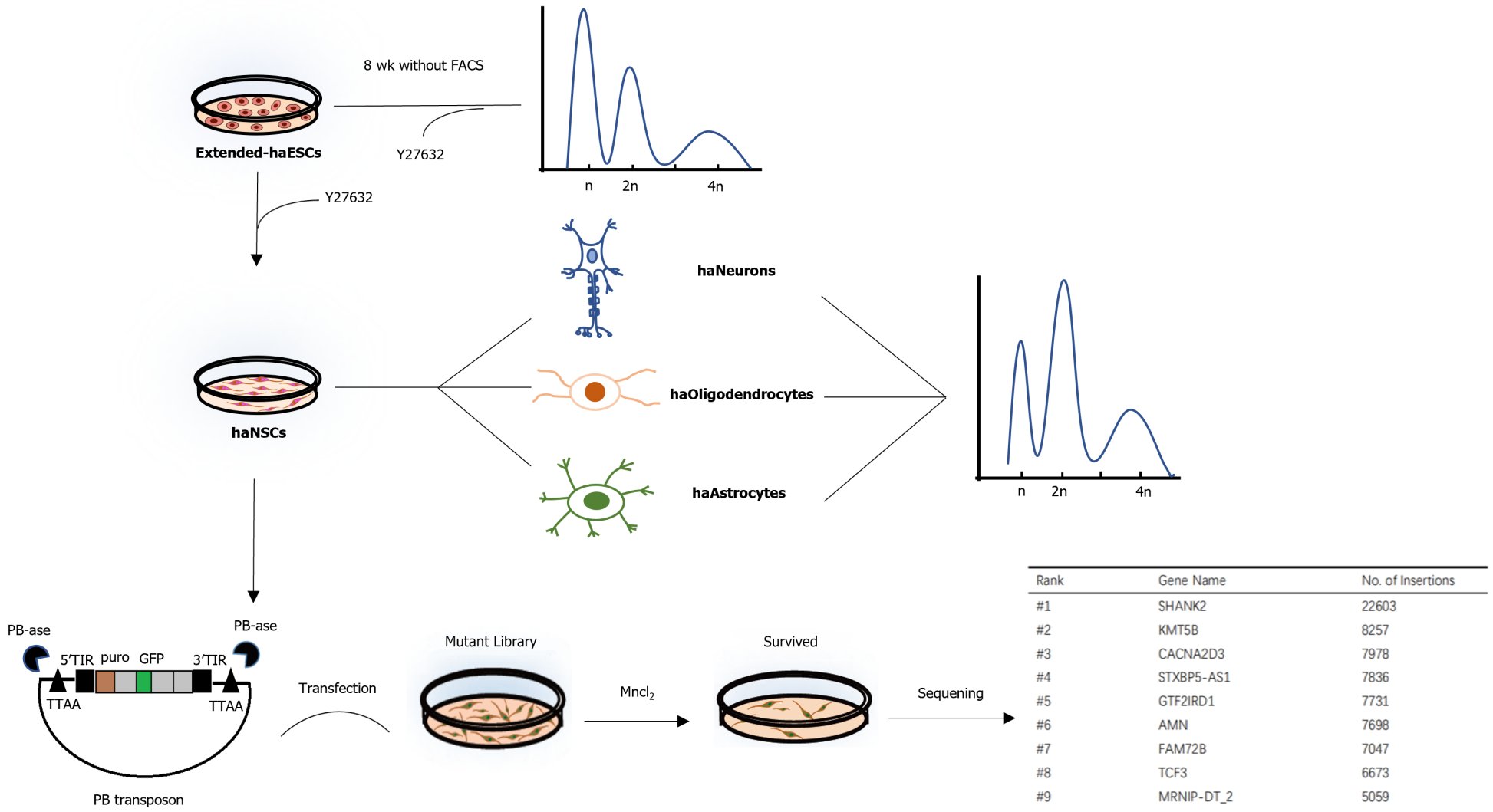


H

Rank	Gene Name	No. of Insertions
#1	SHANK2	22603
#2	KMT5B	8257
#3	CACNA2D3	7978
#4	STXBP5-AS1	7836
#5	GTF2IRD1	7731
#6	AMN	7698
#7	FAM72B	7047
#8	TCF3	6673
#9	MRNIP-DT_2	5059
#10	HRH1	4975

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Figure 5 PiggyBac transgenesis in the derived haploid neural stem cells. A: Schematic overview of PiggyBac (PB) insertion. PB transposons were inserted into the genomes of derived haploid neural stem cells (haNSCs) with the help of PBase; B: Bright field (left) and FITC (right) images of haNSCs after transfection. Scale bar, 100 μm; C: Schematic of the splinkerette PCR. 5' and 3' PB-void *BstYI* was used to digest genomic DNA from derived haNSCs, and T4 DNA ligase was used to form the LEFT and RIGHT cycle. After ligation, the insertion sites were verified by two rounds of nested PCR. The primers are shown as black arrows and listed in Supplementary Table 1; D: Agarose gel electrophoresis was used to analyze the 5' inverted terminal repeat PCR products harvested from transgenic haNSCs; E: Illustration of the sense and antisense; F: Distribution of insertion sites in the genome; G: Gene Ontology enrichment analysis; H: Top 10 most frequently inserted genes in the PB transposon-based mutagenesis.



Rank	Gene Name	No. of Insertions
#1	SHANK2	22603
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#9	MRNIP-DT_2	5059
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Figure 6 Summary graph for this research. Results show that human haploid embryonic stem cells cultured in optimized medium can stably maintain haploidy state and easily generate functional haploid neural stem cells with the help of Y27632 (ROCKi), thus contributing to human lineage-specific genetic screening. At last, we get a gene list relate to $Mn2^{+}$-induced toxicity with the help of this haploid cell line. FACS: Fluorescence activated cell sorting.

ARTICLE HIGHLIGHTS

Research background

Recently, haploid embryonic stem cells (haESCs) have been established in many species and are widely used in forward and reverse genetic screening. Differentiated haploid cell line types in mammals are lacking due to spontaneous diploidization during differentiation that compromises lineage-specific screens.

Research motivation

No human haploid somatic cell line has been generated, although there is an urgent need for lineage-specific screens.

Research objectives

This study aimed to derive haploid neural stem cells (haNSCs) from human extended haESCs by optimizing differentiation methods and verifying human haNSCs to easily carry out lineage-specific screens.

Research methods

Human haploid NSCs were differentiated from human extended haESCs with the help of Y27632 to reduce the diploidization. Neuronal differentiation of haNSCs was performed to examine their neural differentiation potential. Global gene expression analysis was performed to compare haNSCs with diploid NSCs and haESCs. Fluorescence activated cell sorting was conducted to assess the diploidization rate of extended haESCs and haNSCs. Genetic manipulation and screening were utilized to evaluate the significance of human haNSCs as genetic screening tools.

Research results

haNSCs can be generated by differentiation *in vitro*, and they maintain long-term haploidy with a complete genome and multipotency towards neurons and glia. Human haESCs cultured in extended pluripotency medium formed more compact colonies and exhibited more efficient neural differentiation and more stable maintenance of haploidy, which effectively facilitated the derivation of haNSCs. After PiggyBac transfection, there were multiple insertion sites in the haNSC genome and the insertion sites were evenly spread across all chromosomes.

Research conclusions

In this work, we demonstrated that human haNSCs can be generated by differentiation from extended haESCs. This is the first human haploid somatic cell line with a complete genome, acceptable proliferative ability and neural differentiation potential, which provides a cell resource for studies of recessive inheritance and drug targeted screening.

Research perspectives

This cell line will be valuable for genetic screening relevant to the function of the human nervous system and drug targeting research. Future research will be related to identifying key neurogenesis genes with the help of this cell line. Another research focus is to identify the underlying mechanisms of diploidization.

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FOOTNOTES

Author contributions: Sun YP and Wang HS conceived, designed, and supervised the study; Wang HS and Ma XR performed the experiments; Shi H, Zhang N, and Ma NZ analyzed the data; Wang HS, Ma XR, and Jiang ZW wrote the manuscript; Sun YP, Niu WB, and Liu YD revised the manuscript; all authors have read and approve the final manuscript.

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Data sharing statement: The data supporting the results of this research are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

Basic Study

Zinc enhances the cell adhesion, migration, and self-renewal potential of human umbilical cord derived mesenchymal stem cells

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Abstract

BACKGROUND

Zinc (Zn) is the second most abundant trace element after Fe, present in the human body. It is frequently reported in association with cell growth and proliferation, and its deficiency is considered to be a major disease contributing factor.

AIM

To determine the effect of Zn on *in vitro* growth and proliferation of human umbilical cord (hUC)-derived mesenchymal stem cells (MSCs).

METHODS

hUC-MSCs were isolated from human umbilical cord tissue and characterized based on immunocytochemistry, immunophenotyping, and tri-lineage differentiation. The impact of Zn on cytotoxicity and proliferation was determined by MTT and Alamar blue assay. To determine the effect of Zn on population doubling time (PDT), hUC-MSCs were cultured in media with and without Zn for several passages. An i*n vitro* scratch assay was performed to analyze the effect of Zn on the wound healing and migration capability of hUC-MSCs. A cell adhesion assay was used to test the surface adhesiveness of hUC-MSCs. Transcriptional analysis of genes involved in the cell cycle, proliferation, migration, and self-renewal of hUC-MSCs was performed by quantitative real-time polymerase chain reaction. The protein expression of Lin28, a pluripotency marker, was analyzed by immunocytochemistry.

RESULTS
Zn at lower concentrations enhanced the rate of proliferation but at higher concentrations (> 100 μM), showed concentration dependent cytotoxicity in hUC-MSCs. hUC-MSCs treated with Zn exhibited a significantly greater healing and migration rate compared to untreated cells. Zn also increased the cell adhesion rate, and colony forming efficiency (CFE). In addition, Zn upregulated the expression of genes involved in the cell cycle (*CDC20, CDK1, CCNA2, CDCA2*), proliferation (*transforming growth factor β1, GDF5, hypoxia-inducible factor 1α*), migration (*CXCR4, VCAM1, VEGF-A*), and self-renewal (*OCT4, SOX2, NANOG*) of hUC-MSCs. Expression of Lin28 protein was significantly increased in cells treated with Zn.

CONCLUSION
Our findings suggest that zinc enhances the proliferation rate of hUC-MSCs decreasing the PDT, and maintaining the CFE. Zn also enhances the cell adhesion, migration, and self-renewal of hUC-MSCs. These results highlight the essential role of Zn in cell growth and development.

Key Words: Human umbilical cord; Mesenchymal stem cells; Zinc; Cell proliferation; In vitro expansion

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Core Tip: Zinc (Zn) efficiently increased the proliferation of mesenchymal stem cells (MSCs) with a significant increase in the rate of population doublings, and at higher concentrations, it was cytotoxic. MSCs of the test group maintained a higher frequency of mesenchymal progenitors. Upregulation of genes involved in cell cycle, proliferation, pluripotency, and migration, was also observed in test groups. Zn increased the migration capability of MSCs and cell adhesion ability of MSCs. Overall, the results showed that Zn at lower concentrations supported cell growth, division, and proliferation of MSCs while maintaining the MSC-specific characteristics, including pluripotency, migration, and cell adhesion.

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INTRODUCTION

Preclinical and clinical research supports regenerative medicine as a promising therapeutic approach to address degenerative disorders, including cardiovascular diseases, dermal wounds, rheumatic diseases, traumas, immune system diseases, neuronal diseases, and cancers[1,2]. Cell-based therapy approach in regenerative medicine utilizes undifferentiated stem cells and possesses the potential to proliferate clonally and differentiate into other types of cells[3-5]. Among these stem cells, mesenchymal stem cells (MSCs) are the multipotent stem cells that act as immune modulators, lack HLA-DR, and down-regulate the antigen-presenting and co-stimulatory molecules[6]. These immune privilege properties make MSCs a potential candidate in regenerative medicine[7-9]. Currently, more than 1000 registered clinical studies involve MSCs for the regeneration of tissues and organs[10].

MSCs from perinatal sources, such as umbilical cord tissue, umbilical cord blood, and amniotic fluid, are highly proliferative with a sustained tendency of differentiation[11-13]. Though MSCs can be isolated from various body tissues, their low number has created a need for *in vitro* expansion. Clinically, 20 to 100 × 10^6 MSCs are required for a single therapeutic dose, which minimally takes 4 to 8 wk for *in vitro* expansion[13]. The expansion period and the quality of MSCs depend on the isolation and cell culture protocols. In addition, the biological properties of MSCs are influenced by the patient's clinical history, including age and genetic makeup[14]. Non-physiological conditions, including cell density, oxygen level, passage number, culture medium ingredients, and senescence are also being reported to alter biological characteristics of MSC like stemness, morphological features, migration, and proliferative potential[13].

Zinc (Zn) is a trace element that is widely reported for its role in growth and proliferation of cells, namely osteoblasts (MC3T3-E1 cells)[15], endothelial cells[16], smooth muscle cells[17], CD8+T cells[18,19]. Its deficiency is directly proportional to stem cell proliferation[20]. Plasma contains more than 1% of the total Zn[21]. Biologically, Zn is involved in cell catalytic, structural, and regulatory functions[22]. It acts as a cofactor of more than 300 enzymes[23].

Based on the role of Zn in cell growth and proliferation, we hypothesized that the addition of Zn in the growth medium would significantly increase the growth and proliferation rate of MSCs, which will be helpful in their *in vitro* expansion for therapeutic purposes. To the best of our knowledge, no study has addressed the effects of Zn on human umbilical cord (hUC)-MSCs. Thus, for the first time, we anticipate the potential role of Zn in the proliferation, migration, morphology, doubling time, and gene expression profile of hUC-MSCs.

MATERIALS AND METHODS

Collection, Processing, and Culturing of Human Umbilical Cord Tissue Samples

Human umbilical cord tissue samples were collected during elective C-sections of healthy donors under formal consent from Zainab Panjwani Memorial Hospital, Karachi. The independent ethical committee of PCMD, ICCBS, University of Karachi, for human subjects has approved the protocol (IEC-009-UCB-2015). The samples were collected in phosphate buffer saline (PBS) containing 0.5% EDTA as an anticoagulant, and processed as reported previously[24,25]. The cord tissue was washed with PBS to remove blood clots and debris. After washing, the tissue was chopped into 3 mm^3 pieces or explants and transferred into T75 culture flask containing 10-12 mL Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1 mmol/L sodium pyruvate. The flask was incubated at 37 °C in a humidified incubator set at 5% CO_2. The culture medium was changed at the interval of three days. After 10-15 days, an outgrowth of cells was noted from the explants, that was allowed to grow and proliferate. hUC-MSCs up to passage 5 were used in the experiments.

Characterization of MSCs

MSCs were characterized based on tri-lineage differentiation, immunophenotyping, and immunocytochemistry.

Tri-lineage differentiation: For differentiation towards adipogenic, chondrogenic, and osteogenic lineages, the cells at passage P3 were cultured in a 6-well plate and grown in DMEM until they achieved 60%-70% confluency. Subsequently, DMEM was replaced with an adipogenic induction medium (200 µM indomethacin, 1 µM dexamethasone, and 10 µM insulin), osteogenic induction medium (10 µM beta glycerophosphate, 50 µM ascorbic phosphate, and 0.1 µM dexamethasone), and chondrogenic induction medium [20 ng transforming growth factor β1 (TGFβ1), 10 ng insulin, 100 µM ascorbic acid, and 1 µM dexamethasone]. Cells in induction media were incubated for three weeks. After incubation, cells were fixed with 4% Paraformaldehyde (PFA) and stained with Oil red O, Alcian blue, and Alizarin red S stain to detect oil droplets, glycosaminoglycan content, and calcium deposited in induced adipocytes, chondrocytes, and osteocytes, respectively. Images were captured under a bright field microscope with a CCD camera (Ti2, Nikon, Japan).

Immunophenotyping: Immunophenotyping was performed for cell surface markers CD73, Vimentin, CD105, and CD45 (Cat No. 562245, BD Bioscience). MSCs were incubated in blocking solution with primary antibodies at recommended dilutions for 2 h at 37 °C, and finally with secondary antibody Alexa Flour 546 of 1:200 dilution for 1 h. Cells were analyzed on a flow cytometer (BD FACS Celesta, Becton Dickson, United States).

Immunocytochemistry: MSCs were cultured in a chamber slide with a density of 5000 cells per well. Cells were fixed with 4% PFA for 10 min at room temperature (RT) and then permeabilized with 0.1% Triton X-100. Blocking was performed with 2% bovine serum albumin for 20 min at RT. Primary antibodies for MSC antigens CD73 (550256, BD Pharmingen, United States), CD117 (32-9000, Zymed Laboratories, Inc., United States), CD29 (MAB-1981, Chemicon International, United States), CD105 (560839, BD Pharmingen, United States), Stro1 (14-6688-82, Molecular Probes, Invitrogen, United States), Vimentin (V6389, Sigma-Aldrich, Inc., United States), Lin28 (PA1-096, Molecular Probes, Invitrogen, United States), and CD45 (CBL415, BD Pharmingen, United States) were added at recommended dilutions and kept at 4 °C overnight. Following PBS washing, cells were incubated with Alexa fluor-546 secondary antibody having 1:200 dilutions for 1 h at 37 °C. Nuclei were stained with DAPI for 15 min. Finally, cells were mounted and visualized under a fluorescent microscope (NiE, Nikon, Japan).

MTT Assay

MTT assay was performed, taking different concentrations of $ZnCl_2$ in complete DMEM to determine minimal toxic concentration. MSCs (2000 cells) per well were seeded in a 96-well plate with 200 µL complete DMEM. The medium was aspired, and cells were incubated with 5 µM, 10 µM, 20 µM, 30 µM, 50 µM, 100 µM, 250 µM, and 500 µM concentrations of $ZnCl_2$ for overnight. The next day, the medium was removed, and cells were incubated with 200 µL of MTT dye (0.5 gm/mL stock) for 4 h and incubated at 37 °C with 5% CO_2. Then, the MTT solution was discarded, and 200 µL of DMSO was added to dissolve formazan crystals formed by metabolically active cells. The plate was kept on an orbital shaker for 5 min at RT, and absorbance was measured at 570 nm by spectrophotometer (SpectraMax, United States).

Alamar blue assay for hUC-MSC proliferation kinetics

To analyze MSC proliferation, 2000 cells per well were seeded in a 96-well plate and incubated with different concentrations of $ZnCl_2$ for 24 h, 48 h, and 72 h. After each time point, the medium was removed, and 0.02% Alamar blue solution was added to each well. Fluorescence intensity was measured using a fluorescence spectrophotometer with excitation at 560nm and emission at 600nm (Varioskan LUX, Thermo Scientific). The relative fluorescence unit (RFU) was calculated by the given formula given below:

Difference between treated and untreated (%) = (RFU of treated groups/RFU of untreated group) × 100

Proliferation rate of hUC-MSCs

To analyze the proliferation rate of hUC-MSCs, 10000 cells per well were seeded in a 24-well plate and incubated with 20 µM $ZnCl_2$. When cells attained 70%-80% confluency, they were detached and counted by hemocytometer for the number of population doubling (NPD), and population doubling time (PDT) by the formulae given below:

$NPD = 3.33 \times \log (N_t/N_i)$
$PDT = t \times \log (2)/[\log (N_t) - \log (Ni)]$
Where, N_i = no. of cells plated; N_t = no. of cells harvested; t_i = initial day (0 d) and t (harvesting day).

Colony forming unit assay

MSCs were seeded at 500 cells per cm^2 in a T25 culture flask. After two weeks of incubation, cells were fixed with 4% PFA, followed by incubation with 0.5% crystal violet prepared in 10% methanol. Cells were washed with distilled water, and colonies were visualized at 40 × magnification under bright field microscope. A cluster of 100 cells was considered a colony. Results were analyzed by SPSS software.

Transcriptional profile of genes involved in cell cycle, proliferation, pluripotency, and migration

Cells were harvested for RNA isolation, 1 mL Trizol reagent (15596026, Invitrogen) was added, followed by the addition of 500 µL chloroform for phase separation and centrifugation at 12000 rpm. The aqueous layer was collected and incubated with 1 mL absolute ethanol for 2 h at -80 ˚C. The suspension was centrifuged, and the pellet was rinsed with 70% ethanol. Finally, the pellet was reconstituted in 15 µL nuclease-free water, quantified by nanodrop spectrophotometer, and stored at -20 ˚C. cDNA was synthesized using 1 µg RNA by RevertAid first-strand cDNA synthesis kit (K1622, Thermo Scientific, United States). All the primers were designed by http://www.ncbi.nlm.nih.gov/tool/primerblast and synthesized commercially. quantitative real-time polymerase chain reaction (qPCR) amplification was performed in a triplicate manner using qPCR master mix (A600A, Promega, Madison, WI, United States) for the genes mentioned in Table 1.

Scratch assay

For cell migration, 4000 cells per well were seeded in a 24-well plate containing DMEM and incubated for 24 h. After monolayer formation, the scratch was developed using a 100 µL pipette tip, and cells were incubated with 20 µM $ZnCl_2$. Migration of cells was observed at 2 h, 4 h, 6 h, 8 h, and 24 h of scratch development. Images were captured at different time points under a phase-contrast microscope (Eclipse Ts2, Nikon, Japan) and migration was measured in percentage with the help of the following formula:

% of MSCs migration = [(hi − Δh) ÷ hi] × 100%

Where, hi = the area of scratch measured immediately after scratching, and Δh = is the area of scratch measured at 2, 4, 6, 8, and 24 h.

Cell adhesion assay

To analyze cell adherence in control and test groups, MSCs were harvested, seeded in a culture flask, and cell adhesion was determined by observing MSCs at different time points, *i.e.*, at 0 min (immediately after seeding), after 15 min, 30 min, 1 h, and 2 h. Cell adhesion was evaluated by the given formula:

%Percentage = no. of cells adhered on the flask surface/no. of floating cells in media.

Pluripotency retention of hUC-MSCs

To analyze the pluripotency marker after 20 µM $ZnCl_2$ treatment, MSCs were incubated with the primary antibody of Lin28 at 1:100 dilution and kept at 4 °C overnight. After PBS washing, cells were incubated with Alexa fluor-546 secondary antibody of 1:200 dilutions for 1 h at 37 °C. Nuclei were stained with DAPI for 15 min and then visualized under a fluorescent microscope (NiE, Nikon, Japan).

RESULTS

Human Umbilical Cord Processing and Culturing

MSCs were isolated from human umbilical cord tissue by the explant method (Figure 1A). Human umbilical cord tissue comprises one vein, two arteries, and Wharton's jelly (Figure 1B). MSCs started to outgrow the cord tissue after 10-15 d of cord processing. At this stage, cells adhered to the flask surface and appeared as trigonal shape (Figure 1C and D). After approximately 25 d, cells were observed to be interconnected with each other by their extensions, formed colonies, and exhibited elongated fibroblast-like morphology, which was termed P_0 (passage zero) MSCs (Figure 1E and F).

Characterization of hUC-MSCs

Cells isolated from the umbilical cord possessed adhesive properties and exhibited fibroblast-like morphology (Figure 2A). The isolated population of cells showed positive expression for MSCs specific markers, including CD73, CD117, CD29, CD105, Stro1, Vimentin, and Lin28, while negative for CD45, which is a hematopoietic marker (Figure 2B). Immunophenotypic analysis showed positive expression for CD73 (90%), vimentin (98%), and CD105 (80%), and less than 4% population was positive for CD45 (Figure 2C). The isolated cells successfully differentiated into adipogenic, osteogenic, and chondrogenic lineages as confirmed by Oil red O, Alizarine red S, and Alcine blue staining (Figure 2D).

Cytotoxicity analysis and proliferation kinetics of hUC-MSCs

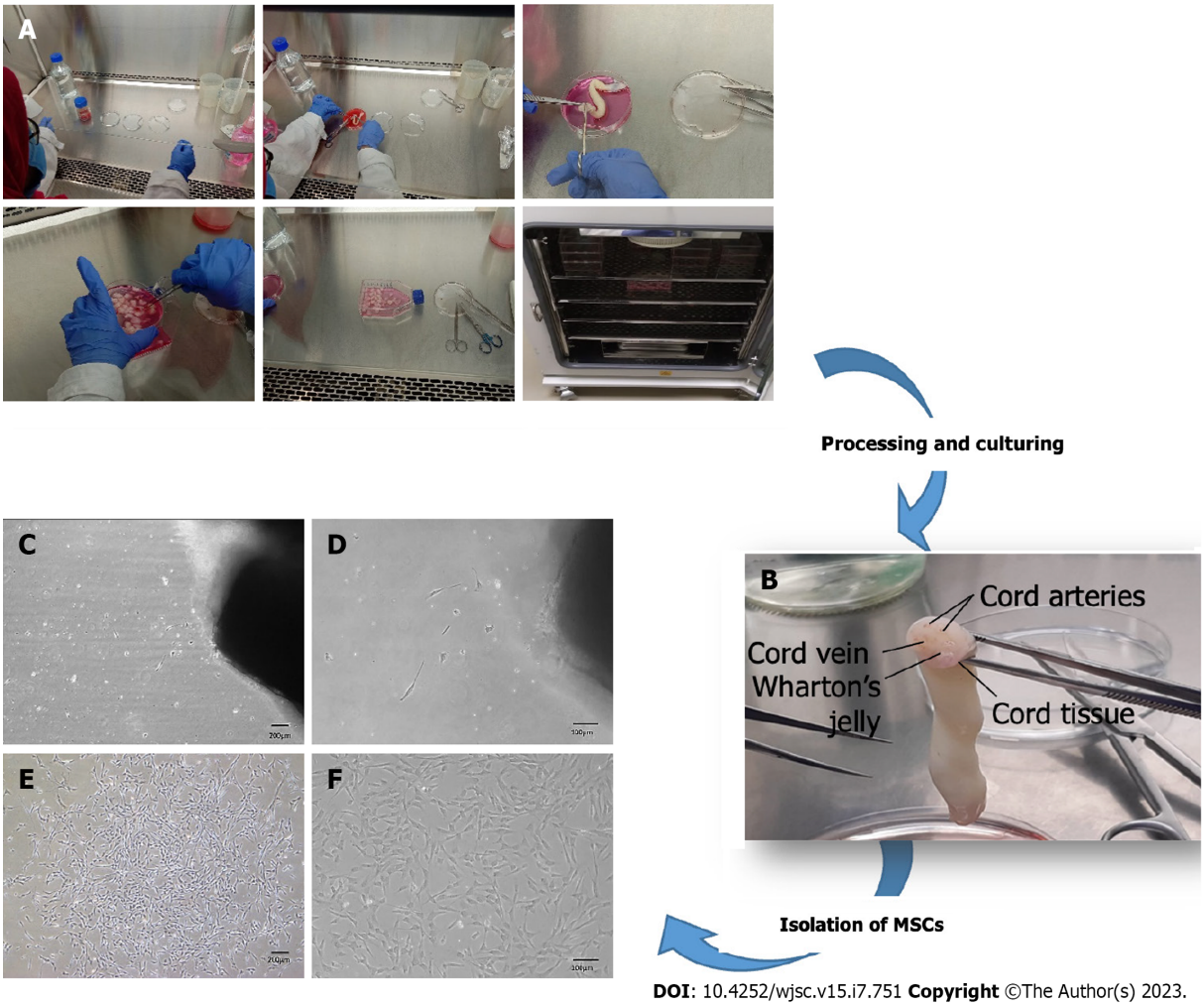
$ZnCl_2$ concentrations ranging from 5 µM-100 µM did not show any cytotoxic effect on cells (Figure 3A and B). $ZnCl_2$ was found to be significantly cytotoxic above 100 µM concentration, and its cytotoxicity was found to be higher in a concen-

Table 1 Primers for cell cycle and proliferation genes

S. No	Genes	Primer sequence (5'-3')	Annealing temperature (ºC)
1	*GAPDH*	(F) CCAGAACATCATCCCTGCCT	58
		(R) CCTGCTTCACCACCTTCTTG	
2	*CDC20*	(F) TCCTGAGTCTGACCATGAGC	58
		(R) CTGAGGTGATGGGTTGGTCT	
3	*CDK1*	(F) GGGGTCAGCTCGTTACTCAA	58
		(R) CACTTCTGGCCACACTTCAT	
4	*CCNA2*	(F) CCAGGAGAATATCAACCCGGA	58
		(R) GGAACGGTGACATGCTCATC	
5	*CDCA2*	(F) TCTGGGAGTAAGCCTGTCTG	58
		(R) AGGAGGTAATTCGGCATGCT	
6	*TGFβ1*	(F) CAAGGCACAGGGGACCAG	58
		(R) CAGGTTCCTGGTGGGCAG	
7	*GDF5*	(F) CACATCCCAAGAGCCCCTTC	58
		(R) GCCCAGGTGAGGAGAAATGG	
8	*HIF1α*	(F) CTCGAGATGCAGCCAGATCT	58
		(R) CCAGAAGTTTCCTCACACGC	
9	*45203*	(F) GATGTCAGGGCTCTTTGTCCA	54
		(R) TACTCTCCCCAGCTTGCTTTG	
10	*SOX-2*	(F) CCCTGCAGTACAACTCCATGAC	54
		(R) GGTAGTGCTGGGACATGTGAAG	
11	*NANOG*	(F) AAGGCAAACAACCCACTTCTG	54
		(R) CCAGTTGTTTTTCTGCCACCT	
12	*CXCR 4*	(F) ACTGAGAAGCATGACGGACA	54
		(R) TCATCTGCCTCACTGACGTT	
13	*VCAM1*	(F) CCTGGGAAGATGGTCGTGAT	54
		(R) AGATGTGGTCCCCTCATTCG	
14	*VEGF-A*	(F) AGCGCAAGAAATCCCGGT	54
		(R) CCCTCCGGACCCAAAGTG	
15	*MMP2*	(F) TTGACGGTAAGGACGGACTC	54
		(R) AGCGGAATGGAAACTTGCAG	

Housekeeping gene: *GAPDH* (Glyceraldehyde-3 Phosphate Dehydrogenase); cell cycle genes: *CDC20* (Cell-Division Cycle Protein 20), *CDK1* (Cyclin-Dependent Kinase 1), *CCNA2* (Cyclin-A2), *CDCA2* (Cell Division Cycle Associated 2); proliferation genes: *TGFβ* (Transforming Growth Factor β1), *1, GDF5* (Growth Differentiation Factor 5), *HIF1α* (Hypoxia-Inducible Factor 1α); pluripotency genes: *OCT-4* (Octamer-Binding Transcription Factor-4), *SOX2* (SRY-box 2), *NANOG*; migration genes: *CXCR 4* (Chemokine Receptor Type 4), *VCAM1* (Vascular Cell Adhesion Molecule1), *VEGF-A* (Vascular Endothelial Growth Factor-A), *MMP2* (Matrix Metallopeptidase 2).

tration-dependent manner. The IC_{50} of $ZnCl_2$ was found at 250 μM concentration (Figure 3A). For proliferation analysis, MSCs were treated with 5-50 μM $ZnCl_2$ for 24, 48, and 72 h. Fluorescence intensity was measured for each group at the respective time after incubating with Alamar blue for 4 h. Results of each temporal group were analyzed by one-way ANOVA between control and test groups. All test groups observed significantly increased proliferation after 48 and 72 h (Figure 4A and B). MSCs in passage 5 were analyzed for NPD and PDT. After 10 d, MSCs of the control and treated group (20 μM $ZnCl_2$), were counted, and significantly higher NPD was observed in 20 μM $ZnCl_2$ treated group (Figure 3C), while PDT reduced significantly in the treated group in contrast to the control group (Figure 3D).



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Figure 1 Processing and culturing of human umbilical cord tissue. A: Demonstration of the pictographic representation of human umbilical cord processing; B: Show cord tissue comprises one vein and two arteries surrounded by Wharton's jelly; C-F: Shows fibroblast-like morphology of mesenchymal stem cells at 4 × and 10 × magnifications at P_0 (C and D), while P_0 at a later stage shows elongated fibroblast-like morphology, which is interconnected with their extensions and is arranged in colonies (E and F). MSCs: Mesenchymal stem cells.

Colony forming unit assay and transcriptional analysis of cell cycle and proliferation genes

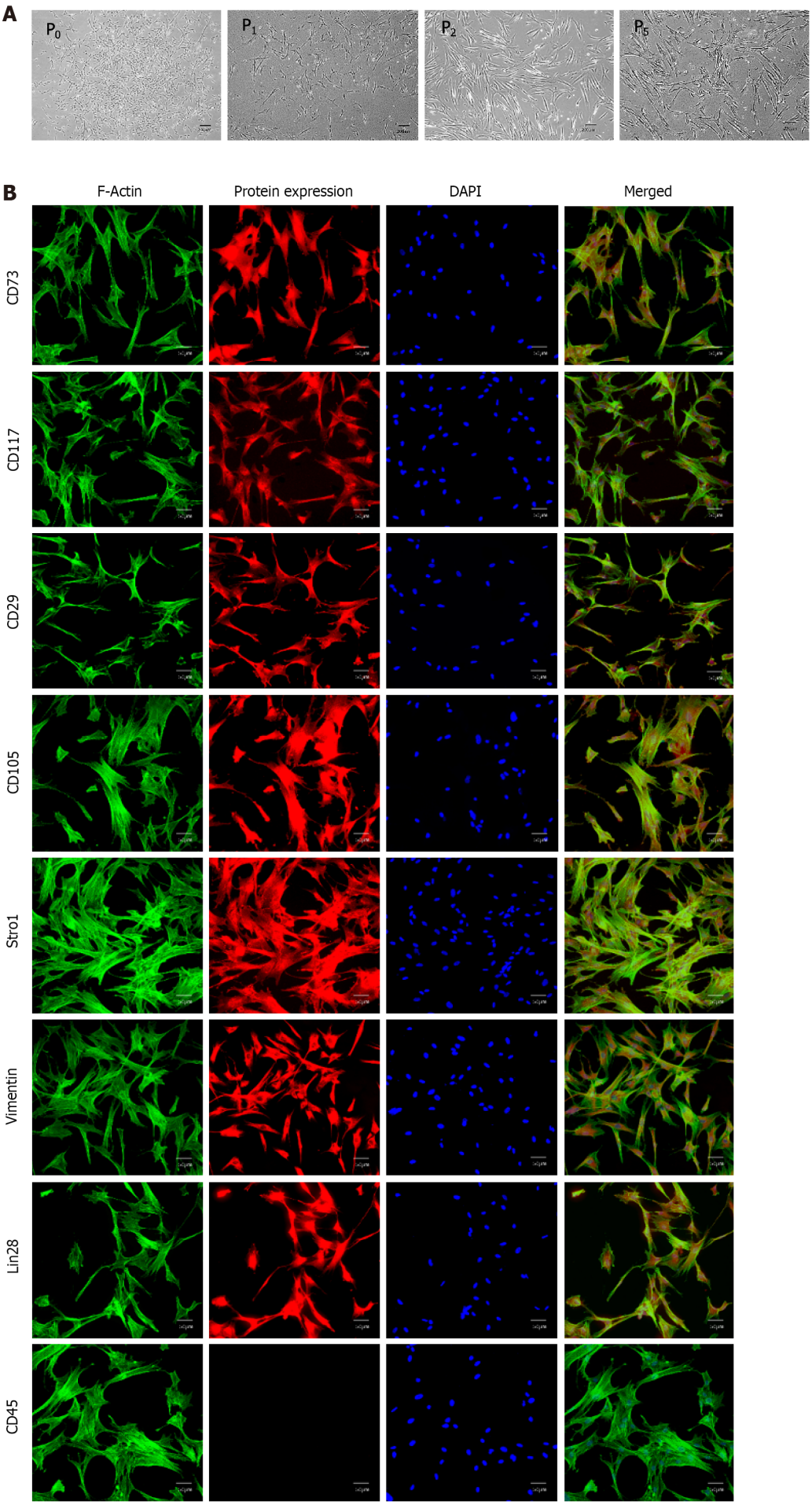
To analyze the presence of mesenchymal progenitors, colony forming unit (CFU) assay was performed. Colonies of hUC-MSCs were observed after 2 wk of cell seeding. The test group supplemented with 20 μM $ZnCl_2$, exhibited a significantly higher number of colonies than the control group (Figure 4A and B). To analyze the transcriptional changes of 20 μM $ZnCl_2$ treated MSCs to the untreated MSCs, fold change regulation ($2^{-\Delta\Delta Ct}$) of cell cycle and proliferation genes was analyzed at 12, 24, 48, and 72 h. Expression of cell cycle genes, *i.e.*, *CDC20, CCNA2,* and *CDCA2,* was comparatively increased in all temporal groups of 20μM $ZnCl_2$, while *CDK1* was upregulated after 24, 48, and 72 h (Figure 4C). Among proliferation genes, expression of *TGFβ1* and *GDF5* was significantly increased in all temporal groups of 20 μM $ZnCl_2$, whereas *hypoxia-inducible factor 1α (HIF-1α)* exhibited a significant upregulation after 24, 48, and 72 h (Figure 4D).

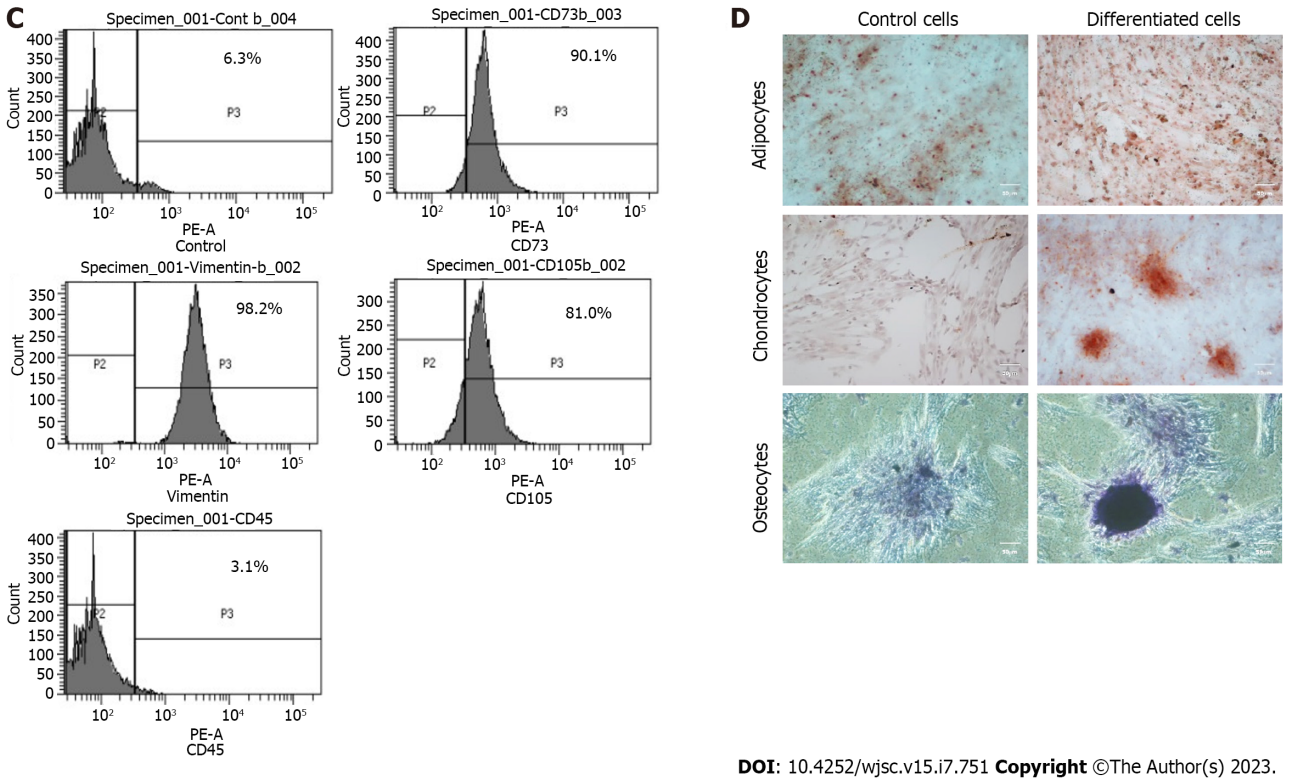
Cell migration assay and transcriptional analysis of genes involved in cell migration

$ZnCl_2$ was also found to facilitate the migration of hUC-MSCs (Figure 5A) and significantly increased ($^bP \leq 0.01$) migration was observed in 20 μM $ZnCl_2$ treated groups for 2-8 h, while scratch was completely healed after 24 h in both groups, *i.e.*, control and $ZnCl_2$ treated groups. The percentage of area healed is plotted against time (Figure 5B). Gene expression analysis of migration factors showed upregulation of *CXCR-4* at all the time points, *i.e.*, 24, 48, and 72 h, while *V-CAM1, VEGF-A* was significantly upregulated after 48 and 72 h. *MMP-2* was found to be upregulated after 24 and 72 h (Figure 5C).

Cell adhesion assay

To analyze the effect of $ZnCl_2$ on the adhesiveness of hUC-MSCs, *in vitro* cell adhesion assay, was performed. A significant increase in the cell adhesion rate of hUC-MSCs in the 20 μM $ZnCl_2$ treated group was noted at 15 min, 30 min,1 h, and 2 h, compared to the control group (Figure 6A). Cell adhesion rate (%) is plotted against time (Figure 6B).


A
P0
P1
P2
P5
B
F-Actin
Protein expression
DAPI
Merged
CD73
CD117
CD29
CD105
Stro1
Vimentin
Lin28
CD45



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Figure 2 Characterization of human umbilical cord-derived mesenchymal stem cells. A: Isolated human umbilical cord-derived mesenchymal stem cells appeared as elongated, fibroblast-like cells which were slightly trigonal at P_0 and then gradually became elongated in latter passages (P_1, P_2, P_5); B: These cells positively expressed CD73, CD117, CD29, CD105, Stro1, Vimentin, and Lin28 while exhibiting negative expression of CD45; C: Cell surface analysis by flowcytometry showed that more than 80% of the cell population was positive for CD73, vimentin, and CD105 while less than 4% were positive for hematopoietic marker *i.e.* CD45; D: The cells were successfully differentiated into adipocytes, osteocytes, and chondrocytes. Oil red O stain, alizarin red S stain, and Alcian blue stain confirmed the presence of lipids vacuoles, calcium deposits, and glycosaminoglycan in adipocytes, osteocytes, and chondrocytes, respectively.

Retention of pluripotency markers

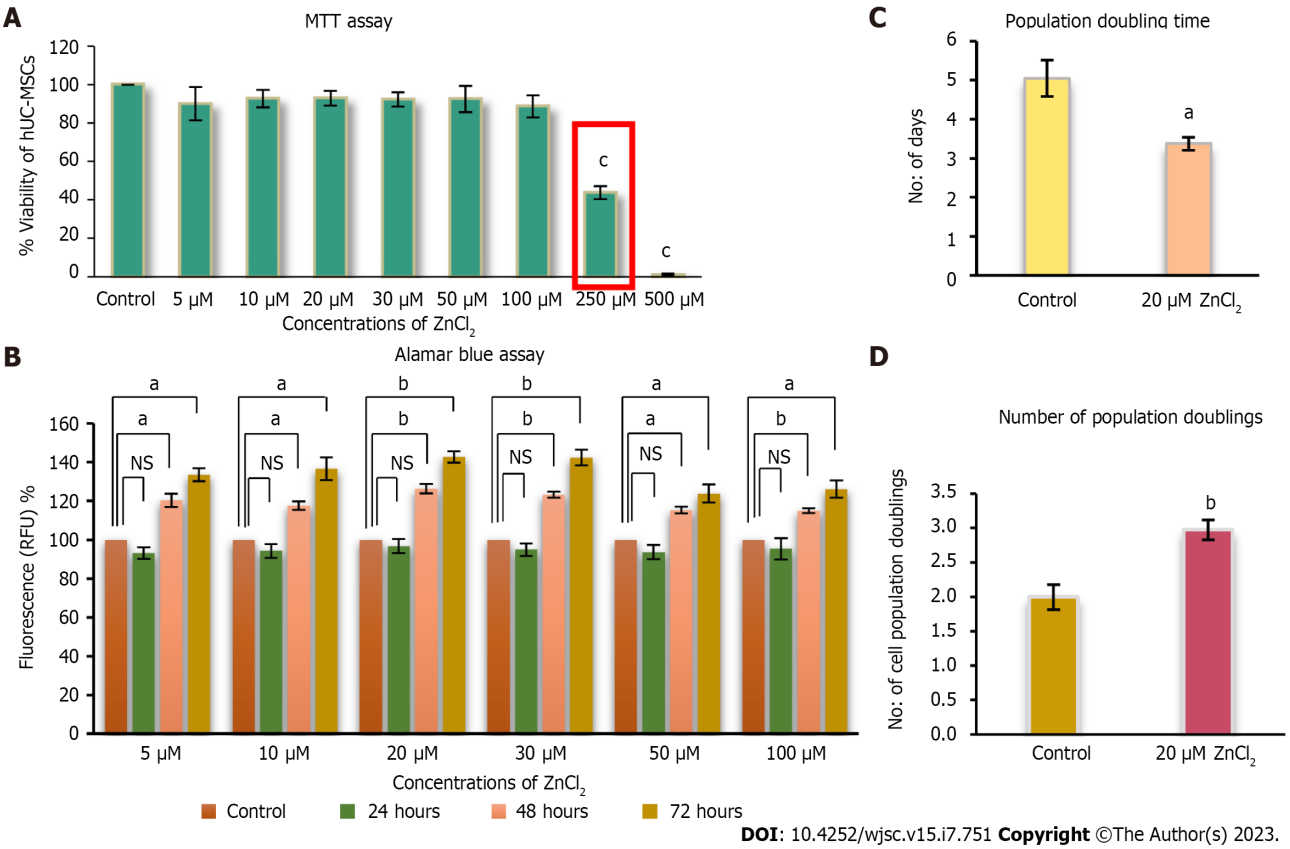
Immunostaining: Immunostaining of 20 μM $ZnCl_2$ treated MSCs showed positive expression of pluripotency marker Lin28 at all the time points (Figure 7A). The quantified mean fluorescence intensity showed a significant increase in expression at 24 h (Figure 7B).

Gene expression analysis: Genes expression analysis showed that *OCT4* was significantly upregulated in all temporal groups (12, 24, 48, and 72 h), of 20 μM $ZnCl_2$. In contrast, the expression of *SOX2* and *NANOG* was significantly higher in temporal groups of 24 and 48 h and then fell to the level of control in 72 h (Figure 7C).

DISCUSSION

Zn is among the essential trace elements that is ubiquitously found in the human body. According to prior investigations, it is one of the trace elements that is majorly involved in cell growth and proliferation[16,17,26]. It stimulates cell division and proliferation by regulating enzymes such as deoxythymidine kinase and/or by regulating hormones including insulin-like growth factor-I (IGF-I) and nerve growth factors[23,27]. Zn acts as a signal molecule for both extracellular and intracellular pathways. It upregulates PI3 kinase pathway which activates AKT in fibroblast cells and transactivates EGF receptor by Src pathway in epithelial cells of the airway. It also regulates the ERK1/2 pathway with the induction of p21 (CiP/WAF1), and cyclin D1 in colonocytes[21]. Studies reported the role of Zn on the proliferation of adipose derived MSCs[23,26]. A recent study reported the effect of Zn on human umbilical cord derived MSCs and showed its importance in MSC survival and differentiation properties. The study suggested that Zn could enhance antioxidative properties of MSCs by regulating Nrf2/Sirt3 signaling pathway[28]. In the present study, we analyzed the effect of $ZnCl_2$ on hUC-MSCs in the context of its proliferation and growth, pluripotency, migration, and cell adhesion properties.

$ZnCl_2$ is reported to have a biphasic effect on the viability and proliferation of the cells and exhibited dose-dependent response, which varies for different cell lines[29]. Most of the studies found $ZnCl_2$ cytotoxic above 100 μM in different dividing cells, including coronary artery endothelial cells, vascular smooth muscle cells, and even MSCs[16,17,23]. Our findings showed cytotoxicity of $ZnCl_2$ above 100 μM, which is in line with the previous study[23]. The proliferation profile of MSCs reported by previous studies gave a bell shape biphasic curve whose peaks vary for $ZnCl_2$ concentrations for different cells. We also obtained a bell shape biphasic curve representing an increase in MSC proliferation with increase of $ZnCl_2$ concentration up to 30 μM, and then gradually decreasing upon further increase. However, up to 100



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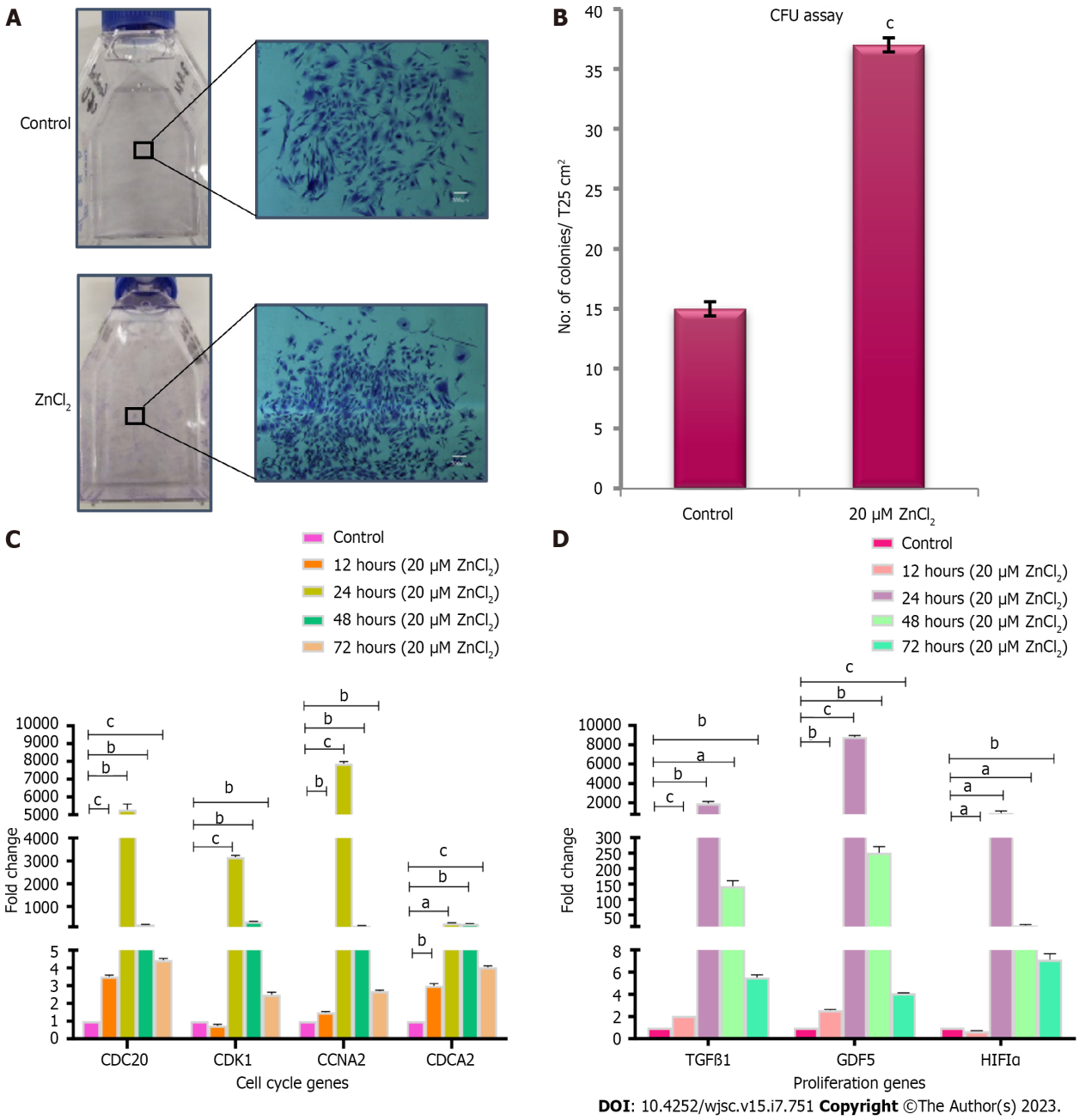
Figure 3 Cytotoxic analysis of $ZnCl_2$ for human umbilical cord-derived mesenchymal stem cells. A: Concentrations of $ZnCl_2$ 5 μM, 10 μM, 20 μM, 30 μM, 50 μM, 100 μM, 250 μM, and 500 μM were analyzed for their cytotoxicity by MTT assay. Concentrations from 5 μM to 100 μM did not show any significant decrease in viability (Percent) of human umbilical cord (hUC)-derived mesenchymal stem cells (MSCs) compared to control. 250 μM reflected IC_{50} value of $ZnCl_2$, and both 250 μM and 500 μM concentrations were found to be significantly ($^cP \leq 0.001$) cytotoxic. Results were analyzed by performing one-way ANOVA and Post-Hoc Bonferroni test; B: Alamar blue assay analysis of hUC-MSCs after 24 h after $ZnCl_2$ did not exhibit any significant difference between control and test groups. But after 48 and 72 h, the proliferation of MSCs was significantly increased ($^aP \leq 0.05$, $^bP \leq 0.01$) in all test groups. For further experiments, 20 μM of $ZnCl_2$ was selected as the test group concentration. C: Test group 20 μM $ZnCl_2$ exhibited significantly higher ($^bP \leq 0.01$) NPD *i.e.*, 2.9 ± 0.14, as compared to the control group, which showed 1.9 ± 0.18. Significantly reduced ($^aP \leq 0.05$) D: PDT was observed in the test group *i.e.*, approximately 3 d ± 0.16 in contrast to the control group having population doubling time of 5 ± 0.46 d. Results were analyzed by independent sample *t*-Test. Data is presented as mean ± SD ($n = 3$) where the significance level is $^aP \leq 0.05$. NS: Not significant.

μM concentration of $ZnCl_2$ gave significantly increased proliferation of MSCs in test groups as compared to control. Increased population doublings (NPD) and reduced PDT were observed in the treatment group in contrast to control MSCs.

MSCs are the progenitor cells that proliferate rapidly[30,31]. MSCs retain their multipotent ability, but the expanded cultured population of MSCs shows heterogeneous morphology of cells with variations in the frequency of early progenitor cells[30]. It is reported that human umbilical cord-derived MSCs exhibit the highest percentage of colony-forming units in contrast to the MSCs derived from bone marrow, adipose tissue, and dental pulp[32]. By CFU assay, we analyzed the impact of Zn on the colony-forming potential of MSCs derived from the human umbilical cord. Our results revealed that $ZnCl_2$ maintains a high pool of early progenitor cells in the MSC population, as many colonies were found in the treated group. $ZnCl_2$ treated group also exhibited denser colonies with spindle-shaped morphology which represented the rapidly self-replicating cells[30]. MSCs of the control group showed broad morphology with less dense colonies, which reflected slow self-replicating cells[30].

Gene expression analysis of treated MSCs exhibited a significant increase in cell cycle genes, including *CDK1, CDC20, CCNA2,* and *CACD2*. The level of CDK1 remains constant in entire cell division, but its activity depends on cyclins that are synthesized at different cell cycle stages[33]. CCNA2 or cyclin A2 is important for G1/S and G2/M transition, thus responsible for cell cycle progression[34]. CDK1 and CDC20 are the key mitotic regulators[35,36]. CDC20 is a cofactor of CPC/C (anaphase-promoting complex/Cyclosome), by activating CPC/C, CDC20 regulates the activity of CDK1 and promotes chromosome segregation by degrading securin, thus responsible for anaphase transition[36]. Whereas CDCA2 is essential for retaining chromatin structure in post-mitotic nuclei and regulates DNA damage responses for mitotic to interphase transition[37]. Upregulation of these genes required at different stages of the cell cycle showed progression of the cell cycle in the test group.

Few growth factors are pleiotropic and are found to be involved in multiple biological functions. For instance, TGFβ is one of them, and previous studies have reported the role of TGFβ in MSCs proliferation[38,39], and differentiation towards chondrogenic lineage; however, it inhibits terminal differentiation[39]. TGFβ1 is found to promote hUC-MSCs



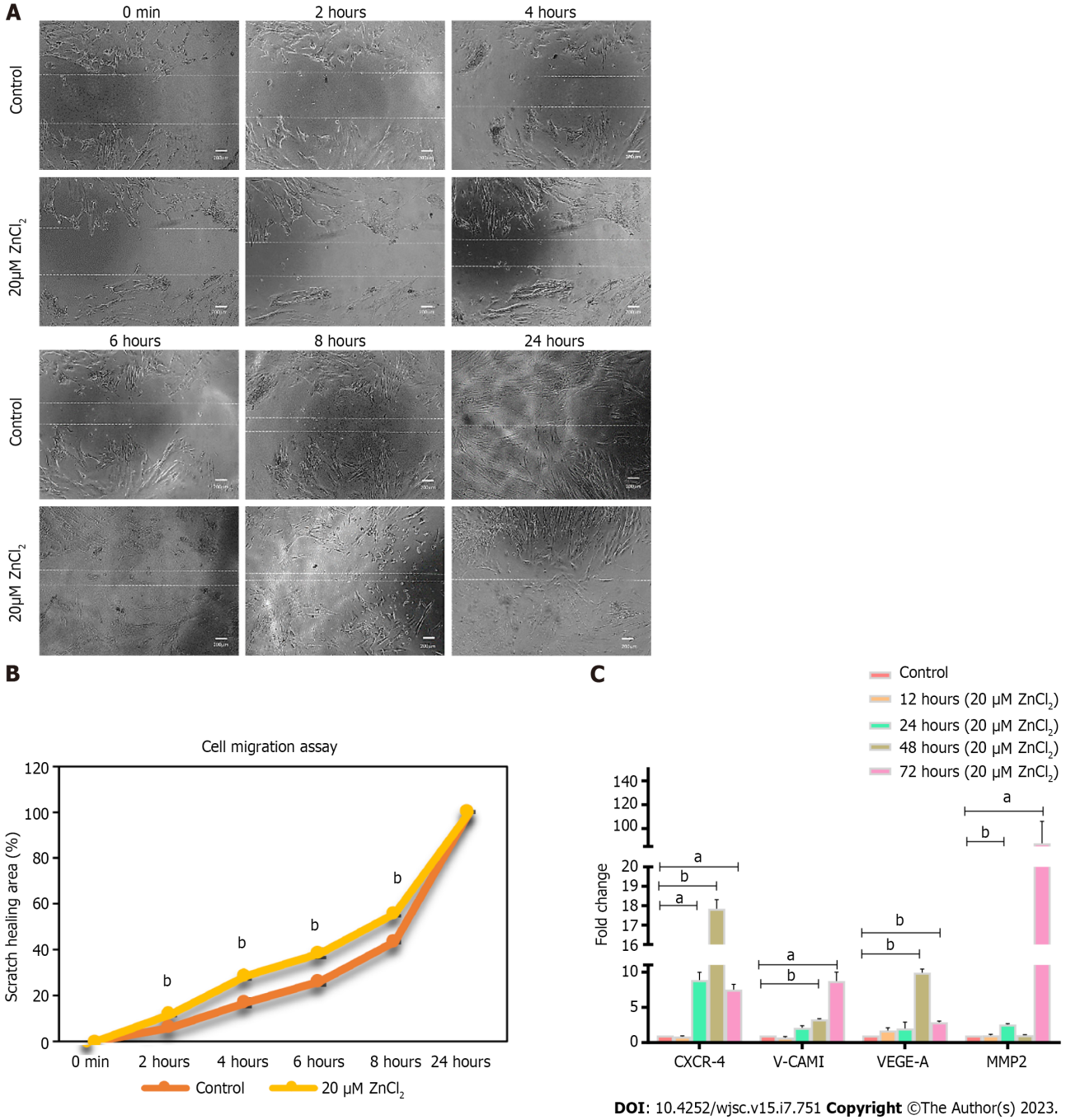
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Figure 4 Colony forming unit assay and transcriptional analysis of cell cycle and proliferation genes. A: Colonies after crystal violet staining in control and test groups. Denser colonies were observed in $ZnCl_2$ treated group. Images were captured at 4 × magnification; B: A significantly higher number ($^cP \leq 0.001$) of colonies was observed in $ZnCl_2$ treated group with a mean number of 37 ± 0.57 colonies per T25 flask as compared to control *i.e.*, 15 ± 0.57 colonies per T25 flask. Results were analyzed by SPSS, performing an independent sample *t*-Test. C: Gene expression of cell cycle genes CDC20, CCNA2, CDCA2 was significantly increased in the test group after 12, 24, 48, and 72 h of treatment with 20 μM $ZnCl_2$. No significant difference was found in CDK1 gene expression after 12, but it was significantly enhanced after 24, 48, and 72 h. This fold change ($2^{-\Delta\Delta Ct}$) increase in genes expression was highest in the 24-h group. D: Gene expression of proliferation genes transforming growth factor β1 and GDF5 were significantly increased in all temporal groups *i.e.* 12, 24, 48, and 72 h, of 20 μM $ZnCl_2$. HIF1α significantly decreased after 12 h but then significantly increased after 24, 48, and 72 h. Results were analyzed by SPSS, performing an independent sample *t*-Test. Data was represented as mean ± SD ($n = 3$) where significance level $^aP \leq 0.05$ ($^aP \leq 0.05$, $^bP \leq 0.01$, $^cP \leq 0.001$).

proliferation without affecting the phenotype of MSCs[38]. Hence, our results are in the context of these findings as the $ZnCl_2$ treated group showed a significant increase in TGFβ1 expression and stemness markers like *OCT4, SOX2*, and *NANOG*.

Few studies have reported the effect of Zinc on the expression of HIF1-α, but there is inconsistency, as some studies on cancer cells proved down-regulation of HIF1-α caused by Zinc[40,41], while in keratinocytes and prostate cells, Zinc is found to stabilize HIF1-α expression[41,42]. Our findings showed enhanced expression of HIF1-α in $ZnCl_2$ treatment group which is in agreement with later studies[41,42]. Overall, the significantly increased expression of *TGFβ1, GDF5,* and *HIF1-α* suggested the increased proliferation and multipotency of MSCs in the test group.

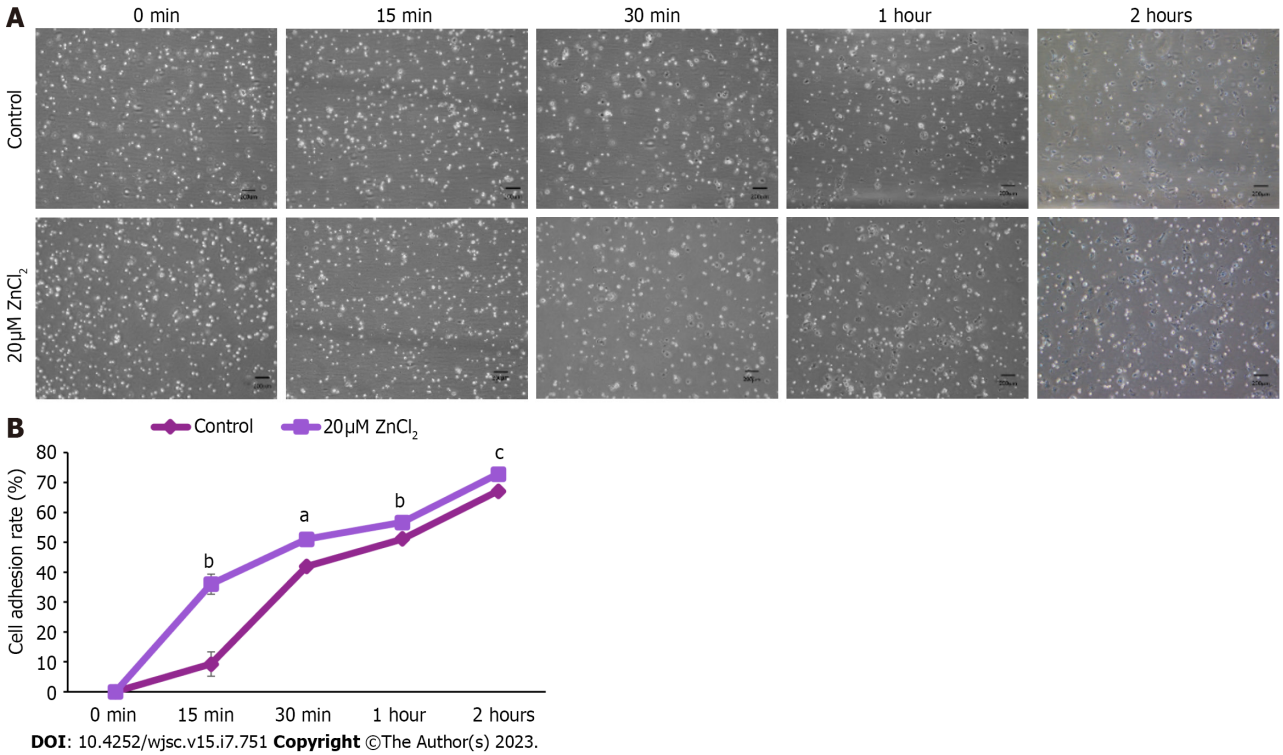
Migration is an important factor that should be considered for better therapeutic effects of MSCs in regeneration and cell therapy. Studies reported that Zn enhances the migratory capability of cells[16,17,23,43]. For example, endothelial



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Figure 5 Effect of $ZnCl_2$ on the migration potential of human umbilical cord-derived mesenchymal stem cells. A: Microscopic examination of human umbilical cord (hUC)-derived mesenchymal stem cells (MSCs) immediately after the scratch was given, *i.e.*, 0 h, and after 2 h, 4 h, 6 h, 8 h, and 24 h. MSCs of the test group (20 μM $ZnCl_2$) exhibited faster migration as compared to control at all time points up to 8 h, and the scratch was completely healed after 24 h in both groups; B: Percentage of the area of scratch healed at time points, *i.e.*, 0 min, 2 h, 4 h, 6 h, 8 h, and 24 h, analyzed by independent sample *t*-test. MSCs exhibited a significantly ($^bP \leq 0.01$) increased migration (Percent) in the test group (20 μM $ZnCl_2$). C: Gene expression analysis of migration factors *CXCR-4, V-CAM 1, VEGF-A,* and *MMP2*, after 12, 24, 48, and 72 h of 20 μM $ZnCl_2$ treatment. *CRCR-4* exhibited significantly increased expression after 24 h, 48 h, and 72 h of treatment. *V-CAM1*, and *VEGF-A* showed significantly higher expression after 48 and 72 h of treatment. *MMP2* showed enhanced expression after 24 and 72 h. Results were analyzed by independent sample *t*-Test. Data was analyzed as mean ± SD ($n = 3$) where significance level $^aP \leq 0.05$ ($^aP \leq 0.05$, $^bP \leq 0.01$, $^cP \leq 0.001$).

cells and smooth muscle exhibited enhanced migration and cell adhesion when treated with $ZnCl_2$. These cells showed upregulation of focal adhesion molecules, including integrin, vinculin, actin, ICAM1, and VEGFA[16,17]. We analyzed the effect of Zn on the migration ability of MSCs by *in vitro* scratch assay, which showed a significant increase in MSC migration after $ZnCl_2$ treatment. These results were further confirmed by gene expression analysis of migration factors, including *CXCR-4, V-CAM1, VEGF-A,* and *MMP2*. These are involved in chemotaxis, cell rolling, and cell invasion and play their role in different phases of cell migration[44]. CXCR-4 and VEGF-A are the early migration markers, where CXCR-4 is a chemokine receptor, and VEGF is a growth factor, and both are involved in chemotaxis and trafficking of cells. V-CAM1 is a cell adhesion molecule, whereas MMP2 is required for cell invasion[44]. Here we analyzed these genes to estimate the migration ability of MSCs after $ZnCl_2$ treatment, and we found that after 12 h, only *VEGF-A* showed high



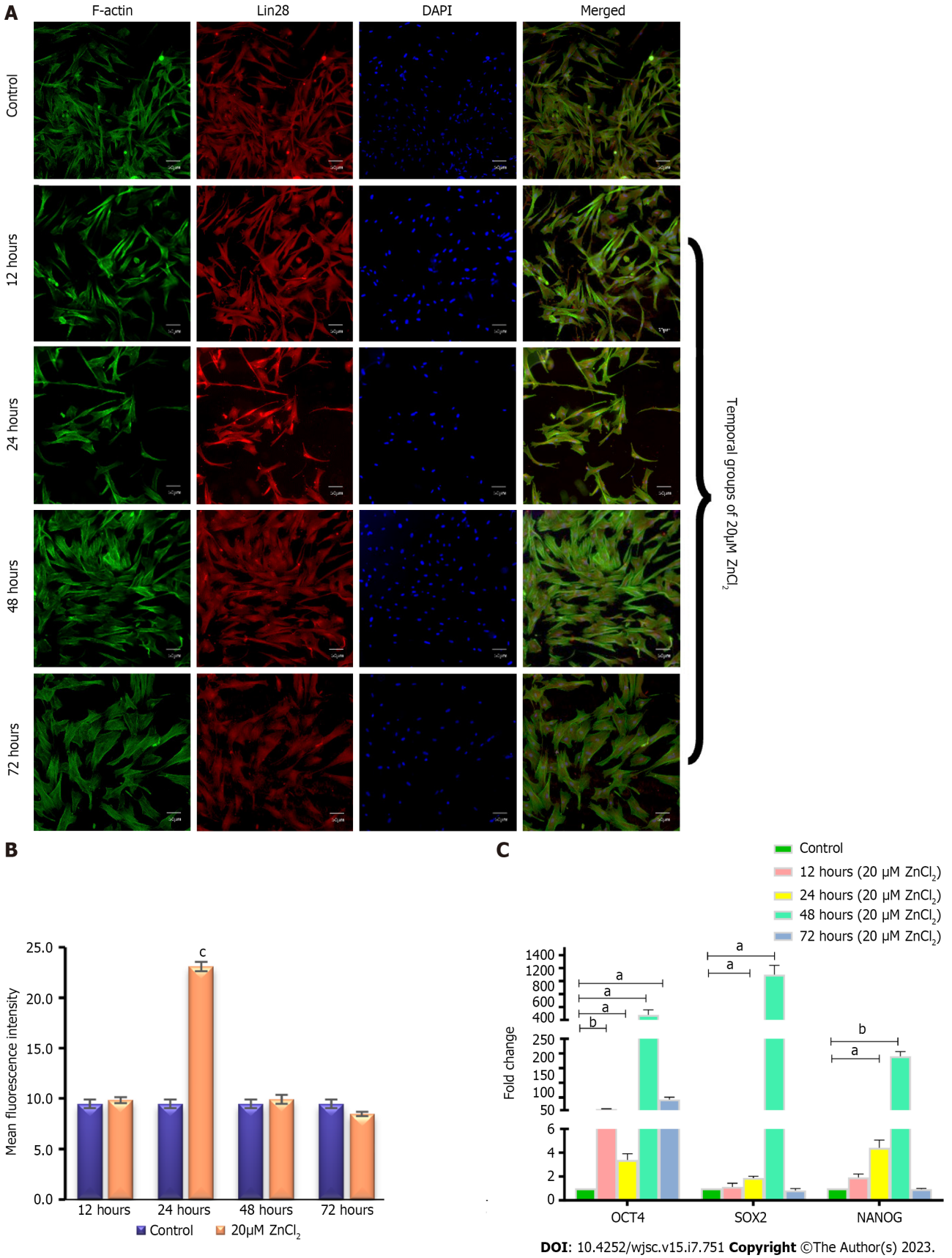
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Figure 6 Effect $ZnCl_2$ on adhesion ability of human umbilical cord-derived mesenchymal stem cells. A: Images of human umbilical cord (hUC)-derived mesenchymal stem cells (MSCs) of the control group and 20 μM $ZnCl_2$ treated groups at 0 min, 15 min, 30 min, 1 h, and 2 h of cell seeding. Bright, rounded, and freely floating cells represent unadhered cells while dark cells represent adhered cells; B: Significant increase was observed in the adhesion ability of hUC-MSCs of the treated group after 15 min ($^bP \leq 0.01$), 30 min ($^aP \leq 0.05$),1 h ($^bP \leq 0.01$), and 2 h ($^cP \leq 0.001$). An independent sample *t*-test was performed to analyze results, and data were represented as mean ± SD ($n = 3$).

expression as compared to control, and after 24 h, all four migration markers exhibited significantly increased expression. After 48 h, *CXCR-4, V-CAM1, and VEGF-A* showed significantly increased expression, and after 72 h all of these genes showed significantly high expression, whereas *MMP2* showed the highest expression. Overall, up-regulation of these genes suggested that Zn also enhanced the expression of migration markers and promoted MSC migration, where fluctuations in their regulation may relate to their role at different stages of cell migration[44]. Studies reported that Zn promotes cell adhesion at lower concentrations in different cell types. Endothelial cells of coronary artery and smooth muscle cells exhibited increased cell adhesion ability when cultured in a $ZnCl_2$ supplemented medium[16,17]. We also found a considerably enhanced cell adhesion rate of MSCs when treated with 20 μM $ZnCl_2$.

Lin28, a pluripotency marker, was analyzed for its protein expression. It is an RNA binding protein that was first characterized in *C.elegans* as a heterochronic gene and was found to control developmental timing. In mammals, two homologs, including Lin28a and Lin28b are present in the cytoplasm as well as in the nucleus[45]. Structurally, Lin28 comprises the N-terminal cold shock domain and two zinc-binding motifs (CCHCx2) located at C-terminal. Lin28 is highly expressed in ESCs, while upon differentiation, its expression is suppressed by miRNAs, namely Let-7 and Lin-4 [45]. It is involved in post-translational modifications of specific subsets of mRNAs. In skeletal muscles, it binds to IGF-2 mRNA[46], while in mouse ESCs it is reported to bind with mRNAs of cell cycle-specific genes[47,48]. OCT4 mRNA is also reported as one of the targets of Lin28 for post-translational modifications[49]. Our results showed positive expression of Lin28 in all temporal groups, however, its expression exhibited fluctuations, *i.e.*, after 24 h of treatment, it was significantly enhanced, and then at later time points, the expression decreased gradually and became approximately similar to control group. It might be because of its post-translational role. That is why it showed higher expression before, *i.e.*, after 24 h than other pluripotency genes that exhibited their higher expression mainly after 48 and 72 h. Altogether, these factors make regulatory circuitry which is regulated through feed-forward loop and autoregulatory mechanism [49], and the fluctuations in their expression at time points may reflect this regulatory circuitry.

OCT4, *SOX2,* and *NANOG*, are the transcription factors that are conserved across mammalian species[50]. All three are highly expressed in ESCs and are responsible for the pluripotency and self-renewal of ESCs[51]. These transcription factors regulate gene expression in a highly cooperative fashion[52]. OCT4 and SOX2 form heterodimers and bind to specific promoters on DNA[53]. NANOG is a homeobox family gene that also forms heterodimer and regulates other pluripotency genes in feedback mechanism[53]. These are also found to have similar role in adult stem cells, including MSCs[11,50,54]. A study carried out by Hu *et al*[55], 2016, saw a significant increase in the expression of *OCT4, SOX2,* and *NANOG* in mouse ESCs when treated with $ZnCl_2$ for 48 h. The study further reported that as ESCs in humans and mice share the same key regulators, *i.e.*, *OCT4, SOX2, NANOG*[55], Zn might exert similar effects regarding the acquaintance and maintenance of the pluripotency in human stem cells. Mnatsakanyan *et al*[56], 2019 also showed significantly increased expression of *SOX2* in mouse ESC after long-term treatment, *i.e.*, 30 days with $ZnCl_2$[56]. In our study, gene



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Figure 7 Protein expression of Lin28 and transcriptional analysis of pluripotency genes. A: Positive expression of mesenchymal stem cells (MSCs) specific marker Lin28 was observed after 12 h, 24 h,48 h, and 72 h of 20 μM $ZnCl_2$ treatment, as visualized by immunocytochemical staining; B: Quantified fluorescent intensities showed that 20 μM $ZnCl_2$ treated MSCs expressed Lin28 protein ($^cP \leq 0.001$); C: Quantitative real-time polymerase chain reaction analysis of pluripotency genes *OCT4, SOX2,* and *NANOG,* exhibited significantly increased ($^aP \leq 0.05$) expression of *OCT4* at 12, 24, 48, and 72 h, while *SOX2,* and *NANOG* showed significantly increased expression at 24 and 48 h ($^aP \leq 0.05$, $^bp \leq 0.01$). Results were analyzed by SPSS, performing an independent sample *t*-test. Data

were analyzed as mean ± SD (n = 3) where significance level $^{a}P \leq 0.05$.

expression analysis of all three genes *OCT4, SOX2, NANOG* showed a significant increase in the test group after 24 and 48 h. After 12 and 72 h of treatment, *SOX2* and *NANOG* did not exhibit any significant difference compared to the control, while *OCT4* represented a significant increase in all temporal groups.

Limitations of this study is that growth kinetics is analyzed by treating cells up to three days with zinc. Further temporal study is required to evaluate the long term effects of zinc. Also the signaling pathways that zinc utilizes to produce its effects are needed to be explored *via* protein quantification. An *in vivo* experimental evaluation is also needed to further support the *in vitro* findings.

CONCLUSION

At lower concentrations, Zn efficiently increased the proliferation of MSCs with a significant increase in the rate of population doublings, and at higher concentrations, it was cytotoxic. MSCs of the test group maintained a higher frequency of mesenchymal progenitors. Upregulation of genes involved in cell cycle, proliferation, pluripotency, and migration, was also observed in test groups. Zn increased the migration capability of MSCs and cell adhesion ability of MSCs. Overall, the results showed that Zn at lower concentrations supported cell growth, division, and proliferation of MSCs while maintaining the MSC-specific characteristics, including pluripotency, migration, and cell adhesion. Hence, Zinc could be a suitable constituent for cell culture medium that would help to enhance the quantity, quality, and rate of *in vitro* proliferation of MSCs for clinical and industrial-scale production. Though fewer studies are reported on Zinc effects on MSCs, further investigations are required to understand the mechanism that Zn utilizes to induce its effects.

ARTICLE HIGHLIGHTS

Research background

Zinc (Zn) is the second most abundant trace element after Fe, present in the human body. It is frequently reported in association with cell growth and proliferation, and its deficiency is considered to be a major disease contributing factor.

Research motivation

Trace elements are required to be supplemented in culturing media that will help to promote proliferation, cell division, and cellular growth, which will result in *in vitro* expansion of mesenchymal stem cells (MSCs) to achieve a desired number of cells for therapeutic dose.

Research objectives

In the present study, we aimed to determine the effect of Zn on *in vitro* growth and proliferation of human umbilical cord (hUC)-derived MSCs.

Research methods

hUC-MSCs were isolated from human umbilical cord tissue and characterized based on immunocytochemistry, immunophenotyping, and tri-lineage differentiation. The impact of Zn on cytotoxicity and proliferation was determined by MTT and Alamar blue assay. To determine the effect of Zn on population doubling time (PDT), hUC-MSCs were cultured in media with and without Zn for several passages. An i*n vitro* scratch assay was performed to analyze the effect of Zn on the wound healing and migration capability of hUC-MSCs. A cell adhesion assay was used to test the surface adhesiveness of hUC-MSCs. Transcriptional analysis of genes involved in the cell cycle, proliferation, migration, and self-renewal of hUC-MSCs was performed by quantitative real-time polymerase chain reaction. The protein expression of Lin28, a pluripotency marker, was analyzed by immunocytochemistry.

Research results

Zn at lower concentrations enhanced the rate of proliferation but at higher concentrations (> 100 μM), showed concentration dependent cytotoxicity in hUC-MSCs. hUC-MSCs treated with Zn exhibited a significantly greater healing and migration rate compared to untreated cells. Zn also increased the cell adhesion rate, and colony forming efficiency (CFE). In addition, Zn upregulated the expression of genes involved in the cell cycle (*CDC20, CDK1, CCNA2, CDCA2*), proliferation (*TGFβ1, GDF5, hypoxia-inducible factor 1α*), migration (*CXCR4, VCAM1, VEGF-A*), and self-renewal (*OCT4, SOX2, NANOG*) of hUC-MSCs. Expression of Lin28 protein was significantly increased in cells treated with Zn.

Research conclusions

Our findings suggest that zinc enhances the proliferation rate of hUC-MSCs decreasing the PDT, and maintaining the CFE. Zn also enhances the cell adhesion, migration, and self-renewal of hUC-MSCs. These results highlight the essential role of Zn in cell growth and development.

Research perspectives

Zinc at lower concentrations supported cell growth, division, and proliferation of MSCs while maintaining the MSC-specific characteristics, including pluripotency, migration, and cell adhesion. This will lead to produce large number of MSCs required for *in vivo* implanation in shorter period of time.

FOOTNOTES

Author contributions: Sahibdad I performed experiments and wrote the original manuscript; Khalid S helped in experimentation and writing; Chaudhry GR evaluated and analyzed the data, and reviewed and edited the manuscript; Salim A evaluated and analyzed the data and reviewed the manuscript; Begum S evaluated and analyzed the data; Khan I conceived and designed the studies, evaluated and analyzed the data, and finalized the manuscript.

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Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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ORIGINAL ARTICLE

Basic Study

Injectable hydrogel made from antler mesenchyme matrix for regenerative wound healing *via* creating a fetal-like niche

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Abstract

BACKGROUND

Scar formation and loss of cutaneous appendages are the greatest challenges in cutaneous wound healing. Previous studies have indicated that antler reserve mesenchyme (RM) cells and their conditioned medium improved regenerative wound healing with partial recovery of cutaneous appendages.

AIM

To develop hydrogels from the antler RM matrix (HARM) and evaluate the effect on wound healing.

METHODS

We prepared the hydrogels from the HARM *via* enzymatic solubilization with pepsin. Then we investigated the therapeutic effects of HARM on a full-thickness cutaneous wound healing rat model using both local injections surrounding the wound and topical wound application.

RESULTS

The results showed that HARM accelerated wound healing rate and reduced scar formation. Also, HARM stimulated the regeneration of cutaneous appendages and blood vessels, and reduced collagen fiber aggregation. Further study showed that these functions might be achieved *via* creating a fetal-like niche at the wound site. The levels of fetal wound healing-related genes, including Collagen III and TGFβ3 treated with HARM were all increased, while the expression levels of

Collagen I, TGFβ1, and Engrailed 1 were decreased in the healing. Moreover, the number of stem cells was increased in the fetal-like niche created by HARM, which may contribute to the regeneration of cutaneous appendages.

CONCLUSION

Overall, we successfully developed an injectable hydrogel made from antler RM matrix for the regenerative repair of full-thickness cutaneous wounds. We uncovered the molecular mechanism of the hydrogels in promoting regenerative wound healing, and thus pave the way for HARM to be developed for the clinic use.

Key Words: Hydrogel; Antler reserve mesenchyme; Regenerative wound healing; Fetal-like niche

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Core Tip: Our study developed an injectable hydrogel made from antler reserve mesenchyme (a tissue suitable for stem cells) matrix for the regenerative repair of full-thickness cutaneous wounds. Moreover, we uncovered the molecular mechanism of the hydrogels in promoting regenerative wound healing, and thus pave the way for HARM to be developed for the clinic use.

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INTRODUCTION

As the biggest organ, skin serves multiple critical protective functions, including preventing harmful microorganism invasion, minimizing body fluid evaporation, and blockage of UV damage[1,2]. Healing in adult skin wounds usually results in scar formation, and scar consists of thick bundles of collagen fibers in the dermis and lacks functional cutaneous appendages (such as hair follicles and sebaceous glands). Scarring can result in patients experiencing bodily discomfort, including heat intolerance, pruritus, thermoregulatory and sensory disabilities, and psychological distress[3,4]. Therefore, realization of regenerative wound healing is the dream in clinics.

Biomaterials, especially the extracellular matrix (ECM) hydrogels, have become a promising strategy for treating severe skin injury[5-7]. ECM provides specific physical and chemical cues that regulate cell behaviors such as cell survival, proliferation, differentiation, and migration[8,9]. Thus far, quite a few types of ECM hydrogels from different animal tissues have been developed, including pig skin, bladder, and human placenta[10,11], and applied for inducing appendage regeneration in adult rat/mouse skin injury models. These hydrogels, however, have limited potential to maintain proliferation, differentiation, and migration of the cells resident in them, resulting in insufficient regeneration of nerves, blood vessels and cutaneous appendages in the healing wounds[10,11]. Therefore, the development of more effective alternative ECM hydrogels is highly desirable.

Deer antlers are the only mammalian organ that can fully regenerate after being lost[12-14]. Antler regeneration begins with regenerative healing of the wounds left after the previous antler casting. Studies show that this regenerative wound healing depends entirely on the adjacent pedicle periosteum (PP) or the PP-derived reserve mesenchyme (RM)[15,16]. The RM cells are a type of adult mesenchymal stem cell but with some embryonic stem cell properties[12,13]. The RM-derived paracrine factors are potential candidates that stimulate cell proliferation, migration and regenerative wound healing[17]. Recently, we applied RM cells through injection to treat rat cutaneous wounds and found that the healed skin consisted of basket-wave-like collagen fibers and numerous cutaneous appendages[18], thus at least partially achieving regenerative wound healing. The ability of the RM cells to stimulate regenerative wound healing is believed to rely on paracrine factors, because the RM cells did not survive in rats for more than 14 d[18]. Subsequently, we topically applied RM-cell-conditioned medium to treat the full-thickness wounds in rats and found that outcome of the healed wounds was comparable to that of using RM cells in a regenerative manner[19]. Therefore, we speculated that the ECM hydrogels prepared from the RM tissue should have similar if not better effects, as the ECM is the secretion of RM cells. Importantly, as a novel injectable hydrogel, antler RM matrix (HARM) is simpler to apply compared to RM cells or RM cell conditioned medium, as it does not need other carrier materials. At the same time, it can also be used as a carrier material for other factors for skin injury treatment.

This study reported the effects of HARM on the healing of full-thickness cutaneous wounds in rats. HARM had a stronger potential to promote skin cell proliferation and migration compared with the hydrogels from porcine urinary bladder matrix (HPUB, as a control), resulting in better wound healing quality. We believe that the regenerative wound healing achieved using the HARM is highly likely through creating a fetal-like niche in the wound area. Thus, HARM has the potential to be developed as a treatment for regenerative wound healing in clinics.

MATERIALS AND METHODS

Preparation of HARM

The preparation of HARM is described in the previous study[5]. Briefly, three 2-branch-antlers were collected from three healthy sika deer (*Cervus nippon*) about 30 d after casting of the previous hard antler buttons in a commercial deer farm (Purchased from Dongao Deer Farm). RM tissue located in the antler growth center of each antler tip was sampled and cut into small pieces (1 mm^3) using two handheld scalpel blades and rinsed with normal saline (NS), ground in liquid nitrogen to obtain RM powder. The RM powder was further mixed with pepsin in 0.01 M HCl with the ratio of 10:1:1 (w:w:v) and the solution was kept shaking at a constant rate of 200 rpm for 48 h at 25 °C. The final pH of the resultant solution (3.0 to 4.0) was adjusted to 7.4 by adding 0.1 M NaOH to inactivate pepsin. HARM was formed when 10 × PBS was added into the solution and stored at 37 °C for 1 h (Supplementary Figure 1A). The predetermined concentration of the hydrogels was made by adding cool 1 × PBS (4 °C). HPUB, a previously reported ECM hydrogel[20], was prepared by the same procedure as HARM.

Surface morphology of the hydrogels was visualized using a scanning electron microscope (SEM, JEO JSM-5600LV, Japan) at a voltage of 10 kV. Lyophilized-HARM (Supplementary Figure 1B) were sputter-coated with a thin layer of gold before observation.

Two-dimensional cell culture

The cell lines of human umbilical vein endothelial cells (HUVEC) and NIH 3T3 fibroblasts were cryopreserved in our laboratory. The mouse adipose mesenchymal stem cells (ADSC) and bone marrow mesenchymal stem cells (BMSC) were obtained from Prof. Yimin Wang of China-Japan Union Hospital of Jilin University. The cells were expanded in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin in an incubator at 37°C, 5% CO_2.

3D cell culture and related assays

Hydrogels were sterilized with UV for 30 min; then cells (1 × 10^5/mL) were mixed into the hydrogels and seeded in 96-well plates incubated at 37 °C with 5% CO_2 for 30 min. The growth medium was then added to each hydrogel and changed every 2 d. The related cells (HUVEC, NIH 3T3, ADSC, BMSC, ADSC, and BMSC) and assays were listed in Table 1.

Creation and treatment of full-thickness wounds in a rat model

Thirty 8-wk-old female SD rats (200-220 g/rat) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China) and were raised under standard laboratory animal feeding conditions. All the rats received humane care, and all study protocols and procedures were approved by the Animal Ethics Committee of Changchun Sci-Tech University (Approval No. CKARI2020012). For skin wounding, rats were anesthetized with 3% pentobarbital sodium (30 mg/kg), and then the hair was removed from the area to be operated (dorsal surface). A 12-mm diameter skin biopsy punch was used to cut the full-thickness skin and make wounds.

The model rats were randomly divided into three groups (HARM, HPUB, and PBS), with each group having 10 rats. HARM was diluted with cold 1 × PBS to the final concentration of 20 μg/mL before use. HARM was administered *via* injection around the wound margins (25 μL/injection × 4 injections) together with topical application over each wound (100 μL/wound) once every 5 d in the first 15 d, thereafter *via* local injection (100 μL) only in the remaining 15 d. The wound healing status was photographed every 5 d and the healing area was calculated using Image-Pro Plus software. PBS solution and HPUB were used as negative control (N-CTRL) and positive control (P-CTRL), respectively. The healed tissues were collected on 15 d post wounding (DPW) and 30 DPW respectively, and each tissue sample was divided into two halves: (1) For protein and RNA extraction; and (2) fixed in 4% paraformaldehyde for histological examination.

Histology

Healed skin tissue was embedded in paraffin and sliced into 5 μm sections. The sections were stained with hematoxylin and eosin (HE), Masson, or immunofluorescence (IF). All antibodies used in IF were listed in Supplementary Table 1. Sections stained with HE and Masson were photographed using the Digital Imaging Scanning System (Precipoint M8; Freising, Germany); and stained with IF and photographed using Fluorescence Microscopes (BX63, Olympus, Japan). The collagen fibers (blue area on micrograph) in Masson staining and the positive expression (fluorescence area on microphage) of the target genes in IF staining were analyzed statistically using Image-Pro Plus software; then the ratio of the target area to the whole area was calculated.

RNA isolation and quantitative real time PCR

Trizol reagent (Sigma-Aldrich, United States) was used for total RNA isolation from the healed skin tissue samples. cDNA was prepared *via* reverse transcription of RNA. cDNA, primers (Supplementary Table 2) and SYBR premix (Roche, Basel, Switzerland) were mixed on quantitative real time PCR (qRT-PCR) using the qTOWER ^{3}G (Analykit Jena AG, Jena, Germany). Results of mRNA quantification were normalized against GAPDH and calculated using the $^{\Delta\Delta}$Ct method. Every experiment was repeated three times.

Western blot

Total proteins were isolated from the healed tissues: 20 μg of total protein was separated *via* polyacrylamide SDS gel and then transferred to a polyvinylidene fluoride membrane (Millipore, MA). After blocking in 5 % (w/v) non-fat milk, the membrane was first incubated with primary antibodies (Engrailed 1, Bioss; GAPDH, ProteinTech) at 4 °C overnight and

then with HRP-conjugated secondary antibody at 25 °C for 2 h. The bands were visualized using an ECL system and quantified using Image J software.

Statistical analysis

Results were expressed as mean ± SEM. Multi-comparisons were made using ANOVA in the package of GraphPad Prism 9.0. Significant differences were evaluated using *t*-test and one-way ANOVA. The difference was considered statistically significant ($^{a}P < 0.05$), or highly significant ($^{b}P < 0.01$, or $^{c}P < 0.001$).

RESULTS

Characterization of HARM

We prepared HARMs (Figure 1A), which had typical gel properties (Figure 1B; Supplementary Figure 1). The effects of HARM on cell proliferation were evaluated *in vitro*. Firstly, the optimum concentration of HARM was determined based on cell proliferation rates. The results showed that proliferation rates of HUVEC, NIH 3T3, ADSC, and BMSC cultured in the medium containing 20 μg/mL HARM all reached their peaks across the concentration range from 0 to 40 μg/mL 7 d after culture (Figure 1C). Further, the proliferation rate of ADSC cultured in 20 μg/mL HARM was significantly higher than in N-CTRL ($P < 0.05$; Figure 1D), and that of BMSC cultured in 20 μg/mL HARM was significantly higher than N-CTRL and P-CTRL ($P < 0.001$; Figure 1E). The differences in cell proliferation rate between cells cultured in HARM and those cultured in N-CTRL or P-CTRL were increased at 10 d after further culture. These results suggest that HARM has great potential to stimulate cell proliferation *in vitro*.

HARM accelerates wound healing rate

To determine the effects of HARM on the rate and quality of cutaneous wound healing, HARM (20 μg/mL) was used to treat full-thickness skin wounds in rats (Figure 2A). The results showed that the areas of the wound in the HARM group were significantly smaller than those in the N-CTRL and P-CTRL groups ($P < 0.001$; Figure 2B and C) on 5 or 10 DPW; wound healing in the HARM group was completed on 20 DPW, but those in N-CTRL and P-CTRL groups were not completed until 30 DPW. These results suggest that HARM can effectively accelerate wound healing rate in the full-thickness skin wounds in rats.

HARM improves the quality of wound healing

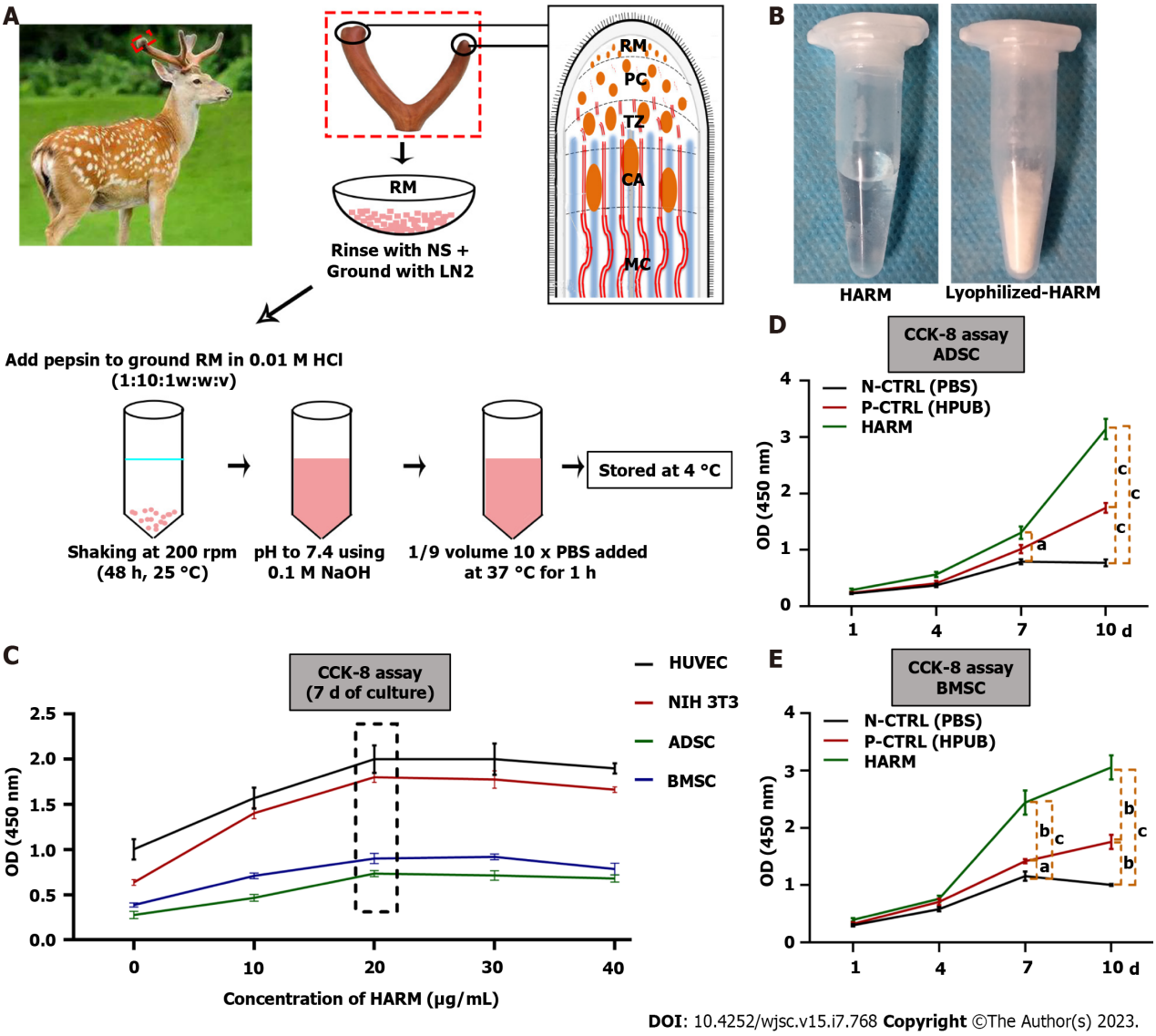
Quality of wound healing was examined at histological level. The results showed significantly more appendages in the HARM group than in the N-CTRL or P-CTRL groups ($P < 0.001$; Figure 3A and B). The collagen fibers of healed skin in the HARM group were fewer and thinner than those in the N-CTRL group ($P < 0.001$; Figure 3A and C) on 15 DPW; moreover, the collagen fibers in the HARM group consisted of basket-wave-like collagens, which were drastically different from the thick-bundle-like collagens in the N-CTRL and P-CTRL groups. Likewise, healed skin in the HARM group on 30 DPW was similar to that in the N-CTRL and P-CTRL groups ($P < 0.01$; Figure 3A, D, and E). The expression levels of CD31 (the surface marker of neovascular endothelial cells[21]) and Ki67 (the marker of cell proliferation and division[22]) in the HARM group were significantly more than those in the N-CTRL or P-CTRL groups both on 15 DPW and 30 DPW (CD31: $P < 0.001$; Figure 3F-H); Ki67: ($P < 0.001$; Supplementary Figure 2). Overall, the results suggest that the HARM treatment can effectively improve the quality of wound healing in rats, and the effects are even better than the HPUB treatment.

HARM promotes the expression of fetal wound healing-related genes in rats

The expression levels of IL10 and TGFβ3, the fetal wound healing-related genes[23,24] were detected in the healed tissues. The results showed that expression levels of IL-10 and TGFβ3 in the healed skin of the HARM group were significantly higher than in those of the N-CTRL or P-CTRL groups on 15 DPW ($P < 0.001$; Figure 4A-C). Likewise, on 30 DPW, these gene expressions had a similar trend to those on 15 DPW ($P < 0.01$; Figure 4A-C). We further evaluated the status of expression of some other fetal wound healing-related genes using qRT-PCR. The results showed that the healed skin of the HARM group favored expression of fetal wound healing genes including collagen III (15 DPW: $P < 0.001$, $P < 0.001$; 30 DPW: $P < 0.01$, $P < 0.01$) and TGFβ3 (15 DPW: $P < 0.01$, $P < 0.01$; 30 DPW: $P < 0.001$, $P < 0.001$); but did not favor expression of scar healing genes including collagen I (15 DPW: $P < 0.01$, $P < 0.05$; 30 DPW: $P < 0.001$, $P < 0.01$) and TGFβ1 (15 DPW: $P < 0.01$, $P < 0.01$) when compared with those in the N-CTRL and P-CTRL groups to varying degrees (Figure 4D, E, G and H). The ratios of collagen III to collagen I and TGFβ3 to TGFβ1 in the healed tissue of the HARM group were all significantly higher than in those of N-CTRL or P-CTRL groups ($P < 0.05$; Figure 4F and I). Furthermore, the expression of Engrailed 1, activated in adult wound healing and prevented in fetal wound healing[25], was also down-regulated with the HARM treatment in the healed tissues (Figure 4J and K). These results suggest that HARM promotes regenerative wound healing which may be achieved *via* creating a fetal-like wound healing niche.

HARM promotes stem cell recruitment and/or plasticity maintenance

Next, the effects of the fetal-like wound healing niche created by HARM on recruitment of stem cells and maintenance of plasticity were detected. IF staining results showed that healed skin of the HARM group expressed significantly higher CD73 and CD90 than that of the N-CTRL or P-CTRL groups ($P < 0.01$, $P < 0.001$; Figure 5A-C) on 15 DPW. Likewise, on 30 DPW, a similar expression trend was observed as on 15 DPW ($P < 0.001$; Figure 5A, D and E). These results suggest that



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Figure 1 Preparation of hydrogels from the antler reserve mesenchyme matrix and its effects on cell proliferation *in vitro*. A: Location of reserve mesenchyme (RM) and fabrication of hydrogels from the antler reserve mesenchyme matrix (HARM); B: HARM (top) and lyophilized-HARM (bottom); C: Screening of the optimal concentration of HARM on cell [HUVEC, NIH 3T3, adipose mesenchymal stem cells (ADSC), and bone marrow mesenchymal stem cells (BMSC)] proliferation rate; D and E: Comparison of HARM with hydrogels from porcine urinary bladder matrix (HPUB) on cell (ADSC and BMSC) proliferation rate. Note that the concentration of 20 μg/mL HARM had the strongest stimulation over the others on proliferation rate of HUVEC, NIH 3T3, ADSC, and BMSC; moreover, proliferation rates of ADSC and BMSC in HARM were stronger than in N-CTRL (PBS) or in P-CTRL (HPUB). RM: Reserve mesenchyme; PC: Pre-cartilage; TZ: Transitional zone; CA: Cartilage; NS: Normal saline; LN2: Liquid nitrogen; HARM: Hydrogels derived from antler reserve mesenchyme; HPUB: Hydrogels from porcine urinary bladder matrix; HUVEC: Human umbilical vein endothelial cells; NIH 3T3: Mouse embryonic fibroblasts; ADSC: Adipose mesenchymal stem cells; BMSC: Bone marrow mesenchymal stem cells; N-CTRL: Negative control; P-CTRL: Positive control. $n = 3$; mean ± SEM; statistically significance set at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

HARM may promote recruitment of stem cells or maintenance of plasticity which grows in the fetal-like wounding healing niche.

DISCUSSION

Scarring in wound healing is still a challenge in the clinic, where the goal is regeneration. The regeneration of functional cutaneous appendages and organized collagen is a complex process involving multiple factors and cells related to the microenvironment. Keeping the cells that regenerate the injured tissues in the optimum environment may be a practical approach to induce cutaneous regeneration and prevent abnormal scar formation. Although many approaches, such as synthetic hydrogels or ECM hydrogels, have been evaluated to improve the environment during wound healing, the outcomes have been unsatisfactory. This study is the first to report the role of an acellular ECM hydrogel prepared from stem cell tissue (antler RM) in cutaneous wound healing. Through a full-thickness rat model, we found that HARM improved the regeneration of cutaneous appendages and blood vessels and reduced the aggregation of disorganized

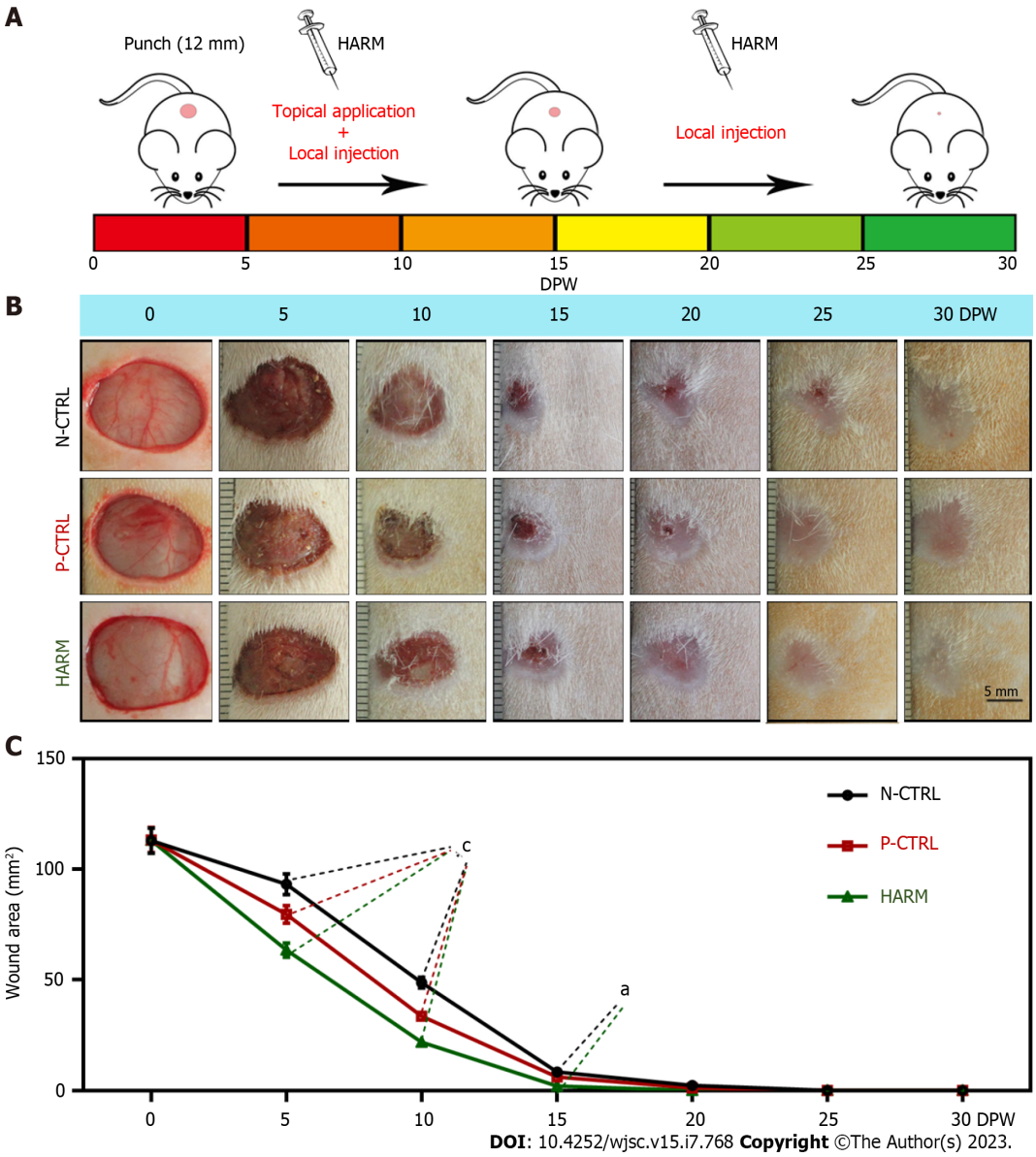


Figure 2 Effects of hydrogels from the antler reserve mesenchyme matrix on wound healing rate in full-thickness skin defects in rats. A: Experimental procedure; B: Macroscopically observation of the wound healing status; C: Quantification of the wound area using the Image J software according to macroscopical images. Note that the fastest wound healing rate and the smallest scar area were observed in the hydrogels from the antler reserve mesenchyme group. DPW: Days post-wounding; HARM: Hydrogels derived from antler reserve mesenchyme; N-CTRL: Negative control; P-CTRL: Positive control. Mean ± SEM; statistical significance set at $^{a}P < 0.05$, $^{c}P < 0.001$.

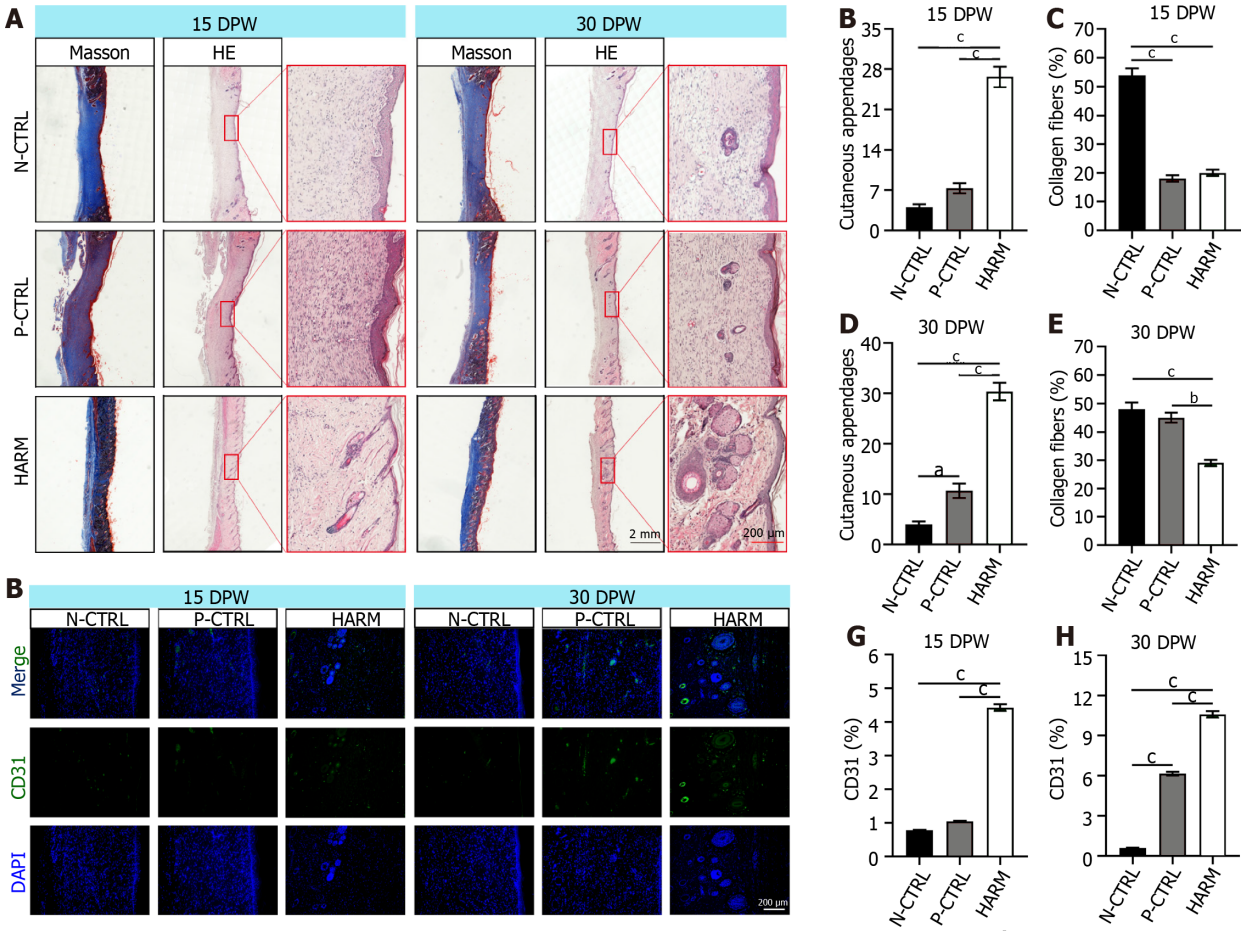
collagen fiber, which may be achieved *via* creating a fetal-like niche at the wound site.

Injectable hydrogels, as a promising strategy, are being used with increasing frequency for the treatment of wound healing. They have several desirable features, including targeted delivery by minimally invasive techniques, ability to fill an irregularly shaped space quickly, and polymerization to form a support structure suitable for host cell infiltration and remodeling[26]. The injectable hydrogels derived from naturally occurring biologic materials are claimed to possess superior biocompatibility and bioactivity compared to their synthetic counterparts, which are usually composed of the ECM of decellularized tissues, and can be partially digested with pepsin, solubilized, and polymerized in situ to form a hydrogel[20,27,28]. Intact ECM hydrogels retain numerous molecular constituents found in the native tissue, such as cell adhesion proteins and growth factors, and these hydrogels support a constructive, site appropriate, remodeling response when implanted in a variety of anatomical sites[29]. It is possible that a hydrogel formed from enzymatically degraded and solubilized ECM may maintain some of the biological activity found in the intact ECM. ECM hydrogels have been prepared from various tissues, including urinary bladder[20], dermis[27], cardiac tissues[28], adipose tissue[30], small intestine[31], and skeletal muscle[32]. The hydrogels derived from these tissue types differ in their composition and activity. However, a common problem is the lack of practical factors to stimulate tissue regeneration/repair, although they do induce a certain level of repair on tissue injury. Therefore, selecting suitable tissue for ECM hydrogel preparation is essential, especially if the tissue is rich in regeneration-stimulated factors; a tissue suitable for stem cell survival may be suitable and advantageous. In the annual regeneration of deer antlers, the RM tissue layer in the growth center is responsible for the growth of deer antlers with a growth rate of up to 2.75 cm/d[33]. RM-derived cells have the character-

Table 1 *In vitro* experiment design

Test purposes	Cell type	Culture system					Culture time	Test type		Required reagents
Screen the optimum concentration of HARM on cell culture	HUVEC NIH 3T3 ADSC BMSC	HARM (0 μg/mL)	HARM (10 μg/mL)	HARM (20 μg/mL)	HARM (30 μg/mL)	HARM (40 μg/mL)	7 d	CCK-8 assay		CCK-8 (Beyotime, Shanghai, China)
Compare the performance of HARM to HUPB on cell proliferation	ADSC BMSC	HARM (20 μg/mL)	P-CTRL (HUPB, 20 μg/mL)		N-CTRL (PBS)		1, 4, 7, 10 d	CCK-8 assay	Nuclear staining	CCK-8 (Beyotime, Shanghai, China)

HUVEC: Human umbilical vein endothelial cells; NIH 3T3: Mouse embryonic fibroblasts; ADSC: Adipose mesenchymal stem cells; BMSC: Bone marrow mesenchymal stem cells; HARM: Hydrogels from antler reserve mesenchyme; HUPB: Hydrogel from porcine urinary bladder matrix; P-CTRL: Positive control; N-CTRL: Negative control.

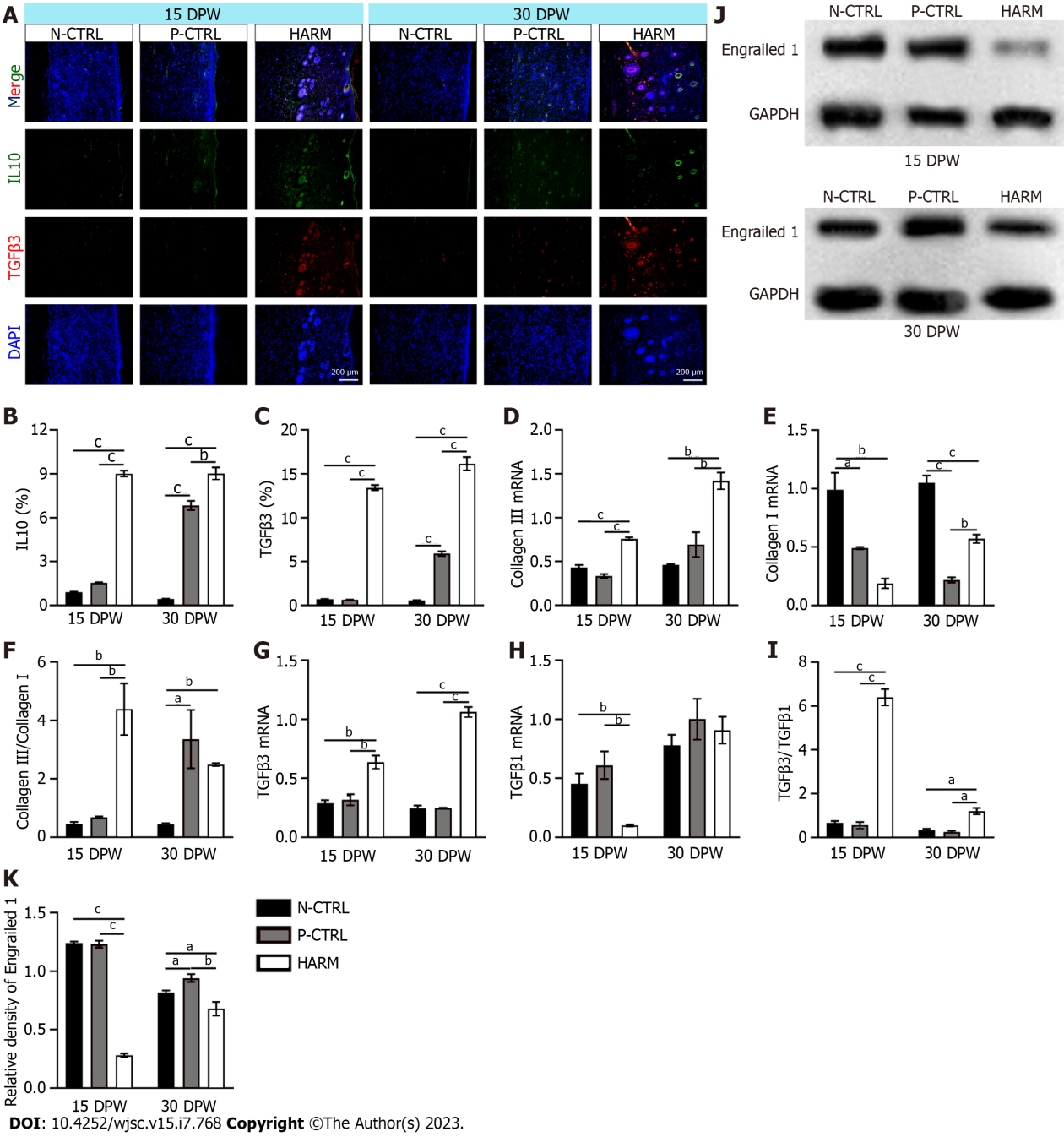


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Figure 3 Effects of hydrogels from the antler reserve mesenchyme on the quality of wound healing. A: Histological sections of the healed skin stained with HE and Masson; B-E: Quantification of cutaneous appendages and the collagen fiber accounts (blue area in Masson staining); F: CD31 immunofluorescence staining; G and H: Expression percentage of CD31. Note that the highest number of cutaneous appendages, least account of collagen fibers, and highest percent were found in the HARM group in the healed tissues compared to the N-CTRL and P-CTRL groups. DPW: Days post-wounding; HARM: Hydrogels derived from antler reserve mesenchyme; N-CTRL: Negative control; P-CTRL: Positive control. $n = 3$; mean ± SEM; statistical significance set at $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

istics of both mesenchymal and embryonic stem cells with strong abilities of proliferation/division and paracrine function, and low immunogenicity[12,13,17].

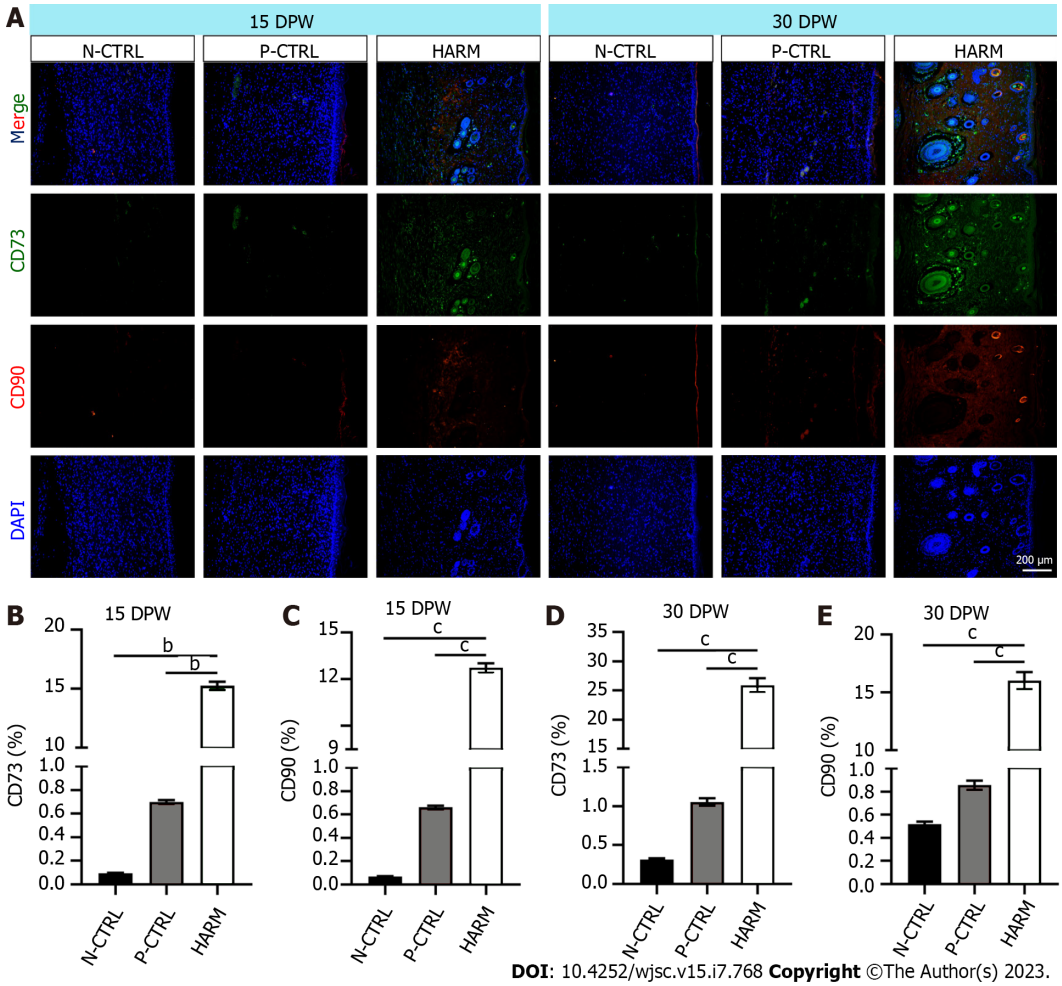
In the present study, we developed a hydrogel from the antler RM matrix, which may retain similar components and characteristics of the RM tissue. It can be kept as a solution at 4 °C and quickly transitions to a hydrogel at 37 °C (average



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Figure 4 Effects of hydrogels from the antler reserve mesenchyme on the expression of typical genes related to fetal wound healing in healed skin of rat wounds. A: IL 10 and TGFβ3 immunofluorescence staining; B and C: Expression percentages of IL-10 and TGFβ3; D-I: mRNA expression levels of collagen III (D), collagen I (E), TGFβ3 (G), and TGFβ1 (H) *via* quantitative real time PCR assay; the ratios of collagen III/collagen I and TGFβ3/TGFβ1 (F and I); J and K: Protein expression of Engrailed 1 in the healed skin *via* Western blot assay. Note that hydrogels from the antler reserve mesenchyme up-regulated the expression levels of IL10, TGFβ3, and collagen III; but down-regulated the expression levels of collagen I, TGFβ1, and Engrailed 1. IL 10: Interleukin 10; TGFβ1: Transforming growth factor β1; TGFβ3: Transforming growth factor β3; DPW: Days post-wounding; HARM: Hydrogels derived from antler reserve mesenchyme; N-CTRL: Negative control; P-CTRL: Positive control. $n = 3$; mean ± SEM; statistical significance set at $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

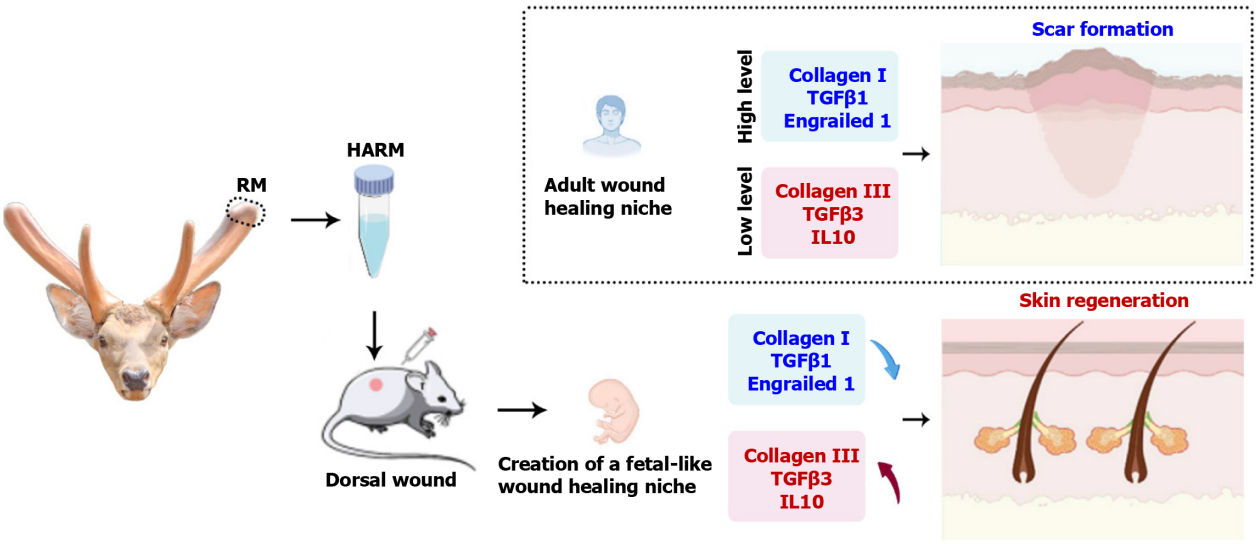
human body temperature; Supplementary Figure 1A). This is clinically important since the biomaterial as a solution can be used in the injection method and quickly reach a gel state that protects the integrity of the tissue and allows for native cells to adhere and grow. Importantly, HARM exhibits pores on the order of 100 μm (Supplementary Figure 1), providing an ideal space for cell division and proliferation. Further, we found that the concentration of 20 μg/mL HARM showed the strongest stimulation over the other concentrations on the proliferation rate of HUVEC, NIH 3T3, ADSC, and BMSC; moreover, proliferation rates of ADSC and BMSC cultured in HARM *in vitro* and cutaneous cells treated with HARM *in vivo* were both stronger than the previously reported hydrogel, HPUB. In general, our results demonstrated the advantage of HARM for cell proliferation, which is essential for reconstructing the lamellar stromal structure using acellular biomaterial.



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Figure 5 Effects of hydrogels from the antler reserve mesenchyme on the expression of stem cell markers in healed skin of rat wounds. A: CD73 and CD90 immunofluorescence staining; B and C: Expression percentages of CD73 and CD90 on 15 DPW; D and E: Expression percentages of CD73 and CD90 on 30 DPW. Note that the HARM group had the highest percent of CD73 and CD90 compared to the other two groups. DPW: Days post-wounding; HARM: Hydrogels derived from antler reserve mesenchyme; N-CTRL: Negative control; P-CTRL: Positive control. n = 3; mean ± SEM; statistically significant at $^{b}P < 0.01$, $^{c}P < 0.001$.

Based on the above characteristics of HARM, we used the full-thickness rat wound to evaluate the effects of HARM on wound healing. As expected, HARM effectively promoted the healing rate and improved the healing quality; these effects were better overall than the HPUB. To the best of our knowledge, this is the first report that a hydrogel derived from a tissue matrix can promote not only the healing rate but also healing quality. However, hydrogels from other tissue matrices can only promote wound healing[34,35]. The microenvironment of wound healing has been reported to be critical in influencing the type of healing. An ideal microenvironment, such as fetal wound healing, results in scarless regenerative healing; a normal microenvironment, such as adult wound healing, results in scarring[23,24]. There are many differences between fetal and adult wound healing, such as inflammatory reactions, growth factors and collagen synthesis[23,24]. It has been reported that IL10 expression is up-regulated in fetal wounds without scars, and wound healing occurs with scar formation in fetal mice with IL-10 deficiency. However, in clinical trials by intradermal injection of recombinant human IL-10 into the wound edge for 1 mo, IL-10 treatment reduced scar formation[36]. In the present study, we found that the expression of IL10 was increased in the healed tissues that had been treated with HARM. TGFβ, including TGFβ1, TGFβ2, and TGFβ3, signals are required for wound repair and regeneration. TGFβ1 causes scar formation after skin injury; on the contrary, TGFβ3 has an anti-fibrotic effect because the ratio of TGFβ3/TGFβ1 in fetal wounds was found to be higher than that in adults[36-38]. We also found that TGFβ3 was increased by HARM in the wound, while TGFβ1 was the opposite. Fetal wound healing differs from adult wound healing in collagen synthesis in terms of the speed of deposition, variation in collagen type ratios, and quantity of collagen. Most striking is the persistence of excess collagen III over collagen I, with healed wounds in the fetus; higher levels of collagen III yield smaller, reticular structures with more cross-linking than collagen I and contribute toward scarless wound healing. In the present study, we found that collagen III was increased by HARM in the wound, while TGFβ1 was the opposite. Engrailed 1 is another typical gene that is activated in adult wound healing, and preventing the activation would inhibit the scarring and achieve fetal-like wound healing[25]. This gene was also down-regulated with HARM treatment in the healed tissues. Above all, the HARM treatment favored expressions of fetal wound healing genes, including IL10, collagen III, and TGFβ3; but did not favor expression of scar healing genes, including collagen IS, TGFβ1, and Engrailed 1,



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Figure 6 Hydrogels from the antler reserve mesenchyme promoted regenerative wound healing *via* creating a fetal-like niche. FMT inhibition. hydrogels from the antler reserve mesenchyme up-regulated the expression levels of fetal wound healing-related genes [interleukin 10, transforming growth factor β3 (TGFβ3), and collagen III]; but down-regulated the expression levels of adult wound healing-related genes (collagen I, TGFβ1, and Engrailed 1). HARM: Hydrogels derived from antler reserve mesenchyme; IL 10: Interleukin 10; TGF: Transforming growth factor.

in the healed tissues. Thus, we speculate that the effects of HARM on regenerative wound healing may be achieved *via* creating a fetal-like niche.

CONCLUSION

This study prepared a new injectable hydrogel using a stem cell tissue matrix, the antler reserve mesenchymal matrix. The therapeutic effects of the hydrogels were evaluated using full-thickness rats. The results revealed that the hydrogels effectively increased the healing rate and improved the healing quality of the wound, including regeneration of the cutaneous appendages and formation of the basketweave-like pattern of collagens. Further study showed that these effects of the hydrogels were likely achieved through creating a niche similar to the regeneration of fetal skin, thereby promoting skin regeneration without scars (Figure 6). Overall, we believe that our prepared hydrogels may have clinical benefits for stimulating regenerative wound healing, especially those large cutaneous wounds caused by burns, scalds, or machinery.

ARTICLE HIGHLIGHTS

Research perspectives

It believes that hydrogels from the antler reserve mesenchyme matrix (HARM) may have clinical benefits for stimulating regenerative wound healing, especially those large cutaneous wounds caused by burns, scalds, or machinery.

Research conclusions

This study prepared a new injectable hydrogel from antler reserve mesenchyme (RM) for regenerative wound healing *via* creating a fetal-like niche.

Research results

HARM was successfully prepared from antler RM. Through a full-thickness rat model, it was found that HARM improved the regeneration of cutaneous appendages and blood vessels and reduced the aggregation of disorganized collagen fiber, which may be achieved *via* creating a fetal-like niche at the wound site.

Research methods

The HARM was prepared *via* enzymatic solubilization with pepsin. Then the therapeutic effects of HARM on a full-thickness cutaneous wound healing rat mode were investigated.

Research objectives

To develop an injectable hydrogel made from antler RM matrix for the regenerative repair of full-thickness cutaneous wounds, which may have clinical benefits for stimulating regenerative wound healing, especially those large cutaneous wounds caused by burns, scalds, or machinery.

Research motivation

Deer antlers are the only mammalian organ that can fully regenerate after being lost. Antler regeneration begins with regenerative healing of the wounds left after the previous antler casting. Studies show that this regenerative wound healing depends entirely on the adjacent pedicle periosteum (PP) or the PP-derived RM.

Research background

Scarring in wound healing is still a challenge in the clinic, where the goal is regeneration. Keeping the cells that regenerate the injured tissues in the optimum environment may be a practical approach to induce cutaneous regeneration and prevent abnormal scar formation. Although many approaches, such as synthetic hydrogels or extracellular matrix hydrogels, have been evaluated to improve the environment during wound healing, the outcomes have been unsatisfactory.

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FOOTNOTES

Author contributions: Li CY and Zhang GK designed and supervised the research; Zhang GK, Ren J, and Li JP performed the research and analyzed the data; Wang DX and Zhang GK contributed to animal experiments; Ren J contributed to histopathological experiments; Li JP and Wang SN contributed to cell experiments; Zhang GK, Shi LY, and Li CY wrote and edited the paper; and all authors read and approved the final manuscript.

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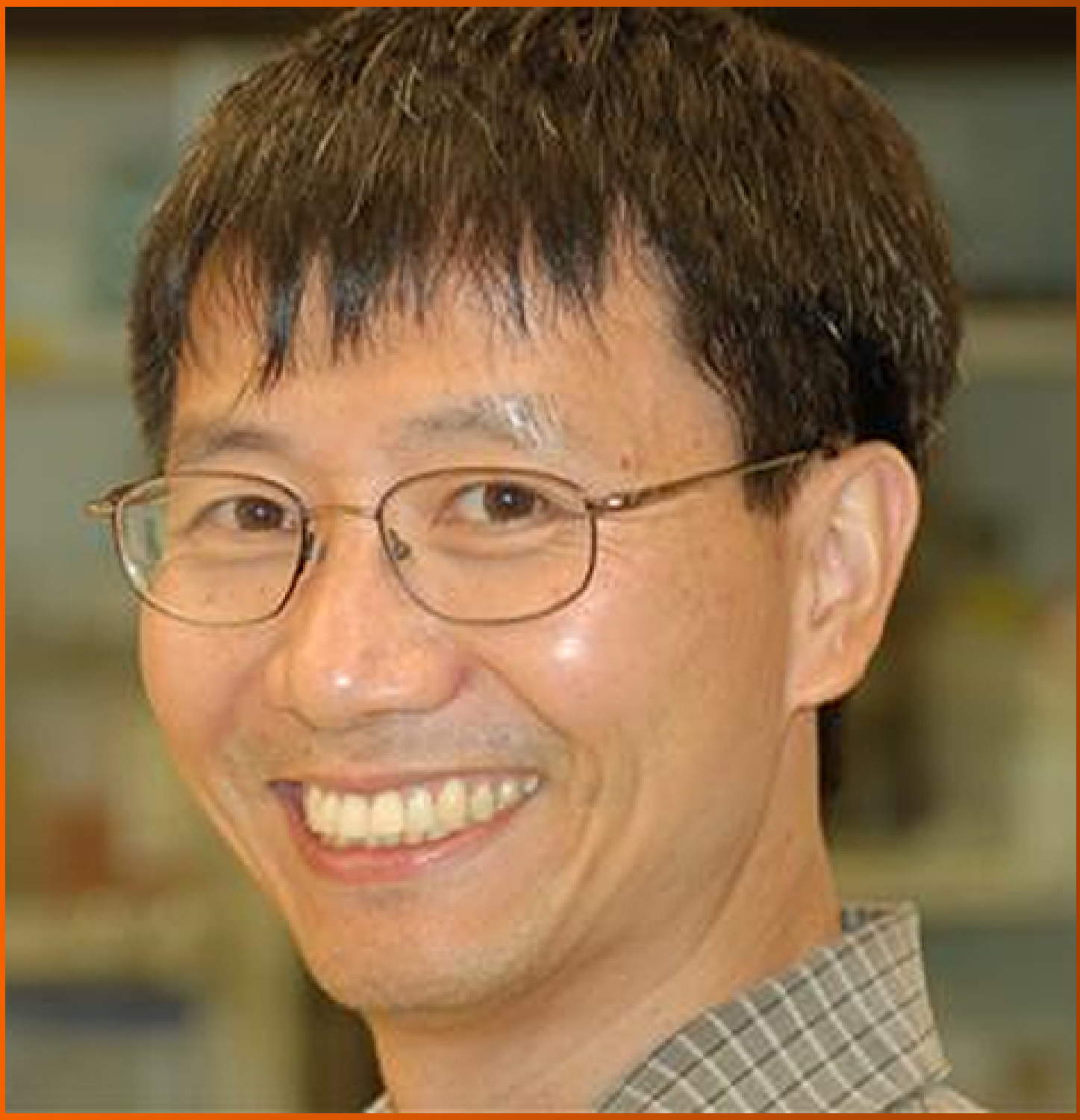


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AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

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EDITORIAL

Mastering the craft: Creating an insightful and widely-cited literature review

Shengwen Calvin Li

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Abstract

The art of constructing an insightful literature review manuscript has witnessed an exemplar in the work of Oz *et al* (2023), wherein concept progression harmoniously merges with figures and tables. Reflecting on retrospective data science, it is evident that well-cited articles can wield a transformative influence on the Journal Citation Reports Impact Factor score, as exemplified by Robert Weinberg's landmark on cancer (Hanahan and Weinberg, 2011). Here, we aim to spotlight a commendable contribution by Tuba Oz, Ajeet Kaushik, and Małgorzata Kujawska in this issue while pivoting towards identifying the hallmarks of a subpar literature review-elements that hinder rather than promote advancement. The hurdles and roadblocks encountered within subpar literature reviews are multifold. Anticipation of emerging trends, identification of challenges, and exploration of solutions remain conspicuously absent. Original Contributions fail to surface amidst the vast sea of pre-existing literature, with noticeable gaps amplified by the lack of illustrative figures and tables. The manuscript, at times, assumes a skeletal form, reflecting an attempt to accommodate an excess of references, leading to convoluted sentences laden with citations. In contrast, a potent solution lies in adopting a comprehensive approach. A nuanced and critical evaluation of sources can culminate in a robust discussion, surpassing the mere summarization of conclusions drawn by others. This approach, often dismissed, holds the potential to elevate clarity, coherence, and logical flow, ultimately inviting engaged readership and coveted citations. The critical necessity of integrating visionary insights is underscored and achieved through a rigorous analysis of pivotal concepts and innovative ideas. Examples can be harnessed to elucidate the application of these solutions. We advocate a paradigm shift, urging literature review writers to embrace the readers' perspective. A literature review's purpose extends beyond providing a comprehensive panorama; it should illuminate avenues for concept development

within a specific field of interest. By achieving this balance, literature reviews stand to captivate a devoted readership, paving the way for manuscripts that are both widely read and frequently cited. The pathway forward requires a fusion of astute analysis and visionary insights, shaping the future of literature review composition.

Key Words: Literature review; Concept progression; Data science; Journal Citation Reports Impact Factor; Original contributions; Comprehensive approach; Clarity and coherence; Visionary insights; Reader engagement; Concept development

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Core Tip: This manuscript highlights a remarkable literature review, demonstrating a seamless fusion of concept progression with figures and tables. Reflecting on data science, it reveals how influential articles can impact the Journal Citation Report Impact Factor. We focus on identifying elements hindering effective literature reviews. Key challenges include absent trend anticipation, gaps in original contributions, and skeletal content. To address this, we propose a comprehensive approach involving critical evaluation, fostering clarity, coherence, and reader engagement. Integration of visionary insights and examples further enhances the impact, emphasizing the importance of concept development and paving the way for influential literature reviews.

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INTRODUCTION

As an editor-in-chief and academic editor, I have not read an insightful literature review until now[1], which came with concept progression well illustrated in figures and tables. Second, retrospective data science revealed a few well-cited articles elevated a Journal Citation Reports Impact Factor score tremendously, *e.g.*, the hallmarks of Cancer by Robert Weinberg, accumulating 69346 citations[2]. Thus, I would like to point out a good one in this issue by Tuba Oz, Ajeet Kaushik, and Małgorzata Kujawska, which might help extrapolate some elements from their writing. However, rather than talk about good elements, I wanted to illustrate the list of inferior elements: The elements that construct a sub-standard literature review, as my conviction was that spotting roadblocks and putting forth measures to tackle those help take success to a higher level.

HURDLES AND ROADBLOCKS

The manuscript text lacked anticipation of any trends, failed to identify or provide answers to particular challenges or problems, and did not identify any leading problems or exceptional opportunities in the sub-field. Given the abundance of existing literature in the field, no original contributions were apparent in the content. Additionally, there seemed to be general gaps in the material, particularly evident in the absence of figures and tables.

Certain sections of the text appeared to be very sketchy, giving the impression that the manuscript was significantly cut down to include over 100 references, and specific sentences crowded cited up to 10 references in one phrase without details on what was about each reference. Instead of merely describing conclusions drawn by other authors, *i.e.*, regurgitating the known facts and data, the authors did not propose their insightful themes based on updating the article by engaging in a more thorough analysis of key concepts and ideas to explore uncharted water, by asking new questions and offering innovative speculations.

The final obstacle in our pursuit was intricately related to the meticulous referencing of the primary data articles. It went beyond the straightforward act of merely acknowledging the sources; it encompassed the intricate task of accurately framing distinct data sets and explicitly attributing terminologies to their original sources. This presented a distinct challenge: The emergence of crowd-group-article citing, where up to seven citations were amalgamated within a single sentence or even a solitary phrase.

CIRCUMVENTING HURDLES IN THE ROAD LEADS TO WELL-READ AND FREQUENTLY-CITED MANUSCRIPTS IN A LITERATURE REVIEW

This flush-out thinking would involve drawing on a range of relevant sources to produce a detailed and insightful discussion. Such critical analysis would be preferable to the alternative of simply summarizing the conclusions drawn by

others. If addressed, all the above elements, particularly the visionary element, might lead to better clarity, coherence, and logical flow, which earns readership and citations. I could illustrate how to overcome these hurdles with examples. My conviction: Advancements in literature review manuscripts require vision, foresight, assiduity, tenacity, determination, and the unwavering courage to go where others fear and hesitate. Why not? Why not consider the unimaginable? It is often claimed that success comprises 99% hard work and 1% inspiration: Both are essential, and neither is a minus.

"Imagination is far more important than knowledge". These profound words, which sprang from the mind of a genius named Albert Einstein, reverberate through the halls of time, lighting a flame of inspiration that guides us beyond the limitations of what we know. Einstein tells us that the power of imagination is the lighthouse that lights up new horizons, which is a crucial point to remember in a society where facts and data often take center stage.

Consider the following: Every spectacular discovery, every extraordinary creation, and every creative leap began in the limitless world of the human imagination. Knowledge provides us with the instruments to comprehend, but imagination gives us the wings to transcend. Such a wing's power propels us sky-high to bird-view unexplored territory and invites us to dance on the edge of the known while welcoming us to embrace the mysteries that lie beyond.

Then there are inquiries, which are unassuming yet powerful forces that propel forward-growth. "Asking questions is more important than knowledge", remarked Albert Einstein. How accurately this is said! The very act of questioning, of attempting to understand the complexities of the cosmos, is a declaration of both our intrinsic curiosity and our unyielding willpower to learn more. Questions kindle the flame of discovery, taking us down roads we've never traveled before and opening the door to worlds we would never have imagined were possible.

Imagine a world in which people simply accepted what they already knew without any attempt to learn more or to satisfy their natural curiosity. In such a world, there would be no development or invention, and people would just sit about doing nothing productive. But we are not content to live in that reality because we are driven by an insatiable appetite to learn more, to understand better, and to elevate ourselves beyond the confines of the present. This hunger compels us to seek out new information and new perspectives.

The words of Albert Einstein are a tribute to the reality that knowledge is a basis but that imagination and inquiry are the wings that propel us to new heights. Knowledge is a foundation. Therefore, let us not be afraid to fantasize beyond the bounds of what is already known. Let us use the power of imagination to conceive of worlds that have not yet been seen and possibilities that have not yet been investigated. And let us never stop asking questions; for by doing so, we create the path for a future that is brighter, bolder, and bursting with the grandeur of human potential. Let us never stop asking questions.

Along the line of referencing, the challenge was not only ensuring accurate citations but also preserving the content's clarity and flow. The risk of overwhelming the reader with an excess of citations was ever-present, demanding a judicious selection process. Each citation had to be carefully evaluated for its relevance and its ability to enhance the understanding of the topic under discussion. Navigating this obstacle required a delicate balance between comprehensive acknowledgment and maintaining the coherence of the narrative. It necessitated the establishment of a coherent thread that seamlessly interwove the multitude of sources while upholding the integrity of the information presented. The complexity lay in recognizing that each citation represented a distinct facet of the overarching concept, contributing a unique perspective to the discourse. It necessitated precision in language, strategic distribution of citations, and a steadfast commitment to maintaining clarity. This intricate dance between referencing and readability was a testament to our dedication to scholarly rigor while delivering information in a manner that was accessible and engaging to our audience. Through this process, we not only conquered the hurdle of crowd-group citing but also upheld the standards of meticulous scholarship in our work.

THE EXAMPLES

The first classical example is the literature review "the hallmarks of Cancer" by Robert Weinberg and his collaborator Douglas Hanahan. They proposed six elements, including maintaining proliferative signaling, evading growth inhibitory signals, resisting programmed cell death, facilitating perpetual replication potential, inducing the formation of new blood vessels (angiogenesis), and triggering processes of cellular invasion and metastasis as the hallmarks of cancer in 2010[3], earning 41039 citations. Two underlying causes are fundamental to acquiring these hallmark traits: Genomic instability, which drives the rapid accumulation of genetic variants, and inflammation, which supports several hallmark activities. Beyond the boundaries of the cancer cells themselves, tumors add another level of complexity due to the presence of a group of cells that, on the surface, appear normal but are actually recruited to help the tumor develop its distinctive traits. This environment is known as the "tumor microenvironment". It is hoped that acknowledging these concepts' broad applicability would substantially impact the development of cutting-edge methods for battling human cancer.

Over the past ten years, there has been a remarkable surge of innovation in our understanding of fundamental concepts beyond the original six-element hallmarks. This period of intellectual evolution has unveiled a pair of promising new frontiers that hold the key to widespread applicability. These cutting-edge developments, akin to beacons illuminating uncharted territory, encompass the manipulation of energy metabolism and the art of outwitting the immune system's defenses, resulting in eight hallmarks of Cancer by Robert Weinberg, accumulating 69346 citations[2].

The canvas of knowledge has been enriched by an exciting brushstroke, as reprogramming the intricate dance of energy within cells emerges as a captivating endeavor. Imagine orchestrating a symphony within the microscopic realm, fine-tuning the metabolic pathways that govern life's essence as spatiotemporal regulation[4]. This spatial omics biology endeavor promises not only to enhance our ability to engineer solutions to a spectrum of challenges but also to unlock the potential for revitalizing weary cells, paving the way for novel therapies and rejuvenation.

In parallel, we witness a daring expedition into the realm of immune evasion-a pursuit that mirrors the cunning strategies of spies and adventurers. Just as an artful rogue slips through the clutches of pursuers, scientists are unraveling the enigmatic mechanisms that allow specific cells to evade the immune system's watchful eye, in particular, Carl June's invention of chimeric antigen receptor T cells immunotherapy (5702 citations)[5] in a designer fashion[6]. This tantalizing avenue opens avenues to fortifying our defenses against diseases, enabling medical interventions to bypass the barricades that often hinder treatment progress.

Together, these eight emergent pillars of insight symbolize a profound transformation in our comprehension of cancer's intricate dance. They beckon us to a landscape where creativity interlaces with knowledge, inviting us to craft innovative solutions, reinvigorate cellular vitality, and conquer challenges once deemed insurmountable. As we embark on this exhilarating voyage of discovery, the horizons of possibility stretch ever wider, promising a future enriched by the fusion of imagination and scientific achievement. All of the above visional elements earned 69346 citations, much higher than their previous 2000's article, which had 41039 citations on August 7, 2023, reflecting the conceptual development that attracts the increased readership.

The second example of a highly specialized and small domain is my own research to illustrate some visional elements by proposing new terminologies. We applied a new method that has been well used (1207 citations)[7]. We presented two new terminologies, the caveolin scaffolding domain (CSD) (969 citations)[8] and the CSD binding motif (CBM) (1129 citations)[9]. Both CSD and CBM have been well studied, which led to assistance to therapeutic development like albumin-bound (nab) paclitaxel (nab-paclitaxel; Abraxane), a $2.9 billion platform of cancer drug delivery by Patrick Soon-Shiong[10]. They further found that elevated levels of CAV1/2 expression are linked to poorer disease-free survival (DFS) and overall survival (OS) outcomes among individuals treated with paclitaxel. In contrast, among patients treated with nab-paclitaxel, heightened CAV1/2 expression appears to correlate with improved pathologic complete response rates while not imposing a significant negative impact on DFS or OS when compared to individuals with lower CAV1/2 expression levels[11]. This example manifested the conceptual development that prompted therapeutic development, followed by clinical validations.

The third approach is to propose a broad application of an envisioned technology. We hypothesized that "Hunting down the dominating subclone of cancer stem cells as a potential new therapeutic target in multiple myeloma: An artificial intelligence perspective" (16 citations)[12]. We also postulated "A biological global positioning system (bGPS): Considerations for tracking stem cell behaviors in the whole body", with eight elements to fulfill stem cell therapies (74 citations)[13]. Specifically, in response to monitoring the detrimental effects of stem cell therapies, we have outlined an all-encompassing bGPS designed to monitor the trajectory of transplanted stem cells. This system incorporates eight essential components, each meticulously tailored to ensure the efficacy of stem cell transplantation (SCT). These components encompass the following: (1) Sensitivity for single cell detection; (2) real-time positioning; (3) an inducible system; (4) retractable; (5) targeted and durable; (6) monitoring cell fate; (7) compliant with the FDA GMP guidelines for clinical applications; and (8) quantification capacity. By integrating these eight critical attributes, our proposed biological GPS not only addresses the aforementioned challenges but also provides a comprehensive framework for effectively tracking and assessing the transplanted stem cells during SCT. This holistic approach underscores our commitment to establishing a robust and clinically compliant solution that ensures the success of stem cell transplantation and holds the potential to enhance the field of regenerative medicine significantly. Future experimentation is needed to achieve the predictions.

The final approach could be to point out that some limitations exist in the current trend of studies. We pointed out that "Cancer genomic research at the crossroads: Realizing the changing genetic landscape as intratumoral spatial and temporal heterogeneity becomes a confounding factor" (44 citations)[14], thereby conjuring up hope for improvements through identifying potentials and challenges (76 citations)[15]. The manuscript led to sum up with specific solutions, such as "Control dominating subclones for managing cancer progression and posttreatment recurrence by subclonal switchboard signal: Implication for new therapies"-a key to two Nature articles and related column of "News & View" on the issue (28 citations)[16]. All of these thoughtful articles prompted the spatial and temporal intervention strategies of various diseases with the integration of machine learning models[17].

THE METRICS

We want to emphasize that while journal impact factors matter less, the impact of being cited does. An insightful remark during a prestigious award presentation caught attention: Physicists with 2000 citations or more fall within the top 1% globally. This figure corresponds to around 210 citations annually over a decade. Interestingly, citation thresholds vary by field, and approximately 44% of published manuscripts remain uncited. Strikingly, almost half of published manuscripts go uncited, and just a single citation places a manuscript near the upper half. Accumulating 10 citations elevates an article into the top quartile while surpassing 100 citations propels a paper to the top 1.8%. The crucial takeaway is that the average number of citations per manuscript tends to remain below 10 (Refer the details to Scott D. Weingart, MD: https://Lucbeaulieu.com/2015/11/19/how-many-citations-are-actually-a-lot-of-citations/) and (https://renaissance.stonybrookmedicine.edu/emergencymedicine/faculty/Weingart).

The database Thomson Reuters Web of Science's average number of citations per article published in scientific journals is as follows (URL: Web of Science Core Collection-Clarivate) using algorithms[18] and based on 4 million authors and 26 million scientific papers that span 15 years and 118 scientific disciplines[19] (Table 1).

Table 1 Average number of citations per article

Disciplines	Citations
Life sciences and medicine	6 to 30 citations
Physical sciences and engineering	5 to 20 citations
Social sciences	5 to 15 citations
Humanities, including literature, history, and philosophy	1 to 5 citations

CONCLUSION

In summary, authors should keep the readers' perspective in mind: Why did they come to read a literature review? A literature review should shed new light on concept development in a particular field. Not only does it offer a comprehensive snapshot of an area of interest, but it also provides promising directions for exploring questions. Both are essential; neither is minor to drum up an enthusiastic readership, leading to well-read and frequently-cited manuscripts in a literature review.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Interferon-gamma and tumor necrosis factor-alpha synergistically enhance the immunosuppressive capacity of human umbilical-cord-derived mesenchymal stem cells by increasing PD-L1 expression

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Abstract

BACKGROUND

The immunosuppressive capacity of mesenchymal stem cells (MSCs) is dependent on the "license" of several proinflammatory factors to express immunosuppressive factors such as programmed cell death 1 ligand 1 (PD-L1), which determines the clinical therapeutic efficacy of MSCs for inflammatory or immune diseases. In MSCs, interferon-gamma (IFN-γ) is a key inducer of PD-L1 expression, which is synergistically enhanced by tumor necrosis factor-alpha (TNF-α); however, the underlying mechanism is unclear.

AIM
To reveal the mechanism of pretreated MSCs express high PD-L1 and explore the application of pretreated MSCs in ulcerative colitis.

METHODS
We assessed PD-L1 expression in human umbilical-cord-derived MSCs (hUC-MSCs) induced by IFN-γ and TNF-α, alone or in combination. Additionally, we performed signal pathway inhibitor experiments as well as RNA interference experiments to elucidate the molecular mechanism by which IFN-γ alone or in combination with TNF-α induces PD-L1 expression. Moreover, we used luciferase reporter gene experiments to verify the binding sites of the transcription factors of each signal transduction pathway to the targeted gene promoters. Finally, we evaluated the immunosuppressive capacity of hUC-MSCs treated with IFN-γ and TNF-α in both an *in vitro* mixed lymphocyte culture assay, and *in vivo* in mice with dextran sulfate sodium-induced acute colitis.

RESULTS
Our results suggest that IFN-γ induction alone upregulates PD-L1 expression in hUC-MSCs while TNF-α alone does not, and that the co-induction of IFN-γ and TNF-α promotes higher expression of PD-L1. IFN-γ induces hUC-MSCs to express PD-L1, in which IFN-γ activates the JAK/STAT1 signaling pathway, up-regulates the expression of the interferon regulatory factor 1 (IRF1) transcription factor, promotes the binding of IRF1 and the PD-L1 gene promoter, and finally promotes PD-L1 mRNA. Although TNF-α alone did not induce PD-L1 expression in hUC-MSCs, the addition of TNF-α significantly enhanced IFN-γ-induced JAK/STAT1/IRF1 activation. TNF-α up-regulated IFN-γ receptor expression through activation of the nuclear factor kappa-B signaling pathway, which significantly enhanced IFN-γ signaling. Finally, co-induced hUC-MSCs have a stronger inhibitory effect on lymphocyte proliferation, and significantly ameliorate weight loss, mucosal damage, inflammatory cell infiltration, and up-regulation of inflammatory factors in colitis mice.

CONCLUSION
Overall, our results suggest that IFN-γ and TNF-α enhance both the immunosuppressive ability of hUC-MSCs and their efficacy in ulcerative colitis by synergistically inducing high expression of PD-L1.

Key Words: Human umbilical-cord-derived mesenchymal stem cells; Programmed cell death 1 ligand 1; Immunomodulation; Interferon-gamma; Tumor necrosis factor-alpha; Ulcerative colitis

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Core Tip: Our study showed that interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) significantly induced programmed cell death protein 1 ligand 1 (PD-L1) expression in human umbilical-cord-mesenchymal stem cells (hUC-MSCs), and pretreated hUC-MSCs exhibited stronger immunomodulatory capacity. Signaling pathway analysis showed that TNF-α up-regulated IFN-γ receptor expression in hUC-MSCs through nuclear factor kappa-B pathway, and then promoted IFN-γ -mediated activation of JAK/STAT1/interferon regulatory factor 1 pathway and PD-L1 expression. In ulcerative colitis mice, hUC-MSCs pretreated with IFN-γ and TNF-α exhibited stronger immunosuppressive ability through high expression of PD-L1, effectively inhibited the inflammation in the colon of mice.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent adult stem cells present in multiple tissues, including the umbilical cord, bone marrow, and fat tissue. MSCs can self-renew by dividing and differentiating into multiple tissues, including bone, cartilage, muscle, fat cells, and connective tissue[1]. In addition, MSCs have a strong immunomodulatory capacity, exerting both anti-inflammatory and pro-inflammatory effects[2]. Owing to their immunosuppressive function, MSCs are widely used to treat various inflammatory and autoimmune diseases, including inflammatory bowel disease, rheumatoid arthritis, pulmonary fibrosis, and systemic sclerosis[3-6]. However, the current clinical efficacy of MSCs transplantation (MSCT) is highly variable among individuals, even for the same autoimmune disease[7]. In general, the immunosuppressive function of MSCs depends on the licensing of the proinflammatory factor-mediated immunological microenvironment[8,9]. The individual differences in the clinical efficacy of MSCT might be explained by the substantial individual

variations in the pro-inflammatory factor-mediated immunological microenvironment at different stages of disease development in patients with inflammatory or autoimmune diseases. Therefore, to improve the therapeutic efficacy of MSCT in these diseases, there is an urgent need to explore the licensing mechanism of the immunosuppressive function of MSCs and to establish an optimal strategy for inducing and maintaining immunosuppressive MSCs.

It is currently believed that immunosuppressive MSCs induced by pro-inflammatory factors inhibit the activity of various immune cells by secreting many soluble immunosuppressive factors, such as indoleamine 2,3-dioxygenase (IDO). They also express many immunosuppressive receptors, such as programmed cell death 1 ligand 1 (PD-L1), which functions in a cell-to-cell contact manner[10,11]. Programmed cell death 1 (PD-1) is an immune-inhibitory receptor expressed in activated T cells, which binds to PD-L1 and can inhibit proliferation, induce apoptosis, and restore the subgroup balance of T cells[12]. PD-1/PD-L1 are important immune checkpoint molecules that play a pivotal role in tumor immune escape[13]. Emerging evidence suggests that PD-1/PD-L1 dysfunction is also involved in the immune hyperactivity observed in multiple autoimmune and inflammatory diseases[14,15]. Additionally, PD-L1 plays an important role in the immunosuppressive capacity of MSCs, inhibiting the activation of the immune response in a variety of ways. This includes reducing the release of inflammatory factors, the proliferation and activation of T cells, and the Th17-mediated autoimmune response, as well as augmenting the generation of regulatory T cells (Tregs)[15-17]. Therefore, to improve the therapeutic efficacy of MSCs for inflammatory or autoimmune related diseases, there is an urgent need to clarify the regulatory mechanism of PD-L1 expression in MSCs and to establish an optimal strategy for inducing and maintaining this expression.

Interferon-gamma (IFN-γ) alone has been suggested to upregulate PD-L1 expression in MSCs at low levels, although the specific mechanism remains unclear[18]. It is also unclear whether a combination of proinflammatory factors can synergistically enhance PD-L1 expression in MSCs and their immunosuppressive function. Notably, researchers have recently demonstrated that the immunosuppressive capacity of MSCs depends on stimulation by IFN-γ, and the effect can be amplified further by cytokines, such as tumor necrosis factor-α (TNF-α)[4,19]. In addition, Li *et al*[20] recently reported that TNF-α can enhance the IFN-γ-induced PD-L1 expression in hepatocellular carcinoma cells. Such findings suggest that TNF-α could also enhance IFN-γ-induced PD-L1 expression in MSCs. In the present study, we first confirmed that IFN-γ induced human-umbilical-cord-MSCs (hUC-MSCs) express PD-L1 through the JAK/STAT1/IRF1 pathway. Second, we observed that TNF-α synergistically enhanced PD-L1 expression induced by IFN-γ in hUC-MSCs. TNF-α also upregulated IFN-γ receptor 1 (IFNGR1) expression through the nuclear factor kappa-B (NF-κB) signaling pathway to promote IFN-γ/IFNGR/STAT1/ interferon regulatory factor 1 (IRF1)/PD-L1 signaling axis activation. Finally, based on an *in vitro* lymphocyte mixing experiment and an *in vivo* dextran sulfate sodium (DSS)-induced acute colitis mouse model, we found an optimal strategy of enhancing the immunosuppressive capacity of hUC-MSCs by licensing with IFN-γ and TNF-α. The results not only elucidate the specific molecular mechanism by which the IFN-γ and TNF-α combination enhances the immunosuppressive function of MSCs, but also provide a theoretical basis for improving the therapeutic efficacy of MSCT in future clinical applications.

MATERIALS AND METHODS

Culture and treatment of hUC-MSCs

All hUC-MSCs were provided by the FuMei Stem Cell Biotechnology Company (Chongqing, China). The hUC-MSCs used in the present study were identified by flow cytometry and three-lineage differentiation culture. The results showed that the hUC-MSCs had the correct molecular phenotype and three-lineage differentiation potential (Supplementary Figure 1). All hUC-MSCs used in the experiment were derived from passages 3 to 7. hUC-MSCs were cultured and stimulated in serum-free medium (LONZA). To measure the induction of PD-L1 expression, hUC-MSCs were incubated with human IFN-γ, TNF-α or both (Peprotech, United States). After 4 h of culture, the cells were harvested for mRNA expression analysis using quantitative real-time PCR (qRT-PCR). After 24 h of culture, they were harvested for protein expression analysis by western blot or flow cytometry.

Quantitative Real-time PCR

Total RNA was extracted from hUC-MSCs using Trizol (Sigma, T9424), and reverse transcribed into cDNA using HiScript® II Q RT SuperMix for qRT-PCR (+gDNA wiper) (Vazyme, China). The qRT-PCR assay was performed using ChamQ Universal SYBR qRT-PCR Master Mix (Vazyme, China). All qRT-PCR experiments were independently repeated three times. All primer sequences used for qRT-PCR are listed in Supplementary Table 1.

Flow cytometry

The hUC-MSCs were prepared as single-cell suspensions and stained for 30 min at room temperature. PD-L1-FITC antibody (BD Pharmingen, 558065), IFNGR1-PE antibody (BD Pharmingen, 558937), FITC isotype control antibody (BD Pharmingen, 555742), or PE isotype control antibody (BD Pharmingen, 555749) were used to quantify the expression of PD-L1 or IFNGR1 in hUC-MSCs. CD29-PE antibody (BD Pharmingen, 555443), CD34-PE antibody (BD Pharmingen, 555822), CD44-PE antibody (BD Pharmingen, 555479), CD45-PE antibody (BD Pharmingen, 555483), CD73-PE antibody (BD Pharmingen, 550257), CD90-PE antibody (BD Pharmingen, 555596), and CD105-PE antibody (BD Pharmingen, 560839) were used to identify hUC-MSCs. Our gating strategy was to select active hUC-MSCs groups. Flow cytometry was performed using a NovoCyte flow cytometer (ACEA Biosciences).

Western blot analysis

Western blot assay was performed to observe changes in relative protein content. Whole cell proteins were extracted from cells using radioimmunoprecipitation assay strong lysate (Beyotime Biotechnology, P0013). The cell lysates from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked with 5% skim milk in TBST for 60 min, incubated with primary antibodies and then with secondary antibodies conjugated with horseradish peroxidase (HRP), and developed using a chemiluminescence HRP substrate (Millipore, WBKLS0100). The primary antibodies used were PD-L1, IFNGR1, JAK1, p-JAK1, JAK2, p-JAK2, STAT1, p-STAT1, IRF1, IKKα, IKKβ, p-IKKα/β, IKBα, p-IKBα, p65, p-p65, and β-tubulin (1:1000, CST). The secondary antibodies were goat anti-rabbit IgG and goat anti-mouse IgG (1:10000, CST).

Signaling inhibition assay

To investigate the signaling pathways activated by IFN-γ or TNF-α in hUC-MSCs, we used the reagents: Ruxolitinib (10 μM) and BAY11-7082 (10 μM) (Selleckchem). After 30 min of pretreatment with the inhibitors, hUC-MSCs were co-cultured with IFN-γ, TNF-α, or IFN-γ plus TNF-α for 4 h or 24 h. The cells were collected for further investigation. The control cells were treated with these cytokines and DMSO.

Small interfering RNA assay

To identify the key pathway molecules involved in the inducible expression of PD-L1 or IFNGR1, a specific Small interfering RNA (siRNA) assay was performed. The siRNA synthesized by RiboBio Company (Guangzhou, China) have proven effective and were thus used in the siRNA assay (Supplementary Figure 2). The siRNAs were transfected into hUC-MSCs using Lipofectamine RNAiMAX transfection reagent (Life Technologies, United States) according to the manufacturer's protocol. After 24 h of transfection, the hUC-MSCs were treated with cytokines for 4 h or 24 h. They were then harvested and subjected to qRT-PCR analysis or western blot.

Gene promoter activity assay

Vector plasmids, IRF1 promoter plasmids, p65 promoter plasmids, PD-L1 promoter plasmids, IFNGR1 promoter plasmids, PD-L1 promoter point mutation plasmids, and IFNGR1 promoter point mutation plasmids were purchased from OBiO Technology (Shanghai). 293T cells were cultured in plates and transfected with the plasmids by DNA transfection reagent (NEOFECT, China) according to the manufacturers' instructions. Fluorescence intensity was determined using a Dual Luciferase Reporter Gene Assay Kit (Beyotime) according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8) assay. The hUC-MSCs were plated in six-well plates (1×10^5 cells per well) and cultured for 24 h. Subsequently, the CCK-8 reagent (Beyotime Biotechnology, China) was added to each well, according to the manufacturer's instructions. The absorbance of each well at 450 nm was measured using an ELX808 microplate reader (Biotek, United States).

Cell apoptosis assay

Apoptotic hUC-MSCs were detected using the Annexin V-FITC/PI Apoptosis Detection Kit and Annexin V-APC/PI Apoptosis Detection Kit (KeyGEN BioTECH, China). The hUC-MSCs were plated in six-well plates (1×10^5 cells per well) and cultured for the indicated time. After TNF-α stimulation for 24 h, all hUC-MSCs were stained by Annexin V and PI, according to the manufacturer's instructions. Afterward, the cells were evaluated using the NovoCyte flow cytometer. The percentage of apoptotic hUC-MSCs was determined by the sum of Annexin V positive cells.

IFNGR1 overexpression assay

IFNGR1-overexpressing lentiviruses were purchased from Shanghai OBiO Technology. The lentivirus infection procedures were modified from a previously published protocol[21]. After IFNGR1-overexpressed hUC-MSCs were successfully constructed using IFNGR1-overexpressing lentivirus, we used small interfering RNA (siRNA) to interfere with the p65 expression in cells. PD-L1 mRNA expression was detected by qRT-PCR assays after induction by IFN-γ or TNF-α for 4 h.

PBMCs isolation and proliferation assays

Peripheral blood samples were obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated using a human peripheral blood lymphocyte separation tube (Dakewe, China), activated by monoclonal anti-human CD3 antibody (Acro, United States), and stained with 10 mmol/L 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Beyotime, China). CFSE-labeled PBMCs (1×10^6 cells/well) were co-incubated with hUC-MSCs at a ratio of 10:1 in the presence of 100 IU/mL IL2. After co-culturing for 3 d, the proliferation of PBMCs was analyzed using flow cytometry.

DSS-induced colitis and treatment

C57BL/6J mice were purchased from the Weitong Lihua Experimental Animal Technology Company (China). 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 2 wk prior to experimentation. Mice were randomly assigned to six experimental groups: WT, DSS, DSS + MSCs, DSS + IT-MSCs (IT-MSCs: hUC-MSCs treated with IFN-γ and TNF-α), DSS + IT-MSCs + iso antibody, and IT-MSCs + PD-L1 antibody (atezol-

izumab, Selleck). In the DSS, DSS + MSCs, DSS + IT-MSCs, DSS + IT-MSCs + iso antibody, and IT-MSCs + PD-L1 antibody groups, acute colitis was induced in mice by the addition of 4% (w/v) DSS (40 kDa, MP Biomedicals) to the drinking water for 7 d, until euthanasia. The WT group received drinking water only. All mice were weighed daily. On day 3, mice from the DSS + MSCs, DSS + IT-MSCs, DSS + IT-MSCs + iso antibody, and IT-MSCs + PD-L1 antibody groups were transfused with 1 × 10^6 hUC-MSCs *via* the tail vein, and the other mice were transfused with equal amounts of saline *via* the tail vein. Mice in the IT-MSCs group were transfused *via* the tail vein with 1 × 10^6 hUC-MSCs previously treated with IFN-γ and TNF-α for 24 h. The hUC-MSCs of the DSS + IT-MSCs + iso antibody group and the DSS + IT-MSCs+PD-L1 antibody group were treated with IFN-γ, TNF-α, isotype antibody or PD-L1 antibody for 24 h. After seven days of induction, the mice were sacrificed by cervical dislocation, and their colons were collected.

Colon histopathology analysis

For histological analysis, colon samples were fixed with 4% paraformaldehyde (Beyotime, P0099) for 24 h and embedded in paraffin for hematoxylin and eosin staining, as previously described[22]. We evaluated the histopathological scores of colon tissue using a combination of inflammatory cell infiltration and epithelial damage to the colon[22].

Colon immunohistochemistry analysis

Immunohistochemistry was performed on paraffin embedded sections of colon samples. The sections were incubated with anti-CD4 (Abcam, 1:1000) or anti-CD11b (Abcam, 1:4000) at 4 °C overnight and with anti-rabbit IgG and HRP-linked antibody (CST, 1:50) for 60 min. Immunoreactivity was analyzed with a microscope (Olympus CKX31). The total number of CD4 or CD11b positive cells in each of four nonadjacent fields of view was counted per colon sample.

Myeloperoxidase activity assay

We performed a myeloperoxidase (MPO) activity assay to determine the number of neutrophils in the colon tissue. The MPO activity kit was purchased from Jiancheng (Nanjing, China, A044-1-1). We determined the level of MPO activity in the colon tissue following the protocol recommended by the manufacturer. The absorbance of each well was evaluated using an ELX808 microplate reader.

Enzyme linked immunosorbent assay

The concentrations of TNF-α, IFN-γ, and IL-6 secreted by mice with acute colitis were determined by enzyme linked immunosorbent assay (ELISA). The TNF-α, IFN-γ, and IL-6 ELISA kits were purchased from Boster (Wuhan, Hubei Province, China), and were used according to the manufacturer's instructions. The absorbance of each well was evaluated using an ELX808 microplate reader.

Statistical analysis

The results are shown as mean ± SD. Statistical analyses were performed using GraphPad Prism v8.4.2 (GraphPad Software Inc., San Diego, CA, United States). Student's unpaired t-test and one-way analysis of Variance were used to determine significant differences between groups. Statistical significance was set at $P < 0.05$.

RESULTS

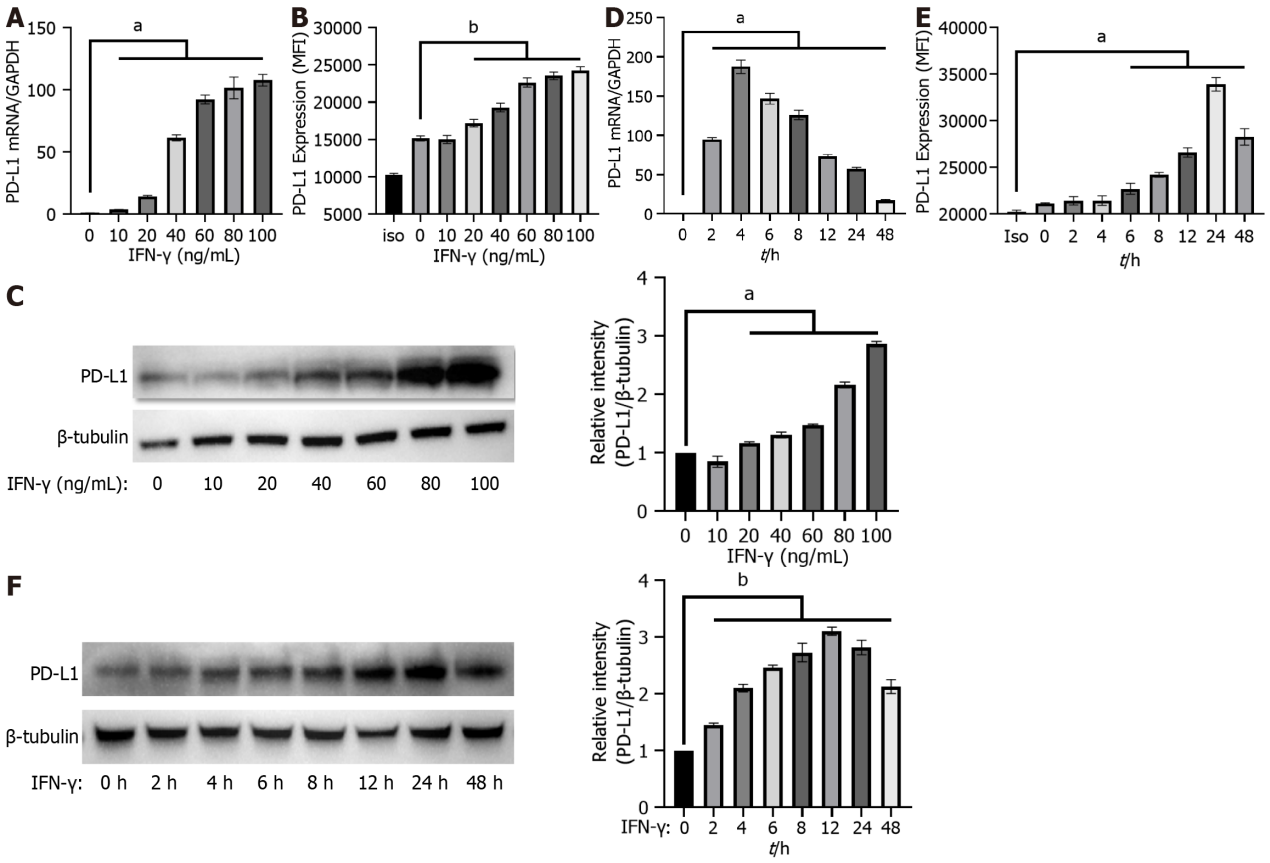
IFN-γ induced the expression of PD-L1 in the hUC-MSCs

IFN-γ induces MSCs to express PD-L1, which exerts an immunosuppressive effect. In the present study, we further investigated the effect of IFN-γ on PD-L1 expression in hUC-MSCs. And hUC-MSCs were treated with either different concentrations of IFN-γ or fixed concentrations of IFN-γ for different time periods. PD-L1 expression in hUC-MSCs was detected using qRT-PCR, western blot, and flow cytometry. After treating hUC-MSCs with variable concentrations of IFN-γ for 24 h, the results showed that when the concentration of IFN-γ was 20 ng/mL, PD-L1 expression increased significantly; it was the highest under 100 ng/mL IFN-γ (Figure 1A-C). Moreover, PD-L1 mRNA expression induced by 20 ng/mL IFN-γ began to increase 2 h after stimulation, and was the highest at 4 h (Figure 1D). The total and membrane protein levels of PD-L1 in hUC-MSCs peaked at 24 h (Figure 1E and F). Based on the results above, we used 20 ng/mL IFN-γ stimulation for 4 h for follow-up qRT-PCR experiments, and 20 ng/mL IFN-γ stimulation for 24 h for western blot or flow cytometry experiments. The results confirmed that IFN-γ induced PD-L1 expression in hUC-MSCs.

IFN-γ upregulated PD-L1 expression through the JAK/STAT1/IRF1 pathway in hUC-MSCs

A previous study showed that IFN-γ increased PD-L1 expression in tumor cells by activating the JAK/STAT/IRF1 pathway[23]. To confirm this finding, we performed signaling inhibition and siRNA interference experiments. We found that ruxolitinib (10 μM), a JAK/STAT signaling pathway inhibitor, significantly reduced IFN-γ-induced PD-L1 mRNA expression (Figure 2A; Supplementary Figure 3A). The results of western blot and flow cytometry showed that ruxolitinib also significantly reduced the levels of IFN-γ-induced PD-L1 membrane protein and total protein (Figure 2B and C). The results suggest that IFN-γ could induce PD-L1 expression through the JAK/STAT pathway in hUC-MSCs.

To further confirm that the JAK/STAT pathway mediates the IFN-γ-induced effect on hUC-MSCs expressing PD-L1, we analyzed the total protein and the phosphorylation levels of key proteins in the JAK/STAT signaling pathway in hUC-MSCs. Western blot results showed that IFN-γ promoted JAK1/2 phosphorylation, STAT1 phosphorylation, IRF1 expression, and PD-L1 expression, while ruxolitinib reversed all these effects (Figure 2C). To confirm the findings, we



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Figure 1 Interferon-gamma induced the expression of programmed cell death protein 1 ligand 1 in human umbilical-cord-mesenchymal stem cells. A-C: Programmed cell death protein 1 ligand 1 (PD-L1) expression was measured by quantitative real-time PCR (qRT-PCR), flow cytometry, and western blot in human umbilical-cord-mesenchymal stem cells (hUC-MSCs) treated with 0, 10, 20, 40, 60, 80 or 100 ng/mL interferon-gamma (IFN-γ) for 24 h; D-F: PD-L1 expression was measured by qRT-PCR, flow cytometry, and western blot in hUC-MSCs treated with 20 ng/mL IFN-γ for 0, 2, 4, 6, 8, 12, 24 or 48 h. Data were represented as mean ± SEM of $n = 3$. Iso: Isotype; C: Control; $^{a}P < 0.01$; $^{b}P < 0.001$. IFN-γ: Interferon-gamma; PD-L1: Programmed cell death protein 1 ligand 1.

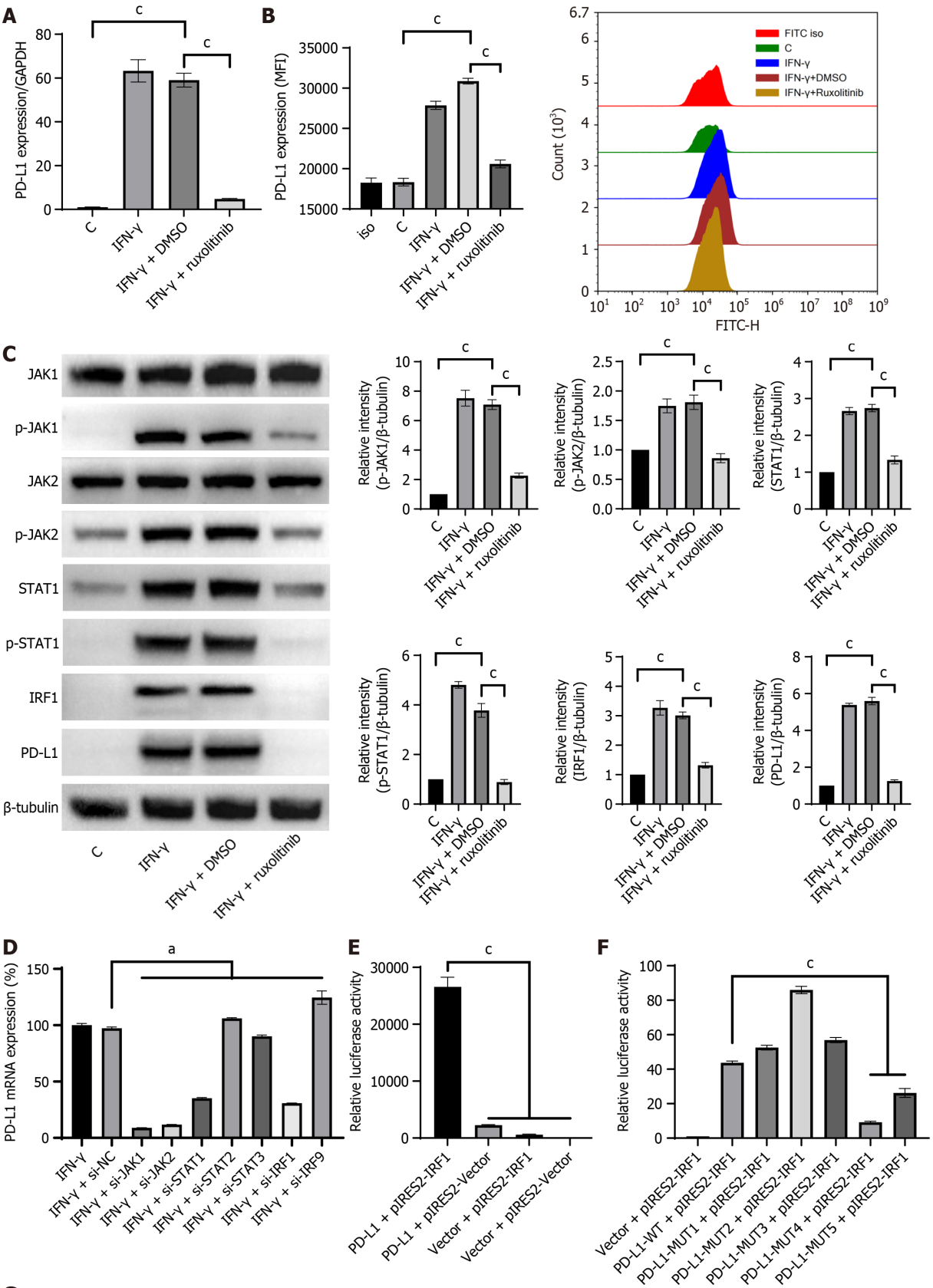
performed an siRNA interference assay using the effective siRNAs for JAK1/2, STAT1/2/3, and IRF1/9, and observed that the siRNAs remarkably inhibited the PD-L1 expression induced by IFN-γ (Figure 2D). The results indicate that IFN-γ induced PD-L1 expression in hUC-MSCs *via* the JAK/STAT1/IRF1 pathway.

IRF1 is a key transcription factor in the JAK/STAT pathway. We constructed a dual-luciferase reporter system to confirm that IRF1 binds to the PD-L1 promoter to promote PD-L1 mRNA expression. In 293T cells, relative luciferase activity was weak when transfected with IRF1 or the PD-L1 promoter plasmid; it was the strongest when the two were co-transfected (Figure 2E). We then predicted the potential binding sites of the proximal part of the PD-L1 gene to IRF1 and identified the five most likely binding sites. Point mutant plasmids were constructed at the five binding sites to assess luciferase reporter activity. We co-transfected a WT-PD-L1 promoter plasmid and five IRF1 binding site mutant PD-L1 promoter plasmids into 293T cells with an IRF1 promoter plasmid and a Renilla luciferase plasmid. We measured the relative luciferase activity 24 h after transfection (Figure 2F). The locations of the binding sites in the PD-L1 promoter are shown in Figure 2G. According to the results, mutations at sites 4 or 5 reduced IFN-γ-induced PD-L1 promoter activity, with site 4 having the greatest effect (Figure 2F). These findings indicate that IFN-γ activated the JAK/STAT pathway in hUC-MSCs and induced IRF1 expression; IRF1, in turn, bound to the PD-L1 promoter, thereby promoting PD-L1 transcription.

TNF-α synergistically enhanced IFN-γ-induced PD-L1 expression in hUC-MSCs

As mentioned above, the mechanism by which TNF-α synergistically enhances IFN-γ-induced PD-L1 expression has not been elucidated. To study the effect of TNF-α alone or in combination with IFN-γ on PD-L1 expression in hUC-MSCs, we performed the following experiments. First, hUC-MSCs were stimulated with seven different concentrations of TNF-α (0 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, 60 ng/mL, 80 ng/mL, and 100 ng/mL) for 24 h, or with the experimentally designed highest concentration (100 ng/mL) of TNF-α for different time periods (0 h, 2 h, 4 h, 6 h, 12 h, 24 h, and 48 h). Subsequently, the membrane expression of PD-L1 was detected by flow cytometry. Our results demonstrated that TNF-α stimulation alone did not affect PD-L1 expression in hUC-MSCs (Figure 3A and B).

Subsequently, to analyze the effect of TNF-α on hUC-MSCs viability, the proliferation and apoptosis of hUC-MSCs treated with TNF-α was tested using cell proliferation and apoptosis assays. We found that TNF-α had no effect on the proliferation or apoptosis of hUC-MSCs (Figure 3C and D). We then studied the effect of combined treatment with IFN-γ

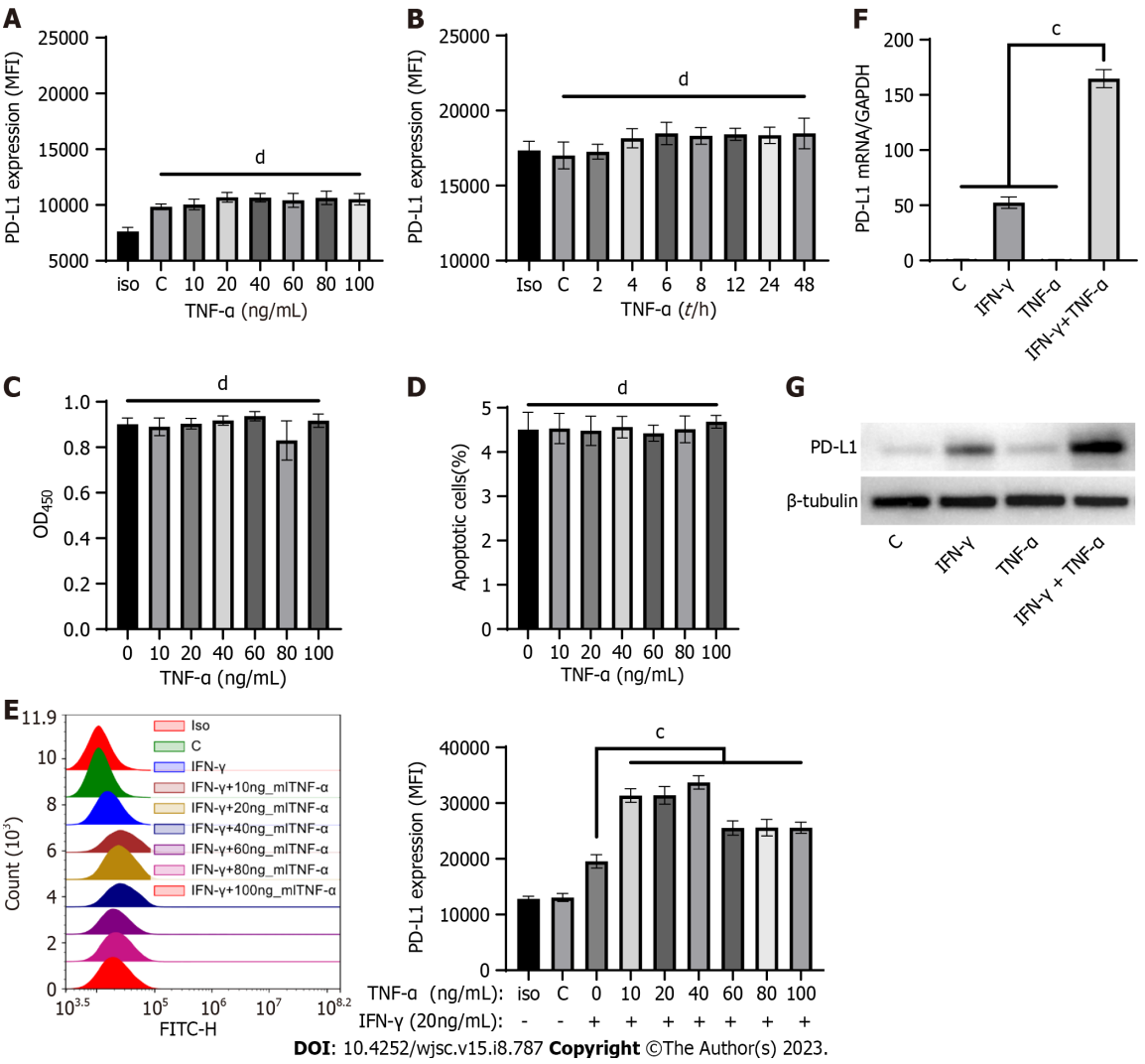


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site 1: -951 ttagaaaaagagaaaaaaaag- 930
site 2: -615 agtttcattatg- 603
site 3: -508 aaaaaccaaagc- 496
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site 5: -151 atacctaaactgaaagcttcc- 130

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Figure 2 Interferon-gamma upregulated programmed cell death protein 1 ligand 1 expression *via* the JAK/STAT1/ interferon regulatory

factor 1 pathway in human umbilical-cord-mesenchymal stem cells. A: Programmed cell death protein 1 ligand 1 (PD-L1) expression was quantified by quantitative real-time PCR (qRT-PCR) in human umbilical-cord-mesenchymal stem cells (hUC-MSCs) treated with a combination of IFN-γ with or without the JAK inhibitor ruxolitinib (10 μM) for 4 h; B: PD-L1 expression was quantified by flow cytometry in hUC-MSCs treated with a combination of IFN-γ with or without ruxolitinib (10 M) for 24 h; C: Activation of the JAK/STAT/interferon regulatory factor 1 (IRF1) pathway and PD-L1 expression were analyzed by western blot in hUC-MSCs treated with IFN-γ (20 ng/mL) with or without ruxolitinib (10 M) for 24 h; D: The PD-L1 expression was quantified by qRT-PCR in hUC-MSCs transfected with siRNA against JAK1, JAK2, STAT1, STAT2, STAT3, IRF1, or IRF9 and subsequently treated with IFN-γ for 4 h. The expression of PD-L1 in hUC-MSCs of the IFN-γ + si-NC (negative control) group was used as the standard (100%); E: PGL4.10-PD-L1 promoter, pIRES2-IRF1, and Renilla luciferase plasmids were transfected together or separately into 293T cells. Cells were lysed 24 h after transfection and luciferase activity was measured; F: pIRES2-IRF1 plasmid and Renilla luciferase plasmid were transfected into 293T cells with the PGL4.10-PD-L1 promoter plasmid or IRF1 binding site mutant PGL4.10-PD-L1 promoter plasmids. Cells were lysed 24 h after transfection and luciferase activity was measured; G: Predicted binding sites of IRF1 on the PD-L1 promoter. Data were represented as mean ± SEM of n = 3. C: Control; Iso: Isotype; $^{a}P < 0.01$; $^{c}P < 0.0001$. IFN-γ: Interferon-gamma; PD-L1: Programmed cell death protein 1 ligand 1.



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Figure 3 Tumor necrosis factor-alpha synergistically enhanced interferon-gamma-induced programmed cell death protein 1 ligand 1 expression in human umbilical-cord-mesenchymal stem cells. A: Programmed cell death protein 1 ligand 1 (PD-L1) expression was measured by flow cytometry in human umbilical-cord-mesenchymal stem cells (hUC-MSCs) treated with 0, 10, 20, 40, 60, 80 or 100 ng/mL tumor necrosis factor-alpha (TNF-α) for 24 h; B: PD-L1 expression was measured by flow cytometry in hUC-MSCs treated with 100 ng/mL TNF-α for 0, 2, 4, 6, 8, 12, 24, or 48 h; C: Proliferation was measured by CCK8 in hUC-MSCs treated with 0, 10, 20, 40, 60, 80 or 100 ng/mL of TNF-α for 24 h; D: Apoptotic hUC-MSCs were measured by flow cytometry in hUC-MSCs treated with 0, 10, 20, 40, 60, 80 or 100 ng/mL TNF-α for 24 h. The histogram shows the quantification results pertaining to the percentage of apoptotic cells (Annexin V^{+} cells) in each group; E: PD-L1 expression was quantified by flow cytometry in hUC-MSCs after 24 h of incubation with IFN-γ (20 ng/mL) and 0, 10, 20, 40, 60, 80 or 100 ng/mL TNF-α; F: PD-L1 expression was measured by quantitative real-time PCR in hUC-MSCs treated with IFN-γ (20 ng/mL), TNF-α (10 ng/mL), or a combination of IFN-γ (20 ng/mL) and TNF-α (10 ng/mL) for 4 h; G: PD-L1 expression was analyzed by western blot in hUC-MSCs treated with IFN-γ (20 ng/mL), TNF-α (10 ng/mL), or a combination of IFN-γ (20 ng/mL) and TNF-α (10 ng/mL) for 24 h. Data were represented as mean ± SEM of n = 3. C: Control; Iso: Isotype; $^{b}P < 0.001$; $^{c}P < 0.0001$; $^{d}P > 0.05$. IFN-γ: Interferon-gamma; PD-L1: Programmed cell death protein 1 ligand 1; TNF-α: Tumor necrosis factor-alpha.

and TNF-α on PD-L1 expression in hUC-MSCs. We stimulated hUC-MSCs with 20 ng/mL IFN-γ and different concentrations of TNF-α (0 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, 60 ng/mL, 80 ng/mL, and 100 ng/mL) and detected PD-L1 expression using flow cytometry. The results showed that 10 ng/mL TNF-α enhanced IFN-γ-induced PD-L1 expression significantly; however, the enhanced effect of TNF-α did not appear to be concentration-dependent (Figure 3E). Furthermore, qRT-PCR and western blot results showed that the mRNA and total protein levels of PD-L1 increased significantly when hUC-MSCs were simultaneously stimulated with 20 ng/mL IFN-γ and 10 ng/mL TNF-α when compared with IFN-γ or TNF-α alone (Figure 3F and G). Based on the results, 10 ng/mL TNF-α was used in follow-up experiments. In addition, we investigated the effects of IFN-γ (20 ng/mL) or TNF-α (10 ng/mL) stimulation individually or in combination on the proliferation or apoptosis of hUC-MSCs. Our results showed that the stimuli were unable to exert any effect on apoptosis or proliferation of hUC-MSCs at the current concentrations (Supplementary Figure 3B and C). The findings suggest that TNF-α alone had no effect on PD-L1 expression; however, TNF-α could synergistically increase IFN-γ-induced PD-L1 expression in hUC-MSCs.

TNF-α improved PD-L1 expression by promoting the activation of JAK/STAT1/IRF1 pathway induced by IFN-γ

As previous studies have demonstrated that IFN-γ induces hUC-MSCs to express PD-L1 through the JAK/STAT1/IRF1 pathway and TNF-α induces various downstream biological effects through activation of the NF-κB pathway[24], we explored the molecular mechanism by which TNF-α enhances IFN-γ-induced PD-L1 expression using signaling inhibition experiments. We treated hUC-MSCs with ruxolitinib and the NF-κB inhibitor BAY11-7082, and then detected PD-L1 mRNA levels after combined treatment with IFN-γ and TNF-α for 4 h. Our results showed that both BAY11-7082 and ruxolitinib inhibited PD-L1 expression induced by a combination of IFN-γ and TNF-α (Figure 4A). Ruxolitinib displayed a stronger inhibitory effect, almost completely inhibiting the induction of PD-L1 mRNA expression, while BAY11-7082 only partially inhibited the expression (Figure 4A). Flow cytometry and western blot assays also showed that ruxolitinib completely inhibited PD-L1 protein expression in hUC-MSCs treated with IFN-γ and TNF-α (Figure 4B and C). Furthermore, we performed an siRNA interference assay using STAT1 siRNA and IRF1 siRNA. The siRNA against STAT1 and IRF1 decreased PD-L1 expression induced by IFN-γ or TNF-α in hUC-MSCs (Figure 4D and E). The results indicated that PD-L1 gene transcription is also induced through the JAK/STAT1/IRF1 pathway when stimulated by IFN-γ and TNF-α.

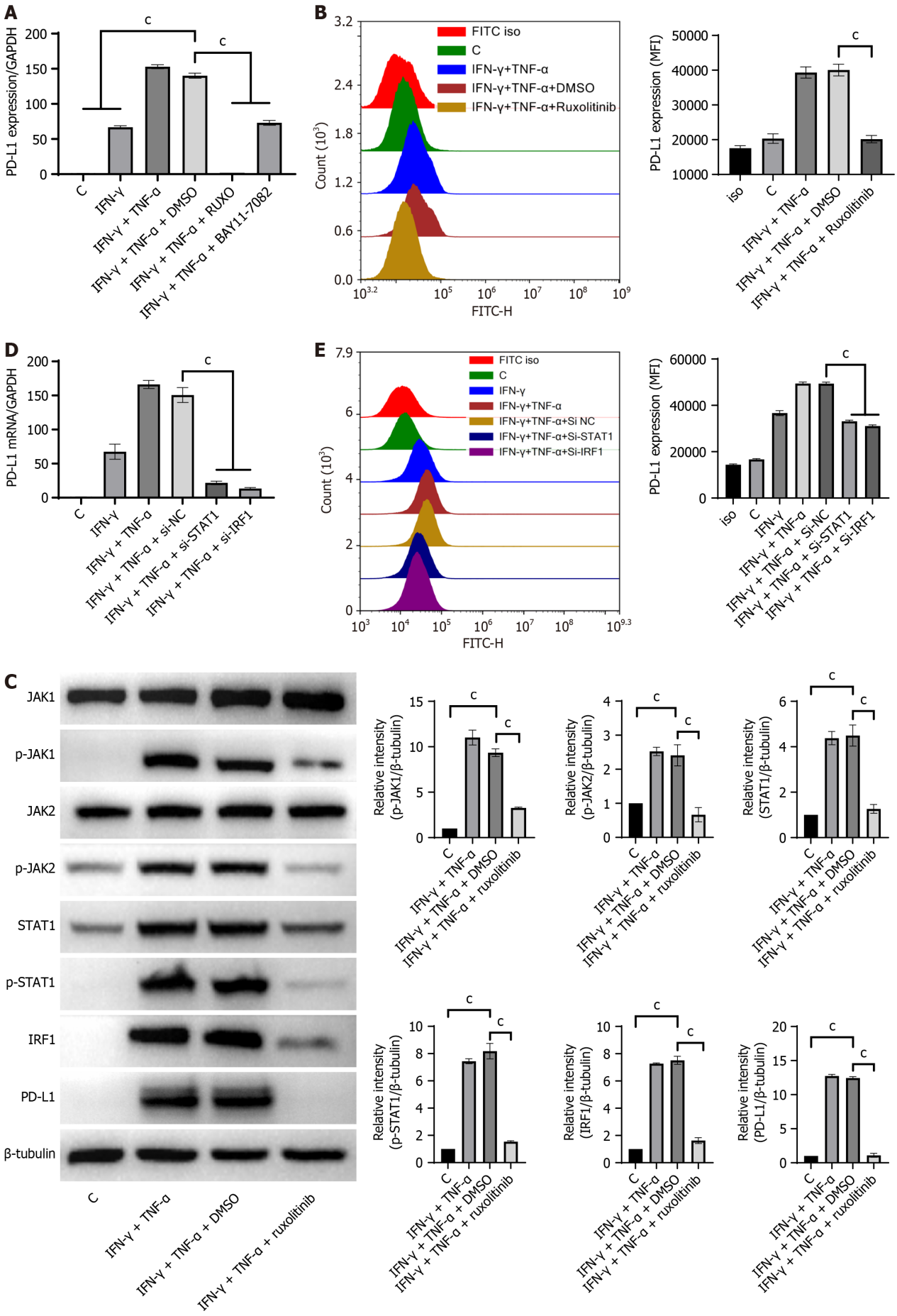
To investigate whether TNF-α affects the activation of the JAK/STAT1/IRF1 pathway, we analyzed the activation of the JAK/STAT1/IRF1 pathway in hUC-MSCs treated with IFN-γ and TNF-α. The results showed that the activation level of the JAK/STAT1/IRF1 pathway was significantly higher following stimulation with IFN-γ and TNF-α (Figure 4F). Additionally, ruxolitinib significantly reduced the phosphorylation levels of JAK1/2 and STAT1, as well as the expression levels of IRF1 and PD-L1, in hUC-MSCs induced by IFN-γ and TNF-α (Figure 4C). The results revealed that the activation of the JAK/STAT1/IRF1 pathway was significantly stronger when co-stimulated with IFN-γ and TNF-α, thus leading to higher PD-L1 expression.

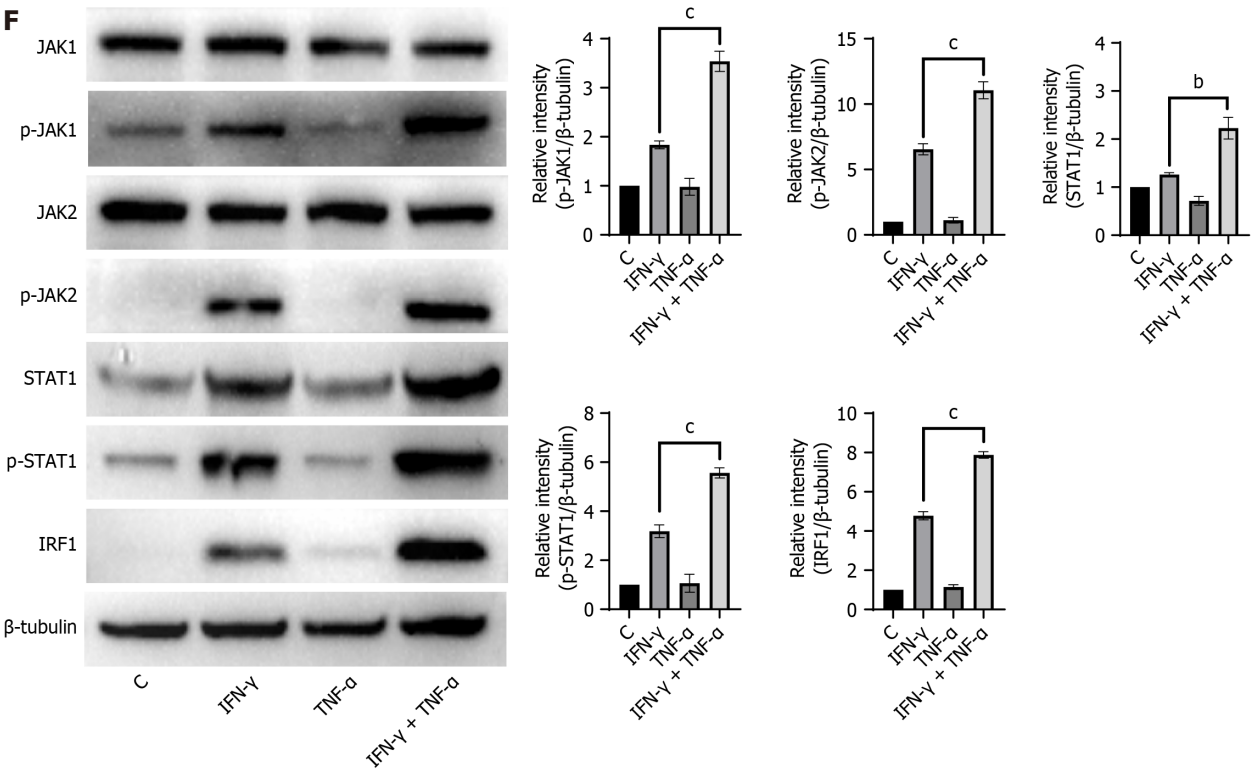
TNF-α synergistically amplified IFN-γ-induced JAK/STAT1/IRF1 signaling activation by upregulating NF-κB-mediated IFNGR1 expression

IFNGR1/2 acts as the membrane receptor of IFN-γ and transmits an activation initiation signal to the JAK/STAT1/IRF1 pathway, which is the key player in PD-L1 expression[23]. We speculated that the higher activation level of the JAK/STAT1/IRF1 pathway might be caused by TNF-α induced IFNGR1/2 expression. As predicted, TNF-α upregulated the expression of IFNGR1 mRNA and protein and the expression of IFNGR2 mRNA (Figure 5A-C). Notably, since no effective anti-IFNGR2 antibody is available, only IFNGR1 expression was detected in western blot experiments. Additionally, the siRNA for IFNGR1/2 decreased PD-L1 mRNA and protein expression in hUC-MSCs induced by IFN-γ and TNF-α (Figure 5D-F). Furthermore, western blot results showed that IFNGR1 siRNA reduced JAK1/2 and STAT1 phosphorylation, as well as IRF1 expression (Figure 5F). The results suggest that TNF-α amplifies the IFN-γ-induced activation of the JAK/STAT1/IRF1 pathway by upregulating IFNGR1/2 expression.

The NF-κB pathway inhibitor BAY11-7082 partially impaired the expression of PD-L1 induced by the combination of IFN-γ and TNF-α (Figure 4A; Supplementary 3D). This suggests that the NF-κB pathway is involved in the regulation of PD-L1 expression induced by IFN-γ and TNF-α. Therefore, we examined the expression of IFNGR1 and the activation of the NF-κB pathway in hUC-MSCs induced by TNF-α. As predicted, TNF-α upregulated IFNGR1 expression by activating the NF-κB pathway, while BAY11-7082 decreased the phosphorylation levels of IKK-α/β, IKB-α, and p65, as well as the expression of IFNGR1 (Figure 5G; Supplementary Figure 4A). To explore NF-κB pathway activation by TNF-α in hUC-MSCs, we assessed nuclear and cytoplasmic p65 and IκB expression after TNF-α treatment. The expression of IκB in the cytoplasm was reduced at 60 min and then restored, while the expression of p65 in the nucleus was significantly increased at 60 min (Figure 5H; Supplementary Figure 4B). These findings indicate that TNF-α can promote NF-κB pathway activation in hUC-MSCs, in addition to enhancing p65 translocation to the nuclei. Moreover, when the expression of p65 was downregulated by siRNA, the effect of TNF-α on the expression of IFNGR1 and PD-L1 induced by IFN-γ was significantly reduced (Figure 5I-K). Importantly, we found that IFNGR1-overexpressing lentivirus restored PD-L1 expression that was reduced by p65 siRNA (Figure 5L). Furthermore, TNF-α upregulated IFNGR1 expression through the NF-κB pathway, and synergistically amplified the activation of JAK/STAT1/IRF1 induced by IFN-γ, finally resulting in an increase in PD-L1 expression in hUC-MSCs.

To confirm that p65 binds to the IFNGR1 promoter to activate IFNGR1 expression, we constructed a luciferase reporter system with NF-κB-driven activity. In 293T cells, higher luciferase activity was observed when p65 and IFNGR1 promoter plasmids were co-transfected into the cells than when these were transfected separately (Figure 5M). As the proximal part of the IFNGR1 gene contained only one p65 binding site, we constructed an IFNGR1 promoter plasmid with a point


A
PD-L1 expression/GAPDH
C
IFN-γ
IFN-γ + TNF-α
IFN-γ + TNF-α + DMSO
IFN-γ + TNF-α + RUXO
IFN-γ + TNF-α + BAY11-7082
B
FITC iso
C
IFN-γ+TNF-α
IFN-γ+TNF-α+DMSO
IFN-γ+TNF-α+Ruxolitinib
Count (10³)
FITC-H
PD-L1 expression (MFI)
iso
IFN-γ + TNF-α + Ruxolitinib
D
PD-L1 mRNA/GAPDH
IFN-γ + TNF-α + si-NC
IFN-γ + TNF-α + si-STAT1
IFN-γ + TNF-α + si-IRF1
E
IFN-γ
IFN-γ+TNF-α+Si NC
IFN-γ+TNF-α+Si-STAT1
IFN-γ+TNF-α+Si-IRF1
C
JAK1
p-JAK1
JAK2
p-JAK2
STAT1
p-STAT1
IRF1
PD-L1
β-tubulin
IFN-γ + TNF-α + ruxolitinib
Relative intensity (p-JAK1/β-tubulin)
Relative intensity (p-JAK2/β-tubulin)
Relative intensity (STAT1/β-tubulin)
Relative intensity (p-STAT1/β-tubulin)
Relative intensity (IRF1/β-tubulin)
Relative intensity (PD-L1/β-tubulin)



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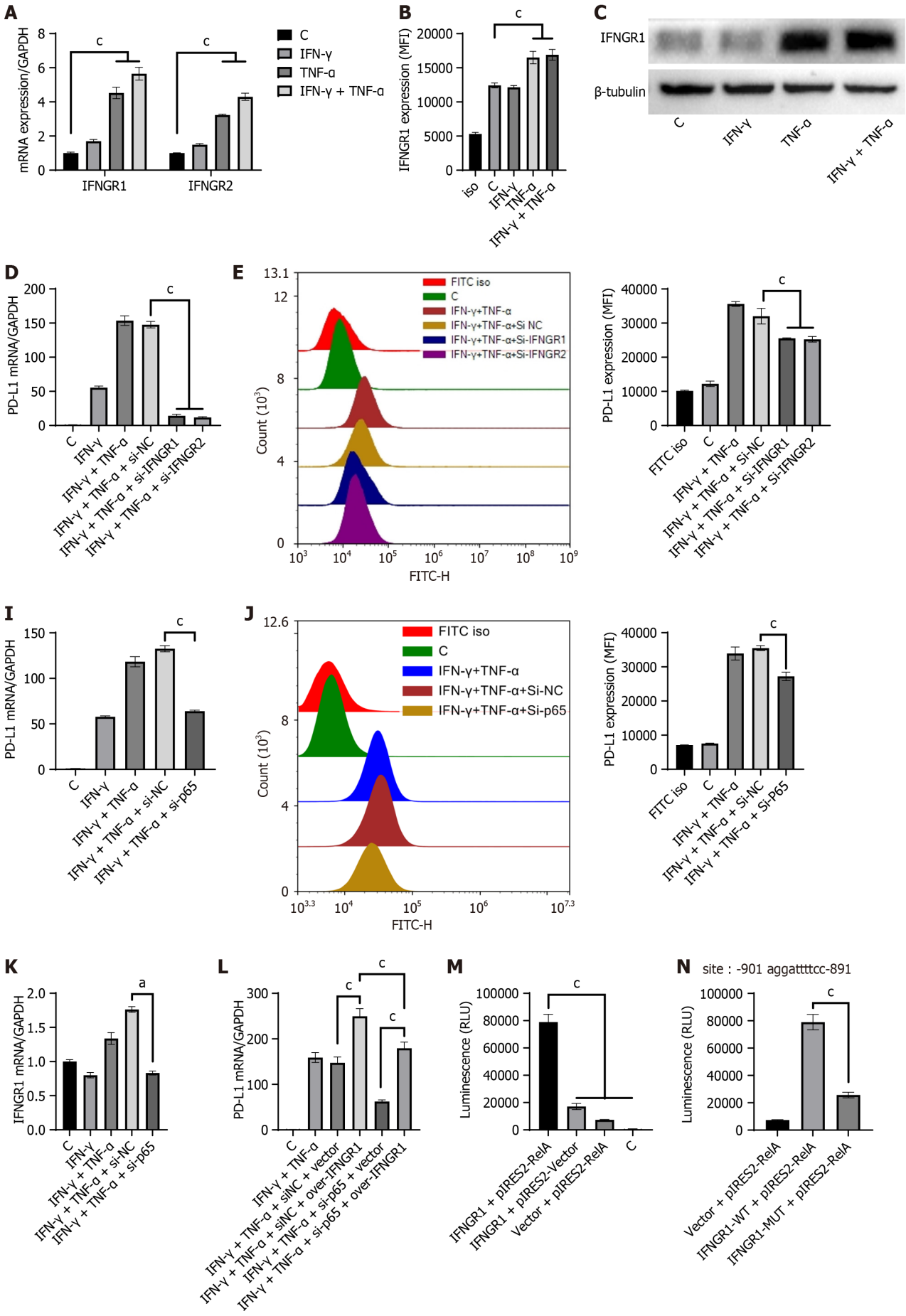
Figure 4 Tumor necrosis factor-alpha increased programmed cell death protein 1 ligand expression by promoting interferon-gamma-induced activation of the JAK/STAT1/interferon regulatory factor 1 pathway. A: Programmed cell death protein 1 ligand (PD-L1) expression was quantified by quantitative real-time PCR (qRT-PCR) in human umbilical-cord-mesenchymal stem cells (hUC-MSCs) after 4 h of incubation with IFN-γ and tumor necrosis factor-alpha (TNF-α) in the presence or absence of ruxolitinib (10 μM) or the NF-κB inhibitor BAY11-7082 (10 μM); B: PD-L1 expression in hUC-MSCs was quantified by flow cytometry after 24 h of incubation with IFN-γ and TNF-α in the presence or absence of ruxolitinib (10 μM); C: Activation of the JAK/STAT1/interferon regulatory factor 1 (IRF1) pathway and the expression of PD-L1 were analyzed by western blot in hUC-MSCs after 24 h of incubation with IFN-γ and TNF-α in the presence or absence of ruxolitinib (10 μM); D and E: PD-L1 expression was quantified by quantitative real-time PCR and flow cytometry in hUC-MSCs transfected with siRNA of NC, STAT1, or IRF1 and subsequently treated with IFN-γ and TNF-α for 4 h or 24 h; F: Activation of the JAK/STAT1/IRF1 pathway was analyzed by western blot in hUC-MSCs treated with IFN-γ (20 ng/mL), TNF-α (10 ng/mL), or a combination of IFN-γ (20 ng/mL) and TNF-α (10 ng/mL) for 24 h. Data were represented as mean ± SEM of n = 3. C: Control; Iso: Isotype; $^{c}P < 0.0001$. IFN-γ: Interferon-gamma; PD-L1: Programmed cell death protein 1 ligand 1; TNF-α: Tumor necrosis factor-alpha.

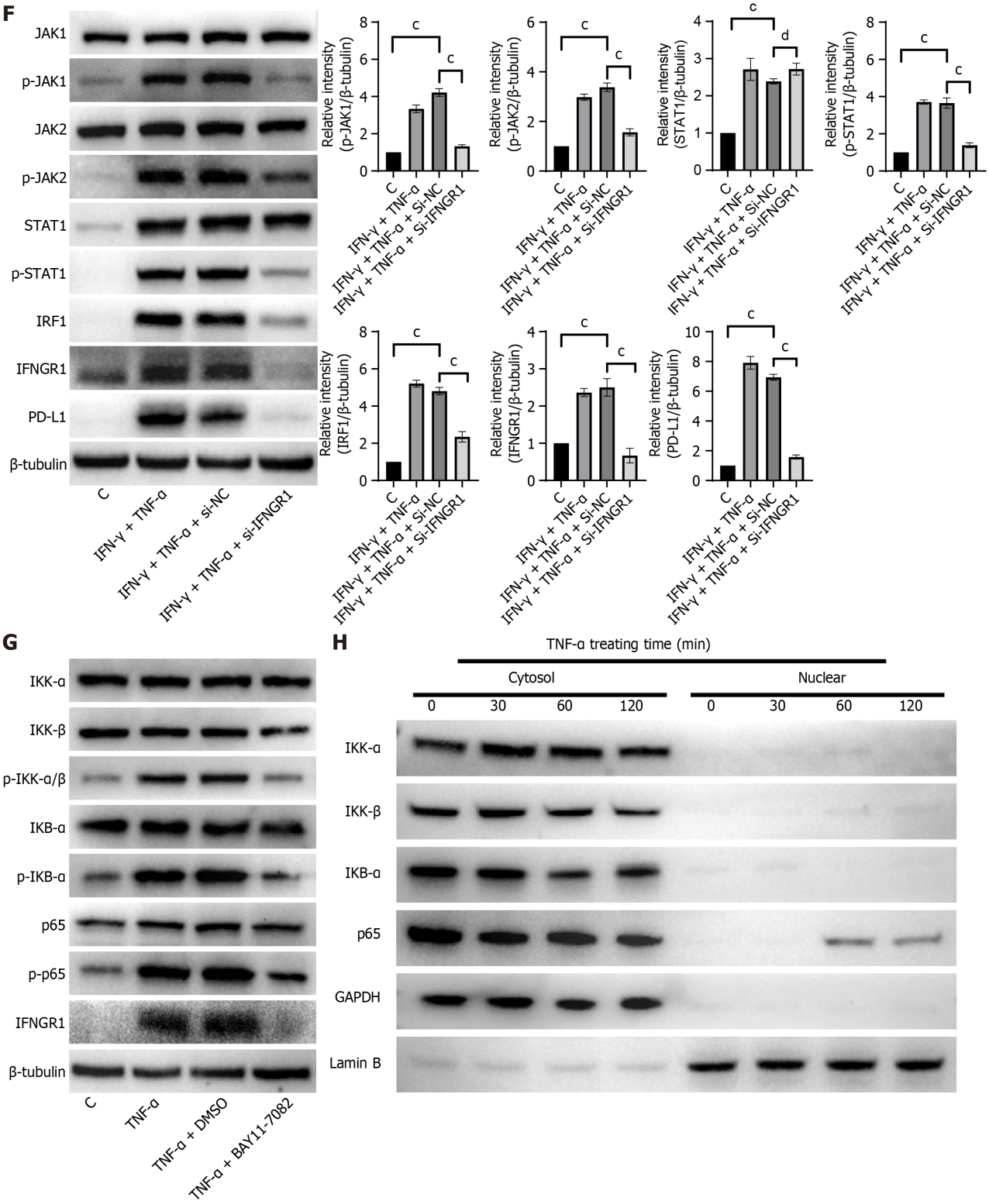
mutation at this binding site. We found that this p65 binding site mutant plasmid displayed weaker luciferase activity in response to TNF-α stimulation compared to the WT-type IFNGR1 promoter plasmid (Figure 5N). The results indicate that TNF-α induces NF-κB p65 activation of the IFNGR1 promoter.

Synergy of IFN-γ and TNF-α enhanced the immunosuppressive capacity of hUC-MSCs by inducing PD-L1 expression

To explore whether the high expression of PD-L1 in hUC-MSCs induced by a combination of IFN-γ and TNF-α could enhance their immunosuppressive function, we first conducted a T lymphocyte proliferation inhibition experiment *in vitro*. The results showed that PBMCs co-cultured with hUC-MSCs that were pre-treated with IFN-γ and TNF-α had higher CFSE fluorescence intensity than that in other groups, which indicated that IFN-γ- and TNF-α-pretreated hUC-MSCs showed a stronger inhibitory effect on PBMCs proliferation (Figure 6A). Meanwhile, the PD-L1 antibody eliminated the inhibitory effect of hUC-MSCs pre-treated with IFN-γ and TNF-α on PBMCs proliferation (Figure 6A). The results suggest that IFN-γ and TNF-α-pretreated hUC-MSCs, with high PD-L1 expression, exhibited a stronger inhibitory effect on T cell proliferation.

We further explored the therapeutic efficacy of hUC-MSCs pre-treated with a combination of IFN-γ and TNF-α in mice with DSS-induced colitis. Colitis was induced in mice by feeding them DSS added to drinking water, and hUC-MSCs were transplanted according to the schematic diagram shown in Figure 6B. Compared with the wild-type hUC-MSCs treatment group, hUC-MSCs pretreated with a combination of IFN-γ and TNF-α displayed a better therapeutic efficacy in DSS-induced mice, including mitigated bodyweight loss (Figure 6C); improved colonic morphology (Figure 6D); relieved colonic ulcer, mucosal edema, epithelial cell injury, and inflammatory cell infiltration (Figure 6E); lower histopathological scoring of colon tissue (Figure 6F); inhibited infiltration of immune cells including $CD4^+$ T cells, $CD11b^+$ macrophages, and neutrophils (lower MPO activity) in colon tissue (Figure 6G and 6H); more Treg ($Foxp3^+$) cells in colon tissue (Figure 6I), and significantly decreased levels of inflammatory factors such as TNF-α, IFN-γ, and IL-6 in colon tissue (Figure 6J). However, PD-L1 antibody treatment significantly inhibited such effects. In addition, no adverse reactions related to hUC-MSCs transplantation were observed. The results suggest that the combination of IFN-γ and TNF-α synergistically enhanced the immunosuppressive capacity of hUC-MSCs by upregulating the expression of PD-L1,


A
mRNA expression/GAPDH
C
IFN-γ
TNF-α
IFN-γ + TNF-α
IFNGR1
IFNGR2
B
IFNGR1 expression (MFI)
iso
C
IFN-γ
TNF-α
IFN-γ + TNF-α
C
IFNGR1
β-tubulin
C
IFN-γ
TNF-α
IFN-γ + TNF-α
D
PD-L1 mRNA/GAPDH
C
IFN-γ
IFN-γ + TNF-α
IFN-γ + TNF-α + si-NC
IFN-γ + TNF-α + si-IFNGR1
IFN-γ + TNF-α + si-IFNGR2
E
FITC iso
C
IFN-γ+TNF-α
IFN-γ+TNF-α+Si NC
IFN-γ+TNF-α+Si-IFNGR1
IFN-γ+TNF-α+Si-IFNGR2
Count (10³)
FITC-H
PD-L1 expression (MFI)
FITC iso
C
IFN-γ + TNF-α
IFN-γ + TNF-α + Si-NC
IFN-γ + TNF-α + Si-IFNGR1
IFN-γ + TNF-α + Si-IFNGR2
I
PD-L1 mRNA/GAPDH
C
IFN-γ
IFN-γ + TNF-α
IFN-γ + TNF-α + si-NC
IFN-γ + TNF-α + si-p65
J
FITC iso
C
IFN-γ+TNF-α
IFN-γ+TNF-α+Si-NC
IFN-γ+TNF-α+Si-p65
Count (10³)
FITC-H
PD-L1 expression (MFI)
FITC iso
C
IFN-γ + TNF-α
IFN-γ + TNF-α + Si-NC
IFN-γ + TNF-α + Si-P65
K
IFNGR1 mRNA/GAPDH
C
IFN-γ
IFN-γ + TNF-α
IFN-γ + TNF-α + si-NC
IFN-γ + TNF-α + si-p65
L
PD-L1 mRNA/GAPDH
C
IFN-γ + TNF-α
IFN-γ + TNF-α + siNC + vector
IFN-γ + TNF-α + siNC + over-IFNGR1
IFN-γ + TNF-α + si-p65 + vector
IFN-γ + TNF-α + si-p65 + over-IFNGR1
M
Luminescence (RLU)
IFNGR1 + pIRES2-RelA
IFNGR1 + pIRES2-Vector
Vector + pIRES2-RelA
C
N
site : -901 aggattttcc-891
Luminescence (RLU)
Vector + pIRES2-RelA
IFNGR1-WT + pIRES2-RelA
IFNGR1-MUT + pIRES2-RelA



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Figure 5 Tumor necrosis factor-alpha synergistically amplified interferon-gamma-induced JAK/STAT1/interferon regulatory factor 1 signaling activation by upregulating NF-κB-mediated interferon-gamma receptor 1/2 expression. A: The expression of NF-κB-mediated interferon-gamma receptor 1/2 (IFNGR1/2) were quantified by quantitative real-time PCR (qRT-PCR) in human umbilical-cord-mesenchymal stem cells (hUC-MSCs) treated with IFN-γ and tumor necrosis factor-alpha (TNF-α) alone or in combination for 4 h; B and C: IFNGR1 expression was quantified by flow cytometry or WB in hUC-MSCs treated with IFN-γ and TNF-α alone or in combination for 24 h; D and E: PD-L1 expression was quantified by qRT-PCR or flow cytometry in hUC-MSCs transfected with siRNA against NC, IFNGR1, or IFNGR2, and subsequently treated with IFN-γ and TNF-α for 4 h or 24 h; F: Activation of the JAK/STAT1/interferon regulatory factor 1 pathway and the expressions of PD-L1 and IFNGR1 were analyzed by western blot in hUC-MSCs after 24 h of incubation with IFN-γ and TNF-α in the presence or absence of IFNGR1 siRNA; G: Activation of the NF-κB pathway and expression of IFNGR1 were analyzed by western blot in hUC-MSCs after 24 h of incubation with TNF-α in the presence or absence of the NF-KB inhibitor BAY11-7082 (10 μM); H: IKKα, IKKβ, p65 and IκBα protein levels in the cytoplasm and nucleus of hUC-MSCs after TNF-α stimulation were measured through western blot; I: IFNGR1 expression was quantified by qRT-PCR in hUC-MSCs transfected

with p65 siRNA and subsequently treated with IFN-γ and TNF-α for 4 h; J and K: PD-L1 expression was quantified by qRT-PCR or flow cytometry in hUC-MSCs transfected with p65 siRNA and subsequently treated with IFN-γ and TNF-α for 4 h or 24 h; L: hUC-MSCs were infected with IFNGR1 overexpressed lentivirus and transfected with siRNA of p65, and induced with IFN-γ and TNF-α for 4 h, and then PD-L1 expression was quantified by qRT-PCR; M: PGL4.10-IFNGR1 promoter plasmid, pIRES2-p65 plasmid, and Renilla luciferase plasmid were transfected together or separately into 293T cells. Cells were lysed 24 h after transfection and luciferase activity was measured; N: pIRES2-p65 plasmid and Renilla luciferase plasmid were transfected into 293T cells with the PGL4.10-IFNGR1 promoter plasmid or the p65 binding site mutant PGL4.10-IFNGR1 promoter plasmid. Cells were lysed 24 h after transfection and luciferase activity was measured. The predicted binding sites of p65 to the IFNGR1 promoter are above the graph. Data were represented as mean ± SEM of $n = 3$. C: Control; Iso: Isotype; [b]$P < 0.001$; [c]$P < 0.0001$; [d]$P > 0.05$. IFN-γ: Interferon-gamma; PD-L1: Programmed cell death protein 1 ligand 1; TNF-α: Tumor necrosis factor-alpha.

yielding a better therapeutic efficacy in DSS-induced mice.

DISCUSSION

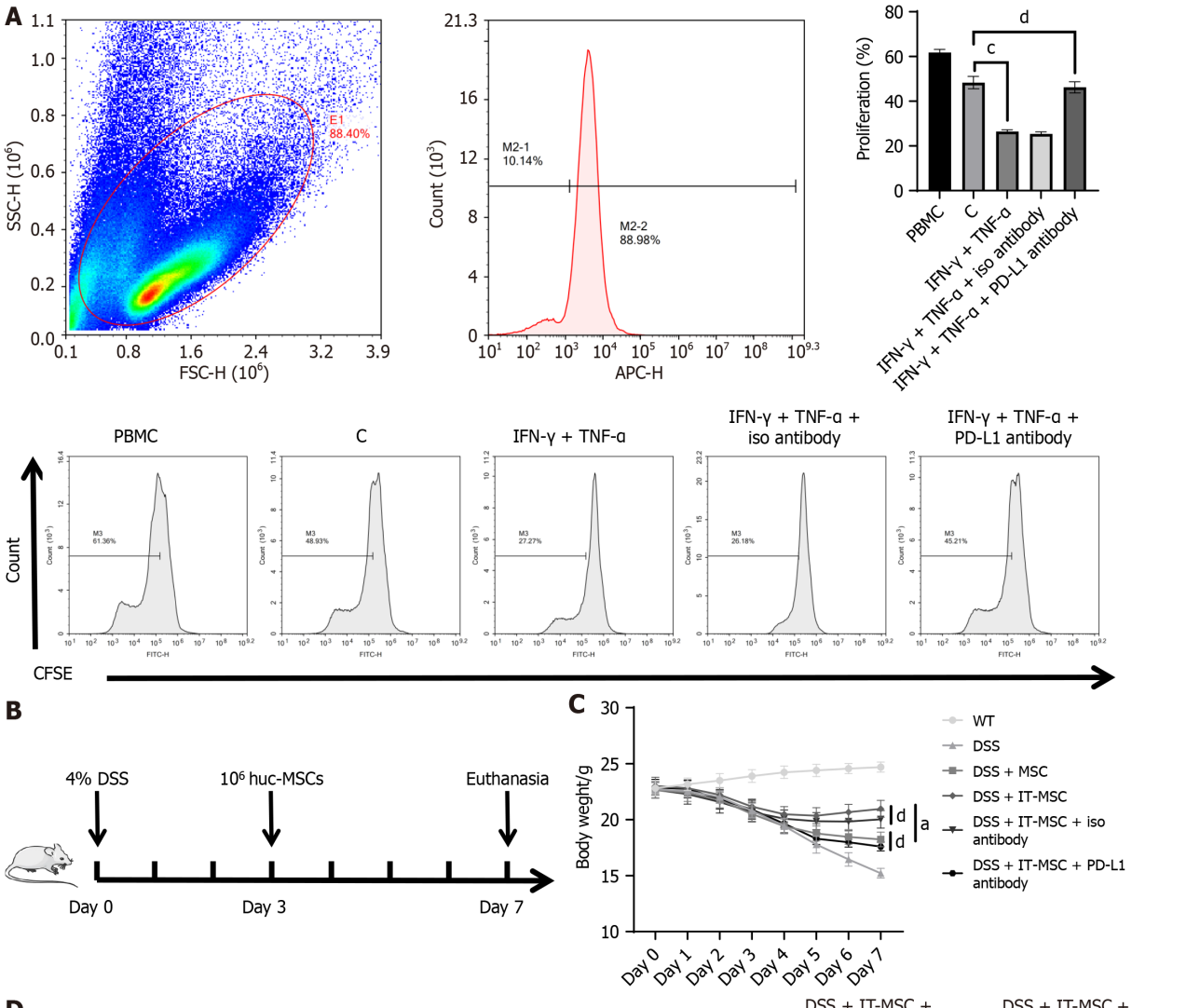
MSCs are usually derived from the umbilical cord, bone marrow, and adipose tissue. In the present study, hUC-MSCs were selected based on multiple considerations. First, as a type of medical waste, umbilical cord tissue is easier to obtain with less ethical controversy. A large number of MSCs are easily obtained from umbilical cord tissue by mature isolation methods. Second, hUC-MSCs have greater proliferative capacity and immunomodulatory ability than other types of MSCs[25,26]. Third, the major histocompatibility complex I (MHC-I) expression level and the immunogenicity of hUC-MSCs is lower. Hence, although the biosafety of MSCs has been a matter of great concern, hUC-MSCs have become the main source of cells for mesenchymal stem cell transplantation therapy[27]. Therefore, as hUC-MSCs have greater clinical application potential, they were selected for use in the study. In DSS-induced colitis mice, MSCs not only significantly improved the colonic lesions of mice but also ameliorated the tumorigenesis of colitis[28]. In a clinical trial, researchers followed up 34 patients with ulcerative colitis who received hUC-MSCs for up to two years, and no long-term complications such as malignant tumors and immune rejection were observed during follow-up[29]. Similarly, except for the therapeutic effect of hUC-MSCs on colitis mice, no MSC-related side effects or toxicities were observed in the present study. Overall, MSCs have a high safety profile in the treatment of ulcerative colitis.

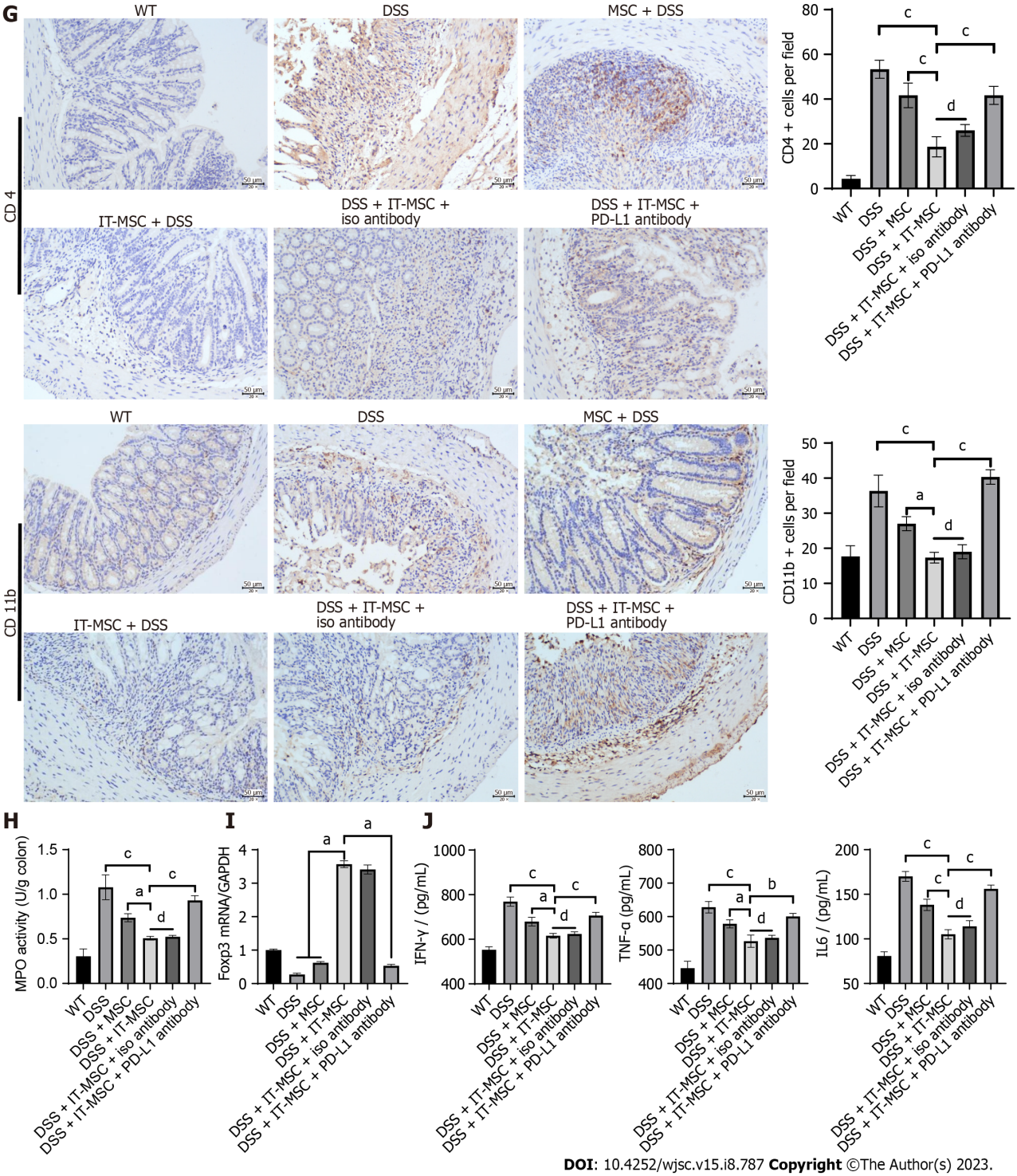
PD-L1 plays an important role in the immunosuppressive capacity of hUC-MSCs. Although the mechanism of PD-L1 expression has been thoroughly elucidated in tumors[30], it remains to be explored in MSCs. The cytokines that induce MSCs to express PD-L1 include IFN-γ, IL25, and IL27, and the relevant pathways are STAT1, JNK/STAT3, and JAK/STAT1[31-33]. We performed this study to investigate the mechanism of PD-L1 expression in MSCs under IFN-γ and TNF-α stimulation and to find a way to enhance the immunosuppressive capacity of MSCs. In the present study, we confirmed that IFN-γ induces PD-L1 expression through the JAK/STAT1/IRF1 pathway in hUC-MSCs. TNF-α upregulates the expression of IFNGR1/2 *via* the NF-κB pathway, which promotes the activation of the JAK/STAT1/IRF1 pathway induced by IFN-γ, significantly increasing PD-L1 expression. Furthermore, synergistic IFN-γ and TNF-α induction of high PD-L1 expression in hUC-MSCs strongly inhibited T-cell proliferation[33]. In mice with DSS-induced colitis, tail vein infusion of pretreated hUC-MSCs reduced disease activity, colonic tissue damage, cytokine levels, and inflammatory cell infiltration. Thus, combined treatment with IFN-γ and TNF-α synergistically induces high PD-L1 expression in hUC-MSCs, which strengthens the immunosuppressive capacity of hUC-MSCs (Figure 7).

PD-L1 expression has been extensively studied in inflammatory diseases and tumors. In tumor cells, various pathways are involved in IFN-γ-induced PD-L1 expression[30]. For instance, IFN-γ induces PD-L1 expression *via* the JAK/STAT/IRF1 pathway in tumor cells[34,35]. Similarly, studies have shown that PD-L1 expression in MSCs can be induced by IFN-γ mediated by STAT1. Based on these studies, we speculated that these signals are similar, and that PD-L1 expression induced by IFN-γ in hUC-MSCs might have occurred through the JAK/STAT1/IRF1 pathway. To verify our hypothesis, we conducted signaling pathway inhibitor, RNA interference, and luciferase reporter gene experiments. Our results showed that IFN-γ activates the JAK/STAT1 pathway and promotes IRF1 binding to the PD-L1 promoter, increasing PD-L1 mRNA expression. The results reveal the molecular mechanism by which IFN-γ induces the expression of PD-L1 in hUC-MSCs in detail.

In tumor cells, TNF-α co-stimulation with IL4 or IFN-γ increased PD-L1 expression, and the effect was associated with the NF-κB pathway[20,36]. HCC cells induced by IFN-γ and TNF-α had higher PD-L1 expression than those induced by IFN-γ alone, and had stronger resistance to adaptive immunity[20]. Such findings suggest that TNF-α enhances PD-L1 expression induced by IFN-γ and NF-κB signaling might be involved. In our results, we found that TNF-α significantly increased IFN-γ-induced PD-L1 expression in hUC-MSCs. Importantly, we demonstrated that TNF-α upregulated IFNGR1 expression through the NF-κB pathway, which enhanced IFN-γ/IFNGR/JAK/STAT1/IRF1 signaling axis activation, significantly upregulating PD-L1 expression. In addition, we found that trends of PD-L1 expression in two others common MSCs pretreated with IFN-γ and TNF-α were similar to those of hUC-MSCs (Supplementary Figure 4C). The results suggest that IFN-γ and TNF-α synergistically induce high PD-L1 expression, which may be universally present in MSCs from different sources. Another issue to consider is that IFN-γ or TNF-α may affect cell survival. Therefore, the effect of such cytokines on the proliferation and apoptosis of hUC-MSCs was evaluated. Additionally, the results showed that IFN-γ and TNF-α stimulation alone or in combination had no effect on the proliferation and apoptosis of hUC-MSCs (Figure 3C and D; Supplementary Figure 3B and C).

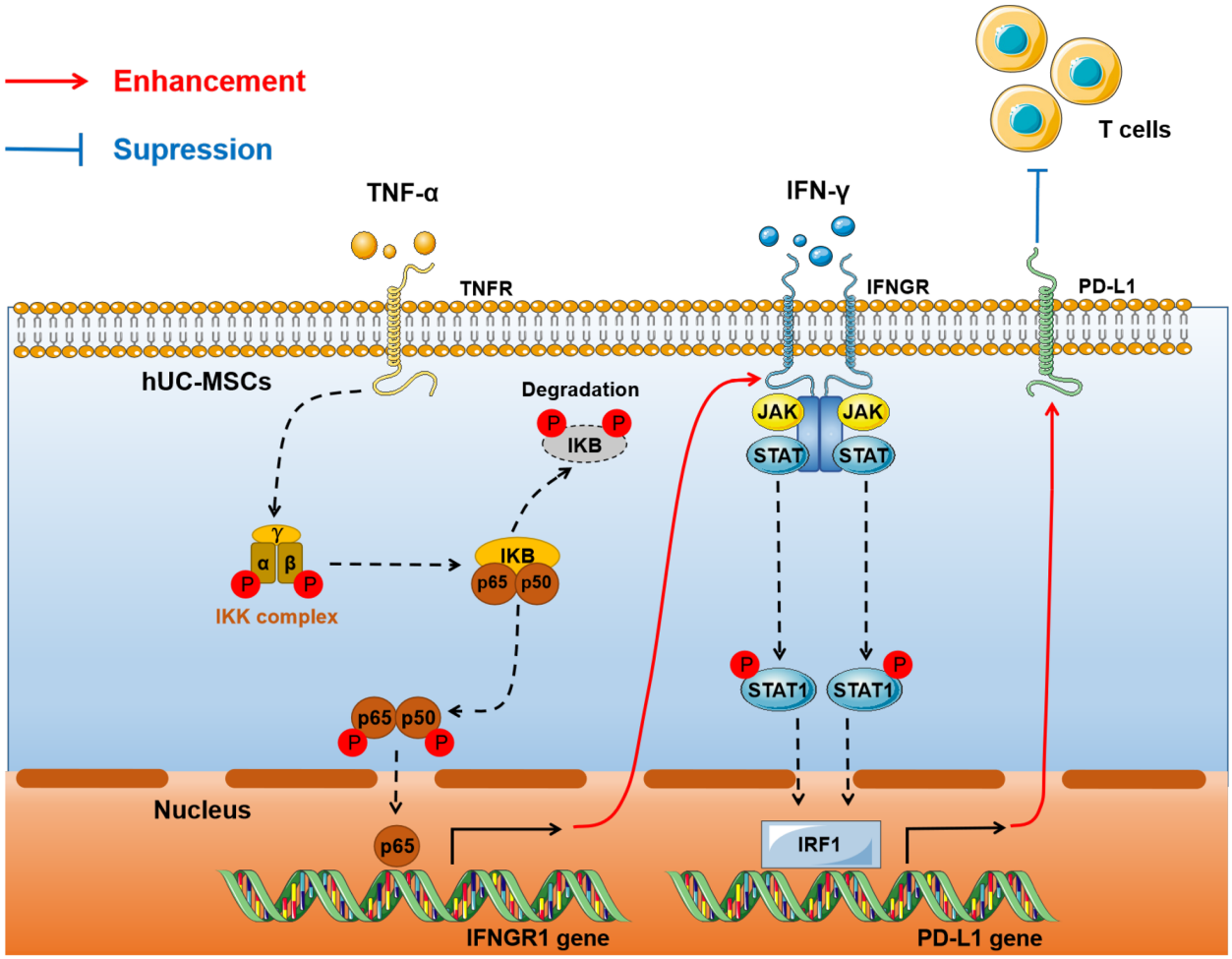
Ulcerative colitis is an autoimmune disease with complex mechanisms[37]. PD-L1 is involved in the pathogenesis of colitis by targeting T cells[38]. The high PD-L1 expression in colitis regulates cytokine production in colonic dendritic


A
SSC-H (10^6)
FSC-H (10^6)
E1
88.40%
Count (10^3)
APC-H
M2-1
10.14%
M2-2
88.98%
Proliferation (%)
PBMC
C
IFN-γ + TNF-α
IFN-γ + TNF-α + iso antibody
IFN-γ + TNF-α + PD-L1 antibody
Count
CFSE
B
4% DSS
10^6 huc-MSCs
Euthanasia
Day 0
Day 3
Day 7
C
Body weight/g
WT
DSS
DSS + MSC
DSS + IT-MSC
DSS + IT-MSC + iso antibody
DSS + IT-MSC + PD-L1 antibody
D
WT
DSS
DSS + MSC
DSS + IT-MSC
DSS + IT-MSC + iso antibody
DSS + IT-MSC + PD-L1 antibody
1 cm
E
WT
DSS
MSC + DSS
IT-MSC + DSS
DSS + IT-MSC + iso antibody
DSS + IT-MSC + PD-L1 antibody
F
Pathological score



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Figure 6 Synergy by IFN-γ and tumor necrosis factor-alpha enhanced the immunosuppressive capacity of human umbilical-cord-mesenchymal stem cells by inducing programmed cell death protein 1 ligand expression. A: The CFSE fluorescence intensity of CFSE-labeled peripheral blood mononuclear cells (PBMCs) was detected after co-culture with human umbilical-cord-mesenchymal stem cells (hUC-MSCs) with different treatments for 3 d. The CFSE fluorescence intensity of CD3-positive PBMCs on day 0 was used as the reference (1.29%). The histogram shows the quantification results pertaining to the mean fluorescent intensity of CFSE in each group; B: Schematic diagram of dextran sulfate sodium-induced colitis and MSCs transplantation therapy in mice; C: Bodyweight of mice in each group during the disease process (n = 6); D: Macroscopic appearance of the colon in each group; E: Colon sections embedded in paraffin were stained with hematoxylin and eosin for light microscopy assessment. Scale bar, 50 μm; F: Histopathological score of colons from each group was assessed for disease severity (n = 3); G: Infiltration cells expressing CD4 or CD11b in colon tissue sections from each group were analyzed by immunohistochemical staining (n = 3). Scale bar, 50 μm; H: Myeloperoxidase activity in the colon was determined (n = 3); I: The colonic mRNA expression of Treg (Foxp3) from each group were analyzed by quantitative real-time PCR (n = 3); J: Levels of inflammatory factors in colon tissue homogenates from each group were determined using ELISA (n = 3). IT-MSCs refer to hUC-MSCs pretreated with IFN-γ and tumor necrosis factor-alpha for 24 h. C: Control; $^{a}P < 0.01$; $^{b}P < 0.001$; $^{c}P < 0.0001$; $^{d}P > 0.05$.



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Figure 7 Molecular mechanism of interferon-gamma and tumor necrosis factor-alpha synergistically upregulated expression of programmed cell death protein 1 ligand in human umbilical-cord-mesenchymal stem cells to inhibit T cells. IFN-γ: Interferon-gamma; PD-L1: Programmed cell death protein 1 ligand 1; TNF-α: Tumor necrosis factor-alpha; hUC-MSCs: Human umbilical-cord-mesenchymal stem cells; IRF1: Interferon regulatory factor 1.

cells, inhibits Th1/Th17 cell activity, and promotes Treg cell proliferation[38-40]. Therefore, DSS-induced colitis models are often used to evaluate the efficacy of drugs and the immunomodulatory ability of MSCs[22,41,42]. We used T-cell proliferation inhibition experiments *in vitro* to confirm that hUC-MSCs treated with IFN-γ and TNF-α have a stronger immunosuppressive capacity than untreated hUC-MSCs. Furthermore, we evaluated the immunosuppressive capacity of hUC-MSCs treated with IFN-γ and TNF-α in a DSS-induced mouse colitis model and found that they exhibited fewer disease symptoms and less tissue damage, as well as lower inflammatory cell infiltration, lower inflammatory factors, and greater Treg cells recruitment than untreated hUC-MSCs. Moreover, we performed RNA-seq analysis on hUC-MSCs treated with IFN-γ and TNF-α. We found that with a combination of IFN-γ and TNF-α treatment, hUC-MSCs also expressed IDO, TSG-6, and other immunosuppressive molecules (data not shown). The results suggest that hUC-MSCs treated with IFN-γ and TNF-α have a stronger immunomodulatory capacity than untreated hUC-MSCs, perhaps because hUC-MSCs can express PD-L1 as well as other immunosuppressive molecules. This idea is worthy of further investigation.

In recent years, MSCT therapy has been rising in popularity for the treatment of various inflammatory diseases due to the immunosuppressive ability of MSCs. However, due to differences in both the sources of MSCs and the techniques of cell extraction and culture, the efficacy of MSCT in patients is not stable. Based on the present study, it is likely that the abundance of immunosuppressive molecules expressed by MSCs plays an important role. Therefore, this study provides two possible solutions to improve the efficacy of MSCT in diseases. On the one hand, MSCs can express more immunosuppressive molecules (PD-L1) after co-induction with lower concentrations of inflammatory factors (IFN-γ and TNF-α) without impairing cell viability. On the other hand, the efficacy of MSCT can be improved by detecting and screening for MSCs with high expression levels of immunosuppressive molecules before transplantation. In future studies, to compensate for some of the limitations of this study in a translational application, it is necessary to investigate the effect of more combinations of inflammatory factors on PD-L1 expression in MSCs, and also investigate the immunosuppressive molecules induced by other inflammatory factors. More research in this area will contribute to the translational application of MSCT in clinical practice.

CONCLUSION

In the present study, we found that IFN-γ and TNF-α synergistically induce a high level of PD-L1 expression in hUC-MSCs, and we elucidated the molecular mechanism involved. More importantly, both *in vitro* and *in vivo* experiments confirmed that INF-γ- and TNF-α-pretreated hUC-MSCs with these high PD-L1 expression levels have strong immunosuppressive capacity and, therefore, have great potential for clinical application in the treatment of autoimmune diseases.

ARTICLE HIGHLIGHTS

Research background

The immunosuppressive capacity of Mesenchymal stem cells (MSCs) is dependent on the "license" of several proinflammatory factors to express immunosuppressive factors such as programmed cell death 1 ligand 1 (PD-L1), which determines the clinical therapeutic efficacy of MSCs for inflammatory or immune diseases. In MSCs, interferon-gamma (IFN-γ) is a key inducer of PD-L1 expression, which is synergistically enhanced by tumor necrosis factor-alpha (TNF-α), but this; however, the mechanism is unclear.

Research motivation

The efficacy of MSCs transplantation (MSCT) is limited by the expression of immunosuppressive molecules of MSCs, including PD-L1. However, the mechanism of IFN-γ alone or in combination with TNF-α inducing MSCs high expression of PD-L1 remains unclear.

Research objectives

To reveal the mechanism of high PD-L1 expression in MSCs pretreated with IFN-γ and TNF-α and explore the application prospects of MSCs with high PD-L1 expression in ulcerative colitis.

Research methods

We assessed PD-L1 expression in human umbilical cord-MSCs (hUC-MSCs) induced by IFN-γ and TNF-α, alone or in combination. Additionally, we performed signaling inhibition and RNA interference experiments to elucidate the specific cross-talk signaling pathways between IFN-γ and TNF-α. Finally, we evaluated the immunosuppressive capacity of hUC-MSCs treated with IFN-γ and TNF-α in an *in vitro* mixed lymphocyte culture assay, and *in vivo* in mice with dextran sulfate sodium (DSS)-induced acute colitis.

Research results

Our results showed that IFN-γ upregulated PD-L1 expression in hUC-MSCs to enhance their immunosuppressive activities through the JAK/STAT1/interferon regulatory factor 1 (IRF1) pathway. TNF-α synergistically enhanced IFN-γ-induced PD-L1 expression in hUC-MSCs by upregulating nuclear factor kappa-B (NF-κB)-mediated expression of IFN-γ receptor 1 to activate JAK/STAT1/IRF1 signaling. Furthermore, high PD-L1 expression in hUC-MSCs induced by IFN-γ and TNF-α significantly attenuated DSS-induced acute colitis in mice.

Research conclusions

Our study revealed that IFN-γ and TNF-α synergistically induce the high PD-L1 expression of hUC-MSCs through the JAK/STAT1/IRF1 pathway and the NF-κB pathway, which effectively improves the immunosuppressive capacity of hUC-MSCs.

Research perspectives

The results of the present study could facilitate the optimization of clinical application strategies of MSCT.

FOOTNOTES

Author contributions: Chen Z and Yao MW contributed equally to this work; Chen Z and Yao MW designed all the project and the experiment; Chen Z, Ao X, Guo W, Xing W, Shen ZL, and Lian QZ developed the methodology; Chen Z, Yao MW, and Ao LQ performed the *in vivo* experiments; Chen Z, Yao MW, Li Z, Li SD, and Lu WY performed the *in vitro* experiments; Chen Z and Ao X wrote the manuscript; Xu X, Ao X, and Chen Z reviewed and edited the paper; and all authors read and approved the final manuscript.

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Constitutive aryl hydrocarbon receptor facilitates the regenerative potential of mouse bone marrow mesenchymal stromal cells

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Abstract

BACKGROUND

Bone marrow mesenchymal stromal cells (BMSCs) are the commonly used seed cells in tissue engineering. Aryl hydrocarbon receptor (AhR) is a transcription factor involved in various cellular processes. However, the function of constitutive AhR in BMSCs remains unclear.

AIM

To investigate the role of AhR in the osteogenic and macrophage-modulating potential of mouse BMSCs (mBMSCs) and the underlying mechanism.

METHODS

Immunochemistry and immunofluorescent staining were used to observe the expression of AhR in mouse bone marrow tissue and mBMSCs. The overexpression or knockdown of AhR was achieved by lentivirus-mediated plasmid. The osteogenic potential was observed by alkaline phosphatase and alizarin red staining. The mRNA and protein levels of osteogenic markers were detected by quantitative polymerase chain reaction (qPCR) and western blot. After coculture with different mBMSCs, the cluster of differentiation (CD) 86 and CD206 expressions levels in RAW 264.7 cells were analyzed by flow cytometry. To explore the underlying molecular mechanism, the interaction of AhR with signal transducer and activator of transcription 3 (STAT3) was observed by co-immunoprecipitation and phosphorylation of STAT3 was detected by western blot.

RESULTS

AhR expressions in mouse bone marrow tissue and isolated mBMSCs were detected. AhR overexpression enhanced the osteogenic potential of mBMSCs

while AhR knockdown suppressed it. The ratio of CD86+ RAW 264.7 cells cocultured with AhR-overexpressed mBMSCs was reduced and that of CD206+ cells was increased. AhR directly interacted with STAT3. AhR overexpression increased the phosphorylation of STAT3. After inhibition of STAT3 *via* stattic, the promotive effects of AhR overexpression on the osteogenic differentiation and macrophage-modulating were partially counteracted.

CONCLUSION
AhR plays a beneficial role in the regenerative potential of mBMSCs partially by increasing phosphorylation of STAT3.

Key Words: Aryl hydrocarbon receptor; Bone marrow mesenchymal stromal cells; Osteogenesis; Macrophage; Signal transducer and activator of transcription 3; Interaction

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Core Tip: Aryl hydrocarbon receptor (AhR) was positively expressed in murine bone marrow tissue and bone marrow mesenchymal stromal cells (BMSCs). *In vitro*, overexpression of AhR enhanced the osteogenic potential of mouse BMSCs. Additionally, AhR-overexpressed BMSCs had an increased ability to polarize macrophages to an anti-inflammatory phenotype. While knockdown of AhR showed the opposite effects. Mechanistically, the beneficial effects of AhR were partially dependent on increased phosphorylation of signal transducer and activator of transcription 3. This study suggests that AhR might be a target for achieving optimal bone regeneration in mouse BMSCs-based tissue engineering.

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INTRODUCTION

Cell-based tissue engineering is an important method for the treatment of bone defects. Bone marrow mesenchymal stromal cells (BMSCs) are one of the most commonly used seed cells. The osteogenic potential of BMSCs is the premise of their applications in bone regeneration[1]. Additionally, the crosstalk between BMSCs and immune cells like macrophages has been recognized as a critical element in achieving ideal bone tissue repair[2]. Plenty studies have demonstrated that BMSCs are able to trigger a functional switch in macrophages from pro-inflammatory classically activated macrophages (M1) to anti-inflammatory alternatively activated macrophages (M2)[3]. In turn, macrophage polarization is essential for the osteogenic potency of BMSCs. M2 macrophages promote the osteogenesis of BMSCs by secreting pro-regenerative cytokines[4], while M1 macrophages suppress the process[5]. Accordingly, approaches to enhance the osteogenic potential and macrophage-modulating capacity of BMSCs, such as genetic engineering to express specific genes, are continuously being explored.

Aryl hydrocarbon receptor (AhR) is a member of the helix-loop-helix transcription factor superfamily[6]. Historically, AhR has been recognized as a nuclear receptor that responds to environmental toxic stimuli. Recently, increasing number of studies have demonstrated that AhR is an essential modulator in bone turnover[7] and immune responses[8]. AhR can be activated by chemosynthetic agonists such 6-formyl (3,2-b) carbazole (FICZ)[9]. In our previous studies, AhR signaling was suppressed in periodontitis, and FICZ alleviated the inflammatory responses by activating AhR and promoting the phosphorylation of signal transducer and activator of transcription 3 (STAT3)[10]. In another study of our group, FICZ was found to play a beneficial role in the proliferation, osteogenic potential and macrophage-modulation of rat BMSCs and primed cartilage templates[11].

Except for ligand-activated AhR, the role of constitutive unligated AhR in the osteogenic and macrophage-modulating potential of BMSCs has not been investigated. Therefore, the aim of the present study was to: (1) Establish stable AhR-overexpressing or AhR-knockdown mouse BMSCs (mBMSCs); (2) explore the osteogenic differentiation of different mBMSCs; (3) observe the phenotype of macrophages cocultured with different mBMSCs; and (4) investigate the involved molecular mechanism.

MATERIALS AND METHODS

Animal

Six-week-old male C57BL/6 mice were obtained from the Hubei Research Centre of Laboratory Animals (Wuhan, China) and kept in specific pathogen free condition. All experimental protocols were approved by the Institutional Animal Care and Use Committee of School and Hospital of Stomatology, Wuhan University (No. 2020-A08).

Isolation of BMSCs

BMSCs was isolated from 6-week-old male C57BL/6 mice *via* whole femur bone marrow adherent culturing. The femora were excised aseptically, cleaned of soft tissues, and passed through 3 washes with phosphate buffered saline (PBS). The ends of the bones were removed, and the marrow flushed out. The released cells were collected in two 75 cm^2 flasks (Corning) containing 10 mL of 10% fetal bovine serum (Hyclone) in -minimum essential medium. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2, at 37°C. After 72 h, all medium was aspirated and replaced to remove the non-adherent cells. The medium was replaced every 2–3 d.

Immunohistochemistry staining

The mice femurs were collected and fixed with 4% paraformaldehyde and then decalcified in 10% ethylene diamine tetraacetic acid for 6 wk. The tissue was subsequently processed for paraffin embedding and serial 4-mm-thick sections were prepared. Then the sections were dewaxed in xylene and rehydrated through graded ethanol to water. Antigen retrieval was conducted in stomach enzyme antigen repair solution for 30 min at 37 °C. Immunostaining was performed by incubating the sections with anti-AhR (NB300-515, 1:200, Novus) at 4 °C overnight. The slides were then washed with PBS and incubated with secondary antibody (Maxim Biotechnology) for 30 min at 37 °C. Staining was visualized with 3, 3-diaminobenzidine and counterstained with hematoxylin.

Immunofluorescent staining

BMSCs at third passage were seeded in cell dish (801002, NEST). After the cells reached 80% confluence, BMSCs were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 20min. Then the cells were blocked with bovine serum albumin (BSA) for 1 h. Subsequently, the cells were incubated with primary antibody against AhR (NB300-515, 1:100, Novus) at 4 °C overnight. After washing, cells were incubated with dylight 594-conjugated secondary antibody (1:200, A23420, Abbkine) for 1 h at room temperature. Then, the cells were stained with 4',6-diamidino-2-phenylindole staining solution (C1005, Beyotime) for 5 min. Finally, the stained cells were observed and photographed under confocal microscope (Leica-LCS-SP8-STED).

Short hairpin RNA design and plasmid preparation

Based on the published sequence of mouse AhR (NM_013464), certain short hairpin RNA (shRNAs) specifically targeting AhR were designed to knockdown their expressions in mBMSCs. The shRNA sequences are as follows: 5′-CATCGA-CATAACGGACGAAAT-3′ (AhR sense) and 5′-ATTTCGTCCGTTATGTCGATG–3′ (antisense). These plasmid DNAs transcribed shRNAs with loop sequences of 5′-CTCGAG–3′. In parallel, a negative control (NC) sequences were projected (sense: 5′-TTCTCCGAACGTGTCACGT-3′, antisense: 5′-ACGTGACACGTTCGGAGAA-3′), which had no homology with human proteins. The generated oligo DNA was cloned into GV493 vector (hU6-MCS-CBh-gcGFP-IRES-puromycin) (GENECHEM, Shanghai).

AhR overexpression plasmid

The coding sequences of mouse AhR (NM_013464) was cloned into GV358 vector (Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin) (GENECHEM, Shanghai). The empty Ubi-MCS-SV40-EGFP-IRES-puromycin vector was served as NC.

Lentiviral packaging and infection of mBMSCs

The 293T packaging cell line was co-transfected with plasmid and lentiviral helper vectors (GENECHEM, Shanghai). The medium was replaced with fresh dulbecco's modified eagle medium overnight. After another 48 h, the supernatants were collected and filtered. The viral supernatants were concentrated *via* ultracentrifuging at the speed of 25000 rpm for 2 h at 4°C. One day prior to infection, mBMSCs were seeded in 6-well plates at the density of 10^5 cells/well. Then the mBMSCs were infected with lentiviral particles containing plasmids of knockdown-NC (sh-NC), knockdown-AhR (sh-AhR), overexpression-NC (oe-NC) or overexpression-AhR (oe-AhR) *via* multiplicity of infection level of 20. After 12-16 h, the culture medium was refreshed. Then the cells were cultured in complete medium containing 2 mg/mL puromycin.

qPCR

Total RNA from different cell samples were isolated by Trizol reagent (Takara Bio). SYBR Green Reagent (Takara Bio) was used to perform qPCR in a 7500 Fast Real-Time PCR system (Applied Biosystem). The primer sequences used in the study was showed in Table 1. The relative expression levels were calculated using the 2-DDCt method. Three biological replicates were conducted.

Western blot

Total proteins were extracted from cells with radio immunoprecipitation assay buffer supplemented with 1:100 proteinase and phosphatase inhibitors. The proteins were separated by 10% sodium dodecyl sulphate (SDS)-polyvinylidene fluoride membranes (Roche). After blocked with 5% milk, the membranes were incubated with primary antibody at 4°C overnight. Then the membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the bands were visualized with electro-chemi-luminescence detection reagents (Thermo Scientific). Anti-AhR (NB300-515, 1:200, Novus), anti-biomineralization associated [tissue-nonspecific alkaline phosphatase (ALPL)] (11187-1-AP, 1:1000, Proteintech), anti-runt-related transcription factor 2 (RUNX2) (PB0171, 1:2000, Boster), anti-phosphorylated STAT3 (Tyr705) (9145, 1:1000, CST), anti-STAT3 (9139, 1:1000, CST) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (BM3876, 1:5000, Boster) primary antibodies were

Table 1 Primer sequences for quantitative polymerase chain reaction (5'-3')

Gene	Sequences
GAPDH	Forward: TGGAAAGCTGTGGCGTGAT
	Reverse: GTCATCATACTTGGCAGGTTTCT
AhR	Forward: GGCTTTCAGCAGTCTGATGTC
	Reverse: CATGAAAGAAGCGTTCTCTGG
ALPL	Forward: GGGCGTCTCCACAGTAACCG
	Reverse: ACTCCCACTGTGCCCTCGTT
RUNX2	Forward: GAGTCAGATTACAGATCCCA
	Reverse: TGGCTCTTCTTACTGAGAGA

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; AhR: Aryl hydrocarbon receptor; ALPL: Tissue-nonspecific alkaline phosphatase; RUNX2: Runt-related transcription factor 2.

used. Three biological replicates were conducted.

Osteogenic induction, alkaline phosphatase staining and alizarin red staining

For osteogenic induction, mBMSCs at passage 3 from different groups (sh-NC, sh-AhR, oe-NC or oe-AhR) were cultured in medium supplemented with 50 mg/mL ascorbic acid, 10 mmol/L b-glycerophosphate and 10^{-8} mol/L dexamethasone (Sigma). Then, cells were fixed in 4% paraformaldehyde for 10 min and stained using alkaline phosphatase (ALP) Color Development Kit (C3206, Beyotime) following the manufacturer's instructions. For alizarin red staining (ARS), cells were fixed and stained with alizarin red solution (Cyagen) for 10 min. Three biological replicates were conducted.

Co-culture of mBMSCs and RAW264.7 cells

For direct coculture, mBMSCs from all groups (sh-NC, sh-AhR, oe-NC or oe-AhR) were plated in monoculture at the density of 5 10^5 cells/well at 6-well plate. After the adherence of mBMSCs, the macrophage linage RAW264.7 cells (ScienceCell, Shanghai) (1 10^5 cells/well) were seeded into the wells. The cells were harvested after 24 h of coculture.

For indirect coculture, conditional medium from the culture of sh-NC, sh-AhR, oe-NC or oe-AhR mBMSCs were collected. The conditioned medium was harvested and centrifuged for 10 min at 1000 rpm and then frozen at -20 °C until used. The RAW 264.7 was treated with 1:2 fresh medium and conditioned medium for 24 h.

Flow cytometry

The macrophage surface markers of RAW264.7 cells in the coculture system was detected by flow cytometry. Three biological replicates were conducted.

For direct coculture, the mBMSCs and RAW264.7 cells were collected from 6-well plates after coculture for 24 h and washed three times with PBS. The cell suspensions were divided into 1.5 mL Eppendorf micro test tubes (EP tubes) and incubated with blocking 3% BSA. Then the cell suspensions were incubated with allophycocyanin anti-F4/80 (157305, BioLegend), combined with fluorescein isothiocyanate (FITC) anti-cluster of differentiation (CD) 86 (105005, BioLegend) or PerCP/Cy5.5 anti-CD206 (141715, BioLegend) at 4°C for 1 h.

For indirect coculture, the RAW264.7 cells were collected and washed three times with PBS. The cell suspensions were divided into 1.5 mL EP tubes and incubated with blocking 2% BSA. Then the cell suspensions were successively incubated with CD86 (13395-1-AP, Proteintech) or CD206 (18704-1-AP, Proteintech), and FITC conjugated immunoglobulin G (BA1105, Boster).

The above cells were washed three times after incubation and suspended in 500 mL containing 3% fetal bovine serum and then detected by Beckman Coulter CytoFLEX S and analyzed by CytoExpert.

Co-immunoprecipitation

The AhR and p65 protein-protein interaction was detected by co-immunoprecipitation (Co-IP) kit (P2179S, Beyotime) following manufacturer's instructions. The mBMSCs protein sample was extracted from cells with lysis buffer supplemented with protease inhibitor cocktail. The AhR antibody (NB300-515, 1:100, Novus) was incubated with protein A+G beads at room temperature for 2 h and then washed three times using Tris buffered saline. Then the beads-antibody complex was immunoprecipitated with mBMSCs protein sample at 4°C overnight. After washed three times using lysis buffer, the beads-antibody-antigen complex was eluted SDS-polyacrylamide gel electrophoresis sample loading buffer. After magnetic separation, the expression of STAT3 in the supernatant was detected using STAT3 primary antibody (9139, 1:1000, CST) *via* western blot. Three biological replicates were conducted.

STAT3 inhibitor stattic

To testify whether the effects of AhR in osteogenic differentiation and macrophage-modulating was partially STAT3-

dependent or not, a specific STAT3 inhibitor stattic (Selleck) was introduced. In osteogenic induction, oe-NC and oe-AhR mBMSCs were cultured in osteogenic medium supplemented with or without 2 mmol/L stattic. For indirect coculture system of mBMSCs and RAW 264.7 cells, the mixed fresh and conditioned medium from mBMSCs was supplemented with or without 2 mmol/L stattic. Three biological replicates were conducted for each treatment.

Statistical analysis

All data were expressed as the mean ± SD. For comparison between two groups, statistical differences were evaluated by a two-tailed Student's *t* test. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by Tukey's test were conducted. *P*-value < 0.05 was considered statistically significant.

RESULTS

The expression of AhR in bone marrow of mouse femurs and isolated mBMSCs

The immunohistochemistry (IHC) staining showed that AhR was expressed in parts of mouse femur bone marrow (Figure 1A-C). In isolated mBMSCs derived from femurs, AhR expression was positively observed *via* immunofluorescence staining (Figure 1D-I), which was consistent with the IHC results.

AhR overexpression or knockdown in mBMSCs

At 48 h after infection with oe-AhR or sh-AhR and their NC lentivirus (oe-NC and sh-NC), the efficiencies of AhR overexpression or knockdown were confirmed by qPCR and western blot. The qPCR data (Figure 2A) showed that the relative AhR mRNA expression level of the oe-NC and oe-AhR groups were 0.85 ± 0.08 and 4.39 ± 0.23 respectively, and those of the sh-NC and sh-AhR groups were 1.13 ± 0.14 and 0.16 ± 0.01, respectively. The western blot results (Figure 2B and C) demonstrated that the relative AhR/GAPDH protein levels of the oe-NC and oe-AhR groups were (0.29 ± 0.05) and (0.45 ± 0.07), and those of the sh-NC and sh-AhR groups were 0.31 ± 0.11 and 0.04 ± 0.01 respectively. The data indicated that AhR was significantly overexpressed or knocked down in oe-AhR or sh-AhR mBMSCs.

The effect of oe-AhR or sh-AhR on the osteogenic differentiation of mBMSCs

At the 7th day of osteogenic induction, ALP staining showed that AhR overexpression resulted in more positive nodules than the NC (Figure 3A). Consistently, after 14 d of osteogenic induction, the visualization of calcium deposits and mineralized nodules by ARS also demonstrated that AhR overexpression promoted the osteogenic potential of mBMSCs (Figure 3A). In contrast, AhR knockdown suppressed the nodules formation and calcium deposition, as showed by ALP staining and ARS, compared to the NC (Figure 3A).

At the 7th day of osteogenic induction, the mRNA levels of the osteogenic markers ALP, ALPL and RUNX2 in oe-AhR mBMSCs were significantly higher than those in oe-NC mBMSCs (Figure 3B), while those in sh-AhR cells were lower than those in sh-NC cells (Figure 3C). Then, the protein samples were harvested after 7 d of osteogenic induction and were subjected to western blotting. The bands showed increased protein expressions of ALPL and RUNX2 in oe-AhR mBMSCs compared to oe-NC mBMSCs, while sh-AhR inhibited their expressions compared to sh-NC (Figure 3D), which was basically consistent with the mRNA results.

The effect of oe-AhR or sh-AhR on the macrophage-modulating capacity of mBMSCs

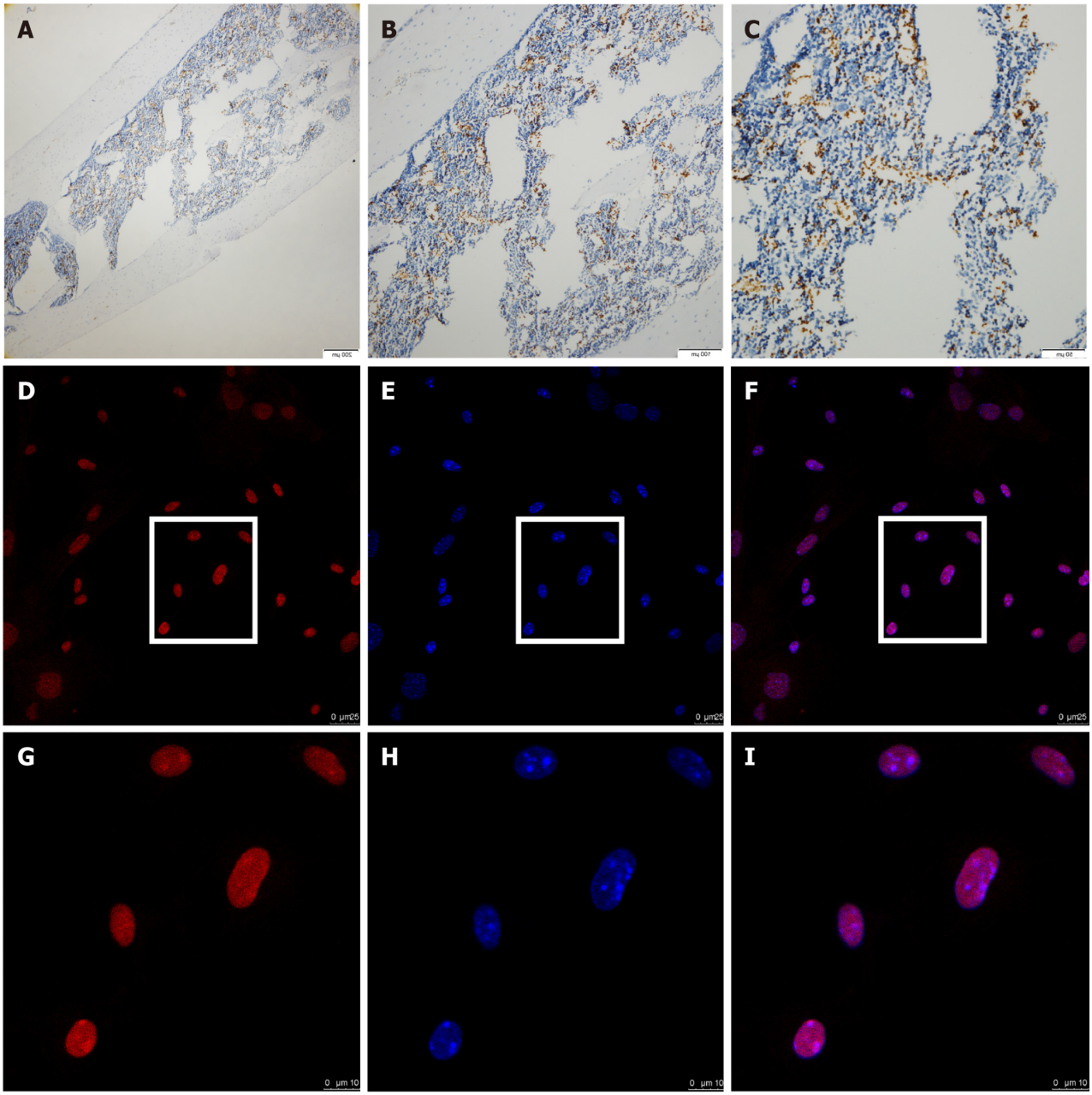
Brightfield images of direct coculture were captured prior to analysis (Figure 4A), and the morphologies of the RAW 264.7 cells and different groups of mBMSCs were similar. In flow cytometry analysis of direct coculture samples, the ratio of M1-like macrophages among RAW 264.7 cells was calculated as CD86 + (Q1-UR)/F4/80 + (Q1-UR + Q1-LR) (Figure 4B). Similarly, the ratio of M2-like macrophages among RAW 264.7 cells was calculated as CD206 + (Q2-UR)/F4/80 + (Q2-UR + Q2-LR) (Figure 4C). Quantitative analysis (Figure 4D) demonstrated that the ratio of CD86+ cells and CD206+ cells was not significantly different between RAW 264.7 cells cocultured with oe-AhR and those cocultured with oe-NC mBMSCs. While the ratio of CD86+ cells among RAW 264.7 cocultured with sh-AhR mBMSCs was significantly higher than that among RAW 264.7 cocultured with sh-NC mBMSCs.

In the indirect coculture system, RAW 264.7 cells were cultured with conditioned medium from different mBMSCs. The CD86 and CD206 expressions were demonstrated using histograms (Figure 5A and B). Quantitative analysis (Figure 5C) showed that the ratio of CD86+ cells was significantly lower and the ratio of CD206+ cells was significantly higher in the oe-AhR group than oe-NC. The ratio of CD86+ cells in the sh-AhR group was significantly higher than that in sh-NC.

The above data suggested that knockdown of AhR in mBMSCs tends to drive macrophages toward the M1-like phenotype and inhibit M2-like polarization. Overexpression of AhR in mBMSCs showed the opposite macrophage-modulating effect.

The molecular mechanism underlying the role of AhR in the osteogenic differentiation and macrophage modulation of mBMSCs

First, the AhR and STAT3 protein-protein interaction was detected *via* Co-IP (Figure 6A). Then, the effect of AhR overexpression or knockdown on the phosphorylation of STAT3 was explored by western blotting (Figure 6B). The results showed that oe-AhR promoted the phosphorylation of STAT3 while sh-AhR suppressed it. To test whether the effects of AhR on osteogenic differentiation and macrophage-modulating were partially STAT3-dependent or not, a specific STAT3



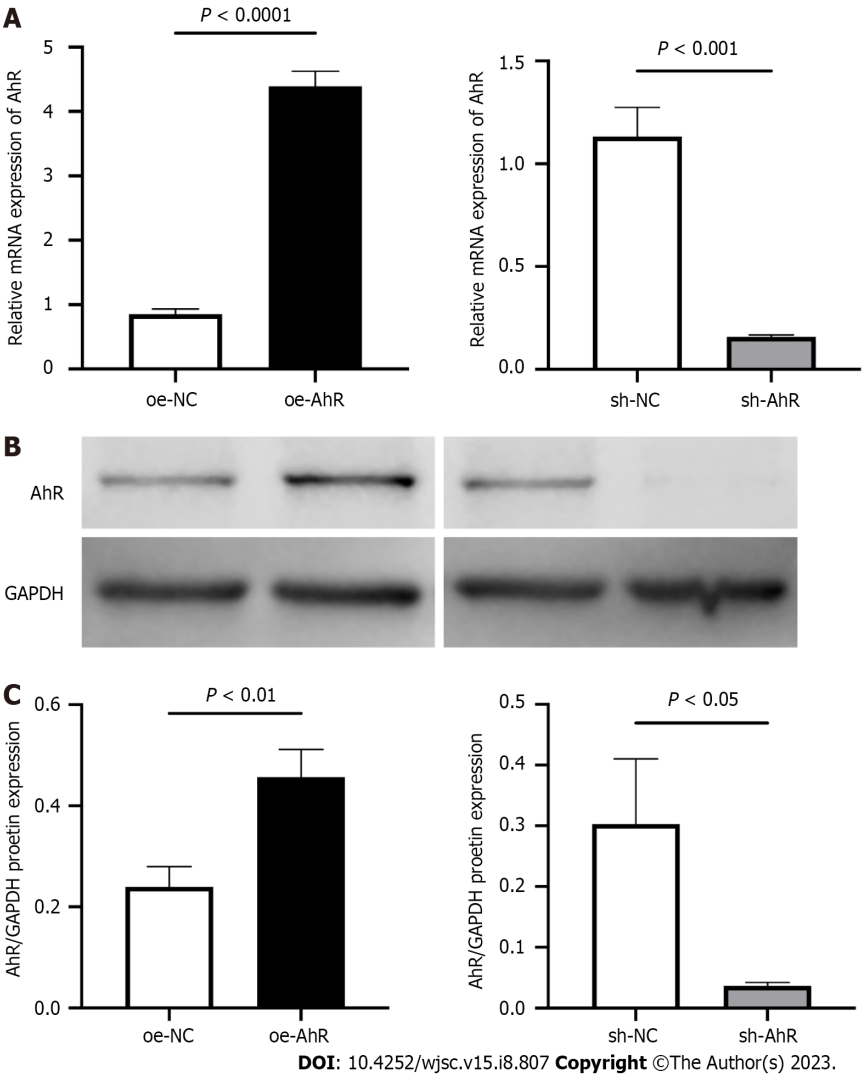
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Figure 1 The expression of aryl hydrocarbon receptor in bone marrow tissue of mouse femur and isolated bone marrow mesenchymal stromal cells. A-C: Immunochemistry staining showed positive aryl hydrocarbon receptor (AhR) expression in bone marrow tissue [bar: 200 mm (left), 100 mm (middle) and 50 mm (right)]. D-I: Immunofluorescence staining of AhR in mouse bone marrow mesenchymal stromal cells *via* confocal microscopy (red: AhR; blue: 4',6-diamidino-2-phenylindole; pink: Merge) [bar: 25mm (D-F) and 10mm (G-I)].

inhibitor, stattic, was introduced. The western blotting lanes testified that 2 mmol/L stattic partially alleviated the AhR overexpression-mediated increase in STAT3 phosphorylation (Figure 6C). ALP staining at the 7th day of osteogenic induction and ARS at the 15th day indicated that 2 mmol/L stattic partially reversed the elevated osteogenic potential mediated by AhR overexpression (Figure 6D). Regarding macrophage modulation, the flow cytometry and quantitative analysis manifested that 2 mmol/L stattic partially reversed the inhibition of CD86 expression and promotion of CD206 in RAW 264.7 cells cultured with conditioned medium from oe-AhR mBMSCs (Figure 6E and F). The above data suggested that AhR promoted the osteogenic and macrophage-modulating potentials of mBMSCs partially by interacting with STAT3 and increasing the phosphorylation of STAT3.

DISCUSSION

Based on the results, and within the limitations of the present study, it could be concluded that: (1) Endogenous unligated AhR promoted osteogenic potential of mBMSCs; (2) endogenous unligated AhR played a positive role in polarizing



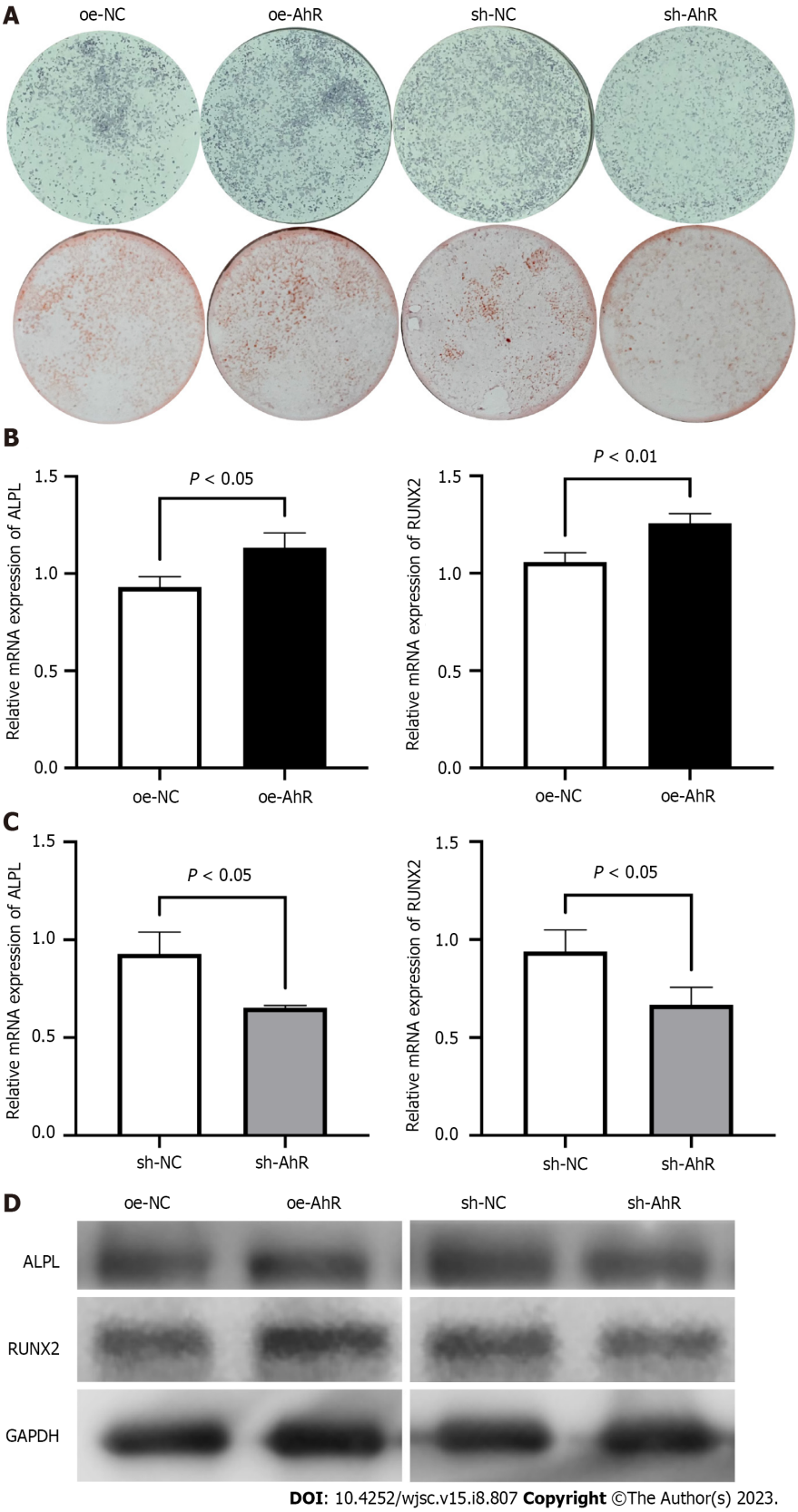
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Figure 2 Overexpression or knockdown of aryl hydrocarbon receptor in mouse bone marrow mesenchymal stromal cells. A: Quantitative polymerase chain reaction data of aryl hydrocarbon receptor (AhR) mRNA level of overexpression-AhR (oe-AhR) or knockdown-AhR (sh-AhR) and their negative controls; B: Representative images of western blot of AhR in oe-AhR or sh-AhR; C: Semi-quantitation of lanes in western blot. AhR: Aryl hydrocarbon receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; oe-NC: Overexpression-negative control; oe-AhR: Overexpression-AhR; sh-NC: Knockdown-negative control; sh-AhR: Knockdown-AhR.

macrophages towards the M2-like phenotype of mBMSCs; and (3) the function of AhR was partially dependent on interacting with STAT3 and increasing the phosphorylation of STAT3. The present study offered new insights into the role of AhR and the involved molecular mechanism in the regenerative potential of mBMSCs, which might be a target for achieving optimal bone regeneration in mBMSCs-based tissue engineering.

The nuclear receptor AhR is positively expressed in bone tissue, including osteoblasts and osteoclasts, and the AhR signaling pathway plays a vital role in bone homeostasis[12]. AhR was previously considered as an environment xenobiotic sensor and mediates oxidative stress[13]. In recent decades, the roles of AhR as a transcription factor in regulating various biological processes have been revealed. Plenty studies have focused on the functions of ligand-activated AhR in biological processes. However, the effects of AhR might vary due to interactions with different ligands. The first identified AhR ligand was a dioxin-like compound (DLC), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which mediates toxic effects after binding to AhR. Then, various kinds of non-dioxin-like endogenous ligands were identified, such as FICZ, 2-(1′-H–indole–3-carbonyl) thiazole–4-carboxylic acid methyl ester, and tryptophan metabolites such as kynurenine, which demonstrated functional diversity[9].

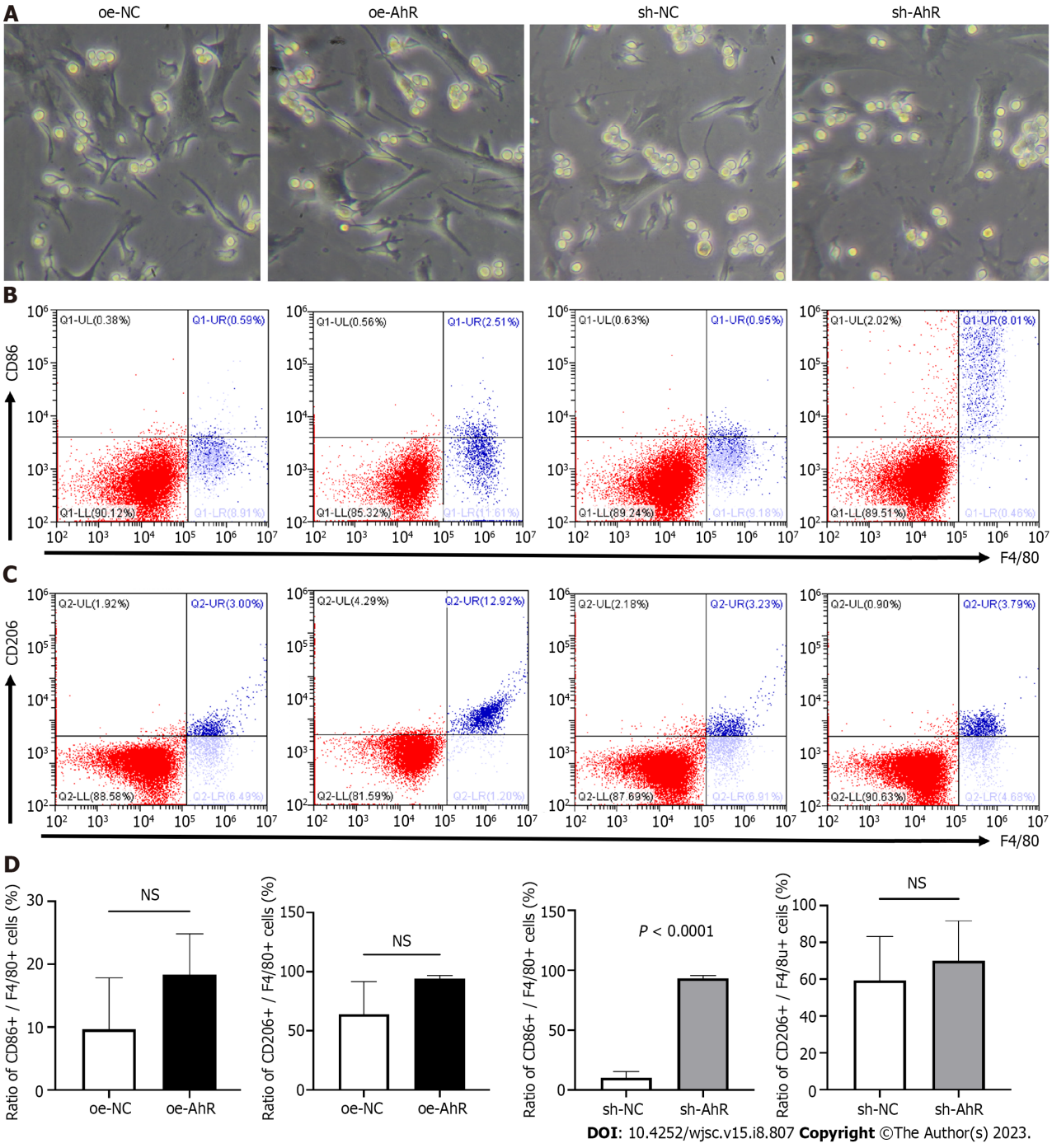
Regarding osteogenic potential, AhR activated by DLC ligands such as TCDD is basically harmful[14], but the effects of endogenous ligands such as FICZ are controversial. In a recent study, two different AhR ligands, benzo[a]pyrene (B[a]P) and FICZ, were analyzed in mice temporomandibular joint osteoarthritis[15]. The above study demonstrated that B[a]P induced mandibular subchondral bone resorption in an AhR-dependent manner. However, FICZ exerted a therapeutic effect and rescued the bone loss *in vivo* at both low (100mg/kg) and high (100mg/kg) concentrations. Moreover, 200ng/mL FICZ promoted the osteogenic differentiation on MC3T3 E1 cells *in vitro*, resulting in more obvious ALP staining and ARS, and increased mRNA expressions of osteogenic markers, including ALP, osteocalcin (OCN) and collagen type I alpha 1. Consistently, in a previous study by our group, 500 nM FICZ enhanced the ALP staining and ARS of rat BMSCs



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Figure 3 The effect of aryl hydrocarbon receptor overexpression or knockdown on the osteogenic differentiation in mouse bone marrow mesenchymal stromal cells. A: Alkaline phosphatase (ALP) staining (upper) at 7th day and Alizarin red staining staining (lower) at 14th day of osteogenic induction; B: Relative mRNA expression of ALP, biomineralization associated [tissue-nonspecific alkaline phosphatase (ALPL)] and runt-related transcription factor 2 (RUNX2) of overexpression-negative control and overexpression-aryl hydrocarbon receptor (AhR) mouse bone marrow mesenchymal stromal cells (mBMSCs); C: Relative ALPL and RUNX2 mRNA expression of knockdown-negative control and knockdown-AhR mBMSCs at 7th day of osteogenic induction; D: Representative images of western blot of ALPL and RUNX2 in mBMSCs of different groups at 7th day of osteogenic induction. oe-NC: Overexpression-negative control; oe-AhR: Overexpression-aryl hydrocarbon receptor; sh-NC: Knockdown-negative control; sh-AhR: Knockdown-aryl hydrocarbon receptor; ALPL: Tissue-nonspecific alkaline

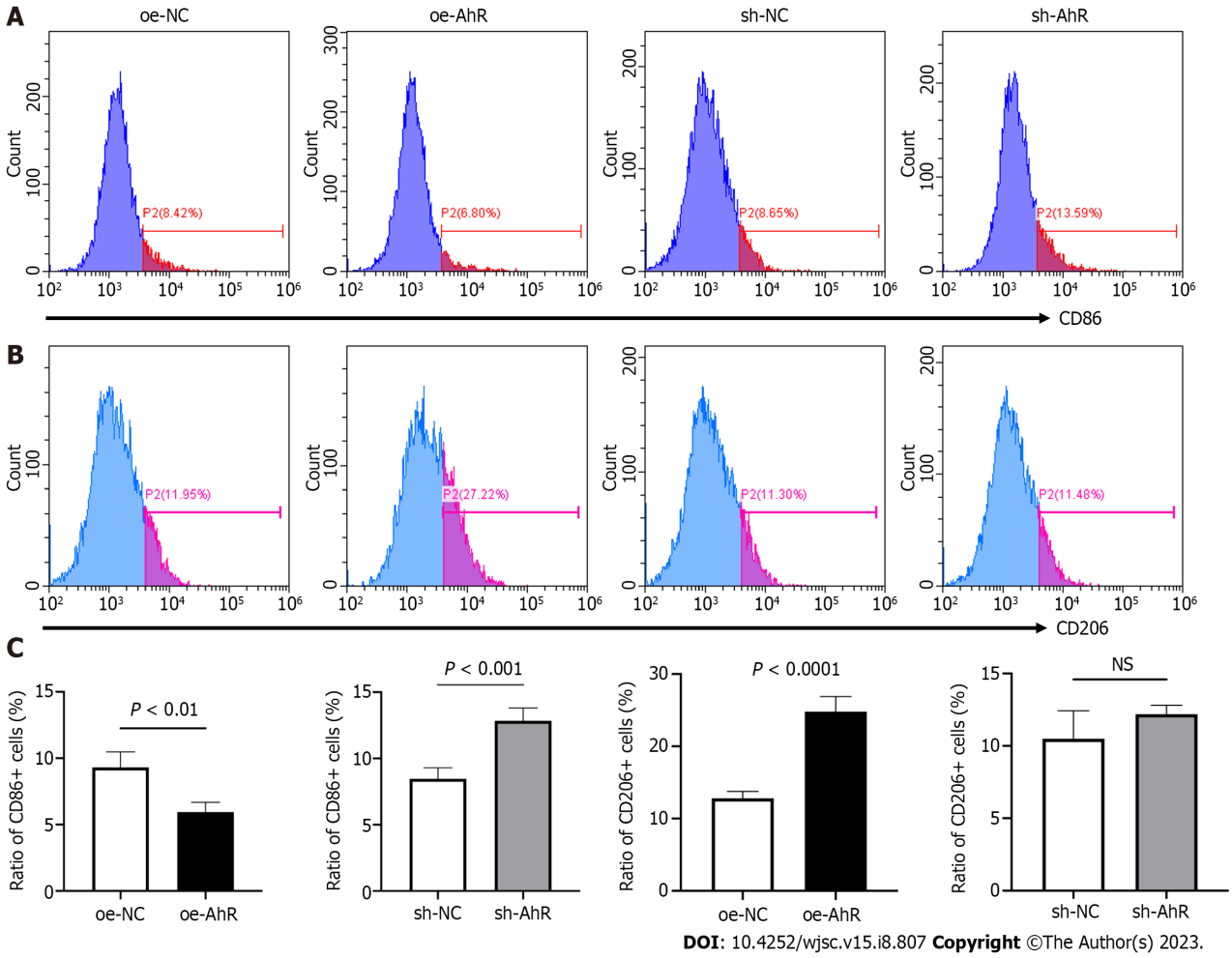
phosphatase; RUNX2: Runt-related transcription factor 2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



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Figure 4 The cluster of differentiation (CD) 86 and CD206 expressions of RAW 264.7 cells that directly co-cultured with different mouse bone marrow mesenchymal stromal cells. A: The brightfield images of the coculture system (10 objective lens); B: The typical four-quadrant images of flow cytometry after direct coculture of RAW 264.7 cells and mBMSCs (X-axis: F4/80; Y-axis: CD86); C: The typical four-quadrant images of flow cytometry after direct coculture of RAW 264.7 cells and mBMSCs (X-axis: F4/80; Y-axis: CD206); D: Quantitative analysis of CD86 + (Q1-UR)/F4/80 + (Q1-UR + Q1-LR) and CD206 + (Q2-UR)/F4/80 + (Q2-UR + Q2-LR) ratios in direct coculture system. oe-NC: Overexpression-negative control; oe-AhR: Overexpression-aryl hydrocarbon receptor; sh-NC: Knockdown-negative control; sh-AhR: Knockdown-aryl hydrocarbon receptor; NS: Not significant.

after osteogenic induction[11]. In addition to ligand-activated AhR, its role in bone ossification was investigated in transgenic mice. In the semistable fracture healing model in mice, the expression of AhR in the healing callus tissue was more than 2-fold higher on the seventh day after fracture than in uninjured samples. On the 14th day after fracture, AhR expression had increased by 10-fold in callus tissue. To determine whether the loss of AhR affects bone healing, the researchers established a tibial fracture model in wild type (WT) mice and AhR knockout (KO) mice and performed micro-computed tomography (micro-CT) scan analysis two weeks later. Mineralized callus tissue in the fracture gap was

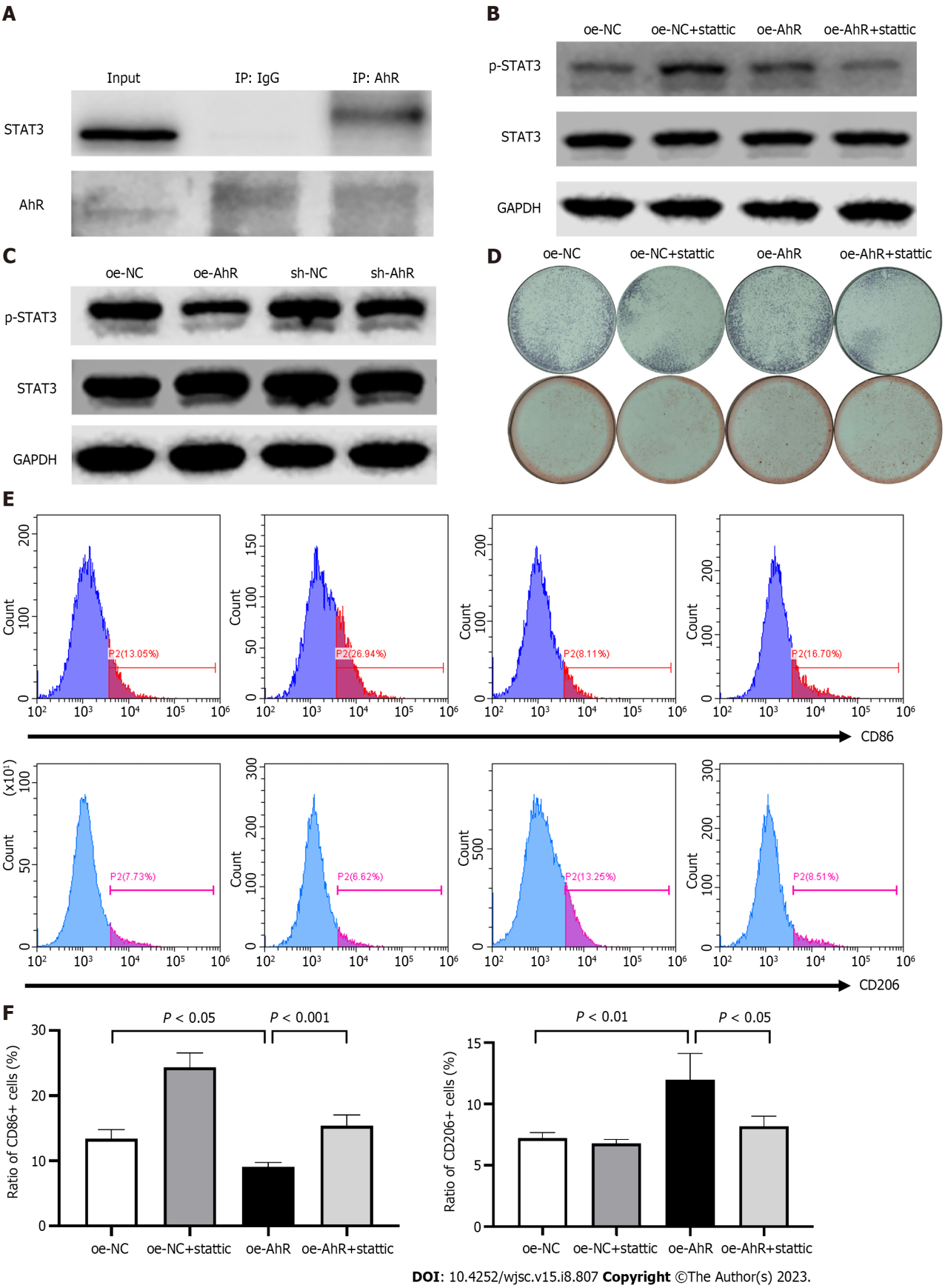


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Figure 5 The cluster of differentiation (CD) 86 and CD206 expressions of RAW 264.7 cells that cocultured with conditioned medium from different mouse bone marrow mesenchymal stromal cells. A: The typical histograms of CD86 expressions of RAW 264.7 cells; B: The typical histograms of CD206 expressions of RAW 264.7 cells; C: Quantitative analysis of ratios of CD86+ and CD206+ cells. oe-NC: Overexpression-negative control; oe-AhR: Overexpression-aryl hydrocarbon receptor; sh-NC: Knockdown-negative control; sh-AhR: Knockdown-aryl hydrocarbon receptor; NS: Not significant.

observed in the former but not the latter mice. At the third week, micro-CT analysis also showed that AhR KO mice had less bone formation than WT mice[7]. In another study[16], the ALP and OCN mRNA expression levels of BMSCs obtained from AhR KO mice were lower than those of WT mice after 8-10 d of osteogenic induction. Interestingly, the ALP and OCN expression patterns in the BMSCs of AhR KO mice after osteogenic induction were rather parallel to the TCDD-suppressed responses in BMSCs from WT mice. The results of present study were consistent with above literatures.

Additionally, AhR has been proven to be involved in modulating immune/inflammatory disease by targeting specific gene expression and altering immune differentiation[8]. Similarly, complex ligand interactions that control AhR function might result in diverse immunologic effects including immunosuppressive or pro-inflammatory downstream functions [17]. In another study, peritoneal macrophages from WT and AhR-null mice were polarized toward the M1 or M2 phenotype by stimulation with lipopolysaccharide/interferon-g or interleukin (IL)-4[18]. The results indicated that AhR-null macrophages presented higher levels of M1 markers including IL-1b, IL-6, IL-12 and tumor necrosis factor-a, and lower levels of M2 markers, including chitinase-like 3 (or called Ym1) and IL-10. It was found that the binding of AhR to the promoters of IL-10 and arginase-1 was increased in macrophages after uptake of apoptotic cells to promote M2 polarization[19]. AhR not only affects the phenotype of macrophages themselves but also influences the results of other cells in regulating macrophage polarization. Treatment with the AhR ligand FICZ attenuated calcium oxalate nephrocalcinosis in a mouse model. Bone marrow-derived macrophages (BMDMs) and calcium oxalate monohydrate (100mg/mL)-treated renal tubular epithelial cells were cocultured in transwell system. FICZ supplement in the system promoted the expression of M2 markers and diminished the expression of M1 markers in BMDMs. The molecular mechanism was that AhR directly targeted downstream microRNA-142a-3p, which suppressed interferon regulatory factor 1 and hypoxia inducible factor 1 alpha by binding to their 3′ untranslated region[20]. In another study, another AhR ligand, TCDD, was added to a coculture system of mBMSCs and macrophages. Treatment of BMSCs with TCDD resulted in a significant increase in M2 markers and a decrease in M1 markers in macrophages. The AhR antagonist CH223191 alleviated the macrophage-modulating effect[21]. In the present study, AhR overexpression in mBMSCs promoted its ability of polarizing macrophages into M2-like phenotype.



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Figure 6 The molecular mechanism of the role of aryl hydrocarbon receptor in osteogenic differentiation and macrophage-modulating in mouse bone marrow mesenchymal stromal cells. A: Co-immunoprecipitation assay showed that aryl hydrocarbon receptor (AhR) and signal transducer and activator of transcription 3 (STAT3) directly interacted in mouse bone marrow mesenchymal stromal cells (mBMSCs); B: Western blot lanes demonstrated that AhR overexpression promoted phosphorylation of STAT3 compared to negative control, while AhR knockdown suppressed it; C: The specific STAT3 inhibitor stattic (2

mmol/L) partially alleviated the promoted STAT3 phosphorylation by AhR overexpression; D: Alkaline phosphatase (upper) and alizarin red staining (lower) staining indicated that 2 mmol/L stattic partially inhibited the elevated osteogenic potential by AhR overexpression; E and F: The histograms of flow cytometry and quantitative analysis manifested that 2 mmol/L stattic partially reversed the CD86 inhibition and CD206 promotion in RAW 264.7 by conditioned medium from overexpression-AhR mBMSCs. AhR: Aryl hydrocarbon receptor; oe-NC: Overexpression-negative control; oe-AhR: Overexpression-AhR; sh-NC: Knockdown-negative control; sh-AhR: Knockdown-AhR; STAT3: Signal transducer and activator of transcription 3; p-STAT3: Phosphorylated STAT3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

It was reported that AhR signaling exhibits considerable crosstalk with other transcription factors, such as those in the nuclear factor-kB family and signal transducer and activator of transcription family[17,22]. AhR can bind to other molecules to undergo a conformational change exposing a nuclear localization signal[17]. STAT3 is critical in regulating immune responses[23] and osteogenic differentiation processes[24]. Our previous study showed that FICZ-stimulated AhR alleviated the inflammatory response in periodontal ligament cells by increasing the phosphorylation of STAT3[10]. The interplay between AhR and STAT3 was explored in various cell types. In A549 cells the interaction of AhR and STAT3 was detected *via* Co-IP. Increased AhR by reduning upregulated the expression of STAT3 and the downstream IL-10, which alleviate severe pneumonia[25]. *Lactobacillus johnsonii* N6.2-derived nano-sized vesicles led to the nuclear translocation of AhR in pancreatic b cells and enhanced the phosphorylation of STAT3 and expression of IL-10, which reduced the apoptosis and improved the expression of genes related to glucose transport[26]. In the present study, the direct AhR and STAT3 interaction was also observed in mBMSCs. Moreover, AhR overexpression upregulated the phosphorylation of STAT3. However, the further research is needed to fully understand the precise molecular mechanisms underlying the AhR-STAT3 interaction.

CONCLUSION

In conclusion, AhR plays a promotive role in the regenerative potential of mBMSCs, including osteogenic differentiation and polarizing macrophages to an anti-inflammatory phenotype. Mechanistically, AhR can interact with STAT3, thereby increasing the phosphorylation level of STAT3. Inhibition of STAT3 partially counteracted the beneficial effect of AhR. Hence, AhR might be a target for achieving optimal bone regeneration in mBMSCs-based tissue engineering.

ARTICLE HIGHLIGHTS

Research background

Bone marrow mesenchymal stromal cells (BMSCs) are one of the most commonly used seed cells in bone tissue engineering. Aryl hydrocarbon receptor (AhR) has been recognized as a nuclear receptor that modulates bone turnover. However, the function of constitutive AhR in BMSCs remains unclear.

Research motivation

To explore whether AhR is involved in the regenerative potential of mouse BMSCs (mBMSCs).

Research objectives

To investigate the role of AhR in the osteogenic and macrophage-modulating potential of mBMSCs and the underlying mechanism.

Research methods

Immunochemistry and immunofluorescent staining were used to observe the expression of AhR in mouse bone marrow tissue and mBMSCs. The overexpression or knockdown of AhR was achieved by lentivirus-mediated plasmid. The osteogenic potential was observed by alkaline phosphatase and alizarin red staining. The mRNA and protein levels of osteogenic markers were detected by quantitative polymerase chain reaction and western blot. After coculture with different mBMSCs, the cluster of differentiation (CD) 86 and CD206 expressions levels in RAW 264.7 cells were analyzed by flow cytometry. To explore the underlying molecular mechanism, the interaction of AhR with signal transducer and activator of transcription 3 (STAT3) was observed by co-immunoprecipitation and phosphorylation of STAT3 was detected by western blot.

Research results

AhR expressions in mouse bone marrow tissue and isolated mBMSCs were detected. AhR overexpression enhanced the osteogenic potential of mBMSCs while AhR knockdown suppressed it. The ratio of CD86+ RAW 264.7 cells cocultured with AhR-overexpressed mBMSCs was reduced and that of CD206+ cells was increased. AhR directly interacted with STAT3. AhR overexpression increased the phosphorylation of STAT3. After inhibition of STAT3 *via* stattic, the promotive effects of AhR overexpression on the osteogenic differentiation and macrophage-modulating were partially counteracted.

Research conclusions

AhR plays a beneficial role in the regenerative potential of mBMSCs partially by increasing phosphorylation of STAT3.

Research perspectives

This study suggested that AhR and its interaction with STAT3 might be a potential candidate target for achieving optimal bone regeneration in mBMSCs-based tissue engineering.

FOOTNOTES

Author contributions: Huang J, Wang YN and Zhou Y designed the study and analyzed the data; Huang J performed the experiments, collected the data, and wrote the manuscript; All authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Wnt signaling pathway inhibitor promotes mesenchymal stem cells differentiation into cardiac progenitor cells *in vitro* and improves cardiomyopathy *in vivo*

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Abstract

BACKGROUND

Cardiovascular diseases particularly myocardial infarction (MI) are the leading cause of mortality and morbidity around the globe. As cardiac tissue possesses very limited regeneration potential, therefore use of a potent small molecule, inhibitor Wnt production-4 (IWP-4) for stem cell differentiation into cardiomyocytes could be a promising approach for cardiac regeneration. Wnt pathway inhibitors may help stem cells in their fate determination towards cardiomyogenic lineage and provide better homing and survival of cells *in vivo*. Mesenchymal stem cells (MSCs) derived from the human umbilical cord have the potential to regenerate cardiac tissue, as they are easy to isolate and possess multilineage differentiation capability. IWP-4 may promote the differentiation of MSCs into the cardiac lineage.

AIM

To evaluate the cardiac differentiation ability of IWP-4 and its subsequent *in vivo* effects.

METHODS

Umbilical cord tissue of human origin was utilized to isolate the MSCs which were characterized by their morphology, immunophenotyping of surface markers specific to MSCs, as well as by tri-lineage differentiation capability. Cytotoxicity analysis was performed to identify the optimal concentration of IWP-4. MSCs were treated with 5 μM IWP-4 at two different time intervals. Differentiation of MSCs into cardiomyocytes was evaluated at DNA and protein levels. The MI rat

model was developed. IWP-4 treated as well as untreated MSCs were implanted in the MI model, then the cardiac function was analyzed *via* echocardiography. MSCs were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) dye for tracking, while the regeneration of infarcted myocardium was examined by histology and immunohistochemistry.

RESULTS

MSCs were isolated and characterized. Cytotoxicity analysis showed that IWP-4 was non-cytotoxic at 5 μM concentration. Cardiac specific gene and protein expression analyses exhibited more remarkable results in fourteen days treated group that was eventually selected for *in vivo* transplantation. Cardiac function was restored in the IWP-4 treated group in comparison to the MI group. Immunohistochemical analysis confirmed the homing of pre-differentiated MSCs that were labeled with DiI cell labeling dye. Histological analysis confirmed the significant reduction in fibrotic area, and improved left ventricular wall thickness in IWP-4 treated MSC group.

CONCLUSION

Treatment of MSCs with IWP-4 inhibits Wnt pathway and promotes cardiac differentiation. These pre-conditioned MSCs transplanted *in vivo* improved cardiac function by cell homing, survival, and differentiation at the infarcted region, increased left ventricular wall thickness, and reduced infarct size.

Key Words: Myocardial infarction; Inhibitor Wnt production-4; Differentiation; Mesenchymal stem cells; Wnt pathway; Cardiac function

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Core Tip: This study highlights the role of Wnt signaling pathway in the differentiation of mesenchymal stem cells (MSCs) into cardiac progenitor cells and the therapeutic potential of MSCs conditioned with inhibitor Wnt production-4 (IWP-4) for the treatment of heart disease. Further studies are required to comprehend the mode of action of IWP-4 on MSCs and to assess its potency and safety in human clinical trials. Nevertheless, this research is an exciting step forward for new treatments for heart diseases and is more focused on the importance of continued investment in the development of innovative therapies for this devastating condition.

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INTRODUCTION

Cardiovascular diseases (CVDs) remain the major mortality cause around the globe[1]. The associated morbidity and mortality may rise to 23.6 million by 2030, which emphasizes the urgent need of addressing the issue of regenerating the damaged heart[2]. CVDs account for a wide range of pathological changes in the blood vessels and heart. Current treatment strategies include inhibitors of beta and aldosterone, angiotensin altering enzyme inhibitors, and biventricular striding. However, heart failure related morbidity and mortality cause temporary success due to these therapeutic interventions and highlight the need for novel therapy that can inhibit and reverse heart dysfunction. Cell therapy emerged in the 1990s and was perceived as a promising strategy to repair an injured heart after myocardial infarction (MI)[3].

Mesenchymal stem cells (MSCs) are the epitome of tissue regeneration due to their easy isolation and existence in almost all types of tissues. They can be differentiated into multiple lineages. Their immunological properties such as immunosuppressive, anti-inflammatory, and immunoregulatory potential make them potent immunotolerant cells[4]. MSCs isolated from the umbilical cord are relatively advantageous over other adult sources owing to their ability to high passaging, expansion on a larger scale, higher anti-inflammatory effects, and more significant senescence retardation[5].

The Wnt signaling pathway comprises a complex network of signaling molecules that play a crucial role in stem cell growth, survival, and differentiation. Wnt antagonists (IWPs) inhibit Wnt ligand release by inhibiting a membrane bound O-acyltransferase, Porcn, responsible for catalyzing Wnt palmitoylation[6]. Several studies reported that the inhibition of Wnt pathway promotes the differentiation of stem cells toward cardiomyocytes. Inhibitor Wnt production-4 (IWP-4) was also reported to produce functional cardiomyocytes from induced pluripotent stem cells and embryonic stem cells[7,8].

In this study, we investigated the role of IWP-4, a potent inhibitor of the Wnt signaling pathway on human umbilical cord derived MSCs. Our results showed that treatment with IWP-4 promotes MSCs differentiation into cardiac progenitor cells *in vitro*, which is a promising step towards the development of stem cell treatment for heart disease. To assess the

therapeutic potential of IWP-4 treated MSCs, cells were transplanted in rat MI model. Our results demonstrated that treatment with IWP-4 improves the efficacy of the MSCs to recover cardiac function of the MI rats, as evidenced by an increase in the ejection fraction. These findings suggest that preconditioning of MSCs with IWP-4 may be a promising approach for the treatment of heart diseases and that targeting Wnt signaling pathway may represent a promising approach for this debilitating condition.

MATERIALS AND METHODS

In vitro studies

Ethical approval: This study was conducted according to the institutional ethical standards outlined in the protocol #ICCBS/IEC-067-HT/UCB-2021/Protocol/1.0, which was approved by the ethical review committee. Healthy donors' umbilical cords were obtained from Zainab Panjwani Memorial Hospital with informed consent after C-section delivery. The cord samples were collected aseptically and transferred into a sterile glass bottle filled with 0.5% EDTA in phosphate buffer saline (PBS) and kept at 4 °C. The samples were processed immediately after collection.

Human umbilical cord tissue processing: The biosafety cabinet class II, type A2 (ESCO, Singapore) was used for sample processing. The cord sample was washed with sterile PBS and then cut into 1-3 mm pieces. These pieces were placed in T-75 flasks containing 10-12 mL complete DMEM (10% fetal bovine serum, 100 U of streptomycin/penicillin, and 1 mmol/L sodium pyruvate). The flasks were kept at 37 °C in a humidified CO_2 incubator. The flask was monitored regularly for cell growth, and the medium was changed after every 3 d. After 10-14 d of initial culture, MSCs from the explant were migrated and adhered to the surface of the flask. When the cells were adequately attached, explants were removed and cells were supplied with fresh medium for further propagation. The cells were labeled as passage zero (Po). When the cells became 80% confluent, they were trypsinized for the next passage using 1× trypsin-EDTA. The experiments were carried out using passages (P_2 to P_3) MSCs.

Characterization of MSCs: The isolated MSCs were characterized based on phase contrast microscopy, immunocytochemistry, immunophenotyping, and trilineage differentiation.

Phase contrast microscopy: Phase contrast microscopy was performed for the morphological analysis of MSCs at different passages. The images were captured with phase contrast microscope (Ti-2, Nikon, Tokyo, Japan).

Immunocytochemistry: Coverslips were placed in a 24-well tissue culture plate and 5000 cells were seeded with 200 µL of complete DMEM in each well, then the plate was incubated in a CO_2 incubator at 37 °C for 24-48 h to allow for monolayer formation. The next day, DMEM was discarded and cells were washed with 1× PBS, and fixed with 200 µL of 4% Paraformaldehyde (PFA) for 10 min. Cells were again washed with PBS and then permeabilized with 300 µL of 0.1% Triton X-100 for 10 min followed by 3 washes with PBS. The cells were then blocked for 1 h at 37 °C using a blocking solution containing 2% BSA, 0.1% Tween-20, and 1× PBS to prevent non-specific binding. After blocking, the monoclonal primary antibodies CD117 (32-9000, Zymed, United States), CD29 (MAB-1981, Chemicon International, United States), Lin28 (PA1- 096, Invitrogen, United States), CD105 (560839, BD Pharmingen, United States), Vimentin (V6389, Sigma-Aldrich, United States), and CD45 (CBL415, BD Pharmingen, United States) were added to each well, and the plate was incubated at 4 °C overnight. The next day, primary antibodies were removed, and cells were washed with 1× PBS. Cells were then incubated with Alexa Fluor 546 conjugated goat anti-mouse secondary antibody (A-11010, Molecular Probes, Invitrogen, United States) at 37 °C for 1 h, followed by washing with 1× PBS. The cells were stained with a 0.5 µg/mL solution of DAPI-PBS (4', 6-diamidino-2-phenylindole) for 15 min at room temperature followed by washing with 1× PBS. Finally, coverslips were placed on a glass slide, and then slides were mounted with 5 µL of aqueous mounting medium. The fluorescence microscope (Nikon Ti2; Nikon, Japan) was used to observe the slides.

Flow cytometry: Immunophenotypic characterization was performed by flow cytometry. MSCs were trypsinized and washed with PBS then resuspended in FACS buffer (1 mmol/L EDTA, 1% BSA, and 0.1% sodium azide). Cells were incubated with primary antibody CD73 (550256, BD Pharmingen, United States), CD105, Vimentin, and CD45 incubated at 37 °C for 2 h. The primary antibody was removed and cells were washed with PBS. Then Alexa Fluor 488 goat anti-mouse secondary antibody was added for 1 h at 37 °C. Cells were washed and analyzed *via* FACS Calibur (Becton Dickinson, United States). For control, unlabeled and isotype labeled cells were used.

Tri-lineage differentiation: For the characterization of MSCs through tri-lineage differentiation, cells were allowed to grow in complete DMEM in a 6-well plate. For the differentiation of MSCs into adipocytes, MSCs were allowed to grow in adipogenic induction medium containing complete DMEM, 1 µM dexamethasone, 10 µg/mL insulin, 200 µM indomethacin, and 0.5 µM isobutyl methylxanthine for 21 d. MSCs were differentiated into osteocytes in osteogenic induction medium containing complete DMEM with 0.1 µM dexamethasone, 0.2 mmol/L L-ascorbic acid-2-phosphate, and 10 mmol/L glycerol-2- phosphate for 28 d. For chondrogenic differentiation, MSCs were allowed to grow in chondrogenic induction medium containing complete DMEM, 1 µM dexamethasone, 20 ng/mL transforming growth factor-β, 100 mmol/L ascorbic acid, and 10 ng/mL insulin for 21 d. Differentiated cells were stained with Oil Red O for the detection of lipid droplets inside the cells for adipocytes. Alizarin Red staining was performed to detect calcium deposits in osteocytes. Alcian Blue staining confirmed the presence of proteoglycans and glycosaminoglycans (GAG).

Cytotoxicity analysis of IWP-4 by JC-1 assay: JC-1 cytotoxicity analysis was performed to determine the cytotoxic effect of IWP-4 on MSCs. MSCs were grown in complete DMEM in T-75 flasks and then treated with the different concentrations (5, 10, 20, 30, 50, and 100 µM) of IWP-4 for 24 h. Cells were trypsinized, washed, and then stained with 500 µL of 10µg/mL JC-1 dye working dilution and incubated for 15 min at 37 °C. Afterwards, MSCs were washed and resuspended in PBS. Data were analyzed with BD CellQuest pro software.

Treatment with IWP-4: The concentration of 5 µM was selected for cardiac differentiation based on JC-1 cytotoxicity assay. Working solutions were prepared from 2 mmol/L IWP-4 stock solution. Complete DMEM was used for the preparation of conditioned medium. 20 µL stock compound was added in 50 mL of media and stored at 4 °C. When cells became 60% confluent, normal media was discarded, and conditioned media was added to the flask and then kept in a CO_2 incubator for seven, and fourteen days. The conditioned medium was replaced every 3 d.

Gene expression of cardiac markers: For cardiac gene expression analysis, RNA was isolated from untreated, seven and fourteen days IWP-4 treated MSCs *via* Trizol method according to the manufacturer's instructions. The concentration and purity of RNA were checked by using Nanodrop2000 (Thermo Fisher Scientific, United States) at 260 nm absorbance. A 260/280 absorbance ratio was used to determine the purity of isolated RNA. cDNA was synthesized from 1 µg of RNA by using RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Fisher Scientific, United States), according to the manufacturer's protocol. Cardiac specific genes were amplified using primer sequences enlisted in Table 1, by quantitative real-time polymerase chain reaction (qPCR) using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, United States). Bright Green 2× qPCR master mix (Applied Biological Materials, Canada) was used for the amplification. For gene amplification, initial denaturation was performed at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and extension at 60 °C for 1 min. The *GAPDH* gene (a housekeeping gene) was used as endogenous control. To analyze the relative gene expression level, the cycle threshold (Ct) value was acquired after the completion of the reaction, and the fold change was calculated using $2^{-\Delta\Delta Ct}$ method.

Gene expression of Wnt pathway genes: For Wnt pathway gene expression analysis, total RNA was isolated and cDNA was prepared and amplified as described above. For gene amplification, initial denaturation was performed at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 s, and extension at 60 °C for 1 min. Wnt specific genes were amplified using primer sequences enlisted in Table 2. The *GAPDH* gene (a housekeeping gene) was used as endogenous control. To analyze relative gene expression level, the Ct value was acquired after completion of the reaction, and the fold change was calculated using $2^{-\Delta\Delta Ct}$ method.

Immunocytochemistry of cardiac protein: To examine protein expression, immunofluorescence staining was performed on both untreated and fourteen days IWP-4 treated MSCs by the same protocol as explained earlier for the characterization of MSCs. Untreated and IWP-4 treated MSCs were washed with PBS and fixed with 4% PFA, and then incubated in cardiac specific antibodies such as α-actinin (sc-17829, Santa Cruz Biotech, United States), Connexin-43 (C-43) (13-8300, Thermo scientific, United States), cardiac troponin I (cTnI) (Ab209809, Abcam, United Kingdom), Desmin (MAB3430 Chemicon International, United States), GATA-4 (sc-25310, Santa Cruz Biotech, United States), Nkx 2.5 (sc-376565, Santa Cruz Biotech, United States). Next, Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (A-11001, Invitrogen, United States) was added followed by washing with 1× PBS. The cell nuclei were counterstained with DAPI in 1× PBS for 15 min at room temperature and washed again with 1× PBS. Protein expression was observed using a fluorescence microscope. Fluorescent images were processed and quantified by ImageJ software.

In vivo studies

Animal care and ethical approval: MI model was developed using Wistar rats weighing between 220-230 g. The study was conducted in compliance with the international guidelines for laboratory animal care and use under protocol number 2021-006, with ethical approval obtained from the Institutional Animal Care and Use Committee at the Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi. The animals were housed in separate cages, in a room maintained at a temperature of 22 °C ± 2 °C, 55 ± 5% relative humidity, and a 12 h light: 12 h dark cycle, with unrestricted access to water and food.

Cardiac model development: To develop the MI model, a metal rod was cooled in liquid nitrogen for 15 min. Rats were anesthetized with xylazine and ketamine (7 mg/kg and 60 mg/kg, respectively), according to their body weights. To initiate artificial ventilation, a rodent ventilator was used and endotracheal intubation was performed. Left thoracotomy was performed through the anterolateral 4th and 5th intercostal space, and a retractor was used to expose the heart. Then the rod was removed from liquid nitrogen and placed on the left ventricle of the heart for 10 s. Successful MI model development was confirmed with the change in color of the infarcted region from red to pale color. The chest cavity was closed with 5-0 suture (Ethicon, United States), and the skin was closed with a 4-0 suture. Trachea was closed with the help of 6-0 suture. Animals were administered diclofenac sodium (25 mg/mL) and antibiotics (penicillin and streptomycin 10000 U/mL) subcutaneously. Animals were observed until they regained consciousness. In the sham control group, the chest cavity was opened and then closed without the exposure of an ultracold rod.

Transplantation of cells in MI model: After model development, one million cells resuspended in 1× PBS were transplanted in the left ventricular wall immediately after infarction. For *in vivo* cell tracking, untreated and IWP-4 treated MSCs were labeled with the red fluorescent dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) according to the manufacturer's instructions and transplanted into the rats.

Table 1 Details of cardiac specific primers

Gene	Primer sequences (5'-3')	Annealing temperatures (°C)	Product size (bp)
α-actinin (F)	5′-CTCTTGCTTCTACCACGCTTT-3′	58.0	297
α-actinin (R)	5′-AGCGTGTTGAAGTTGATCTCC-3′		
cMHC (F)	5′-GAGGAGCAAGCCAACACCA-3′	58.0	106
cMHC (R)	5′-GCAGCTTGTTGACCTGGGA-3′		
Mef2D (F)	5′-GGGGGCTGGAGGAGTTACC-3′	58.0	292
Mef2D (R)	5′-TGGGGGAACGGTGTTGTCA-3′		
MLC-2v (F)	5′-CCTGAGGAAACCATTCTCAACG-3′	58.0	98
MLC-2v (R)	5′-GATGTGCACCAGGTTCTTGTAG-3′		
cTnC (F)	5′-CTCAACCCCAAATCCCCCGA-3′	58.0	148
cTnC (R)	5′-AGGAAGCGGCCATTGGGTAA-3′		
cTnI (F)	5′-GAACATCACGGAGATTGCAGA-3′	58.0	236
cTnI (R)	5′-TCAGTGCATCGATGTTCTTCC-3′		
cTnT (F)	5′-TCCAGAAGGCCCAGACAGAG-3′	58.0	89
cTnT (R)	5′-CACCTTCCTCCTCTCAGCCA-3′		
Ca- channel (F)	5′-GAGAGCACCCCGGCTTC-3′	58.0	164
Ca-channel (R)	5′-GAAGTCCTGCCCCGCTC-3′		
Na-channel (F)	5′-ACTAGGCAATTTGTCGGCTC-3′	58.0	325
Na-channel (R)	5′-GCCGTTCTTGAGCAGGTAAT-3′		
GATA4 (F)	5′-CTGCCCTCCGTCTTCTGC-3′	58.0	286
GATA4 (R)	5′-CTCGCAGGTCAAGGAGCC-3′		
Nkx-2.5 (F)	5′-CAAGTGTGCGTCTGCCTTTC-3′	58.0	106
Nkx-2.5 (R)	5′-CGCGCACAGCTCTTTCTTT-3′		
GAPDH (F)	5′-CAC CAT GGG GAA GGT GAA GG-3′	58.0	274
GAPDH (R)	5′-AGC ATC GCC CCA CTT GAT TT-3′		

Echocardiography: Cardiac functional analysis was performed by echocardiography after 2 and 4 wk of MI with the help of an Echo machine (Aloka, Japan) provided with 7-MHz transducer. Motion-mode (M-mode) and 2D-bright mode (B-mode) were used to scan the parasternal long-axis view of papillary muscles. Left ventricular internal systolic and diastolic dimensions (LVIDs and LVIDd) were calculated using M-mode scans, and averaged three consecutive cardiac cycles were taken for measurements. Percent ejection fraction (%EF) and fractional shortening (%FS), and end diastolic and systolic volumes (EDV and ESV) were calculated by using the formula as given below:

$$FS\ (\%) = (LVIDd - LVIDs)/LVIDd \times 100$$

$$EF\ (\%) = SV/EDV \times 100$$

$$EDV = [7/(2.4) + LVIDd] \times LVIDd^3$$

$$ESV = [7/(2.4) + LVIDs] \times LVIDs^3$$

Heart harvesting and tissue processing for histology: After 4 wk from surgery and cell transplantation, rats were anesthetized, perfused with 1× PBS, and then fixed with 4% PFA. The hearts were removed and placed in 4% PFA overnight, then dehydrated in graded alcohol, and afterward immersed in xylene then xylene-paraffin mixture followed by embedding in paraffin. Microtome sectioning was performed to cut 5 μm thick sections of paraffin blocks. Sections were taken on gelatin coated slides. Hematoxylin-Eosin (H&E) and Masson's trichrome staining were performed according to the manufacturer's instructions. For histological analysis, the stained section images were observed under a bright-field microscope (NiE, Nikon, Japan).

Tracking of DiI labeled cells in MI model: For DiI labeled cell tracking in animals, hearts were perfused with 1× PBS, fixed with 4% PFA for 4 h, and then mold was prepared in optimal cutting temperature medium. Mold was cryosectioned and cells were tracked by observing them under a fluorescent microscope. To assess the long-term survival, distribution, and differentiation of the transplanted cells in the MI model, tissue was permeabilized with triton-X 100, and blocked with blocking solution. The sections were stained with cardiac specific primary antibodies against GATA-4, cTnI, and

Table 2 Details of Wnt pathway primers

Genes	Primer sequences (5'-3')	Annealing temperatures (°C)	Product size (bp)
GAPDH (F)	5'-CAC CAT GGG GAA GGT GAA GG-3'	58.0	274
GAPDH (R)	5'-AGC ATC GCC CCA CTT GAT TT-3'		
Wnt-2 (F)	5'-GTCGGGAATCTGCCTTTGTT-3'	58.0	376
Wnt-2 (R)	5'-GTTTTCCTGAAGTCGGCCAT-3'		
DVL (F)	5'CTATGGATCAGGATTTCGGGGT-3'	58.0	121
DVL (R)	5'-ATCTCGGGTTGGGGATTATCTG-3'		
GSK 3β (F)	5'-TGTGTTGGCTGAGCTGTTACTA-3'	58.0	200
GSK 3β (R)	5'-TGAAATGTCCTGTTCCTGACGA-3'		
β-catenin (F)	5'-TGATATTGGTGCCCAGGGAG-3'	58.0	102
β-catenin (R)	5'-TCCATACCCAAGGCATCCTG-3'		
c-myc (F)	5'-CACTAACATCCCACGCTCTGA-3'	58.0	217
c-myc (R)	5'-CGCATCCTTGTCCTGTGAGTA-3'		
cyc-D (F)	5'-CAGAGGCGGAGGAGAACAAA-3'	58.0	219
cyc-D (R)	5'-CCGGGTCACACTTGATCACT-3'		
c-jun (F)	5'-TGAGCCTACAGATGAACTCTTTCT-3'	58.0	191
c-jun (R)	5'-ACTCAGAGTGCTCCAAATCTCTTA-3'		
TCF (F)	5'-CGAGAAGAGCAGGCCAAGTA-3'	58.0	219
TCF (R)	5'-GAGCACTGTCATCGGAAGGA-3'		
Axin (F)	5'-GCATGGAGGAGGAAGGTGAG-3'	58.0	165
Axin (R)	5'-CCAGGATGCTCTCAGGGTTC-3'		

alpha actinin. Alexa fluor 488 secondary antibody was used to detect primary antibodies. Fluorescent microscope images were captured and quantified using Image J software and plotted using GraphPad Prism software.

Statistical analysis

The statistical analysis was conducted using IBM SPSS Statistics software version 23. For comparison between the two groups, independent sample *t*-test analysis was performed. However, for the comparison between multiple groups, One-way analysis of variance with post-hoc Bonferroni corrections was used. The results were expressed as mean ± SEM, and statistical significance was determined at $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

RESULTS

In vitro studies

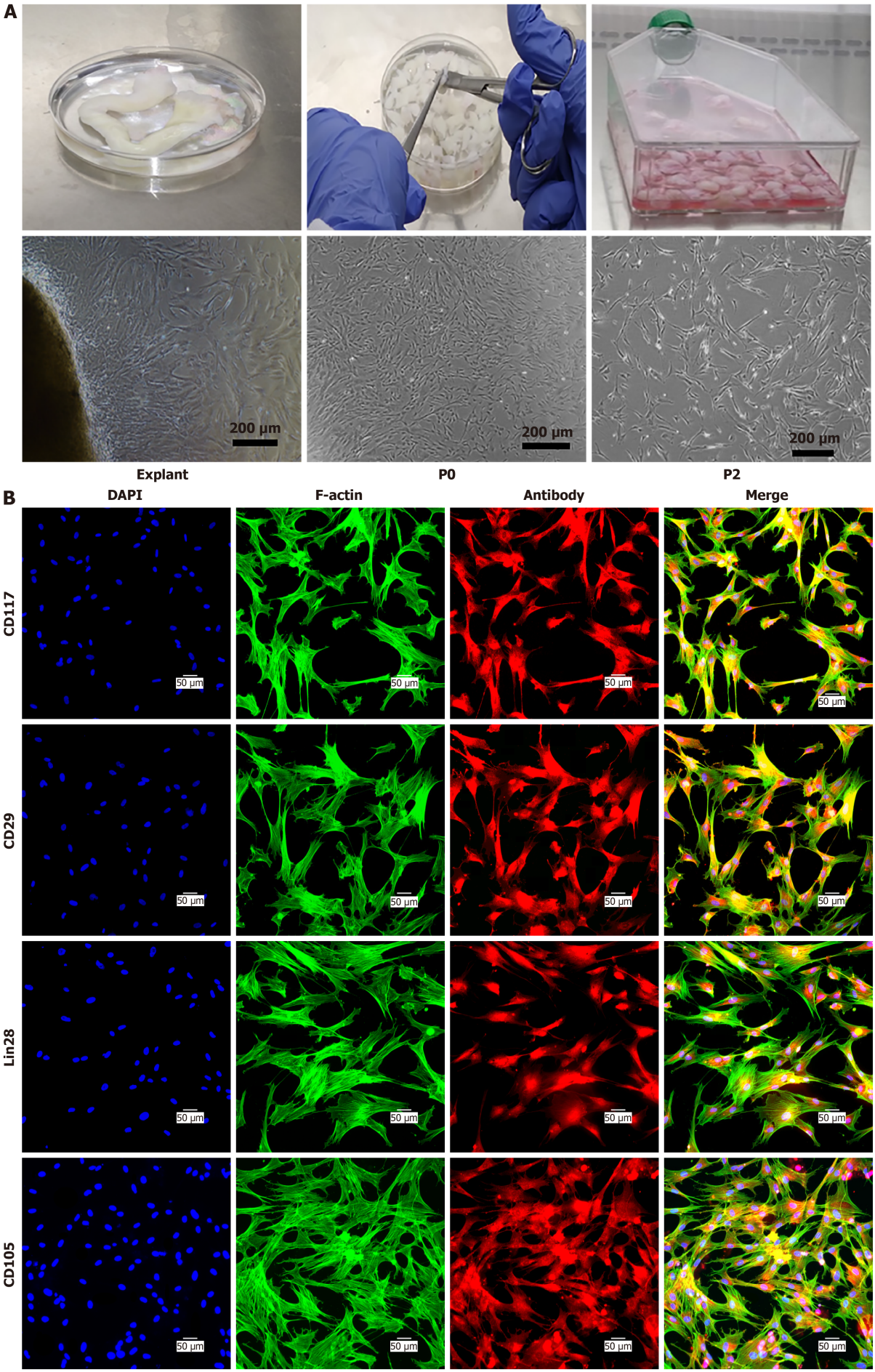
Morphology of human umbilical cord-derived MSCs: MSCs culture exhibited spindle shape morphology. Explant of cord tissue culture showed outgrowth of MSCs after 7-10 d of processing after adhering to the tissue culture flask which then showed gradual expansion and propagation. After 15 d, P_o cells were grown in colonies and possessed elongated, fibroblast like morphology. P_o cells were trypsinized and sub-cultured to get the homogeneous population of MSCs at P_1. Human umbilical cord processing and morphology of MSCs sub-culture at different passages are shown in Figure 1A.

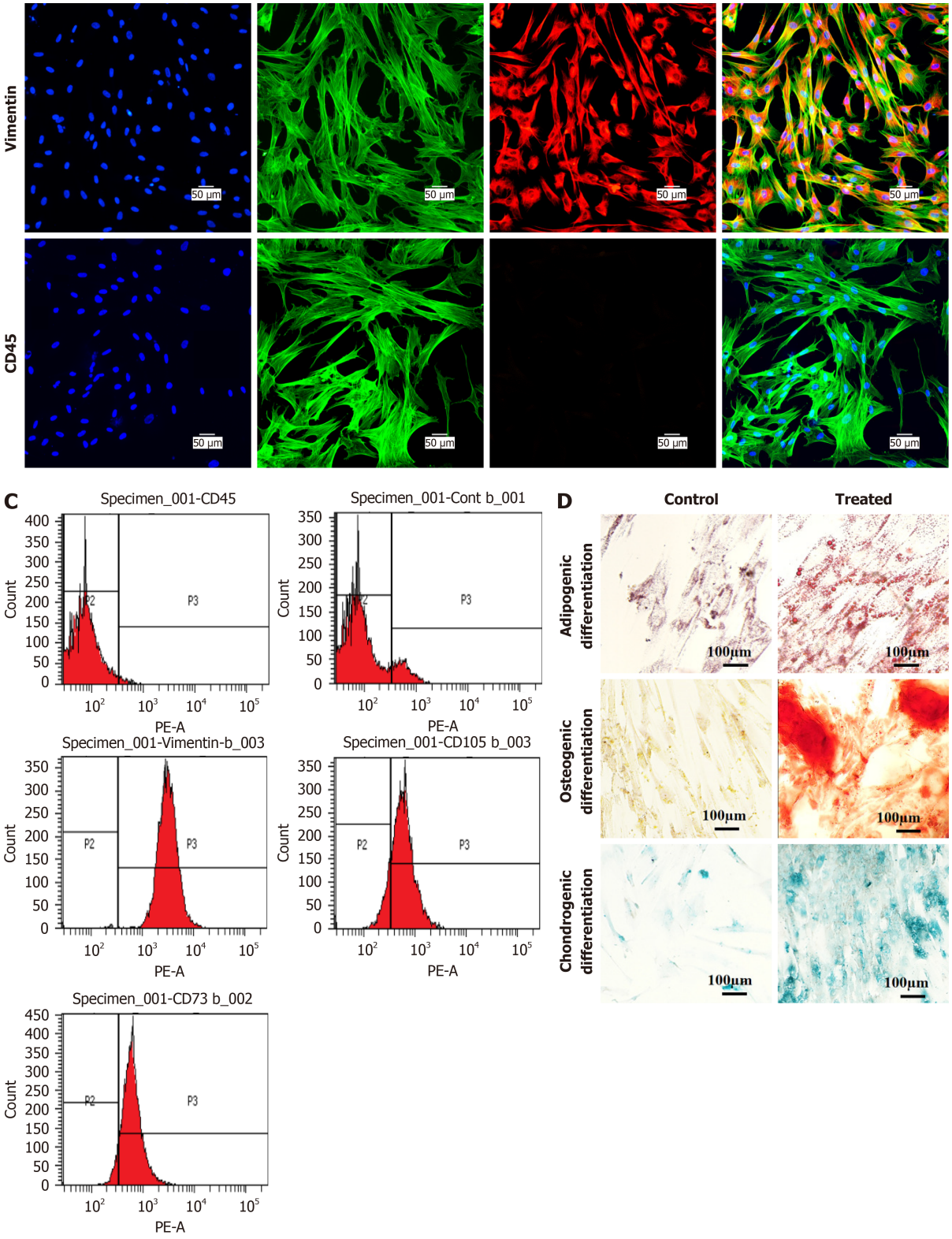
Immunocytochemistry of MSCs: MSCs showed positive expression of specific cell surface markers CD117, CD29, Lin28, CD105, Vimentin, and negative expression of CD45 which is a hematopoietic marker, as shown in Figure 1B.

Flow cytometric analysis

Immunophenotyping was performed with flow cytometry that showed greater than 90% of the cell population expressed CD105, Vimentin, and CD73, while CD45 exhibited negative expression that confirms the presence of MSCs in culture, as shown in Figure 1C.

Tri-lineage differentiation: MSCs possess tri-lineage differentiation potential that was confirmed by differentiating them into adipogenic, osteogenic, and chondrogenic lineages. Mineral deposition confirmed the differentiation of MSCs into


A
200 μm
200 μm
200 μm
Explant
P0
P2
B
DAPI
F-actin
Antibody
Merge
CD117
CD29
Lin28
CD105
50 μm



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Figure 1 Isolation, propagation, and characterization of human umbilical cord-derived mesenchymal stem cells. A: Explant processing and isolation of human umbilical cord-derived mesenchymal stem cells (MSCs). Phase contrast images showing homogenous fibroblast like morphology and growth; B: Characterization of MSCs by immunocytochemistry representing the positive expression of CD117, CD29, Lin28, CD105, Vimentin, and negative expression of CD45; C: Immunophenotyping of MSCs by flow cytometry showing positive expression of CD105, Vimentin, and CD73, and negative expression of CD45; D: Tri-lineages differentiation of MSCs into osteogenic, adipogenic, and chondrogenic lineages.

the osteogenic lineage. Adipogenic differentiation was confirmed by the accumulation of intracellular lipid droplets in treated MSCs, which turn out to be bright red with an Oil Red O stain. Alizarin Red stained differentiated MSCs into orange red color, while large mineral deposits appeared dark red. Chondrogenic differentiation was observed by the

accumulation of GAG that stained blue after Alcian Blue staining. The results are shown in Figure 1D.

Cytotoxicity analysis by JC-1 assay: MSCs treated with different concentrations of IWP-4 were stained with JC-1 dye. Percent cell cytotoxicity was calculated by the shift of cells in the lower right quadrant of density plot in flow cytometer software. JC-1 assay showed that IWP-4 at 5 μmol/L concentration was non cytotoxic for MSCs. The results are shown in Figure 2.

Cardiac gene expression analysis: After fourteen days of treatment of MSCs with IWP-4, cardiac gene expression analysis showed significant upregulation of early and late cardiac genes. After seven days of treatment with IWP-4, myosin light chain-2 (MLC-2v) ($P < 0.01$), and Ca-channel ($P < 0.05$) genes were significantly upregulated, while MHC-β and cardiac troponin C (cTnC) showed non-significant change. However, Na-channel ($P < 0.05$), cardiac troponin T (cTnT), GATA4 ($P < 0.01$), alpha-actinin, cTnI, Mef2D, and Nkx2.5 ($P < 0.001$) showed significant downregulation as shown in Figure 3A. After fourteen days treatment of MSCs with IWP-4, when compared to control, alpha-actinin ($P < 0.05$), MHC-β, MLC-2v, Mef2D, cTnT, cTnC, cTnI, Ca-channel, Na-channel, Nkx2.5, and GATA4 ($P < 0.001$) were significantly upregulated as shown in Figure 3B.

Gene expression analysis of Wnt pathway genes: Wnt pathway gene expression profile was analyzed after seven and fourteen days treatment of MSCs with IWP-4. Gene expression profile showed that seven days treatment of MSCs with IWP-4 inhibit Wnt pathway. When compared to control, Wnt pathway genes, *GSK, DVL, Wnt, TCF, β-catenin,* and downstream transcription factors *C-jun, C-myc, Cyc-D* ($P < 0.001$) were significantly downregulated, while *Axin* showed upregulation ($P < 0.01$) (Figure 4A). After fourteen days treatment of MSCs with IWP-4, Wnt pathway was further downregulated. When compared to control, Wnt pathway genes *DVL, Wnt, and β-catenin* ($P < 0.001$) showed significant downregulation, while negative regulators of pathway *GSK* ($P < 0.01$), and *Axin* ($P < 0.05$) were upregulated. However, downstream transcription factors *C-jun, Cyc-D, TCF* showed non-significant change, while *C-myc* ($P < 0.05$) was upregulated as shown in Figure 4B.

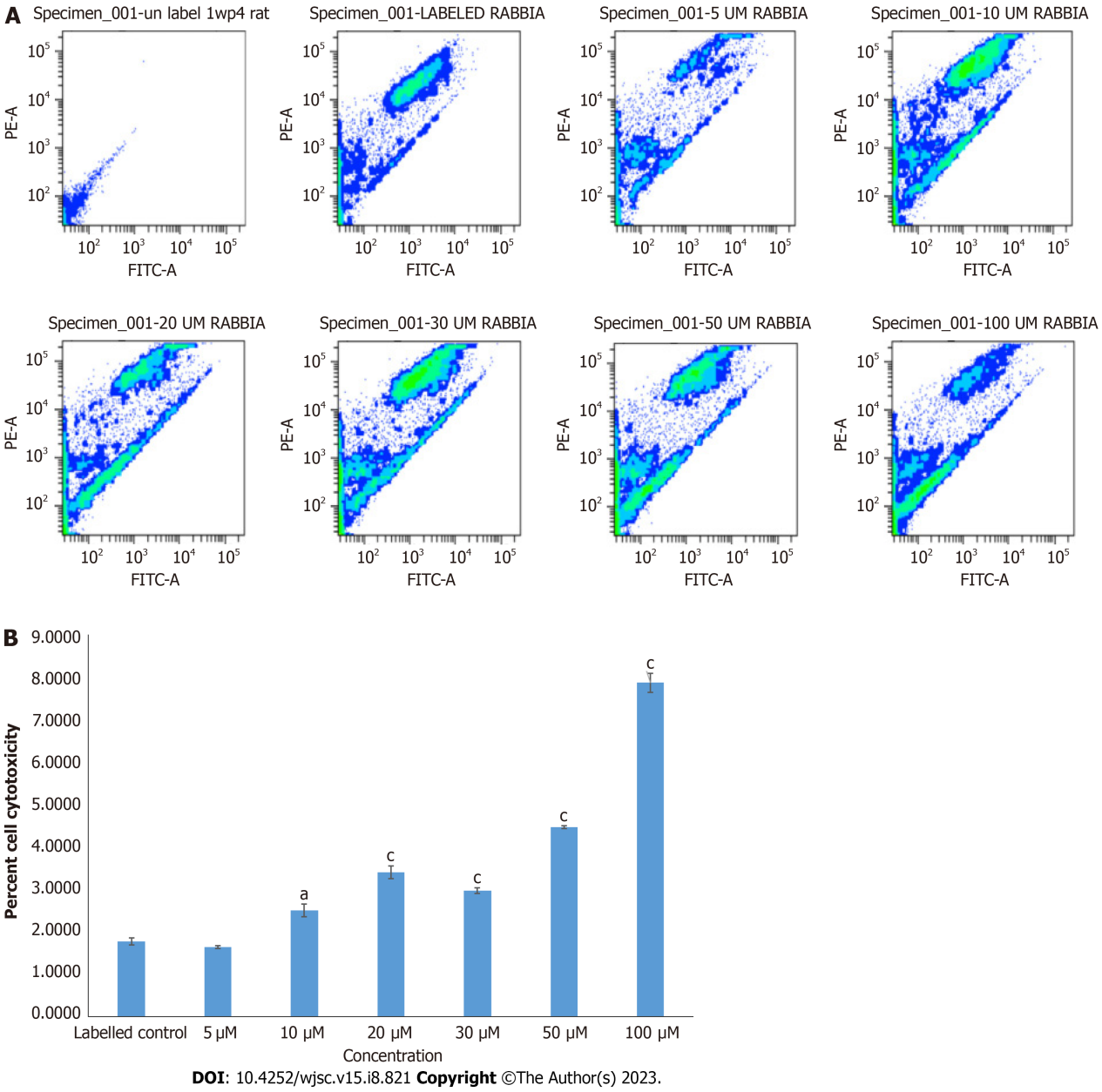
Analysis of differentiated cells: Differentiated MSCs showed cardiac like cell morphology as shown in Figure 5A. Seven days IWP-4 treated MSCs showed positive expression of C-43, cTnI, ($P < 0.001$), desmin, GATA-4, and Nkx2.5 ($P < 0.05$), while alpha-actinin protein expression showed non-significant change. As compared to untreated MSC control, fourteen days IWP-4 treated MSCs showed positive expression of alpha-actinin, C-43, cTnI, desmin, GATA-4, and Nkx2.5 ($P < 0.001$). As compared to seven days IWP-4 treated MSCs, fourteen days IWP-4 treated MSCs showed positive expression of alpha-actinin, C-43, cTnI, desmin, GATA-4, and Nkx2.5 ($P < 0.001$) as shown in Figure 5B and C.

In vivo studies

Cardiac function analysis by echocardiography: Echocardiographic analysis of cardiac function showed decreased left ventricular wall contraction in rats with an infarcted heart (MI group) as compared to the sham control. A significant difference ($P < 0.001$) in the left ventricular systolic and diastolic dimensions, %EF, %FS, and end-systolic and -diastolic volumes were measured after two and four weeks of MI as compared to sham control. The infarcted hearts that received untreated MSCs and IWP-4 treated MSCs showed significant improvement in heart function. As compared to the MI group, left ventricular systolic and diastolic dimensions were significantly decreased ($P < 0.001$) after two and four weeks of transplantation. The %EF and %FS showed significant improvement ($P < 0.001$) in untreated and IWP-4 treated MSCs. End-systolic and -diastolic volumes were also significantly improved ($P < 0.001$) in both the cell transplantation groups as compared to the MI group as shown in Figure 6.

Analysis of isolated rat hearts: Treated and untreated MSCs were transplanted immediately after the MI model development. Hearts were isolated after 4 wk of surgery for histological (Figure 7A and B) and macroscopic analysis (Figure 7C). Macroscopic analysis revealed a major difference in the appearance of hearts in all groups. Hearts from the sham control group appeared normal, containing healthy myocardium, while in the case of MI group, the left ventricular wall of the heart showed a white fibrous scar. In the untreated MSCs transplanted group, the area of white fibrous scar was lower as compared to that of MI group, while in IWP-4 treated MSCs transplanted group, the white fibrous scar was greatly reduced as shown in Figure 7C.

Histological analysis: Cross-sections of heart tissue were differentially stained with Masson's trichrome staining which showed the fibrotic green regions of the collagen deposition in the left ventricle of the MI heart as compared to sham control, while red stained regions showed the normal tissue and myocytes with blue/black stained nuclei. At high magnification, the infarcted region of the MI heart showed fibrous tissue and tightly packed collagen fibrils. The sham control heart section showed healthy myocytes with interconnected cytoplasmic junctions in the left ventricle. The untreated MSCs transplanted group showed reduced fibrosis, while the IWP-4 treated MSCs transplanted group showed complete restoration of the left ventricle and regeneration of the myocytes as shown in Figure 7A and B. Quantification of the total fibrotic area showed that the IWP-4 treated MSCs transplanted group has significantly reduced ($P < 0.001$) fibrosis as compared to the MI model and untreated MSCs transplanted group (Figure 7D). Measurements of left ventricular wall thickness revealed that in the untreated MSCs transplanted group, the restoration of left ventricular wall thickness was non-significant, while in the case of IWP-4 treated MSCs, the left ventricular wall thickness was significantly improved ($P < 0.001$) as compared to the MI group as shown in Figure 7E. H&E staining showed the accumulation of inflammatory cells in the left ventricular region of the infarcted heart in the MI group as compared to the sham control. However, it was improved in untreated MSCs and IWP-4 treated MSCs transplanted group as shown in Figure 8.



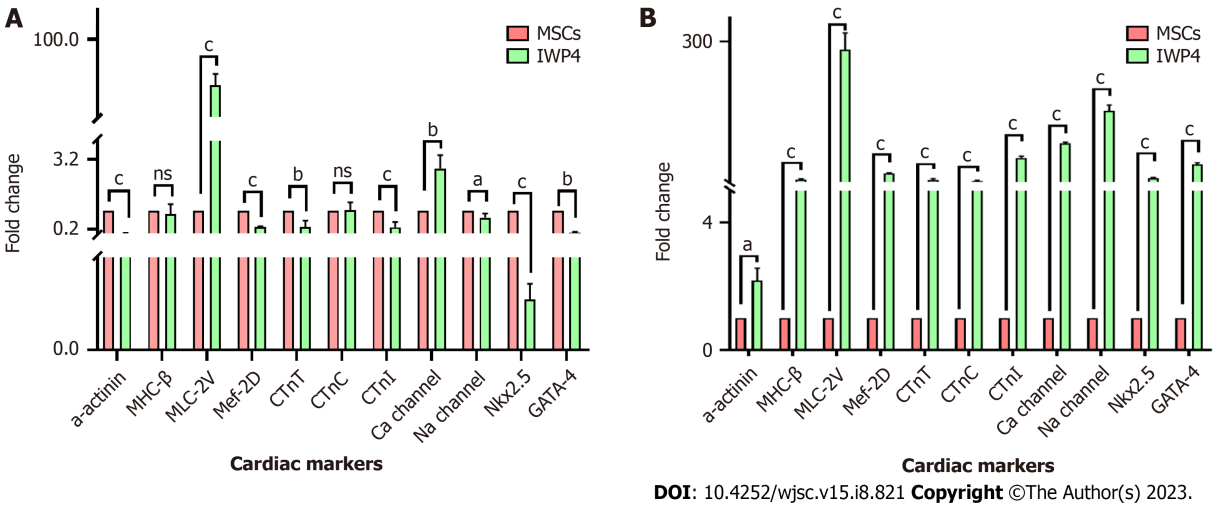
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Figure 2 Cytotoxicity analysis of mesenchymal stem cells treated with different concentrations of inhibitor Wnt production-4 by JC-1 assay. A: Mesenchymal stem cells (MSCs) treated with 5 μM concentration of inhibitor Wnt production-4 showed non-significant number of apoptotic cells, while 10 μM concentration or above showed significant cytotoxic effect on MSCs; B: Quantification of apoptotic and non-apoptotic cells presented in bar graphs. For statistical analysis, One-way ANOVA was used followed by the Bonferroni post-hoc test. Values are expressed as means ± SEM; level of significance is $P < 0.05$ ($^{a}P < 0.05$, $^{c}P < 0.001$).

Tracking of DiI labeled cell: Fluorescence images of DiI labeled untreated MSCs, as well as IWP-4 treated MSCs showed cell distribution and homing in the infarcted myocardium, in both transplanted heart sections. The DiI labeled cells in the cardiac tissue were immunohistochemically stained for cardiac proteins including GATA-4, cTnI, and alpha actinin which showed that the transplanted cells differentiated into cardiac lineage. However, more pronounced cardiac differentiation and cell density were observed in the IWP-4 treated MSCs transplanted group, with GATA-4, cTnI ($P < 0.001$), and alpha actinin ($P < 0.01$) as compared to untreated MSCs group as shown in Figure 9.

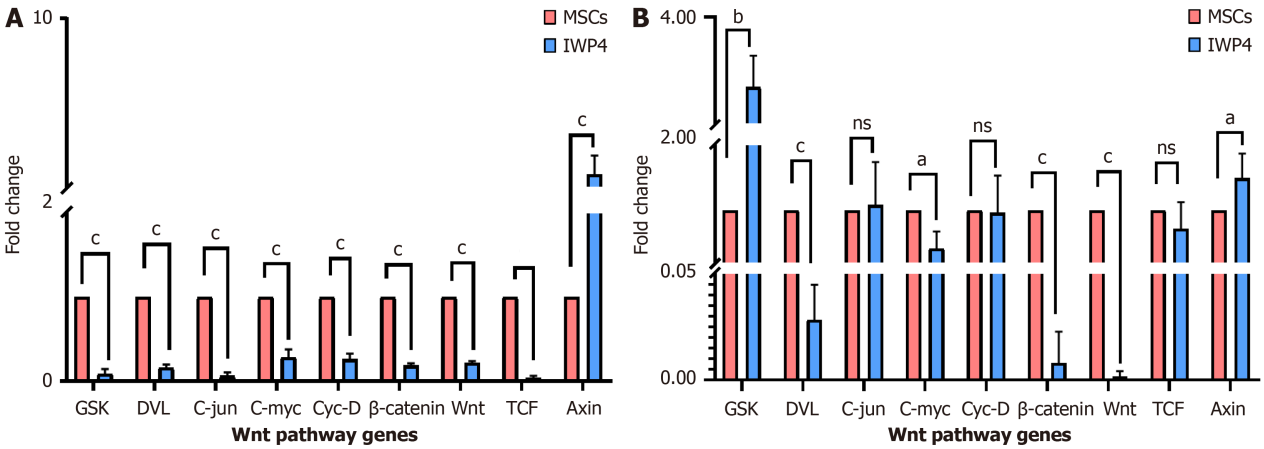
DISCUSSION

In this study, we explored the role of IWP-4 on MSCs in cardiac differentiation *in vitro* and cardiac function restoration *in vivo*. Human umbilical cord derived MSCs are a potential candidate for cell therapy as they are easy to isolate and have a high proliferation rate, low immunogenicity, and immunomodulatory effects. MSCs have various therapeutic advantages that have been explained in several MI models[9]. Unfortunately, transplanted MSCs show poor survival and homing in



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Figure 3 Cardiac markers gene expression analysis by quantitative real-time polymerase chain reaction. A: Seven days treatment of mesenchymal stem cells (MSCs) with inhibitor Wnt production-4 (IWP-4); B: Fourteen days treatment of MSCs with IWP-4 in comparison with untreated control showing significant increase in the expression of early cardiac markers, *GATA-4, Nkx2.5,* and late cardiac markers, *MHC-β, MLC-2v*, *Mef-2D*, *cTnT*, *cTnC*, *cTnI*, *α-actinin*, *Ca-channel* and *Na-channel*. Quantitative two fold ($2^{-\Delta\Delta CT}$) difference of mean is represented by ΔΔCt method. Statistical analysis was performed using an Independent sample t-test. Values are presented as mean ± SEM from three independent biological triplicates; level of significance is $P < 0.05$ ($^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$). IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; NS: No significant.



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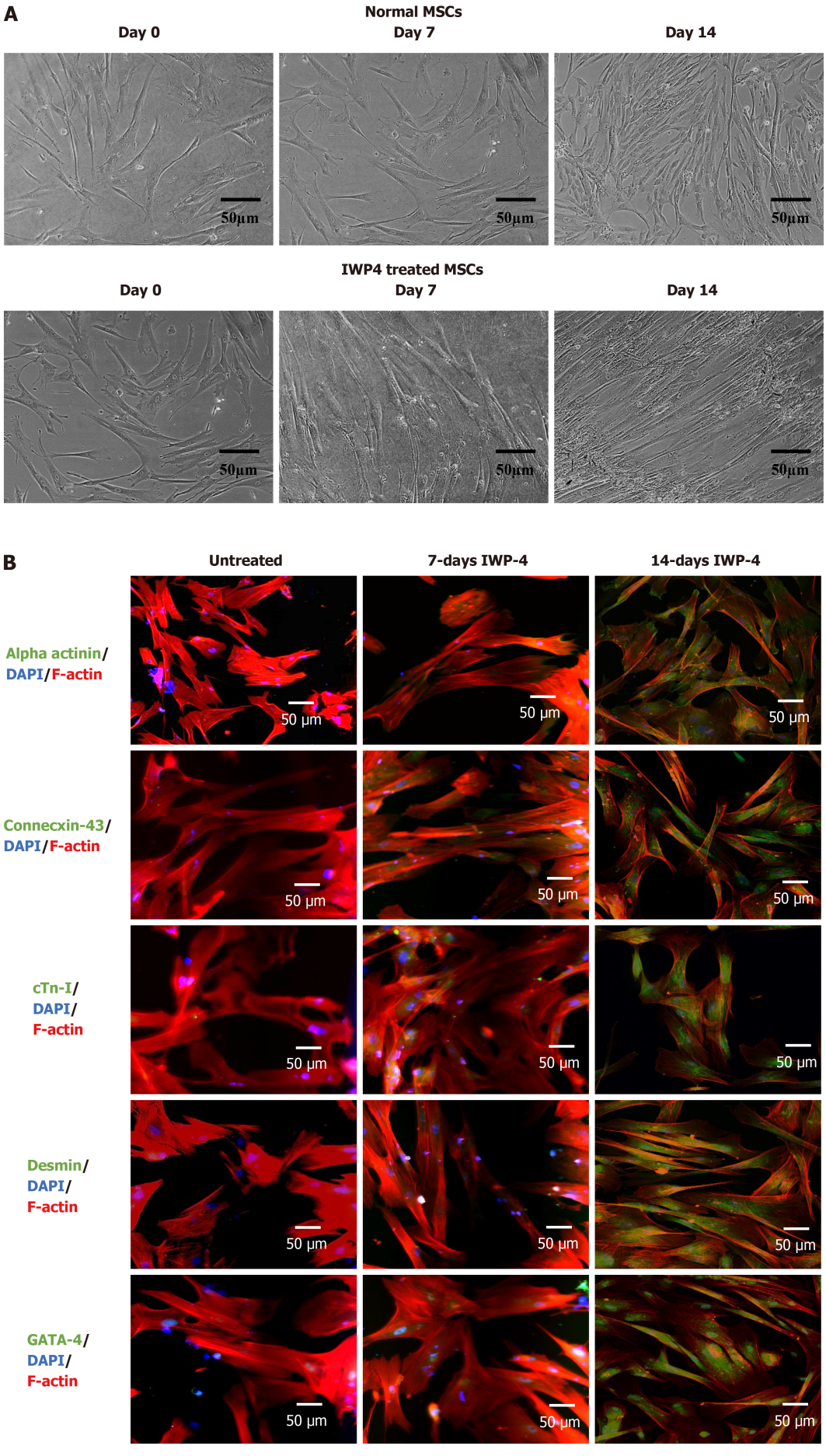
Figure 4 Wnt pathway gene expression analysis: Wnt pathway gene expression analysis by quantitative real-time polymerase chain reaction. Seven days, and fourteen days treatment of mesenchymal stem cells with inhibitor Wnt production-4, in comparison with untreated control showing significant decrease in the expression of Wnt pathway genes, *DVL*, *Wnt*, *TCF*, *Axin*, and *β-catenin*, and downstream transcription factors *C-jun*, *C-myc*, and *Cyc-D*. A: Seven days; B: Fourteen days. *GSK* increased in the fourteen days treatment. Quantitative two fold ($2^{-\Delta\Delta CT}$) difference of mean is represented by ΔΔCt method. Statistical analysis was performed using an Independent sample t-test. Values are presented as mean ± SEM from three independent biological triplicates; level of significance is $P < 0.05$ ($^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$). IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; NS: No significant.

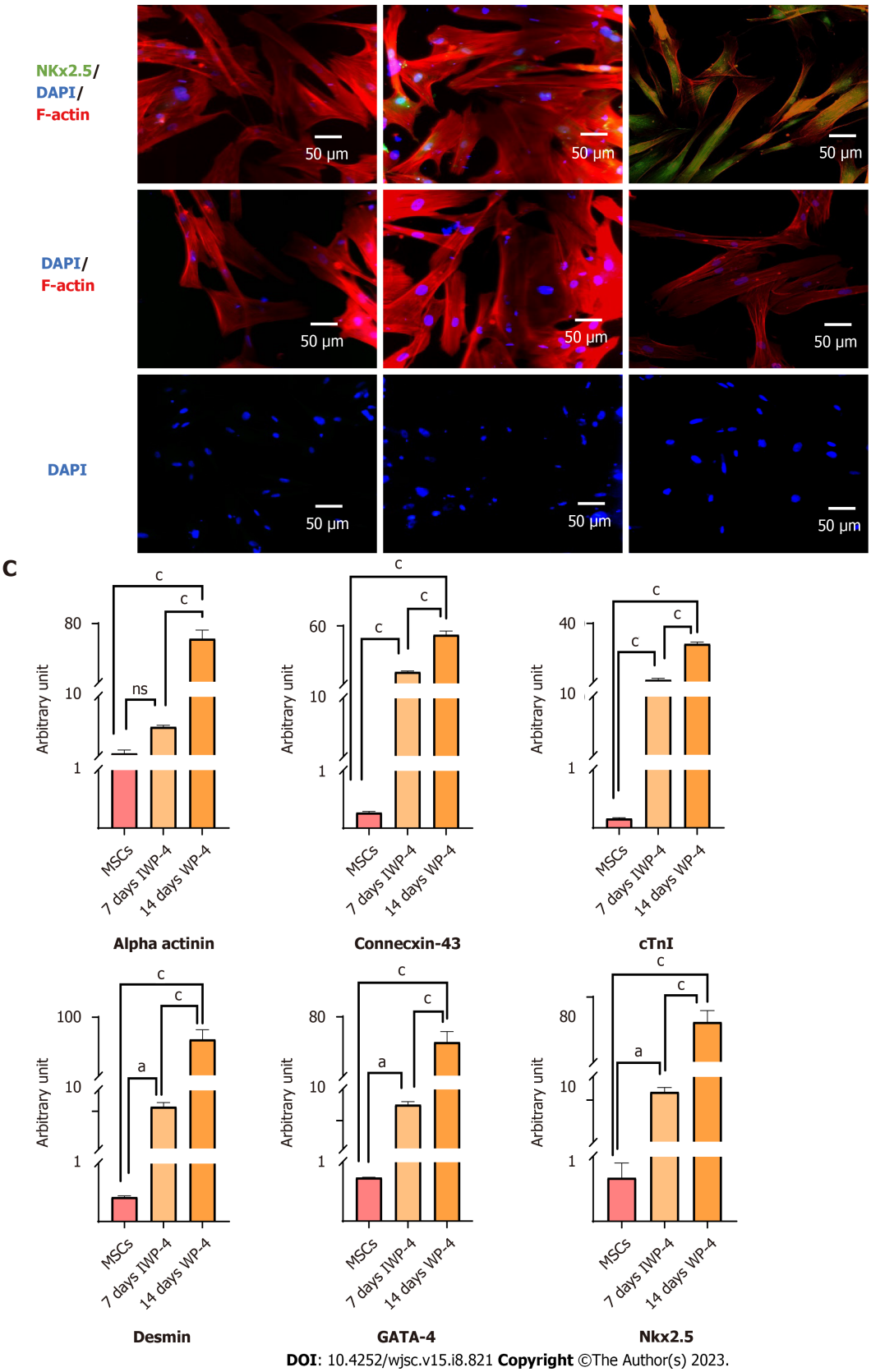
the infarcted myocardium[10], while pre-differentiated MSCs have several advantages over MSCs[11].

Wnt and Smad signaling pathways play an important role in the fate determination of stem cell and their potential for cardiovascular differentiation. Several studies reported that Wnt and Smad signaling pathways play an important role in the fate determination of stem cells and their potential for cardiovascular differentiation. Several studies reported that early application of Wnt/β-catenin signaling pathway inhibitor, IWR1 promotes cardiomyocyte differentiation[12]. Similarly, IWP-4 is a potent small molecule that has been reported to differentiate embryonic stem cells into cardiomyocyte like cells[7]. IWP-4 was also reported to differentiate human umbilical cord derived induced pluripotent stem cells into cardiomyocyte like cells[13].

In this study, IWP-4 was used to differentiate human umbilical cord derived MSCs into cardiomyocyte like cells. First, MSCs were isolated, propagated, and expanded from cord tissue according to the minimum criteria established by the International Society for Cellular Therapy.

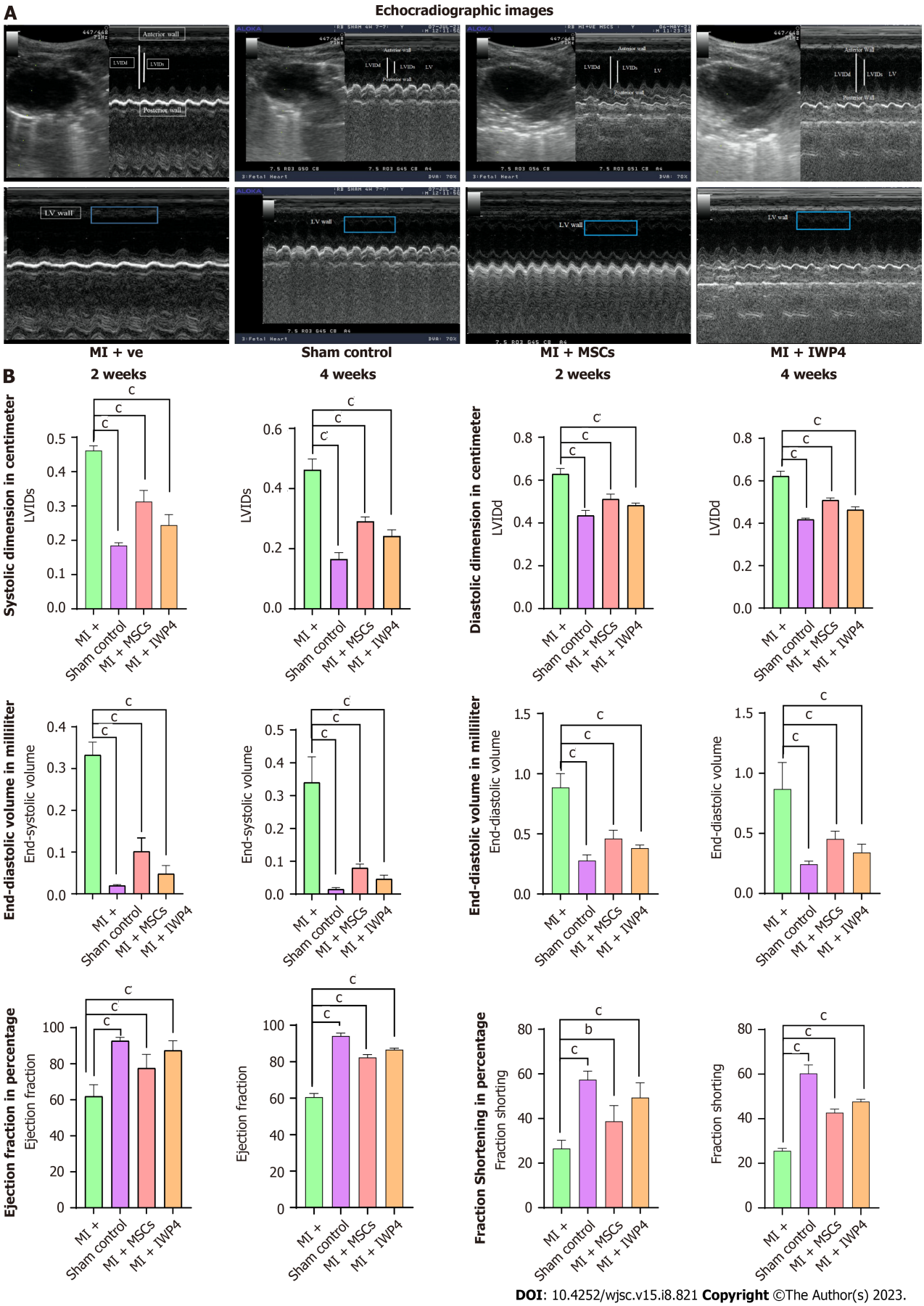
To identify the optimal non-toxic concentration of IWP-4, MSCs were treated with different concentrations of IWP-4. 5 μM concentration was found to be non-cytotoxic after 24 h of treatment and analyzed *via* JC-1 assay.


A
Normal MSCs
Day 0
Day 7
Day 14
50μm
IWP4 treated MSCs
Day 0
Day 7
Day 14
50μm
B
Untreated
7-days IWP-4
14-days IWP-4
Alpha actinin/
DAPI/F-actin
Connecxin-43/
DAPI/F-actin
cTn-I/
DAPI/
F-actin
Desmin/
DAPI/
F-actin
GATA-4/
DAPI/
F-actin
50 μm



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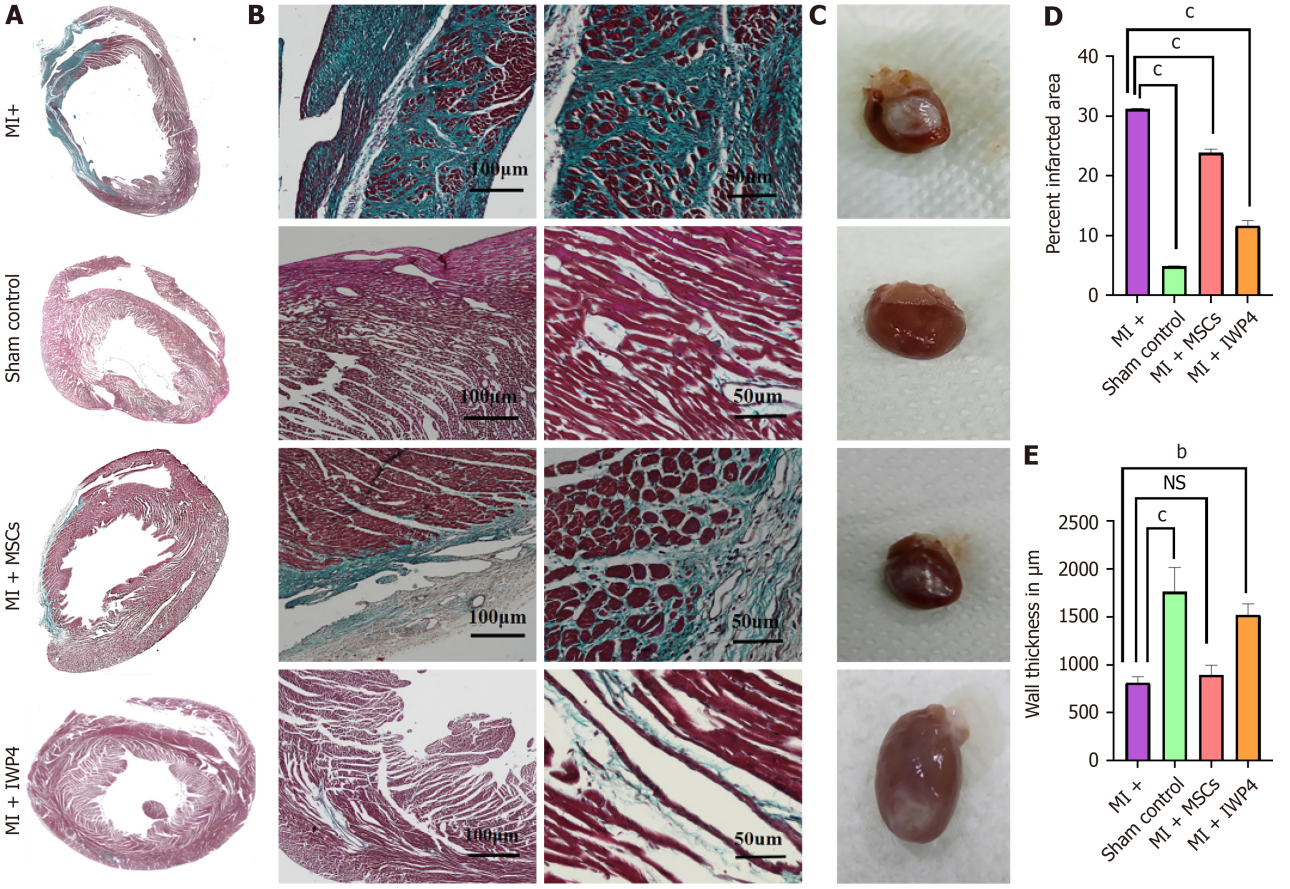
Figure 5 Analysis of fourteen days inhibitor Wnt production-4 treated mesenchymal stem cells. A: Morphology of untreated mesenchymal stem cells (MSCs), seven days inhibitor Wnt production-4 (IWP-4) treated MSCs and fourteen days IWP-4 treated MSCs; B: Immunocytochemical analysis of untreated MSCs, seven days and fourteen days IWP-4 treated MSCs showing positive expression of cardiac-specific proteins *α-actinin*, *connexin-43*, *cTnI*, *Desmin*, *GATA-4*, *Nkx2.5*, secondary control, and DAPI control. Alexa fluor 488 secondary antibody was used for detection, then counterstained with DAPI to stain the nuclei; C: Quantification of fluorescence intensities in untreated MSCs, seven days, and fourteen days IWP-4 treated MSCs. For statistical analysis, One-way ANOVA was used followed by the Bonferroni post-hoc test. Data are presented as mean ± SEM with significance level $P < 0.05$ (where $^{a}P < 0.05$, $^{c}P < 0.001$). IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; NS: No significant.



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Figure 6 Cardiac functional analysis by echocardiography. A: Ultrasound images taken at parasternal long axis showing B and M mode scans of left ventricle in sham control, myocardial infarction (MI+) group, mesenchymal stem cells (MI+MSC) treated group, and inhibitor Wnt production-4 (IWP-4) (MI+IWP-4)

treated groups; B: Bar graphs representing cardiac functional analysis in terms of left ventricular systolic internal dimensions, left ventricular diastolic internal dimensions, end-systolic volume and end-diastolic volume, ejection fraction and fractional shortening, after 2 and 4 wk of MI model development. Both untreated and IWP-4 treated MSC groups showed cardiac functional improvement. However, IWP-4 treated group exhibited more significant results. Statistical analysis was performed using One-way ANOVA followed by Bonferroni post-hoc test. Data are presented as mean ± SEM with significance level $P < 0.05$ (where $^{b}P < 0.01$; $^{c}P < 0.001$). IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; MI: Myocardial infarction; LVIDs: Left ventricular systolic internal dimensions; LVIDd: Left ventricular diastolic internal dimensions.

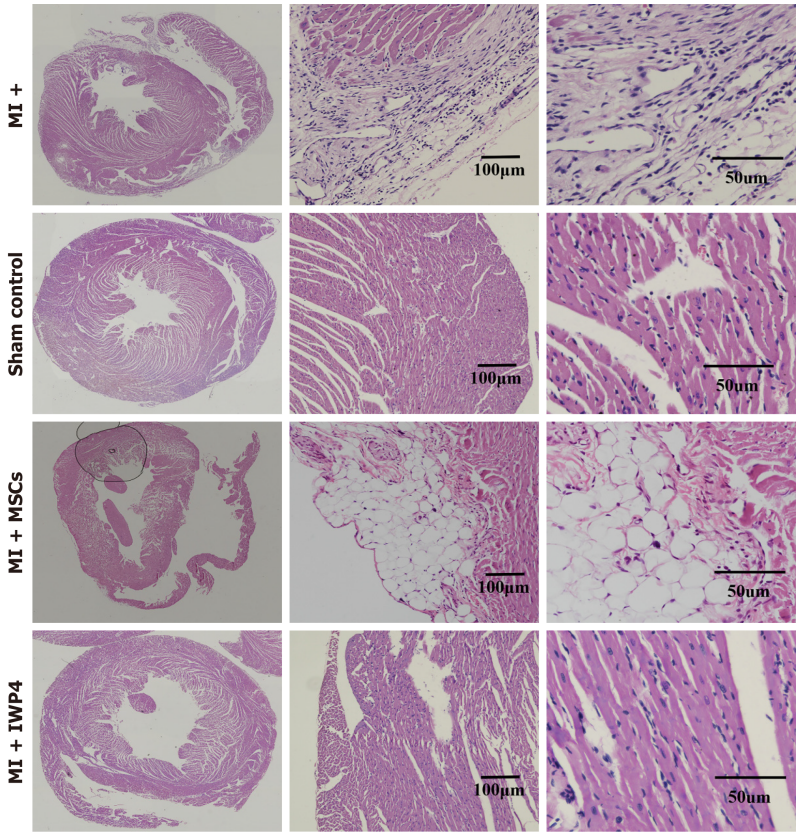


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Figure 7 Histological analysis of heart sections. A: Large scan images showing Masson's trichrome stained transverse section of the whole heart; B: Magnified images showing Masson's trichrome stained sections in sham control, myocardial infarction (MI+), mesenchymal stem cells (MSCs) (MI+MSCs), and inhibitor Wnt production-4 (IWP-4) treated MSCs (MI+IWP-4) groups after 4 wk of MI; C: Images showing isolated rat hearts of sham control, MI+ group, MI+MSC group, and MI+IWP-4 groups; D: Bar graph showing percent infarcted area as compared to MI+ group. Percent infarcted area was significantly decreased in MI+MSC and MI+IWP-4 groups after 4 wk of MI; E: Bar graph showing left ventricular wall thickness as compared to MI+ group; left ventricular wall thickness was non-significant in MI+MSC group and significantly increased in the MI+IWP-4 group after 4 wk of MI. For statistical analysis, One-way ANOVA was used followed by the Bonferroni post-hoc test. Data is presented as mean ± SEM with significance level $P < 0.05$ where ($^{b}P < 0.01$, $^{c}P < 0.001$). IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; MI: Myocardial infarction; NS: No significant.

To confirm the Wnt pathway inhibition, Wnt pathway gene expression profile was analyzed after seven and fourteen days treatment of MSCs with IWP-4. Gene expression profile revealed that seven days treatment of MSCs with IWP-4 inhibits Wnt pathway as compared to untreated MSCs; Wnt pathway genes, *GSK*, *DVL*, *Wnt*, *TCF*, *β-catenin*, and downstream transcription factors *C-jun*, *C-myc*, *Cyc-D* ($P < 0.001$) were significantly downregulated, while *Axin* showed upregulation ($P < 0.01$). However, fourteen days treatment of MSCs with IWP-4, further downregulated the Wnt pathway as compared to untreated control; Wnt pathway genes *DVL*, *Wnt*, *β-catenin* ($P < 0.001$) showed significant downregulation, while negative regulators of the pathway *GSK* ($P < 0.01$), and *Axin* ($P < 0.05$) were upregulated. Downstream transcription factors *C-jun*, *Cyc-D*, *TCF* were non-significant, while *C-myc* ($P < 0.05$) was upregulated.

The Wnt (β-catenin dependent) comprises several proteins that are crucial for embryonic development as well as adult tissue homeostasis. In the absence of Wnt ligands, cytoplasmic β-catenin is captured by the destruction complex including glycogen synthase kinase-3β (GSK3β), axin, adenomatous polyposis coli, and casein kinase 1 (CK1). This destruction complex induces the β-catenin phosphorylation at the amino terminus by CK1 and GSK3β, that results in the ubiquitination and degradation of β-catenin[14]. In the presence of ligands, Wnt binds to its receptor and activates the signaling pathway. This ligand receptor binding recruits the axin and DVL protein towards the cell membrane, thus resulting in the inactivation of the destruction complex. As a result, β-catenin translocate to the nucleus where it binds to the transcription



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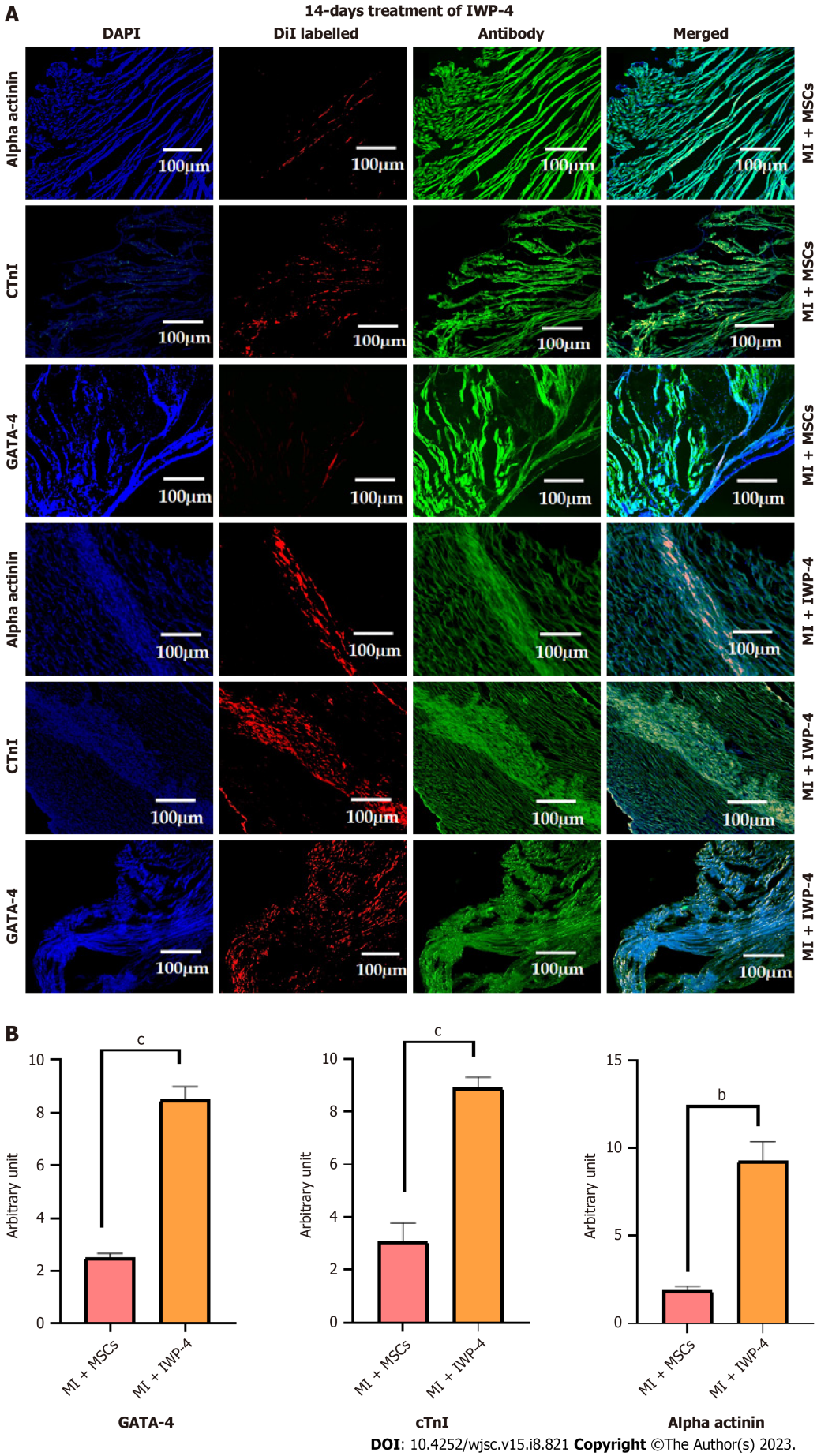
Figure 8 Hematoxylin-Eosin staining of heart section. Large scan images showing Hematoxylin-Eosin (H&E) stained transverse section of the whole heart. Magnified images showing H&E stained sections in sham control, myocardial infarction (MI) group, mesenchymal stem cells (MSCs) (MI+MSC) group, and inhibitor Wnt production-4 (IWP-4) treated MSCs (MI+IWP-4) group after 4 wk of MI. IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; MI: Myocardial infarction.

factor TCF and increases the expression of Wnt target genes[13,15].

In our study, IWP-4 treatment was given for seven and fourteen days. Morphological features of fourteen days treated MSCs showed cardiomyogenic differentiation; the cells were flattened and appeared larger with myotube like structures. To confirm the differentiation of cells toward cardiac lineage, cardiac specific gene expression profile was analyzed through qPCR. In the seven days treated MSCs, only two cardiac genes *MLC-2v* ($P < 0.01$) and *Ca-channel* ($P < 0.05$) showed upregulation, while *Na-channel* ($P < 0.05$), *cTnT*, *GATA4* ($P < 0.01$), *alpha-actinin*, *cTnI*, *Mef2D* and *Nkx2.5* ($P < 0.001$) genes showed significant downregulation; *MHC-β* and *cTnC* were non-significant. However, fourteen days treated MSCs showed significant upregulation of all cardiac genes including *alpha-actinin* ($P < 0.05$), *MHC-β*, *MLC-2v*, *Mef2D*, *cTnT*, *cTnC*, *cTnI*, *Ca-channel*, *Na-channel*, *Nkx2.5* and *GATA4* ($P < 0.001$) as compared to untreated MSCs.

The cardiac markers GATA-4 and Nkx2.5 play important role in the cardiac development[16]. GATA-4 is an important early cardiac transcription factor that regulates several cardiac genes[17]. Nkx2.5 is another important transcription factor that showed high expression in the cardiac progenitor cells and promotes cardiomyocyte maturation[18]. Cardiac troponin exists in three different subunits, cTnT, cTnI, and cTnC. Troponin proteins are linked with tropomyosin which is present in all skeletal and cardiac muscle cells and plays an essential role in cardiac contractile movement. cTnT directly binds with the thin filaments, while cTnI and cTnC bind with cTnT. It is also reported to be circulated into the blood after cardiomyocyte injury[19]. cTnI and cTnT are the essential components of the cardiac troponin complex particularly involved in the formation of cardiomyocyte contractile network and regulate cardiac contraction and relaxation[20,21].

In the human heart, cardiac myosin heavy chain is expressed in two isoforms *i.e.* α-MHC and β-MHC. β-MHC is an important cardiac marker, predominant ventricular isoform, and principal mediator of cardiac contractile function[22]. Any change regarding the expression of MHC isoforms disturbs the cardiac contractile functions[23]. Ventricular MLC-2v is the ventricular cardiac muscle form of myosin light chain 2. MLC-2v is regarded as a ventricular-specific marker of the myocardium. MLC-2v is expressed during early cardiac development in humans[24]. Mef2 is a transcription factor that is important for right ventricle development[25]. Cardiac α-actinin-2, also known as alpha actinin, is a homodimer, Z-disk component of sarcomere responsible for three important functions: Anchoring of thin filaments actin, sarcomere formation, and interaction with titin[26]. The upregulation of cardiac ion channels (sodium and calcium) decides the cell fate to become mature functional cardiomyocytes[27]. Calcium acts as a second messenger in signal transduction pathways as well as necessary for muscular contraction. Calcium is controlled by calcium transport proteins, calcium channels, carriers and pumps[28]. Voltage-gated sodium channels are transmembrane proteins that generate action potentials in neurons and cardiac cells[29].



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Figure 9 Immunohistochemistry of heart tissue section. A: Immunohistochemical images of heart sections showing the transplanted untreated

mesenchymal stem cells (MSCs) and fourteen days inhibitor Wnt production-4 (IWP-4) treated MSCs labeled with red fluorescent DiI dye. Cardiac specific proteins α-actinin, cTnI, and GATA-4 were immunostained for the expression of cardiac proteins. Alexa fluor 488 secondary antibody was used for detection; B: Quantification of the fluorescence intensities of the DiI labeled cells presented in bar graphs. As compared to normal MSCs, fluorescence intensity was significantly increased in case of alpha actinin, GATA-4, and cTnI in the fourteen days IWP-4 treated MSCs group in the infarcted myocardium. For statistical analysis, One-way ANOVA was used followed by the Bonferroni post-hoc test. Data are presented as mean ± SEM with significance level $P < 0.05$ where ($^{b}P < 0.01$, $^{c}P < 0.001$). IWP-4: Inhibitor Wnt production-4; MSC: Mesenchymal stem cell; MI: Myocardial infarction.

Cardiac specific protein expression analysis was also performed to further confirm myogenic differentiation. Cardiac protein expression was analyzed in untreated, and seven and fourteen days treated MSCs. Seven days treated MSCs showed less protein expression of C-43, cTnI, ($P < 0.001$), desmin, GATA-4, and Nkx2.5 ($P < 0.05$), while alpha-actinin protein expression was non-significant. Fourteen days treated MSCs showed significant positive expression of cardiac proteins including alpha-actinin, C-43, cTnI, desmin, GATA-4, and Nkx2.5 ($P < 0.001$). C-43 is a gap junction protein that connects the neighboring cardiac cells. C-43 is abundant and expressed in atrial as well as ventricular cardiomyocytes [30]. Desmin is an important intermediate filament that maintains the cytoskeletal structure and cellular integrity in skeletal, smooth, and cardiac muscles[31]. The presence of cardiac specific proteins further confirmed the differentiation of MSCs into cardiomyocytes, as these proteins are necessary for several developmental processes and functioning of the heart. Hence, a pronounced effect was observed in fourteen days treated MSCs, so it was selected for *in vivo* studies.

To examine the ability of pre-differentiated MSCs to regenerate the infarcted myocardium *in vivo*, a rat MI model was established and fourteen days treated pre-differentiated MSCs were transplanted. In previous studies, it was reported that the pre-differentiated MSCs homed and survived better than the untreated MSCs in the injured myocardium[11,32]. To confirm, untreated MSCs, as well as IWP-4 treated MSCs, were transplanted in the left ventricular wall of the infarcted hearts immediately after MI induction.

For cardiac functional analysis, echocardiography was performed after 2 and 4 wk of MI. Functional studies revealed a significant increase in LVIDd, LVIDs, EDV, and ESV in the MI model; however, FS and EF were significantly decreased as compared to the sham control. These results confirmed the successful MI model development. When compared with the MI group, LVIDd, LVIDs, EDV, and ESV were significantly reduced, while EF and FS were significantly improved after 2 and 4 wk in both untreated and IWP-4 treated MSCs transplanted groups. However, the IWP-4 treated group showed more pronounced results.

For histological analysis, heart tissue was harvested after 4 wk of infarction. The MI heart exhibited a whitish scar with no blood vessels in the infarcted area, while in the IWP-4 transplanted heart, the area of scar was reduced with blood vessels in the repaired myocardium. To observe the histology, and to calculate the fibrotic area and wall thickness, H&E and Masson's trichrome staining were performed. Histological analysis revealed that the fibrotic area was reduced and wall thickness was increased in the untreated MSCs transplanted heart sections, while the IWP-4 treated group showed significantly reduced fibrosis, and improved wall thickness as compared to the MI group. Untreated as well as IWP-4 treated MSCs were labeled with DiI dye to track the localization and homing of cells in the infarcted myocardium, and analyzed through immunohistochemistry. Cardiac functional proteins GATA-4, alpha actinin and cTnI were used for immunohistochemical analysis of the cryosectioned heart that showed cardiac proteins as well as DiI dye expression in the myocardium. Fluorescence intensity calculation revealed the transplanted cell density and significantly higher cardiac protein expression in the IWP-4 treated MSCs group as compared to the untreated group. This data further supports the histological results that revealed a markedly decreased fibrotic area and increased regeneration of the infarcted myocardium. Altogether, the results showed that IWP-4 treated MSCs regenerated myocardium, ventricular remodeling, and improved heart function.

CONCLUSION

This study focused on the treatment of hUC-MSCs with IWP-4 for differentiation of MSCs into cardiomyogenic lineage *via* inhibiting Wnt pathway and their consequent role in the cardiac function restoration in the rat MI model. Fourteen days IWP-4 treatment increases the expression of cardiac markers in MSCs, both at the gene and protein levels, as compared to the untreated control. Transplanted pre-differentiated cells in the rat MI model not only distributed, survived, and homed, but also differentiated into mature cardiomyocytes better than untreated MSCs. The results obtained from the current preclinical study suggests that treatment with the small molecule IWP-4 could be a good option for the differentiation of MSCs into functional cardiomyocytes in clinical trials of MI patients in future.

ARTICLE HIGHLIGHTS

Research background

Cardiovascular diseases particularly myocardial infarction (MI) is a global health complication with high mortality and morbidity rate. As cardiac tissue lacks regeneration potential, so cardiac tissue regeneration using a potent small molecule inhibitor Wnt production-4 (IWP-4) for stem cell fate transition towards cardiomyocytes could be an effective approach.

Research motivation
Inhibition of Wnt pathway is important in stem cell fate determination towards cardiomyocytes. Wnt pathway inhibitor, such as IWP-4, may promote the differentiation of mesenchymal stem cells (MSCs) into cardiac lineage. These pre-conditioned cells may provide better survival, homing and migration capability at the site of injury.

Research objectives
This study was designed to evaluate the IWP-4 cardiac differentiation capability and its subsequent *in vivo* effects.

Research methods
Human umbilical cord-derived MSCs were characterized on the basis of morphology, immunophenotyping of surface markers associated with MSCs and tri-lineage differentiation capability. Isolated MSCs were treated with 5 μM IWP-4 at two different time intervals. Cardiomyogenic differentiation of treated MSCs was evaluated at DNA and protein levels. MI rat model was developed. IWP-4 treated as well as untreated MSCs were implanted in the MI model, and cardiac function was analyzed *via* echocardiography. MSCs were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) dye for tracking, and regeneration of the infarcted myocardium was examined by histology and immunohistochemistry.

Research results
Isolated MSCs were characterized and then pre-conditioned with 5 μM concentration of IWP-4. The cardiac specific gene and protein expression analysis exhibited more remarkable results in fourteen days treated group that was eventually selected for *in vivo* transplantation. Cardiac function was restored in the IWP-4 treated group in comparison to the MI group. Immunohistochemical analysis confirmed the homing of pre-differentiated MSCs that were labeled with DiI cell labeling dye. Histological analysis confirmed the significant reduction in the fibrotic area, and improved the left ventricular wall thickness in the IWP-4 treated MSC group.

Research conclusions
Our data suggest that treatment of MSCs with IWP-4 inhibits Wnt pathway and promotes cardiac differentiation. These pre-conditioned MSCs transplanted *in vivo* improved cardiac function by cell homing, survival, and differentiation at the infarcted region, increased left ventricular wall thickness, and reduced infarct size.

Research perspectives
The study demonstrated that treatment with IWP-4 improves the efficacy of the MSCs to ameliorate the cardiac function of the MI rats, as evidenced by an increase in the ejection fraction. Pre-conditioning of MSCs with IWP-4 may serve as a promising strategy to treat heart disease. Targeting the Wnt signaling pathway may represent a promising therapeutic approach for this debilitating condition.

FOOTNOTES

Author contributions: Muneer R performed experiments and wrote the original manuscript; Qazi R, Fatima A, and Ahmad W helped in experimentation and writing; Salim A evaluated and analyzed the data, and reviewed the manuscript; Dini L evaluated and analyzed the data; Khan I conceived and designed the studies, evaluated and analyzed the data, and finalized the manuscript.

Institutional review board statement: The independent ethical committee of PCMD, ICCBS, University of Karachi, for human subjects has approved the protocol, No. ICCBS/IEC-067-HT/UCB-2021/Protocol/1.0.

Institutional animal care and use committee statement: Ethical approval for the use of animals was obtained from the Institutional Animal Care and Use Committee at the PCMD, ICCBS, University of Karachi with protocol number 2021-006.

Informed consent statement: Informed consent was obtained from the donor.

Conflict-of-interest statement: Authors declare no conflict of interest.

Data sharing statement: No additional data to share.

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ORIGINAL ARTICLE

Basic Study

Quercetin ameliorates oxidative stress-induced senescence in rat nucleus pulposus-derived mesenchymal stem cells *via* the miR-34a-5p/SIRT1 axis

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Abstract

BACKGROUND

Intervertebral disc degeneration (IDD) is a main contributor to low back pain. Oxidative stress, which is highly associated with the progression of IDD, increases senescence of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) and weakens the differentiation ability of NPMSCs in degenerated intervertebral discs (IVDs). Quercetin (Que) has been demonstrated to reduce oxidative stress in diverse degenerative diseases.

AIM

To investigate the role of Que in oxidative stress-induced NPMSC damage and to elucidate the underlying mechanism.

METHODS

In vitro, NPMSCs were isolated from rat tails. Senescence-associated β-galactosidase (SA-β-Gal) staining, cell cycle, reactive oxygen species (ROS), real-time quantitative polymerase chain reaction (RT-qPCR), immunofluorescence,

and western blot analyses were used to evaluated the protective effects of Que. Meanwhile the relationship between miR-34a-5p and Sirtuins 1 (SIRT1) was evaluated by dual-luciferase reporter assay. To explore whether Que modulates tert-butyl hydroperoxide (TBHP)-induced senescence of NPMSCs *via* the miR-34a-5p/SIRT1 pathway, we used adenovirus vectors to overexpress and downregulate the expression of miR-34a-5p and used SIRT1 siRNA to knockdown SIRT1 expression. *In vivo,* a puncture-induced rat IDD model was constructed, and X rays and histological analysis were used to assess whether Que could alleviate IDD *in vivo*.

RESULTS

We found that TBHP can cause NPMSCs senescence changes, such as reduced cell proliferation ability, increased SA-β-Gal activity, cell cycle arrest, the accumulation of ROS, and increased expression of senescence-related proteins. While abovementioned senescence indicators were significantly alleviated by Que treatment. Que decreased the expression levels of senescence-related proteins (p16, p21, and p53) and senescence-associated secreted phenotype (SASP), including IL-1β, IL-6, and MMP-13, and it increased the expression of SIRT1. In addition, the protective effects of Que on cell senescence were partially reversed by miR-34a-5p overexpression and SIRT1 knockdown. *In vivo*, X-ray, and histological analyses indicated that Que alleviated IDD in a puncture-induced rat model.

CONCLUSION

In summary, the present study provides evidence that Que reduces oxidative stress-induced senescence of NPMSCs *via* the miR-34a/SIRT1 signaling pathway, suggesting that Que may be a potential agent for the treatment of IDD.

Key Words: Quercetin; Nucleus pulposus-derived mesenchymal stem cells; Oxidative stress; Senescence; Intervertebral disc degeneration; miR-34a-5p/SIRT1 pathway

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Core Tip: In our article, we provide the evidence that quercetin (Que) can prevent oxidative stress induced senescence of nucleus pulposus-derived mesenchymal stem cells *via* miR-34a/SIRT1 signaling pathway. Moreover, Que could ameliorate the progression of intervertebral disc degeneration (IDD) in rat model. Thus, Que can be considered as a potential agent for the treatment of IDD.

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INTRODUCTION

As a widely known musculoskeletal disorder, low back pain (LBP) is the leading cause of disability and results in a huge economic burden on the family and society[1,2]. Intervertebral disc degeneration (IDD) is a main contributor to LBP, but the etiology of IDD is multifactorial[3]. Therefore, elucidating the underlying molecular mechanisms of IDD will help develop solutions for the prevention and treatment of IDD.

Several previous studies have indicated that oxidative stress and reactive oxygen species (ROS) are highly associated with the progression of IDD[4-7]. While ROS production is inevitable during the metabolic processes of disc cells, the progression of IDD is considered to be the result of disc cell senescence caused by ROS accumulation[7,8]. Recently, stem cell-based endogenous repair has been shown to play a significant role in the repair and regeneration of degenerative intervertebral discs (IVDs)[9,10]. Nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were first reported by Risbud *et al*[11] in 2007, which rendered a basis for the endogenous repair of IDD[11]. We also previously found NPMSCs in normal and degenerated IVDs[12-15]. Wu *et al*[16] indicated that the regenerative potential of NPMSCs decreases with aging and degeneration of IVDs[16]. In addition, Ma *et al*[17] proposed that the decrease in endogenous NPMSCs caused by adverse microenvironments, such as oxidative stress, low nutrition, and inflammation, is one of the main reasons for the failure of endogenous repair of IDD[17]. Therefore, inhibition of oxidative stress-induced senescence of NPMSCs may be of great significance in alleviating IDD.

Quercetin (Que), a natural flavonoid widely present in various plants, has antioxidative stress, anti-inflammatory, and antiaging properties. Que has been used as a senolytic to prevent diverse degenerative diseases[18-20]. Zhu *et al*[21] found that Que reduces senescent cells by selectively killing senescent human epithelial cells and mouse bone marrow mesenchymal stem cells[21]. Feng *et al*[22] reported that Que attenuates oxidative stress-induced apoptosis in rat

chondrocytes and prevents the progression of osteoarthritis in a rat model[22]. Previous clinical studies have also demonstrated that Que reduces the expression levels of senescence-related markers[23].

MicroRNAs (miRNAs) are small double-stranded noncoding RNAs that are approximately 20 nucleotides in length and interfere with RNA translation by binding to the 3'UTR sequence of the target gene RNA. Abnormal expression of miRNAs has been observed in degenerative IVD, indicating that miRNAs play an important role in the pathophysiological process of IDD[24,25]. A previous study has also demonstrated that the expression of miR-34a-5p is significantly upregulated in degenerated IVDs and that miR-34a-5p increases extracellular matrix degradation and cell apoptosis. Sirtuins 1 (SIRT1), the target of miR-34a, is a member of the sirtuin family and plays a significant role in cancer, age-related diseases, and degenerative diseases[26-28]. Several studies have reported that SIRT1 delays the senescence of IVD cells by decreasing oxidative stress and inflammation as well as by improving mitochondrial function[29,30]. However, the protective effect of Que on NPMSCs is still unknown.

In the present study, tert-butyl hydroperoxide (TBHP) was used to trigger oxidative stress in NPMSCs, which is widely accepted as an apoptosis and senescence *in vitro* model. The effects of Que on senescence in NPMSCs under oxidative stress and the role of the miR-34a-5p/SIRT1 pathway were investigated. Finally, the therapeutic effects were also evaluated in a puncture-induced rat IDD model.

MATERIALS AND METHODS

Isolation and culture of NPMSCs

All the procedures performed in this study were approved by the Ethical Committee of the Clinical Medical College of Yangzhou University. Sprague-Dawley (SD) rats (2-4 mo old and weighing 200-300 g) were obtained from Yangzhou University, No. SYXK (Su) 2017-0044. The separation of nucleus pulposus (NP) tissues and the isolation of NPMSCs were performed as previously described[14]. Briefly, the NP tissues obtained from SD rats were carefully separated by a microscope under sterile conditions, and the NP tissue was mechanically fragmented into 1 mm^3 pieces and digested with 0.2% collagenase type II (Gibco, United States) at 37 °C in 5% CO_2 for 12 h. Cells were filtered through a 75-μm cellular filter, centrifuged at 1000 rpm for 3 min, washed with phosphate-buffered saline (PBS), resuspended in Mesenchymal Stem Cell Complete Medium (Cyagen, United States) with 10% fetal bovine serum (FBS; HyClone, United States) and 1% antibiotics (Gibco, United States), and cultured in an incubator at 37 °C with 5% CO_2. NPMSCs were subcultured at a ratio of 1:3 when they reached 80% confluence. The third passages of NPMSCs were used for the subsequent experiments.

Surface marker identification of NPMSCs

NPMSCs were cultured in a 12-well plate with a 25 mm diameter cell slide in complete medium. Cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. Subsequently, cells were blocked with 10% bovine serum albumin for 1 h at room temperature and incubated with the following primary antibodies (1:100) overnight at 4 °C according to the International Society for Cellular Therapy (ISCT): CD34 (ABclonal, China), CD45 (ABclonal, China), CD73 (ABclonal, China), CD90 (ABclonal, China), CD105 (Proteintech, China), and tyrosine kinase with Ig and EGF homology domains 2 (Tie2, a disc NP progenitor marker) (Biodragon, China). Cells were then incubated with FITC- or Cy3-conjugated secondary antibodies (1:200) at room temperature for 1 h in the dark. After treatment with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, the cell slides were observed under a fluorescence microscope (Leica, Wetzlar, Germany).

Cell treatment and cell viability assay

To establish an oxidative stress-induced model of NPMSCs, NPMSCs were cultured in complete medium with different concentrations (0, 25, 50, 100, 200, and 400 μM) of TBHP (Sigma-Aldrich, United States) for 12 h. Cells were pretreated with different concentrations (0, 10, 20, 40, 80, and 160 μM) of Que (MedChem Express, China) for 24 h before the addition of a suitable concentration of TBHP to investigate the suitable dose of Que. A cell counting kit-8 (CCK-8, Beyotime, China) was used to evaluate cell viability according to the manufacturer's instructions. Briefly, 2 × 10^3 cells/well were seeded in 96-well plates and cultured overnight at 37 °C in 5% CO_2. After the cells were treated as described above, a mixture of 10 μL of CCK-8 reagent and 100 μL of fresh medium was added to each well for 1 h at 37 °C. The optical density (OD) value of each well was measured at 450 nm by a microplate reader (Bio-Rad, United States). Cells were divided into the following groups: Control group (untreated), TBHP group (treated with 100 μM TBHP) and Que + TBHP group (treated with 20 μM Que and 100 μM TBHP).

Cell proliferation assay

Cell proliferation was evaluated by an 5-ethynyl-2'-deoxyuridine (EdU) assay (Beyotime, China). Cells (5 × 10^5 cells/well) were seeded in 12-well plates and cultured at 37 °C in 5% CO_2. According to the manufacturer's instructions, NPMSCs were incubated with EdU for 2 h, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 15 min, and incubated with Click Reaction Mixture for 30 min. After washing with PBS, cells were counterstained with Hoechst 33342 in the dark for 10 min. Finally, cells were observed under a fluorescence microscope and analyzed by ImageJ software (NIH, United States).

Senescence-associated β-galactosidase (SA-β-Gal) staining

NPMSCs were seeded in a 6-well plate at a density of 4 × 10^5 cells/well at 37 °C in 5% CO_2. Cells were stained with SA-β-

Gal using a SA-β-Gal staining kit (Beyotime, China) according to the manufacturer's instructions. Cells were fixed with SA-β-gal fixing solution for 15 min at room temperature and then incubated overnight with an SA-β-Gal working solution at 37 °C without CO_2. Cells were observed under a microscope and analyzed by ImageJ software.

ROS assay

According to the manufacturer's instructions provided by a ROS detection fluorescent probe-DHE kit (Keygen, China), NPMSCs were incubated with 20 μM DHE for 30 min at 37 °C. Cells were then observed under a fluorescence microscope and analyzed by ImageJ software.

JC-1 assay for mitochondrial membrane potential

A JC-1 Detection Kit (Keygen, China) was used to measure mitochondrial membrane potential (MMP). Briefly, NPMSCs were cultured in medium containing 2 μM JC-1 dye, a cationic dye, for 20 min. After being washed twice with incubation buffer, cells were cultured in complete medium, observed by fluorescence microscopy, and analyzed by ImageJ software.

Cell cycle assay

The cell cycle phases of TBHP-induced senescent NPMSCs were determined by flow cytometric analyses using a Cell Cycle Detection Kit (Keygen, China). Briefly, NPMSCs were harvested from 6-well plates, washed twice with ice-cold PBS, fixed with 75% ethanol at 4 °C for 2 h, and incubated with a mixed solution of propidium iodide (PI) and RNase A for 30 min in the dark. Flow cytometry (BD Company, USA) was used to analyze the cell cycle phases.

Cell transfection

Adenovirus vectors containing miR-34a-5p mimic, miR-34a-5p inhibitor, and their corresponding negative controls were obtained from GeneChem (Shanghai, China). SIRT1 siRNA and scrambled siRNA were obtained from Gene Pharma (Shanghai, China). NPMSCs (5×10^5/well) were seeded in 6-well plates and then transfected with miR-34a-5p mimic or miR-34a-5p inhibitor using Lipofectamine 2000 (Thermo Fisher, UT, USA) for 12 h at 37 °C according to the manufacturer's protocol. After replacing the medium with fresh complete medium, NPMSCs were cultured for an additional 24 h. Cells were then harvested for subsequent experiments.

Dual-luciferase reporter assay

The TargetScan Human database (https://www.targetscan.org/vert_80/) was used to predict the complementary binding site of miR-34a-5p in the 3'-UTR of SIRT1 mRNA. According to the Lipofectamine 2000 transfection protocol, SIRT1 3'-UTR wild-type plasmids or mutant plasmids were cotransfected with miR-34a-5p mimic and mimic control into cells. After 48 h, a dual-luciferase reporter assay kit was used to detect luciferase activity.

qRT-PCR

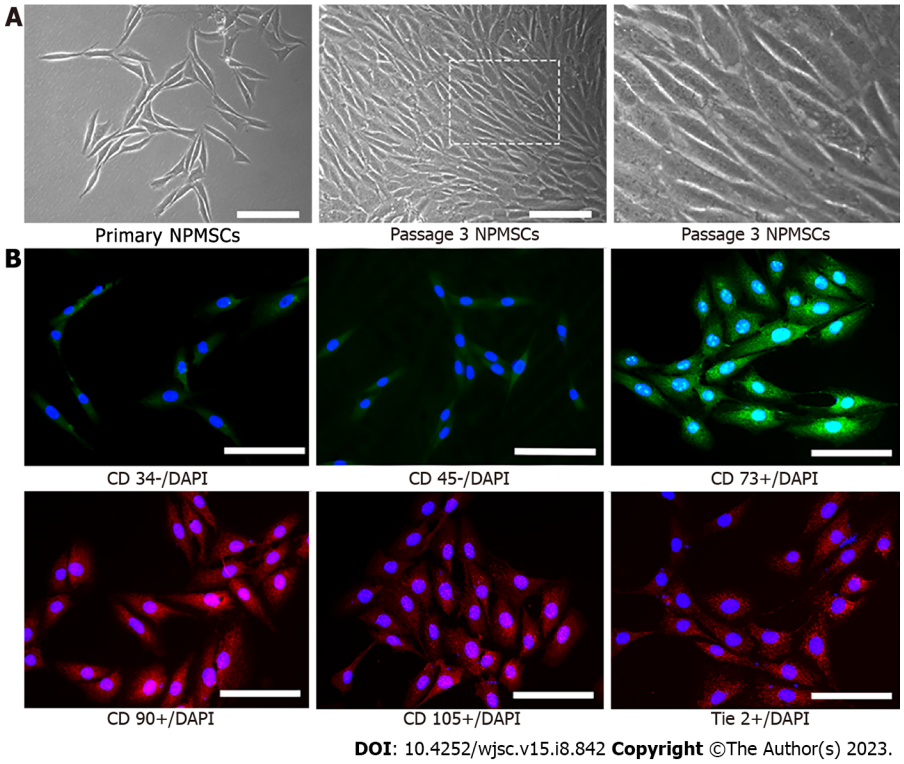
TRIzol reagent (Invitrogen, United States) was used to extract total RNA from NPMSCs. To measure the expression levels of mRNA, RNA was reverse-transcribed into complementary DNA (cDNA) by a Prime Script-RT reagent kit (Vazyme Biotech, China) according to the manufacturer's protocol. cDNA was amplified by SYBR Premix Ex Taq (Vazyme Biotech, China). To measure the expression levels of miR-34a, reverse transcription of RNA and amplification of cDNA were performed using the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme Biotech, China) and miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech, China). The $2^{-\Delta\Delta CT}$ method was used to calculate the expression level of target genes. The expression levels of mRNA or miRNA were normalized to Gapdh or U6. The primers for the target genes used in this study are listed in Table 1.

Western blot analysis

The total protein of NPMSCs was extracted by a Whole Cell Lysis assay (Keygen Biotech, China), and the protein concentration was determined by a BCA protein assay kit (Beyotime, China). Each sample containing 30 μg of protein was separated by 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, United States). After being blocked in 5% skim milk for 2 h at room temperature, the membranes were incubated overnight with the following primary antibodies at 4 °C: P16 (1:1000; Proteintech, China), p21 (1:1000; Proteintech, China), p53 (1:5000; Proteintech, China), SIRT1 (1:1000; ABclonal, China), IL-1β (1:1000; ABclonal, China), IL-6 (1:1000; ABclonal, China), matrix metalloproteinase-13 (MMP-13, 1:1000; ABclonal, China) and Gapdh (1:1000; Servicebio, China). The membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (1:5000; Abcam). The protein bands were observed with an enhanced chemiluminescence system, and the expression of protein was analyzed by ImageJ software.

Puncture-induced rat IDD model

In total, 15 male SD rats (2-4 mo old and weighing 200-300 g) were randomly divided into the following three groups: Control group (n = 5), IDD group (n = 5), and Que group (n = 5). The IDD model of SD rats was established as previously described[13]. Briefly, the rats were anesthetized with pentobarbital, and the tail skin was sterilized by povidone iodine. A 21 G needle was used to puncture the coccygeal IVD (Co 6-7), and the needle was rotated 180° and kept in the disc for 5 s at a depth of 5 mm. After surgery, the Que group was treated with Que (100 mg/kg, 5 mg/mL of Que suspended in 0.5% sodium carboxymethyl cellulose solution) every other day by intragastric administration for 4 wk. The control group and IDD group were treated with 0.5% sodium carboxymethyl cellulose solution for 4 wk.



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Figure 1 Identification of nucleus pulposus derived mesenchymal stem cells. A: Primary nucleus pulposus-derived mesenchymal stem cells (NPMSCs) presented with elongated spindle shape and grew in flower formation; B: NPMSCs exhibited low fluorescent expression of CD34 and CD45, but a high fluorescent expression of CD73, CD90, and CD105. Scar bar = 100 μm.

Radiographic evaluation and histological analysis

At 4 wk postinjury, all rats were placed in a prone position after anesthetization with pentobarbital, and X-ray images were acquired. ImageJ software was used to calculate the disc height index (DHI) as previously described[31]. The tails were harvested after the rats were euthanized with an overdose of sodium pentobarbital. The specimens were fixed in 4% paraformaldehyde for 48 h, decalcified in EDTA for 1 mo, dehydrated in gradient alcohol, and embedded in paraffin. The specimens were cut into 5 μm sections, and the sections were stained with hematoxylin-eosin (HE), Alcian blue, and Safranin O-fast Green. Histological scores were evaluated as previously described[31,32] based on the following scale: 5, normal discs; 6-11, moderately degenerated discs; and 12-14, severely degenerated discs.

Immunofluorescent staining

After the IVD specimens were prepared, a freezing microtome (Leica, Wetzlar, Germany) was used to cut the specimens into 5-μm sections. The sections were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. Subsequently, the sections were blocked with 10% bovine serum albumin for 1 h at room temperature and incubated with the following primary antibodies (1:100) at 4 °C overnight: Collagen type II (ABclonal, China) and aggrecan (ABclonal, China). The sections were then incubated with FITC- or Cy3-conjugated secondary antibodies (1:200) at room temperature for 1 h in the dark. Fluorescence microscopy and ImageJ software were used to observe and analyze the sections.

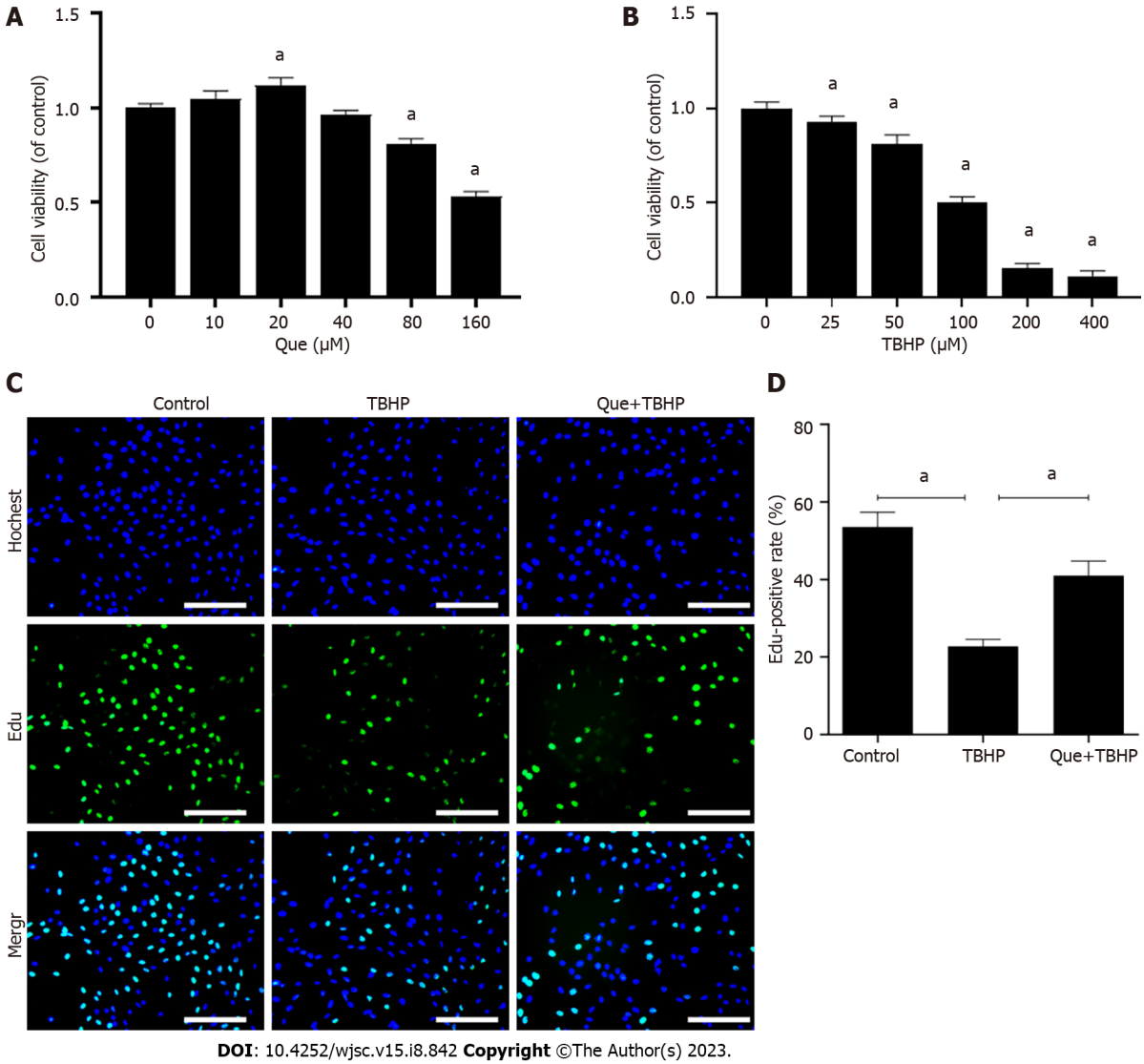
Statistical analysis

All data were analyzed by GraphPad Prism 8 (GraphPad, La Jolla). Data are expressed as the mean ± SD. Student's t test and one-way analysis of variance (ANOVA) was used to analyze the data of two groups and multiple independent groups, respectively. The Kruskal-Wallis H test was used to analyze the histological score. A P value < 0.05 was considered significant.

RESULTS

Characterization of NPMSCs

Elongated spindle-shaped NPMSCs were successfully isolated and cultured from rat IVDs (Figure 1A). Based on the immunofluorescence staining analysis, the MSC-associated markers, namely, CD73, CD90, and CD105 were highly expressed, whereas the expression of CD34 and CD45 was low (Figure 1B). Moreover, the expression of Tie2, a disc NP progenitor marker, was also high in NPMSCs (Figure 1B). These results indicated that NPMSCs isolated from IVD



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Figure 2 Cell viability assay and cell proliferation assay. A: Cell counting kit-8 (CCK-8) results of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) treated with different concentrations of quercetin for 24 h; B: CCK-8 results of NPMSCs treated with different concentrations of tert-butyl hydroperoxide (TBHP) for 24 h; C: EdU assay results of NPMSCs in different groups. Green fluorescence represents cells in a proliferating state, and blue fluorescence represents cell nucleus (scale bar = 200 μm); D: Quantitative analysis of EdU results. Data are represented as mean ± SD. $^{a}P < 0.05$ compared with control group, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

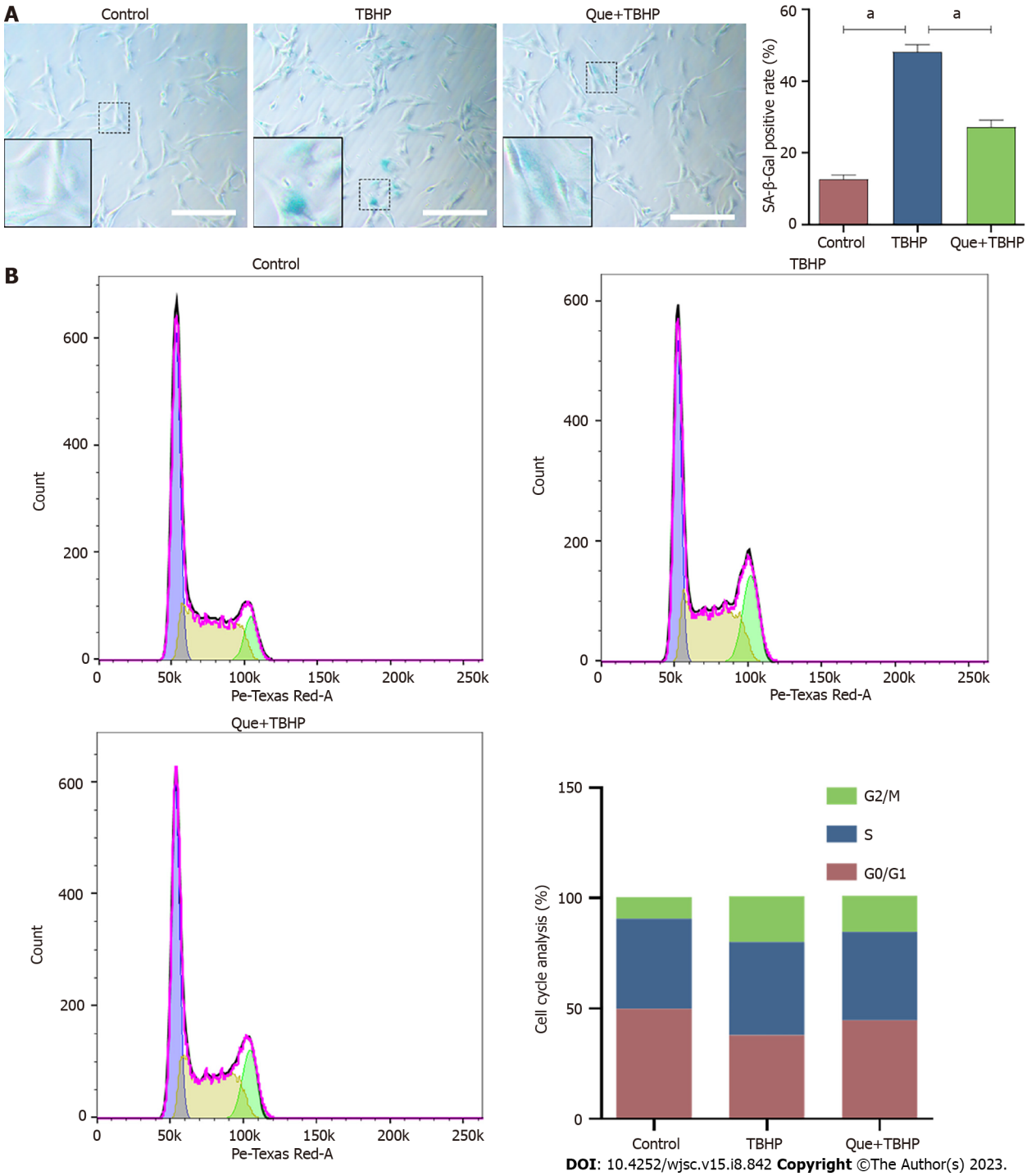
correspond to the standards of stem cells described by ISCT.

Effects of Que and TBHP on the viability and proliferation of NPMSCs

A CCK-8 assay was used to evaluate the effect of Que and TBHP on NPMSC viability. As shown in Figure 2A, Que showed no significant cytotoxic effect at concentrations up to 20 μM for 24 h. Therefore, a concentration of 20 μM Que was selected for the subsequent experiments. NPMSC viability was decreased after treatment with TBHP in a dose-dependent manner (Figure 2B), and 100 μM TBHP was selected for use in subsequent experiments. The effect of Que on NPMSC proliferation was detected by EdU staining. Compared to the control group, the positive rate of EdU in the TBHP group was significantly lower ($P < 0.05$), and the positive rate of EdU was partially increased in the Que + TBHP group ($P < 0.05$) (Figure 2C and D).

Effects of Que on SA-β-Gal staining and the cell cycle in NPMSCs

SA-β-Gal accumulation is an indicator of cellular senescence. Compared to the control group, the number of SA-β-Gal-positive senescent NPMSCs in the TBHP group was significantly higher ($P < 0.05$), while the number of SA-β-Gal-positive cells was decreased in Que + TBHP group (Figure 3A). Compared to the control group, the percentage of NPMSC in the G2/M phase in TBHP group was higher, which indicated cell cycle arrest, but treatment with Que decreased the percentage of NPMSCs arrested in G2/M phase (Figure 3B).



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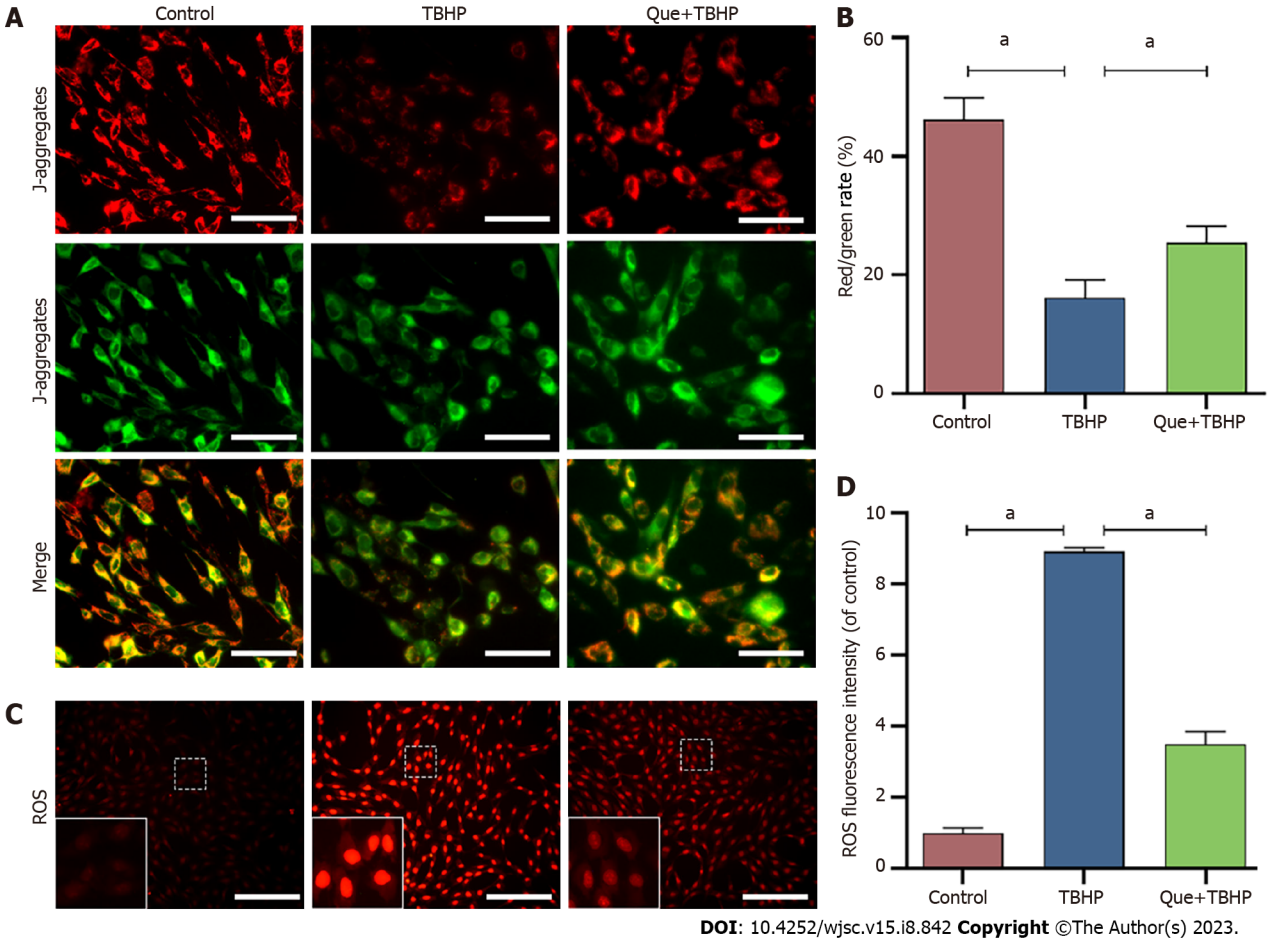
Figure 3 Senescence-associated β-Galactosidase staining assay and cell cycle assay. A: Senescence-associated β-Galactosidase (SA-β-gal) staining results of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) in different groups. Senescent cells exhibit high expression of SA-β-gal in blue staining; B: Cell cycle results of NPMSCs in different groups. Scar bar = 200 μm. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

Effects of Que on MMP and ROS generated in TBHP-treated NPMSCs

When the MMP is high, JC-1 exists in the mitochondria as J-aggregates of polymers, which produce red fluorescence. When the MMP is low, JC-1 is released from the mitochondria matrix and exists in the cytoplasm in the form of monomers, which produce green fluorescence. As shown in Figure 4A and B, the MMP was decreased after TBHP treatment, and this effect was partially reversed by pretreatment with Que. Figure 4C and D show that NPMSCs in the TBHP group had higher ROS levels compared to the control group, and pretreatment with Que partially reversed the effect of TBHP on ROS generation.

Que inhibits oxidative stress-induced senescence of NPMSCs

Cell senescence is broadly characterized by cell cycle arrest and the production of SASP. To explore the effect of Que in



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Figure 4 Mitochondrial membrane potential assay and reactive oxygen species assay. A: Results of Mitochondrial membrane potential (MMP) in different groups detected by fluorescence. Red fluorescence represents the mitochondrial aggregate JC-1 and green fluorescence indicates the monomeric JC-1. Scale bar = 100 μm; B: Quantitative analysis of MMP results; C: Results of ROS in different groups detected by fluorescence. Red fluorescence represents high level of reactive oxygen species assay (ROS). Scale bar = 200 μm; D: Quantitative analysis of ROS results. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin; ROS: Reactive oxygen species assay.

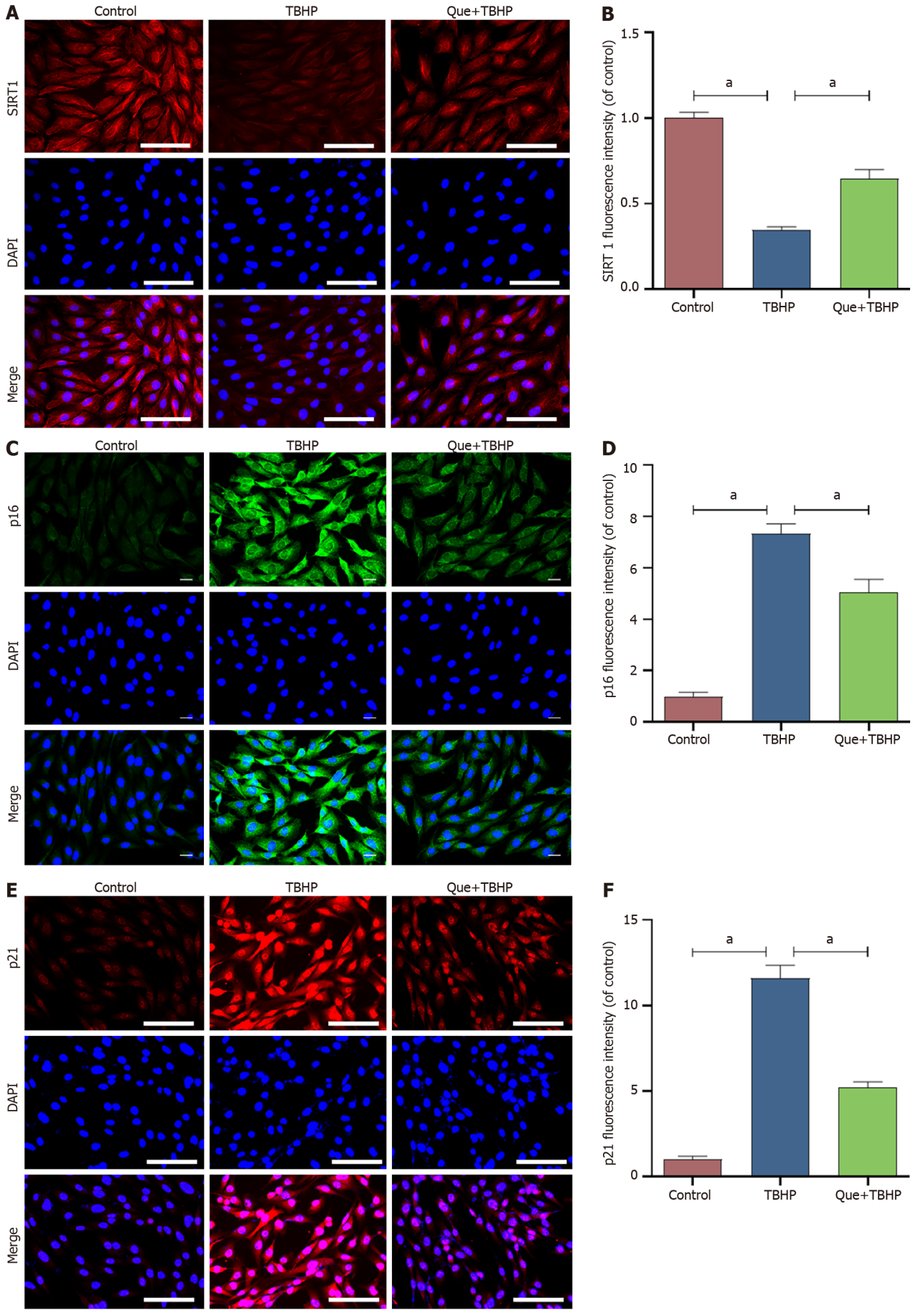
TBHP-induced NPMSCs, western blot analysis and immunofluorescence staining were used to evaluate the expression of SIRT1, cell senescence-related proteins (p16, p21, and p53), and SASP (IL-1β, IL-6, and MMP-13). The expression of SIRT1 was downregulated after TBHP treatment, and this effect was partially reversed by Que pretreatment ($P < 0.05$; Figure 5). Moreover, Que treatment significantly decreased the upregulation of p16, p21, p53, IL-1β, IL-6, and MMP-13 expression induced by TBHP ($P < 0.05$; Figures 5 and 6). Taken together, these results indicated that Que may have a protective role in TBHP-induced oxidative stress injury of NPMSCs.

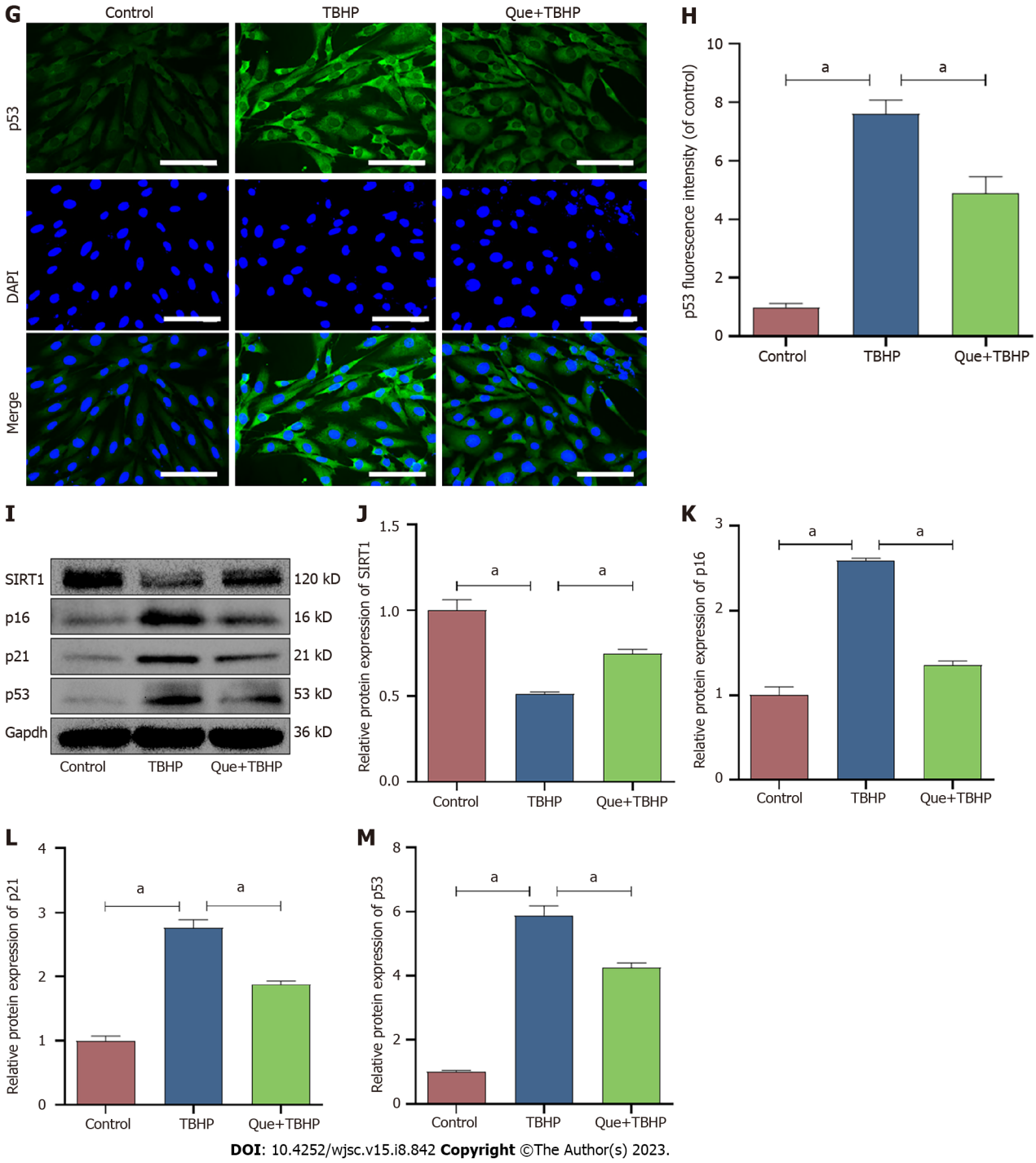
MiR-34a-5p represses SIRT1 expression by targeting the 3'-UTR of SIRT1

According to the TargetScan online prediction database, SIRT1 is a potential target of miR-34a-5p (Figure 7A). A dual-luciferase reporter assay revealed that the miR-34a-5p mimic significantly inhibited the relative luciferase activity in the wild-type SIRT1 reporter vector (WT-SIRT1), whereas there was no significant change in the luciferase activity in the mutant SIRT1 reporter vector (MUT-SIRT1) (Figure 7B). These data indicated that miR-34a-5p inhibits SIRT1 expression by directly binding to the 3UTR of SIRT1. In addition, the mRNA and protein expression levels of SIRT1 were significantly inhibited by overexpression of miR-34a-5p (Figure 7C-E). Conversely, downregulation of miR-34a-5p increased the expression level of SIRT1 (Figure 7F-H). Moreover, the expression of SIRT1 was successfully downregulated by siRNA (Figure 7I-K).

MiR-34a-5p enhances oxidative stress-induced senescence of NPMSCs

According to the qRT-PCR analysis, the expression of miR-34a-5p was increased by TBHP treatment, and it was suppressed and promoted by the miR-34a-5p inhibitor and miR-34a-5p mimic, respectively (Figures 8A and 9A). In contrast, the expression of SIRT1 was suppressed after TBHP treatment, and it was promoted and suppressed and by miR-34a-5p inhibitor and miR-34a-5p mimic, respectively (Figures 8 and 9). The TBHP-enhanced senescence-related proteins (p16, p21, and p53) and SASP (IL-1β, IL-6, and MMP-13) were significantly suppressed after miR-34a-5p knockdown (Figure 8). In contrast, the expression levels of senescence-related proteins and SASP were increased when cells were treated with the miR-34a-5p mimic (Figure 9).


A
Control
TBHP
Que+TBHP
SIRT1
DAPI
Merge
B
SIRT 1 fluorescence intensity (of control)
1.5
1.0
0.5
0.0
a
a
Control
TBHP
Que+TBHP
C
Control
TBHP
Que+TBHP
p16
DAPI
Merge
D
p16 fluorescence intensity (of control)
10
8
6
4
2
0
a
a
Control
TBHP
Que+TBHP
E
Control
TBHP
Que+TBHP
p21
DAPI
Merge
F
p21 fluorescence intensity (of control)
15
10
5
0
a
a
Control
TBHP
Que+TBHP

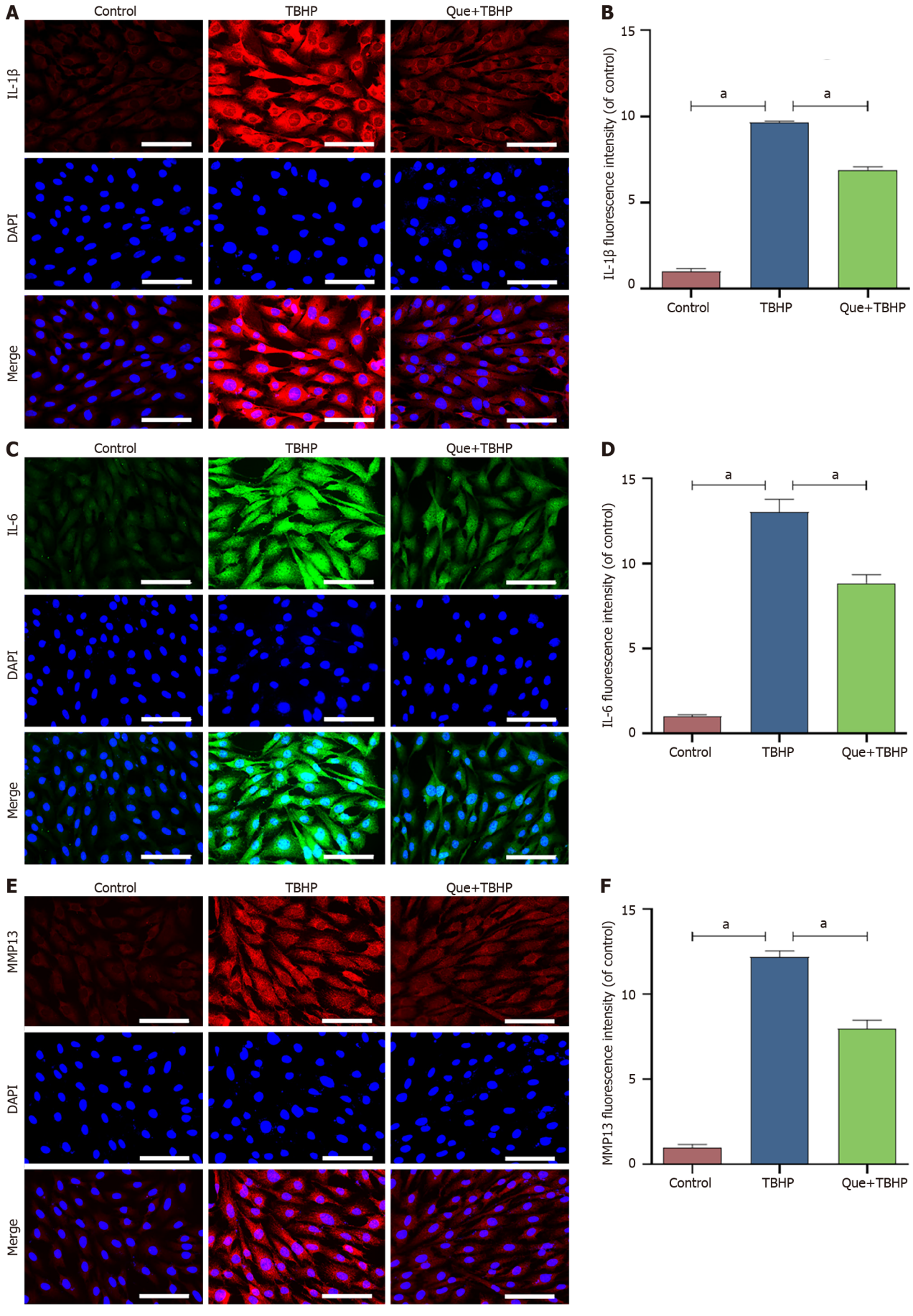


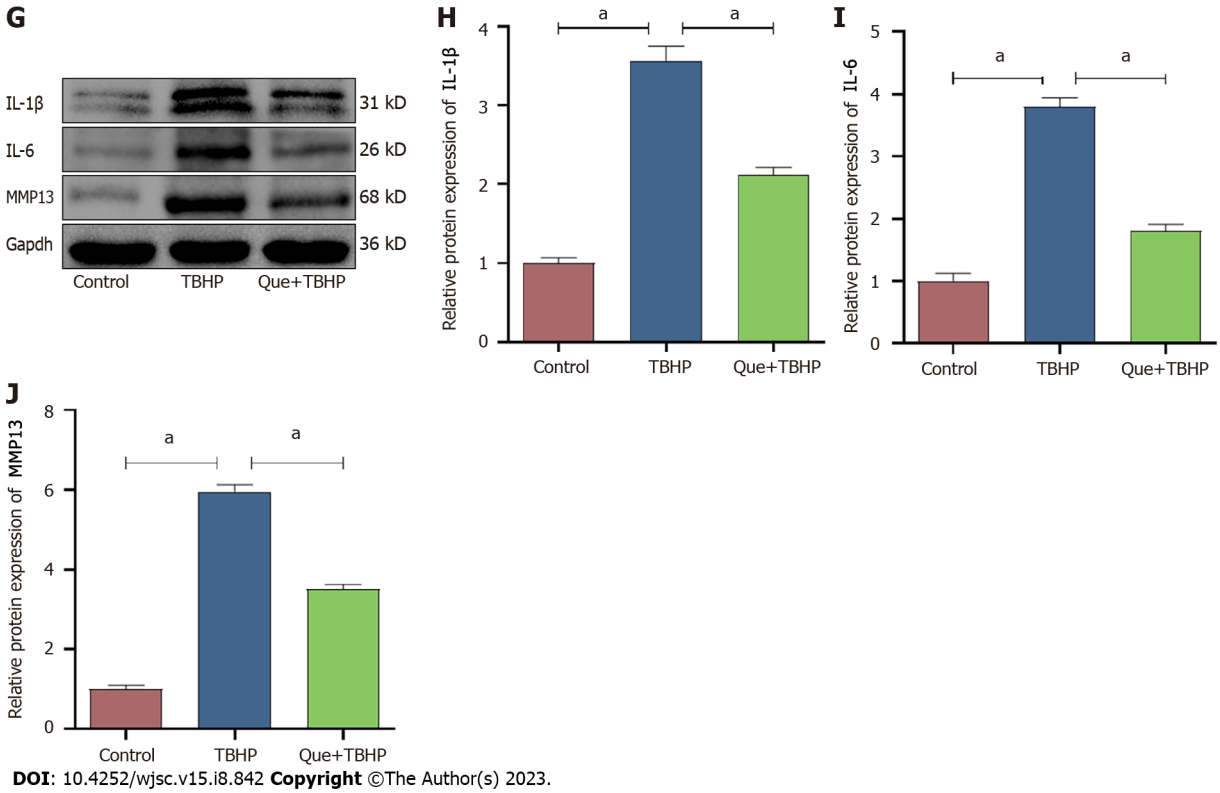
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Figure 5 Quercetin upregulates the expression of SIRT1 and downregulates the expression of p16 and p21 and p53. A and B: Immunofluorescence staining and quantitative analysis of SIRT1; C and D: Immunofluorescence staining and quantitative analysis of p16; E and F: Immunofluorescence staining and quantitative analysis of p21; G and H; Immunofluorescence staining and quantitative analysis of p53; I-M: The protein expressions and quantitative analysis of SIRT1, p16, p21 and p53 in the different groups. Scale bar = 100 μm. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

Que modulates TBHP-induced senescence of NPMSCs via the miR-34a-5p/SIRT1 pathway

To explore the relationship between miR-34a-5p/SIRT1 and oxidative stress-induced senescence in Que-treated NPMSCs, the miR-34a-5p mimic and siSIRT1 were used to investigate whether overexpression of miR-34a-5p and knockdown of SIRT1, respectively, counteracts the effects of Que on the expression of senescence-related proteins (p16, p21, and p53), SASP (IL-1β, IL-6, and MMP-13), and SIRT1. As shown in Figures 10 and 11, Que suppressed the expression of senescence-related proteins and SASP but promoted the expression of SIRT1, whereas overexpression of miR-34a-5p and knockdown of SIRT1 impaired this protective effect in TBHP-treated NPMSCs. These results suggested that Que may protect NPMSCs from TBHP-induced senescence *via* the miR-34a-5p/SIRT1 pathway.


A
Control
TBHP
Que+TBHP
IL-1β
DAPI
Merge
B
IL-1β fluorescence intensity (of control)
15
10
5
0
a
a
Control
TBHP
Que+TBHP
C
Control
TBHP
Que+TBHP
IL-6
DAPI
Merge
D
IL-6 fluorescence intensity (of control)
15
10
5
0
a
a
Control
TBHP
Que+TBHP
E
Control
TBHP
Que+TBHP
MMP13
DAPI
Merge
F
MMP13 fluorescence intensity (of control)
15
10
5
0
a
a
Control
TBHP
Que+TBHP



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Figure 6 Quercetin downregulates the expression of senescence associated secreted phenotype: IL-1β, IL-6 and MMP13. A and B: Immunofluorescence staining and quantitative analysis of IL-1β; C and D: Immunofluorescence staining and quantitative analysis of IL-6; E and F: Immunofluorescence staining and quantitative analysis of MMP-13; G-J: The protein expressions and quantitative analysis of IL-1β, IL-6 and MMP-13 in different groups. Scale bar = 100 μm. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

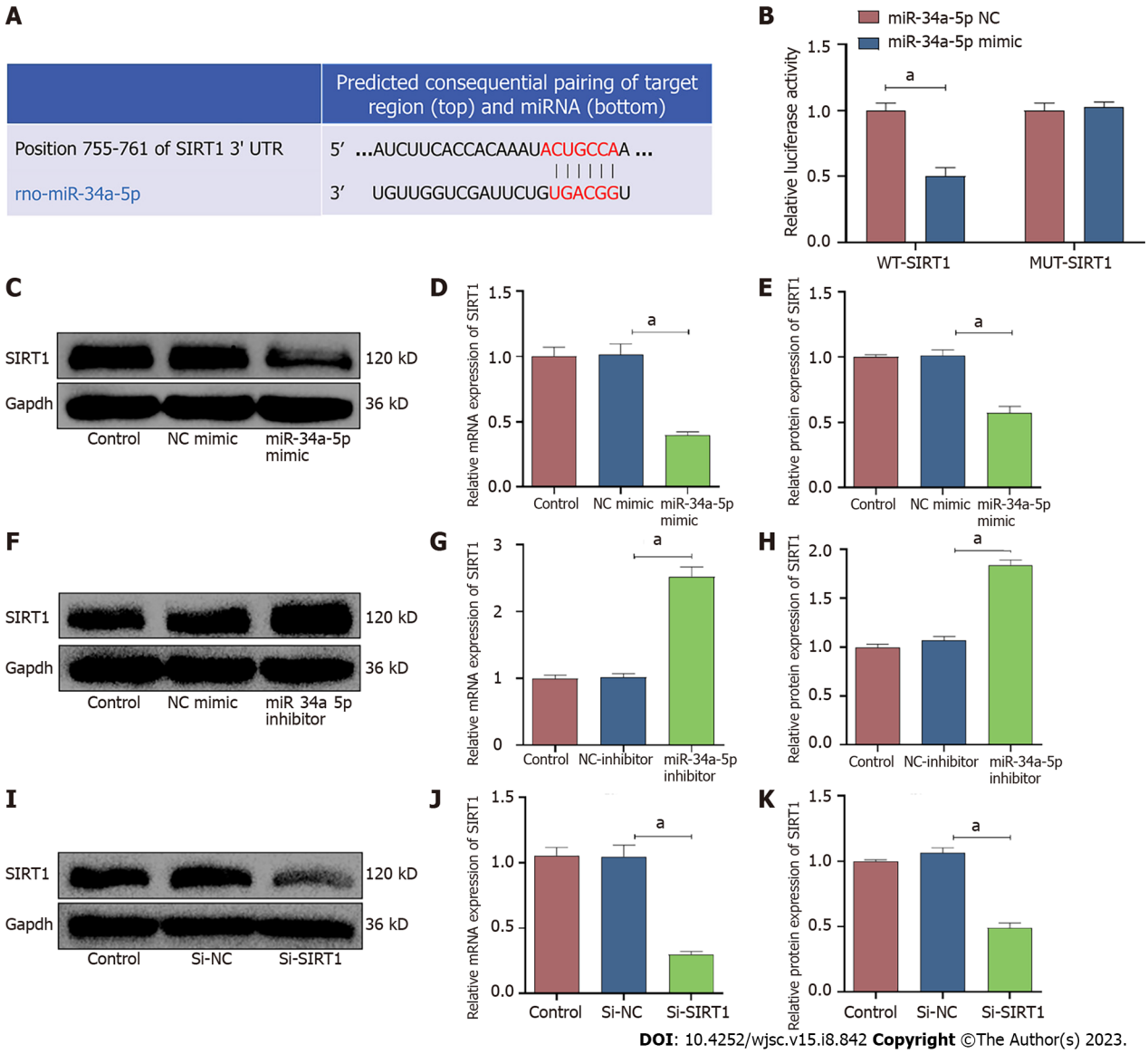
Que ameliorates disc degeneration in a rat model

A puncture-induced rat model was established to investigate the effect of Que in IDD *in vivo*, and disc height was measured by X-ray images at Week 0 and Week 4 after puncture (Figure 12A). Compared to the control group, the DHI of the IDD group was significantly decreased (Figure 12B-E). Interestingly, the DHI of the Que group was significantly higher than that of the IDD group ($P < 0.05$) (Figure 12B-E). According to the Hematoxylin & eosin staining, NP tissues occupied most of the area in the discs, and NP cells were well dispersed in the matrix. The annulus fibrosus (AF) was well organized in the remaining area. In the IDD group, the NP tissues almost disappeared, and the border between NP and AF was severely disrupted (Figure 12F). However, Que treatment alleviated the degeneration and morphological changes in the NP and AF (Figure 12F). The histological score was higher in the IDD group compared to the control group ($P < 0.05$) (Figure 12G). Both Safranin-O Fast Green and Alcian blue staining showed that the proteoglycan matrix area was lower in the IDD group compared to the control group (Figure 12F). However, Que treatment alleviated the proteoglycan decrease compared to the IDD group (Figure 12H). Immunofluorescence was also used to assess the expression of collagen II and aggrecan in the disc tissue. Compared to the control group, the expression of collagen type II and aggrecan was significantly lower in the IDD group, and Que treatment significantly attenuated the decrease in collagen type II and aggrecan compared to the IDD group (Figure 12I and J). Taken together, these results suggested that Que alleviates the IDD process in a puncture-induced rat model.

DISCUSSION

In the present study, the results demonstrated that Que ameliorates oxidative stress-induced senescence in NPMSCs *via* the miR-34a-5p/SIRT1 axis. The *in vivo* results also demonstrated that Que inhibits the expression of senescence-related proteins (p16, p21, and p53) and SASP (IL-1β, IL-6, and MMP-13) as well as promotes the expression of SIRT1, suggesting that it has a therapeutic effect in the IDD rat model.

Several previous studies have found that oxidative stress is highly associated with the progression of IDD[4,7]. Oxidative stress is triggered in the microenvironment of degenerative IVD when the balance between generation and elimination of ROS is broken[33]. ROS not only trigger oxidative damage of the extracellular matrix of IVD but also induce oxidative damage to DNA, proteins, and mitochondria[33]. Moreover, ROS promote the production of ROS in IVD cells, forming a positive feedback loop[5-7,34]. The discovery of NPMSCs provides a theoretical basis for the endogenous repair of IDD[9,11]. The endogenous repair ability of NPMSCs in degenerative IVD is achieved by differentiation into NP

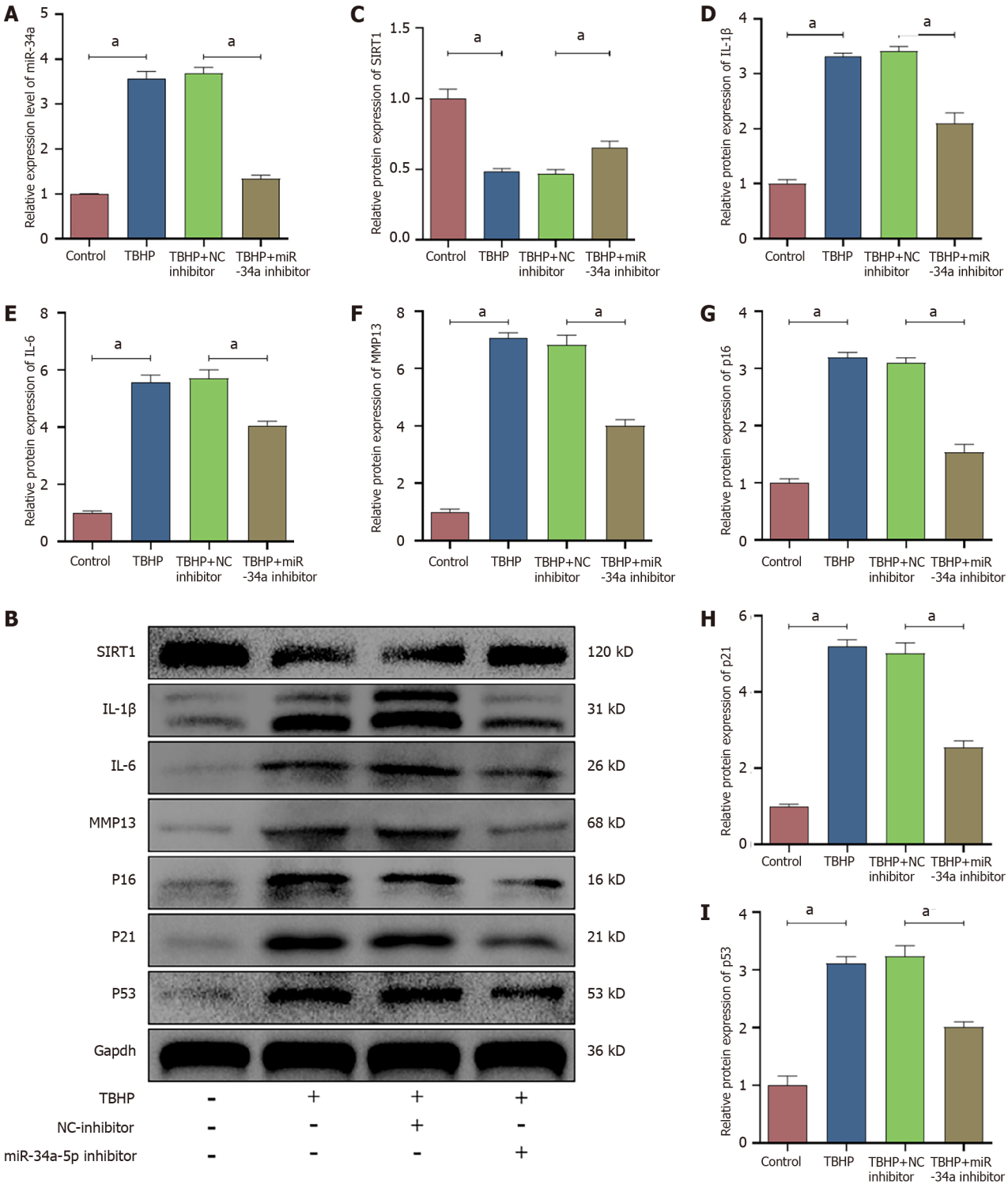


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Figure 7 miR-34a-5p represses SIRT1 expression by targeting the 3'-UTR of SIRT1. A: Schematic of the predicted miR-34a-5p binding sites in the 3'-UTR of SIRT1 mRNA from TargetScan online database; B: Luciferase reporter activity of SIRT1 co-transfected with miR-34a-5p nc or miR-34a-5p mimic; C-E: The protein and mRNA expression level of SIRT1 in nucleus pulposus-derived mesenchymal stem cells (NPMSCs) transfected with miR-34a-5p mimic; F-H: The protein and mRNA expression level of SIRT1 in NPMSCs transfected with miR-34a-5p inhibitor; I-K: The protein and mRNA expression level of SIRT1 in NPMSCs transfected with Si-SIRT1. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide.

cells and inhibiting cell apoptosis and/or senescence[10,35]. Oxidative stress in the microenvironment of IVD induces NPMSC senescence, and senescent NPMSCs decrease multipotency and self-renew ability, thus decreasing the regenerative potential and endogenous repair ability of NPMSCs[17,36-38]. In the present study, we found increased expression of senescence-related proteins (p16, p21, and p53), increased SASP (IL-1β, IL-6, and MMP-13), increased SA-β-Gal-positive senescent cells, decreased expression of SIRT1, decreased proliferation, and decreased viability after TBHP treatment in NPMSCs. Therefore, it is important to understand how to maintain the quantity and quality of NPMSCs under adverse microenvironments in degenerative IVD, such as oxidative stress, hypoxia, nutrient deficiency, and compression.

As a potent antioxidant, Que scavenges ROS, scavenges free radicals, inhibits lipid peroxidation, and inhibits xanthine oxidase activity[18-20,39]. Previous studies have reported that Que is an effective activator of SIRT1[40-42]. Feng *et al*[22] reported that Que attenuates oxidative stress-induced apoptosis of rat chondrocytes *via* the SIRT1/mitogen-activated protein kinase (AMPK) pathway and prevents the progression of osteoarthritis in a rat model[22]. Lou *et al*[43] confirmed that Que inhibits HepG2 cell apoptosis through the p53/miRNA-34a/SIRT1 pathway[43]. To evaluate the effect of Que in TBHP-induced NPMSCs, we pretreated cells with Que (20 μM) for 24 h before the addition of TBHP (100 μM) in the present study. Pretreatment with Que decreased ROS generation, decreased SA-β-Gal activity, and partially restored cell proliferation in oxidative stress induced-NPMSCs. Further, Que significantly reduced the expression of senescence-related proteins (p16, p21, and p53), reduced SASP (IL-1β, IL-6, and MMP-13), and increased the expression of SIRT1. Therefore, these results indicated that Que may have a protective effect against TBHP-induced oxidative stress in

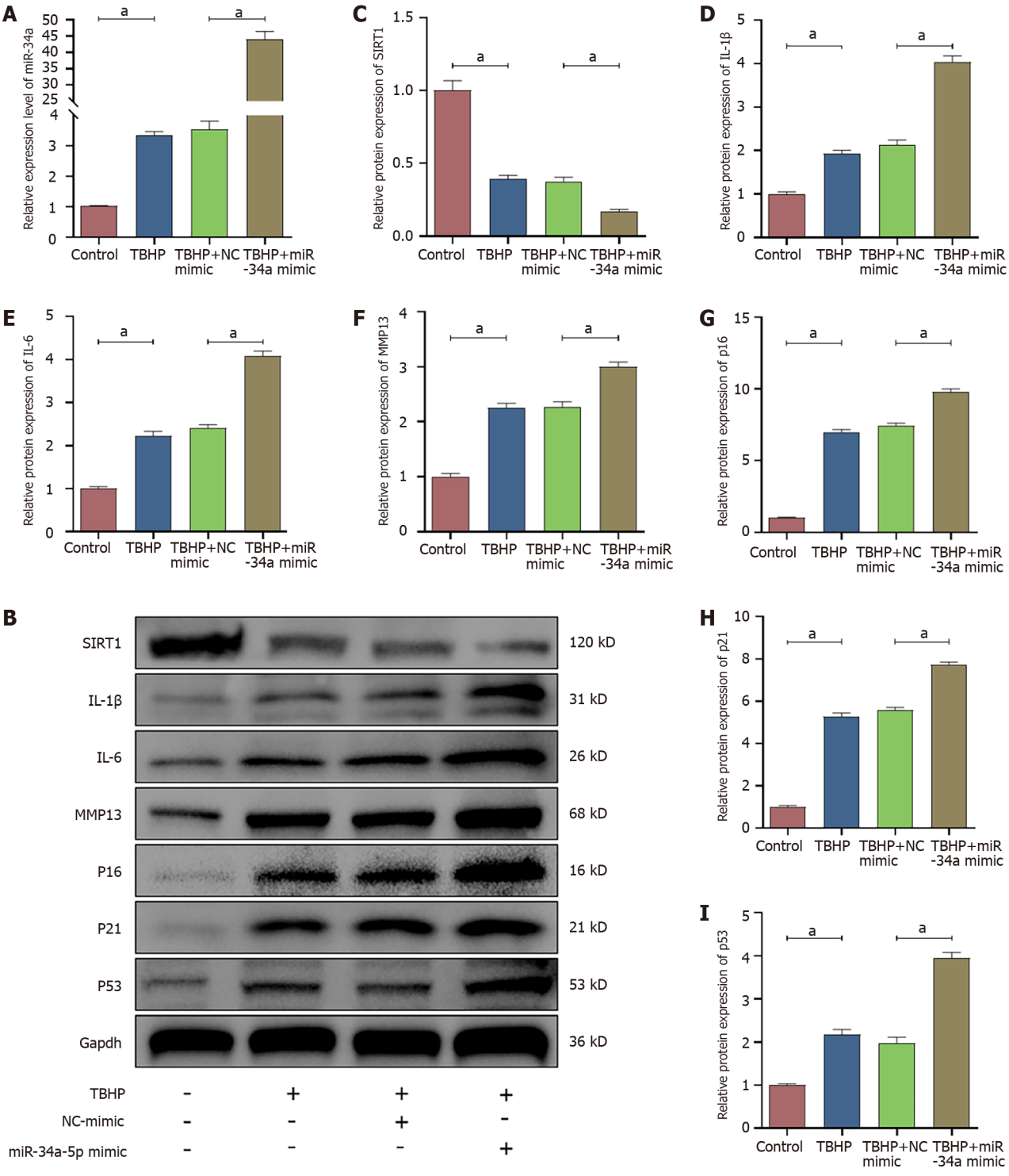


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Figure 8 Downregulation of miR-34a-5p alleviated oxidative stress-induced nucleus pulposus-derived mesenchymal stem cells senescence. A: The RNA levels of miR-34a-5p in nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were transfected with miR-34a-5p inhibitor and treated with tert-butyl hydroperoxide; B: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in NPMSCs transfected with miR-34a-5p inhibitor; C-I: Quantitative analysis of protein expression in the different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide.

NPMSCs.

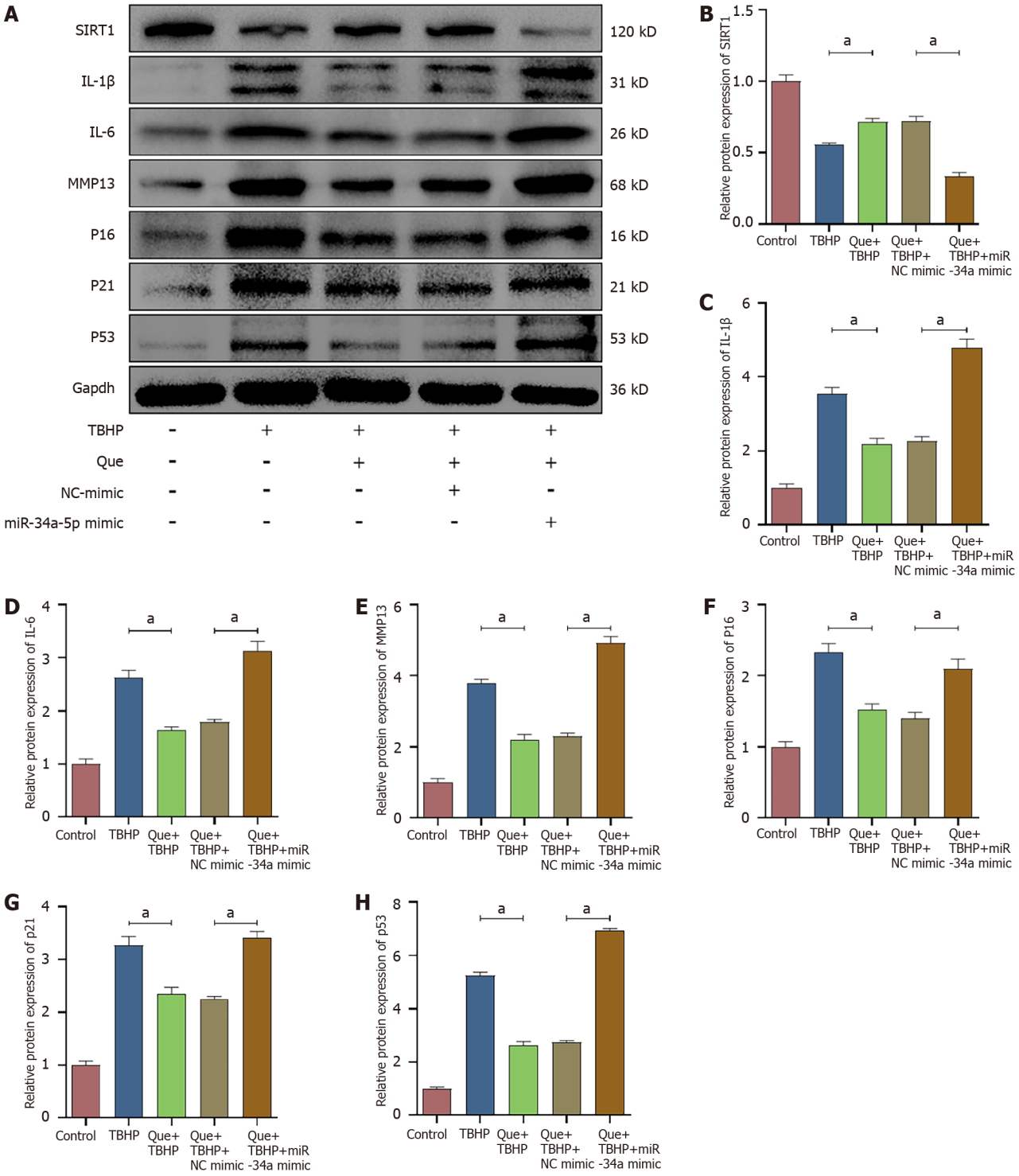
A previous study has also demonstrated that the expression of miR-34a-5p is significantly upregulated in degenerative IVD and that miR-34a-5p increases extracellular matrix degradation and cell apoptosis. Chen *et al*[44] reported that miR-34a increases apoptosis in degenerated cartilage endplate (CEP) chondrocytes. In addition, inhibition of miR-34a reduces cell apoptosis, while upregulation of miR-34a has an opposite effect in CEP cells[44]. In the present study, TBHP significantly upregulated miR-34a-5p expression. Overexpression miR-34a aggravated the oxidative stress damage



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Figure 9 Overexpression of miR-34a-5p enhanced oxidative stress-induced nucleus pulposus-derived mesenchymal stem cells senescence. A: The RNA levels of miR-34a-5p in nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were transfected with miR-34a-5p mimic and treated with TBHP; B: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in NPMSCs transfected with miR-34a-5p mimic; C-I: Quantitative analysis of protein expression in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide.

induced by TBHP in NPMSCs, including increased the expression of senescence-related proteins and SASP as well as and decreased the expression of SIRT1, whereas knockdown of miR-34a had an opposite effect in NPMSCs. Accumulating studies have demonstrated that SIRT1 exerts important function in the progression of IDD. The expression level of SIRT1 in IVD cells decreases with the progression of IDD[45], and SIRT1 expression is negatively correlated with Pfirrmann grade in IDD[46]. It has been reported that SIRT1 decreases the oxidative stress-induced senescence in CEP cells through the p53/p21 pathway[47]. H_2O_2-induced oxidative stress reduces the expression of SIRT1 as the expression of SIRT1 is downregulated with the increasing concentration of H_2O_2, and SIRT1 activation alleviates oxidative stress-induced

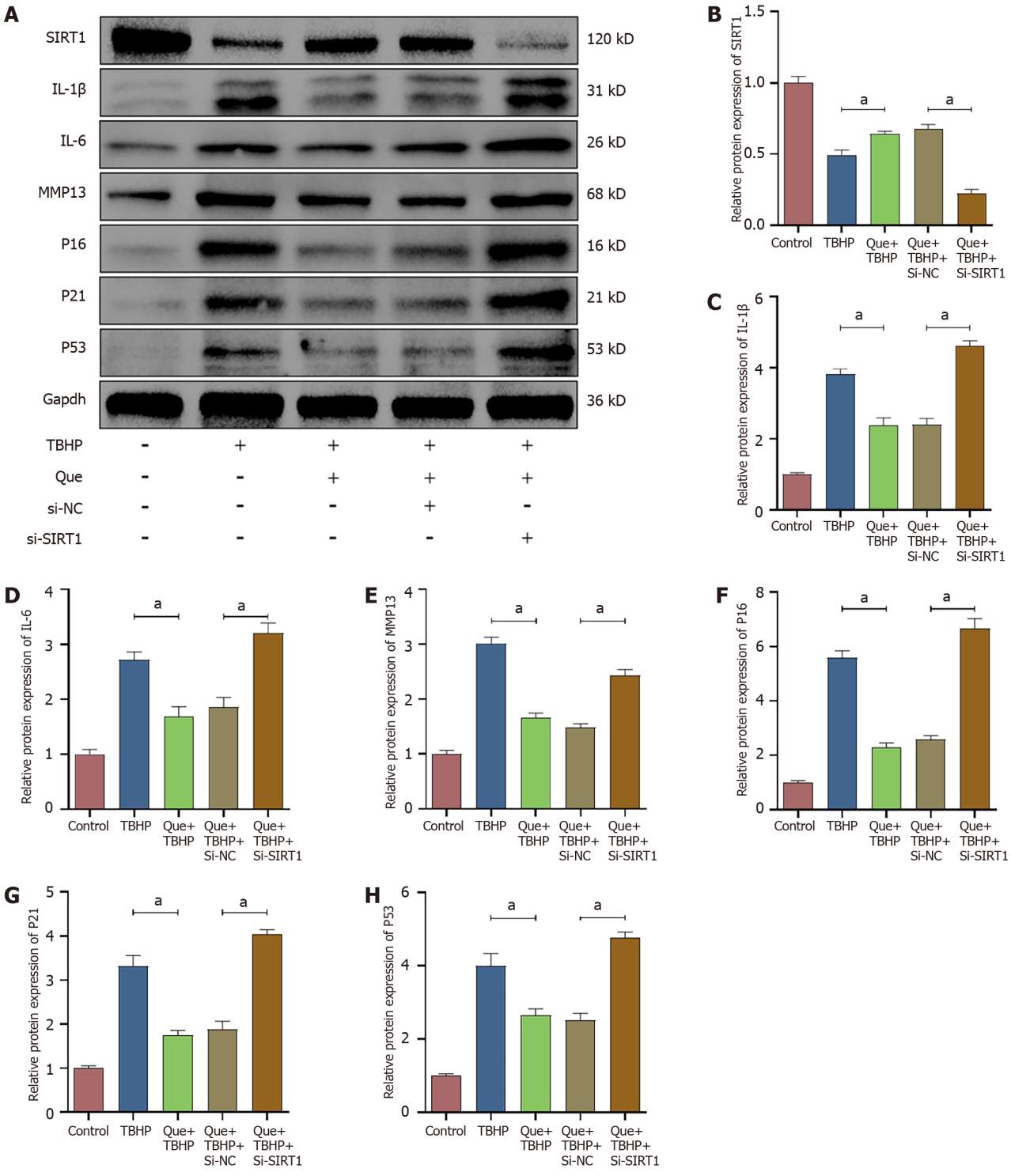


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Figure 10 Quercetin alleviates TBHP-induced senescence *via* miR-34a-5p/SIRT1 axis. A: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in different groups; B-H: Quantitative analysis of protein expression in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as $^aP < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

senescence in NP cells[29,48]. Xia *et al*[49] found that the expression of p16 is downregulated by activating SIRT1 in rat IDD model[49]. The present study found that SIRT1 expression was downregulated in TBHP induced-NPMSCs, which was rescued by Que and inhibiting miR-34a-5p. These results suggested that SIRT1 plays a protective role in TBHP induced-NPMSCs. miR-34a-5p overexpression and SIRT1 downregulation in NPMSCs were utilized to investigate whether Que reduces oxidative stress damage *via* the miR-34a/SIRT1 pathway to delay NPMSC senescence. The results demonstrated that the protective effect of Que is reversed by miR-34a-5p overexpression and SIRT1 knockdown.

In the present study, Que was also administered to IDD animal models to evaluate the protective effect of Que *in vivo*. X-ray analysis indicated the loss of DHI in the IDD model, but Que treatment ameliorated the decreased DHI. Moreover, histological analysis using HE, Alcian blue, and S-O staining as well as immunofluorescence analysis of collagen type II

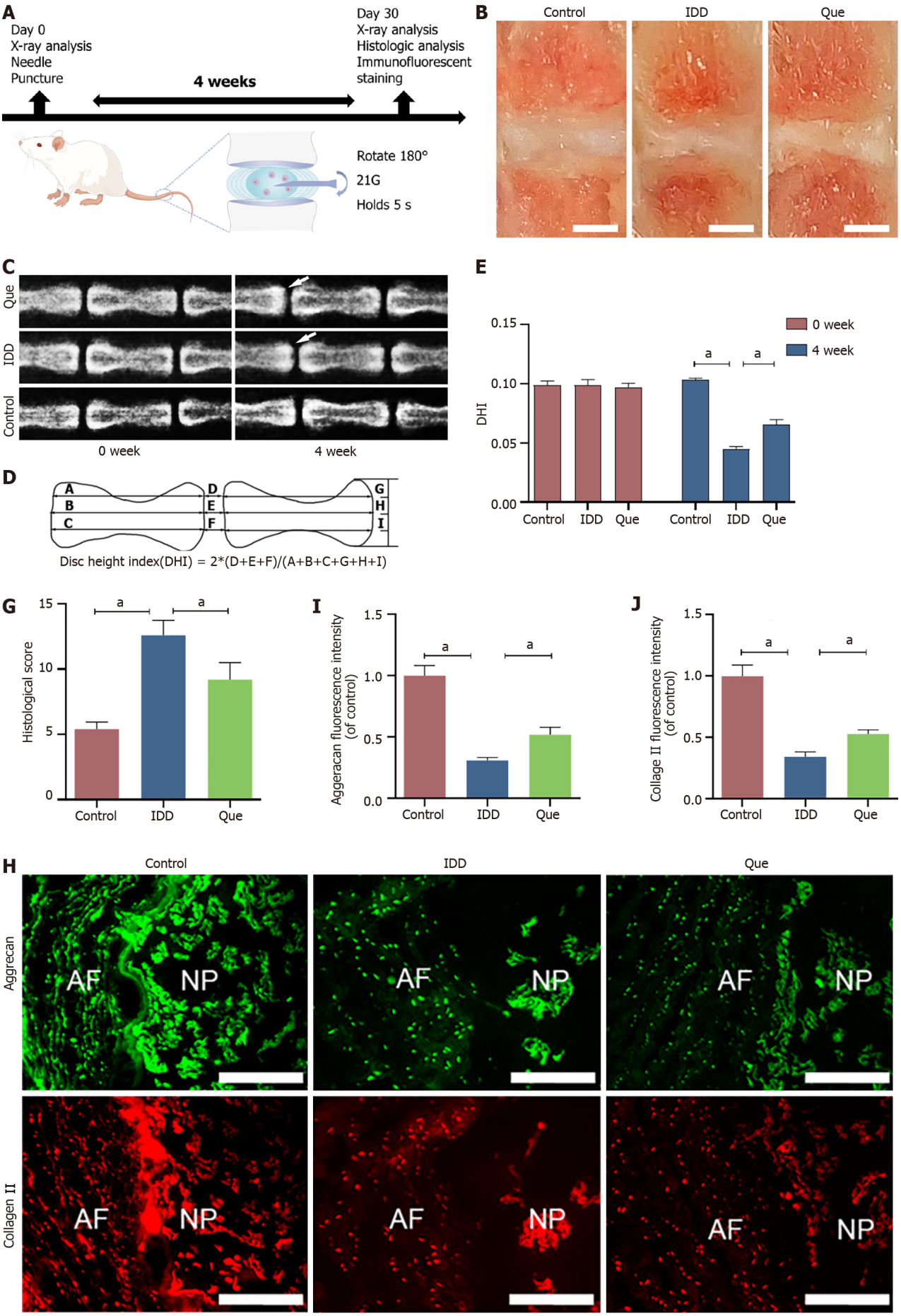


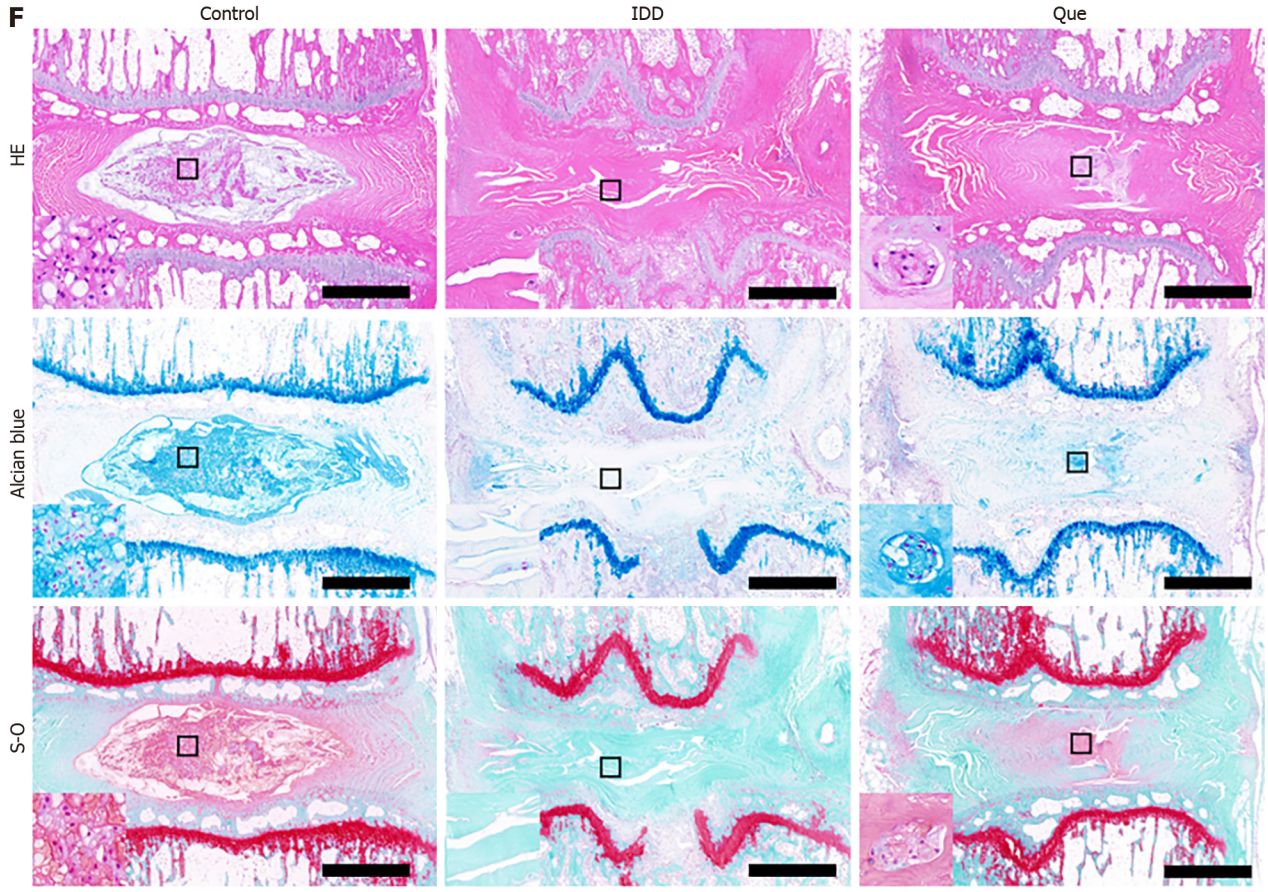
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Figure 11 Quercetin alleviates TBHP-induced senescence *via* miR-34a-5p/SIRT1 axis. A: The protein levels of SIRT1, p16, p21, p53, IL-1β, IL-6 and MMP-13 in different groups; B-H: Quantitative analysis of protein expression in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

and aggrecan confirmed that Que had a positive effect on delaying the degree of IDD.

The present study had several limitations. Previous studies have shown Que has pleiotropic properties. In addition to the activation of SIRT1, Que has anti-inflammatory and antioxidative effects *via* activating nuclear factor erythroid 2-related factor 2 (NRF2)[39], mitogen-activated protein kinase (MAPK)[39], and phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT)[50]. Hence, it remains unknown whether Que plays a role in NPMSC senescence induced by oxidative stress *via* other signaling pathways, suggesting that further study is needed to explore the other mechanisms underlying the protective effect of Que on NPMSCs. Moreover, future clinical studies are needed to evaluate the effect of Que on IDD progress.


A
Day 0
X-ray analysis
Needle
Puncture
4 weeks
Day 30
X-ray analysis
Histologic analysis
Immunofluorescent staining
Rotate 180°
21G
Holds 5 s
B
Control
IDD
Que
C
Que
IDD
Control
0 week
4 week
D
A
B
C
D
E
F
G
H
I
Disc height index(DHI) = 2*(D+E+F)/(A+B+C+G+H+I)
E
DHI
0.15
0.10
0.05
0.00
0 week
4 week
a
a
Control
IDD
Que
Control
IDD
Que
G
Histological score
15
10
5
0
a
a
Control
IDD
Que
I
Aggrecan fluorescence intensity (of control)
1.5
1.0
0.5
0.0
a
a
Control
IDD
Que
J
Collage II fluorescence intensity (of control)
1.5
1.0
0.5
0.0
a
a
Control
IDD
Que
H
Control
IDD
Que
Aggrecan
Collagen II
AF
NP



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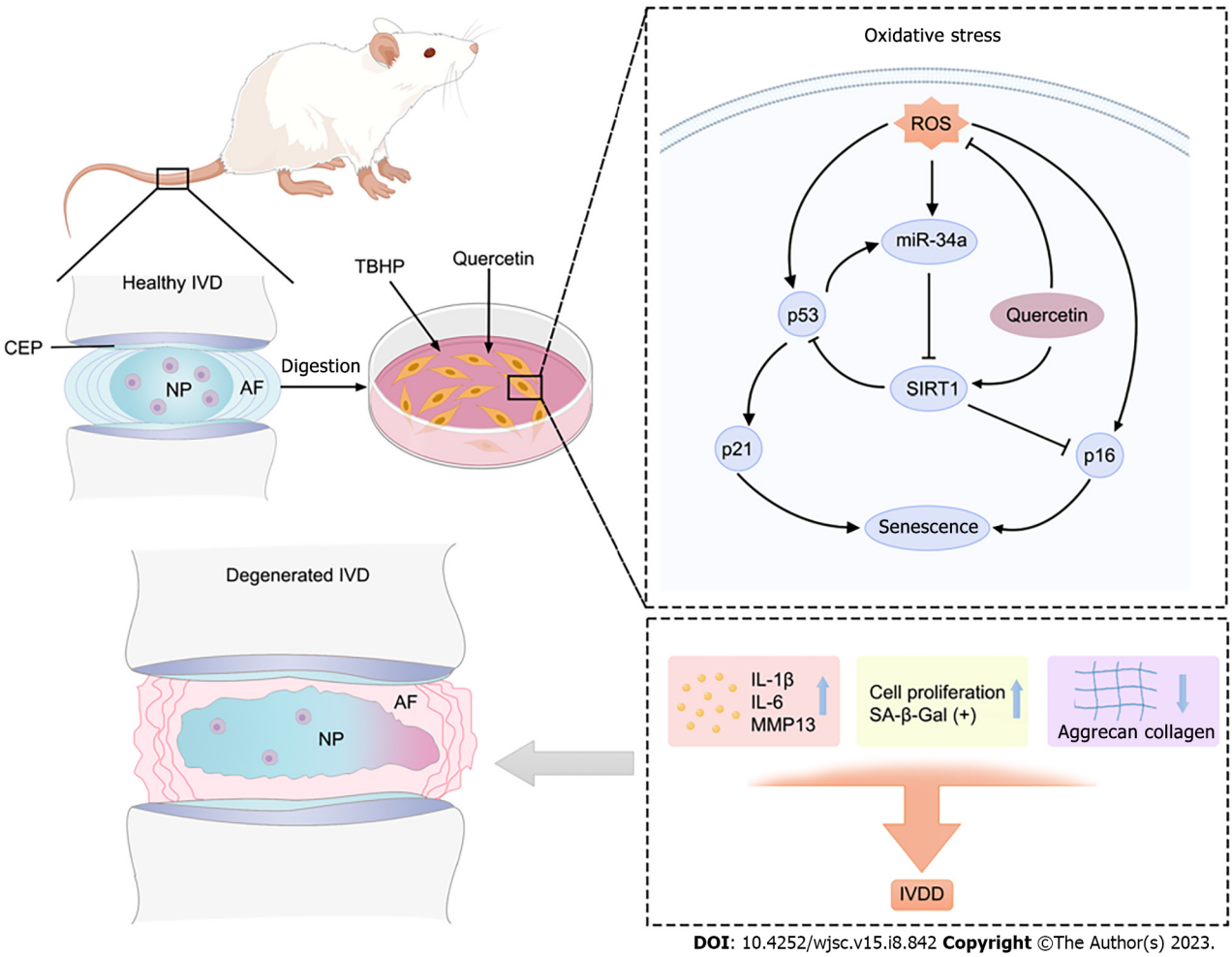
Figure 12 Quercetin ameliorates IVDD *in vivo*. A: Schematic of the basic process of *in vivo* experiment (By Figdraw); B: Macroscopic appearances of rat tail intact disc mid-sagittal sections in control group, intervertebral disc degeneration group and quercetin group (Scar bar = 2 mm); C: The X-ray in different groups at 0 wk and 4 wk after puncturing; D: Measurements of intervertebral disc height index (DHI); E: Quantitative analysis of DHI; F-G: Hematoxylin-eosin staining and quantitative analysis of histological score at 4 wk after puncture in different groups (scale bar = 1mm); H: The expression of aggrecan and collagen type II in different groups (scale bar = 400 μm); I-J: Quantitative analysis of aggrecan and collagen type II in different groups. Data are represented as mean ± SD. Significant differences between groups are indicated as $^{a}P < 0.05$, $n = 3$. HE: Hematoxylin-eosin; NP: Nucleus pulposus; AF: Annulus fibrosus; IDD: Intervertebral disc degeneration; TBHP: Tert-butyl hydroperoxide; Que: Quercetin.

CONCLUSION

In summary, the present study demonstrated that Que prevents oxidative stress-induced senescence of NPMSCs *via* the miR-34a/SIRT1 signaling pathway (Figure 13). Moreover, Que ameliorates the progression of IDD in a rat model. These findings suggested that Que may be a potential agent for the treatment of IDD.

Table 1 Sequences of primers used for real-time polymerase chain reaction

Gapdh	F: CTGGAGAAACCTGCCAAGTATG
	R: GGTGGAAGAATGGGAGTTGCT
SIRT1	F: TGACCTCCTCATTGTTATTGGG
	R: GGCATACTCGCCACCTAACCT
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
miR-34a-5p	F: ACACTCCAGCTGGGTGGCAGTGTCTTAGCT
	R: TGGTGTCGTGGAGTCG



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Figure 13 Schematic representation of the mode of action of quercetin. Quercetin ameliorates oxidative stress-induced senescence in nucleus pulposus-derived mesenchymal stem cells *via* miR-34a-5p/SIRT1 axis. HE: Hematoxylin-eosin; NP: Nucleus pulposus; AF: Annulus fibrosus; IVD: Intervertebral disc; TBHP: Tert-butyl hydroperoxide; ROS: Reactive oxygen species assay. By Figdraw.

ARTICLE HIGHLIGHTS

Research background

Intervertebral disc degeneration (IDD) is a main contributor to low back pain. Oxidative stress, which is highly associated with the progression of IDD, increases senescence of nucleus pulposus-derived mesenchymal stem cells (NPMSCs) and weakens the differentiation ability of NPMSCs in degenerated intervertebral discs (IVDs). Quercetin (Que) has been demonstrated to reduce oxidative stress in diverse degenerative diseases.

Research motivation

An adverse microenvironments of degenerative intervertebral disc such as oxidative stress, low nutrition, and inflammation leads to increased senescence NPMSCs, which severely affects endogenous repair. Therefore, inhibition senescence of NPMSCs may be of great significance in alleviating IDD.

Research objectives

The present study aimed to investigate the role of Que in oxidative stress-induced NPMSC damage and to elucidate the underlying mechanism.

Research methods

In vitro, NPMSCs were isolated from rat tails. Senescence-associated β-galactosidase (SA-β-Gal) staining, cell cycle, reactive oxygen species (ROS), real-time quantitative polymerase chain reaction (RT-qPCR), immunofluorescence, and western blot analyses were used to evaluate the protective effects of Que. Meanwhile the relationship between miR-34a-5p and Sirtuins 1(SIRT1) was evaluated by dual-luciferase reporter assay. To explore whether Que modulates tert-butyl hydroperoxide (TBHP)-induced senescence of NPMSCs *via* the miR-34a-5p/SIRT1 pathway, we used adenovirus vectors to overexpress and downregulate the expression of miR-34a-5p, and used SIRT1 siRNA to knockdown SIRT1 expression. *In vivo*, a puncture-induced rat IDD model was constructed, and X rays and histological analysis were used to assess

whether Que could alleviate IDD *in vivo*.

Research results

We found that TBHP can cause NPMSCs senescence changes, such as reduced cell proliferation ability, increased SA-β-Gal activity, cell cycle arrest, the accumulation of ROS, and increased expression of senescence-related proteins. While abovementioned senescence indicators were significantly alleviated by Que treatment. Que decreased the expression levels of senescence-related proteins (p16, p21, and p53) and senescence-associated secreted phenotype (SASP), including IL-1β, IL-6, and MMP-13, and it increased the expression of SIRT1. In addition, the protective effects of Que on cell senescence were partially reversed by miR-34a-5p overexpression and SIRT1 knockdown. *In vivo*, X-ray, and histological analyses indicated that Que alleviated IDD in a puncture-induced rat model.

Research conclusions

In summary, the present study provides evidence that Que reduces oxidative stress-induced senescence of NPMSCs *via* the miR-34a/SIRT1 signaling pathway, suggesting that Que may be a potential agent for the treatment of IDD.

Research perspectives

We demonstrated Que ameliorates oxidative stress-induced senescence of NPMSCs and delays the progression of IDD. Que may be a potential agent for the treatment of IDD.

ACKNOWLEDGEMENTS

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FOOTNOTES

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META-ANALYSIS

Up-to-date meta-analysis of long-term evaluations of mesenchymal stem cell therapy for complex perianal fistula

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Abstract

BACKGROUND

Local mesenchymal stem cell (MSC) therapy for complex perianal fistulas (PFs) has shown considerable promise. But, the long-term safety and efficacy of MSC therapy in complex PFs remain unknown.

AIM

To explore the long-term effectiveness and safety of local MSC therapy for complex PFs.

METHODS

Sources included the PubMed, EMBASE, and Cochrane Library databases. A standard meta-analysis was performed using RevMan 5.3.

RESULTS

After screening, 6 studies met the inclusion criteria. MSC therapy was associated with an improved long-term healing rate (HR) compared with the control condition [odds ratio (OR) = 2.13; 95% confidence interval (95%CI): 1.34 to 3.38; P = 0.001]. Compared with fibrin glue (FG) therapy alone, MSC plus FG therapy was associated with an improved long-term HR (OR = 2.30; 95%CI: 1.21 to 4.36; P = 0.01). When magnetic resonance imaging was used to evaluate fistula healing, MSC therapy was found to achieve a higher long-term HR than the control treatment (OR = 2.79; 95%CI: 1.37 to 5.67; P = 0.005). There were no significant differences in long-term safety (OR = 0.77; 95%CI: 0.27 to 2.24; P = 0.64).

CONCLUSION

Our study indicated that local MSC therapy promotes long-term and sustained healing of complex PFs and that this method is safe.

Key Words: Complex perianal fistula; Mesenchymal stem cells; Long-term evaluation; Meta-analysis

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Core Tip: The long-term safety and efficacy of mesenchymal stem cell (MSC) therapy for complex perianal fistulas (PFs) remain unknown. So, we explored the long-term effectiveness and safety of local MSC therapy for complex PFs. We found that MSC treatment is a safe and effective method that can significantly improve the long-term healing of complex PFs, and this method confers no risk of MSC-related adverse events.

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INTRODUCTION

A perianal fistula (PF) is an epithelialized tract that connects the luminal surface of the anal canal or rectum with the perianal or perineal skin. It affects approximately 2 in 10000 people and represents a difficult therapeutic challenge and a source of physical and psychological morbidity with a long-term risk of proctectomy[1]. The most severe form is complex PF, which is difficult to manage, as it has a high rate of recurrence and may cause sphincter damage and fecal incontinence. Complex PF is defined as cases with more muscle involvement or anterior fistulas in female patients, as well as recurrent fistulas, suprasphincteric fistulas, extrasphincteric fistulas, horseshoe fistulas, fistulas associated with irritable bowel disease, transsphincteric fistulas that involve greater than 30% of the external sphincter and fistulas associated with preexisting fecal incontinence, inflammatory bowel disease, radiation, malignancy, or chronic diarrhea. PFs are also a probable consequence of Crohn's disease (CD) since as many as 26% of CD patients eventually develop a PF within 20 years after diagnosis[2,3]. Complex PFs in patients with CD have a high recurrence rate and cause a vast range of complications that significantly reduce quality of life (QoL)[4]. In addition, there is a risk of developing a neoplasm in the PF area related to the complexity and perianal disease duration[5]. The results of one study showed that complex PFs may lead to anal cancer in approximately 28% of patients within 20 years after diagnosis[6]. Complex PF is a chronic, recurrent immune-mediated disease with a variety of treatment options. Its exact pathogenesis is unknown. The key long-term therapeutic goals for the treatment of complex PF are to: (1) Resolve fistula discharge; (2) achieve fistula healing; (3) prevent fistula recurrence; (4) maintain fecal continence; (5) avoid long-term diversion (protectomy with stoma), and hence; and (6) improve and maintain QoL for patients. Although current treatments for PFs include a range of medical and surgical options, managing this condition is difficult. Patients with complex PFs tend to have poor treatment outcomes or experience frequent relapses, and most interventions are ineffective in providing long-term healing[7-10]. Immunomodulators can have serious side effects. Additionally, there is a risk of opportunistic infection associated with the use of biological treatments. In more severe cases of complex PF, fecal incontinence can occur, furthering morbidity. Complex PF is often not permanently cured by surgery, leading to multiple procedures and complications such as fecal incontinence. Therefore, there is a need for an effective therapy that provides long-term healing of complex PFs without the risk of fecal incontinence.

In recent years, local injection of mesenchymal stem cells (MSCs) has shown notable promising results in the treatment of PFs[11]. MSCs are a heterogeneous subset of stromal stem cells. They can be isolated from a wide variety of tissues and expanded *in vitro* to obtain large quantities. MSCs are characterized by multilineage differentiation and powerful immunomodulatory effects and are able to mitigate inflammatory states. Complex PF is thought to arise from an epithelial defect, which may be caused by ongoing inflammation. Current treatments cannot maintain long-term healing of the disease. Possible alternative treatments include cell therapy, especially MSC therapy. Local administration is the most performed approach to deliver MSCs. After being delivered directly to fistula tracts, MSCs induce peripheral tolerance and migrate to injured tissues, where they can inhibit the release of proinflammatory cytokines and promote the survival of damaged cells. Consequently, MSCs are capable of repairing damaged tissues and promoting tissue healing, which can lead to long-term fistula healing, significantly improving patients' quality of life. An increasing number of studies have indicated that local MSC therapy is safe and efficacious for complex PFs. In 2020, to evaluate whether local MSC therapy for complex PFs is effective and safe, we conducted a meta-analysis. That study, with a follow-up of 8 wk to 2 years, showed that local MSC therapy for complex PFs was safe and feasible. However, the efficacy and safety evaluation period of the study was short and middle term[12]. Therefore, the long-term efficacy of MSC therapy is unclear. To date, an increasing number of studies have aimed to perform long-term evaluations of MSC therapy for PFs. Thus, based on a previous study[12], this study aimed to explore the long-term effectiveness and safety of MSC therapy for complex PFs (48 wk to 4 years of follow-up after MSC therapy).

MATERIALS AND METHODS

Data source and search strategies

This meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[13]. In August 2022, a search was performed in the PubMed, EMBASE and Cochrane Library databases for clinical studies written in English regarding local MSC treatment in patients with complex PFs. The following search terms were used: "Mesenchymal stem cell'', "mesenchymal stromal cell", "complex perianal fistula", "perianal fistula", "complex perianal Crohn's disease", "Crohn's perianal fistula", "perianal Crohn's disease", "Crohn's disease", "perianal fistulizing Crohn's disease", "cryptoglandular perianal fistula", and "long-term". We also checked the reference lists of the screened full-text studies to identify other potentially eligible trials.

Study selection

Two authors independently assessed studies for inclusion by screening titles and abstracts. The inclusion criteria were: (1) Studies of human subjects; (2) randomized clinical trials (RCTs) and retrospective studies or cohort studies of patients with complex PFs treated with local injection(s) of autologous or allogeneic MSCs from any source; (3) local injection of MSCs or MSCs combined with fibrin glue (FG) for complex PFs; and (4) assessment of the efficacy and/or safety of MSC therapy at least 48 wk after treatment.

Data extraction and quality assessment

A customized data extraction form was used by two authors to extract data. These authors extracted study data features including: (1) Study characteristics (first author, publication year); (2) MSC origin; (3) definition of fistula healing [clinical and/or magnetic resonance imaging (MRI)]; (4) study outcomes; (5) dosage and modalities of intervention administration; (6) adverse events (AEs); and (7) concomitant treatment (anti-TNF). Table 1 lists the basic characteristics of the 6 identified studies. Quality assessment was performed using Review Manager (version 5.3) according to the recommendations from the Cochrane Collaboration. The bias risk assessment tool recommended by Cochrane was used to assess the quality of all enrolled studies. Each item of studies was judged as having a high, low or unclear risk of bias.

Statistical analysis

Based on the included studies, odds ratios (ORs) and corresponding 95% confidence intervals (95%CIs) were calculated to compare the MSC groups and control groups. Heterogeneity was quantified using the I^2 statistic. If the I^2 value was ≤ 50%, heterogeneity was considered low, and we employed a fixed-effect model. If the I^2 value was > 50%, heterogeneity was considered high, and we employed a random-effect model. All statistical analyses were performed using Review Manager (version 5.3). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Literature search and quality assessment

The literature search process is illustrated in Figure 1. Using this search strategy, we identified 462 references related to local MSC treatment for complex PF, of which 358 duplicate articles were removed. After reading the titles and abstracts, we identified 20 articles for full-text review. Ultimately, after all criteria were applied, 6 articles were included in the meta-analysis[14-19]. A summary of the risk of bias in the included articles is presented in Figure 2.

Long-term efficacy of MSCs in complex PF (48 wk to 4 years of follow-up after MSC therapy)

Our meta-analysis included 6 studies that assessed the long-term efficacy of post-MSC treatment. The pooled analysis showed that MSC therapy was associated with an improved long-term healing rate (HR) compared with the control condition (OR = 2.13; 95%CI: 1.34 to 3.38; $P = 0.001$) (Figure 3). The benefit was sustained for at least 48 wk of follow-up after MSC therapy.

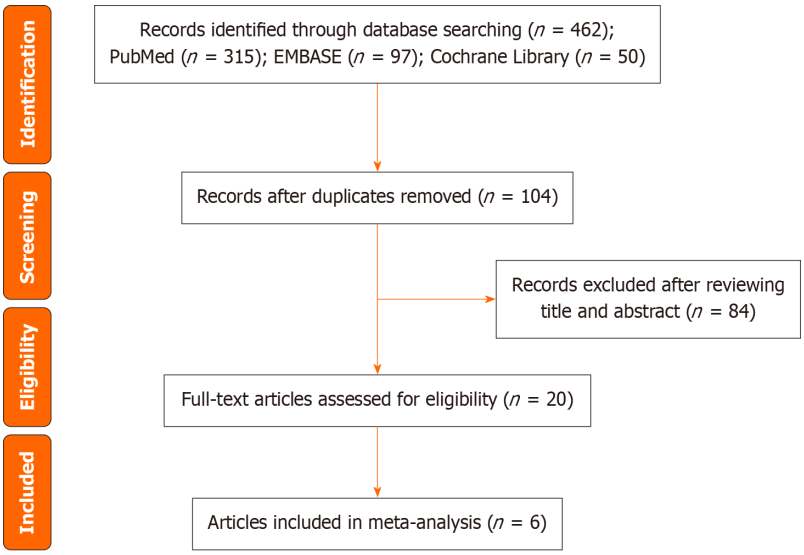
Long-term efficacy of MSCs for complex PF (MSCs + FG vs FG alone)

Cell therapy strategies using MSCs carried in FG have shown promising results in regenerative medicine. FG is a natural polymer involved in the coagulation process. In regenerative medicine, FG can be used as a delivery system for drugs, biomolecules, growth factors and cells. FG also provides a temporary structure that favors angiogenesis, extracellular matrix deposition and cell-matrix interactions and it also FG maintains the local and paracrine functions of MSCs, providing tissue regeneration through less invasive clinical procedures. The biological properties of FG as a growth environment for MSCs have been reported in several studies[20]. Now, local FG combined with MSCs therapy is still a relatively new treatment and has not yet gained popularity. So, the need for the local FG combined with MSCs therapy remains unknown. In our meta-analysis, three studies were included that reported local FG combined with MSCs therapy for PF[14,15,17], with low heterogeneity between the studies ($I^2 = 0\%$). In a fixed-effects model, MSCs plus FG had more long-term efficacy for fistula healing than FG alone (OR = 2.30; 95%CI: 1.21 to 4.36; $P = 0.01$) (Figure 4). So, we think local FG combined with MSCs therapy have synergistic effect on PF.

Table 1 Studies of local mesenchymal stem cells in complex perianal fistulas

Ref.	Cell type and source	Outcome assessment	Study outcomes	Intervention (mean)	AEs	Concurrent use of anti-TNF
Guadalajara *et al* [14], 2012	Autologous, ASCs	Re-epithelialization + MRI	10/18 for MSCs + FG; 3/12 for FG at 1 yr	First: 2 × 10^7 MSCs; second: 4 × 10^7 MSCs	/	Yes
Herreros *et al* [15], 2012	Autologous, ASCs	Re-epithelialization +MRI	24/42 for MSCs; 22/42 for MSCs + FG; 19/51 for FG at 48 wk	First: 2 × 10^7 MSCs; second: 4 × 10^7 MSCs	/	Yes
Garcia-Arranz *et al*[16], 2020	Autologous, ASCs	Re-epithelialization	10/20 for MSCs + FG; 5/19 for FG at 2 yr	First: 10 × 10^7 MSCs; second: 10 × 10^7 MSCs	7/23 for MSCs + FG *vs* 9/21 for FG	Yes
Barnhoorn *et al* [17], 2020	Allogeneic, BMSCs	MRI	8/13 for MSCs; 0/3 for placebo group at 4 yr	A: 1 × 10^7 MSCs; B: 3 × 10^7 MSCs; C: 9 × 10^7 MSCs	/	/
Panés *et al*[18], 2022	Allogeneic, ASCs	Re-epithelialization	23/43 for MSC; 21/46 for saline solution at 156 wk	12 × 10^7 MSCs	/	Yes
Garcia-Olmo *et al* [19], 2022	Allogeneic, ASCs	Re-epithelialization	14/25 for MSCs; 6/15 for saline solution at 104 wk	12 × 10^7 MSCs	3/25 for MSCs *vs* 1/15 for placebo	Yes

AEs: Adverse events; MSCs: Mesenchymal stem cells; ASCs: Adipose-derived mesenchymal stromal cells; BMSCs: Bone marrow-derived mesenchymal stromal cells; FG: Fibrin glue; MRI: Magnetic resonance imaging.



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Figure 1 Study selection process.

MRI as the standard for evaluating fistula healing

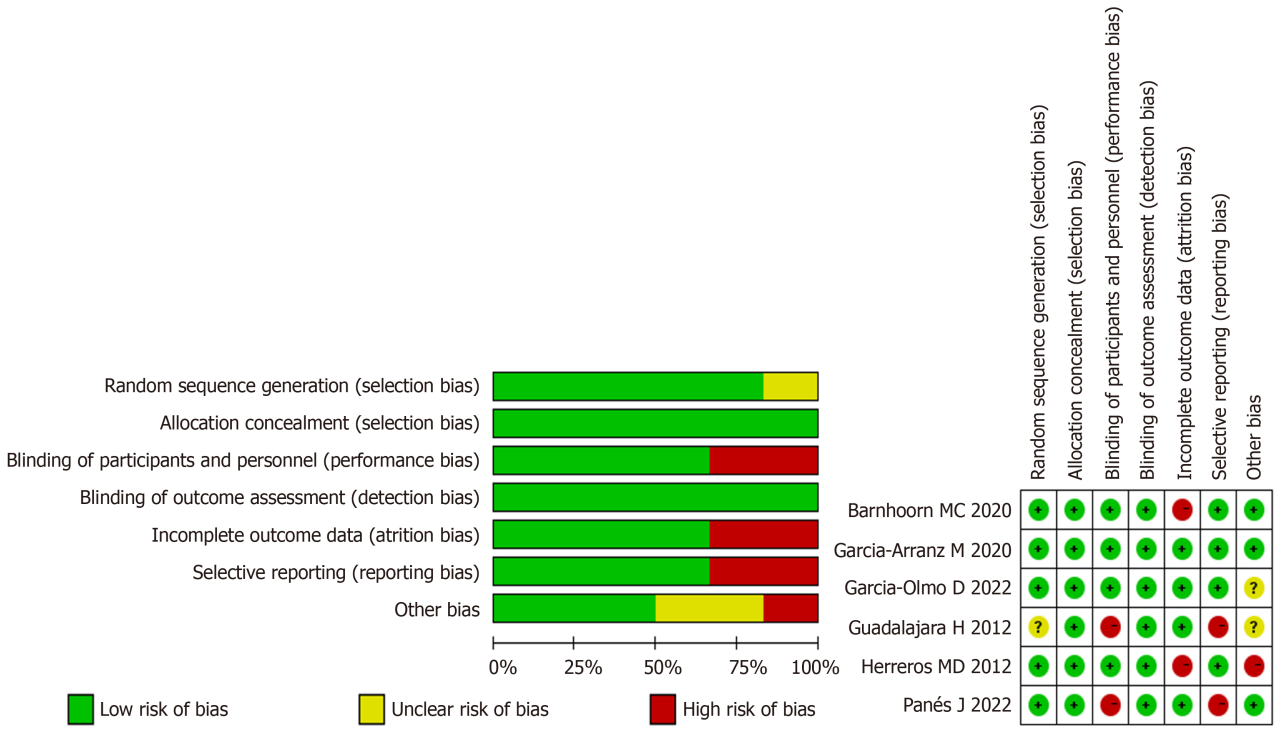
In our meta-analysis, MRI was used to evaluate fistula healing in 3 studies. The pooled analysis showed that MSC therapy was associated with improved long-term HR (OR = 2.79; 95%CI: 1.37 to 5.67; P = 0.005) (Figure 5).

Long-term safety

Only two studies[16,19] in this review assessed the long-term safety of MSC treatment for complex PF. The pooled results demonstrated that MSC treatment did not increase the risk of any long-term adverse or serious AEs (OR = 0.77; 95%CI: 0.27 to 2.24; P = 0.64) (Figure 6). No serious AEs related to MSC therapy were found.

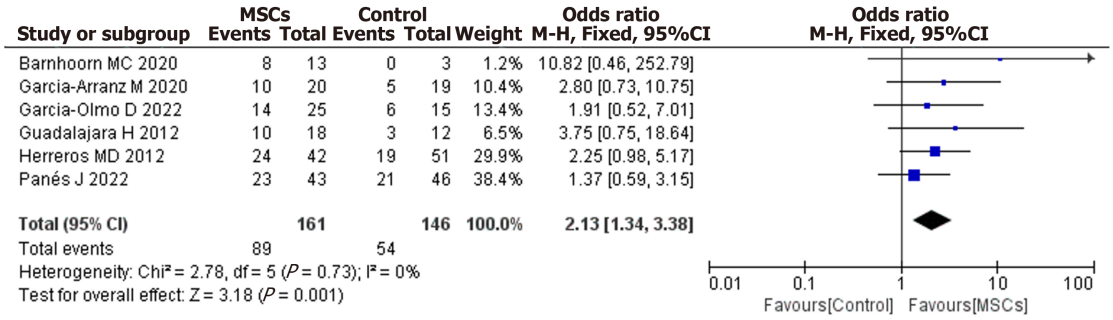
DISCUSSION

During the past decade, cell therapy-based treatments have been developed to manage several digestive tract diseases, including PF[21,22]. Previous research has shown that MSCs have a variety of mechanisms that promote wound healing. These cells also lack substantial immunogenicity and are thus suitable for use across human leukocyte antigen (HLA)



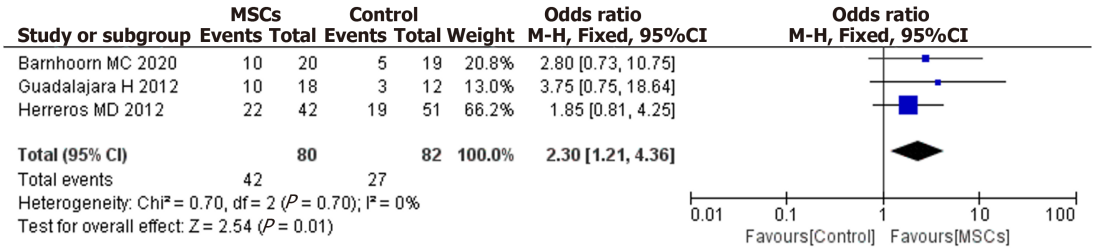
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Figure 2 Risk of bias of the articles included in the meta-analysis.



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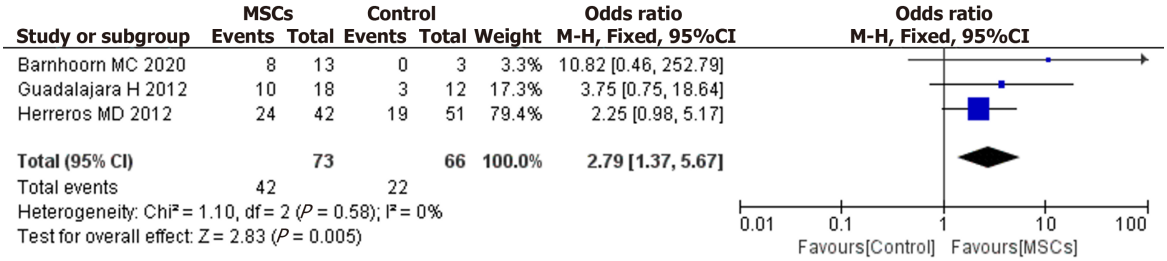
Figure 3 Forest plot showing the long-term effectiveness of mesenchymal stem cells for treating complex perianal fistula. MSCs: Mesenchymal stem cells; CI: Confidence interval.



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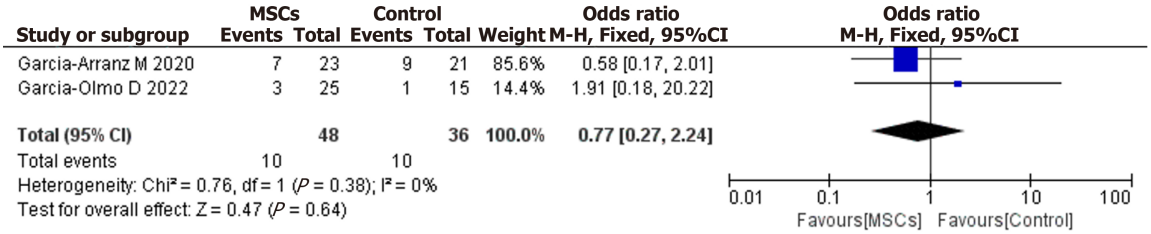
Figure 4 Differences in long-term effectiveness between mesenchymal stem cells plus fibrin glue and fibrin glue groups from meta-analysis. MSCs: Mesenchymal stem cells; CI: Confidence interval.

barriers[23,24]. An increasing number of studies have shown that MSC therapy is a safe and efficacious option for the short-term closure of PFs. However, maintaining continuous long-term fistula closure is also very important. A recurrent fistula is considered more difficult to treat surgically than the original fistula. Recently, an increasing number of studies have aimed to perform long-term follow-up of patients undergoing MSC administration to treat complex PFs. However,



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Figure 5 Forest plot showing the long-term effectiveness of mesenchymal stem cells for treating complex perianal fistula when magnetic resonance imaging is considered the standard for evaluating fistula healing. MSCs: Mesenchymal stem cells; CI: Confidence interval.



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Figure 6 Pooled odds ratio of long-term safety. MSCs: Mesenchymal stem cells; CI: Confidence interval.

there has been no meta-analysis to comprehensively evaluate the long-term effectiveness and safety of MSC treatment. Therefore, this study aimed to find a treatment to maintain long-term PF healing and to provide a basis for clinical application.

To our knowledge, this is the first meta-analysis to evaluate the long-term safety and efficacy of local MSC therapy for complex PFs. Findings from our study show that MSC therapy promotes sustained healing of complex PFs and that this therapy alone or combined with FG treatment can promote the long-term healing of complex PFs (OR = 2.13; 95%CI: 1.34 to 3.38; P = 0.001). The benefit over the control was sustained for at least 48 wk after local injection of MSCs. In our study, the timepoint for the assessment of fistula healing fluctuated greatly (48 wk-4 years), and all included studies showed good long-term healing of fistulas post-MSC treatment. Therefore, we believe that fistula healing persisted after MSC treatment. Barnhoorn *et al*[17] also reported that in addition to high fistula closure rates, MSC-treated patients had a higher QoL after therapy than at baseline. Some studies have also shown that in patients receiving MSC transplantation, the PF closure rate is significantly higher and the time to closure significantly shorter than that with anti-TNF drugs and fistulotomy, and MSC transplantation yields a decreased frequency of recurrence of the disease[25-27]. Therefore, these data confirm that MSC therapy promotes the long-term healing of complex PFs and significantly improves the QoL of patients. In the future, in evaluating MSC therapy for PF, it might be useful to consider work productivity and lifestyle restrictions.

Cell therapy strategies using MSCs carried in FG have shown promising results in regenerative medicine. MSCs have angiogenic, anti-apoptotic and immunomodulatory properties. FG provides a temporary structure that favors angiogenesis, extracellular matrix deposition and cell-matrix interactions. Additionally, FG maintains the local and paracrine functions of MSCs, providing tissue regeneration through less invasive clinical procedures[28]. The use of FG has been found to be uniformly safe, with minimal adverse effects, an early return to normal activity, and no negative impact on continence. One study showed that FG had a short-term effectiveness in the treatment of PF. At week 8 of the study, more than one-third of patients had healed fistulas, and half showed clinical improvement. Most of the patients in clinical remission at week 8 maintained clinical remission at week 16[29]. There was also a study indicating that FG was effective over the long term for the treatment of PF, and nearly 2 years after the use of FG to treat PF, over half of the patients showed clinical signs of remission[30]. Therefore, in association with MSCs, the use of FG has shown promising results in the field of regenerative medicine[31]. However, there is a lack of long-term success data on the combination of FG and other treatments for complex PF. In our study, MSCs plus FG had more long-term efficacy for fistula healing than FG alone (OR = 2.30; 95%CI: 1.21 to 4.36; P = 0.01). Therefore, we believe that the stimulation of the cellular adhesion and growth action of FG and the differentiation ability of MSCs may have a synergistic effect on the healing of fistulas. In our study, all patients received cleaning surgery before MSC treatment. Deep curettage had a positive effect on fistula closure in both groups (MSC recipients and controls). However, Garcia-Arranz *et al*[16] observed an increased number of long-term recurrences among control participants. Therefore, we speculate that the inflammatory focus persists, explaining why "deep curettage" may not provide a lasting resolution. In this scenario, MSCs and their anti-inflammatory and immunomodulatory effects can promote long-term healing.

Questions persist regarding the safety of MSC treatment for PF. Although MSC therapy has not raised any major safety concerns thus far in clinical trials, it is important to evaluate the safety of cell therapy in the long term. Our study

provides evidence that MSC therapy has a good long-term safety profile as a treatment for complex PF (OR = 0.77; 95%CI: 0.27 to 2.24; P = 0.64). In our study, the surgical management was fistula tract curettage and internal opening closure before MSC therapy. Notably, this is a minimally invasive surgery (involving perioperative antibiotic use, anesthesia, antisepsis, internal fistula orifice location, de-epithelization of the fistula tract, cleaning of the cavities and fistula tracts, closure of the internal opening, stem cell handling and resuspension, and cell injection) and does not produce fecal incontinence[32]. In contrast, anti-TNF therapies used for the treatment of PFs are associated with an increased risk of opportunistic infections, and surgical procedures often result in fecal incontinence. Tumorigenicity and ectopic tissue formation are the main concerns with the use of MSCs, and the risk of these SAEs is especially high during long-term MSC treatment. To date, MSCs have not been reported to cause tumors. Nevertheless, neoplasm development may become apparent only after longer follow-up periods. In another study conducted by our group, no neoplasm development was reported over the longest follow-up period of approximately 4 years[17]. To further confirm that neoplasms are not a concern with MSC treatment, biopsies taken from the fistula region were examined in that study. However, publication of these results is still pending, and we can continue the follow-up. While MSCs have not been shown to cause tumors in existing studies, long-term follow-up should be carried out to investigate the risk of cancer development. In the future, more long-term safety data are needed to fully assess the safety aspects of local MSC treatment.

Studies included in our meta-analysis used variable definitions of fistula healing. We believe that defining a healed fistula as complete re-epithelialization of external openings is not objective or accurate, as it does not account for the inside of the entire fistula. To evaluate the efficacy of MSC treatment, we should use more accurate methods for evaluating the inside of the entire fistula. MRI is the reference standard and cornerstone of fistula imaging. It demonstrates high sensitivity and specificity for the number and location of fistula tracts, detecting complexities frequently missed on clinical examination alone[33]. Therefore, MRI plays a crucial role in the evaluation, detection and follow-up of complex PFs[34]. In our meta-analysis, 3 RCTs based fistula healing on a combination of clinical examination and MRI imaging. The results showed that the MSC group had a higher HR than the control group (OR = 2.79; 95%CI: 1.37 to 5.67; P = 0.005). The absence of MRI examinations in some of the included studies at the end of the long-term follow-up is a limitation of this meta-analysis. In the future, if possible, blood should be drawn for standard measurements, and serum should be used to comprehensively evaluate the long-term healing of MSC therapy.

In this study, we aimed to evaluate the long-term efficacy and safety of MSC therapy through a meta-analysis. However, there are some unresolved questions. In the current clinical research, there are two ways to inject stem cells: (1) Systemic (mainly intravenous) injection; and (2) local injection. In our study, all patients received local MSC treatment. We speculate that for localized digestive tract diseases, local application and delivery seems more logical because side effects can be minimized and the cells are kept in direct contact with the at-risk tissue. Therefore, local MSC therapy seems to be a more promising treatment approach for further research. In our study, all eligible patients received a fixed dose of MSCs (one-time local injection or a second dose). Unfortunately, not all of the studies have compared various doses of MSCs or the benefit of repeat injections after the initial treatment. Most patients in our study received two doses of MSCs. Some studies have indicated a relationship between cell dose—or even the number of doses and efficacy[35,36]. In addition, all eligible patients with complex PFs may have branches with multiple tracks involving an extensive area that cannot always be adequately treated with a fixed dose of cells. Perhaps the cell dosage is related to the length of the fistula tracts and cavities. Due to the limitations of studies, it is difficult to provide recommendations on the optimal dose. In future research, we should pay attention to these unresolved questions (such as MSC origin, dosage and modality of intervention) to ensure that PF patients receive optimal treatment.

Our meta-analysis is the first to evaluate the long-term efficacy and safety of MSCs for PF treatment. Inevitably, this article has some limitations: (1) The studies used MSCs of different origins (adipose tissue and bone marrow from autologous as well as allogeneic sources); (2) some included studies defined the healed fistula as re-epithelialization of the external opening of the fistula. This may cause our results to be overestimated; (3) all patients underwent surgical procedures such as deep curettage. This may be beneficial to the short-term clinical remission of the fistulas. However, whether deep curettage will benefit long-term healing remains uncertain; (4) the follow-up period of the included studies varied significantly. Indeed, some of the studies lacked long-term follow-up data. In the future, we need more patients to enter the extended follow-up period so that the long-term safety and efficacy of MSCs can be assessed; and (5) the number of included studies and the sample size were limited, and extrapolation of the meta-analysis results was limited to some extent. So, our study was limited by its multiple centers and heterogeneity in the study inclusion criteria, mesenchymal stem cell origin, dose and frequency of delivery, and definition and time point of fistula healing. In the future, more patients must be evaluated in long-term follow-ups to optimize the efficacy and safety of MSCs for PF treatment.

CONCLUSION

In summary, MSC treatment is a safe and effective method that can significantly improve the long-term healing of complex PFs, and this method confers no risk of MSC-related AEs.

ARTICLE HIGHLIGHTS

Research background

An increasing number of studies have indicated that local mesenchymal stem cell (MSC) therapy is safe and efficacious for complex perianal fistulas (PFs). But, the long-term efficacy of MSC therapy is unclear. To date, an increasing number of studies have aimed to perform long-term evaluations of MSC therapy for PFs.

Research motivation

Local MSC therapy for PFs has shown considerable promise. But, the long-term safety and efficacy of MSC therapy in complex PFs is unknown.

Research objectives

To explore the long-term effectiveness and safety of MSC therapy for complex PFs.

Research methods

PubMed, EMBASE and Cochrane Library databases were searched that reported the long-term evaluation of local MSC therapy for complex PFs. The effectiveness and safety data analysis were conducted using RevMan5.3.

Research results

After screening, 6 studies met the inclusion criteria. MSC therapy was associated with an improved long-term healing rate (HR) compared with the control condition [odds ratio (OR) = 2.13; 95% confidence interval (95%CI): 1.34 to 3.38; P = 0.001]. Compared with fibrin glue (FG) therapy alone, MSC plus FG therapy was associated with an improved long-term HR (OR = 2.30; 95%CI: 1.21 to 4.36; P = 0.01). When magnetic resonance imaging was used to evaluate fistula healing, MSC therapy was found to achieve a higher long-term HR than the control treatment (OR = 2.79; 95%CI: 1.37 to 5.67; P = 0.005). There were no significant differences in long-term safety (OR = 0.77; 95%CI: 0.27 to 2.24; P = 0.64).

Research conclusions

Our study indicated that local MSC therapy promotes long-term and sustained healing of complex PFs and that this method is safe.

Research perspectives

In complex PFs treatment, local MSC therapy should be paid more attention to. Considering that this small number may not be enough to represent the whole complex PFs population, In the future, to improve on the quality of research, future studies should be carefully designed and reported.

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FOOTNOTES

Author contributions: Cheng F and Zhong H developed a search strategy for each database and collected the citations; Hong Z and Li Z performed the assessment of study quality and risk of bias, the data extraction and statistical analysis; Cheng F was major contributors in writing the manuscript; and all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Basic Study

Interferon-γ priming enhances the therapeutic effects of menstrual blood-derived stromal cells in a mouse liver ischemia-reperfusion model

Qi Zhang, Si-Ning Zhou, Jia-Min Fu, Li-Jun Chen, Yang-Xin Fang, Zhen-Yu Xu, Hui-Kang Xu, Yin Yuan, Yu-Qi Huang, Ning Zhang, Yi-Fei Li, Charlie Xiang

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have been used in liver transplantation and have certain effects in alleviating liver ischemia-reperfusion injury (IRI) and regulating immune rejection. However, some studies have indicated that the effects of MSCs are not very significant. Therefore, approaches that enable MSCs to exert significant and stable therapeutic effects are worth further study.

AIM

To enhance the therapeutic potential of human menstrual blood-derived stromal cells (MenSCs) in the mouse liver ischemia-reperfusion (I/R) model *via* interferon-γ (IFN-γ) priming.

METHODS

Apoptosis was analyzed by flow cytometry to evaluate the safety of IFN-γ priming, and indoleamine 2,3-dioxygenase (IDO) levels were measured by quantitative real-time reverse transcription polymerase chain reaction, western blotting, and ELISA to evaluate the efficacy of IFN-γ priming. *In vivo*, the liver I/R model was established in male C57/BL mice, hematoxylin and eosin and TUNEL staining was performed and serum liver enzyme levels were measured to assess the degree of liver injury, and regulatory T cell (Treg) numbers in spleens were determined by flow cytometry to assess immune tolerance potential. Metabolomics analysis was conducted to elucidate the potential mechanism underlying the regulatory effects of primed MenSCs. *In vitro*, we established a hypoxia/reoxygenation (H/R) model and analyzed apoptosis by flow cytometry to investigate the mechanism through which primed MenSCs inhibit apoptosis. Transmission electron microscopy, western blotting, and immunofluorescence were used to analyze autophagy levels.

RESULTS

IFN-γ-primed MenSCs secreted higher levels of IDO, attenuated liver injury, and increased Treg numbers in the mouse spleens to greater degrees than untreated MenSCs. Metabolomics and autophagy analyses proved that primed MenSCs more strongly induced autophagy in the mouse livers. In the H/R model, autophagy inhibitors increased the level of H/R-induced apoptosis, indicating that autophagy exerted protective effects. In addition, primed MenSCs decreased the level of H/R-induced apoptosis *via* IDO and autophagy. Further rescue experiments proved that IDO enhanced the protective autophagy by inhibiting the mammalian target of rapamycin (mTOR) pathway and activating the AMPK pathway.

CONCLUSION

IFN-γ-primed MenSCs exerted better therapeutic effects in the liver I/R model by secreting higher IDO levels. MenSCs and IDO activated the AMPK-mTOR-autophagy axis to reduce IRI, and IDO increased Treg numbers in the spleen and enhanced the MenSC-mediated induction of immune tolerance. Our study suggests that IFN-γ-primed MenSCs may be a novel and superior MSC product for liver transplantation in the future.

Key Words: Mesenchymal stem cells; Cell therapy; Reperfusion injury; T-lymphocytes; Autophagy; Liver

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Core Tip: In this study, we identified a suitable interferon-γ (IFN-γ) priming strategy for menstrual blood-derived stromal cells (MenSCs) in the liver ischemia-reperfusion (I/R) model and proved that primed MenSCs could significantly increase the number of regulatory cells in the spleen by secreting higher levels of indoleamine 2,3- dioxygenase (IDO) and thereby exhibited better immunoregulatory potential. Besides, through metabolomics and related molecular biology experiments, we found that IDO could reduce ischemia-reperfusion injury *via* the AMPK-mammalian target of rapamycin-autophagy axis. Our study suggests that IFN-γ-primed MenSCs may be a novel and superior mesenchymal stem cell product for liver transplantation in the future.

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INTRODUCTION

Liver transplantation is the only therapeutic option that is available to patients with terminal liver failure. Both liver ischemia-reperfusion injury (IRI), which occurs during surgery, and immune rejection, which occurs after surgery, affect the results of liver transplantation[1]. Therefore, there is an urgent need to develop effective strategies for treating IRI and immune rejection.

Mesenchymal stem cells (MSCs) have been used in liver transplantation and have shown certain therapeutic effects[2-4]. For example, MSCs alleviated hepatocellular apoptosis in a mouse liver ischemia-reperfusion (I/R) model *via* PINK1-dependent mitophagy[5]. In a clinical study, the percentage of regulatory T cells (Tregs) and the ratio of Tregs to T helper 17 (Th17) cells, which can be used as indicators of immune tolerance potential, were significantly increased in liver transplant recipients 4 wk after MSC infusion[6]. However, the results of another clinical study showed that the infusion of MSCs into liver transplant recipients before surgery was safe, but only mild changes in the peripheral blood immunoregulatory T cell numbers and natural killer cell numbers were observed in these patients[7]. In addition, the administration of immunosuppressive agents with MSCs showed no synergistic effect compared with the administration of immunosuppressive agents alone in a rat liver transplant study[8]. Therefore, approaches that enable MSCs to exert

more significant protective effects in reducing IRI and inducing immune tolerance in liver transplantation deserve further study.

Priming can induce an anti-inflammatory state in MSCs, causing them to secrete more bioactive molecules and improving their therapeutic effects[9,10]. Interferon (IFN)-γ-primed MSCs have been shown to have more stable and stronger abilities to regulate immune responses and prevent damage in many disease models[11-13]. Priming with IFN-γ increases the mRNA expression of Toll-like receptor 3 and the secretion of immunosuppressive molecules, such as indoleamine 2,3-dioxygenase (IDO), interleukin (IL)-10, hepatocyte growth factor, and kynurenine[14]. IDO, an enzyme involved in tryptophan metabolism, has been widely reported to regulate T-cell immunity, specifically by promoting the differentiation of Tregs, inhibiting Th17 cells, and promoting immune tolerance and immune escape[15,16]. Additionally, compared with control conditions, IFN-γ priming significantly induces IDO expression, enhances the immunoregulatory effect of MSCs on T cells, increases Treg differentiation, and promotes immune tolerance[17].

Menstrual blood-derived stromal cells (MenSCs) have been studied in several clinical and preclinical trials, and their safety and immunomodulatory properties have been fully demonstrated[18-20]. In addition, MenSCs can be collected in a manner that is painless, noninvasive, convenient, and inexpensive, thus, their use in cell-based therapy has high clinical and economic value. In this study, we hypothesized that IFN-γ-primed MenSCs could secrete higher levels of IDO to better induce immune tolerance in a mouse liver I/R model. However, whether priming with IFN-γ affects the viability of MenSCs themselves is unclear. Moreover, whether IFN-γ-primed MenSCs, which represent a new type of modified MSC product, can better mitigate liver injury in the liver I/R model has not been clarified. Therefore, it is worthwhile to further study appropriate priming strategies and whether primed MenSCs can exert better therapeutic effects in the liver I/R model.

MATERIALS AND METHODS

MenSCs

MenSCs were provided by the Innovative Precision Medicine Group (IPM, Hangzhou, China) and cultured as described in our previous study[2-4]. Briefly, the cells were cultured in α-minimum essential medium (Thermo Fisher Scientific, United States) supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher Scientific, United States) and 1% penicillin-streptomycin solution (Thermo Fisher Scientific, United States) in an incubator at 37 °C with 5% CO_2. When the cells reached 80%-90% confluence, they were subcultured with 0.25% trypsin-EDTA (Thermo Fisher Scientific) and passaged 5-8 times before being used in experiments. MenSCs were identified based on their surface marker expression and differentiation potential.

Flow cytometry was performed to detect the surface markers of MenSCs. The cells were collected, washed twice with staining buffer (BD, Biosciences, San Jose, CA), and incubated with antibodies against CD29, CD34, CD45, CD73, CD90, CD105, CD117, and human leukocyte antigen-DR (HLA-DR) (PE, BD, Biosciences, San Jose, United States) at 4 °C for 30 min in the dark. Negative controls (NCs) were established with the corresponding isotype control antibodies. A flow cytometer (ACEA Biosciences, CA, United States) was used to analyze all the cells.

Osteogenic, adipogenic, and chondrogenic differentiation was induced to evaluate the multi-differentiation potential of the cells. Briefly, approximately 5 × 10^5 MenSCs were cultured in 6-well plates for 3-4 wk with osteogenic differentiation medium (Cyagen Biosciences, United States) or adipogenic differentiation medium (Cyagen Biosciences, United States) in an incubator at 37 °C with 5% CO_2 to promote osteogenic or adipogenic differentiation, respectively; the medium was changed every 1-3 d according to the instructions. After fixation with 4% formaldehyde, the cells were incubated with Alizarin Red or Oil Red O solution for 30 min to label calcium or neutral lipids, respectively. Chondrogenic differentiation was performed in a 15 mL centrifuge tube. Approximately 5 × 10^5 MenSCs were centrifuged and cultured with 1 mL of human MSC chondrogenic differentiation medium (Cyagen Biosciences, United States) in an incubator at 37 °C with 5% CO_2 for 3-4 wk; the medium was changed every 2-3 d according to the instructions. After fixation with 4% formaldehyde, the pelleted cells were embedded in paraffin, cut into 4 mm sections, and stained with Alcian blue solution to label cartilage.

Priming of MenSCs and IDO quantification

IFN-γ (Miltenyi Biotec Inc., Auburn, CA, United States) at a concentration of 100 or 200 ng/mL was used to prim MenSCs for 24, 48, or 72 h. After the cells were collected, RNA or protein was extracted to measure IDO expression by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) or western blotting assays, respectively, as described below. Additionally, the supernatants were collected to evaluate the release of IDO using a Human IDO ELISA Kit (Sangon Biotech, Shanghai, China).

Establishment of the mouse liver I/R model and animal grouping

Six- to eight-week-old male C57BL/6J mice (Slac Laboratory Animal Corporation, Shanghai, China) were housed in a specific pathogen-free room (23 ± 2 °C, 12 h light/12 h dark cycle, and 50% humidity) at the Laboratory Animal Center of Zhejiang University with plenty of food and water for two weeks prior to experimentation. All the procedures used for the animal experiments, the number of animals, and the ethics of the animal housing environment were performed according to the 3Rs rule and approved by the Experimental Animal Center of Zhejiang University, and the ethical approval number is ZJU20210299.

Anesthesia: Before all the surgical procedures, 1% pentobarbital sodium (100 μL/10 g intraperitoneal injection) was used to anesthetize the mice (weight 20-23 g)[5]. Surgical procedure: All the mice received a ventral midline incision. Hepatic arteries, portal veins, and bile ducts were clipped with an atraumatic vascular clip to interrupt 70% of the blood supply to the liver. After 1 h of ischemia, the atraumatic vascular clamps were removed to terminate the ischemia; the wounds were sutured layer by layer, and erythromycin ointment was applied to prevent infection. The mice were resuscitated on a 37 °C blanket to maintain their body temperature. Group design: The sample size (total n = 27, and n = 3/group), grouping, and animal experiment schedule were decided based on a similar study published previously[2] and can be found in Figure 1. To establish the short-term I/R model, the mice were randomly divided into the sham group, IR group, IRM group, and IRM (IFN-γ) group (n = 3/group). The model mice in the IR, IRM, and IRM (IFN-γ) groups were administered 100 μL of phosphate buffered saline (PBS) or MenSCs (10^6/100 μL intravenous injection) or IFN-γ-primed MenSCs (10^6/100 μL intravenous injection) 1 h before the surgery. The mice in the sham group received only a ventral midline incision and were sutured after injection of PBS. After 6 h of reperfusion, the mice were euthanized in a classic, quick, and painless manner (150 mg/kg pentobarbital sodium, intraperitoneal injection), and tissue samples were collected for further investigation.

To establish the long-term I/R model, the mice were randomly divided into the sham group, IR group, IRM group, IRM (IFN-γ) group, and IRM (IFN-γ) + 1-methyl-D-tryptophan (1MT) group (n = 3/group). The treatment plan was similar to that used for the short-term model, with the exception that 1MT (2 mg/mL; MCE, New Orleans, LA, United States) was added to the drinking water of the mice in the IRM (IFN-γ) + 1MT group, and the mice in this group had continuous access to this supplemented water starting from the day of the surgery (day 0) until the seventh day (day 7). The mice in the other groups were given normal water without 1MT. All the long-term I/R model mice were euthanized (150 mg/kg pentobarbital sodium, intraperitoneal injection) on day 7 for tissue collection.

Assessment of liver injury

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using the ALT Kit and AST Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, to assess the liver injury. Liver injury was also assessed by hematoxylin and eosin staining. Sections were examined under a microscope, and Suzuki's injury criteria (Supplementary Table 1) were utilized to determine the histological damage score by a researcher who was blinded to the group of the immunohistochemistry sections.

qRT-PCR

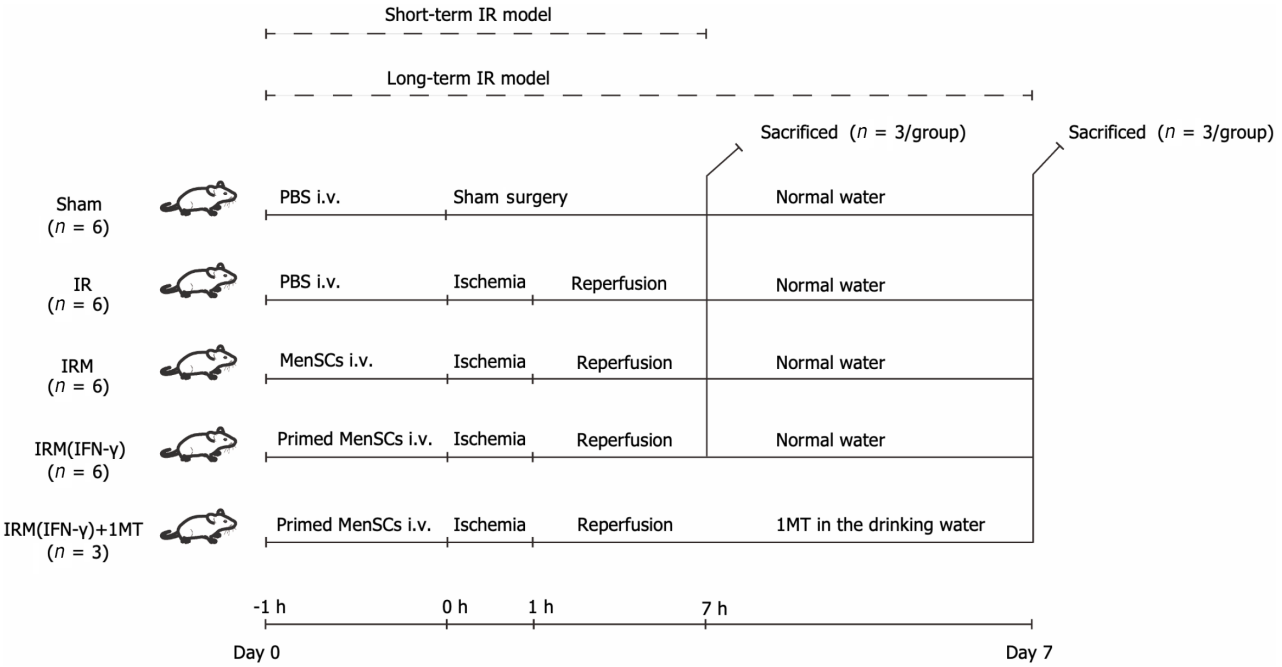
To confirm the effects on the transcriptome, RNA was extracted from cells using the RNA Extraction Kit (TaKaRa, Beijing, China). Prime Script TMRT Master Mix and TB Green Premix Ex Taq Bulk (TaKaRa, Beijing, China) were used for qRT-PCR. First, Buffer RL was added to the cells to lyse them, and the lysis solution was transferred to a gDNA Eraser Spin Column to remove impurities and gDNA. Then, 70% ethanol was added to the filtrate, and the mixture was moved to an RNA spin column to capture the RNA. Then, the RNA spin column was cleaned with Buffer RWA and Buffer RWB. The RNA solution was obtained by eluting the RNA with RNase-free dH_2O. Then, we mixed an appropriate amount of RNA with 5 × A solution and RNase-free dH_2O to a final volume of 10 μL, and reverse transcription was performed to obtain cDNA. Then, the TB Green mix (12.5 μL), forward PCR primer (10 μM × 0.5 μL, Supplementary Table 2), reverse PCR primer (10 μM × 0.5 μL, Supplementary Table 2), DNA template (2 μL), and 9.5 μL ddH_2O were mixed in a final volume of 25 μL. The qRT-PCR conditions were as follows: 95 °C for 30 s ; 40 cycles of 95 °C for 5 s and 60 °C for 30 s for 2 step PCR; and 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s for dissociation. The melting curves were determined, and the cycle threshold (CT) values of the β-actin (ACTB) transcripts were used to normalize the CT values of the IDO transcripts. The qRT-PCR data were analyzed using the ΔΔCT method.

Western blotting

Liver tissues and cells were lysed with a moderate amount of lysis buffer, which was composed of radioimmunoprecipitation assay buffer (RIPA Lysis Buffer, 10 ×, Merck KGaA, Darmstadt, Germany), a protease and phosphatase inhibitor cocktail for mammalian cell and tissue extracts (Beyotime, Shang Hai, China) and ddH_2O, on ice for 30-60 min. Equal amounts of proteins were subjected to 12% or 8% SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, United States). The membranes were incubated with 5% nonfat milk for 1 h and then with the primary antibodies overnight at 4 °C; the primary antibodies included antibodies against IDO (16630, Cell Signaling Technology, United States), LC3 (12741, Cell Signaling Technology, United States), AMPKα (total) (5831, Cell Signaling Technology, United States), p-AMPKα (50081, Cell Signaling Technology, United States), mammalian target of rapamycin (mTOR) (total) (2983, Cell Signaling Technology, United States), p-mTOR (5536, Cell Signaling Technology, United States), and β-actin (ACTB) (8227, Abcam, United States). The membranes were then washed three times with Tris-buffered saline with Tween 20 (TBST) and incubated with the secondary antibody [anti-rabbit immunoglobulin G (IgG), Sigma, United States or anti-mouse IgG, Sigma, United States] for 1 h at room temperature. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence with a Fluor Chem Systems imager (Bio-Rad, CA, United States). The intensities of the bands were determined using ImageJ software.

Transmission electron microscopy

Liver samples were cut into 1-mm^3 pieces and fixed with 2.5% glutaraldehyde overnight at 4 °C. After fixation and dehydration, all the samples were stained with osmic acid and uranyl acetate. Then, we used an ultramicrotome to slice the samples and obtain ultrathin sections. All the samples were imaged with a transmission electron microscope (Talos



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Figure 1 Animal experiment grouping and timeline. Detailed descriptions are provided in the Materials and Methods. In brief, we established short-term and long-term liver ischemia-reperfusion mouse models to explore the therapeutic potential of menstrual blood-derived stromal cells (MenSCs) and primed MenSCs. MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; 1MT: 1-methyl-D-tryptophan; IR: Ischemia-reperfusion; IRM: Combination treated with ischemia-reperfusion and menstrual blood-derived stromal cells; IRM (IFN-γ): Combination treated with ischemia-reperfusion and interferon-γ-primed menstrual blood-derived stromal cells; PBS: Phosphate buffered saline; i.v.: Intravenous injection.

L120C, Thermo Fisher, Carlsbad, CA, United States) to confirm and monitor autophagy, and the number of autophagic vacuoles in each cell was quantified.

Establishment of the hypoxia/reoxygenation model in L02 cells and grouping

Under normoxic conditions, the L02 cells (FuHeng Biology, Shanghai, China) were cultured in 1640 complete medium (RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin) in a typical incubator at 37 °C with 5% CO_2. To simulate liver IRI, the L02 cells were subjected to hypoxia/reoxygenation (H/R). Briefly, after 6 h of culture in FBS-free 1640 medium in a 2.5 L sealed culture tank (MGC, Tokyo, Japan) under hypoxic conditions, the cells were transferred to atypical incubator for 4 h to allow reoxygenation. The hypoxic conditions in the sealed culture tank (94% N_2, 5% CO_2, and 1% O_2) were maintained by AnaeroPack-Anaero-5% (MGC, Tokyo, Japan).

Group design: The cells that were cultured under normal oxygen conditions were divided into the NC group, 3-methyladenine (3MA) group, 1MT group, MenSCs group, and MenSC (IFN-γ) group. The cells that were cultured under H/R conditions were divided into the HR group, HR + 3MA group, HR + 1MT group, HRM group, HRM (IFN-γ) group, HRM (IFN-γ) + 3MA group, and HRM (IFN-γ) + 1MT group.

The NC group consisted of cells cultured in 1640 complete medium without any other treatment. The cells in the 3MA group and 1MT group were cultured in 1640 complete medium with 10 mmol/L 3MA (an autophagy inhibitor, MCE, New Orleans, LA, United States) or 1 mmol/L 1MT (an IDO inhibitor, MCE, New Orleans, LA, United States). The cells in the MenSCs group and MenSC (IFN-γ) group were cocultured with MenSCs or MenSC (IFN-γ), respectively, in 1640 complete medium using a coculture chamber as described previously. The HR group, HR + 3MA group, HR + 1MT group, HRM group, and HRM (IFN-γ) group, were subjected to treatments that were similar to those used in the experiments conducted under normal oxygen conditions, but H/R treatment was added. In addition to the HRM (IFN-γ) treatment, the cells in the HRM (IFN-γ) + 3MA group and HRM (IFN-γ) + 1MT group received 3MA and 1MT in the medium, respectively.

Quantitative GFP-LC3 analysis

L02 cells in every group were transfected with pCMV-GFP-LC3 (Beyotime, Shang Hai, China) for 24 h before treatment. We used a confocal laser scanning microscope (Olympus Corporation, Japan) to detect fluorescence emission. Intense punctate GFP-LC3 aggregates in the nucleus and cytoplasm were considered to indicate autophagy, whereas diffuse distribution of GFP-LC3 was considered to indicate non-autophagic conditions. We counted the number of intense punctate GFP-LC3 aggregates in each cell to indicate the number of autophagosomes.

Treg analysis

The mononuclear cells in the spleen were harvested by gradient centrifugation using Ficoll 1.084 (Cytiva, Logan, UT, United States). FACS was performed with 3 fluorochrome-conjugated antibodies: Anti-CD4 monoclonal antibody (APC,

17-0041-83, eBioscience, CA, United States), anti-CD25 monoclonal antibody (PE, 12-0251-82, eBioscience, CA, United States), and anti-FOXP3monoclonal antibody (FITC, 11-5773-82, eBioscience, CA, United States). Nuclear permeabilization was performed with a FoxP3/transformation factor permeabilization buffer (00-5523-00, eBioscience, CA, United States) before the anti-FoxP3 antibody was added to the cells for incubation. Cell fluorescence was evaluated using a flow cytometer (ACEA Biosciences, CA, United States). The percentage of $CD4^+$ $CD25^+$ cells among all mononuclear cells and the percentage of $FoxP3^+$ cells among the $CD4^+CD25^+$ cells were recorded, and the product of these percentages was used to represent the percentage of Tregs among all mononuclear cells.

Statistical analysis

The metabolomics data were analyzed using the free online Majorbio choice platform (cloud.majorbio.com). Other data were analyzed using Prism version 5.0 (GraphPad, United States). The data are shown as the means ± SDs or means ± SEMs. One-way ANOVA was used for comparisons of three or more groups. A P value of <0.05 was considered to indicate a significant difference ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$, and $^dP < 0.0001$), and P values ≥ 0.05 were considered to indicate no significant difference (NS).

RESULTS

Characterization of MenSCs

Primary MenSCs were cultured and exhibited adherent growth after approximately 24 h, and obvious cell proliferation was observed as soon as the cells became round and fusiform. According to a phenotypic analysis, MenSCs expressed CD29, CD73, CD90, and CD105, but they did not express CD45, CD80, CD117, or HLA-DR (Figure 2A). Specific staining showed that MenSCs could undergo osteogenic, chondrogenic, and adipogenic lineage differentiation (Figures 2B-D). The MenSCs that were used in this study met the International Society for Cellular Therapy criteria for the definition of multipotent MSCs[21].

MenSC priming with IFN-γ

In this study, we hypothesized that IFN-γ-primed MenSCs could secrete higher levels of IDO to induce stronger immune tolerance in a liver I/R model. Therefore, to determine the appropriate priming strategy, we evaluated the safety and efficacy of priming by measuring the degree of cell apoptosis and the levels of IDO, respectively.

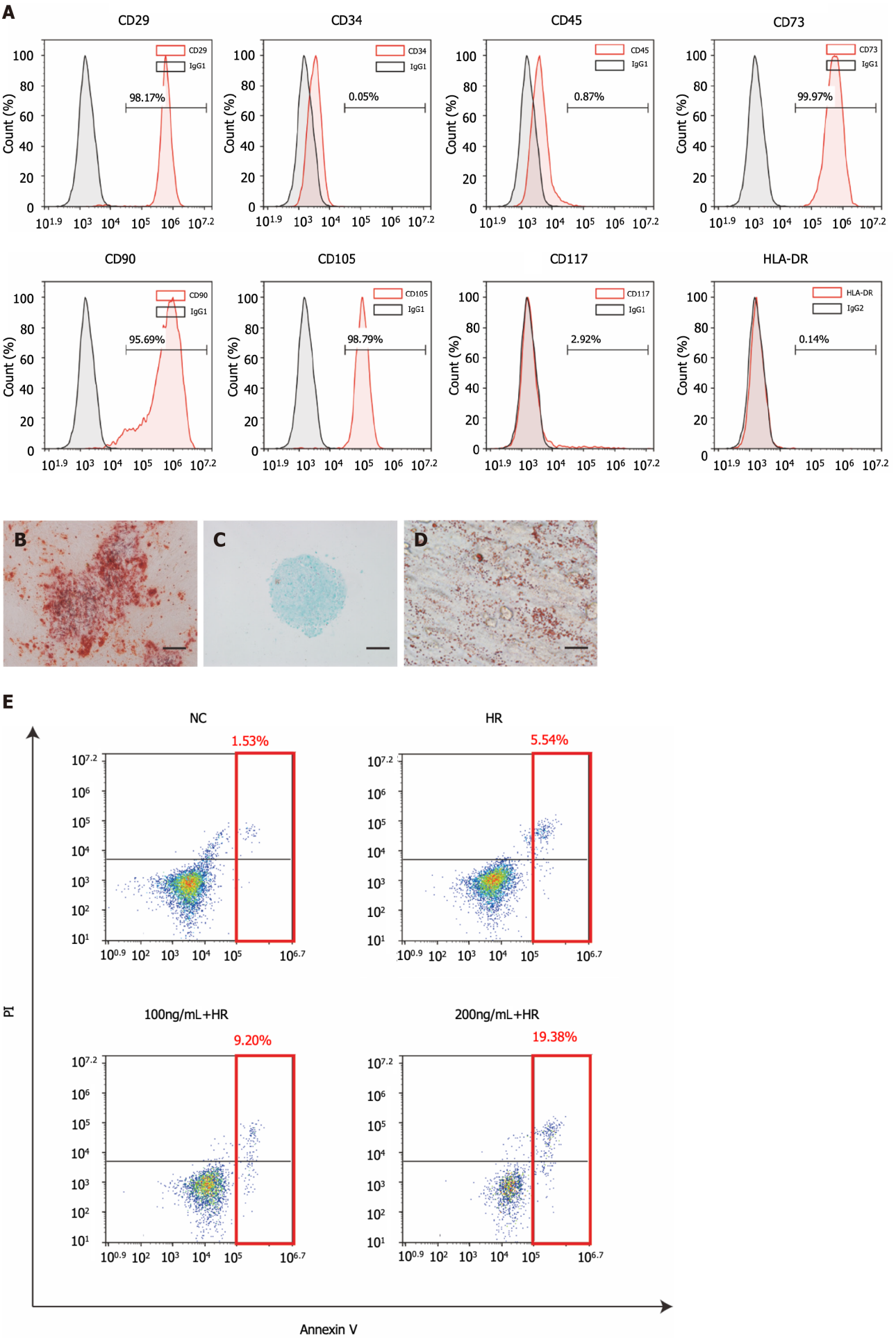
MenSCs were treated with 100 ng/mL IFN-γ (low concentration) or 200 ng/mL IFN-γ (high concentration) to determine the appropriate concentration for treatment. To accurately mimic the environment in which MenSCs perform their biological functions, we exposed MenSCs to H/R 72 h after priming[14,18]. Then, the cells were stained with Annexin V and propidium iodide (PI), and the proportions of apoptotic MenSCs in the different treatment groups were analyzed by flow cytometry. We found that H/R slightly increased stem cell apoptosis, but the difference was not significant (Figures 2E and F). Moreover, MenSCs that were primed with low and high IFN-γ concentrations were less resistant to H/R-induced apoptosis, and the overall proportions of apoptotic cells reached approximately 10% and 17%, respectively (Figures 2E and F). To maintain a relatively good state of MenSCs after intervention, we used the lower concentration of 100 ng/mL to prim MenSCs in the subsequent experiments.

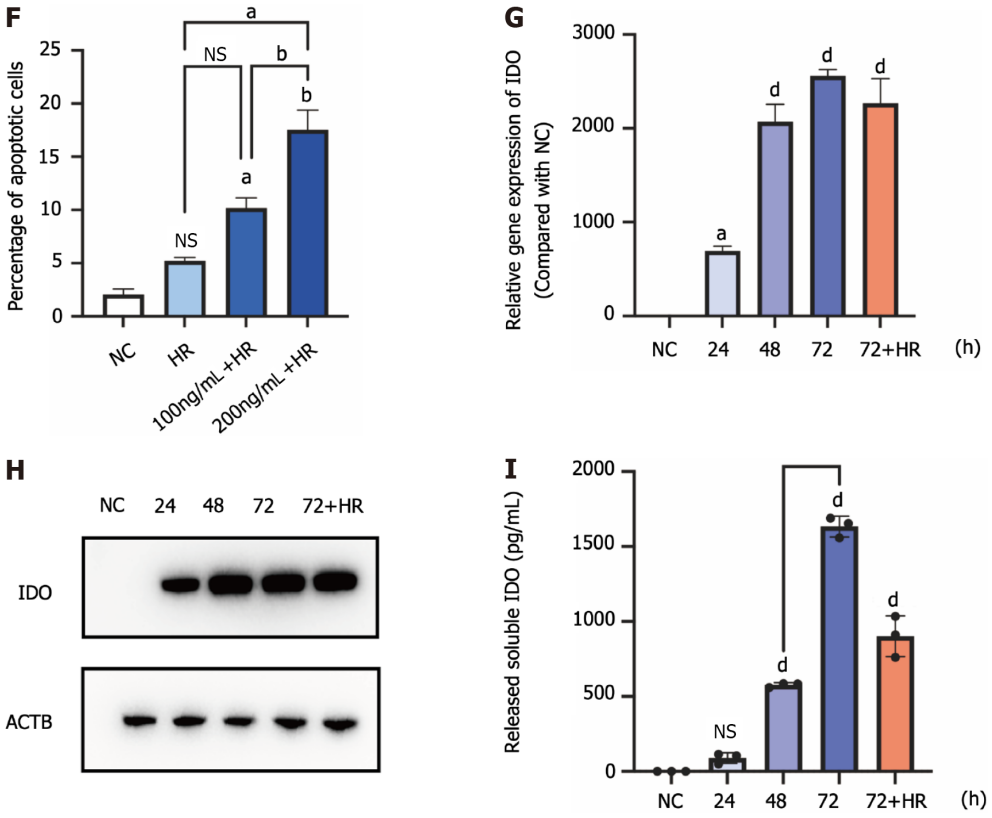
As previously mentioned, IDO secreted by MSCs plays a key role in the regulation of T cells, and this metabolite can induce the differentiation of Tregs and thus promote immune tolerance[15,16]. Therefore, we used the IDO levels as an indicator to evaluate the efficacy of priming. The mRNA and protein levels of IDO in MenSCs were measured by qRT-PCR and western blotting, respectively, and the concentrations of IDO that were secreted by MenSCs were measured by ELISA. These results showed that the expression of IDO in MenSCs was upregulated 24 h after IFN-γ priming and reached a high level at 72 h. In addition, IDO continued to be expressed at a high level in MenSCs under H/R treatment conditions after the removal of IFN-γ (Figures 2G and H). Measurement of the IDO levels by ELISA showed that IDO was gradually secreted into the cell culture medium after priming, and when IFN-γ was removed after 72 h of priming, a considerable amount of IDO continued to be secreted by MenSCs under H/R treatment conditions (Figure 2I).

These results indicated that MenSCs could be maintained in a relatively good cell state when exposed to H/R after treatment with a low concentration of IFN-γ. Additionally, IFN-γ priming for 72 h promoted the expression and continuous secretion of IDO by MenSCs. Therefore, we chose 100 ng/mL IFN-γ for 72 h as the strategy for priming MenSCs to ensure that priming would be safe and effective.

IFN-γ-primed MenSCs attenuated liver IRI in the mouse short-term I/R model

The grouping and timeline of the animal experiments are shown in Figure 1. We administered MenSCs and IFN-γ-primed MenSCs (primed MenSCs) to short-term I/R model mice to explore their therapeutic potential. We found that the mice in the IR group showed significant hepatocyte injury after 6 h of reperfusion; this injury manifested as vacuole formation, increased inflammatory cell infiltration, disordered hepatic lobules, and tissue necrosis (Figure 3A), and these effects were accompanied by elevated serum levels of AST and ALT, which are indicators of liver function (Figures 3E and F), and relatively high Suzuki's injury scores (Figure 3B). However, the administration of MenSCs and primed MenSCs resulted in decreased ALT and AST levels, ameliorated IRI, and decreased Suzuki's injury scores (Figures 3A, B, E and F). Additionally, caspase-3 and TUNEL staining of liver tissue sections were performed to observe apoptosis. We reached the same conclusion, namely, that both MenSCs and IFN-primed MenSCs could attenuate I/R-induced hepatocyte apoptosis (Figures 3C and D). Moreover, these results also showed that primed MenSCs exerted a better therapeutic effect than


A
CD29
CD29
IgG1
98.17%
CD34
CD34
IgG1
0.05%
CD45
CD45
IgG1
0.87%
CD73
CD73
IgG1
99.97%
CD90
CD90
IgG1
95.69%
CD105
CD105
IgG1
98.79%
CD117
CD117
IgG1
2.92%
HLA-DR
HLA-DR
IgG2
0.14%
Count (%)
B
C
D
E
NC
1.53%
HR
5.54%
100ng/mL+HR
9.20%
200ng/mL+HR
19.38%
PI
Annexin V



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Figure 2 Characterization of menstrual blood-derived stromal cells and menstrual blood-derived stromal cells primed with interferon-γ. A: The surface marker expression of menstrual blood-derived stromal cells (MenSCs) was assessed by flow cytometry analysis; B: The osteogenic differentiation potential of MenSCs. Scale bar 200 μm; C: The chondrogenic differentiation potential of MenSCs. Scale bar 200 μm; D: The adipogenic differentiation potential of MenSCs. Scale bar 200 μm; E: MenSC apoptosis was detected by flow cytometry. The red box indicates the sum of late and early apoptotic cells, which represent the overall level of apoptosis; F: The overall level of apoptosis was analyzed; G: After MenSC priming with 100 ng/mL interferon-γ for 24 h, 48 h, 72 h, and 72 h + hypoxia/reoxygenation, the mRNA levels of indoleamine 2,3-dioxygenase (IDO) in MenSCs were determined by quantitative real-time reverse transcription polymerase chain reaction analysis; H: The protein level of IDO in MenSCs was determined by western blotting; I: The level of IDO secreted by MenSCs into the cell culture supernatant was measured by ELISA. The data are shown as the means ± SEMs, $n = 3$. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. NS represents not statistically significant. All *P* values were obtained by one-way ANOVA. HLA-DR: Human leukocyte antigen-DR; IgG: Immunoglobulin G; NC: Negative control; HR: Hypoxia/reoxygenation; PI: Propidium iodide; IDO: Indoleamine 2,3-dioxygenase.

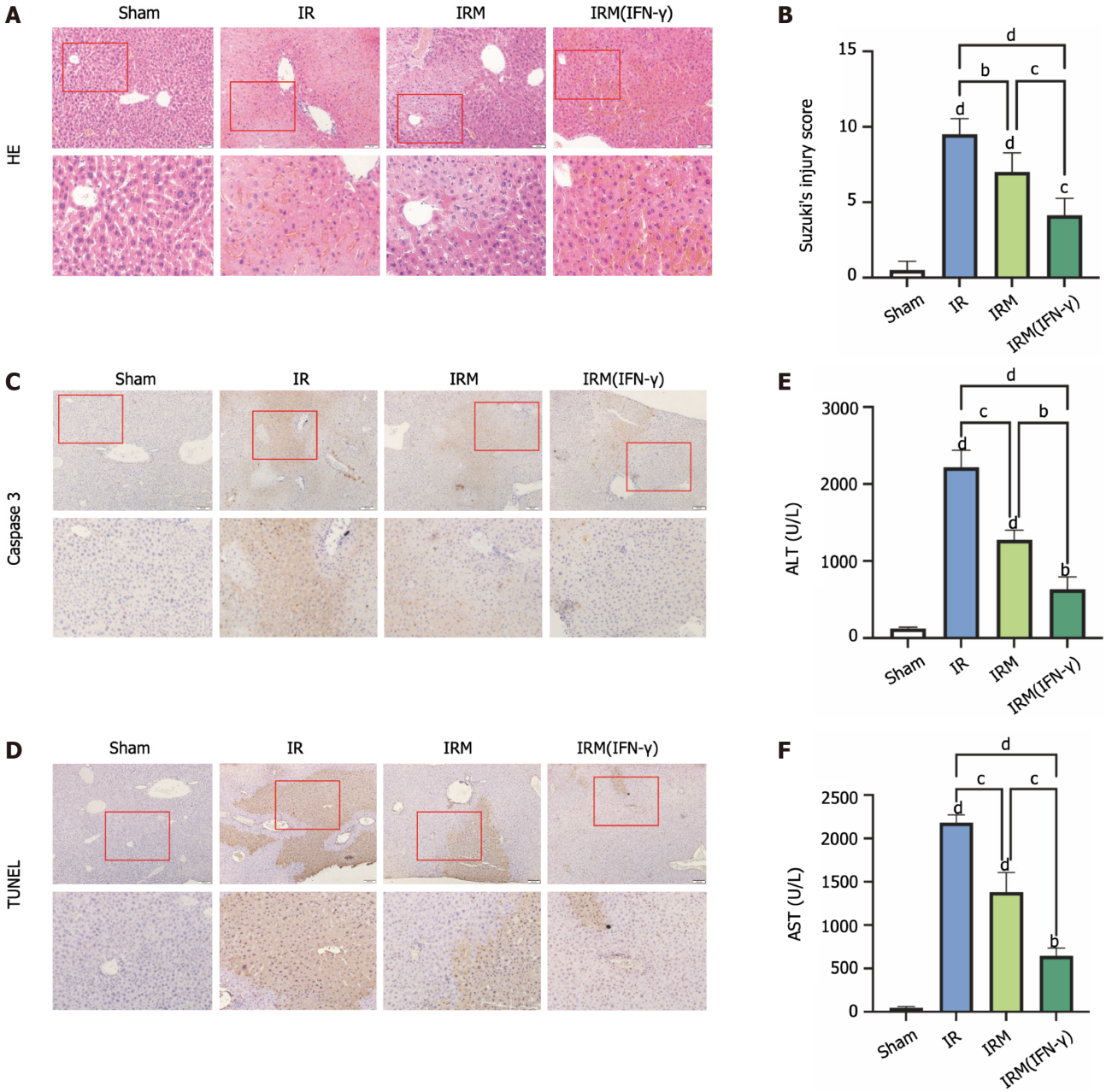
untreated MenSCs (Figures 3A-F).

IFN-γ-primed MenSCs reduced hepatocellular damage and increased the Treg numbers in the mouse long-term I/R model

As IFN-γ-primed MenSCs could exert a better therapeutic effect in the short-term model, we used 7 d of reperfusion after I/R to establish a long-term model in order to further explore the long-term effects of primed MenSCs, particularly their potential to induce immune tolerance.

We found that after 7 d of ischemia, the liver tissues of the mice showed obvious edema, a large cavity, and necrosis, and these conditions resulted in high Suzuki's injury scores. However, MenSCs and particularly IFN-γ-primed MenSCs significantly inhibited these changes (Figures 4A and B). Additionally, we determined the Treg numbers in the spleen to assess the immune tolerance of the mice. To determine the number of Tregs, we analyzed the percentage of cells that were positive for CD4, CD25, and Foxp3. Tregs accounted for approximately 1% of the total lymphocyte population in the sham and IR groups, and primed MenSCs increased the percentage of Tregs in the lymphocyte population to a great extent (Figures 4C and D). However, although untreated MenSCs also increased the percentage of Tregs, no significant difference was found compared with the sham group (Figures 4C and D). To verify the correlation between the stronger immunomodulatory ability of primed MenSCs and IDO, we added 1MT to the drinking water of some mice and found that 1MT clearly reversed the increase in the percentage of Tregs in the spleen and inhibited the protective effect of primed MenSCs on the liver (Figures 4A-D).

These results indicated that after 7 d of reperfusion, IFN-γ-primed MenSCs exerted a stronger and more stable effect on reducing liver injury and increasing the percentage of Tregs in the spleen compared with untreated MenSCs. These stronger therapeutic effects were closely associated with IDO.

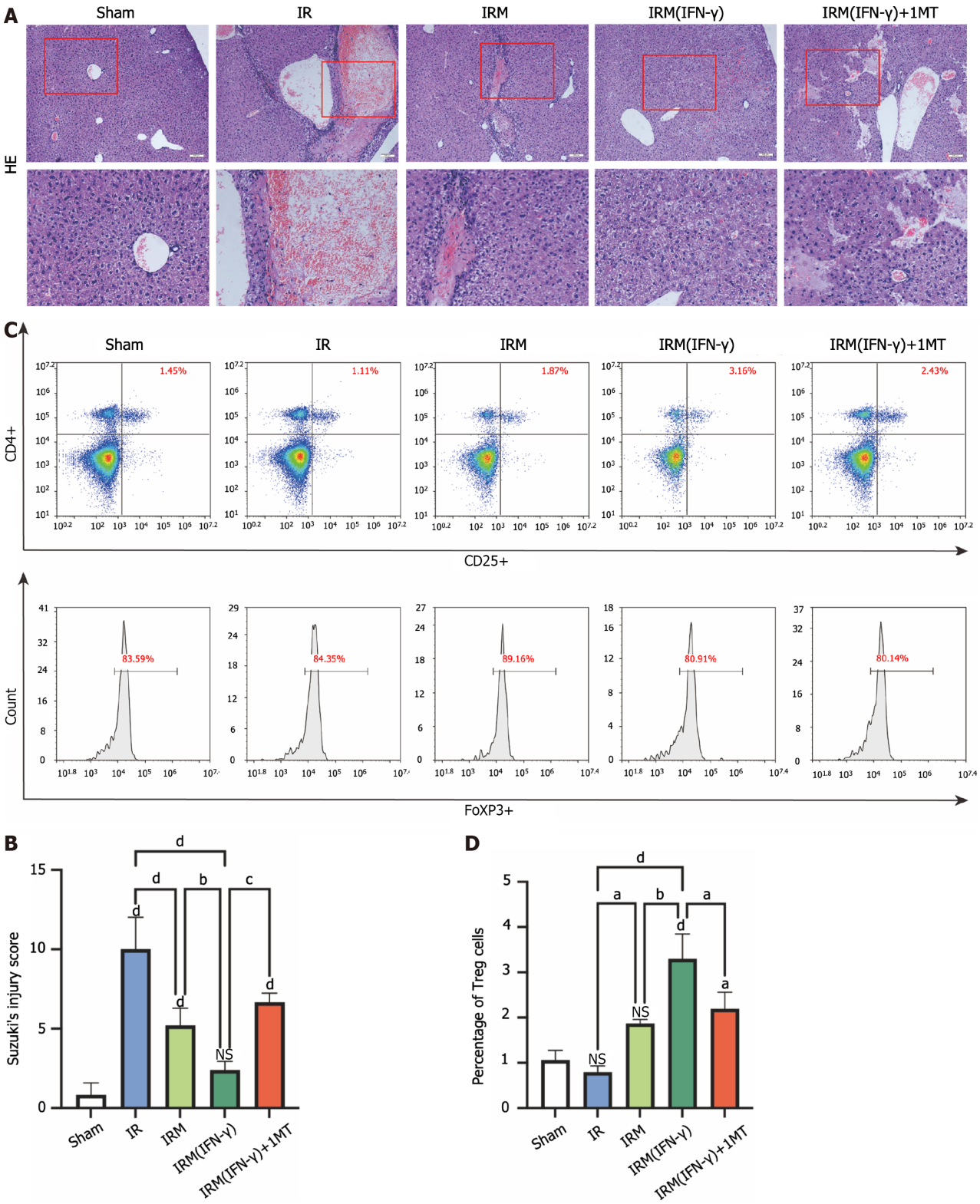


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Figure 3 Interferon-γ-primed menstrual blood-derived stromal cells attenuated liver ischemia-reperfusion injury in the mouse short-term ischemia-reperfusion model. A: Representative images of histological liver sections stained with hematoxylin and eosin (hematoxylin and eosin staining, magnification 200 ×, images in the second row are partial magnifications of regions indicated by the red box); B: The degree of liver injury in each group was determined according to Suzuki's injury score. The data are expressed as the means ± SEMs (n = 10/group); C: Representative images of liver sections from each group stained for caspase-3 to evaluate the apoptosis level (magnification 200 ×, images in the second row are partial magnifications of regions indicated by the red box); D: Representative liver sections from each group stained with TUNEL were obtained to evaluate the apoptosis level (magnification 200 ×, images in the second row are partial magnifications of regions indicated by the red box); E: The serum alanine aminotransferase levels were measured to indicate liver function levels, the data are expressed as the means ± SEMs (n = 3/group); F: The serum aspartate aminotransferase levels were measured to indicate liver function levels, and the data are expressed as the means ± SEMs (n = 3/group). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. NS represents not statistically significant. All P values were obtained by one-way ANOVA. MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; IR: Ischemia-reperfusion; IRM: Combination treated with ischemia-reperfusion and menstrual blood-derived stromal cells; IRM (IFN-γ): Combination treated with ischemia-reperfusion and interferon-γ-primed menstrual blood-derived stromal cells; HE: Hematoxylin and eosin; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

IFN-γ-primed MenSCs promoted autophagy in mouse livers

As described above, we verified that the more significant immunomodulatory effect of primed MenSCs was indeed related to IDO. Strikingly, the more significant effect of primed MenSCs in alleviating liver injury was also associated with IDO. To explore the specific mechanism through which primed MenSCs ameliorate IRI, metabolomics sequencing of mouse liver samples from the short-term model was performed.



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Figure 4 Interferon-γ-primed menstrual blood-derived stromal cells reduced hepatocellular damage and increased the Treg numbers in the mouse long-term ischemia-reperfusion model. A: Representative images of histological liver sections stained with hematoxylin and eosin were obtained (hematoxylin and eosin staining, magnification 200×, images in the second row are partial magnifications of regions indicated by the red box); B: The degree of liver injury in each group was determined according to Suzuki's injury score. The data are expressed as the means ± SEMs (n = 3-5/group); C: The numbers of CD4, CD25, and FoxP3 positive cells (regulatory T cells) in the spleen were determined by flow cytometry to estimate the ability of primed menstrual blood-derived stromal cells to induce immune tolerance; D: The percentages of Tregs are expressed as the means ± SEMs (n = 3/group). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. NS represents not statistically significant. All P values were obtained by one-way ANOVA. MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; 1MT: 1-methyl-D-tryptophan; IR: Ischemia-reperfusion; IRM: Combination treated with ischemia-reperfusion and menstrual blood-derived stromal cells; IRM (IFN-γ): Combination treated with ischemia-reperfusion and interferon-γ-primed menstrual blood-derived stromal cells; HE: Hematoxylin and eosin; Treg: Regulatory T cell.

PCA and PLS-DA of samples from the two groups were performed to evaluate the degree of aggregation and dispersion of samples within a single group as well as between different groups, and the results indicated that the data could be used for subsequent analysis (Supplementary Figures 1A-D). A statistical heatmap of 45 metabolites that differed between the IR group and IRPM group [IRM (IFN-γ) group] was generated, and the results are also shown in the form of a volcano plot (Figures 5A and B). Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of the differential metabolites revealed that the differential metabolites are involved in a variety of biological processes, mainly including lipid metabolism, amino acid metabolism, signaling molecule transduction, and membrane transport (Figure 5C). To further elucidate the main biological functions of the differential metabolites, we performed KEGG pathway enrichment analysis of these differential metabolites, and the results showed that the differential metabolites were mainly enriched in autophagy (Figure 5D). Relative quantification of the level of the differential metabolite phosphatidyl ethanolamine (PE), which is related to autophagy, showed that treatment with IFN-γ-primed MenSCs increased the levels of PE in the livers (Figure 5E). The main role of PE in autophagy is mediating the formation of the autophagy membrane marker LC3II from LC3I. A higher PE level is related to a higher level of autophagy[22-25]. Therefore, we hypothesized that primed MenSCs may increase the level of autophagy in mouse liver tissues.

To further verify this hypothesis, we observed the autophagic bodies in mouse liver cells by transmission electron microscopy (TEM) and measured the expression of autophagy-related proteins in liver tissues by western blotting to evaluate the autophagy levels. MenSCs, and primed MenSCs increased the number of autophagosomes in liver tissues, and the increase was most obvious in the IRM (IFN-γ) group (Figures 6A and B). Consistent with the TEM results, LC3II expression was increased after I/R, suggesting that I/R could increase the level of autophagy in hepatocytes (Figures 6C and D). Moreover, the autophagic flux was amplified by MenSCs and particularly by primed MenSCs (Figures 6C and D). Additionally, we observed the changes in the AMPK pathway and the mTOR pathway, which are two classical pathways that are upstream of autophagy, and we found that I/R could promote AMPK phosphorylation and inhibit mTOR phosphorylation; in other words, I/R could activate the AMPK pathway and inhibit the mTOR pathway (Figures 6C and D). Moreover, most of these effects were enhanced by MenSCs and particularly primed MenSCs (Figures 6C and D).

In conclusion, we demonstrated that primed MenSCs amplified I/R-induced autophagy in the mouse livers through metabolome sequencing analysis and molecular biology experiments. However, the relationship between the enhanced autophagy and IDO and whether autophagy is related to the therapeutic effect of MenSCs in alleviating IRI still need to be further clarified.

IFN-γ-primed MenSCs decreased H/R-induced hepatocyte apoptosis via autophagy and IDO

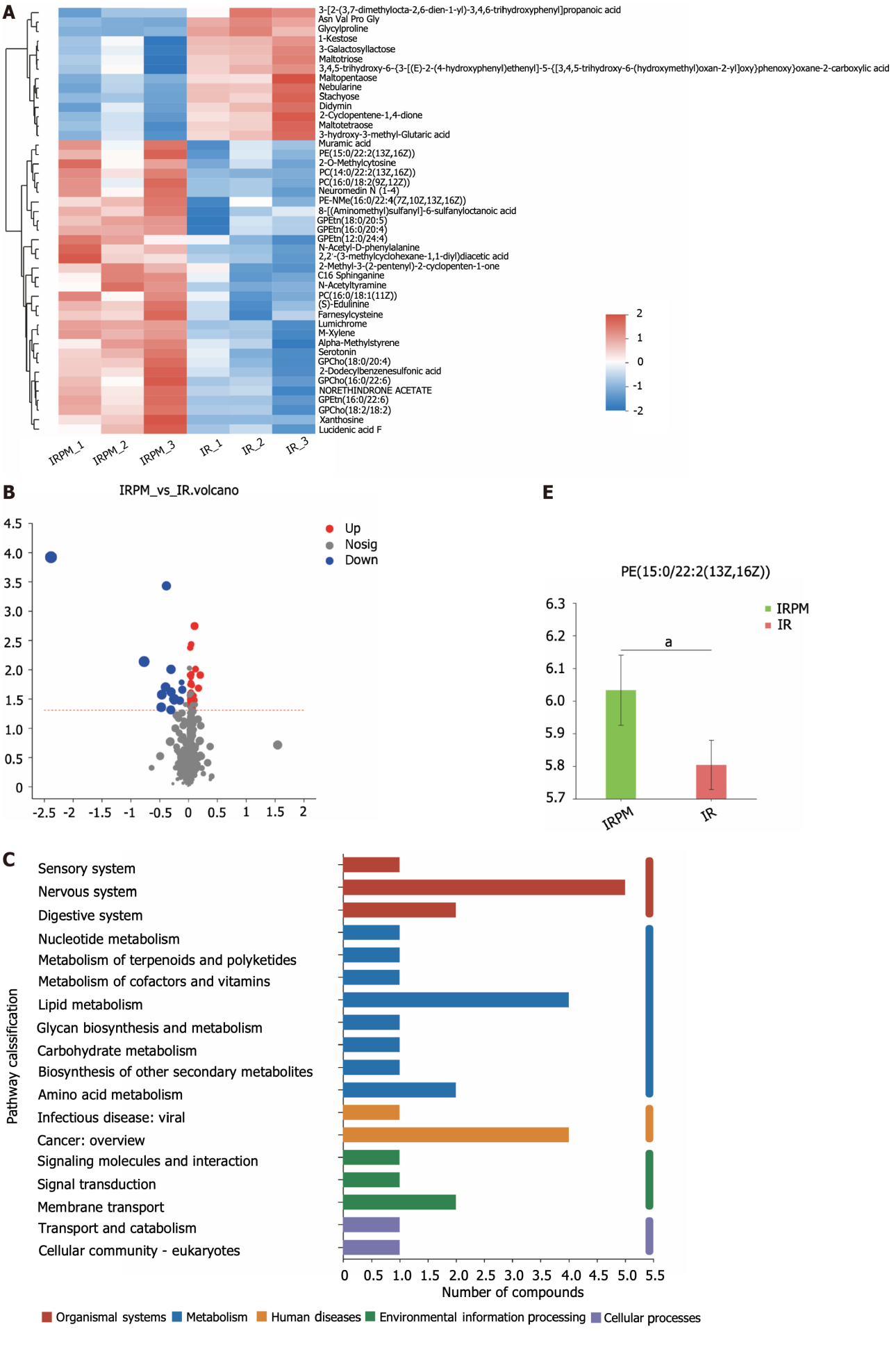
To further investigate the relationship among the therapeutic effect of primed MenSCs, IDO, and enhanced autophagy, we established an *in vitro* H/R model in L02 cells with the corresponding treatment conditions.

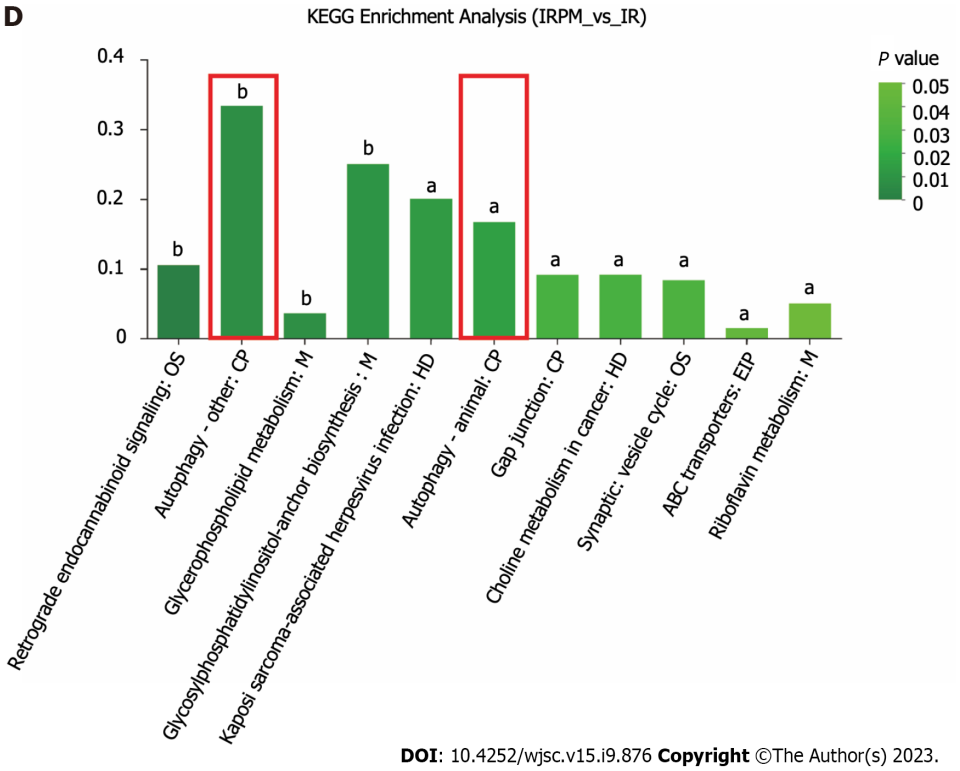
First, we exposed L02 cells to H/R for different times to determine an approximately appropriate H/R time. Autophagy-related protein levels were measured by western blotting. The AMPK pathway activation that was caused by hypoxia gradually decreased with increases in the reoxygenation time, and primed MenSCs delayed this process (Supplementary Figure 2A). In addition, we observed that autophagy-related protein expression changed in a time-dependent manner in the mouse model (Supplementary Figure 2B). To allow significant comparisons, we selected 6 h of hypoxia and 4 h of reoxygenation as the H/R conditions.

Apoptotic cells were identified by PI/Annexin V staining, and flow cytometry was performed to analyze cells that were exposed to different treatments. Cells in the upper right corner represent late apoptotic cells, cells in the lower right corner represent early apoptotic cells, and the sum of these two populations represents the overall level of apoptosis. We treated LO2 cells with 3MA (an autophagy inhibitor), 1MT (an IDO inhibitor), MenSCs, and primed MenSCs under normoxic conditions. These treatments alone did not significantly affect apoptosis under normoxic conditions (Figures 7A and B). After H/R, significant L02 cell apoptosis was observed, and the total apoptotic cell proportion accounted for approximately 14.89% of the overall cell population. After treatment of the cells with 3MA to inhibit autophagy under H/R conditions, the total apoptotic cell proportion accounted for approximately 45.06% of the overall cell population (Figures 7A and B). This finding indicated that H/R induced L02 cell apoptosis, and inhibition of autophagy significantly increased H/R-induced apoptosis. In addition, we also observed that the proportion of apoptotic cells was significantly reduced after coculture with MenSCs and particularly with primed MenSCs under H/R conditions, and this result indicated that stem cells can protect L02 cells from H/R-induced apoptosis (Figures 7A and B). A comparison of the HRM (IFN-γ) and HRM (IFN-γ) + 3MA groups revealed that the protective effect of primed MenSCs was significantly reduced when autophagy was inhibited (Figures 7A and B). This finding indicated that primed MenSCs may protect L02 cells against H/R by enhancing autophagy. In addition, a comparison of the HRM (IFN-γ) and HRM (IFN-γ) + 1MT groups showed that inhibition of IDO by 1MT also inhibited the protective effect of primed MenSCs (Figures 7A and B). These results indicated that autophagy reduced H/R-induced apoptosis and that the more powerful protective effect of IFN-γ-primed MenSCs was closely associated with autophagy and IDO.

IDO secreted by primed MenSCs enhanced autophagy by inhibiting the mTOR pathway and activating the AMPK pathway

As mentioned above, primed MenSCs attenuated H/R-induced apoptosis *via* IDO and autophagy. To further investigate whether IDO is related to autophagy, we used 1MT to inhibit IDO and observed changes in autophagy. L02 cells were transfected with pCMV-GFP-LC3, exposed to the indicated treatments and then observed under a fluorescence microscope. We counted the number of intense punctate GFP-LC3 aggregates in each cell to assess autophagy. The results showed that H/R increased the level and aggregation of GFP-LC3 in cells, and this phenomenon was amplified by MenSCs and particularly by primed MenSCs (Figures 8A and B). In addition, the effect of primed MenSCs on GFP-LC3


A
3-[2-(3,7-dimethylocta-2,6-dien-1-yl)-3,4,6-trihydroxyphenyl]propanoic acid
Asn Val Pro Gly
Glycylproline
1-Kestose
3-Galactosyllactose
Maltotriose
3,4,5-trihydroxy-6-{3-[(E)-2-(4-hydroxyphenyl)ethenyl]-5-{[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}phenoxy}oxane-2-carboxylic acid
Maltopentaose
Nebularine
Stachyose
Didymin
2-Cyclopentene-1,4-dione
Maltotetraose
3-hydroxy-3-methyl-Glutaric acid
Muramic acid
PE(15:0/22:2(13Z,16Z))
2-O-Methylcytosine
PC(14:0/22:2(13Z,16Z))
PC(16:0/18:2(9Z,12Z))
Neuromedin N (1-4)
PE-NMe(16:0/22:4(7Z,10Z,13Z,16Z))
8-[(Aminomethyl)sulfanyl]-6-sulfanyloctanoic acid
GPEtn(18:0/20:5)
GPEtn(16:0/20:4)
GPEtn(12:0/24:4)
N-Acetyl-D-phenylalanine
2,2'-(3-methylcyclohexane-1,1-diyl)diacetic acid
2-Methyl-3-(2-pentenyl)-2-cyclopenten-1-one
C16 Sphinganine
N-Acetyltyramine
PC(16:0/18:1(11Z))
(S)-Edulinine
Farnesylcysteine
Lumichrome
M-Xylene
Alpha-Methylstyrene
Serotonin
GPCho(18:0/20:4)
2-Dodecylbenzenesulfonic acid
GPCho(16:0/22:6)
NORETHINDRONE ACETATE
GPEtn(16:0/22:6)
GPCho(18:2/18:2)
Xanthosine
Lucidenic acid F
2
1
0
-1
-2
IRPM_1
IRPM_2
IRPM_3
IR_1
IR_2
IR_3
B
IRPM_vs_IR.volcano
Up
Nosig
Down
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
-2.5 -2 -1.5 -1 -0.5 0 0.5 1 1.5 2
E
PE(15:0/22:2(13Z,16Z))
IRPM
IR
6.3
6.2
6.1
6.0
5.9
5.8
5.7
a
IRPM
IR
C
Pathway calssification
Sensory system
Nervous system
Digestive system
Nucleotide metabolism
Metabolism of terpenoids and polyketides
Metabolism of cofactors and vitamins
Lipid metabolism
Glycan biosynthesis and metabolism
Carbohydrate metabolism
Biosynthesis of other secondary metabolites
Amino acid metabolism
Infectious disease: viral
Cancer: overview
Signaling molecules and interaction
Signal transduction
Membrane transport
Transport and catabolism
Cellular community - eukaryotes
0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5
Number of compounds
Organismal systems
Metabolism
Human diseases
Environmental information processing
Cellular processes



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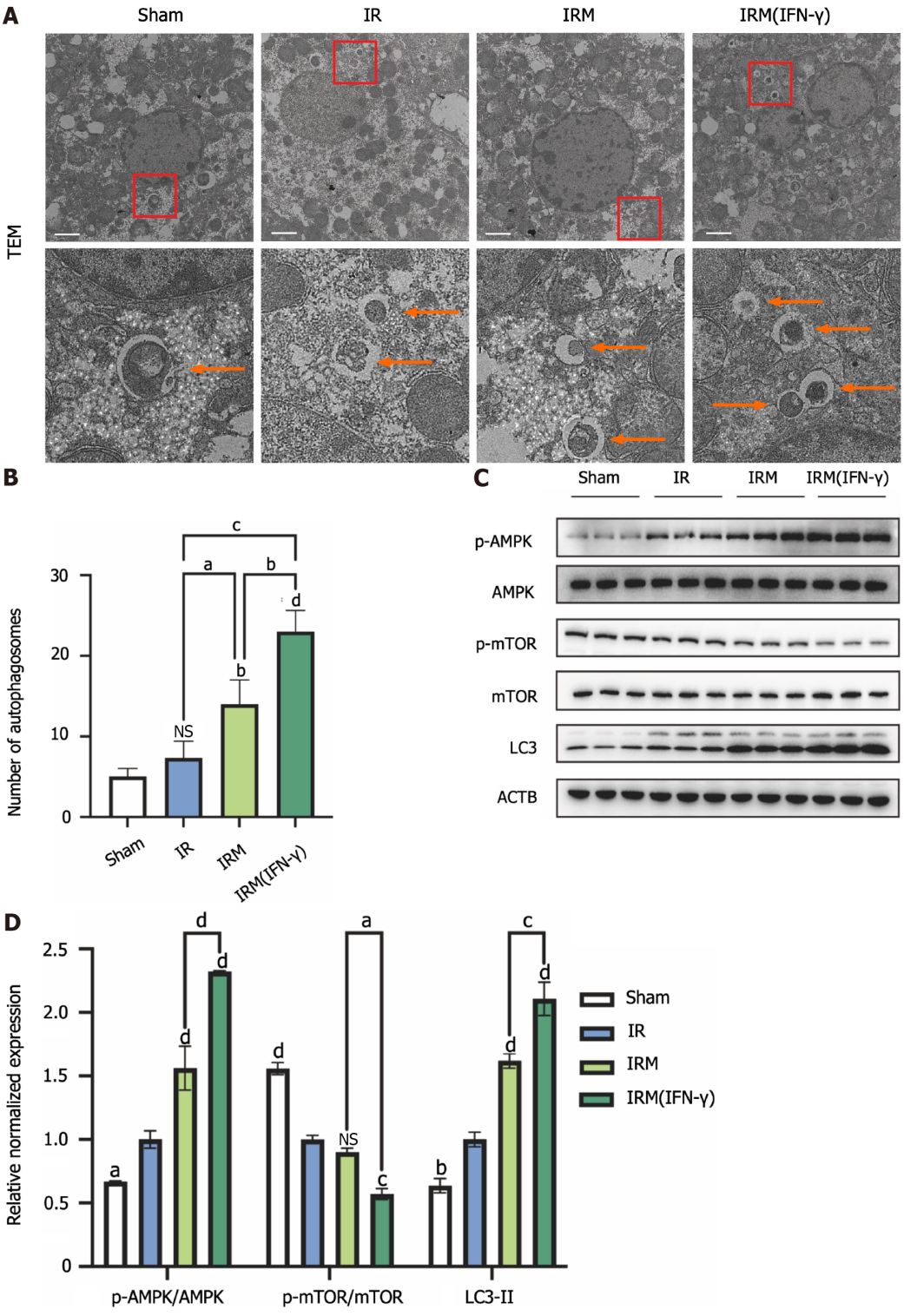
Figure 5 Metabolomic data analysis. A: Heatmap of 45 differential metabolites between the ischemia-reperfusion (IR) group and IRM (interferon-γ) group; B: Volcano plot of 45 differential metabolites; C: The relevant pathways in which the differential metabolites are involved were analyzed by comparing the information in the Kyoto Encyclopedia of Genes and Genomes database; D: The main biological functions of differential metabolites were elucidated by pathway enrichment analysis; E: Relative quantitation of the differential metabolite phosphatidyl ethanolamine was performed to measure the effect of primed menstrual blood-derived stromal cells on the level of autophagy in liver tissues. MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; IR: Ischemia-reperfusion; IRM(IFN-γ) or IRPM: Combination treated with ischemia-reperfusion and interferon-γ-primed menstrual blood-derived stromal cells; PE: Phosphatidyl ethanolamine; OS: Organismal systems; M: Metabolism; HD: Human diseases; CP: Cellular processes; EIP: Environmental information processing; KEEG: Kyoto Encyclopedia of Genes and Genomes.

punctate aggregation was reversed by 1MT, which suggested that IDO was related to the promotion of autophagy (Figures 8A and B). Then, we measured the changes in autophagy-related protein expression by western blotting to further explore how IDO affects autophagy. The results showed that H/R could inhibit the mTOR pathway, activate the AMPK pathway, and increase autophagy, and these effects were enhanced by MenSCs and particularly by primed MenSCs (Figures 8C and D); furthermore, the enhanced effect of primed MenSCs on autophagy was reversed by the IDO inhibitor 1MT (Figures 8C and D). These results indicated that IDO secreted by primed MenSCs enhanced autophagy by inhibiting the mTOR pathway and activating the AMPK pathway.

DISCUSSION

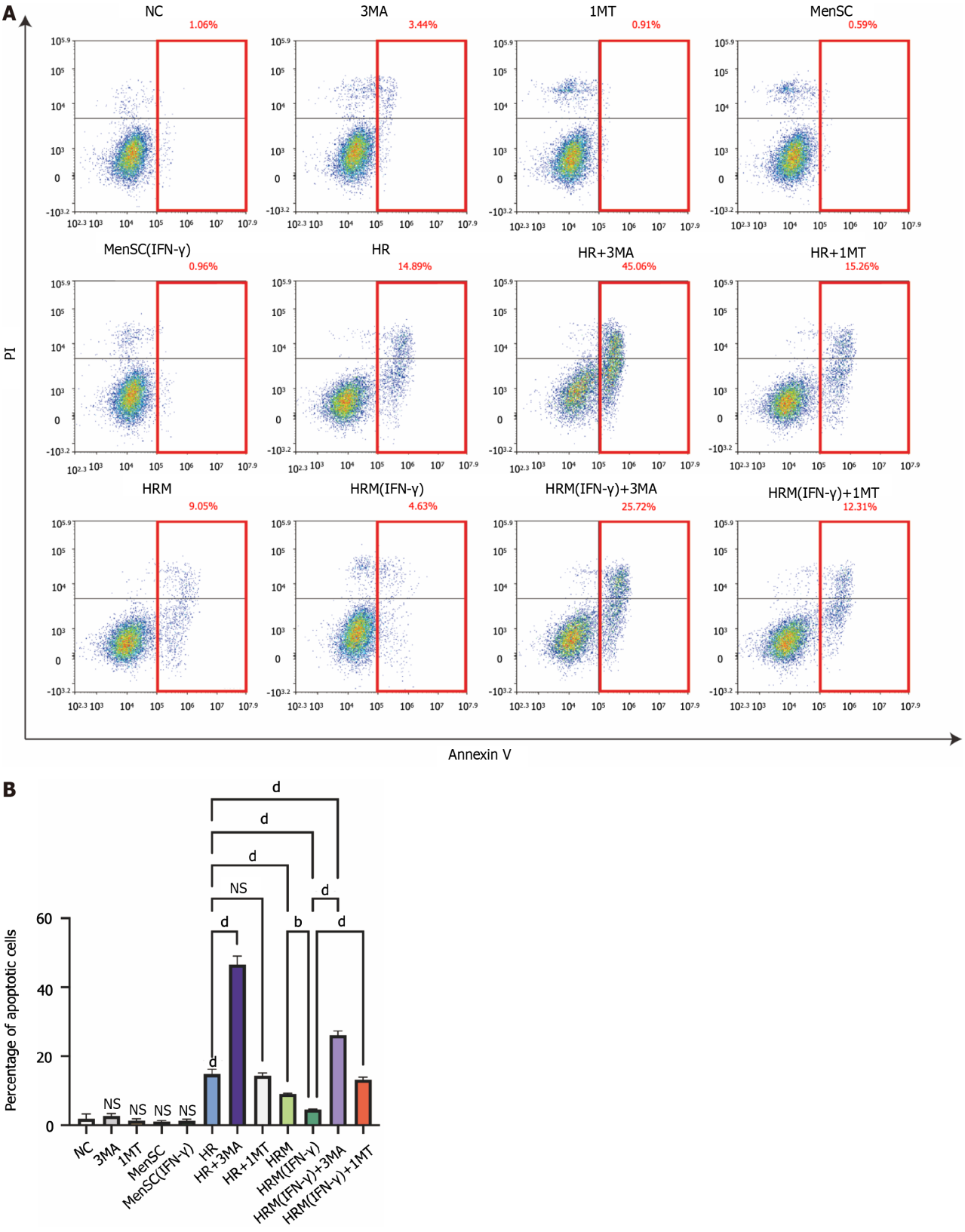
In this study, we found that IDO secreted by IFN-γ-primed MenSCs enhanced the therapeutic effect of MenSCs in the mouse liver I/R model, and the related mechanisms are shown in Figure 9. However, some studies have indicated that the combination of IFN-γ and tumor necrosis factor-alpha (TNF-α) could more efficiently promote the secretion of IDO by MSCs[12]. Although our study showed that priming with 100 ng/mL IFN-γ for 72 h was sufficient to initiate IDO secretion by MenSCs, priming with multiple inflammatory factors to generate better and more functional MenSCs still has a very high experimental value. However, considering that the high concentration of IFN-γ decreased the tolerance of MenSCs to H/R-induced injury (Figures 2E and F), we hypothesized that the priming of MenSCs with multiple inflammatory cytokines may cause more severe damage. Consequently, the side effects of these inflammatory cytokines on MSCs need to be further investigated *in vitro* and *in vivo*.

In addition to priming MSCs with some inflammatory factors, such as transforming growth factor-β, IL-17, TNF-α, and IFN-γ, to promote the secretion of immune regulatory cytokines[26-28], MSCs could also be modified by gene editing to overexpress some immune regulatory factors and thus enhance their therapeutic effects. In one study, MSCs that overexpressed IDO were more effective in alleviating injury in dilated cardiomyopathy in mice and further enhanced the responses of Tregs and Th2 cells[29]. Therefore, subsequent studies may examine the differences in safety, efficacy, and cost between these two approaches to identify the more suitable method of MSC modification.



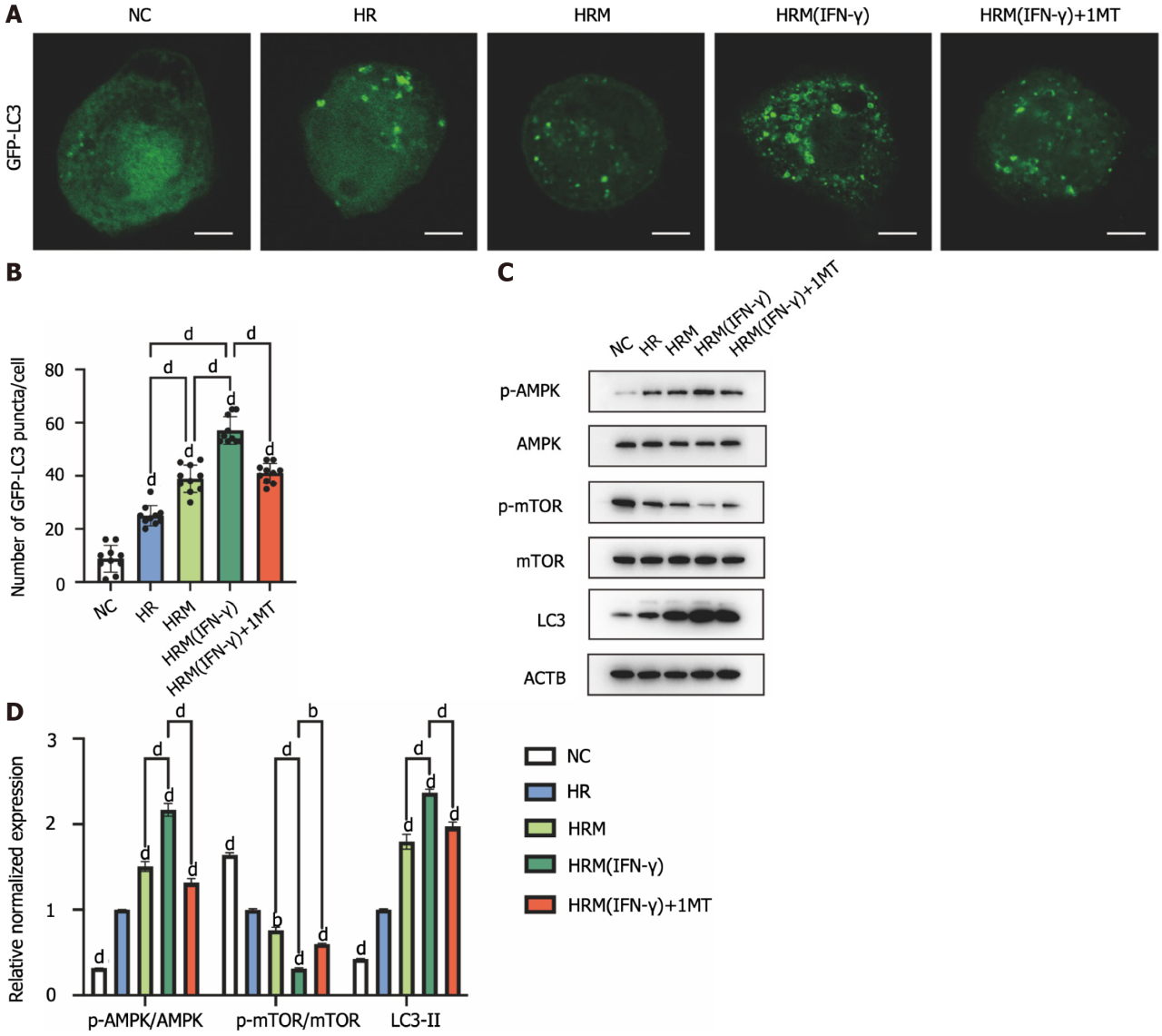
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Figure 6 Interferon-γ-primed menstrual blood-derived stromal cells enhanced autophagy in mouse livers. A: Representative TEM images were used to determine the number of autophagic vacuoles in liver tissues from each experimental group. Scale bar 2 μm. The images in the second row are partial magnifications of the regions indicated by the red box; B: The number of autophagosomes was counted by TEM. The data are expressed as the means ± SEMs (n = 3/group); C: The expression of p-AMPK, AMPK, p-mammalian target of rapamycin (p-mTOR), mTOR, LC3, and ACTB in liver tissues was determined by western blotting; D: The relative normalized expression was quantified based on the intensities of the western blot bands (n = 3/group). The data are presented as the means ± SEMs. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. NS represents not statistically significant. All P values were obtained by one-way ANOVA. MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; IR: Ischemia-reperfusion; IRM: Combination treated with ischemia-reperfusion and menstrual blood-derived stromal cells; IRM (IFN-γ): Combination treated with ischemia-reperfusion and interferon-γ-primed menstrual blood-derived stromal cells; TEM: Transmission electron microscopy; AMPK: The AMP-activated protein kinase signaling pathway; mTOR: Mammalian target of rapamycin; Tregs: Regulatory T cells.



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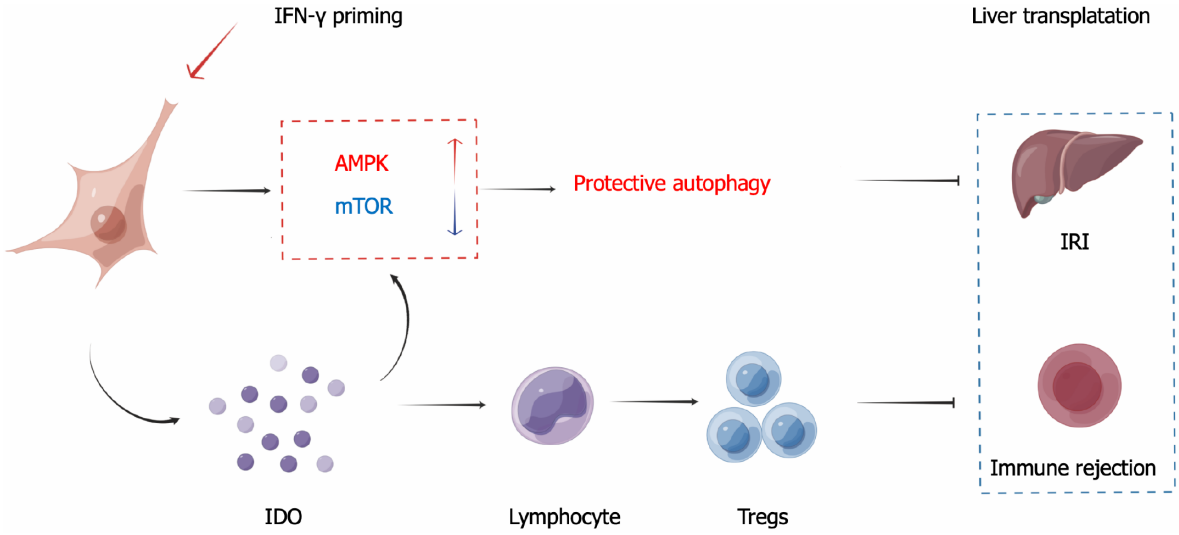
Figure 7 interferon-γ-primed menstrual blood-derived stromal cells decreased hypoxia/reoxygenation-induced cell apoptosis *via* autophagy and indoleamine 2,3-dioxygenase. A: Apoptosis of L02 cells after different treatments was analyzed; B: The sum of the percentage of early apoptotic cells and late apoptotic cells was analyzed. The total percentage of apoptotic cells (sum of the percentage of early apoptotic cells and late apoptotic cells) was analyzed. The data are shown as the mean ± SEM, $n = 3$; $^{b}P < 0.01$, $^{d}P < 0.0001$. NS represents not statistically significant. All P values were obtained by one-way ANOVA. PI: Propidium iodide; NC: Negative control; 3MA: 3-methyladenine; 1MT: 1-methyl-D-tryptophan; MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; HR: Hypoxia/reoxygenation; HRM: Combination treated with hypoxia/reoxygenation and menstrual blood-derived stromal cells; HRM (IFN-γ): Combination treated with hypoxia/reoxygenation and I interferon-γ-primed menstrual blood-derived stromal cells.



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Figure 8 Indoleamine 2,3-dioxygenase secreted by interferon-γ-primed menstrual blood-derived stromal cells enhanced autophagy by inhibiting the mammalian target of rapamycin pathway and activating the AMPK pathway. A: Immunofluorescence images of GFP-LC3. Scale bar 2 μm; B: The number of intense punctate GFP-LC3 aggregates in each cell was counted to determine the level of autophagy in each group. The data are expressed as the means ± SEMs (n = 10/group); C: The expression of p-AMPK, AMPK, p-mammalian target of rapamycin (p-mTOR), mTOR, LC3, and ACTB in L02 cells was determined by western blotting; D: The relative normalized protein expression was quantified based on the intensities of the western blot bands (n = 3/group). The data are presented as the means ± SEMs. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. NS represents not statistically significant. All P values were obtained by one-way ANOVA. NC: Negative control; 3MA: 3-methyladenine; 1MT: 1-methyl-D-tryptophan; MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; HR: Hypoxia/reoxygenation; HRM: Combination treated with hypoxia/reoxygenation and menstrual blood-derived stromal cells; HRM (IFN-γ): Combination treated with hypoxia/reoxygenation and I interferon-γ-primed menstrual blood-derived stromal cells; AMPK: AMP-activated protein kinase; mTOR: the Mammalian target of rapamycin signaling pathway.

To date, 79 clinical trials involving the use of MSCs to prevent or treat graft-*versus*-host disease (GVHD) have been conducted[30]. Most of the studies demonstrated the safety and efficacy of MSCs, but some studies showed that MSCs did not exert the desired protective effect; differences in the immunomodulatory effects of MSCs may be related to the origin and the dose of MSCs that were used. One study compared the therapeutic potential of MSCs from different origins in a mouse GVHD model and found that bone marrow-derived MSCs (BM-MSCs) induced a rapid proinflammatory response before exerting immunosuppressive effects and that BM-MSCs were less capable of inducing Treg differentiation than umbilical cord blood-derived MSCs (UC-MSCs)[31]. Therefore, differences in the origin of MSCs might be a reason why some MSCs did not exert the expected effects in some studies[7,8]. It has also been suggested that subsequent studies on MSCs may focus on the differences in the therapeutic potential of MSCs that are derived from different sources. Moreover, another study showed that a double dose of UC-MSCs exerted a better therapeutic effect than a single dose of UC-MSCs in a rat dilated cardiomyopathy model[29]. Consequently, in addition to improving MSCs, selecting the right MSC source and the right MSC dose is also crucial for research.



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Figure 9 Schematic depiction of the mechanism. Ischemia-reperfusion injury (IRI) and immune rejection are two important factors that affect the prognosis of liver transplantation. After priming menstrual blood-derived stromal cells (MenSCs) with interferon-γ, the MenSCs secreted high levels of indoleamine 2,3-dioxygenase (IDO). On the one hand, MenSCs and IDO enhanced the AMPK-mammalian target of rapamycin-autophagy axis to reduce IRI; on the other hand, IDO promoted the differentiation of lymphocytes into Tregs to reduce immune rejection. IRI: Ischemia-reperfusion injury; MenSCs: Menstrual blood-derived stromal cells; IFN-γ: Interferon-γ; IDO: Indoleamine 2,3-dioxygenase; AMPK: AMP-activated protein kinase signaling pathway; mTOR: Mammalian target of rapamycin signaling pathway; Tregs: Regulatory T cells.

This study found that IDO secreted by IFN-γ-primed MenSCs was found to activate the AMPK pathway and inhibited the mTOR pathway, which resulted in inducing of protective autophagy in hepatocytes. However, the specific mechanism through which IDO activates the AMPK pathway or inhibits the mTOR pathway remains unclear. IDO is a metabolic enzyme that can promote tryptophan metabolism and produce kynurenine. Some studies have shown that IFN-γ priming and IDO overexpression can promote tryptophan depletion and kynurenine accumulation, and these phenomena induce autophagy in cervical cancer cells and promote phagocytosis by macrophages[32]. Studies have also shown that a protective autophagic response and amino acid metabolism, which are driven by IFN-γ-mediated induction of IDO enzyme activity, could inhibit antibody-mediated inflammatory kidney injury in a mouse model of nephrotoxic serum nephritis[33]. Another study showed that glucose metabolism is also related to the T cell inhibition caused by MSCs and IDO[34]. In this study, we found that primed MenSCs increased the relative expression of the autophagy-related metabolite PE in mouse livers, and corresponding experiments proved that primed MenSCs indeed promoted autophagy through IDO; however, we did not conduct targeted metabolomics analyses to quantify the absolute expression of PE. In addition, how IDO causes changes in the levels of PE are unclear. Therefore, further study is needed to determine how IDO triggers autophagy. Notably, tryptophan catabolism by IDO could alter inflammatory responses and favor T-cell tolerance in cancer[35,36]. Although there is no evidence showing that MSCs can promote tumor growth and our previous study showed that MSCs can inhibit the growth of hepatocellular carcinoma through epigenetic regulation[37], the use of primed MSCs in cancer patients may still be controversial.

In addition, because IDO secreted by MSCs could increase the Tregs to induce immune tolerance, as has been observed in several studies[14,17], we paid more attention to the potential of primed MenSCs to reduce liver IRI in this study, and only used the number of Tregs as an indicator to evaluate the MSC-induced - immune tolerance potential. Therefore, although primed MenSCs significantly increased the numbers of Tregs, it is still necessary to verify whether the increased Tregs could alleviate IRI[38] or better induce immune tolerance in the liver transplantation model. Besides, recent studies have shown that macrophage activation and neutrophil infiltration also play important roles in liver IRI[39,40], and the regulation of MSCs to different types of immune cells deserves further study.

Notably, untreated MenSCs could also activate the AMPK pathway, inhibit the mTOR pathway and enhance autophagy in the I/R or H/R models; however, untreated MenSCs secreted little IDO (Figures 2G-I). This finding indicates that the effect of MenSCs in enhancing autophagy is not entirely dependent on IDO and may be related to other mechanisms. How stem cells enhance autophagy in recipient cells is still worth further study.

CONCLUSION

IFN-γ-primed MenSCs could exert a better therapeutic effect in a liver I/R model than untreated MenSCs by secreting higher levels of IDO. On the one hand, MenSCs and IDO enhanced the AMPK-mTOR-autophagy axis to reduce IRI; on the other hand, IDO increased the Treg numbers in the spleen and enhanced the ability of MenSCs to induce immune tolerance. Our study suggests that IFN-γ-primed MenSCs may be a novel and improved MSC product for liver transplantation in the future.

ARTICLE HIGHLIGHTS

Research background

Ischemia-reperfusion injury (IRI) and immune rejection are two important factors that affect the prognosis of liver transplantation. Mesenchymal stem cells (MSCs) have been used in liver transplantation and showed certain beneficial effects.

Research motivation

Some studies have indicated that the effects of MSCs are not very significant. Therefore, there is an urgent to find a new way to make MSCs exert better therapeutic effect.

Research objectives

In this study, we attempted to enhance the therapeutic potential of human menstrual blood-derived stromal cells (MenSCs) in a mouse liver ischemia-reperfusion (I/R) model through interferon-γ (IFN-γ) priming strategies and explore the specific mechanisms.

Research methods

After determining the appropriate priming strategies, we applied MenSCs and primed MenSCs in the short-term and long-term mouse liver I/R models, and compared their therapeutic effects. In order to further explore the specific mechanism, we performed a metabolomic analysis of mouse liver samples.

Research results

IFN-γ-primed MenSCs secreted higher levels of indoleamine 2,3-dioxygenase (IDO), attenuated liver injury, and increased regulatory T cell (Treg) numbers in the mouse spleens to greater degrees than untreated MenSCs. Metabolomics and autophagy analyses proved that primed MenSCs more strongly induced protective autophagy in the mouse livers. Further experiments proved that IDO enhanced the protective autophagy by inhibiting the mammalian target of rapamycin (mTOR) pathway and activating the AMPK pathway.

Research conclusions

IFN-γ-primed MenSCs exerted better therapeutic effects in the liver I/R model through secreting higher IDO levels. IDO, on the one hand, increased Treg numbers in the spleen; on the other hand, activated the AMPK-mTOR-autophagy axis to reduce IRI.

Research perspectives

Our study suggests that IFN-γ-primed MenSCs may be a novel and superior MSC product for liver transplantation in the future, and the specific mechanism of how MSCs or IDO enhance autophagy in recipient cells is still worth further study.

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FOOTNOTES

Author contributions: Zhang Q, Zhou SN, Chen LJ, Xu ZY, and Xiang C designed the research study; Zhang Q, Zhou SN, Fu JM, Fang YX, Xu HK, Yuan Y, Huang YQ, Zhang N, and Li YF performed the research; Zhang Q, Zhou SN, and Chen LJ analyzed the data and wrote the manuscript; and all authors have read and approved the final manuscript.

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Laboratory Animal Welfare and Ethics Committee of Zhejiang University (ZJU-IACUC), and the ethics code is ZJU20210299.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: All data mentioned in the article can be obtained by contacting the corresponding author.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

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ORIGINAL ARTICLE

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Mechanism of adipose-derived mesenchymal stem cell exosomes in the treatment of heart failure

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Abstract

BACKGROUND

Heart failure (HF) is a global health problem characterized by impaired heart function. Cardiac remodeling and cell death contribute to the development of HF. Although treatments such as digoxin and angiotensin receptor blocker drugs have been used, their effectiveness in reducing mortality is uncertain. Researchers are exploring the use of adipose-derived mesenchymal stem cell (ADMSC) exosomes (Exos) as a potential therapy for HF. These vesicles, secreted by cells, may aid in tissue repair and regulation of inflammation and immune responses. However, further investigation is needed to understand the specific role of these vesicles in HF treatment.

AIM

To investigate the mechanism of extracellular vesicles produced by ADMSC s in the treatment of HF.

METHODS

Exogenous surface markers of ADMSCs were found, and ADMSCs were cultured.

RESULTS

The identification of surface markers showed that the surface markers CD44 and CD29 of adipose-derived stem cells (ADSCs) were well expressed, while the surface markers CD45 and CD34 of ADSCs were negative, so the cultured cells were considered ADSCs. Western blotting detected the Exo surface marker protein, which expressed CD63 protein but did not express calnexin protein, indicating that ADSC-derived Exos were successfully extracted.

CONCLUSION

The secretion of MSCs from adipose tissue can increase ATP levels, block cardiomyocyte apoptosis, and enhance the heart function of animals susceptible to HF. The inhibition of Bax, caspase-3 and p53 protein expression may be related to

this process.

Key Words: Adipose-derived mesenchymal stem cell exosomes; Heart failure; Cardiomyocyte apoptosis

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Core Tip: Our study highlights the potential of mesenchymal stem cell exosomes (Exos) from adipose tissue as a promising therapy for heart failure (HF). Administration of adipose-derived mesenchymal stem cell Exos improved cardiac function, evidenced by increased ATP content and enhanced parameters like ejection fraction, fractional shortening, and stroke volume. Furthermore, adipose-derived stem cells (ADSCs)-Exo reduced serum levels of b-type natriuretic peptide and atrial natriuretic peptide, associated with HF progression, and exhibited anti-apoptotic effects by regulating pro- and anti-apoptotic proteins in cardiac tissue. These findings suggest that ADSCs-Exo could prevent cardiomyocyte death and inhibit HF progression.

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INTRODUCTION

Heart failure (HF), which is mainly caused by impaired ventricular diastolic or systolic function, is characterized by arterial ischemia or venous congestion. As the final stage of many diseases, HF is a significant public health issue that needs to be addressed globally[1,2]. Numerous studies have found that HF develops and is directly correlated with cardiac remodeling, while cardiomyocyte apoptosis is an important factor leading to myocardial remodeling[3,4]. Although the clinical treatment of HF has been effective in recent years, the prognosis for patients is generally dismal, and hospitalization and fatality rates are still high. We review recent progress in the diagnosis and treatment of chronic HF. Digoxin has been used clinically for more than 200 years and is an essential drug in the treatment of chronic HF. However, the results of the DIG study confirmed that digoxin did not reduce or increase mortality compared with that of the placebo but was superior to placebo in reducing hospitalization rates for HF. In recent years, with the accumulation of ELITEII, OPTIMAAL and VALIANT studies, especially the results of recent CHARM trials, the role of angiotensin receptor blocker drugs in the treatment of HF has been improved. In particular, there is clear evidence that candesartan and valsartan reduce mortality and disability. The recently published HEAAL study showed that losartan 150 mg daily (high-dose group) was more effective than losartan 50 mg daily (low-dose group) in the treatment of HF. Therefore, it is difficult to explore the pathogenesis of HF and find an effective treatment for HF. Mesenchymal stem cells (MSCs) comprise cells with a wide range of differentiation potential and include MSCs from adipose tissue, umbilical cord, bone marrow, and other sources[5]. Because adipose tissue is more abundant than other MSCs and benefits from simple access and consumption, it has been widely studied by an increasing number of scholars[6,7]. However, adipose-derived MSCs (ADMSCs) have relatively high requirements during storage and transportation, which causes the survival rate of these cells during transplantation to significantly decline. Therefore, the clinical use of adipose-derived stem cells (ADSCs) has certain limitations. Exosomes (Exos) are lipid bilayer vesicles secreted by cells with a diameter of 30-200 nm. In addition to being secreted into cells, these Exos can also be stored for a long time in any environment at 4 °C and easily transported. The discovery of Exos provides a new direction for the clinical use of ADSCs[8,9]. All cells secrete Exos in both normal and pathological states. Exos are mainly involved in intercellular communication and regulate a series of physiological processes in target cells. Exos from different cell types have different functions. Some studies have found that ADSC Exos have the functions of tissue repair and regeneration, inflammation inhibition and immune regulation. However, the effect of ADSC Exos on HF has not been confirmed. The goal of this research is to investigate how ADMSC Exos work to treat HF.

MATERIALS AND METHODS

Animal experiment

The license number for Jinan Pengyue Experimental Animal Breeding Co., LTD sale's of the rats used in this investigation was SCXK (Lu) 2019-0003. There were 30 rats with a body weight of 180-220 g Wistar and 20 C57 mice, all of which were male specific pathogen-free (SPF) grade. All the experimental animals were kept in the SPF animal room, drinking water freely and providing animal feed according to the standard. The room temperature was controlled at 20 °C to 26 °C, and the humidity was controlled at 50%-60%, ensuring that the indoor environment was controlled at 12 h/12 h alternating day and night.

Reagents and instruments

Mouse adipose stem cell complete medium (Suzhou Saiye Biotechnology Co., LTD.); antibodies against Bcl-2, Bax, and caspase-3 (Cell Signaling Technology, United States); hematoxylin staining and eosin staining (Beijing Yili Fine Chemicals Co., LTD.); ELISA kit (Wuhan Fien Biotechnology Co., LTD.); polyvinylidene fluoride (PVDF) transfer membrane (Millipore, United States); goat anti-rabbit IgG antibody that was HRP-labeled (Shanghai Biyuntian Biotechnology Co., LTD.); IE33 heart color exclusive Opo ultrasound instrument (Philips, Netherlands); RM2245 microtome and DW4-B biological microscope (Leica, Germany).

Extraction and culture of ADSCs

Ten C57 mice were sacrificed in a sterile environment, and the subcutaneous adipose tissue of the mice was taken and cut into pieces until the tissue was erosive and placed in a centrifuge tube. Then, 0.5% collagenase type I was added to the centrifuge tube, and the centrifuge tube was digested for 45 min in the dark. After that, an equal volume of α-MEM medium was added to the centrifuge tube to terminate digestion. The mixture in the centrifuge tube was filtered through a 200-mesh sterile steel sieve, and the filter was collected and centrifuged at 15000 r/min for 5 min. The supernatant was thrown away, and an equal volume of red blood cell lysate was added to the centrifuge tube to lyse the cells. After 5 min, the cells were centrifuged again, and then α-MEM complete medium was added to the centrifuge tube to obtain the cell suspension, which was completely spread in the culture dish. After 24 h, the cells began to grow adherent, and all the nonadherent cells were removed. Under a microscope, the cell morphology was observed, and the cell surface expression markers CD29, CD44, CD34 and CD45 were detected by a cell loss analyzer.

Extraction and identification of Exos derived from ADSCs

Fourth-generation ADSCs with a fusion degree of 80% were used, and serum-free MEM was used instead of complete medium. Cell supernatant was collected after 48 h of culture. The cells received the same amount of Exo extraction reagent and were cultured overnight at 4 °C. Then, the cells were placed into a centrifuge tube and centrifuged at 15000 r/min for 30 min to separate the Exos. The morphology of the Exos was identified by transmission electron microscopy, and the protein expression related to the Exos was determined by western blotting.

Grouping and model preparation

Thirty Wistar rats received 7 d of adapted feeding. Using a random number system, the rats were divided into groups that were not given any instructions. (Control group), HF rat model group (HF group), and HF rat model treated with ADSC-derived Exo intervention group (ADSCS-Exo group). Each group included ten rats. Except for the control group, the other rats were intraperitoneally injected with 0.8 mg/kg doxorubicin solution at a dose of 3 mg/kg in the first three weeks and 2 mg/kg in the second 3 wk. For six weeks, the control group of rats received weekly intraperitoneal injections of the same volume of NaCl solution. After the intervention, the cardiac function indices of the rats in each group were measured. The establishment of the rat model of HF was demonstrated by a left ejection fraction (LVEF) of 45%. Rats in the ADSC-Exo group were injected with 100 μL of ADSC-derived Exos suspension at a concentration of 0.2 mg/mL through the tail vein, and identical doses of phosphate buffered saline (PBS) solution were injected into the tail veins of the rats in the control and HF groups. Rats in each group were injected once every 2 d for two consecutive times.

Determination of cardiac function indices

After the drug intervention, the rats in each group were fasted for 12 h and allowed to drink water freely. The rats were fixed in the supine position after receiving an intraperitoneal injection of 20% urethane to induce anesthesia. By using color Doppler echocardiography, cardiac function markers, such as left ventricular ejection fraction, were found. Three consecutive cardiac cycles were used to test each group's LVEF, left ventricular fractional shortening (LVFS), and stroke volume (SV), with the average value being used.

Serum b-type natriuretic peptide and atrial natriuretic peptide levels were measured

The abdominal aortas of rats in each group were sampled for 5 mL of blood. After standing for 2 h at 3000 rpm and 4 °C, the blood samples were centrifuged for 10 min. According to the directions on the ELISA kit, serum b-type natriuretic peptide (BNP) was found. According to the directions on the ELISA kit, BNP and atrial natriuretic peptide (ANP) serum levels were discovered.

The pathological changes in myocardial histopathology were detected by hematoxylin and eosin staining

The rats in each group were killed by cutting the neck and chest to remove the blood stains on the surface of the heart disease. After placing the heart in ice, all tissues except the left ventricular myocardial tissue were removed. Dry filter paper was used to cut part of the left ventricular apex group. After dehydration, the heart was fixed in 4% paraformaldehyde solution, made transparent, and embedded in paraffin sections with a thickness of 4 μm. Hematoxylin and eosin (HE) staining, neutral resin and biopsy tissue were used to observe each group of sections under the microscope to find any abnormal myocardial changes.

Flow cytometry was used to detect cardiomyocyte apoptosis

By using a mechanical process, a single-cell suspension was created. Put AGAR in 200 stainless steel net, cut up the myocardial tissue in 2 net, rub while washed with PBS solution, after being fully knead, collected in AGAR suspension again through 300 mesh sieve, collected after cell suspension in centrifuge tube, in a speed of 2000 r centrifugal 5 min per

minute, suck out and discard the supernatant, collected after the cells. The cells were resuspended with PBS solution at 4 °C, then placed in the centrifuge tube again under the same conditions as above, washed and adjusted the cell density to 1 × 10^5/ mL. The cells were resuspended in 300 μL binding buffer, and after that, the cells were gently mixed with 5 L of Annexin V-FITC. The cells were cultured at room temperature for fifteen minutes, and then 5 μL PI and 200 μL binding buffer were added. To determine which group of cardiomyocytes had undergone apoptosis, the centrifuge tube was placed into a flow cytometer.

Comparison of ATP content in myocardial tissue of rats in each group

One hundred milligrams of rat myocardial tissue was taken, 500 μL of perchloric acid was added, and at room temperature for fifteen minutes, 200 μL of supernatant was taken, and 120 μL of 1 mol/L sodium hydroxide (NaOH) was added. Following a 5-min, 5000 r/min, 4 °C centrifugation, ATP content in rat myocardial tissue was detected by high performance liquid chromatography.

Western blotting was used to detect the protein expression of Bcl-2, Bax, caspase-3 and p53 in myocardial tissue

Fifty milligrams of myocardial tissue from each rat was lysed on ice until tissue homogenization was performed by centrifugation. The DAB technique was used to determine the protein content in the supernatant. The concentrations of the concentration and separation gel were 5% and 8%, respectively. The sample protein was added to the gel well, and the membrane was transferred after electrophoresis for 10 min. After 2 h of incubation, 5% blocking solution was added to the sample, after which it was activated and transported to the PVDF membrane, and the PVDF membrane was blocked for one hour. Overnight, the primary Bcl-2, Bax, caspase-3, and p53 antibodies were added. HRP-labeled secondary antibodies were added and incubated for another 2 h. The PVDF membrane was detected by ECL and imaged.

Statistical methods

SPSS 26.0 software was utilized for the statistical evaluation. The mean and standard deviation of the data were written as (mean ± SD). One-way analysis of variance was used to compare the groups. The LSD-*t* test was used for homogeneity of variance, and the nonhomogeneity of variance was corrected using the *t* test. At $P < 0.05$, statistics were deemed significant.

RESULTS

Morphological changes of ADSCs

ADSCs began to grow adherent after 24 h of primary culture, and the cell fusion rate was 80% at 5-7 d. The size of cells in the first generation was inconsistent, and the morphology was mostly spindle, round and polygon. At the fourth passage, the morphology of ADSCs gradually changed to a long spindle shape, which was similar to that of fibroblasts and arranged in a spiral shape, and the nuclei became significantly larger (Figure 1).

Identification of ADSC surface markers

The ADSC surface markers CD44 and CD29 were positively expressed, and the expression rates were 99.4% and 94.8%, respectively. The ADSC surface markers CD45 and CD34 were negative, with expression rates of 0.026% and 0.213%, respectively. Thus, the cultured cells were ADSCs (Figure 2).

Morphology and protein identification of ADSC-derived Exos

The Exos were spotted using transmission electron microscopy to have a round shape and an unbroken cell membrane (Figure 3A). Western blot detection of Exo surface marker protein showed that CD63 protein was expressed, but Calnexin protein was not expressed, indicating that ADSC-derived Exos were successfully extracted (Figure 3B).

Comparison of cardiac function in each group

Rats in the HF group had considerably lower LVEF, LVFS, and SV values than those in the control group ($P < 0.05$). The LVEF, LVFS, and SV of rats in the ADSCS-EXO group were significantly greater than those in the HF group ($P < 0.05$) (Table 1).

Comparison of BNP and ANP levels in the serum of rats in each group

BNP levels in serum were significantly higher in the HF group of rats and ANP compared to those in the control group ($P < 0.05$). Rats in the ADSCS-Exo group had significantly lower serum levels of BNP and ANP than the HF group ($P < 0.05$) (Table 2).

Comparison of myocardial histopathological changes in each group

The control group cardiomyocytes had a distinct, orderly, and closely packed structure. There was no degeneration or necrosis of the cells and no edema or congestion in the interstitium. The configuration of the cardiomyocytes in the HF group was noticeably different from that of the control group, with obvious swelling, cell degeneration or even necrosis, myocardial fiber breakage, and obvious interstitial edema. In contrast to the HF group, the myocardial tissue of the ADSCS-Exo group was greatly enhanced, with loose arrangement of cardiomyocytes, reduced swelling and necrotic cells (Figure 4).

Table 1 Comparison of left ejection fraction, left ventricular fractional shortening and stroke volume in each group (mean ± SD, %)

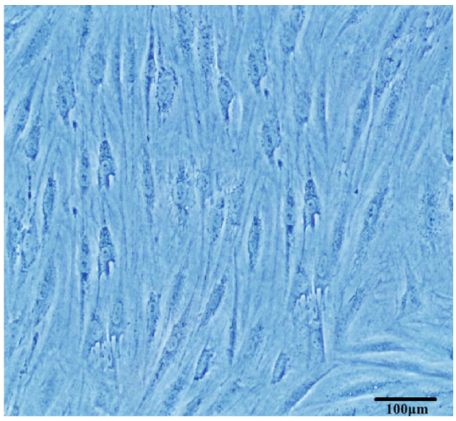
Group	*n*	LVEF	LVFS	SV
Control group	10	83.26 ± 3.41	50.01 ± 2.63	0.27 ± 0.03
HF group	10	40.16 ± 2.37[a]	28.65 ± 1.84[a]	0.13 ± 0.02[a]
ADSCs - exobiology group	10	70.85 ± 2.86[a,b]	40.27 ± 2.16[a,b]	0.22 ± 0.03[a,b]
F value		580.8	229.2	68.64
P value		< 0.0001	< 0.0001	< 0.0001

[a]*P* < 0.05, compared to control group.
[b]*P* < 0.05, compared with heart failure group.
ADSC: Adipose-derived stem cells; HF: Heart failure; LVEF: Left ejection fraction; LVFS: Left ventricular fractional shortening; SV: Stroke volume.

Table 2 Comparison b-type natriuretic peptide of and atrial natriuretic peptide contents in serum of rats in each group (mean ± SD, pg/mL)

Group	*n*	BNP	ANP
Control group	10	153.62 ± 10.42	123.46 ± 8.11
HF group	10	518.37 ± 15.91[a]	326.54 ± 11.42[a]
ADSCs - exobiology group	10	225.43 ± 13.65[a,b]	151.27 ± 9.34[a,b]
F value		2044	1283
P value		< 0.0001	< 0.0001

[a]*P* < 0.05, compared to control group.
[b]*P* < 0.05, compared with heart failure group.
ADSC: Adipose-derived stem cells; HF: Heart failure; BNP: B-type natriuretic peptide; ANP: Atrial natriuretic peptide.



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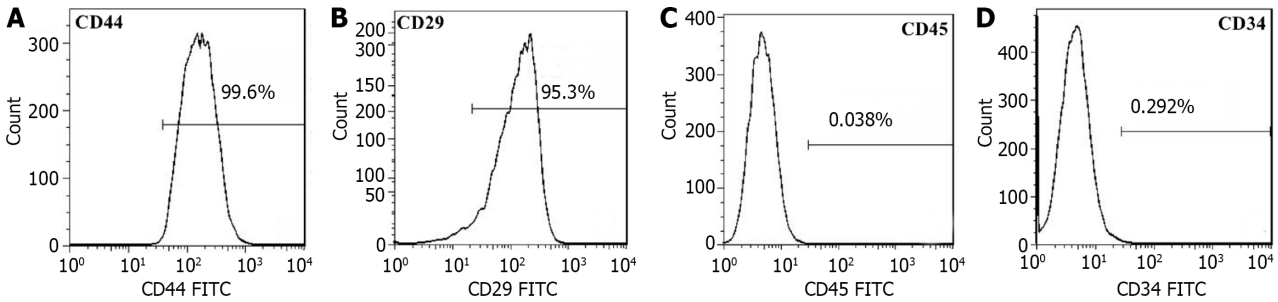
Figure 1 Morphology of adipose-derived stem cells at passage 4 (× 100).

Comparison of cardiomyocyte apoptosis rate in each group

The rate of cardiomyocyte apoptosis in the HF group was much greater than that in the control group ($P < 0.05$). The cardiomyocyte apoptosis rate of the ADSC-Exo group was much lower than that of the HF group ($P < 0.05$) (Figure 5).

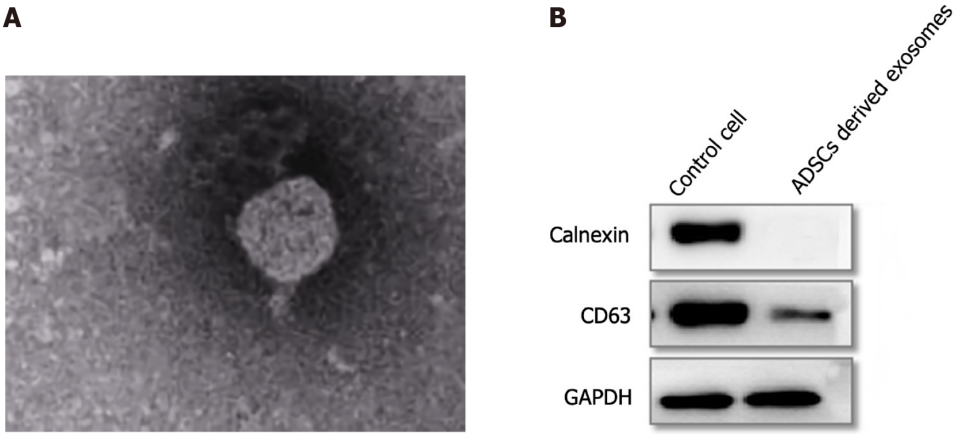
Comparison of ATP content in myocardial tissue of rats in each group

Rats in the HF group had considerably lower myocardial ATP content than those in the control group ($P < 0.05$). Rats in the ADSCS-Exo group had considerably more ATP in their cardiac tissue than the HF group ($P < 0.05$) (Figure 6).



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Figure 2 Detection of cell surface markers by flow cytometry. A: Positive expression of CD44; B: Positive expression of CD44; C: Negative expression of CD45; D: Negative expression of CD34.



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Figure 3 Morphology and protein identification of adipose-derived stem cell-derived exosomes. A: Adipose-derived stem cell (ADSC)-derived exosome morphology seen using transmission electron microscopy (× 15000); B: Surface detection of ADSC-derived exosomes by western blotting. ADSC: Adipose-derived stem cell.

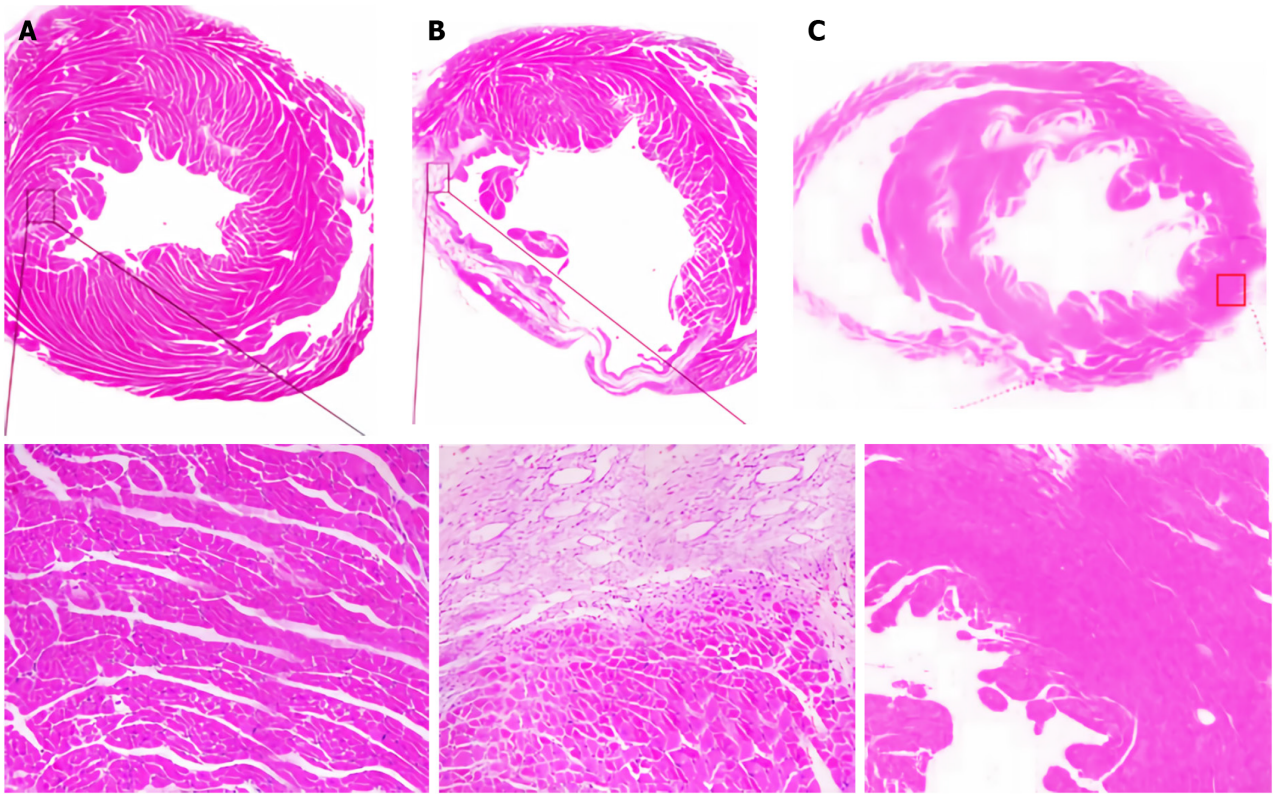
Comparison of protein expression of Bcl-2, Bax, caspase-3 and p53 in myocardial tissue of rats in each group

Bcl-2 protein expression was considerably decreased relative to the control group in the HF group, and the HF group's Bax, caspase-3, and expression levels of the p53 protein were much greater than those of the control group. p53, caspase-3, and Bax protein levels were all substantially lower in the HF group than in the control group, while Bcl-2 protein expression was substantially greater in the ADSC-Exo group than in the HF group ($P < 0.05$) (Figure 7).

DISCUSSION

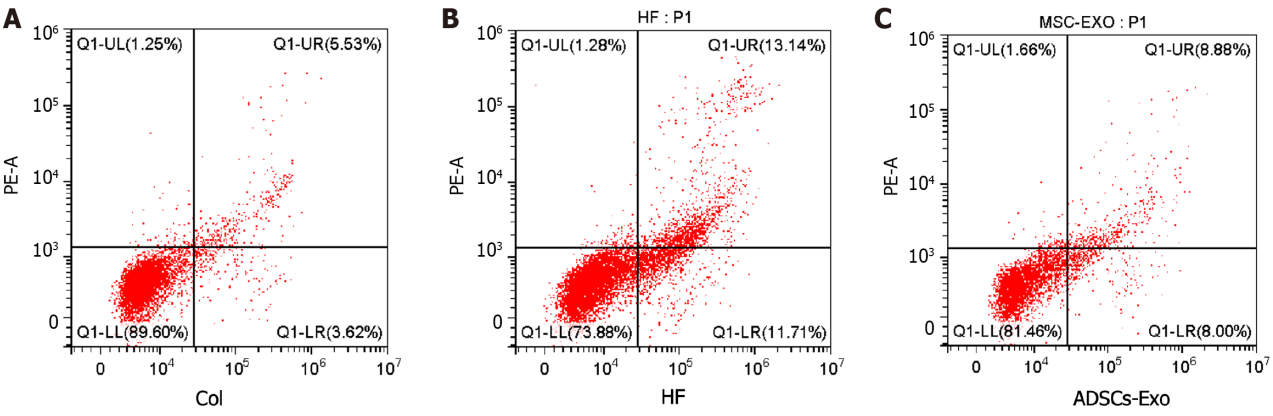
Current research suggests that HF is a chronic, naturally progressing condition[10]. HF is a disease with a pathogenesis induced by aberrant neuroendocrine system activation, which results in ventricular remodeling and cardiomyocyte death. When cardiomyocyte apoptosis occurs, the myocardium will not only maintain myocardial function due to a large amount of cell loss but also lead to myocardial failure and gradual deterioration when the number of cardiomyocytes is lost, thus forming a vicious cycle of pathological processes[11]. Therefore, by enhancing pump performance, prevention of cardiomyocyte apoptosis can successfully lower the incidence of myocardial remodeling and ease the onset and development of HF. Doxorubicin is a common anthracycline in clinical practice. Doxorubicin has been used to treat a variety of tumors, but its use is limited due to its dose-dependent and delayed cardiotoxicity[12]. Doxorubicin can often cause irreversible damage to the heart, including HF[13]. Doxorubicin was injected intraperitoneally to create a rat HF model for this investigation. LVEF < 45% was detected by cardiac color Doppler ultrasound, demonstrating the establishment of the rat model of cardiac failure. Current research on the molecular mechanism by which doxorubicin causes myocardial damage shows that doxorubicin can interact with DNA and inhibit protein synthesis-induced myocardial cell apoptosis, but there is currently no effective treatment for HF caused by adriamycin; thus, exploring new drugs for HF is a hotspot in current clinical studies[14].

ADSCs can grow rapidly in culture medium without excessive nutrient requirements, have strong proliferation and passage ability and are relatively stable in the genetic process, with multiple differentiation potential. Currently, it has been clinically found that ADSCs can purify ADSC-derived Exos in the supernatant of ADSC culture in either hypoxic or normoxic environments, and the diameter of the Exos is significantly larger than that of previously reported Exos[15].



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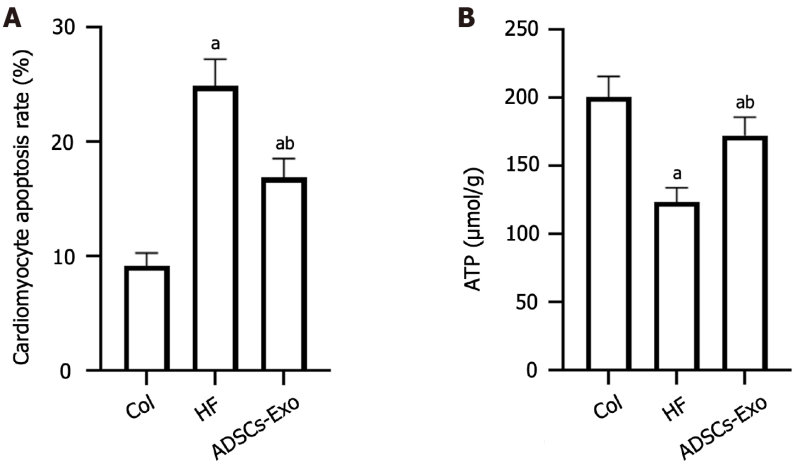
Figure 4 Hematoxylin and eosin staining of rat myocardial tissue. A: Myocardial tissue of rats in control group; B: Myocardial tissue of rats in heart failure group; C: Myocardial tissue of rats in adipose-derived stem cell-exosome group; Col: Control; HF: Heart failure; ADSCs-Exo: Adipose-derived stem cell-exosome.



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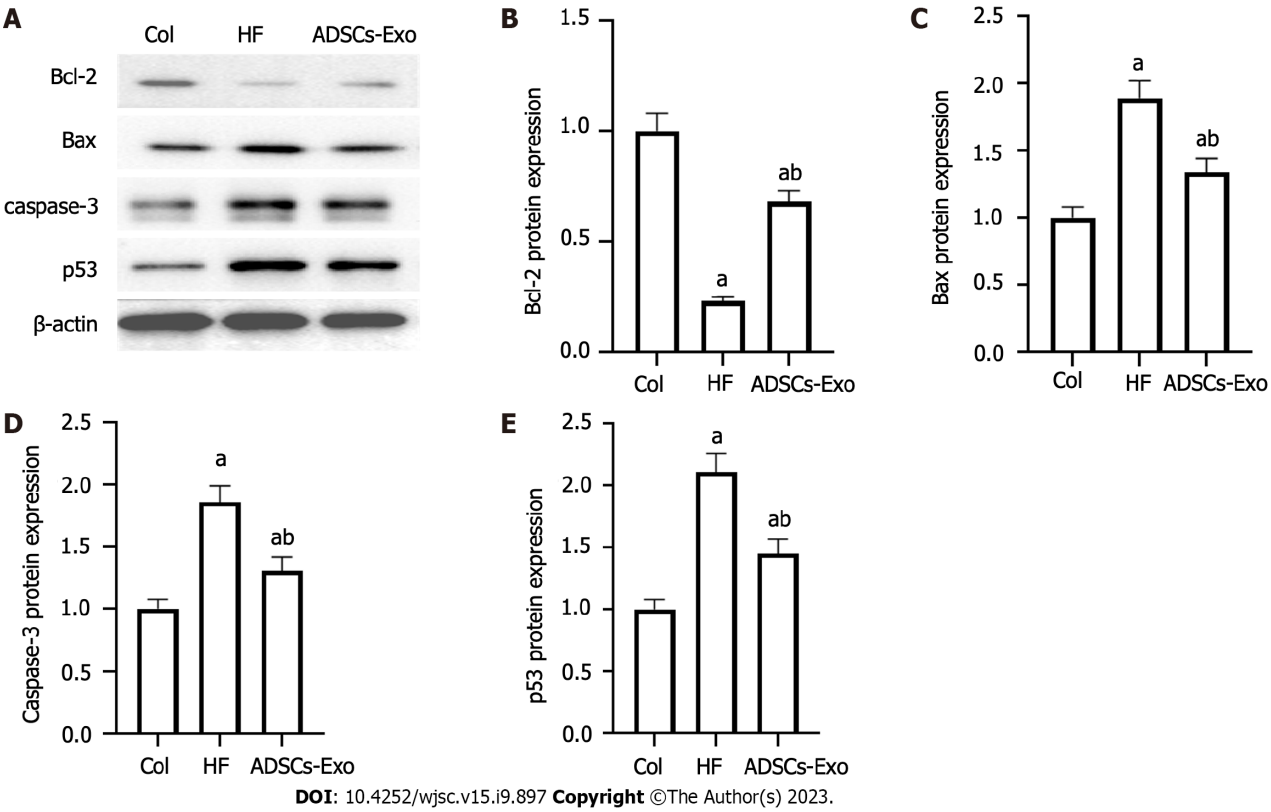
Figure 5 Comparison of cardiomyocyte apoptosis rate in each group. A: Cardiomyocyte apoptosis rate in control group; B: Cardiomyocyte apoptosis rate in heart failure group; C: Cardiomyocyte apoptosis rate in adipose-derived stem cell-exosome group. Col: Control; HF: Heart failure; ADSCs-Exo: Adipose-derived stem cell-exosome.

Numerous studies have found that Exos derived from ADSCs can promote vascular regeneration, wound healing, scar repair, and nerve regeneration and regulate immunity, the inflammatory response and tumor growth[16]. Some scholars have found that through the study of a myocardial ischemia model[17], ASC-derived Exos can inhibit cardiomyocyte apoptosis and alleviate myocardial structural remodeling. In this study, the surface markers of ADSC-Exos were identified, and Exos may be effectively extracted from adipose-derived mesenchymal cells and adipose tissue. After injecting ADSC-Exos into HF rats, it was found that the LVEF, LVFS and SV of rat heart function indices were significantly increased, suggesting that ADSC-Exos could alleviate cardiac function in rats with HF. Sun *et al*[18] discovered that Exos from MSCs generated from adipose tissue may treat old HF rats, and the mechanism may be due to miR-423-mediated control of 5p in the PI3K/Akt signaling pathway.



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Figure 6 Comparison of ATP content in myocardial tissue of rats in each group. A: Apoptosis rate of cardiomyocytes in three groups of rats; B: ATP content in myocardial tissue of rats in three groups. [a]*P* < 0.05 compared with the control group; [b]*P* < 0.05 compared with the heart failure group. Col: Control; HF: Heart failure; ADSCs-Exo: Adipose-derived stem cell-exosome.



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Figure 7 Comparison of the protein expression levels of Bcl-2, Bax, caspase-3, and p53 in the myocardial tissue of rats in each group. A: Myocardial tissue protein electrophoresis detection; B: Protein expression levels of Bcl-2; C: Protein expression levels of Bax; D: Protein expression levels of caspase-3; E: Protein expression levels of p53. [a]*P* < 0.05 compared with the control group; [b]*P* < 0.05 compared with the heart failure group. Col: Control; HF: Heart failure; ADSCs-Exo: Adipose-derived stem cell-exosome.

ANP is mainly synthesized by the atrium, where it is stored and secreted. ANP levels are frequently elevated in a number of illnesses, including HF, coronary heart disease, and chronic pulmonary obstruction, and elevated concentrations suggest poor prognosis. The findings of this investigation demonstrated that rats in the HF group had considerably higher serum levels of BNP and ANP, and serum BNP and ANP concentrations of rats after ADSCS-Exo intervention were significantly decreased, suggesting that by reducing the serum concentrations of BNP and ANP in HF-prone rats, ADSCS-Exo can enhance cardiac function. Chen and Li[19] showed that rats with HF have significantly higher serum levels of ANP and BNP, which can improve the cardiac function of patients by reducing the levels of ANP and BNP. The heart needs energy metabolism to sustain its typical physiological processes and energy needs because the most

direct source of energy for cellular functions is ATP. Some researchers have found that compared that of normal people, ATP in HF patients is significantly reduced by 30%-40%[20]. The findings of this investigation demonstrated that ADSC-Exos can enhance the amount of ATP in HF rat cardiac tissue, demonstrating that when HF occurs, ADSC-Exos can increase the amount of ATP in myocardial tissue and then maintain the energy required for normal myocardial activity by improving the dysfunction of mitochondrial energy metabolism. These results were consistent with the above studies.

The transition from compensatory to decompensatory HF is marked by cardiomyocyte apoptosis, which can be controlled by numerous genes associated with apoptosis, including the pro- and apoptotic protein Bcl-2. Bcl-2 protein family members that have received much research are Bcl-2 and Bax. The Bcl-2 to Bax ratio is highly associated with cell survival or death. By preserving mitochondrial integrity, cytochrome C cannot be released when Bcl-2 is present. By decreasing mitochondrial membrane potential, Bax can cause the release of cytochrome C and certain apoptotic precursors into the cytoplasm, and cytochrome C can form apoptotic bodies by activating APAF-1 and caspase-9. Cytochrome C can induce apoptosis by activating caspase-3 and caspase-7. P53 is a pro-apoptotic transcription factor that can mediate the induction of cardiomyocyte apoptosis and accelerate the progression of HF. When HF occurs, DNA is damaged in cardiomyocytes, and p53 protein is activated, which can accelerate the development of HF by inducing cardiomyocyte apoptosis by upregulating the competitive binding of Bax protein to Bcl-2 and lowering the Bcl-2 to Bax ratio by downregulating the expression of Bcl-2. The results of this study showed that ADSC-Exos could inhibit the expression of Bax, caspase-3 and p53 proteins in cardiac tissue and thereby improve cardiac function by inhibiting cardiomyocyte apoptosis. Sun *et al*[21] demonstrated that ADSC-Exo treatment can boost the production of the protein Bcl-2 while decreasing the presence of Bax and caspase-3 in cardiomyocytes and upregulate the ratio of Bcl-2/Bax to inhibit cardiomyocyte apoptosis, enhancing heart function in rats suffering from cardiomyocyte apoptosis.

CONCLUSION

In conclusion, our findings suggest that MSC Exos derived from adipose tissue have the potential to improve heart health in rats with HF. This finding is supported by the observed increase in ATP content and improvement in cardiac function parameters, such as LVEF, LVFS, and SV. In addition, the administration of ADSC-Exos was associated with a significant reduction in serum BNP and ANP levels, indicating a potential cardioprotective effect. Furthermore, ADSC-Exo treatment suppressed the expression of proteins involved in apoptosis, including Bax, caspase-3, and p53, while promoting the expression of the anti-apoptotic protein Bcl-2 in cardiac tissue.

These findings imply that ADSC-Exos may prevent cardiomyocyte death and inhibit the progression of HF. The ability of ADSC-Exos to modulate key factors involved in cell survival and apoptosis suggests their potential therapeutic application for treating HF. However, further research is needed to fully understand the underlying mechanisms and to optimize the dosage and administration protocol. Nevertheless, this study provides valuable insights into the potential use of ADSC-Exos as a novel therapeutic strategy for HF.

ARTICLE HIGHLIGHTS

Research background

This paper discusses the significant public health issue of heart failure (HF) and the limited efficacy of current treatment options. It explores the use of digoxin and angiotensin receptor blockers in HF treatment, with conflicting results regarding mortality reduction. The paper introduces mesenchymal stem cells (MSCs), specifically adipose-derived MSCs (ADMSCs), as a potential solution. However, storage and transportation requirements limit their clinical use. Exosomes (Exos), lipid bilayer vesicles secreted by cells, are proposed as an alternative. These Exos can be stored and transported easily and have shown potential in tissue repair, inflammation inhibition, and immune regulation. The aim of the research is to investigate the use of ADMSC Exos for treating HF.

Research motivation

The research motivation of the paper is to address the global public health issue of HF and the limitations of current treatment options. The authors aim to explore the potential of ADMSC Exos as a novel therapy for HF. By investigating the effects of these Exos on heart function, the researchers seek to provide insights into new diagnostic and treatment strategies for chronic HF.

Research objectives

The research objectives of the paper are to review the current state of diagnosis and treatment for chronic HF, explore the potential use of ADMSC Exos as a therapy, investigate the limitations of ADMSCs for clinical use, examine the functions of MSC Exos, and evaluate their effectiveness in treating HF. The ultimate goal is to contribute to the development of more effective diagnostic and treatment strategies for HF.

Research methods

The exogenous surface markers of adipose derived MSCs were found and adipose derived MSCs were cultured.

Research results

The identification of surface markers showed that the surface markers CD44 and CD29 of adipose-derived stem cells (ADSCs) were well expressed, while the surface markers CD45 and CD34 of ADSCs were negative, so the cultured cells were ADSCs. Western blotting detected the Exo surface marker protein, which expressed CD63 protein but did not express calnexin protein, indicating that ADSCs derived Exos were successfully extracted.

Research conclusions

The secretion of MSCs from adipose tissue can increase ATP level, block cardiomyocyte apoptosis, and enhance the heart function of animals susceptible to HF. The inhibition of Bax, caspase-3 and p53 protein expression may be related to this process.

Research perspectives

Current research suggests that HF is a chronic condition caused by neuroendocrine system activation, leading to ventricular remodeling and cardiomyocyte death. Preventing cardiomyocyte apoptosis is crucial in managing HF. Doxorubicin, a chemotherapy drug, can cause heart damage. ADSC Exos have shown potential in promoting heart regeneration and immune regulation. They can inhibit cardiomyocyte apoptosis, alleviate structural remodeling, and improve cardiac function. ADSC Exos may achieve these effects through the PI3K/Akt signaling pathway. Elevated levels of atrial natriuretic peptide (ANP) are associated with HF, and ADSC Exos can reduce ANP and b-type natriuretic peptide concentrations, improving cardiac function. ADSC Exos can also increase ATP content in cardiac tissue to maintain normal myocardial activity. They inhibit the expression of apoptotic proteins while promoting anti-apoptotic protein expression, thus improving cardiac function. Overall, ADSC Exos have therapeutic potential for HF, but further research is needed.

FOOTNOTES

Author contributions: Wang L, Zhang JJ, and Wang SS contributed equally to this work and are co-first authors. Wang L, Zhang JJ, and Wang SS proposed the overall research goal and designed the research plan and model design, conducted statistical processing and analysis of the data, and are responsible for writing the first draft of the paper; Wang L, Zhang JJ, Wang SS, and Li L conducted feasibility analysis, review and supervision of the experiment; and are responsible for the review, revision and quality control of the paper; and all authors determined the final draft of the paper.

Institutional animal care and use committee statement: This study was approved by the Experimental Animal Ethics Committee of Fourth Medical Center of PLA General Hospital.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Basic Study

Multiomics reveal human umbilical cord mesenchymal stem cells improving acute lung injury *via* the lung-gut axis

Lu Lv, En-Hai Cui, Bin Wang, Li-Qin Li, Feng Hua, Hua-Dong Lu, Na Chen, Wen-Yan Chen

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Abstract

BACKGROUND

Acute lung injury (ALI) and its final severe stage, acute respiratory distress syndrome, are associated with high morbidity and mortality rates in patients due to the lack of effective specific treatments. Gut microbiota homeostasis, including that in ALI, is important for human health. Evidence suggests that the gut microbiota improves lung injury through the lung-gut axis. Human umbilical cord mesenchymal cells (HUC-MSCs) have attractive prospects for ALI treatment. This study hypothesized that HUC-MSCs improve ALI *via* the lung-gut microflora.

AIM

To explore the effects of HUC-MSCs on lipopolysaccharide (LPS)-induced ALI in mice and the involvement of the lung-gut axis in this process.

METHODS

C57BL/6 mice were randomly divided into four groups (18 rats per group): Sham, sham + HUC-MSCs, LPS, and LPS + HUC-MSCs. ALI was induced in mice by intraperitoneal injections of LPS (10 mg/kg). After 6 h, mice were intervened with 0.5 mL phosphate buffered saline (PBS) containing 1 × 10^6 HUC-MSCs by intraperitoneal injections. For the negative control, 100 mL 0.9% NaCl and 0.5 mL PBS were used. Bronchoalveolar lavage fluid (BALF) was obtained from anesthetized mice, and their blood, lungs, ileum, and feces were obtained by an aseptic technique following CO_2 euthanasia. Wright's staining, enzyme-linked immunosorbent assay, hematoxylin-eosin staining, Evans blue dye leakage assay,

immunohistochemistry, fluorescence *in situ* hybridization, western blot, 16S rDNA sequencing, and non-targeted metabolomics were used to observe the effect of HUC-MSCs on ALI mice, and the involvement of the lung-gut axis in this process was explored. One-way analysis of variance with post-hoc Tukey's test, independent-sample Student's *t*-test, Wilcoxon rank-sum test, and Pearson correlation analysis were used for statistical analyses.

RESULTS

HUC-MSCs were observed to improve pulmonary edema and lung and ileal injury, and decrease mononuclear cell and neutrophil counts, protein concentrations in BALF and inflammatory cytokine levels in the serum, lung, and ileum of ALI mice. Especially, HUC-MSCs decreased Evans blue concentration and Toll-like receptor 4, myeloid differentiation factor 88, p-nuclear factor kappa-B (NF-κB)/NF-κB, and p-inhibitor α of NF-κB (p-IκBα)/IκBα expression levels in the lung, and raised the pulmonary vascular endothelial-cadherin, zonula occludens-1 (ZO-1), and occludin levels and ileal ZO-1, claudin-1, and occludin expression levels. HUC-MSCs improved gut and BALF microbial homeostases. The number of pathogenic bacteria decreased in the BALF of ALI mice treated with HUC-MSCs. Concurrently, the abundances of *Oscillospira* and *Coprococcus* in the feces of HUS-MSC-treated ALI mice were significantly increased. In addition, *Lactobacillus*, *Bacteroides*, and *unidentified_Rikenellaceae* genera appeared in both feces and BALF. Moreover, this study performed metabolomic analysis on the lung tissue and identified five upregulated metabolites and 11 downregulated metabolites in the LPS + MSC group compared to the LPS group, which were related to the purine metabolism and the taste transduction signaling pathways. Therefore, an intrinsic link between lung metabolite levels and BALF flora homeostasis was established.

CONCLUSION

This study suggests that HUM-MSCs attenuate ALI by redefining the gut and lung microbiota.

Key Words: Acute lung injury; Human umbilical cord mesenchymal cells; Lipopolysaccharide; Microflora; Untargeted metabolomics; Toll-like receptor 4/nuclear factor kappa-B signaling pathway

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Core Tip: The results of this study suggest that human umbilical cord mesenchymal cells (HUC-MSCs) inhibit the inflammatory cytokine expression levels in serum and the lung of mice with acute lung injury (ALI), which may be achieved by redefining the gut and lung microbiota. This study not only provides a scientific basis for the pathophysiological mechanisms and clinical application of HUC-MSCs, but it also provides new ideas for the development of therapeutic strategies for ALI.

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INTRODUCTION

Acute lung injury (ALI), whose final severe stage is defined as acute respiratory distress syndrome (ARDS), is caused by various pathogenic factors, including acute pneumonia, sepsis, severe trauma, and acute pancreatitis[1]. It is primarily characterized by pulmonary edema and acute inflammation[2], and has high morbidity and mortality rates in patients due to the lack of effective patient-specific treatments[3]. Therefore, ALI has received much attention from the academic community, and significant developments have been made in understanding its pathophysiological mechanisms; however, clinically available treatments for ALI are still limited[4]. Lipopolysaccharide (LPS), a common pathogenic factor associated with ALI, is the main constituent of the Gram-negative bacterial cell wall[5]. A previous study found that LPS induced lung tissue damage and increased the expression of inflammatory factors in the bronchoalveolar lavage fluid (BALF)[6]. Animal models of LPS-induced lung injury are commonly used to study ALI[7]. Interestingly, LPS-induced ALI mice have a gut microbiota imbalance, and improvement in gut microbiota homeostasis can ameliorate lung inflammation of ALI mice and inhibit Toll-like receptor 4 (TLR4)/nuclear factor kappa-B (NF-κB) signaling in the lungs [8], suggesting the possible involvement of the intestinal microbiota in ALI.

A prospective observational cohort study focused on the relationship between lung microbiota and ALI and found that BALF microbiota can predict clinical outcomes in critically ill patients, especially the enrichment of gut-associated bacteria in BALF[9]. There is increasing evidence that the effects of host-microorganism interactions extend well beyond the local environment and influence the responses of peripheral tissues[7]. Homeostasis of the gut microbiota is important for human health, including its modulatory effects on ALI[10]. A study, based on 16S rRNA amplicon and metagenomic sequencing, found that the composition of the gut microbiota has a significant impact on wasting and death

in mice with ALI, suggesting the importance of the lung-gut microbiota crosstalk in lung injury[11]. Moreover, the depletion of gut microbes using an antibiotic cocktail improves lung injury and decreases interleukin (IL)-6 levels in the BALF of ALI mice[12]. A clinical cohort study found that the gut microbiome composition in patients with coronavirus disease 2019 (COVID-19), a respiratory illness, was significantly altered compared with that in non-COVID-19 individuals, irrespective of whether patients had received medication, and that the gut microbiome composition was correlated with disease severity[13]. Additionally, intestinal diseases alter the composition of the pulmonary microbiota and their metabolites[14]. Therefore, changes in the abundance and composition of the gut microbiota may be key to improving ALI *via* the lung-gut microflora. Recently, the impact of gut microbiota homeostasis on lung diseases has come into focus.

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various specialized cell types, including osteoblasts, chondrocytes, and adipocytes[15]. They originate from the mesoderm and are widely derived from adult stem cells with multi-differentiation potential, and can be isolated from a variety of tissues, such as the bone marrow, umbilical cord, amniotic membrane, adipose tissue, and skeletal muscle[16]. In addition, they have been shown to have beneficial effects in ALI[17]. Human umbilical cord mesenchymal cells (HUC-MSCs) have gained popularity in stem cell research and applications because of their specific advantages, including easy availability, abundance, lack of tumorigenicity, and ethical compliance[18]. Ahn *et al*[19] found that HUC-MSCs improved chronic lung disease and bronchopulmonary dysplasia in premature infants, without any transplantation-related adverse outcomes. Additionally, HUC-MSCs improved lung injury and inhibited the pro-inflammatory cytokine levels in the lungs of LPS-induced ALI mice[20]. HUC-MSC treatment ameliorated lung inflammation and fibrosis in bleomycin-induced pulmonary fibrosis mice[21]. Furthermore, researchers have reported that intraperitoneal infusions of HUC-MSCs improve colitis by reshaping the diversification of the gut microbiota[22]. Additionally, the gut microbiota of pulmonary hypertension mice was reversed by MSC treatment[23]. Studies on the HUC-MSC treatment of inflammation-related diseases are developing rapidly; however, their mechanisms of action are poorly understood. Therefore, by analyzing the lung-gut microbiota and lung metabolomics, this study aimed to explore the mechanisms underlying the amelioration of ALI by HUC-MSCs to provide a scientific basis for the clinical application of HUC-MSCs and a direction for the development of therapeutic strategies for ALI/ARDS.

MATERIALS AND METHODS

Animals and cells

A total of 72 6-8-wk-old male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a standard specific pathogen-free environment. The mice were isolated and allowed to adapt for one week. All procedures involving animals were approved and supervised by the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center. Mycoplasma free HUC-MSCs (HUM-iCell-e009) were purchased from iCell Bioscience Inc. (Shanghai, China) and cultured in a specialized medium (PriMed-iCELL-012; iCell Bioscience, China) containing supplements at 37 °C in a 5% CO_2 incubator. The purity of HUC-MSCs was assessed by flow cytometry and was typically greater than 90%. Cell identification was performed by iCell Bioscience Inc.

Animal model of ALI

The random number method was used to divide mice into four groups, namely, sham, sham + MSCs, LPS, and LPS + MSCs groups, with 18 mice in each group. The 36 randomly selected mice were intraperitoneally injected with 100 mL of LPS (10 mg/kg) to induce ALI[7], and sham mice were administered 100 mL of 0.9% NaCl as controls. After 6 h, half of the ALI mice and half of the sham mice were given 0.5 mL of phosphate buffered saline (PBS) containing HUC-MSCs (1×10^6 cells/mL) by intraperitoneal injections[24], and the other half of the ALI mice and sham mice were given 0.5 mL of 0.9% NaCl. The mice were reared under standard laboratory conditions. The experiments were carried out by expert technicians who were blinded to the animal grouping.

Sample preparation

Three days after HUC-MSC intervention in ALI mice, BALF was obtained after the mice were anesthetized with isoflurane as reported by Wu *et al*[24]. Briefly, the trachea was flushed five times with PBS *via* a 20-gauge catheter, and all liquids were collected. Subsequently, all mice were euthanized with CO_2, and the blood, lungs, ileum, and feces were obtained using aseptic techniques. The lungs (one lung was evenly divided into four) from three mice in each group were taken, weighed, and then baked in an oven at 80 °C for 48 h to determine the dry weight to calculate the ratio of wet lung weight to dry lung weight (W/D weight ratio) as reported by Li *et al*[25]. In addition, a part of the remaining lung and ileum were made into paraffin blocks separately, and the other were stored at 80 °C for further analysis. The BALF was divided into two parts for inflammation cytokine detection and 16S rDNA sequencing, respectively.

Detection of immune cells and inflammatory cytokines

The protein concentration in the BALF was measured using the BCA assay (Beyotime, China). Additionally, the cells in BALF were precipitated and resuspended, and mononuclear cells and neutrophils were counted after Wright's staining. The levels of tumor necrosis factor (TNF)-α (ml002095-1, Enzyme-linked, China), IL-1β (ml063132-1, Enzyme-linked, China), and IL-6 (ml002293-1, Enzyme-linked, China) in the serum, lungs, and ileum were measured by enzyme-linked

Table 1 Information of antibodies used in this study

Antibody	Dilution ratio	Manufacturer	Country	Cat. No.
VE-cadherin antibody	1:2000	Affinity	United States	AF6265
ZO-1 antibody	1:5000	Proteintech	United States	21773-1-AP
Occludin antibody	1:1000	Affinity	United States	DF7504
TLR4 antibody	1:2000	Affinity	United States	AF7017
Myd88 antibody	1:1000	Affinity	United States	AF5195
p-NF-κB p65 antibody	1:2000	Affinity	United States	AF2006
NF-κB p65 antibody	1:2000	Affinity	United States	AF5006
p-IκBα antibody	1:2000	Affinity	United States	AF2002
IκB antibody	1:2000	Affinity	United States	AF5002
Claudin-1 antibody	1:3000	Affinity	United States	AF0127
Anti-rabbit IgG, HRP-linked antibody	1:6000	Cell signaling technology	United States	7074
Anti-mouse IgG, HRP-linked antibody	1:6000	Cell signaling technology	United States	7076
β-actin antibody	1:10000	Affinity	United States	AF7018

VE: Vascular endothelia; ZO-1: Zonula occludens-1; TLR4: Toll-like receptor; Myd88: Myeloid differentiation factor 88; NF-κB: Nuclear factor kappa-B; IκB α: Inhibitor α of nuclear factor kappa-B; HRP: Horse radish peroxidase.

immuno sorbent assay (ELISA) according to the manufacturer's instructions.

Histopathological observation

Hematoxylin-eosin (H&E) staining was performed to observe the histopathology of the lungs and ileum. Briefly, the lungs and ileum were fixed in 10% formalin. The tissues were then placed in paraffin and sectioned, and sections were stained with H&E. The injury of lungs and ileum were scored in a blinded fashion as previously reported[26,27].

Measurement of alveolar-capillary permeability

Three mice were randomly selected from each group for the Evans blue dye leakage assay to explore the function of the lung tissue endothelial barrier as previously reported[28]. This assay was performed using 25 mg/kg of 0.5% blue dye (Sigma, United States) injected into mice *via* the tail vein 2 h before isoflurane anesthesia. After the heart of the anesthetized mice was exposed, the left ventricle was intubated and flushed with 4% normal saline. Subsequently, the mice were euthanized using CO_2. Lungs were homogenized in 2 mL PBS and treated with formamide (Sigma, United States) at 60 °C for 24 h. Finally, the concentration of Evans blue dye in the lung tissue was determined at 620 nm using a microplate reader (CMaxPlus, Molecular Devices, United States).

Immunohistochemistry

Immunohistochemistry (IHC) was performed as described by Peng *et al*[29]. Paraffin sections of lung tissue were deparaffinized, permeabilized, and blocked. The sections were then incubated at 4 °C overnight with anti-TLR4 antibody (1:100 dilution; Affinity, United States). After washing with PBS, the slides were incubated with goat anti-rabbit IgG HRP (Abcam, United States) at 37 °C for 30 min. The slides were washed again, mounted with DAPI (Vector Laboratories, Burlingame, CA), and examined using an E100 fluorescence microscope (Nikon, Japan).

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was performed on slides of ileum and lung tissues as previously described[30], using a pan-bacteria FITC-labeled probe (EUB338) and an RNA FISH kit (Genepharma, China). After DAPI staining, the slides were visualized under an inverted fluorescence microscope (Ts2-FL; Nikon, Japan). Images were acquired using Micro-Manager and analyzed using ImageJ/FIJI software (National Institutes of Health, United States, version 1.53c).

Western blot analysis

The antibodies used for western blot (WB) are shown in Table 1. The lung and ileum tissues were homogenized in a lysis solution for the extraction of proteins. After centrifugation and supernatant collection, the samples were uniformly concentrated and denatured. Briefly, sodium dodecyl sulfate-gel electrophoresis and membrane transfer were performed for 20 μg protein per group as reported by Li *et al*[3]. The membranes were incubated with primary antibodies overnight and then with secondary antibodies for 1 h. Finally, the membranes were subjected to chemiluminescence reactions and protein levels were measured by enhanced chemiluminescence. ImageJ/FIJI software was used for semiquantitative analysis.

16S rDNA sequencing

High-throughput 16S rDNA sequencing was performed to analyze the gut and pulmonary microflora by BioDeep Co., Ltd (Suzhou, China). A sequencing library was prepared using the TruSeq Nano DNA LT Library Prep Kit of Illumina. A NovaSeq6000 system (PE250, Illumina, United States) was used for sequencing as reported by Yi *et al*[31]. Then the QIIME2 DADA2 and OmicStudio platforms[32] were used to analyze the data.

Metabolomics for lung tissue

The non-targeted metabolomics approach was performed by PANOMIX Biomedical Tech Co., Ltd. (Suzhou, China). The samples were treated with 75% methanol-chloroform (9:1) and 25% water for sonication and centrifugation to extract the metabolites. The samples (2 μL) were analyzed by liquid chromatography-mass spectrum (MS) detection with the Vanquish UHPLC System (Thermo, United States). Additionally, an Orbitrap Exploris mass spectrometer (Thermo, United States) was used for mass spectrometry analysis. After data acquisition, the Proteowizard package (v3.0.8789) and the R xcms package were used to preprocess the data. Metabolites were identified on the public databases such as the Human Metabolome Database[33], MassBank (https://massbank.eu/MassBank/), Lipid Maps (https://Lipidmaps.org/), mzCloud (https://www.mzcloud.org/), Kyoto Encyclopedia of Genes and Genomes (KEGG)[34], and self-building repository, and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) analysis was performed to screen the differential metabolites.

Statistical analysis

Statistical analyses were performed using SPSS (version 16.0; IBM, Armonk, NY, United States). Data from multiple *in vivo* experiments were analyzed using one-way analysis of variance (ANOVA) with a post-hoc Tukey test. The independent-samples Student's *t*-test was used for comparison between the two groups. The Wilcoxon rank-sum test was conducted to explore the microflora with a significant difference in abundance. Pearson's correlation analysis was used to explore the intrinsic associations. Additionally, the Variable Importance for the Projection (VIP) of OPLS-DA was used to screen for metabolites with biomarker potential (VIP > 1). Two-way Orthogonal Partial Least Squares (O2PLS) analysis was used to explore the links between BALF microbiota and lung metabolism. The threshold for significance was $P < 0.05$ for all tests.

RESULTS

HUC-MSC treatment alleviates lung injury and inflammation in ALI mice

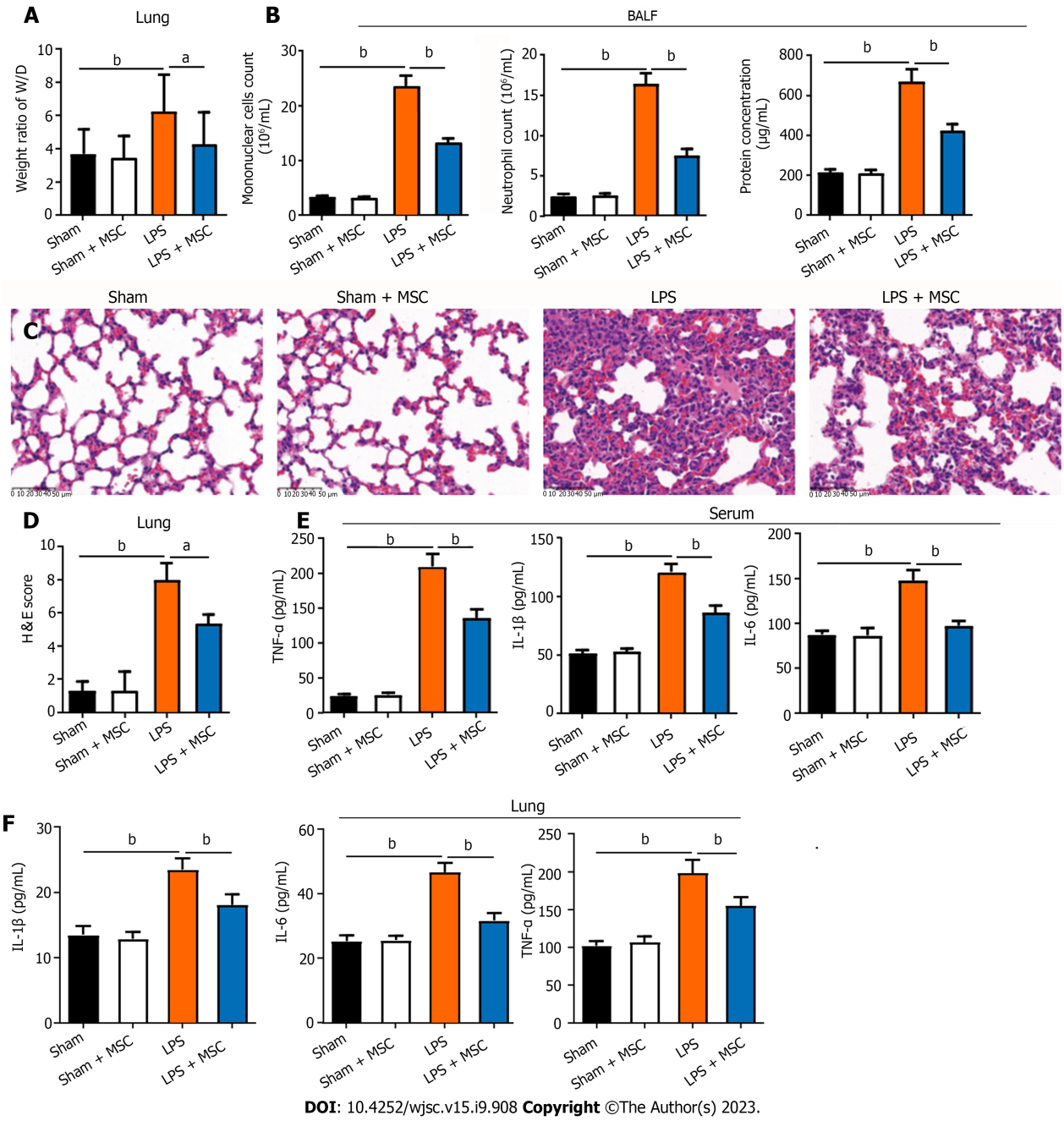
This study used LPS to induce ALI in mice and then collected their lungs and BALF to explore the ameliorating effect of HUC-MSCs on ALI by H&E staining and ELISA. ALI mice had a higher lung W/D weight ratio and more mononuclear cells and neutrophils than sham mice ($P < 0.01$), whereas HUC-MSC treatment on ALI mice decreased the lung W/D weight ratio ($P < 0.05$), mononuclear cell and neutrophil counts, and protein concentration ($P < 0.01$) (Figures 1A and B). Additionally, compared to sham mice, the lung of ALI mice had markedly thickened alveolar septa with significant inflammatory cell infiltration; however, HUC-MSC treatment alleviated the degree of alveolar septal thickening and inflammatory cell infiltration in ALI mice (Figure 1C). Likewise, the score of lung injury in the LPS group was higher than those in the sham ($P < 0.01$) and LPS + MSC groups ($P < 0.05$) (Figure 1D). Moreover, ALI mice had significantly increased levels of TNF-α, IL-1β, and IL-6 in their serum and lung tissues ($P < 0.01$) (Figures 1E and F). In particular, the above-mentioned inflammatory factor levels were decreased in the ALI mice treated with HUC-MSCs ($P < 0.01$) (Figures 1E and F).

HUC-MSC treatment improves endothelial barrier function and integrity in the lungs of ALI mice

In addition, this study observed the endothelial barrier function and integrity of the lungs using Evans blue, WB, and IHC assays. As shown in Figure 2A, ALI mice had a higher concentration of Evans blue dye in the lungs than the sham mice, and HUC-MSC treatment reduced Evans blue concentration in the lungs of ALI mice ($P < 0.01$). In particular, the levels of endothelial barrier-associated proteins, such as vascular endothelial (VE)-cadherin, zonula occludens-1 (ZO-1), and occludin, were markedly decreased in ALI mice ($P < 0.05$ or $P < 0.01$); however, HUC-MSC treatment reversed the expression levels of these proteins ($P < 0.01$) (Figures 2B-D). In addition, this study explored the signal intensity of the TLR4/myeloid differentiation factor 88 (Myd88)/NF-κB signaling pathway. The TLR4, Myd88, p-NF-κB/NF-κB, and p-inhibitor α of NF-κB (p-IκBα)/IκBα expression levels in the lung were all increased in ALI mice compared to the sham mice ($P < 0.01$); however, in ALI mice treated with HUC-MSCs, the expression levels of these proteins were decreased ($P < 0.05$ or $P < 0.01$) (Figures 2E-I). Similarly, TLR4 detected by IHC had a strong positive expression in the lungs of ALI mice, whereas HUC-MSC treatment attenuated the positive expression of TLR4 ($P < 0.05$ or $P < 0.01$) (Figures 2J and K).

HUC-MSC treatment improves injury, endothelial barrier integrity, and bacterial translocation in the ileum of ALI mice

Studies have reported not only lung function injury but also intestinal dysfunction in ALI[17]. Therefore, the ileum was examined using H&E staining, ELISA, WB, and FISH. The ileal tissue of ALI mice had shorter and ruptured villi with significant inflammatory cell infiltration compared to sham mice, whereas these ileal injuries in ALI mice treated with HUC-MSCs were improved ($P < 0.05$ or $P < 0.01$) (Figure 3A). In addition, LPS treatment on mice markedly increased the ileal TNF-α, IL-1β, and IL-6 levels ($P < 0.01$), whereas HUC-MSC treatment inhibited them ($P < 0.01$) (Figure 3B). The suppressed expression of endothelial barrier-associated proteins, including claudin, ZO-1, and occludin by LPS was



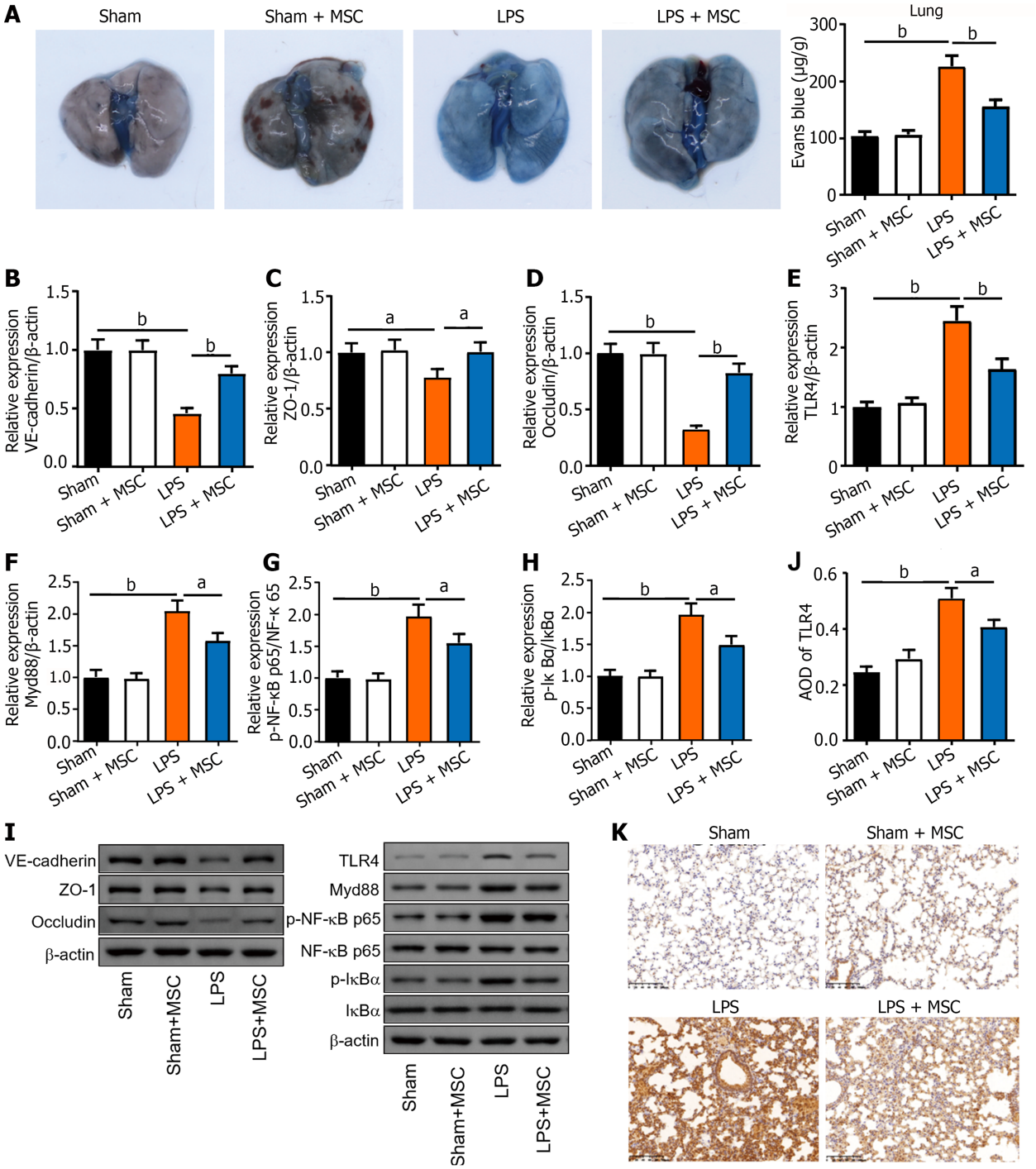
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Figure 1 Human umbilical cord mesenchymal stem cells attenuate lipopolysaccharide-induced lung injury and inflammation in acute lung injury mice. A: In acute lung injury (ALI) mice, the wet-to-dry (W/D) weight ratio of the lung was higher than that of human umbilical cord mesenchymal stem cell (HUC-MSC)-treated ALI mice (n = 3); B: ALI mice had more mononuclear cells and neutrophils, and higher protein concentration in bronchoalveolar lavage fluid (BALF) than sham mice, while HUC-MSC treatment decreased these parameters (n = 12); C: Representative images of hematoxylin-eosin (H&E)-stained lung tissues (× 400, scale = 50 μm); D: Lung histopathological damage in mice (H&E staining; n = 3); E: Serum tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and lipopolysaccharide levels were higher in ALI mice and HUC-MSC treatment decreased their levels (n = 12); F: TNF-α, IL-1β, and IL-6 levels in the lungs of ALI mice were hugely increased than those in sham mice, while HUC-MSC treatment in ALI mice decreased them (n = 12). $^{a}P < 0.05$, $^{b}P < 0.01$. LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell; BALF: Bronchoalveolar lavage fluid; H&E: Hematoxylin-eosin; IL: Interleukin; TNF: Tumor necrosis factor.

enhanced by HUC-MSC treatment in ALI mice ($P < 0.05$) (Figures 3C and D). Furthermore, the EUB338 counts of the ileum epithelium and lungs in ALI mice were measured to observe the lung translocation of gut bacteria, and it was found that they were increased in ALI mice, whereas HUC-MSC treatment reduced them ($P < 0.01$) (Figures 3E-H).

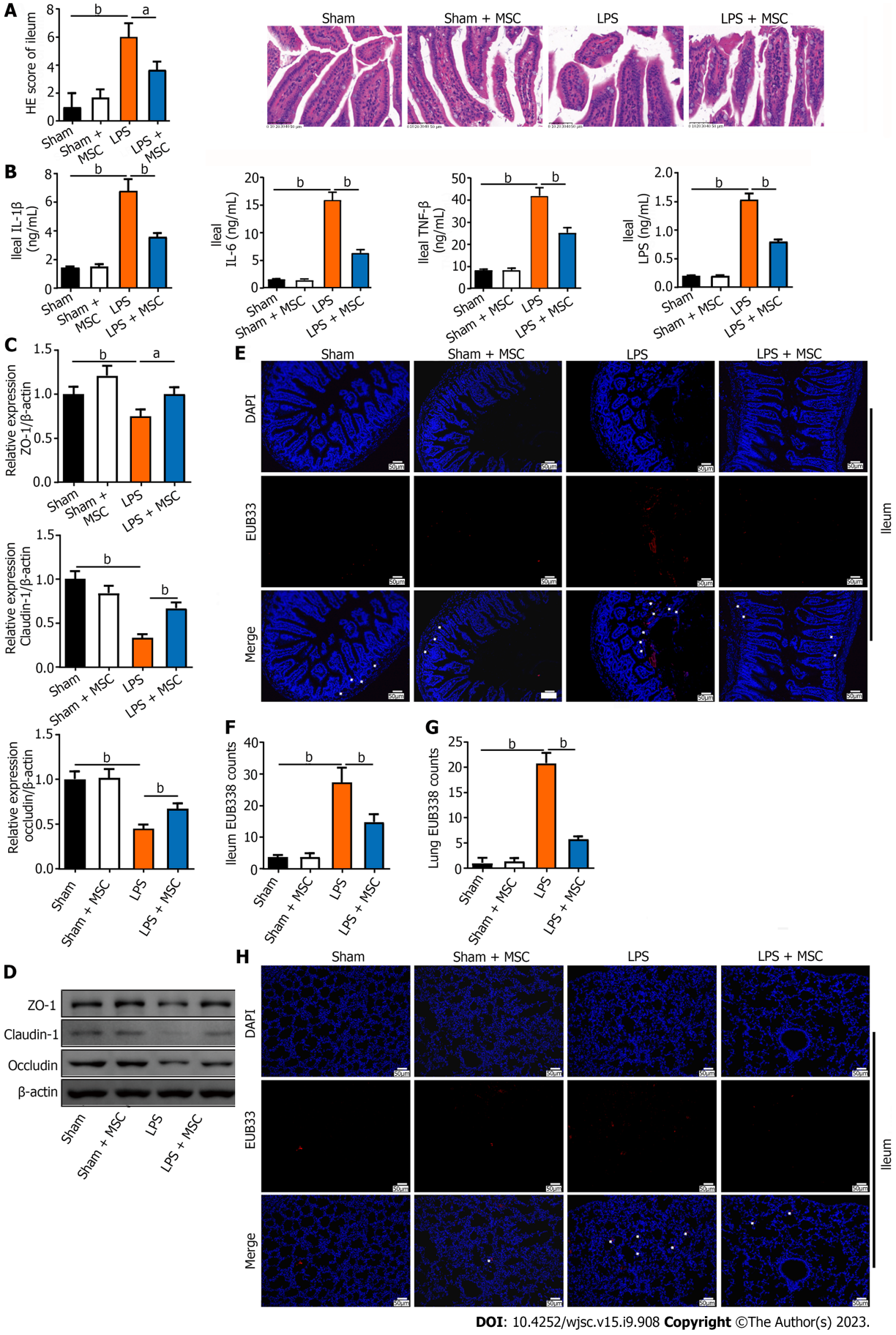
HUC-MSC treatment attenuates ALI via regulating lung-gut microbiota homeostasis

16S rDNA sequencing was used to explore the effect of the lung and gut microbiota on the HUC-MSC-mediated amelioration of ALI. BALF and fecal samples were collected for 16S rDNA sequencing. After homogenizing the sequencing depth of each group, 48 BALF and fecal samples from the four groups were identified at the genus level, with an average of 48.25 units in each group. Figures 4A and B shows the top 20 flora with the highest average abundance at the genus level. The Shannon and Simpson indices of alpha diversity reflect richness and community evenness,



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Figure 2 Human umbilical cord mesenchymal stem cells improve endothelial barrier function *in vivo* and regulate related protein expression. A: The Evans blue assay was carried out to assess lung endothelial barrier function in acute lung injury (ALI) mice (n = 3). The ALI mice [lipopolysaccharide (LPS) group] had the largest amount of Evans blue contents; human umbilical cord mesenchymal stem cell (HUC-MSC) treatment in ALI mice (LPS + MSC group) decreased the contents in the lung; B-H: LPS treatment in mice inhibited the expression of vascular endothelial-cadherin (B), zonula occludens-1 (C), and occludin (D) and raised the expression of Toll-like receptor 4 (TLR4) (E), myeloid differentiation factor 88 (F), p-nuclear factor kappa-B (NF-κB)/NF-κB (G), and p-inhibitor α of NF-κB (IκBα)/IκBα (H) (n = 3), while HUC-MSC treatment antagonized the effects of LPS; I: Representative western blot bands of the above proteins; J and K: Statistical results of immunohistochemistry and its representative images. It was showed that TLR4 was highly expressed in the lung tissue of ALI mice and HUC-MSC treatment could decrease its expression (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$. LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell; LPS: Lipopolysaccharide; VE: Vascular endothelial; ZO-1: Zonula occludens-1; TLR4: Toll-like receptor 4; Myd88: Myeloid differentiation factor 88; NF-κB: Nuclear factor kappa-B; IκBα: Inhibitor α of nuclear factor kappa-B.



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Figure 3 Human umbilical cord mesenchymal stem cells improve histopathology, inflammation, and endothelial barrier integrity of the

ileum in acute lung injury mice. A: Ileum tissue injury in acute lung injury (ALI) mice was observed after hematoxylin-eosin staining. ALI mice had severe ileum tissue injury while human umbilical cord mesenchymal stem cell (HUC-MSC) treatment improved such injury (n = 3); B: Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and lipopolysaccharide (LPS) levels in the ileum were measured by ELISA (n = 12), and ALI mice had higher levels of TNF-α, IL-1β, IL-6, and LPS compared to sham mice, while HUC-MSC treatment decreased the levels of these factors; C: Zonula occludens-1 (ZO-1), claudin-1, and occludin levels were measured to observe the integrity of the ileum barrier (n = 3); D: Representative western blot bands of ZO-1, claudin-1, and occludin; E-H: Bacterial translocation was determined by fluorescence *in situ* hybridization, and the EUB338 counts in the ileum epithelium and lung per field were quantified (n = 3). $^{a}P < 0.05$, $^{b}P < 0.01$. MSC: Mesenchymal stem cell; LPS: Lipopolysaccharide; ZO-1: Zonula occludens-1; HE: Hematoxylin-eosin; IL: Interleukin; TNF: Tumor necrosis factor.

respectively. According to the Kruskal-Wallis rank-sum test, the Shannon index showed no significant differences among the groups (P = 0.056) (Figure 4C). Additionally, the Bray-Curtis distance of beta diversity reflects microbial diversity between groups and was analyzed by principal coordinates analysis, which revealed that the projection distance of the LPS + MSC group on the coordinate axis was closer to that of the negative control group than that of the LPS group (Figure 4C). Similarly, the Simpson index showed no significant differences among the groups (P = 0.058), and the projection distance of the gut microflora was closer to that of the negative control group than to that of the LPS group (Figure 4D).

Subsequently, the Wilcoxon rank-sum test was performed to explore the microflora with a significant difference in abundance (marked microflorae) (Figures 4E and F). In particular, there were 21 microflorae with upregulated abundance and 12 microflorae with downregulated abundance in the BALF of mice in the LPS + MSC group compared to the LPS group ($P < 0.05$) (Figure 4E), and 17 microflorae with upregulated abundance and 3 microflorae with downregulated abundance in feces ($P < 0.05$) (Figure 4F). The 33 marked microflorae with the largest upregulation or downregulation of operational taxonomic units in the BALF of ALI mice with/without HUC-MSC treatment are shown in Figure 5A, and 20 marked microflorae in feces are shown in Figure 5B. *Rhizobiales* had the largest log2 fold change (FC) in the BALF of mice in the LPS + MSC group compared to the LPS group [log2(FC) = 9.3264, P = 0.0284], and *Elizabethkingia* had the lowest log2FC in the BALF of mice in the LPS + MSC group compared to that of the LPS group [log2(FC) = -5.1799, P = 0.028] (Figure 5A). In fecal samples, the log2FC of *unclassified_Bacteroidales* was the highest in the marked microflorae of the LPS + MSC group compared to that of the LPS group [log2(FC) = 4.7549, P = 0.027], and that of the unidentified_F16 was the lowest [log2(FC) = -4.6328, P = 0.012] (Figure 5B).

Furthermore, the Pearson's correlation analysis was used to analyze the correlation between marked microflorae of the gut and lungs, and most of the bacteria in the BALF and feces had a strong or extremely strong correlation (Figure 6). The *Desulfovibrio* genus in feces was positively correlated with *Stenotrophomonas* in BALF ($P < 0.05$) (Supplementary Table 1).

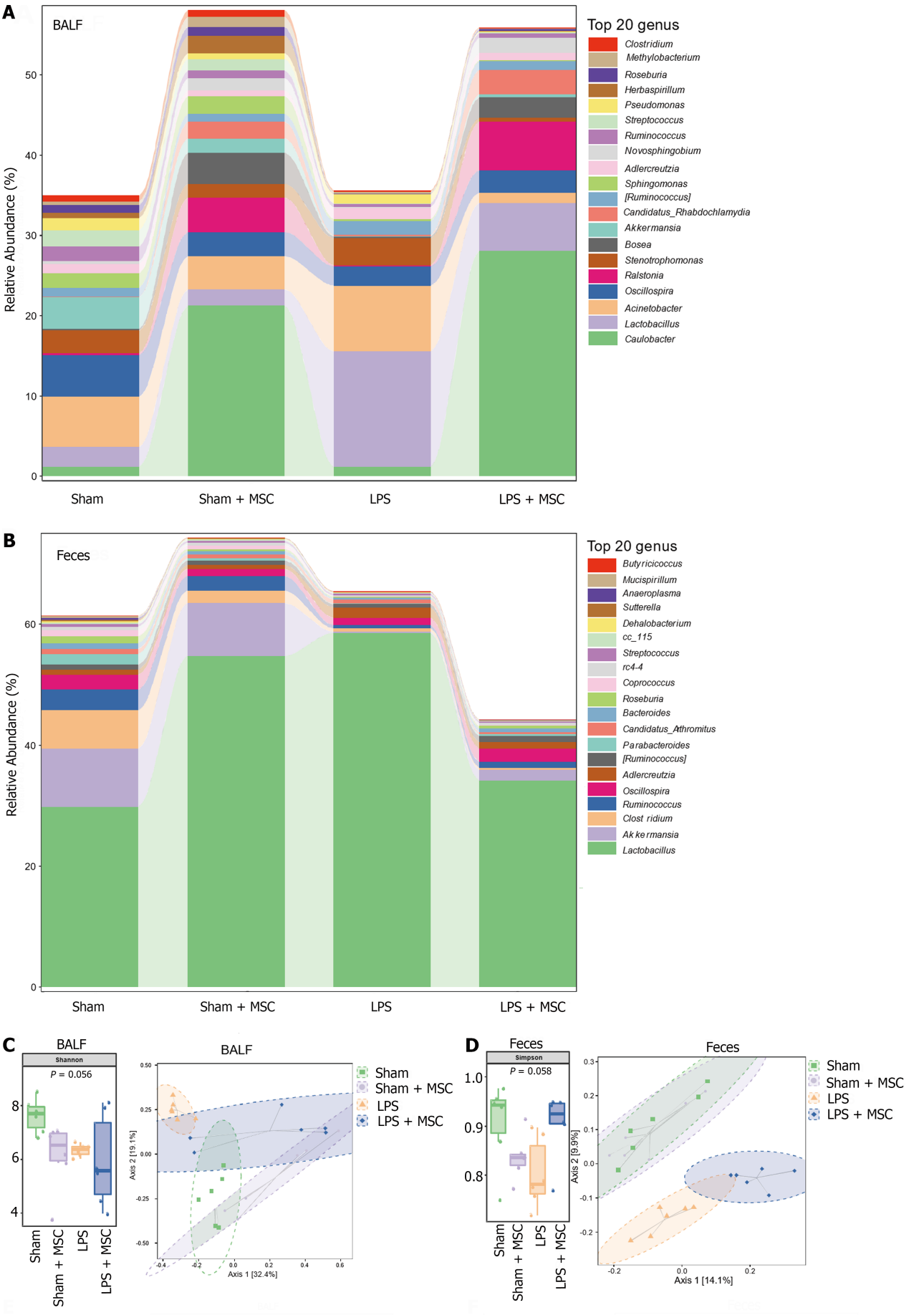
HUC-MSC treatment regulates metabolic profile of lung tissue in ALI mice

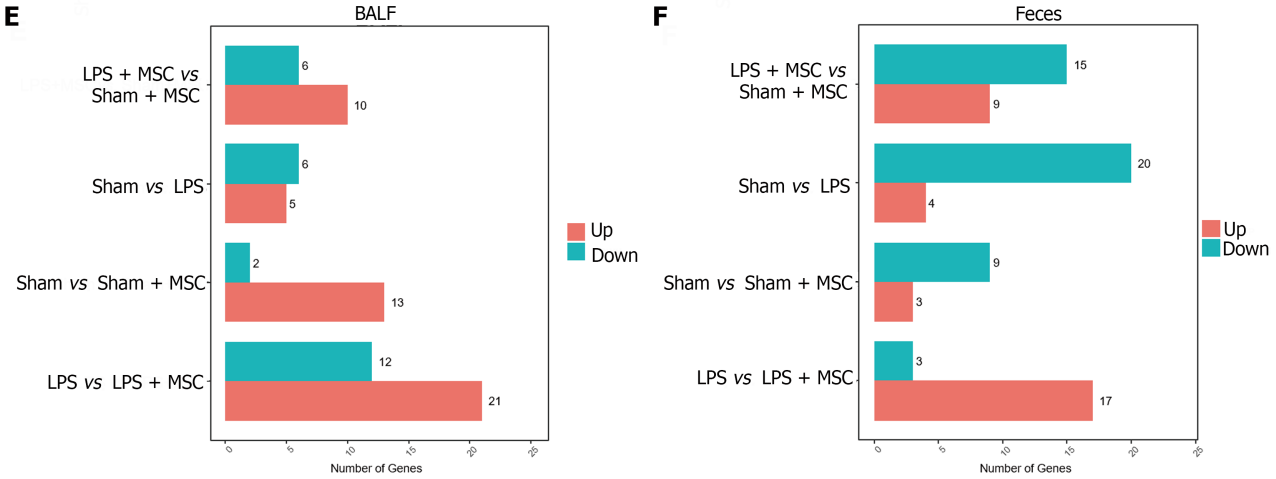
The base peak chromatograms in the positive and negative modes of the four groups showed similar trends, suggesting good repeatability and reliable results (Figure 7A). Additionally, Partial Least Squares-Discriminant Analysis (PLS-DA) was used to distinguish metabolite differences between groups in the positive/negative mode. In particular, all blue Q2 positions in the permutation test chart were lower than the original points on the far right, suggesting that the PLS-DA models were valid (Figures 7B and C). In the positive mode, MS analysis identified a total of 1206 common biomarkers between the sham and LPS + MSC groups, 362 between the LPS and sham + MSC groups, 347 between the LPS and sham groups, 343 between the LPS + MSC and LPS groups, and 181 between the sham + MSC and sham groups (Figure 7D). In the negative mode, this study identified 905 common biomarkers between the sham and LPS + MSC groups, 476 between the LPS and sham + MSC groups, 225 between the LPS and sham groups, 112 between the LPS + MSC and LPS groups, and 139 between the sham + MSC and sham groups (Figure 7E).

Subsequently, precise screening of the metabolic profiles by MS/MS was performed to eliminate false positives. This study identified four upregulated metabolites (3-succinoylpyridine, nicotinamide ribotide, aldosterone, and phenylacetic acid) and one downregulated metabolite (lithocholic acid) in the sham + MSC group compared to the sham group. In addition, KEGG enrichment analysis suggested that these differential metabolites might participate in nicotinate and nicotinamide metabolism, aldosterone-regulated sodium reabsorption, and aldosterone synthesis and secretion pathways (Figures 8A-C). Additionally, 12 markedly upregulated metabolites (hydroxyindole, xanthine, and N-acetyl-L-aspartic acid, *etc.*) and 20 markedly downregulated metabolites (prostaglandin E2, argininosuccinic acid, and guanine, *etc.*) were identified in the LPS group compared to the sham group, and these metabolites were predicted to be potentially involved in alanine, aspartate, and glutamate metabolism, oxidative phosphorylation, the cAMP signaling pathway, and so on (Figures 8A, D and E). In particular, five upregulated metabolites (anabasine, IMP, lidocaine, salicylic acid, and propionylcarnitine) and 11 downregulated metabolites (N-acetylleucine, guanosine, guanine, *etc.*) were identified in the LPS + MSC group compared to the LPS group (Figures 8A and F). They were related to purine metabolism and the taste signaling transduction pathways ($P < 0.001$) (Figure 8G).

Significant correlations between microbes in BALF and metabolites in lung tissue

To explore the role of HUC-MSCs in regulating the microflora of the lung-gut axis to improve ALI, we performed an O2PLS correlation analysis on the expression of microflora in BALF and the expression of metabolites in the lungs to determine the microflora involved in the improvement of ALI by HUC-MSCs and their impact on metabolism. In Figure 9A, the top 25 bacteria and top 25 metabolites are shown with large absolute joint loading values, suggesting that they have a large weight in the improvement of ALI following HUC-MSC treatment. Subsequently, these flora and metabolites were analyzed using correlation analysis (Supplementary Table 1). Significantly related bacteria and metabolites were screened based on $P < 0.05$. The number of metabolites that significantly correlated with BALF microbes


A
BALF
Relative Abundance (%)
0
10
20
30
40
50
Sham
Sham + MSC
LPS
LPS + MSC
Top 20 genus
Clostridium
Methylobacterium
Roseburia
Herbaspirillum
Pseudomonas
Streptococcus
Ruminococcus
Novosphingobium
Adlercreutzia
Sphingomonas
[Ruminococcus]
Candidatus_Rhabdochlamydia
Akkermansia
Bosea
Stenotrophomonas
Ralstonia
Oscillospira
Acinetobacter
Lactobacillus
Caulobacter
B
Feces
Relative Abundance (%)
0
20
40
60
Sham
Sham + MSC
LPS
LPS + MSC
Top 20 genus
Butyricicoccus
Mucispirillum
Anaeroplasma
Sutterella
Dehalobacterium
cc_115
Streptococcus
rc4-4
Coprococcus
Roseburia
Bacteroides
Candidatus_Athromitus
Parabacteroides
[Ruminococcus]
Adlercreutzia
Oscillospira
Ruminococcus
Clost ridium
Ak ke rmansia
Lactobacillus
C
BALF
Shannon
P = 0.056
4
6
8
Sham
Sham + MSC
LPS
LPS + MSC
BALF
Axis 2 [19.1%]
Axis 1 [32.4%]
Sham
Sham + MSC
LPS
LPS + MSC
D
Feces
Simpson
P = 0.058
0.8
0.9
1.0
Sham
Sham + MSC
LPS
LPS + MSC
Feces
Axis 2 [9.9%]
Axis 1 [14.1%]
Sham
Sham + MSC
LPS
LPS + MSC



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Figure 4 Microflora homeostasis in bronchoalveolar lavage fluid and feces of acute lung injury mice (*n* = 6). A and B: Relative abundance of top 20 genera of microflora in bronchoalveolar lavage fluid (BALF) and feces; C and D: The within-sample richness and evenness (alpha diversity) were statistically analyzed by Shannon index, Simpson index, and the bray-Curtis based principal coordinates analysis of similarity coefficients in the BALF and feces of different groups (beta diversity); E and F: The Wilcoxon rank sum test was used to explore microflorae with a significant difference in abundance on OmicShare Tools. The number of differential microflorae in lipopolysaccharide (LPS) + mesenchymal stem cells (MSCs) group *vs* sham + MSC group, sham group *vs* LPS group, sham group *vs* sham + MSC group, and LPS group *vs* LPS + MSCs group is presented. The length of the red bar indicates the number of up-regulated microflorae and the length of the blue-green bar indicates the number of down-regulated microflorae. LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell; BALF: Bronchoalveolar lavage fluid.

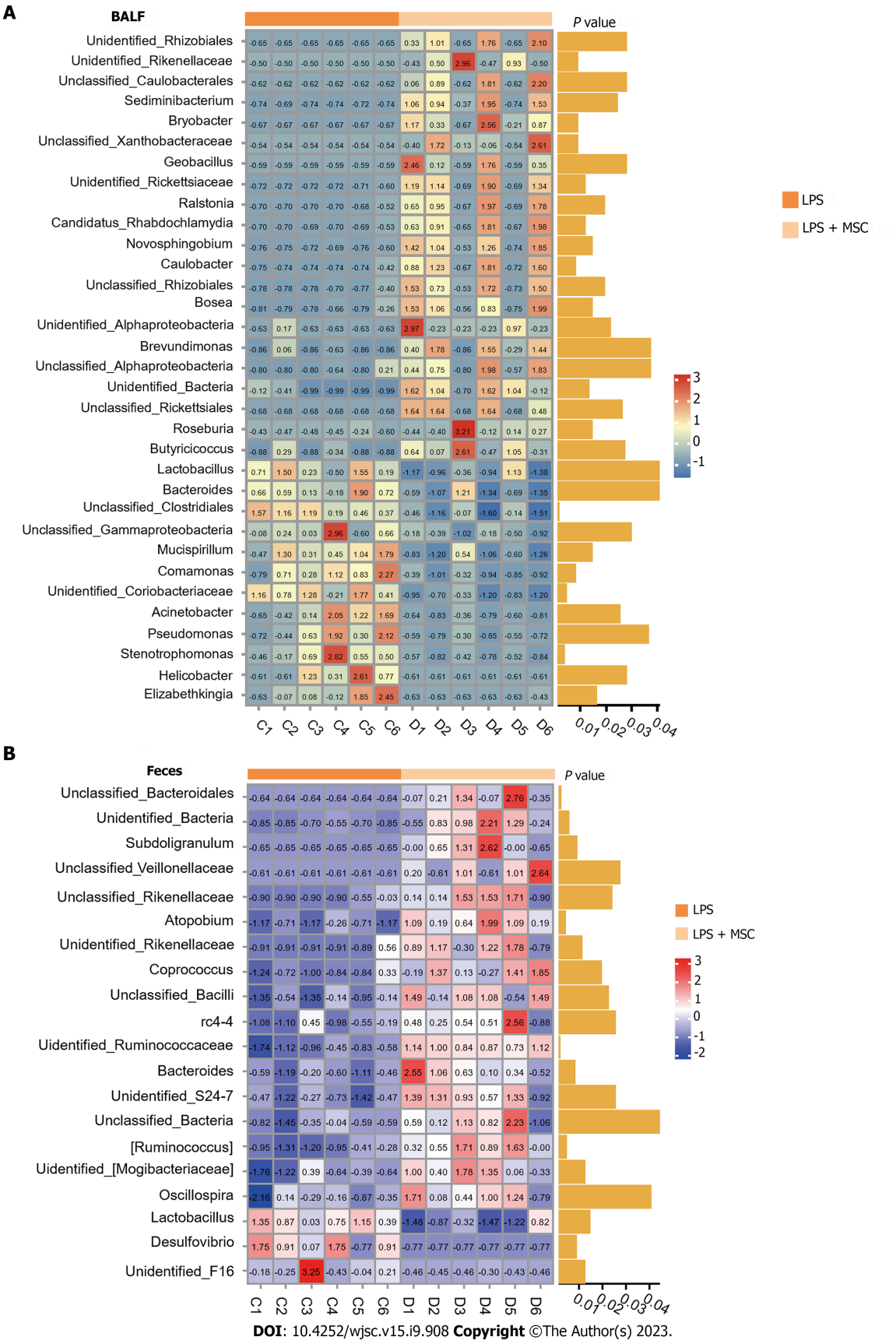
is shown in Figure 9B. Additionally, metabolites with a large role in the improvement of ALI by HUC-MSC treatment were subjected to KEGG enrichment analysis and were found to be mainly involved in the signaling pathways of drug metabolism-other enzymes, tyrosine metabolism, autophagy-animal, and endocytosis (Figure 9C).

DISCUSSION

MSCs have emerged as a promising therapeutic strategy for inflammatory diseases, owing to their low immunogenicity, ability to stabilize immunity, and ability to ameliorate inflammatory responses[35]. Moreover, HUC-MSCs not only ameliorate acute and chronic pneumonia, but also enteritis[36,37]. This study found that HUC-MSCs improved pulmonary edema, alleviated pathological damage to the lungs and ileum, and inhibited the levels of inflammatory cells in the BALF and inflammatory factors in the serum, BALF, lungs, and ileum. Furthermore, HUC-MSC treatment of ALI mice improved endothelial barrier integrity in the lungs and ileum. Endothelial permeability is regulated by intercellular junctions including adherens junctions and tight junctions, which are composed of cell junction proteins including occludin, claudin 1, VE-cadherin, *etc*[38]. Claudin-1 in the ileum and VE-cadherin, ZO-1, and occludin in the lung and ileum play key roles in maintaining vascular integrity. This decrease indicated endothelial barrier disruption in the lungs and ileum. In this study, HUC-MSC treatment of ALI mice advanced lung VE-cadherin, ZO-1, and occludin expression signal intensity and upregulated claudin-1, ZO-1, and occludin expression levels in the ileum. Collectively, treatment of ALI mice with HUC-MSCs ameliorates lung and ileal barrier integrity.

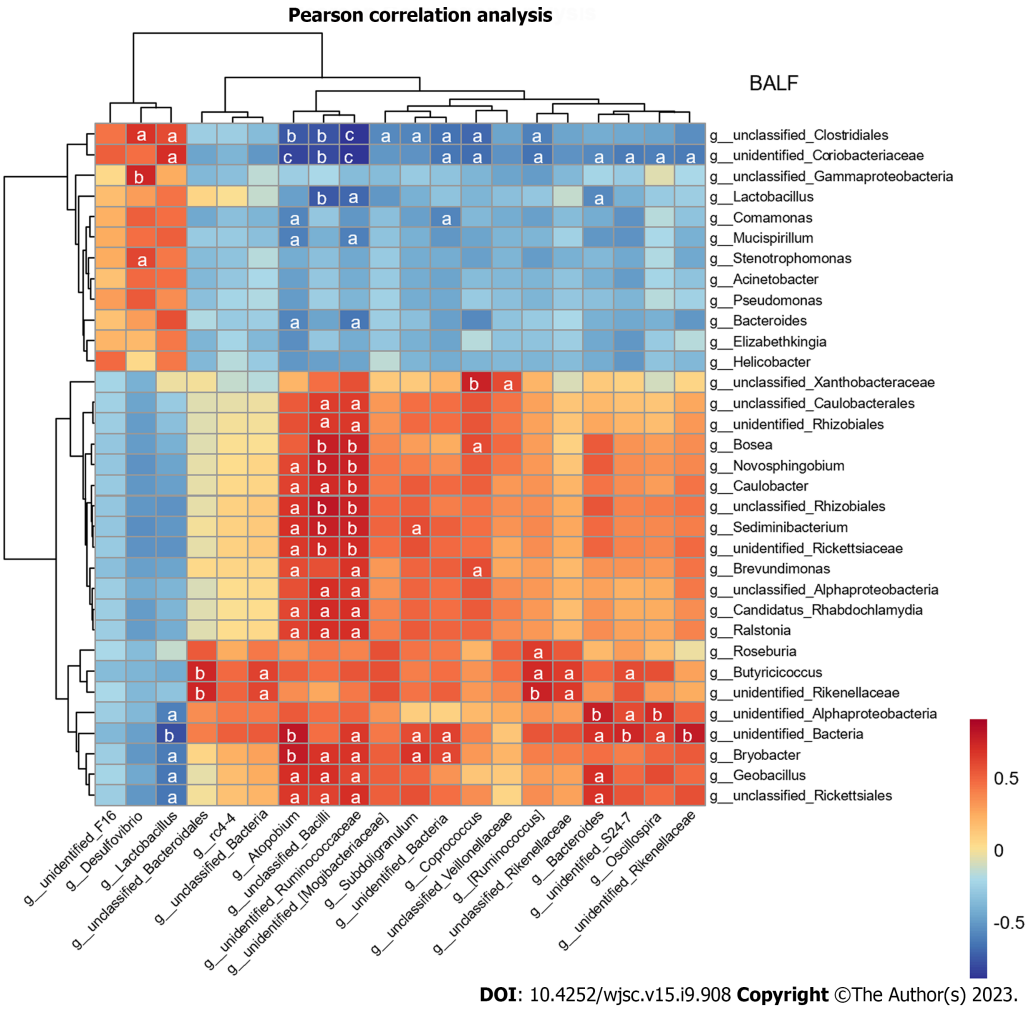
Inflammatory factors and cells promote ALI injury. A study reported that TNF-α could promote M1 macrophage activation which has pro-inflammatory effects in sepsis-related ALI[39]. Neutrophil overactivation promotes the development of inflammation and injury in ALI[40]. Microvascular endothelial barrier dysfunction, the main pathophysiological feature of ARDS/ALI, induces capillary leakage and edema, which further intensifies inflammatory injury, thus causing high morbidity and mortality[41]. Botros and colleagues reported that stabilizing the endothelial barrier during inflammation alleviated inflammatory responses, edema, and lung injury in mice with ALI[42]. Interestingly, the inhibition of the TLR4 signaling pathways is related to the integrity of the pulmonary endothelial barrier. One study found that TLR4 knockdown decreased the sensitivity to particulate matter-induced pulmonary edema in ALI mice and increased the signal expression intensity of VE-cadherin[43]. Furthermore, MSC treatment in paraquat-induced ALI rats downregulated the TLR4 and NF-κB protein levels in the lungs[44]. Besides, LPS facilitates TLR4 activation to recruit MyD88 and thereby activates NF-κB to promote the production of pro-inflammatory factors such as TNF-α and IL-6[45]. Similarly, this study reported the inhibitory effect of HUC-MSCs on TLR4, Myd88, and NF-κB in the lungs of LPS-induced ALI mice, which suggests that HUC-MSCs may mitigate microvascular endothelial barrier dysfunction and inflammation in ALI *via* the TLR4/Myd88/NF-κB signaling pathway.

There is a correlation between microbiota levels and lung diseases[46]. By analyzing the 16S rDNA of microflora in BALF, we observed a decrease in some pathogenic bacteria. For example, the *Stenotrophomonas* genus was decreased in the BALF of mice in the LPS + MSC group. One study has reported that *Stenotrophomonas* maltophilia is commonly found



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Figure 5 Differences in genus abundance of different microflorae in bronchoalveolar lavage fluid and feces of acute lung injury mice treated with human umbilical cord mesenchymal stem cells (*n* = 6). A and B: Heatmaps showing the differences in the genus abundance of different microflorae in bronchoalveolar lavage fluid and feces. Red represents the genus that is more highly abundant; blue/purple represents the genus that is less abundant; white/yellow indicates no difference in expression between groups. Abundance is homogenized by Z-score. The value displayed in the middle of the grid is log2 (fold change). LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell; BALF: Bronchoalveolar lavage fluid.



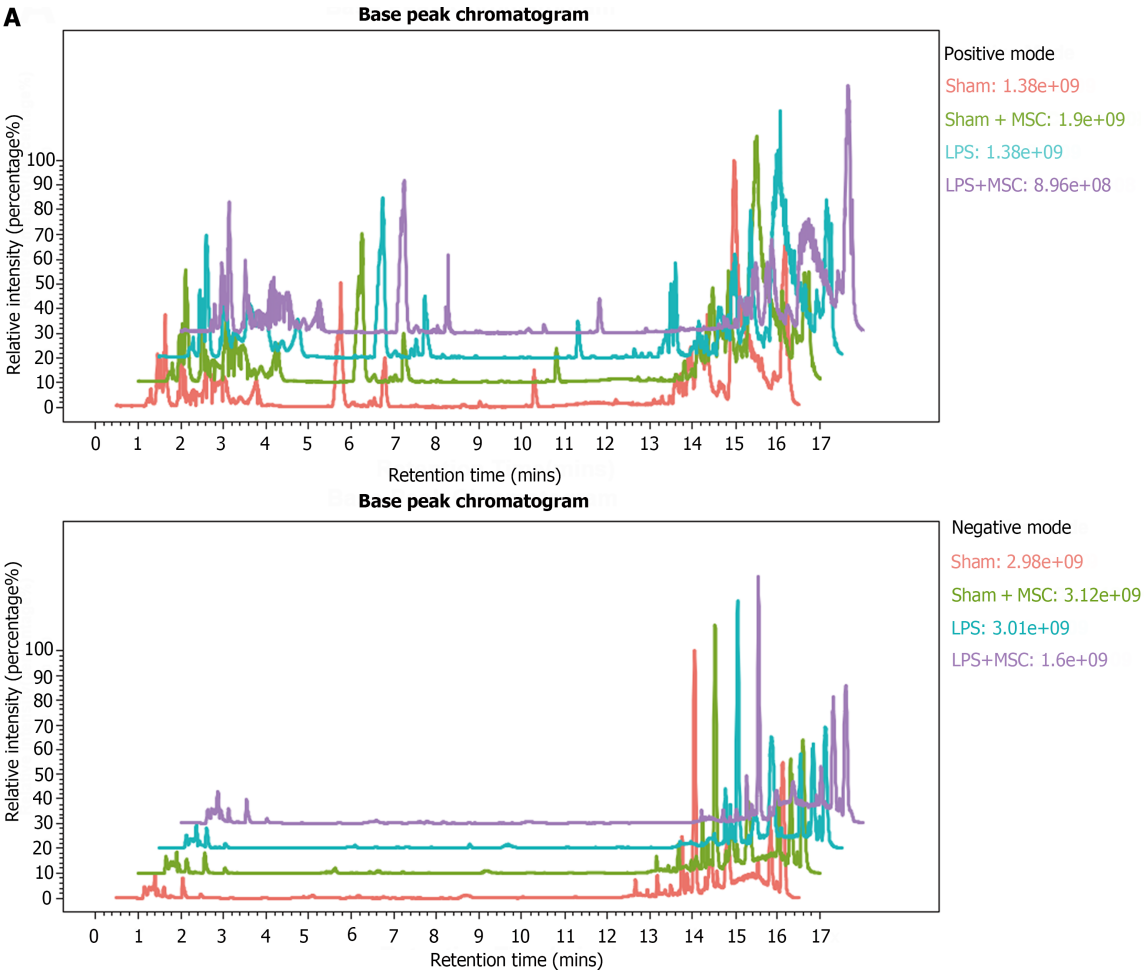
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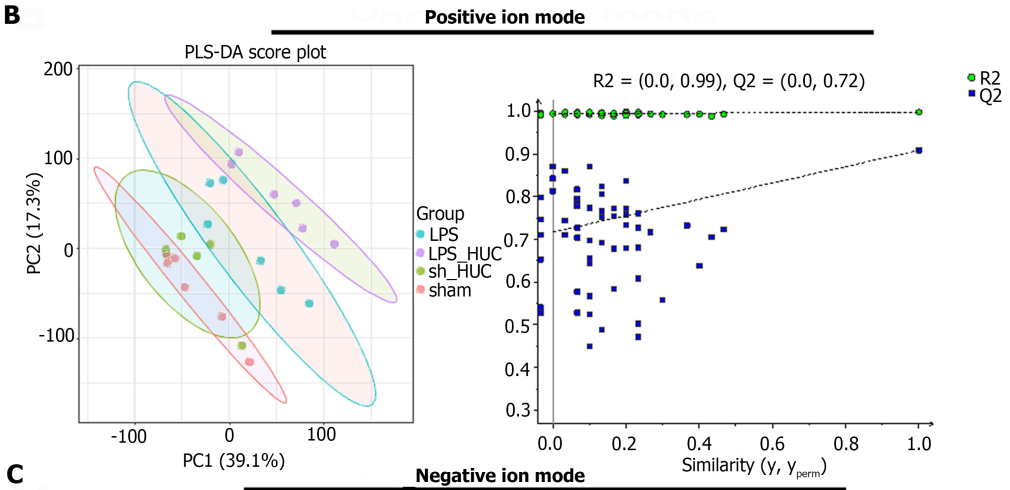
Figure 6 Correlation analysis between gut and lung microflorae. Person correlation analysis was used to analyze the correlation between marked microflorae of the gut and lung with OmicsStudio Tools. Red indicates positive correlation and blue indicates negative correlation. $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ *vs* lipopolysaccharide group. BALF: Bronchoalveolar lavage fluid.

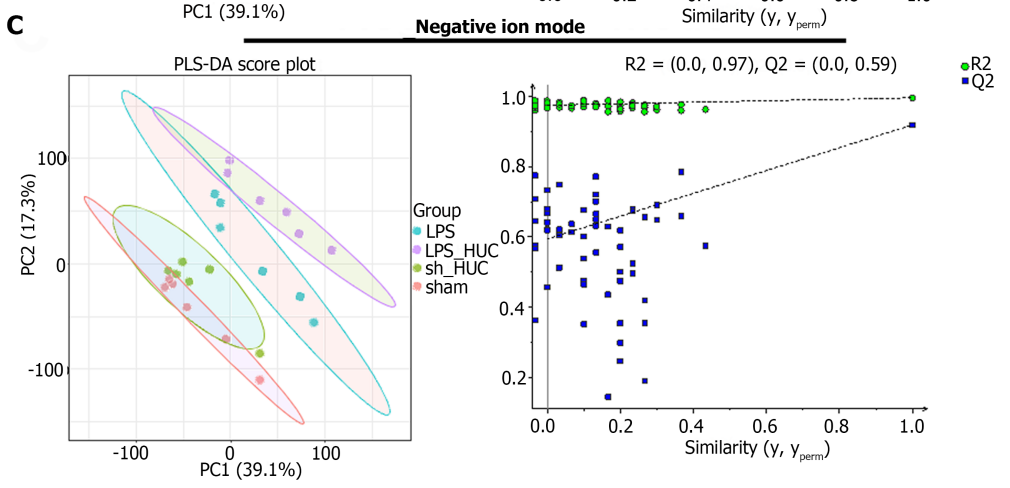
in respiratory tract infections[47]. Additionally, the *Comamonas* genus is an important opportunistic pathogen in human [48]. The genus *Elizabethkingia* has recently emerged as the cause of life-threatening infections in humans, most commonly causing meningitis[49]. *Acinetobacter Baumannii* infections have also been linked to ventilator-associated pneumonia[50]. These findings suggest that HUC-MSCs ameliorate ALI by inhibiting the abundance of pathogenic bacteria. Additionally, by analyzing the microflora in the feces, *Oscillospira* and *Coprococcus* genus abundance in the LPS + MSC group was increased. *Oscillospira*, a common genus of gut bacteria, positively correlates with gut microbiota diversity[51]. Moreover, butyrate, a product of the *Coprococcus* genus, is thought to be able to participate in anti-inflammatory processes[52]. Thus, *Oscillospira* and *Coprococcus* may be involved in the restorative effects of HUC-MSC treatment on gut microflora homeostasis. Additionally, some bacteria have contradictory roles in various diseases. The *Mucispirillum* genus is associated with Crohn's disease-like colitis in immunodeficient mice and is linked to health promotion in immunocompetent hosts[53]. In this study, the improvements in ALI may have been correlated with the abundance of microflora.

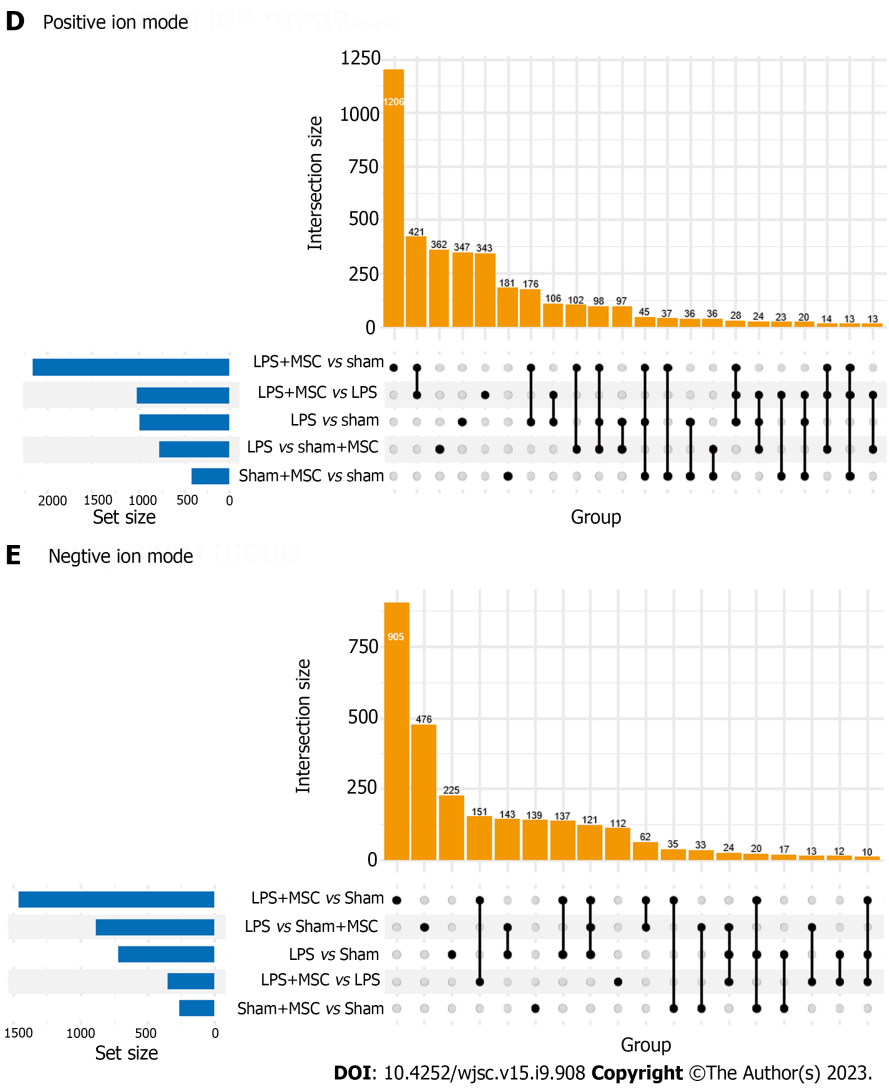
Correlation analysis of the gut and lung microflora noticed that the *Desulfovibrio* genus was positively correlated with *Stenotrophomonas*. The *Desulfovibrio* genus is a candidate microbe that induces weight loss of mice with ALI and is directly related to survival[11]. This study not only noted bacteria that strongly correlated with the downregulation of pathogenic bacterial abundance in BALF but also those that were present in both the gut and lungs. *Lactobacillus, Bacteroides,* and *unidentified_Rikenellaceae* genera appeared in the feces and BALF. Moreover, the *Bacteroides* in feces were significantly related to *Lactobacillus* in the BALF. Significant changes in their abundance may be associated with the mechanism of the lung-gut axis. Although the *Lactobacillus* genus is a beneficial flora most of the time in colitis[54], *Lactobacillus rhamnosus* GG treatment in patients with severe pneumonia does not improve the clinical outcomes[55], suggesting that changes in the abundance of *Lactobacillus* may be a consequence of the improvement of ALI by HUM-MSCs and could be used as biomarkers. *Lactobacillus, Bacteroides,* and *unidentified_Rikenellaceae* genera are potential biomarkers for evaluating the treatment efficacy of HUC-MSCs.

Moreover, lung tissue metabolomics was performed in this study, and the composition of metabolites was different in sham mice and ALI mice; HUM-MSC treatment in ALI mice changed the lung metabolite composition. The results of the metabolomic analysis suggest that HUM-MSC treatment alters the bile secretion pathway. Bile and its nuclear receptor farnesoid X are involved in inflammatory liver and bowel diseases[56]. In addition, this study verified the correlation


A
Base peak chromatogram
Relative intensity (percentage%)
Retention time (mins)
Positive mode
Sham: 1.38e+09
Sham + MSC: 1.9e+09
LPS: 1.38e+09
LPS+MSC: 8.96e+08
Base peak chromatogram
Relative intensity (percentage%)
Retention time (mins)
Negative mode
Sham: 2.98e+09
Sham + MSC: 3.12e+09
LPS: 3.01e+09
LPS+MSC: 1.6e+09


B
Positive ion mode
PLS-DA score plot
PC2 (17.3%)
PC1 (39.1%)
Group
LPS
LPS_HUC
sh_HUC
sham
R2 = (0.0, 0.99), Q2 = (0.0, 0.72)
R2
Q2
Similarity (y, y_perm)


C
Negative ion mode
PLS-DA score plot
PC2 (17.3%)
PC1 (39.1%)
Group
LPS
LPS_HUC
sh_HUC
sham
R2 = (0.0, 0.97), Q2 = (0.0, 0.59)
R2
Q2
Similarity (y, y_perm)



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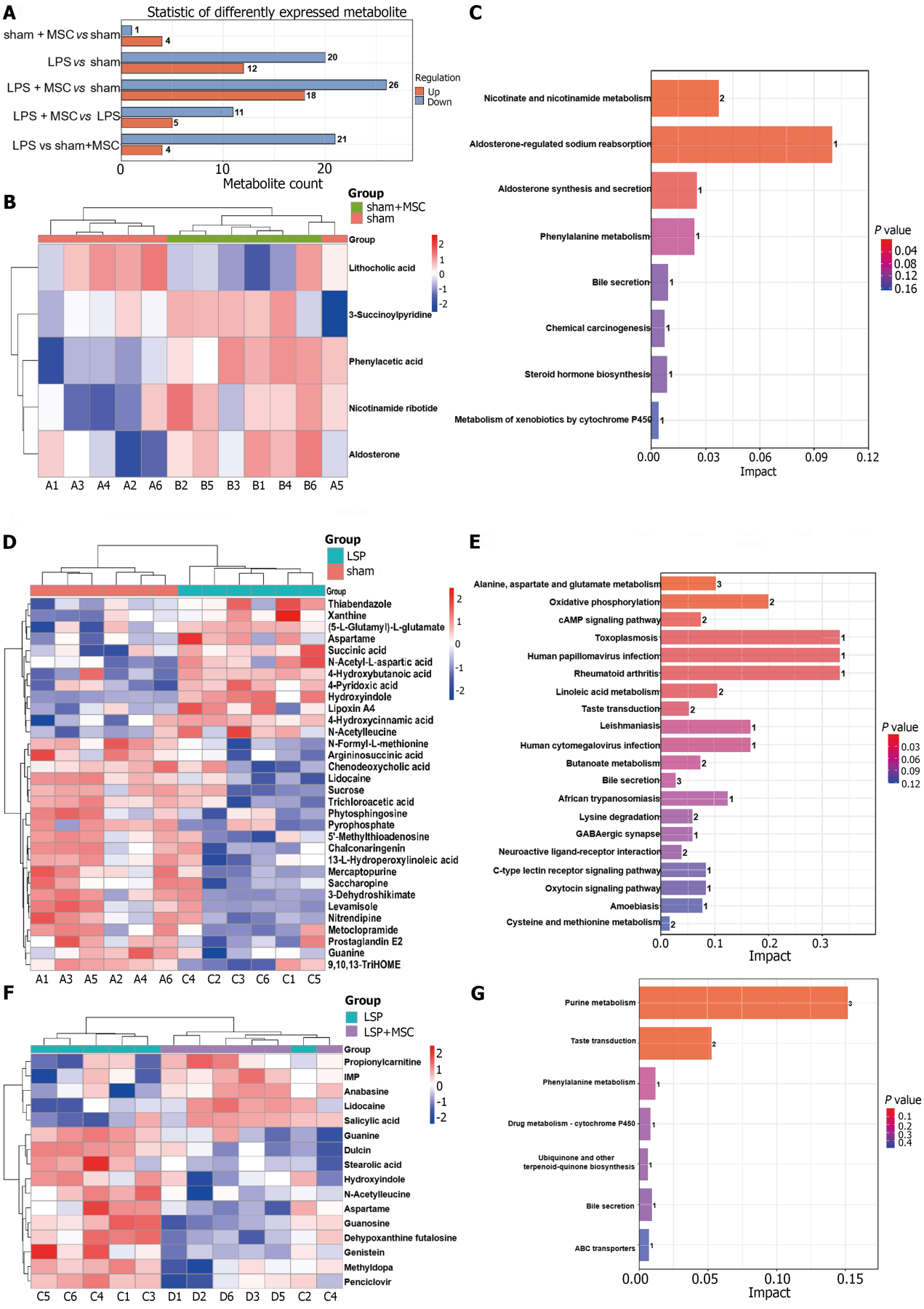
Figure 7 Metabolomics for the lung (*n* = 6). A: Base peak chromatogram in positive/negative mode; B and C: The Partial Least Squares-Discriminant Analysis was used to distinguish metabolite differences between groups in positive/negative mode; D and E: Upset Venn diagrams were used to count the number of common and unique differential metabolites in different groups. The bar chart at the bottom left represents the total number of differential metabolites between two groups. The bar chart above shows common metabolites. The results are based on mass spectrum analysis data. LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell.

between the BALF microflora and lung tissue metabolites using the O2PLS method. *Haemophilus* has a pivotal role in improving ALI mice by HUC-MSCs. One study reported that non-typeable *Haemophilus influenzae* induces neutrophilic inflammation in severe asthma[57]. Moreover, Yue *et al*[58] found that autophagy can combat the inflammation caused by *Haemophilus parasuis*, acting as a cellular defense mechanism. Similarly, KEGG enrichment results of the high-impact metabolites predicted by O2PLS suggested that autophagy-related pathways may play a critical role in HUC-MSC treatment in ALI mice. The exploration of lung metabolites contributes to the biological mechanism and biomarker discovery of HUM-MSCs treatment in ALI.

Naturally, this study only examined the correlation between microarray and metabolomics in the lung and gut and cannot have conclusive evidence to confirm that the lung-gut axis microbiota is a crucial factor behind the ability of HUC-MSCs to improve ALI. Animal and clinical studies are necessary to validate the role of gut and lung microorganisms in the improvement of ALI by HUC-MSCs. As depicted in Figure 10, this research showcases the crucial involvement of the lung-intestinal axis in safeguarding the lungs and intestines of ALI rats treated with HUC-MSCs, highlighting the interconnectedness of the lung-intestinal microbiota and metabolites.

CONCLUSION

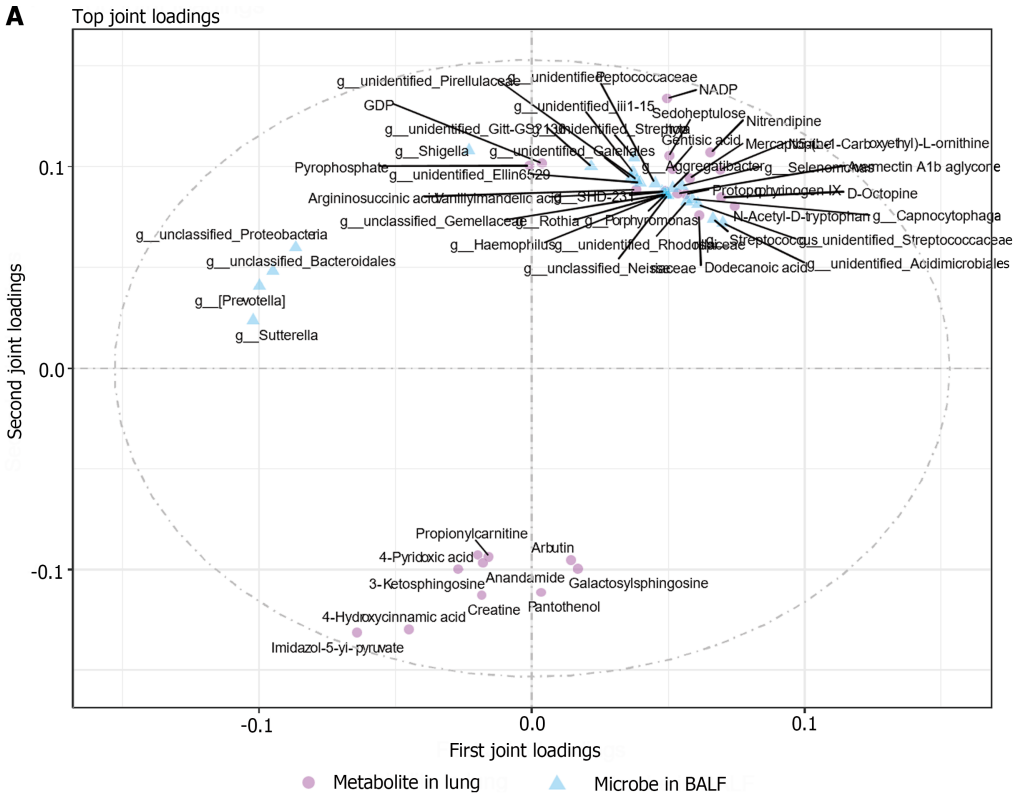
This study showed that HUM-MSC treatment of ALI improved the edema, tissue injury, and endothelial barrier function of the lung; upregulated the VE-cadherin, ZO-1, and occludin levels in the lung; and inhibited the inflammatory cytokine expression levels in the serum and lung. Moreover, HUM-MSC treatment of ALI attenuated the expression of the TLR4/Myd88/NF-κB signaling pathway in the lung tissue. In addition, HUM-MSC treatment of ALI mice improved ileal histopathological damage, reduced the levels of inflammatory factors, promoted ZO-1, claudin-1, and occludin protein

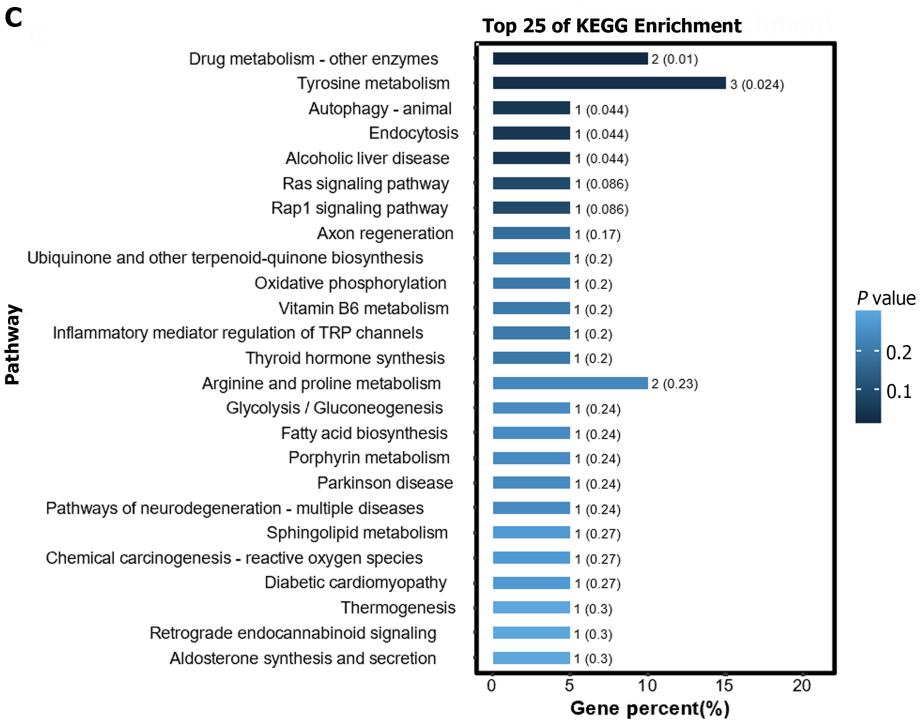


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Figure 8 Screening of differently expressed metabolites in acute lung injury mice treated with human umbilical cord mesenchymal stem

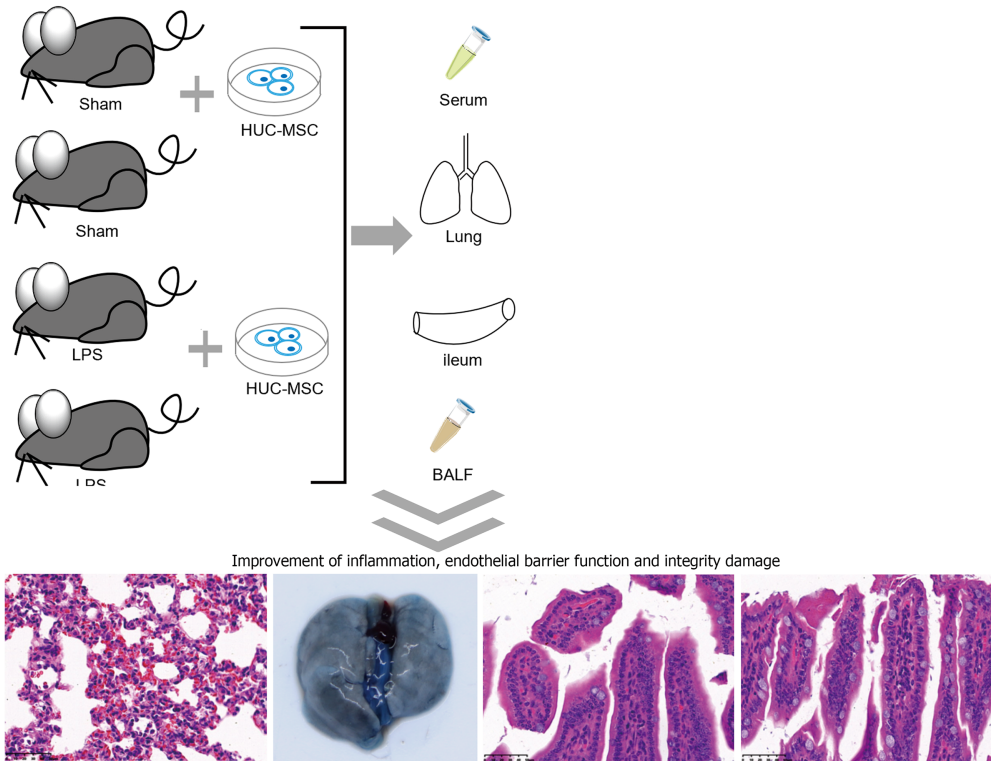
cells (*n* = 6). A: Statistic of differently expressed metabolites under liquid chromatography-tandem mass spectrometry mode; B: Heatmaps of sham + mesenchymal stem cells (MSCs) group *vs* sham group; C: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by differently expressed metabolites of sham + MSC group *vs* sham group; D: The lipopolysaccharide (LPS) group *vs* sham group were used to analyze and display differently expressed metabolites; E: KEGG pathway enriched by differently expressed metabolites of LPS group *vs* sham group; F: Heatmaps of LPS group *vs* LPS + MSC group; G: KEGG pathways enriched by differently expressed metabolites of sham + MSC group *vs* sham group. LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell.





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Figure 9 Extent of correlation between lung metabolites and bronchoalveolar lavage fluid microflorae. A: Top 25 lung metabolites and top 25 bronchoalveolar lavage fluid (BALF) microflorae were analyzed by the two-way Orthogonal Partial Least Squares analysis. A larger absolute value in a coordinate indicates a greater degree of association. Circles represent metabolites in lung tissues and squares represent microflora in BALF; B: The number of lung metabolites which were related with BALF microflorae; C: Kyoto Encyclopedia of Genes and Genomes enrichment analysis of top 25 BALF microflorae with strong association. KEGG: Kyoto Encyclopedia of Genes and Genomes; BALF: Bronchoalveolar lavage fluid.



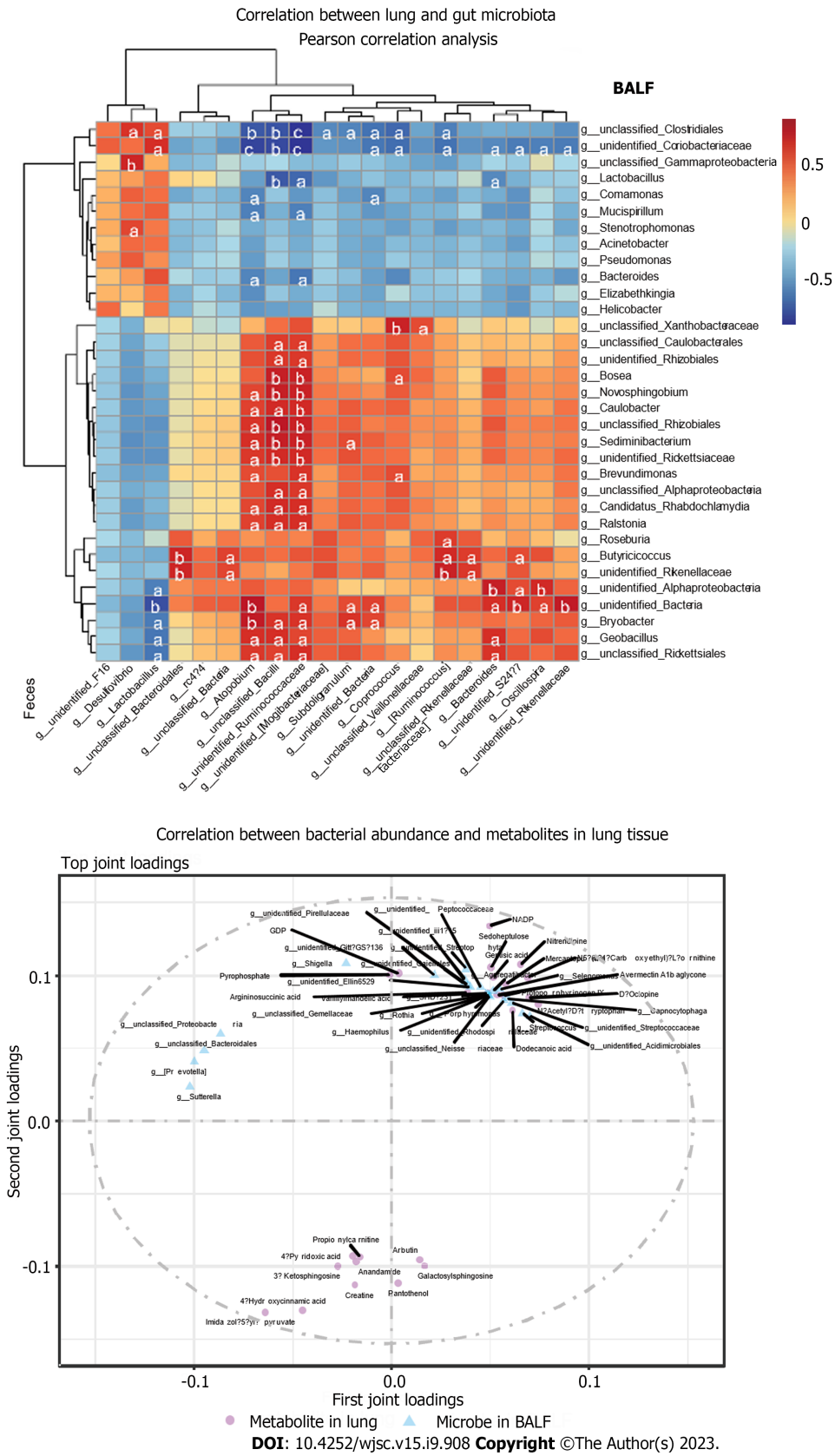


Figure 10 Graphical abstract. LPS: Lipopolysaccharide; MSC: Mesenchymal stem cell; BALF: Bronchoalveolar lavage fluid.

expression, and decreased EUB338 counts in the lung and ileum. In particular, this study found that the gut and lung microflora and metabolites were significantly different between ALI mice untreated and treated with HUM-MSCs. There was a correlation between the abundances of the gut and lung microflora. *Lactobacillus*, *Bacteroides*, and *unidentifiedden-tified_Rikenellaceae* genera are potential biomarkers for evaluating the treatment efficacy of HUC-MSCs. Additionally, this study contributes to the biological mechanism and biomarker discovery of HUM-MSC treatment of ALI by the combined

analysis of lung tissue metabolomics and microbiota, providing a scientific basis for the biological mechanism and clinical application of HUC-MSCs and new ideas for the development of therapeutic strategies for ALI.

ARTICLE HIGHLIGHTS

Research background

Acute lung injury (ALI) has high morbidity and mortality rates and needs effective treatment. Research has found that the gut microbiota improves lung injury through the lung-gut axis. Human umbilical cord mesenchymal cells (HUC-MSCs) can improve ALI.

Research motivation

Although HUC-MSCs can improve ALI, their biological mechanism of action is not yet clear.

Research objectives

To explore changes in the microbiota in the lung-gut axis and the relationship with HUC-MSC treatment.

Research methods

C57BL/6 mice were used to establish an ALI animal model by intraperitoneal injections of lipopolysaccharide. Wright's staining, ELISA, hematoxylin-eosin staining, Evans blue dye leakage assay, immunohistochemistry, fluorescence *in situ* hybridization, and western blot were used to observe the improvement of ALI mice by HUC-MSCs. High-throughput 16S rDNA sequencing was used to observe the microbiota homeostases in the lung-gut axis. The non-targeted metabolomics was used to explore changes in lung tissue metabolites.

Research results

HUC-MSCs ameliorated histopathological damage in the lung and ileum of ALI mice. HUC-MSC treatment improved inflammation, endothelial barrier integrity, and bacterial translocation in the lungs and ileum of ALI mice. HUC-MSCs regulated lung-gut microbiota homeostasis. HUC-MSC treatment regulated the metabolic profile in the lung and ileum of ALI mice.

Research conclusions

This study shows the improvement of changes in the lung and ileum of ALI mice by HUC-MSCs, and suggests a correlation between HUM-MSC-improved ALI and gut and lung microbiota homeostases.

Research perspectives

This study explores the biological mechanism of HUC-MSCs in improving ALI from the perspective of the correlation between the microbiota in the lung-gut axis and lung tissue metabolites, providing a research basis for HUC-MSC treatment.

FOOTNOTES

Author contributions: Cui EH, Lv L, and Wang B conceived and designed the research; Lv L, Li LQ, and Lu HD acquired the data; Cui EH, Li LQ, and Hua F analyzed and interpreted the data; Lv L, Lu HD, Chen WY, and Chen N performed statistical analysis; Cui EH and Wang B obtained the funding; Lv L and Cui EH drafted the manuscript; Cui EH, Wang B, and Hua F revised the manuscript for important intellectual content; and all authors approved the final version of the article.

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Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals (Approval No. ZJEY-20220721-02).

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ORIGINAL ARTICLE

Basic Study

Integrin beta 3-overexpressing mesenchymal stromal cells display enhanced homing and can reduce atherosclerotic plaque

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Abstract

BACKGROUND

Umbilical cord (UC) mesenchymal stem cell (MSC) transplantation is a potential therapeutic intervention for atherosclerotic vascular disease. Integrin beta 3 (ITGB3) promotes cell migration in several cell types. However, whether ITGB-modified MSCs can migrate to plaque sites *in vivo* and play an anti-atherosclerotic role remains unclear.

AIM

To investigate whether ITGB3-overexpressing MSCs ($MSCs^{ITGB3}$) would exhibit improved homing efficacy in atherosclerosis.

METHODS

UC MSCs were isolated and expanded. Lentiviral vectors encoding ITGB3 or green fluorescent protein (GFP) as control were transfected into MSCs. Sixty male apolipoprotein $E^{-/-}$ mice were acquired from Beijing Vital River Lab Animal Technology Co., Ltd and fed with a high-fat diet (HFD) for 12 wk to induce the formation of atherosclerotic lesions. These HFD-fed mice were randomly separated into three clusters. GFP-labeled MSCs ($MSCs^{GFP}$) or $MSCs^{ITGB3}$ were transplanted into the mice intravenously *via* the tail vein. Immunofluorescence staining, Oil red O staining, histological analyses, western blotting, enzyme-linked immunosorbent assay, and quantitative real-time polymerase chain

reaction were used for the analyses.

RESULTS

ITGB3 modified MSCs successfully differentiated into the "osteocyte" and "adipocyte" phenotypes and were characterized by positive expression (> 91.3%) of CD29, CD73, and CD105 and negative expression (< 1.35%) of CD34 and Human Leukocyte Antigen-DR. In a transwell assay, $MSCs^{ITGB3}$ showed significantly faster migration than $MSCs^{GFP}$. ITGB3 overexpression had no effects on MSC viability, differentiation, and secretion. Immunofluorescence staining revealed that ITGB3 overexpression substantially enhanced the homing of MSCs to plaque sites. Oil red O staining and histological analyses further confirmed the therapeutic effects of $MSCs^{ITGB3}$, significantly reducing the plaque area. Enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction revealed that MSC^{ITGB3} transplantation considerably decreased the inflammatory response in pathological tissues by improving the dynamic equilibrium of pro- and anti-inflammatory cytokines.

CONCLUSION

These results showed that ITGB3 overexpression enhanced the MSC homing ability, providing a potential approach for MSC delivery to plaque sites, thereby optimizing their therapeutic effects.

Key Words: Atherosclerosis; Inflammation; Integrin beta 3; Mesenchymal stem cells; Arg-Gly-Asp structure; Umbilical cord

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Core Tip: Mesenchymal stem cell (MSC) transplantation is considered a new treatment for atherosclerosis. However, research regarding homing of MSCs to atherosclerotic lesions is insufficient. Here, we transplanted integrin beta 3 (ITGB3)-overexpressing MSCs into a mouse model of atherosclerosis. ITGB3-overexpressing MSCs were more greatly accumulated in atherosclerotic plaques. These MSCs prevented plaque progression by shifting the local cytokine profile. The use of ITGB3-overexpressing MSCs may be a novel tool to treat atherosclerosis.

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INTRODUCTION

Atherosclerosis is a serious public health problem and the most commonly diagnosed cardiovascular disease in the general population[1]. Implementing early detection of atherosclerosis[2] with systemic pharmacological treatments[3] and percutaneous coronary intervention[4] has contributed to substantial progress in the treatment of atherosclerosis-related diseases[5,6]. Despite medical improvements, the incidence of atherosclerosis-related diseases remains high[7]. Atherosclerotic plaques are still associated with high mortality rates in patients with high-risk or visible advanced plaque disease. Therefore, more studies are needed to discover and explore effective molecules and targets for treatments.

Mesenchymal stem cell (MSC) transplantation accomplished improvements in experimental studies of atherosclerosis [8-11]. MSCs have the ability to secrete many cytokines that mitigate vascular inflammation and regulate the local microenvironment owing to the effects of their secreted anti-inflammatory factors within the vascular plaque[12-14]. However, some limitations preclude the translation of stem cell therapy into clinical applications[15,16], such as stem cells accurately homing to plaques. To determine this, we need to understand the specific mechanisms that underlie the migration and adhesion of MSCs under physiological and pathological conditions. Some studies have attempted to genetically decorate MSCs with specific receptors required for efficient homing. For example, Shahror *et al*[17] found that overexpression of fibroblast growth factor 21 considerably increased MSC migration to and adhesion in the injured brain tissue. Moreover, overexpression of C-X-C chemokine receptor 5 (CXCR5) increased the migratory capability of MSCs toward CXCL13, which changed most substantially in animal models of contact allergy, accompanied by decreases in inflammatory cellular infiltration and reduced levels of pro-inflammatory cytokine production[18]. It is well established that damaged tissues release specific types of cytokines and chemokines. Therefore, elucidating the interrelationships between tissue-specific chemokines and matching receptors on MSCs could offer novel ways of promoting homing and treatment effects of these cells.

The integrin family of receptors, a major family of migration-promoting receptors, plays an important role in the crosstalk between cells and their surroundings[19,20]. Arg-Gly-Asp (RGD) site-containing proteins and corresponding integrin receptors constitute the primary recognition system for cell adhesion[21,22]. Many inflammatory cytokines, such as intercellular cell adhesion molecule-1 (ICAM-1), osteopontin (OPN), and vascular cell adhesion molecule-1 (VCAM-1), contain an RGD motif in their structure[23,24]. The primary sequence of integrin beta 3 (ITGB3), a well-preserved region in all integrin beta subunits, has been called the RGD crosslinking region[25]. Based on this information, we genetically

modified MSCs using *ex vivo* lentiviral transduction to overexpress ITGB3. In the current study, we first demonstrated that ITGB3-overexpressing MSCs ($MSCs^{ITGB3}$) showed enhanced chemotaxis toward plaque tissues *in vivo* and inflammatory cells *in vitro*. Compared with green fluorescent protein (GFP)-labeled MSCs ($MSCs^{GFP}$), $MSCs^{ITGB3}$ reduced the formation of atherosclerotic plaques in homozygous apolipoprotein-E $(ApoE)^{-/-}$ mice, suggesting that ITGB3 enhances homing of modified stem cells to plaque tissue, thereby promoting their therapeutic efficacy in the $ApoE^{-/-}$ mouse model of atherosclerosis.

MATERIALS AND METHODS

Isolation of human umbilical cord MSCs

Human umbilical cord (UC) samples were collected from three healthy donors. All the donors provided written informed consent. MSCs were isolated and cultured from the UCs as reported previously[26]. Briefly, the UC tissue was collected from healthy pregnant women undergoing labor. After removing the UC tissue's arteries and veins, the remaining Wharton's jelly was cut into small pieces for patch cultures. The tissue fragments were cultured at 37 °C and 5% CO_2 in mesenchymal stem cell medium (MSC medium; Sciencell, Carlsbad, CA, United States) containing 5% fetal bovine serum (FBS), stem cell growth supplements, penicillin, and streptomycin. After 10-15 d, fibroblast-like MSCs migrated out of the tissue patches. The studies involving pregnant participants were reviewed and approved by The Ethical Committee of The Second Hospital of Hebei Medical University (approval number: 2021-R496).

Raw264.7, vascular smooth muscle cell, and primary microvascular endothelial cell cultures

The murine macrophage cell line Raw264.7 was purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, the Chinese Academy of Science (Shanghai, China). Vascular smooth muscle cells were isolated from mouse aortas and cultured in low-glucose Dulbecco's modified Eagle's medium containing 10% FBS. Lung primary microvascular endothelial cells were obtained from the lungs of 4-week-old C57 mice through two series of immunoselection with CD31- and CD102-conjugated magnetic beads using a previously described procedure[27] and subsequently cultured in endothelial cell medium containing 5% FBS and endothelial cell growth supplements.

Lentiviral vector construction and transduction

Lentiviral vector (LV) encoding ITGB3 and GFP as control were generated and packaged by Hanbio Biosciences (Shanghai, China). All LVs were used for MSC transfection with an infection multiplicity of 30-40. MSCs were plated at a density of 3-5 × 10^5 cells/cm^2 in each six-well plate, depending on the subsequent use. When MSCs reached 40%-50% confluence, the transfection was performed in the presence of polybrene (Cyagen Biosciences, Santa Clara, CA, United States). Following transfection for 72 h, the transfection effectiveness of the virus was checked through fluorescent staining.

AS model induction and MSC delivery

$ApoE^{-/-}$ mice (7–8-week-old) were acquired from Beijing Vital River Lab Animal Technology Co., Ltd (Beijing, China). Sixty male $ApoE^{-/-}$ mice were housed in Hebei Medical University and fed with standard chow and drinking water for 1 wk to adapt to the new surroundings. Then, all animals were fed a high-fat diet (HFD) for 12 wk to induce the formation of atherosclerotic lesions. These HFD-fed mice were randomly separated into three clusters and received the following treatment: (1) HFD mice (n = 20) were injected 200 μL of phosphate-buffered saline (PBS) into the caudal vein every week from week 9, four times in total; (2) MSC^{GFP} mice (n = 20) receiving 200 μL of PBS containing 1 × 10^6 $MSCs^{GFP}$ intravenously through the tail vein every week from week 9; and (3) MSC^{ITGB3} mice (n = 20) receiving 200 μL of PBS containing 1 × 10^6 $MSCs^{ITGB3}$ intravenously through the tail vein every week from week 9. The animals were euthanized after 12 wk of HFD, the aorta and aortic sinus were collected, and histological samples were processed for subsequent atherosclerosis assessment. At the same time, blood samples were collected to determine lipid levels. All animal procedures complied with the Guide for the Care and Use of Experimental Laboratory Animals and were approved by the Animal Care Committee of The Second Hospital of Hebei Medical University (No. 2021-R496).

Echocardiographic assessment

At the end of week 12, 10 mice were gas-anesthetized *via* a facemask and kept on a minimal dose of isoflurane (1.0%-2.0%). The mice were placed in a supine position and maintained spontaneous breathing with isoflurane insufflation throughout the echocardiographic assessment. Echocardiographic data were recorded by a VisualSonics Vevo2100 Imaging System (Fujifilm Visual Sonics Inc., Tokyo, Japan) with an EZ-SA800 Single Animal System (E-Z Systems Inc., Bethlehem, PA, United States). Ascending aorta functions were evaluated from the parasternal view of the long axis. The diameter of the systolic aorta was measured at the peak anterior movement point of the ascending aorta, and the diameter of the diastolic aorta was measured using a Q-wave (end-diastolic) echocardiogram. The average diameter measurements of five consecutive heart cycles were selected for data analysis. Meanwhile, left ventricle function and heart chamber dimensions were also evaluated, including the left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS), using accompanying software. The data were recorded and analyzed.

Murine aortic tissue culture

Murine aortic rings were cultured as described previously[28]. Briefly, after 12 wk of HFD, external organs and tissues

were removed. Then, the whole aortic vessel was separated from the adipose and surrounding tissue. The aorta was divided into 2-4 mm rings and cultured in low-glucose Dulbecco's modified Eagle's medium supplemented with 5% FBS. Aortic rings were maintained at 37 °C in 5% CO_2 for 24 h for further experiments.

Hematoxylin and eosin staining, Masson's trichrome staining, and oil red O staining

The aortic arch and aortic valve were harvested after 12 wk of HFD. The animals were euthanized, and the left ventricles were cannulated and injected with PBS containing heparin. Thereafter, the aortic arch and aortic valve were separated, embedded in optimal cutting temperature compound, shortly frozen in liquid nitrogen, and sliced. Subsequently, hematoxylin and eosin (HE) staining was performed to assess plaque size. The cross-sectional areas of the plaque were measured with Image-Pro Plus (IPP) 6.0 software (Media Cybernetics Corp., Rockville, MD, United States). Collagen depositions were assessed using Masson's trichrome staining. Average values were determined from at least three sections in each sample.

For oil red O (ORO) staining, the fixed whole aorta was incubated with 0.3% ORO solution (Sigma-Aldrich, St Louis, MO, United States) for 20 min and, then, washed with 60% isopropanol. Frozen aortic arch and aortic valve sections were incubated with ORO for 5 min, washed with PBS, and counterstained with hematoxylin. The atherosclerotic lesions were photographed using a light microscope (Carl Zeiss, Jena, Germany) and quantified using IPP 6.0 software.

Cell viability assay

The effects of LV transfection on MSC viability were evaluated using cell counting kit 8 (CCK-8) assays as described previously[29]. Briefly, MSCs, $MSCs^{ITGB3}$, and $MSCs^{GFP}$ were seeded into 96-well plates at a density of 1×10^4 cells/well, respectively. After 24 h, CCK-8 was added to each well and incubated at 37 °C for 1 h. The absorbance was measured at 450 nm using a VersaMax (Ocean Springs, MI, United States) microplate scanner.

Chemotaxis assays

Chemotaxis assays were operated in 24-well transmembrane chambers with 8-μm pore filters. To test the ability of stem cells to migrate toward defined chemokines, Raw264.7, vascular smooth muscle cells, and primary microvascular endothelial cells were separately seeded into the lower chamber (1×10^5 cells/well). After achieving confluence, serum-free cells were stimulated with tumor necrosis factor-α (TNF-α, 20 ng/mL) for 24 h. Then MSCs, $MSCs^{GFP}$, and $MSCs^{ITGB3}$ were respectively plated into the upper chamber at 2×10^4 cells/well. After incubation for 24 h, the upper layer of cells was removed, and the lower layer was stained with crystal violet.

For the vascular atherosclerotic plaque, mice were sacrificed after week 12. Aortic rings were separately cultured for 24 h in Dulbecco's modified Eagle's medium with 5% FBS. Next, MSCs, $MSCs^{GFP}$, and $MSCs^{ITGB3}$ were respectively plated into the upper chamber at 2×10^4 cells/well. The medium for re-suspending MSCs matched the medium in the lower chamber. After incubation for 24 h, non-migrated cells were removed from the upper surface, and the cells that had migrated across the membrane to the lower surface were fixed with 4% paraformaldehyde and stained with crystal violet. For each membrane, five fields of view were imaged and analyzed. Each experiment was repeated at least three times, and the number of migrated cells was expressed as the mean ± standard error of the mean (SEM) of total cell counts per field.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA of cells or tissue samples was extracted using TRIzol reagent, according to the manufacturer's protocol. cDNA was synthesized using the M-MLV First Strand Kit. Quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green qPCR SuperMix were performed using an ABI 7500. qRT-PCR analyses were repeated at least three times. The qRT-PCR data were standardized to β-actin expression, using the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Supplementary Table 1. The average threshold cycle for each gene was determined from at least three independent experiments.

Western blot analysis

Cell or tissue lysates were extracted with lysis buffer. Equal amounts of protein were separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% bovine serum albumin for 1 h at room temperature, incubated with specific antibodies against VCAM-1, ICAM-1, OPN, and GAPDH at 4 °C overnight, and, then, incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G for 1 h at room temperature. Antigen-antibody complexes were assessed using the GE ImageQuant™ LAS 4000 detection system (GE Healthcare Inc., Chicago, IL, United States). The protein bands of interest were quantified with IPP 6.0 software.

Statistical analyses

Data analysis was performed with GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, United States). Data are displayed as mean ± SEMs, and each independent experiment was repeated three times. The statistical significance of differences between two groups was determined using the unpaired Student's t-test, and comparisons of more than two groups were performed using a one-way analysis of variance. For all statistical comparisons, significance was considered at $P < 0.05$.

RESULTS

Inflammatory factors containing the RGD motif are highly upregulated in atherosclerotic vessels

Atherosclerosis is a chronic inflammatory disease of the arteries with high expression of adherence molecules. We tested the expression of VCAM-1, ICAM-1, and OPN because studies have shown that they contain the RGD motif that can bind to the ITGB3 receptor[19,20,30]. Our results demonstrated that the protein expression levels of VCAM-1, ICAM-1, and OPN were six to eight times higher in atherosclerotic than in control blood vessels (Figure 1A). Similarly, qRT-PCR and immunofluorescence staining also confirmed that the three adhesion factors considerably increased at sites of inflammation (Figure 1B–D).

The ITGB3 receptor is scarcely expressed on human UC-derived MSCs, and ITGB3 overexpression by MSCs does not affect other MSC characteristics

Inflammatory factors that are highly expressed at plaque sites and matching receptors expressed on the surface of MSCs play an important role in guiding stem cells. Thus, we analyzed the expression of the ITGB3 receptor using western blot, qRT-PCR, and fluorescence staining methods. First, human MSCs at the third passage had very low expression of the ITGB3 receptor at the protein and mRNA levels (Figure 2A and B). Second, immunofluorescence staining confirmed that MSCs barely expressed the ITGB3 receptor (Figure 2C and D). To further determine whether overexpression of ITGB3 receptor in MSCs can promote their homing capability toward plaque tissues, MSCs were transfected with LVs encoding ITGB3 and GFP ($MSCs^{ITGB3}$ for short) or GFP ($MSCs^{GFP}$ for short). We found that both $MSCs^{ITGB3}$ and $MSCs^{GFP}$ highly expressed GFP, confirming the stable expression of the GFP reporter gene (Figure 2C). In contrast to $MSCs^{GFP}$ and MSCs, $MSCs^{ITGB3}$ showed substantially stronger expression of ITGB3 at the protein, mRNA, and fluorescence staining levels (Figure 2A–D). Moreover, ITGB3 overexpression did not affect stem cell viability according to CCK8 analyses (Figure 2E). To further verify whether transfection of ITGB3 affects the differentiation properties of MSCs, we observed several typical stem cell markers using flow cytometry and qRT-PCR analyses. The results showed that in $MSCs^{ITGB3}$, MSC-specific markers (CD73, CD105, and CD29), but not hemopoietic stem cell antigens (CD34 and human leukocyte antigen-DR), were highly expressed (Figure 2F and G). The differentiation of $MSCs^{ITGB3}$ into adipocytes and osteoblasts was separately evaluated using ORO and Alizarin red staining (Figure 2H and I). Moreover, marker genes in induced adipocytes and osteoblasts were verified through qRT-PCR (Figure 2J and K). These findings confirmed that transfected MSCs still have stem cell properties.

ITGB3 enhances MSC migration toward inflammatory sites in vitro and in vivo

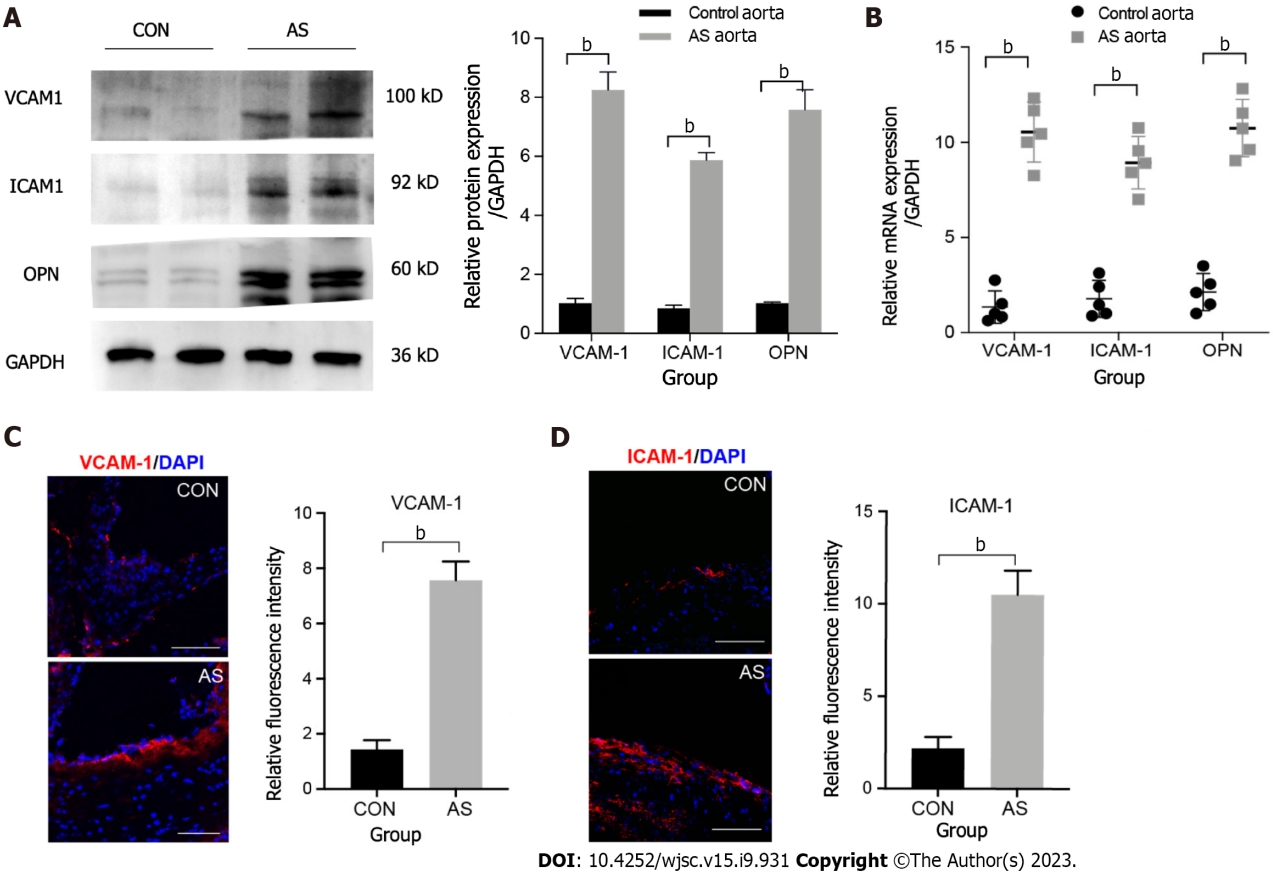
ITGB3 is vital for cell migration, adhesion, and invasion[20-22,31]. Therefore, we next checked whether $MSCs^{ITGB3}$ could promote MSC migration *in vitro* and *in vivo*. The *in vitro* cell chemotaxis assay showed that in the presence of TNF-α, $MSCs^{ITGB3}$ displayed a significantly increased migration toward the bottom chamber, especially if it contained macrophages (Figure 3A and B). However, in the absence of TNF-α, all cells showed similar, very low, nonspecific migration toward the lower, cell-containing chamber (Figure 3A and B). We also performed transwell-based chemotaxis assays using atherosclerotic aorta samples *in vitro* (Figure 3C and D). $MSCs^{ITGB3}$ had the highest migratory activity, indicating that the atherosclerotic plaque secreted inflammatory factors which were chemoattractants for $MSCs^{ITGB3}$, but not for $MSCs^{GFP}$ (Figure 3C and D). Next, we observed whether MSCs migrate to plaque sites *in vivo* following intravenous infusion. $MSCs^{ITGB3}$ and $MSCs^{GFP}$ were injected into HFD mice through the tail vein every week from weeks 9 to 12. The aortas were collected from each group at the end of week 12 for in situ fluorescence staining. The results showed that $MSCs^{ITGB3}$ were highly aggregated at sites of inflammation, whereas $MSCs^{GFP}$ had less positive staining at such sites (Figure 3E and F). Meanwhile, the qRT-PCR results also revealed that there was a higher GFP expression in the MSC^{ITGB3} group when compared to the GFP expression in the $MSCs^{GFP}$ group (Figure 3G). However, there were negligible MSCs in the myocardium, and no difference between the two groups was observed (Supplementary Figure 1). These data suggested that ITGB3 overexpression promoted the targeted migration of MSCs to plaque sites *in vivo*, possibly by targeting inflammatory factors containing the RGD structure.

$MSCs^{ITGB3}$ reduce the progression of atherosclerotic plaques in $ApoE^{-/-}$ mice fed an HFD

To explore the effects of $MSCs^{ITGB3}$ on the progression of atherosclerosis, the whole aorta, aortic root, and aortic arch were stained with 0.5% ORO, and the stained areas were quantified to determine the atherosclerotic plaque area. The stained area in the aorta was substantially decreased in the MSC^{ITGB3} group when compared with that of the HFD and MSC^{GFP} groups (Figure 4A and B). The stained areas in the aortic root and aortic arch decreased in the MSC^{ITGB3} group compared with the corresponding in the HFD and MSC^{GFP} groups (Figure 4C). HE and Masson's trichrome staining analyses of sections taken from the aortic root and aortic arch demonstrated that $MSCs^{ITGB3}$ inhibited aortic plaque formation and collagen deposition more effectively than $MSCs^{GFP}$ (Figure 4D and E). Aortic stiffness is the earliest detectable evidence of changes in arterial wall function. Echocardiographic results showed that both systolic and diastolic diameters were considerably increased while the vessel wall thickness was decreased in the MSC^{ITGB3} group (Figure 4F). However, left ventricular EF and FS were not different among the three groups (Supplementary Figure 2). The number of macrophages in atherosclerotic plaques at the aortic arch was determined by F4/80 staining. The F4/80 staining-positive areas were notably reduced in the MSC^{ITGB3} group compared with the HFD group (Figure 4G).

$MSCs^{ITGB3}$ do not alter the biochemical parameters of $ApoE^{-/-}$ mice fed an HFD

To evaluate the effects of $MSCs^{ITGB3}$ on biochemical features *in vivo*, we recorded changes in mouse body weight and



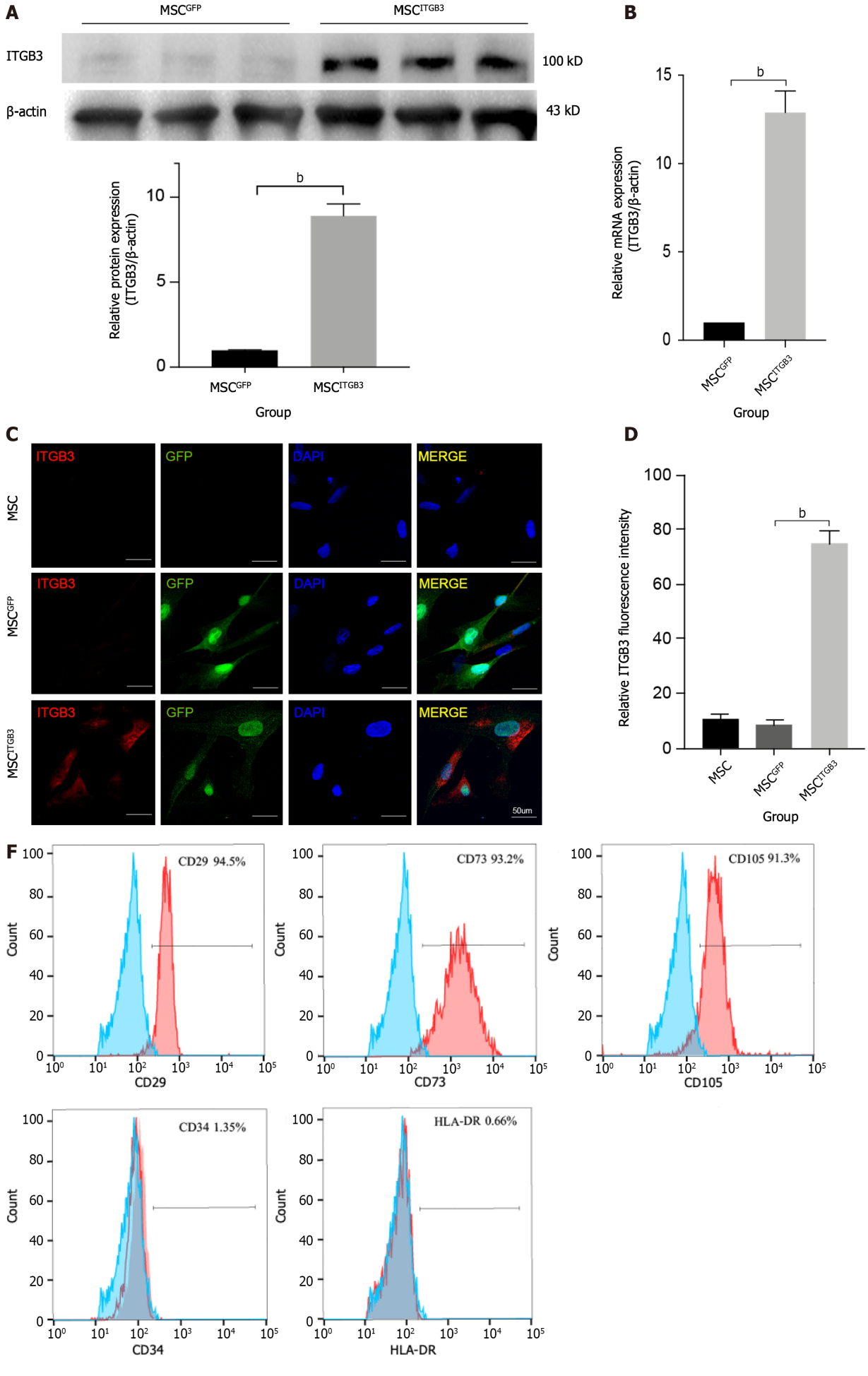
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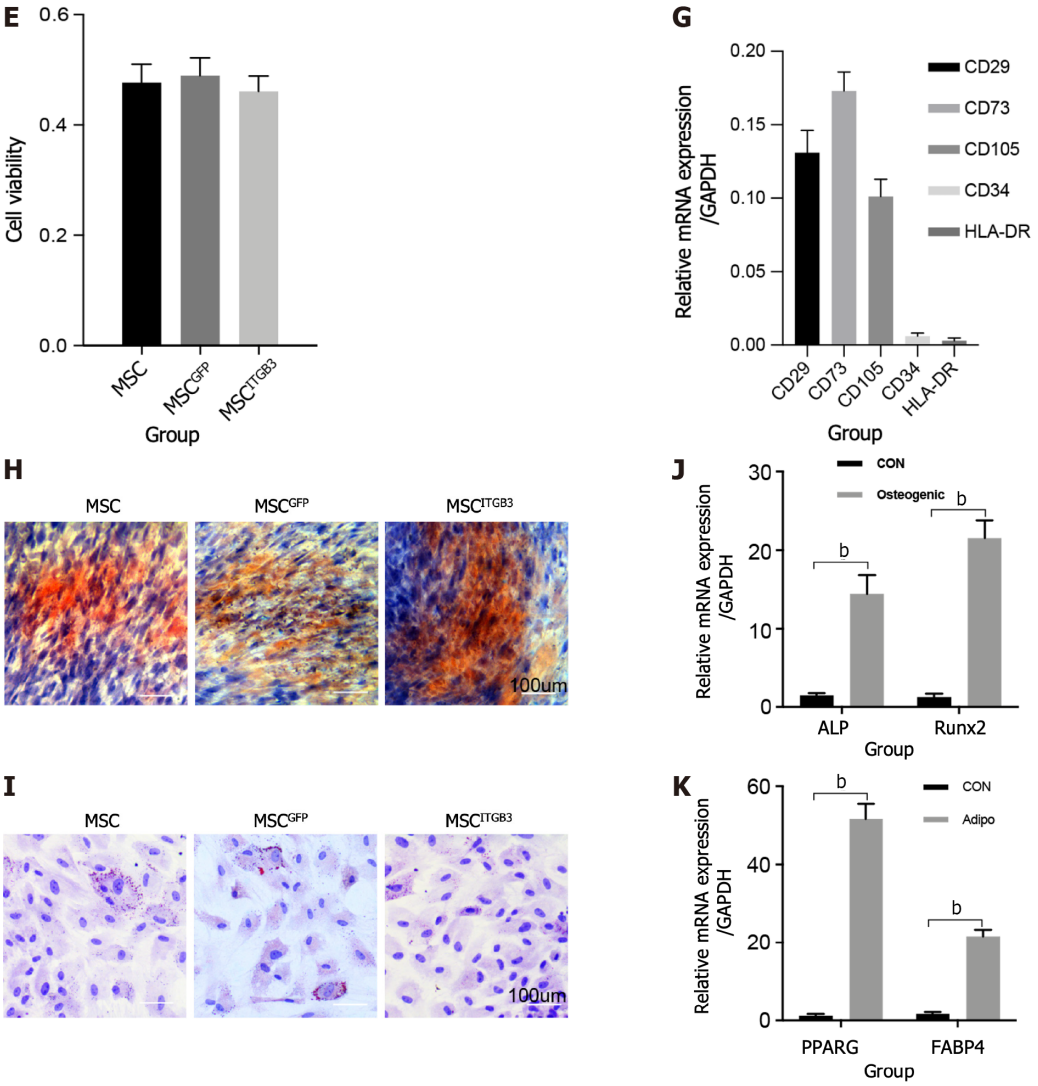
Figure 1 Expression of inflammatory factors in the vascular atherosclerotic plaque. A: Expression of mouse vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and osteopontin (OPN) expression in total tissue lysates of normal and atherosclerotic (AS) aorta analyzed using western blot. GAPDH was used as the internal control. The experiment was repeated thrice with tissues isolated from independent mice; a representative blot is shown; B: Expression levels of various inflammatory factors involved in atherosclerosis analyzed using quantitative real-time polymerase chain reaction of mRNA samples extracted from normal and AS vessels of three independent mice. Data are presented as the mean ± SEM for each group. Fold change represents the expression of each inflammatory factor in AS vessel of a mice fed with high fat diet for 12 wk compared with that in normal blood vessel; C: Representative images of normal and AS vascular sections stained for VCAM1 (red) and ICAM1 (red). The experiment was repeated three times with tissues isolated from independent mice; a representative image is shown. Nuclei were visualized by DAPI staining (blue). Scale bars = 100 mm; D: Representative images of normal and AS vascular sections stained for ICAM1 (red). The experiment was repeated three times with tissues isolated from independent mice; a representative image is shown. Nuclei were visualized by DAPI staining (blue). Scale bars = 100 μm. $^{b}P < 0.001$. AS: Atherosclerotic; OPN: Osteopontin; qRT-PCR: Quantitative real-time polymerase chain reaction; SEM: Standard error of the mean; VCAM-1: Vascular cell adhesion molecule-1; ICAM-1: Intercellular cell adhesion molecule-1.

measured blood lipid concentrations. The results showed that the body weight did not vary among the three groups during the experiment (Supplementary Figure 3A). Plasma lipid analyses, including those measuring total cholesterol, high-density lipoprotein cholesterol, triglyceride, and low-density lipoprotein cholesterol levels, were performed on terminal blood samples obtained by cardiac puncture but showed no statistical differences among the HFD, MSC^{GFP}, and MSC^{ITGB3} groups (Supplementary Figure 3B). These results suggest that intravenous injection of stem cells did not interfere with lipid metabolism in mice receiving an HFD.

MSCs^{ITGB3} modulate cytokine expression in the AS mouse model

The levels of inflammatory factors are positively correlated with the degree of inflammation in atherosclerosis. Cytokines play a pivotal role in chronic inflammatory diseases[31]. They affect the expression of adherence molecules, permeability of endothelial cell, as well as proliferation and migration of inherent cells in blood vessels, all of which are associated with atherosclerosis. Interleukin (IL)-1β, an established driver of atherosclerotic disease[32], is involved in the entire process of atherosclerotic lesion progression[33]. Advanced atherosclerotic plaques can be alleviated and stabilized through IL-1β inhibition. After observing that MSC^{ITGB3} treatment can reduce the plaque area, we further investigated whether MSC^{ITGB3} infusion can decrease the levels of pro-inflammatory factors or increase the levels of anti-inflammatory factors in the serum and aorta tissue. Our results showed that pro-inflammatory factors, including TNF-α, IL-1β, and IL-6, showed a significant downward trend in mice who had received MSC^{ITGB3} or MSC^{GFP} treatment. However, the treatment effect of MSCs^{ITGB3} was more pronounced than that of MSCs^{GFP}. Likewise, mice receiving MSCs^{ITGB3} or MSCs^{GFP} showed significantly increased levels of anti-inflammatory factors, such as IL-4, IL-10, and tumor necrosis factor-stimulated gene-6 in the serum and aorta tissue (Figure 5). These results indicate that MSC^{ITGB3} treatment primarily reduces pathological inflammatory responses by improving the balance between secreted pro- and anti-inflammatory factors.


A
MSCGFP
MSCITGB3
ITGB3
β-actin
100 kD
43 kD
Relative protein expression (ITGB3/β-actin)
b
Group
B
Relative mRNA expression (ITGB3/β-actin)
C
MSC
MSCGFP
MSCITGB3
ITGB3
GFP
DAPI
MERGE
50um
D
Relative ITGB3 fluorescence intensity
F
CD29 94.5%
CD73 93.2%
CD105 91.3%
CD34 1.35%
HLA-DR 0.66%
Count
CD29
CD73
CD105
CD34
HLA-DR



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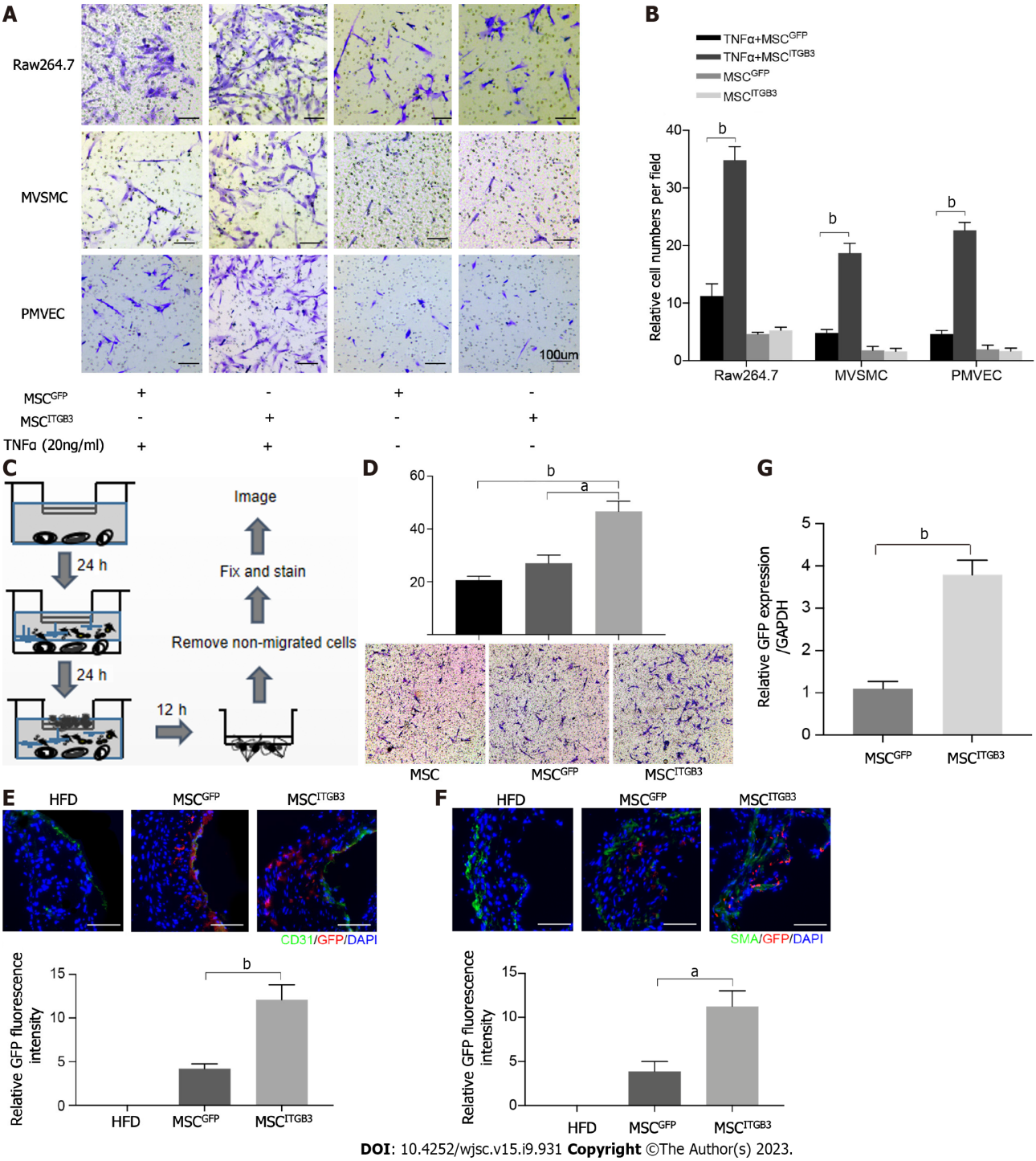
Figure 2 Expression of integrin subunit beta 3 on human umbilical cord-derived mesenchymal stem cells. A: Western blot; B: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of Integrin subunit beta 3 (ITGB3) expression; C: Representative immunofluorescence image; D: Quantification of ITGB3 and green fluorescence protein staining in untransfected and transfected mesenchymal stem cells (MSCs); E: Cell viability of untransfected and transfected MSCs determined by CCK8 assay. The experiment was repeated three times; F and G: Analysis of cell surface markers on MSC^{ITGB3} using flow cytometry to determine whether they have pluripotent characteristics; H: Osteogenic differentiation of MSC^{ITGB3} identified using Alizarin red staining; I: Oil Red O staining shows differentiation of MSC^{ITGB3} into adipocytes; J: qRT-PCR analysis of marker genes in induced osteoblasts; K: qRT-PCR analysis of marker genes in induced adipocyte. $^{b}P < 0.001$. GFP: Green fluorescence protein; ITGB3: Integrin subunit beta 3; MSC: Mesenchymal stem cell; ORO: Oil Red O; qRT-PCR: Quantitative real-time polymerase chain reaction.

DISCUSSION

In this study, adhesion molecules containing the RGD motif were highly expressed in an atherosclerosis model, and transfection of ITGB3 into MSCs improved their targeting capability. As expected, intravenous injection of MSCs^{ITGB3} substantially reduced atherosclerotic plaque in the ApoE$^{-/-}$ HFD mouse model.

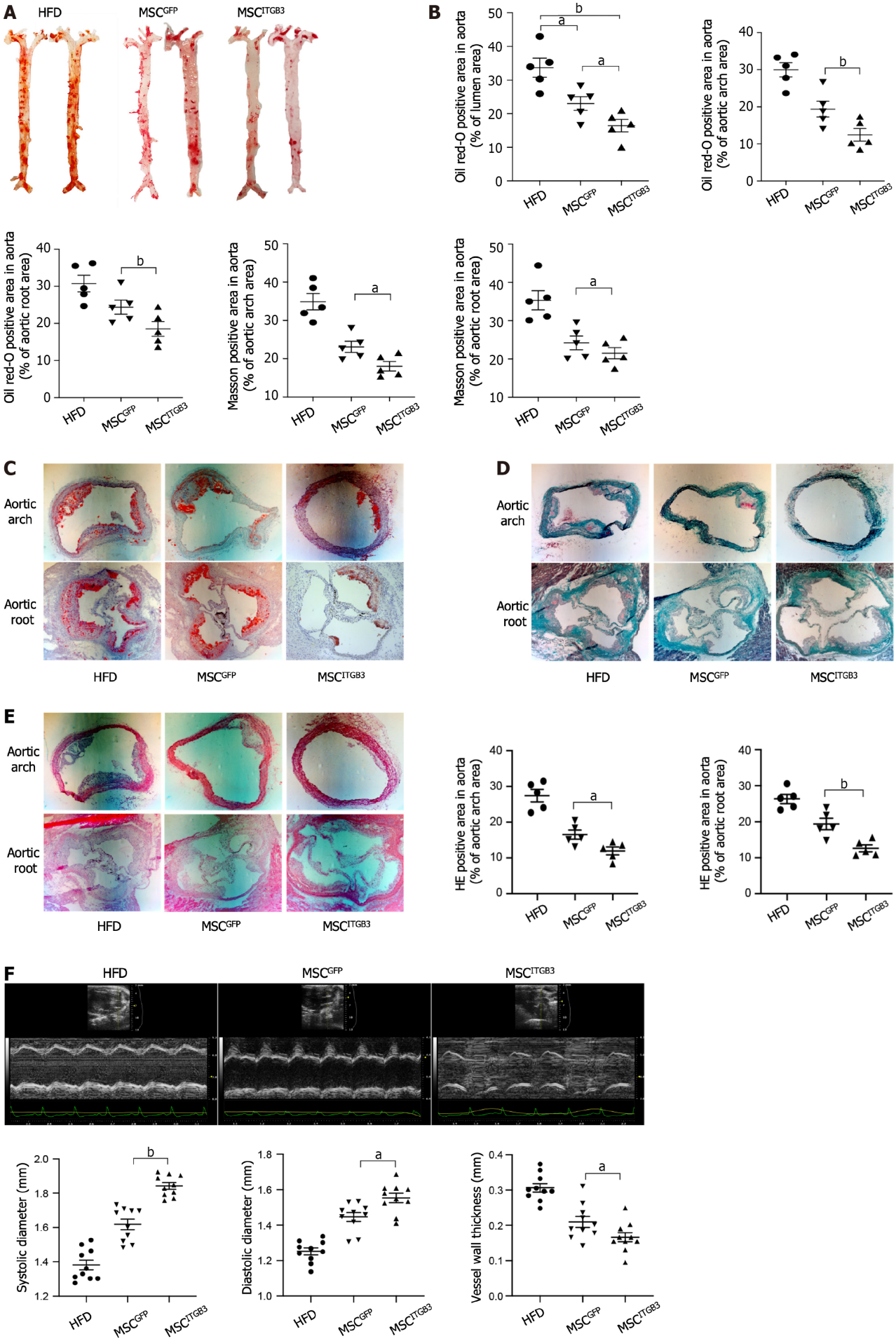
Owing to their adaptive immunomodulatory properties and superior secretory potential, MSCs have attracted extensive attention in the treatment of atherosclerotic diseases[8,14]. The route of MSC administration is critical for their therapeutic efficiency, and systemic delivery *via* intravenous infusion is the primary approach for many cell therapies. However, several problems occur with this approach, and one of the major hurdles is insufficient cell engraftment into the damaged tissue[34]. It is well known that the therapeutic level of MSCs mainly depends on intercellular interactions and their regulation by the local microenvironment. Therefore, the therapeutic effect of intravenously injected MSCs may be closely related to their localization. It is predicted that the effectiveness of cell therapy can be improved by increasing local recruitment, further promoting tissue repair. However, methods to increase intercellular interactions that promote local engraftment of MSCs remain unclear.

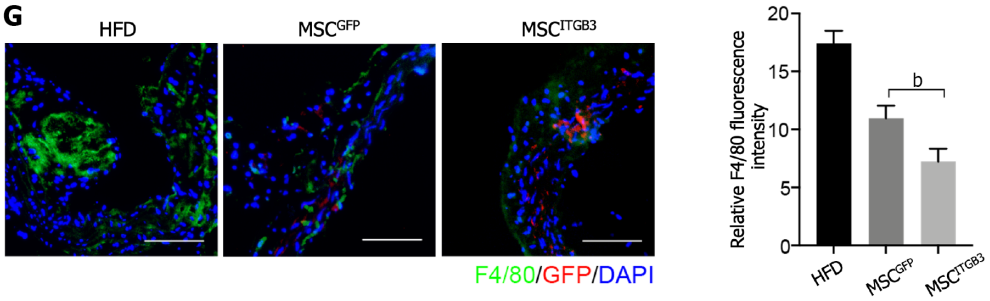
Integrins, as transmembrane receptors, mediate cell connections by integrating Ig superfamily counterreceptors (ICAM-1 and VCAM-1) on adjacent cells[20]. Adhesion molecules and integrin receptors have been identified to facilitate cell migration toward target organs and adhesion in target tissues. Therefore, intravenous injection of MSCs^{ITGB3} may



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Figure 3 Integrin subunit beta 3 enhances mesenchymal stem cell migration *in vitro* and *in vivo*. A: *In vitro* migration of integrin subunit beta 3 (ITGB3)-overexpressing mesenchymal stem cell (MSC) (MSCs^{ITGB3}) toward lower Raw264.7, MVSMC and PMVEC stimulated by tumor necrosis factor α. Scale bars = 100 μm; B: Quantification of migrated cells. Data are presented as the mean ± SEM; C: Experimental scheme of chemotactic assays with mouse atherosclerotic vascular samples. The blood vessel was divided into three parts. For each of the experiments, each part was cultured for 24 h in medium. To perform the assay, a suspension of 2 × 10^5 MSCs, 2 × 10^5 MSCs^{GFP}, or 2 × 10^5 MSCs^{ITGB3} in medium were seeded in the upper chamber. Following incubation for 24 h, non-migrated cells were removed; D: Representative image and quantification of migrated cells stuck in the porous membrane or the lower layer of the transwell stained with 0.1% crystal violet; E: MSCs^{ITGB3} and MSCs^{GFP}, both expressing green fluorescence, were intravenously injected into mice. The expression of green fluorescent protein (GFP) and CD31 was examined by frozen staining. GFP-positive cells were quantified per microscopic field of vascular staining in triplicate mice; F: The expression of GFP and SMA were examined by frozen staining. GFP-positive cells were quantified per microscopic field of vascular staining in triplicate mice. Scale bars = 100 μm; G: Expression levels of GFP in atherosclerosis analyzed using quantitative real-time polymerase chain reaction of mRNA samples extracted from MSC^{GFP} and MSC^{ITGB3} vessels of three mice. Data are presented as the mean ± SEM for each group. The data are representative of three independent experiments. $^aP < 0.05$, $^bP < 0.001$. GFP: Green fluorescent protein; ITGB3: Integrin subunit beta 3; MSC: Mesenchymal stem cell; qRT-PCR: Quantitative real-time polymerase chain reaction; SEM: Standard error of the mea; TNF: Tumor necrosis factor.


A
HFD
MSCGFP
MSCITGB3
B
Oil red-O positive area in aorta (% of lumen area)
Oil red-O positive area in aorta (% of aortic arch area)
Oil red-O positive area in aorta (% of aortic root area)
Masson positive area in aorta (% of aortic arch area)
Masson positive area in aorta (% of aortic root area)
C
Aortic arch
Aortic root
D
E
HE positive area in aorta (% of aortic arch area)
HE positive area in aorta (% of aortic root area)
F
Systolic diameter (mm)
Diastolic diameter (mm)
Vessel wall thickness (mm)



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Figure 4 Injection of mesenchymal stem cell^{ITGB3} attenuates atherosclerotic *in vivo*. A: Representative *en face* images of mouse aortas stained with Oil Red O (ORO); B: Quantification of the atherosclerotic plaque area as a ratio of the stained area to the total area of the aorta; C: Representative section of mouse aortic arch and aortic root stained with ORO. The ORO-positive area in the aortic root is shown as the ratio of the stained area to the total area of the aortic sinus; D: Representative section of mouse aortic arch and aortic root stained with Masson staining. The Masson staining-positive area in the aortic arch and aortic root is shown as the ratio of the stained area to the total area of the aortic sinus; E: Representative section of mouse aortic arch and aortic root stained with hematoxylin eosin (HE) staining. The HE staining-positive area in the aortic arch and aortic root is shown as the ratio of the stained area to the total area of the aortic sinus; F: Ultrasonography of the aorta in each group, including representative images, systolic diameter, diastolic diameter, and vessel wall thickness; G: Representative images of aortic arch section stained with anti-F4/80 antibody. The F4/80-positive area in the atherosclerotic plaque is shown as the ratio of the stained area to the total area of the atherosclerotic plaque. Scale bar = 100 μm. $^{a}P < 0.05$, $^{b}P < 0.001$. AS: Atherosclerotic; HE: Hematoxylin eosin; MSC: Mesenchymal stem cell; ORO: Oil Red O.

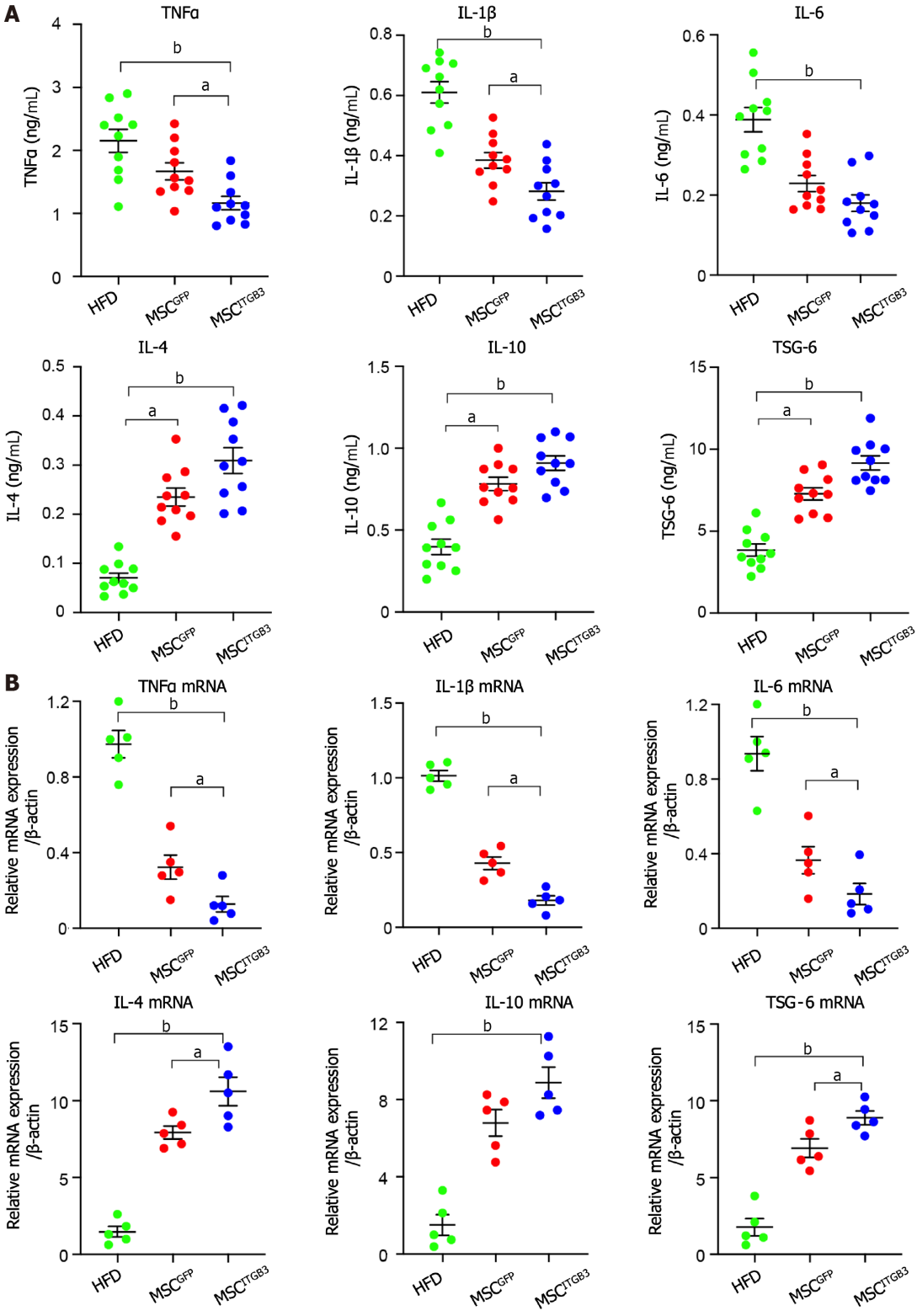
provide a new approach to improve stem cell homing. Previous studies have also verified that MSCs with altered chemokine receptors, such as CXCR5[18], integrin α4[35], and CCR5 and CXCR6[36], significantly increased cell motion to lesion sites and enhanced the therapeutic effect of MSCs. MSC homing to a target organ requires the proper integration of interactions of chemokines secreted by injured tissue and adhesion molecules with matching receptors on MSCs. Therefore, determining disease-specific ligand expression profiles will provide the precise signal necessary for MSC homing. In our study, we elucidated that the ITGB3/RGD motif axis is a specific regulator for MSC homing in atherosclerosis. Adhesion molecules containing the RGD motif, such as VCAM-1, ICAM-1, and OPN, are highly expressed in atherosclerotic plaque and are involved in a variety of immune functions, including T cell activation, migration, and extravasation[37]. High VCAM-1 and ICAM-1 expression levels have also been reported in the inflamed tissue, including vascular plaque[38]. We found that the RGD structure acts as an important feature of the plaque tissue that chemoattracts the migration of MSCs^{ITGB3} to lesions *in vivo*. Moreover, our findings demonstrated that the ITGB3-RGD motif axis is the atherosclerosis-specific regulator for MSC homing. Factors containing RGD structures can be observed in various inflammatory tissues, including the site of atherosclerotic lesions[39]. However, the expression of ITGB3 was almost absent on the surface of MSCs. Thus, MSCs^{ITGB3} may serve as a disease-specific therapeutic tool to improve MSC homing when treating atherosclerosis. MSC transplantation and homing appear to improve atherosclerosis *via* different mechanisms. There is a substantial amount of evidence indicating that transplanted MSCs primarily promote local microenvironmental improvement through their paracrine secretory effects[40-42]. The anti-inflammatory cytokines secreted by MSCs can reduce inflammation and modulate immune response while enhancing normal cell survival and differentiation[9,11]. It has also been documented that the anti-inflammatory properties of MSCs are primarily mediated by extracellular vesicles (EVs)[8,43]. Various disease models, such as those involving endothelial cell senescence[44], autoimmune diseases[45], arterial stiffness and hypertension[46], and lung injury[47], have demonstrated that EVs derived from MSCs can mitigate cell death, prevent apoptosis, and enhance recovery. In this regard, as a result of the continuous release of EVs in the vicinity of the damage site, transplantation of MSCs overexpressing ITGB3 with improved migratory capability could play a more effective role in treating atherosclerotic disease.

A previous study found that ITGB3 stimulated the progression of human pancreatic ductal adenocarcinoma *via* activation of the STAT3 pathway[48]. Furthermore, ITGB3 promoted cell senescence and profibrotic changes through p53 signaling activation and secretion of transforming growth factor beta in cultured tubular cells[49]. However, these studies have not examined the specific effect of ITGB3 overexpression on MSC migration and homing *in vivo*. Further research is necessary to elucidate the underlying mechanisms of how overexpression of ITGB3 intensifies homing abilities of MSCs.

This study has certain limitations. First, the MSCs^{ITGB3} were generated by lentiviral infection. As lentiviral vectors can randomly integrate into the genome resulting in deleterious mutations, their therapeutic application in humans is limited by an inherent risk of tumorigenesis. Second, other suitable methods are needed to increase the expression of ITGB3. Third, the current experiment did not specifically investigate the survival time of MSCs^{ITGB3} *in vivo*. The next step will be to trace the target location and lifespan of MSCs^{ITGB3} through *in vivo* imaging technology.

CONCLUSION

Collectively, we genetically modified MSCs *in vitro* and found that ITGB3 overexpression substantially upregulated MSC migration, aggregation in plaque sites, and immunomodulatory properties of MSCs *in vivo*. Although the therapeutic potential of modified MSCs in atherosclerosis has not yet been translated into clinical practice, our work improves our



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Figure 5 Mesenchymal stem cell^{ITGB3} modulated cytokine expressions in an atherosclerotic mouse model. A: Serum pro-inflammatory factors tumor necrosis factor (TNF)α, interleukin (IL)-1β, and IL-6 and anti-inflammatory factors IL-4, IL-10, and tumor necrosis factor-stimulated gene-6 (TSG-6) levels measured using ELISA; B: mRNA expression of pro-inflammatory factors TNFα, IL-1β, and IL-6 and anti-inflammatory factors IL-4, IL-10, and TSG-6 in aortic tissue, quantified using quantitative real-time polymerase chain reaction. Expression is shown relative to the expression of housekeeping genes (β-actin). All values are expressed as mean ± SEM. $^{a}P < 0.05$, $^{b}P < 0.001$. ELISA: Enzyme-linked immunosorbent assay; IL: Interleukin; MSC: Mesenchymal stem cell; qRT-PCR: Quantitative real-time polymerase chain reaction; SEM: Standard error of the mean; TNF-α: Tumor necrosis factor-a; TSG-6: Tumor necrosis factor-stimulated gene-6.

understanding of the adhesion molecule–integrin receptor axis, as well as of stem cell features, and may provide novel insights into the rapid and targeted delivery of MSCs to disease sites, thereby optimizing their therapeutic effects.

ARTICLE HIGHLIGHTS

Research background

Umbilical cord mesenchymal stem cell (MSC) transplantation is a potential therapeutic intervention for atherosclerotic vascular disease. Integrin beta 3 (ITGB3) promotes cell migration in several cell types. However, whether ITGB3-modified MSCs can migrate to plaque sites *in vivo* and play an anti-atherosclerotic role remains unclear.

Research motivation

Atherosclerosis is a serious public health problem and more treatment options are needed to explore and identify effective molecules and targets.

Research objectives

The objective of our study was to evaluate the chemotaxis ability of ITGB3-overexpressing MSCs toward inflammatory cells *in vitro* and plaque tissues *in vivo,* promoting their therapeutic efficacy in the atherosclerosis mouse model.

Research methods

Umbilical cord MSCs were isolated and expanded. Lentiviral vectors encoding ITGB3 or green fluorescent protein (GFP) as control were transfected into MSCs. Male apolipoprotein $E^{-/-}$ mice were fed with a high-fat diet (HFD) for 12 wk to induce the formation of atherosclerotic lesions. The HFD-fed mice were randomly separated into three clusters. GFP-labeled MSCs ($MSCs^{GFP}$) or $MSCs^{ITGB3}$ were transplanted into the mice intravenously *via* the tail vein. Immunofluorescence staining, Oil red O staining, histological analyses, western blotting, enzyme-linked immunosorbent assay, and quantitative real-time polymerase chain reaction were used for the analyses. Statistical evaluation between two groups was determined using the unpaired Student's t-test, and comparisons of more than two groups were performed using a one-way analysis of variance.

Research results

$MSCs^{ITGB3}$ successfully differentiated into the "osteocyte" and "adipocyte" phenotypes and were characterized by positive expression (> 91.3%) of CD29, CD73, and CD105 and negative expression (< 1.35%) of CD34 and human leukocyte antigen-DR. $MSCs^{ITGB3}$ showed significantly faster migration than $MSCs^{GFP}$. ITGB3 overexpression had no effects on MSC viability, differentiation, and secretion. Immunofluorescence staining revealed that ITGB3 overexpression substantially enhanced the homing of MSCs to plaque sites. Oil red O staining and histological analyses further confirmed the therapeutic effects of $MSCs^{ITGB3}$, significantly reducing the plaque area. Enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction revealed that MSC^{ITGB3} transplantation considerably decreased the inflammatory response in pathological tissues by improving the dynamic equilibrium of pro- and anti-inflammatory cytokines.

Research conclusions

The study demonstrated that ITGB3 overexpression enhanced the MSC homing ability, providing a potential approach for MSC delivery to plaque sites, thereby optimizing their therapeutic effects.

Research perspectives

The ITGB3-modified MSCs can migrate the plaque sites and play an anti-inflammation role, which may be an effective strategy to treat vascular atherosclerotic related diseases.

FOOTNOTES

Author contributions: Cui W and Han M designed and coordinated the study; Hu HJ, Xiao XR, Li T, Liu DM and Geng X performed the experiments and acquired and analyzed data; Cui W interpreted the data; Hu HJ and Liu DM wrote the manuscript; all authors revised the manuscript and approved the final version of the article.

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Institutional animal care and use committee statement: Laboratory Animal Use and Management Committee has been carefully discussed and voted on May 27, 2021 for the subject animal-related content, 10 voters, 10 people suggested a formal experiment, and

made the following recommendations. The design is reasonable, in line with the animal requirements. The ethics committee agreed to carry out a formal experiment.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Basic Study

Enhanced wound healing and hemostasis with exosome-loaded gelatin sponges from human umbilical cord mesenchymal stem cells

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Abstract

BACKGROUND

Rapid wound healing remains a pressing clinical challenge, necessitating studies to hasten this process. A promising approach involves the utilization of human umbilical cord mesenchymal stem cells (hUC-MSCs) derived exosomes. The hypothesis of this study was that these exosomes, when loaded onto a gelatin sponge, a common hemostatic material, would enhance hemostasis and accelerate wound healing.

AIM

To investigate the hemostatic and wound healing efficacy of gelatin sponges loaded with hUC-MSCs-derived exosomes.

METHODS

Ultracentrifugation was used to extract exosomes from hUC-MSCs. Nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and western blot techniques were used to validate the exosomes. *In vitro* experiments were performed using L929 cells to evaluate the cytotoxicity of the exosomes and their impact on cell growth and survival. New Zealand rabbits were used for skin irritation experiments to assess whether they caused adverse skin reactions. Hemolysis test was conducted using a 2% rabbit red blood cell suspension to detect whether they caused hemolysis. Moreover, *in vivo* experiments were carried out by implanting a gelatin sponge loaded with exosomes subcutaneously in Sprague-Dawley (SD) rats to perform biocompatibility tests. In addition, coagulation index test was conducted to evaluate their impact on blood coagulation. Meanwhile, SD rat liver defect hemostasis model and full-thickness skin defect model were used to study whether the gelatin sponge loaded with exosomes effectively stopped bleeding and promoted wound healing.

RESULTS

The NTA, TEM, and western blot experimental results confirmed that exosomes

were successfully isolated from hUC-MSCs. The gelatin sponge loaded with exosomes did not exhibit significant cell toxicity, skin irritation, or hemolysis, and they demonstrated good compatibility in SD rats. Additionally, the effectiveness of the gelatin sponge loaded with exosomes in hemostasis and wound healing was validated. The results of the coagulation index experiment indicated that the gelatin sponge loaded with exosomes had significantly better coagulation effect compared to the regular gelatin sponge, and they showed excellent hemostatic performance in a liver defect hemostasis model. Finally, the full-thickness skin defect healing experiment results showed significant improvement in the healing process of wounds treated with the gelatin sponge loaded with exosomes compared to other groups.

CONCLUSION

Collectively, the gelatin sponge loaded with hUC-MSCs-derived exosomes is safe and efficacious for promoting hemostasis and accelerating wound healing, warranting further clinical application.

Key Words: Human umbilical cord mesenchymal stem cells; Exosomes; Gelatin sponge; Safety; Hemostasis; Wound healing

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Core Tip: In this study, we loaded exosomes derived from human umbilical cord mesenchymal stem cells onto a gelatin sponge, a common hemostatic substance in clinics, to stop bleeding and promote wound healing. The fabricated material appears relatively safe, provides better hemostatic activity than gelatin sponge alone, and promotes good wound healing.

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INTRODUCTION

In recent years, biomaterials have increasingly been used in clinical practice. Notably, gelatin sponges have been widely used in various surgical procedures owing to their excellent characteristics. Gelatin sponges are highly accessible, cheap, and easy to use, allowing use as a dry material or impregnated with saline or thrombin prior to use. Furthermore, gelatin sponges exhibit a robust absorption capacity and can absorb approximately 40 times their own weight of water or liquid and expand to 200% of their original volume, thus increasing the local platelet concentration[1]. Moreover, the expanded gelatin particles help limit blood flow and provide a packing effect in the limited space, thereby promoting the formation of blood clots during wound healing.

Gelatin sponges were first used in neurosurgery in the 1940s and have since been widely used in various surgical procedures. In recent years, many new applications have been developed for absorbable gelatin sponges. Wang *et al*[2] have fabricated an Au/Ag gelatin sponge by adding Au/Ag to the gelatin matrix, which improved its antibacterial performance. Yang *et al*[3] have used a cocktail of ropivacaine, dexamethasone, and vitamin B12 to impregnate a gelatin sponge for promoting leg pain relief and functional recovery after percutaneous endoscopic lumbar discectomy. Notably, Jogo *et al*[4] have loaded gelatin sponge particles with 5% ethanolamine oleate iopamidol and used the mixture to examine the effect of retrograde occlusion of veins in gastric varices. Incorporating colloidal Ag into gelatin sponges was beneficial in promoting bone healing in infected skull defects[5]. Accordingly, gelatin sponges can be used to promote wound healing and combined with various drugs or substances to improve their therapeutic effects.

Mesenchymal stem cells (MSCs) have been shown to markedly improve wound closure, angiogenesis, and wound healing. However, stem cell therapy is complex, expensive, and time-consuming. Recent studies have demonstrated the effectiveness of stem cell-derived exosomes in treating wounds. Exosomes are emerging as a new mode of intercellular communication. Additionally, exosomes play an important role in wound repair[6]. Exosomes are membrane-derived vesicles interacting with target receptor cells, enabling inter cellular communication[7]. Growing evidence suggests that exosomes are crucial in coagulation, intercellular signaling, and waste metabolism[8]. The biological characteristics of exosomes derived from MSCs are similar to those of umbilical cord (UC)-MSCs, making them valuable for tissue repair.

Recently, an increasing number of studies have explored the combined application of exosomes and biological materials. Wang *et al*[9] have fabricated an injectable, viscous, heat-sensitive, multifunctional polysaccharide-based wound dressing capable of sustained exosomes release to accelerate wound healing by stimulating the angiogenic process of the wound tissue. Tao *et al*[10] have combined exosomes released by synovial MSCs overexpressing miR-126-3p with chitosan, demonstrating that this strategy could be used to treat skin wounds. Xu *et al*[11] have assembled exosomes on chitosan/silk hydrogel sponges based on platelet-rich plasma to examine wound healing in a diabetic rat model, revealing their capacity to promote wound healing during diabetes. Furthermore, Nooshabadi *et al*[12] have employed an exosome-loaded chitosan hydrogel to study wound healing and dermal reconstruction in mice. The authors found that the exosome-loaded chitosan hydrogel exhibited a wound closure capacity of approximately 83.6% and a high degree of

re-epithelization[12]. Based on the above literature, novel dressings made by combining exosomes and a variety of biological materials afford better effects than conventional dressings.

In the present study, we loaded a common clinically used gelatin sponge with exosomes to synthesize a novel gelatin sponge with improved characteristics. The safety and effectiveness of the improved gelatin sponge in hemostasis and promotion of healing were studied both *in vivo* and *in vitro*. The findings will provide novel avenues for further research on the potential applications of human UC MSCs (hUC-MSCs)-derived exosomes in tissue repair. Furthermore, our study provided evidence for the feasibility of using a bioscaffold synthesized by combining exosomes and biological materials simply and non-invasively to stop bleeding and promote wound healing, thereby supporting the further use of exosomes for clinical applications.

MATERIALS AND METHODS

Statement of animal treatment

All animals were obtained from Changsha Tianqin Biotechnology Co., Ltd. (Changsha, China) and were housed under ordinary conditions. The animals were maintained under a 12-h light/12-h dark cycle at stable temperature (22-26 °C) and humidity (50%-70%). They were given free access to food and water throughout the study period.

Extraction and characterization of exosomes derived from hUC-MSCs

hUC-MSCs were purchased from Guangxi TaiMeiRenSheng Biotechnology Co. Ltd. Fourth-passage cells at logarithmic phase were inoculated in T175 air-permeable cell culture flasks with a dedicated medium (HUXUC-90011, Cyagen). The cells were cultured at 37 °C in an incubator containing 5% CO_2. When the adherent cells reached a confluence of 80%-90%, the used cell medium was discarded and replaced with a serum-free medium. After 48 h, cells were centrifuged and cell supernatants were collected. Exosomes in the cell supernatants were extracted by ultracentrifugation. Impurities in the cell supernatants were removed by centrifugation at 300 × *g* for 10 min, followed by the removal of dead cells by centrifugation at 2000 × *g* for 20 min; then, cell debris was removed by centrifugation at 10000 × *g* for 30 min. Subsequently, cell supernatants were filtered using a 0.22 μM membrane filter and centrifuged in an ultracentrifuge (Beckman, America) for 120 min at 120000 × *g*, and the supernatants were discarded. The precipitated exosomes were re-suspended in phosphate buffered saline (PBS) and centrifuged again for 120 min at 120000 × *g*, and the exosomes precipitate were finally re-suspended in filtered PBS. All centrifugations were performed at 4 °C.

Exosomes were identified using a nanoparticle tracking analyzer (ZetaView, Particle Metrix, Germany), which was used to measure their concentration and particle size. A transmission electron microscope (HT-7700, Hitachi High-tech Company) was used to observe the shape and size of exosomes. Western blot was used to detect the surface marker proteins, CD9, Tsg101, and Calnexin, in exosomes. The protein concentration of hUC-MSCs-derived exosomes was determined using a BCA protein quantitation kit (Zoman, Biotechnology, ZD301). After adding the required proportion of loading buffer, the sample was heated at 95 °C for protein denaturation. Protein from each sample was separated on a sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk for 2 h, the membranes were incubated overnight with anti-CD9 (20597-1-AP, Proteintech), anti-Tsg101 (28283-1-AP, Proteintech), and anti-Calnexin (10427-2-AP, Proteintech) antibodies. Subsequently, the secondary antibody (SA00001-2, Proteintech) was added, followed by incubation for 1.5 h. Band visualization was achieved using a chemiluminescence kit (P0018FM, Beyotime, China) and observed on a chemiluminescent imaging system (MiniChemi 610, Sagecreation, China).

Characterization of gelatin sponge loaded with hUC-MSCs derived-exosomes

An absorbable gelatin sponge was purchased from Jiangxi Xiangen Medical Technology Development Co., Ltd. It was sliced into appropriate sizes, and immersed in the exosome solution mixed with trehalose, to ensure that the gelatin sponge fully absorbed the exosomes. Subsequently, the loaded gelatin sponge was freeze-dried and characterized *via* scanning electron microscopy.

Cell viability assay

The effect of the hUC-MSCs-derived exosomes on mouse epithelioid fibroblasts (L929) was evaluated using the CCK-8 assay. Briefly, L929 cells were inoculated into 96-well plates and divided into control, sponge extract, and exosome groups. The cell density was adjusted to 10000 cells/well and then incubated at 37 °C in a 5% CO_2 incubator for 12 h. Subsequently, the medium in the exosome group was adjusted to ensure that each well contained 6 × 10^6 particles of exosomes and then incubated at 37 °C under 5% CO_2 for a further 6 h. After 6 h, 10 μL of CCK-8 reagent was added to all groups, followed by incubation in the dark for 1 h. Finally, the absorbance of each well was measured at 450 nm using a microplate reader.

Hemolysis assay

Briefly, 5 mL of fresh blood from New Zealand rabbits was stirred in one direction with a glass rod, followed by fibrin removal; then, 20 mL of normal saline was added. The mixture was mixed well and centrifuged at 2000 rpm for 10 min. The supernatant was discarded, normal saline was added, and the process was repeated 3-4 times until the supernatant was clear. Finally, the supernatant was discarded, and 1 mL of fresh blood was added to 49 mL of normal saline to obtain a 2% rabbit red blood cell suspension for subsequent use. The assay was divided into negative control, positive control,

sponge, and exosome-loaded sponge groups. Subsequently, 500 μL of the 2% rabbit red blood cell suspension was added to each group, and an equal volume of liquid was added (normal saline was added to the negative control group, and Triton X-100 with a mass fraction of 0.5% was added to the positive control group. Gelatin sponge extract at a concentration of 10 mg/mL was added to the sponge group, and gelatin sponge extract with 1×10^9 exosomes was added to the exosome-loaded sponge group). Then, the solutions were mixed evenly and incubated at 37 °C for 3 h. The samples were then centrifuged at 3000 rpm for 3 min. The optical density (OD) of the supernatant was measured at a wavelength of 545 nm, and the hemolysis rate was calculated by photography. The hemolysis rate was calculated as $(OD_{sam} - OD_{neg})/(OD_{pos} - OD_{neg}) \times 100\%$ (1)[13].

Skin irritation test

The skin irritation test was performed using three New Zealand rabbits. The hair on their backs was carefully removed. Four specific areas on their skin were identified and treated with different fluids. They were blank group without any articles, NaCl group with 0.9%NaCl solution, sponge group with 10 mg/mL sponge extract, and exosome group with 5×10^8 exosomes solution. The application was repeated every 24 h until absorption was complete. The rabbits were closely monitored to assess any changes in their skin condition, such as redness, swelling, inflammation, or other related symptoms. After 72 h, skin samples were harvested from treated sites for histopathological examination to observe any signs of inflammation in the skin tissue.

Histocompatibility assay

Eighteen male Sprague-Dawley (SD) rats were divided into blank control, sponge, and exosome-loaded sponge groups. After anesthesia with 10% chloral hydrate, the hair on their back was removed, the subcutaneous bags were excised, and 1 cm × 1 cm × 0.5 cm of either gelatin sponges or gelatin sponges loaded with exosomes were implanted into them, while the blank control group was not implanted with any gelatin sponge. The adjacent skin was collected for pathological analysis on day 7 and 14, respectively, to evaluate the *in vivo* biocompatibility of the gelatin sponges.

Whole blood coagulation index test

The gelatin sponges were cut into 1 cm × 1 cm × 0.5 cm sections, and preheated in an oven at 37 °C for 5 min. In the sponge and exosome groups, the gelatin sponges were infiltrated with 100 μL of normal saline and 100 μL of exosome solution containing 5×10^8 exosomes, respectively. Next, 100 μL of rat whole blood was added to each gelatin sponge in both groups for adequate absorption, followed by 10 μL of 0.2 M calcium chloride solution, and incubated at 37 °C for 15 min. Subsequently, 25 mL of deionized water was used to rinse blood from the gelatin sponge surface without coagulation. Finally, the absorbance (Abs1) of the washed liquid was measured at 540 nm by using a microplate reader. For the blank control (Abs0), 100 μL of whole blood was added to 25 mL of deionized water, and the blood coagulation index (BCI) was calculated using the formula: $BCI\ (\%) = Abs1/Abs0 \times 100\%$ (2).

Hemostatic assay using rat liver defect hemostasis model

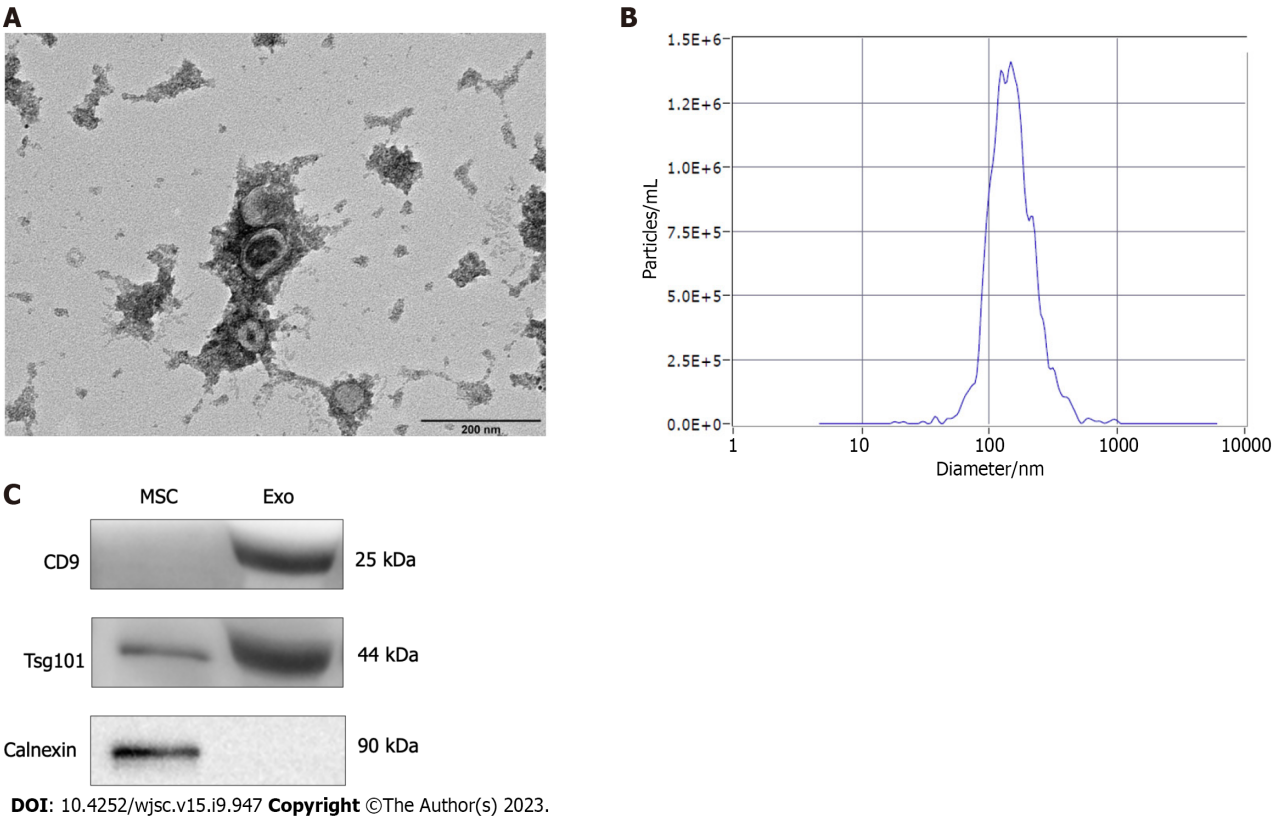
The hemostatic capacity of the exosome-loaded gelatin sponge was evaluated using a rat liver defect hemostasis model [14]. Twelve SD rats were divided into sponge and exosome-loaded sponge groups (n = 6 rats/group), with an equal distribution of male and female rats. After anesthesia induction with 10% chloral hydrate, an abdominal incision was made to expose the liver, and the tissue fluid around the liver was carefully removed using a dry cotton ball to prevent inaccurate blood weight measurements. The liver was placed on a pre-weighed filter paper, and a 0.5 cm long and 0.2 cm deep wound was created on the liver using a scalpel. The gelatin sponge loaded with exosomes was immediately placed at the bleeding site and bleeding was observed. After the bleeding stopped completely, the bleeding time was recorded, and the blood-absorbed filter paper was weighed. The weight difference between the front and rear of the filter paper was considered as the weight of blood loss from the rat liver.

Effect of exosomes-loaded gelatin sponge on healing of skin full-thickness defect model

Eighteen male SD rats (220-280 g) were randomly divided into control, sponge, and exosome-loaded sponge groups. After anesthesia induction with 10% chloral hydrate, the hair on the back of rats was removed, and a full-thickness skin defect wound (approximately 1.5 cm × 1.5 cm) was created on their backs. Photographs were taken to record the original area, and the wound was covered with either a gelatin sponge or an exosome-loaded gelatin sponge, and wrapped with a transparent dressing; the control group received only a transparent dressing. On day 7 and 14 post the procedure, the gauze was opened to observe and record wound healing. All wounds were photographed, the area was analyzed using Image J software (National Institutes of Health, Bethesda, MD), and skin samples around the wounds were collected and fixed with 4% paraformaldehyde for pathological analysis.

Masson staining and immunohistochemistry

Masson's trichrome staining was used to determine collagen content and wound maturity. Image J software was used to analyze the areas of interest. The collagen content was quantified. Ki67 was used to evaluate local whole-cell proliferation and CD31 staining was used to evaluate angiogenesis. Sections were incubated with antibodies against Ki67 (1:600, #AF0198, Affinity) or CD31 (1:1000, Cat No.28083-1-AP, Proteintech). Subsequently, the secondary antibody was added (2118D1104, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.). Six randomly selected areas were imaged using an inverted microscope for each specimen, and the number of Ki67- and CD31- positive cells/positive blood vessels was detected using image J software.



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Figure 1 Characterization of human umbilical cord mesenchymal stem cell-derived exosomes. A: Morphology of the exosomes assessed by transmission electron microscopy. Scale bar = 200 nm; B: Grain size of the exosomes; C: Surface protein markers of exosomes. MSC: Mesenchymal stem cell; Exo: Exosome.

Statistical analysis

SPSS 26.0 (IBM Corp., Armonk, NY, United States) and GraphPad Prism 8.0 (GraphPad Software Inc.; San Diego, CA, United States) were used for statistical analyses. Paired or unpaired *t*-tests were used for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among three or more groups. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of exosomes

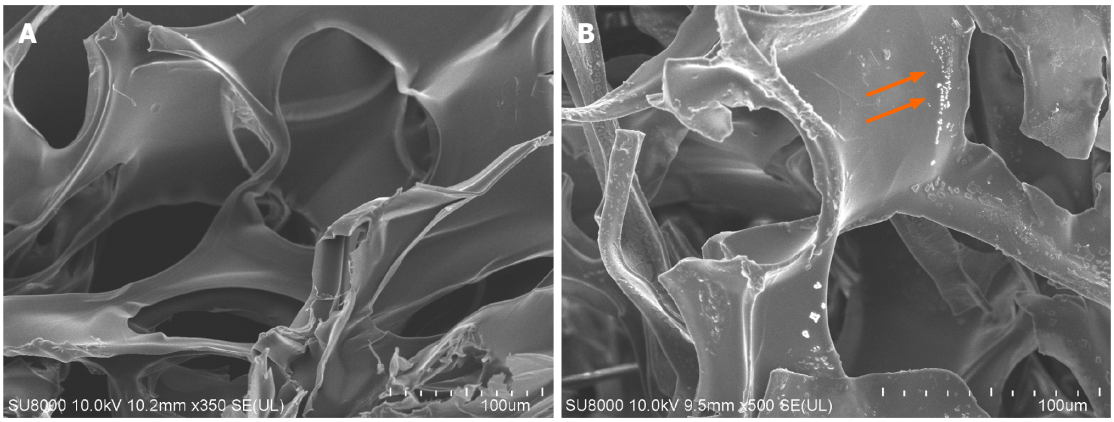
We successfully isolated exosomes from hUC-MSCs by ultracentrifugation. After successful separation, the morphology of the exosomes was observed by using TEM, and a typical cup-shaped structure was observed (Figure 1A). The exosome concentration and particle size were measured using a nano-particle size analyzer. The average exosome particle size was 144.7 nm, consistent with the particle size of exosomes (30-150 nm) (Figure 1B). Western blot results showed that the extracted exosomes successfully expressed the surface marker proteins CD9 and Tsg101, but not Calnexin (Figure 1C).

Characterization of gelatin sponge loaded with exosomes

The exosomes on the gelatin sponge were detected *via* scanning electron microscopy. The gelatin sponge with exosomes was coarser than the sponge without exosomes, and exosome particles could be observed (Figures 2A and B).

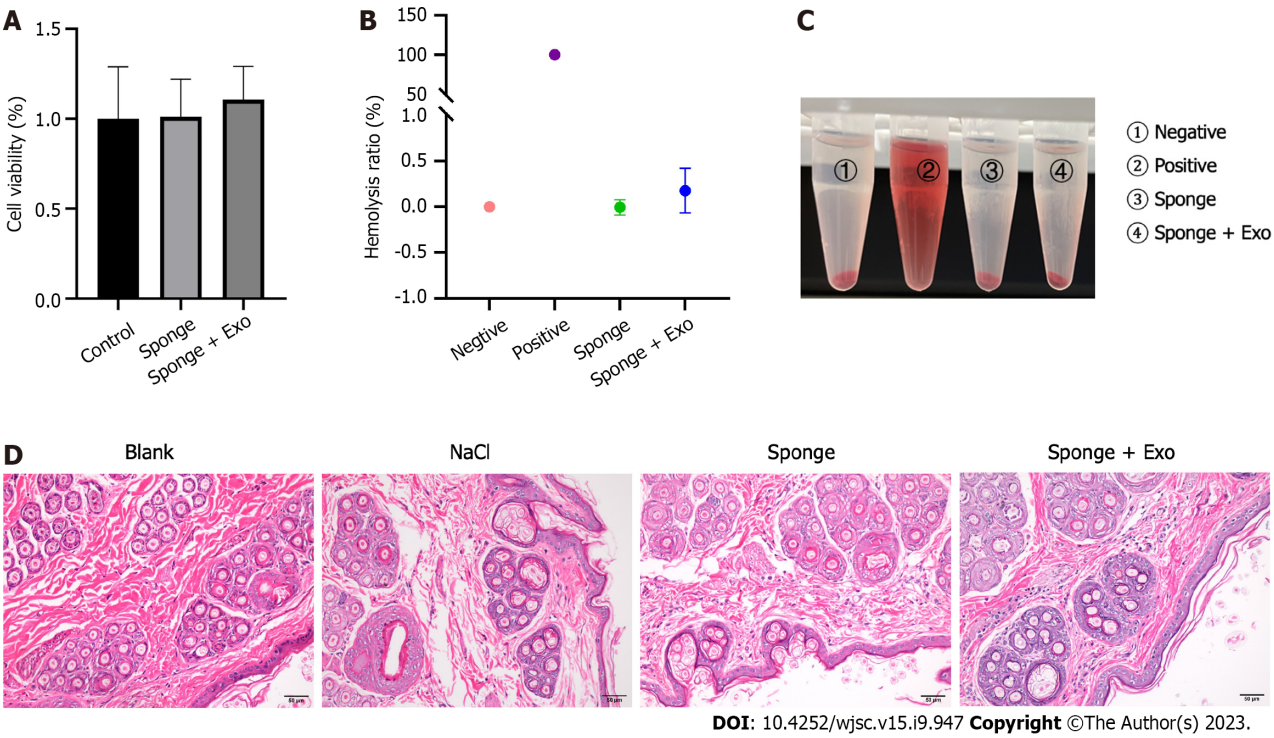
Cytotoxicity and biocompatibility of gelatin sponge loaded with exosomes

The gelatin sponge loaded with exosomes did not induce significant toxicity toward L929 cells as the cells remained viable following exposure to them (Figure 3A). Moreover, the hemolytic assay showed that no hemolysis was observed with the naked eye using the gelatin sponge loaded with exosomes (Figure 3C). Measuring the OD value of the solution and calculating the hemolysis rate, we found that the hemolysis rates of the gelatin sponge and exosome-loaded gelatin sponge in whole blood were -0.01% and 0.18%, respectively, which were within the permissible range for biological materials (less than 5%), indicating that the exosome-loaded gelatin sponge was safe for use (Figure 3B). All experimental rabbits had an intact epidermis, with normal sebaceous glands, hair follicles, and hair structures; there were no notable pathological changes, such as inflammatory cell infiltration, tissue congestion, and edema (Figure 3D).



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Figure 2 Characterization of gelatin sponge loaded with exosomes. A: SEM image of the gelatin sponge; B: SEM images of the gelatin sponge loaded with exosomes. The orange arrows show the exosomes.



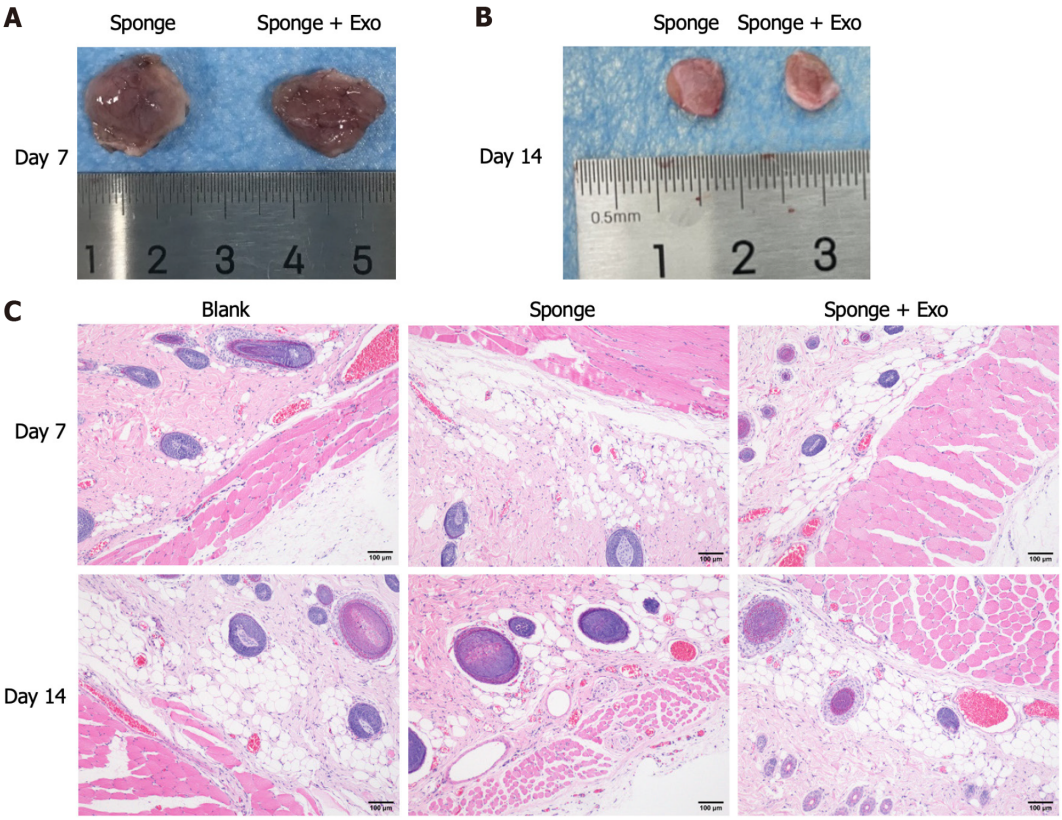
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Figure 3 Biosafety of gelatin sponge loaded with exosomes. A: Cell viability of gelatin sponge loaded with exosomes; B and C: Hemolysis ratio of gelatin sponge loaded with exosomes; D: Representative diagram of pathological condition of rabbit skin after stimulation with exosome and sponge extract. Scale bar = 50 μm. Exo: Exosome.

To investigate the *in vivo* biocompatibility of gelatin sponge loaded with exosomes, we implanted the gelatin sponge into the dorsal subcutaneous space of SD rats, and observed the condition of the implant on day 7 and day 14 (Figures 4A and B). A portion of the gelatin sponge was retained in the body on day 7 and day 14; however, the gelatin sponge on day 14 was obviously smaller than that on day 7, indicating that the gelatin sponge could be degraded in the body. Adjacent skin tissue samples were collected for pathological analysis. After 7 and 14 d of implantation, there was no obvious inflammatory response around the tissue, and no obvious tissue fibrosis or necrosis was observed (Figure 4C), indicating that the gelatin sponge loaded with exosomes showed good biocompatibility.

Hemostatic effect of gelatin sponge loaded with exosomes

We evaluated the clotting ability of gelatin sponge and gelatin sponge loaded with exosomes. Our findings showed that the BCI values of the gelatin sponge and exosome-loaded gelatin sponge groups were 45.63% and 31.47%, respectively, indicating that both groups had significant clotting ability, and the clotting ability of the exosome-loaded gelatin sponge was significantly higher than that of the gelatin sponge group (Figure 5A).



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Figure 4 Biocompatibility of gelatin sponge loaded with exosomes. A and B: The size of the gelatin sponge remaining on day 7 and day 14; C: Hematoxylin and eosin staining of the adjacent skin tissues in the dorsal subcutaneous space of SD rats after 7 and 14 d of implantation. Scale bar = 100 μm. Exo: Exosome.

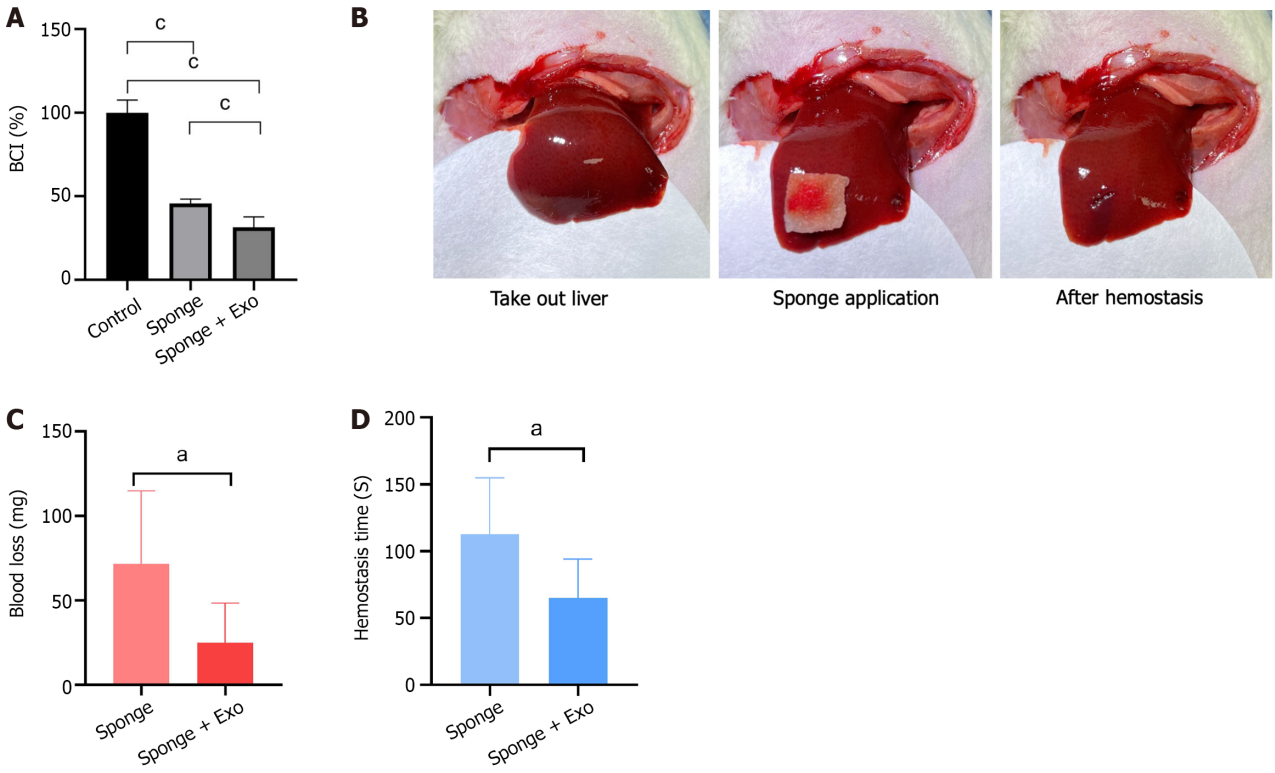
After establishing a liver defect model, we covered the wound with a gelatin sponge and recorded blood loss and hemostasis time. The blood loss and hemostasis time of the gelatin sponge group loaded with exosomes were significantly lower than those of the gelatin sponge group, indicating that the gelatin sponge loaded with exosomes had a better hemostatic effect than the conventional gelatin sponge (Figures 5B-D).

Effect of gelatin sponge loaded with exosomes in promoting wound healing

After establishing a rat full-thickness skin defect model, the wound was covered with a gelatin sponge, and the gauze was opened on day 7 and day 14 post the procedure to observe and record the wound healing status. The wound size of all groups decreased with time. Moreover, the wound size of the gelatin sponge group loaded with exosomes was smaller than that of the other two groups, indicating that the gelatin sponge loaded with exosomes promoted wound healing better than the conventional gelatin sponge (Figures 6A and B). Histopathological results showed that on day 14, the gelatin sponge loaded with exosomes group demonstrated better regeneration of hair follicles and sebaceous glands than the other two groups (Figure 6C).

Masson's trichrome staining was performed on the skin adjacent to the wound to evaluate the formation and distribution of collagen, which is important for promoting wound healing. Microscopic observations showed that collagen fibers in the skin of all three groups were relatively few on day 7. However, on day 14, the collagen fibers in the skin of the three groups gradually increased along with gradual healing of the wound (Figure 7A). Statistical analysis showed that the collagen fibers in the exosome-loaded gelatin sponge group were significantly higher than those in the other two groups on day 14; on day 7, the collagen fibers in the gelatin sponge and exosome-loaded gelatin sponge groups were significantly higher than those in the control group (Figure 7B). These findings suggested that the exosome-loaded gelatin sponge promoted wound healing by promoting collagen fiber synthesis.

To explore the possible mechanism of action of gelatin sponges loaded with exosomes, we employed immunohistochemical techniques to evaluate angiogenesis and cell proliferation in the granulation tissue. To evaluate angiogenesis, we stained the skin near the wound for CD31. On day 7 and day 14, the number of CD31-positive blood vessels in the exosome-loaded gelatin sponge group was significantly higher than that in the control group; on day 14, this number was significantly higher than that in the gelatin sponge group (Figures 7C and D). Subsequently, we detected the proliferation of whole cells of granulation tissue in the skin near the wound and found that the proportion of Ki67-positive cells in the exosome-loaded gelatin sponge group was significantly higher than that in the control and gelatin sponge groups on day 7 and day 14. However, there was no statistically significant difference in the proportion of Ki67-positive cells between the gelatin sponge and control groups (Figures 7E and F). These results indicated that the gelatin sponge loaded with exosomes could promote cell proliferation and angiogenesis, thereby accelerating wound healing.



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Figure 5 Hemostatic effect of gelatin sponge loaded with exosomes. A: Clotting ability of exosome-loaded gelatin sponge; B: Experimental process of hemostasis in liver injury model; C: Hepatic blood loss in rats; D: Time required for hemostasis. $^{a}P < 0.05$, $^{c}P < 0.001$. $n = 6$. Exo: Exosome.

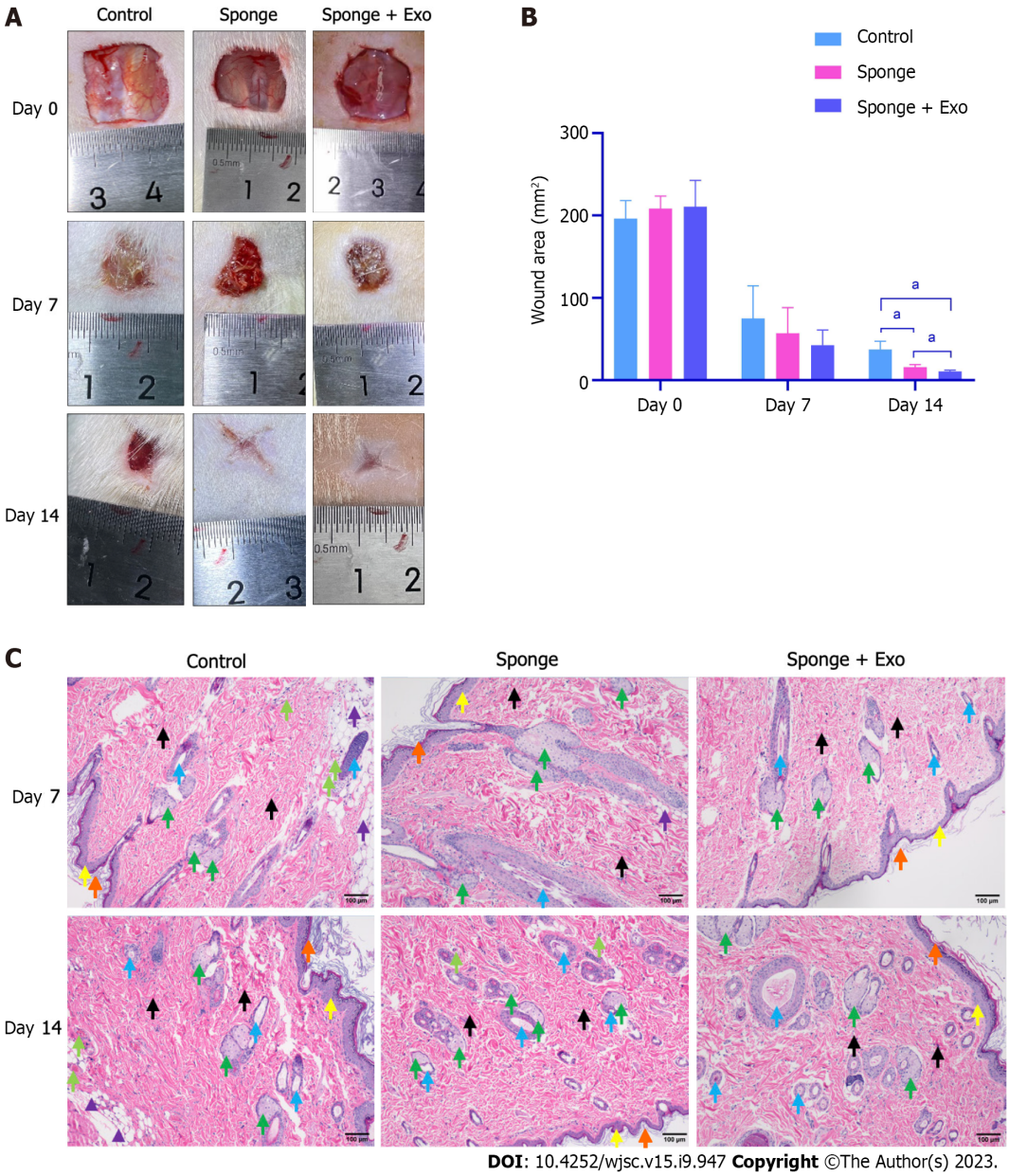
DISCUSSION

Wound healing presents an important challenge for both clinicians and patients. Poor wound healing can negatively impact the patient's quality of life and exacerbate pain, stress, and depression[15,16]. Therefore, methods that effectively promote wound healing are particularly important. Several studies have demonstrated that MSCs accelerate wound closure by promoting skin cell migration, angiogenesis, epithelization, and granulation tissue formation. However, the clinical application of MSCs faces ethical issues, and there is a lack of long-term follow-up evidence regarding their safety. Therefore, alternative cell-free therapies are required. With the emergence of the paracrine hypothesis, the therapeutic applications of exosomes have become more extensive. Exosomes exhibit functions similar to MSCs[17]. Given their immunogenicity, compared with that of MSCs, exosomes can be considered a good alternative[18]. Recently, an increasing number of studies have shown that stem cell-derived exosomes contain mRNA, microRNAs, growth factors, and various other proteins, and potentially participate in processes such as hemostasis, angiogenesis, and wound healing[19,20]. For example, exosomes derived from hUC-MSCs containing miR-21, miR-23a, miR-125b, and miR-145 inhibit fibroblast proliferation and reduce scar formation during wound healing[21].

In the current study, we proposed a new method for loading exosomes into gelatin sponges to achieve wound healing. Our analyses showed that exosome-loaded gelatin sponge was safe and reliable for use. *In vivo* and *in vitro* experiments showed that exosome-loaded gelatin sponge exhibited a good hemostatic effect and promoted the formation of wound granulation tissue, collagen deposition, and angiogenesis, thereby promoting wound healing. Although several studies have shown that exosomes are well-tolerated in animal models, their clinical safety needs to be verified. Considering the goal of exosome-loaded gelatin sponges for clinical application, the safety of exosome-loaded gelatin sponges was verified in this study. We confirmed that the exosome-loaded gelatin sponge was safe for use, as determined by hemolysis, skin irritation, and histocompatibility experiments, consistent with the results of Sun *et al*[22]. These findings provide further evidence for the clinical application of exosomes.

Healing of skin injuries is a complex process that mainly includes four stages: Hemostasis, inflammation, proliferation, and remodeling[23]. The first stage involves hemostasis and clot formation to prevent blood loss. The second stage is the inflammatory stage, which includes coagulation, phagocytosis, removal of foreign bodies, and recruitment of growth factors and anti-inflammatory cells to injury site. The third stage involves proliferation, including fibrous proliferation, angiogenesis, and cell migration. Finally, fibroblasts continue to secrete collagen during the remodeling phase[24,25]. Type I collagen replaces type III collagen at the wound site. Subsequently, scar formation occurs *via* apoptosis.

In this study, we conducted *in vitro* and *in vivo* experiments to verify the potential role of a gelatin sponge loaded with exosomes in coagulation and hemostasis. We found that the coagulation index of the gelatin sponge loaded with exosomes was significantly lower than that of the control group and the gelatin sponge group, thereby suggesting that the gelatin sponge loaded with exosomes had significant coagulation ability; this finding was also confirmed in the liver

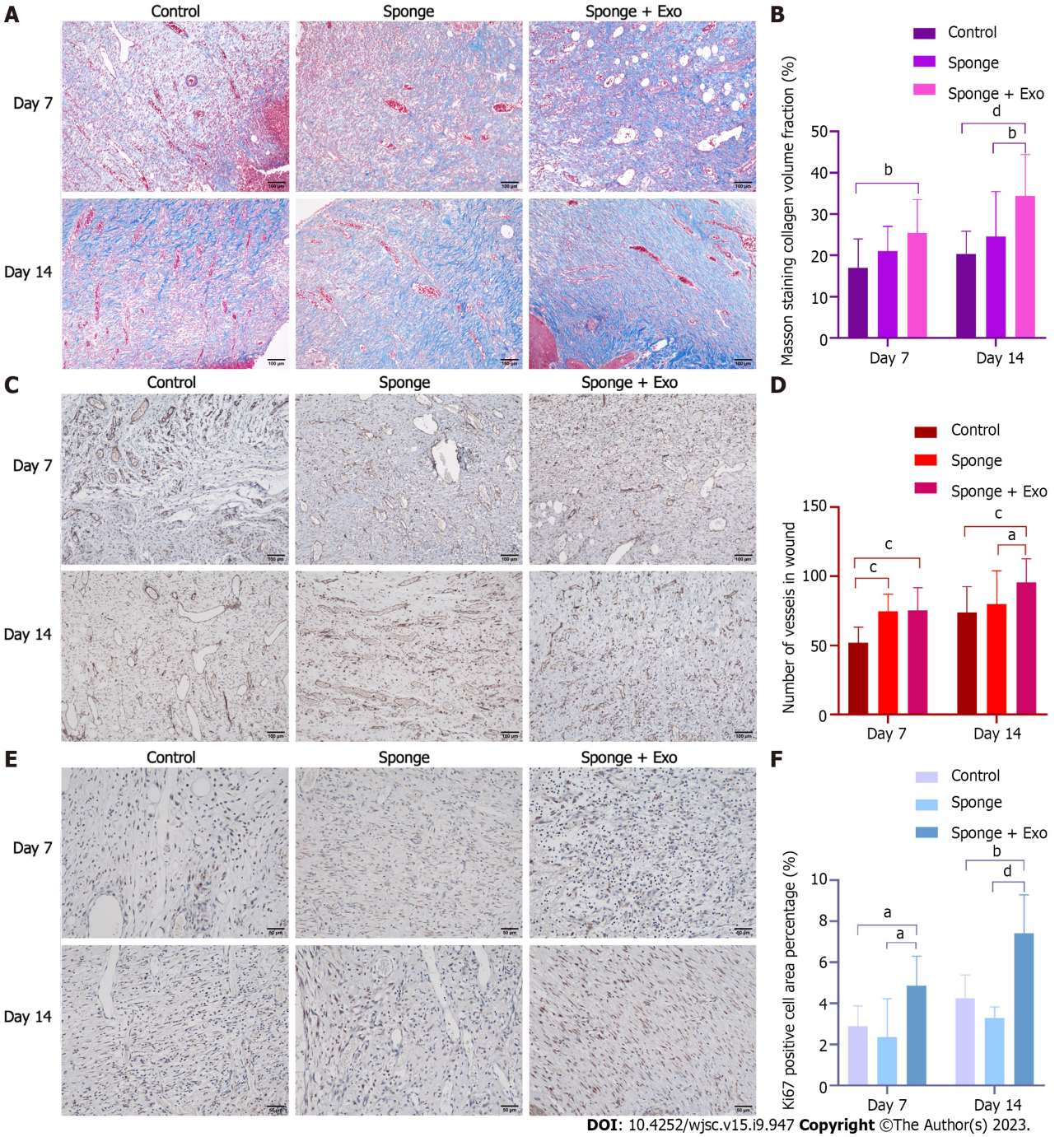


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Figure 6 Wound healing effect of gelatin sponge loaded with exosomes. A: Representative images of rat wounds; B: Statistical analysis of the wound area; C: Hematoxylin and eosin-stained wound tissue sections as observed under a light microscope. The orange arrows indicate the stratum corneum, the yellow arrows indicate the granular layer, the green arrows indicate the sebaceous gland, the blue arrows indicate the hair follicle, the black arrows indicate the collagen fibers, the light green arrows indicate the blood vessels, and the purple arrows indicate the subcutaneous fat. Scale bar = 100 μm. $^{a}P < 0.05$. $n = 3$. Exo: Exosome.

injury hemostasis experiment, where the gelatin sponge group loaded with exosomes reduced bleeding in rats and shortened the bleeding time. These data proved that the gelatin sponge loaded with exosomes afforded a better hemostatic effect than the conventional gelatin sponge, and had a significant effect in promoting wound healing during this stage of hemostasis.

Angiogenesis is essential for wound repair. Blood vessels provide progenitor cells, oxygen, and nutrients to maintain proliferation and remodeling of the wound site[26]. CD31 is an endothelial cell marker demonstrating the degree of tissue vascularization. In the current study, we evaluated angiogenesis by immunohistochemical staining for CD31 in the skin adjacent to the wound. The results showed that the number of CD31-positive blood vessels in the exosome-loaded gelatin sponge group was significantly higher than that in the control and gelatin sponge groups, indicating that the exosome-loaded gelatin sponge had a better angiogenic effect than the conventional gelatin sponge. This may be related to the provascular effects of exosomes. Many studies have shown that exosomes participate in angiogenesis signaling pathways and affect the occurrence, development, and maturation of blood vessels[27]. Zhang *et al*[28] have extracted exosomes from induced pluripotent stem cells and studied their effects on the proliferation, migration, and angiogenesis of hUC blood venous endothelial cells. They found that in a rat model of full-thickness skin defects, exosomes could promote not only angiogenesis at the wound site but also the maturation of blood vessels[28]. Fat-derived exosomes can also be ingested by the vascular endothelium to promote blood vessel formation *in vivo* and *in vitro*[29].



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Figure 7 Gelatin sponge loaded with exosomes promotes collagen formation, cell proliferation, and angiogenesis during the wound healing process. A: Masson-stained wound tissue sections as observed. Scale bar = 100 μm; B: Statistical analysis of the percentage of collagen volume with respect to tissue volume; C: CD31-positive vessels in wound tissue sections. Scale bar = 100 μm; D: Statistical analysis of CD31-positive vessels; E: Ki67-positive cells in wound tissue sections. Scale bar = 50 μm; F: Statistical analysis of Ki67 positive cell area percentage. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. Exo: Exosome.

Masson's trichrome staining is a classical technique used to distinguish collagen and muscle fibers. In the current study, we performed Masson's trichrome staining on the skin near the wounds of rats. We found that the collagen fibers produced in the exosome-loaded gelatin sponge group were significantly higher than those in the gelatin sponge and control groups at the end of the observation period. This indicates that the exosome-loaded gelatin sponge could promote the formation of collagen fibers. This finding is consistent with that of Kim *et al*[30], who employed exosomes to directly treat human skin tissue and found that exosomes can facilitate the migration of fibroblasts and nearby collagen synthesis, as well as promote the synthesis of collagen and elastin in human skin tissue. Ki67 is a marker of cell proliferation, and, in general, angiogenesis begins with endothelial cell proliferation, followed by their isolation, migration, adhesion, and differentiation from adjacent tissues[31]. Cell proliferation and migration are the two key steps in angiogenesis. In this study, we detected the proliferation of integral cells of granulation tissue in the skin near the wound and found that the proportion of Ki67-positive cells in the exosome-loaded gelatin sponge group was significantly higher than that in the

control and gelatin sponge groups. This finding was consistent with the results reported by Wang *et al*[9], who found that in diabetic rats, the expression of the Ki67 in the FEP@exo group was significantly higher than that in the FEP and control groups. These observations may be related to the promotion of cell proliferation. Moreover, the application of exosomes derived from hUC-MSCs has been explored in a rat skin burn model, revealing that exosomes could dose-dependently promote fibroblast proliferation[6,32].

In summary, using an exosome-loaded gelatin sponge for wound healing could be safe and reliable and promote wound healing by promoting cell proliferation, collagen fiber formation, and blood vessel formation.

CONCLUSION

In conclusion, our study has proposed a novel method that uses hUC-MSC-derived exosomes loaded onto gelatin sponges for wound healing, which afforded good hemostatic and wound healing-promoting effects. The exosome-loaded gelatin sponge is safe and could provide new avenues for the clinical application of exosomes. Gelatin sponges are a common hemostatic material used in clinical practice. Moreover, they are inexpensive and readily available. Therefore, combining exosomes and gelatin sponges has strong clinical adaptability and is conducive to the clinical transformation and application of exosomes.

ARTICLE HIGHLIGHTS

Research background

Hemostasis and wound healing are one of the common problems in clinics which need to be paid attention to. Gelatin sponge is often used as a hemostatic material in clinics. Exosomes have been proved to play an important role in wound repair. Therefore, it is worth studying whether the combination of exosomes derived from human umbilical cord mesenchymal stem cells (hUC-MSCs) and a gelatin sponge could promote hemostasis and wound healing more efficiently.

Research motivation

Poor wound healing would contribute a negative impact on patients' quality of life and aggravate pain, stress and depression, so it is of great significance to find ways to effectively promote wound healing.

Research objectives

The present study aimed to investigate the hemostatic and wound healing efficacy of a gelatin sponge loaded with hUC-MSCs-derived exosomes.

Research methods

After the exosomes were extracted and characterized by ultracentrifugation, we loaded the exosomes on a gelatin sponge. Then, *in vitro* and *in vivo* experiments, including cell viability assay, hemolysis assay, skin irritation test, and histocompatibility assay were performed to verify the safety of the exosome-loaded gelatin sponge. Subsequently, whole blood coagulation index test, hemostatic assay using a rat liver defect hemostasis model, and full-thickness skin defect healing promoting test were performed to verify the effects of the exosome-loaded gelatin sponge in hemostasis and wound healing.

Research results

We successfully extracted exosomes from hUC-MSCs. The safety experiments showed that the gelatin sponge loaded with exosomes would not cause abnormal proliferation of L929 cells, hemolysis, or irritation to skin and tissues. In addition, the exosome-loaded gelatin sponge had a better hemostatic effect than the traditional gelatin sponge, which can promote the formation of collagen fibers and blood vessels around the wound and increase the proportion of Ki67-positive cells, thus promoting the wound healing.

Research conclusions

In a word, gelatin sponge loaded with hUC-MSCs-derived exosomes is safe. It is better than traditional gelatin sponge in stopping bleeding and promoting wound healing.

Research perspectives

The gelatin sponge loaded with exosomes derived from hUC-MSCs may be a potential material to stop bleeding and promote wound healing.

FOOTNOTES

Author contributions: Qi ZQ and Hu XM contributed to the study conception and design; Hu XM, Wang CC, and Xiao Y contributed to data collection, analysis, and interpretation; Hu XM contributed to manuscript writing; Hu XM, Wang CC, Xiao Y, Jiang P, and Liu Y performed the animal experiments; Qi ZQ, Hu XM, and Xiao Y contributed to the conception, design, financial support, and final approval of the manuscript; and all the authors have read and approved the final manuscript.

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REVIEW

Potential of dental pulp stem cells and their products in promoting peripheral nerve regeneration and their future applications

Wen-Bo Xing, Shu-Ting Wu, Xin-Xin Wang, Fen-Yao Li, Ruo-Xuan Wang, Ji-Hui He, Jiao Fu, Yan He

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Abstract

Peripheral nerve injury (PNI) seriously affects people's quality of life. Stem cell therapy is considered a promising new option for the clinical treatment of PNI. Dental stem cells, particularly dental pulp stem cells (DPSCs), are adult pluripotent stem cells derived from the neuroectoderm. DPSCs have significant potential in the field of neural tissue engineering due to their numerous advantages, such as easy isolation, multidifferentiation potential, low immunogenicity, and low transplant rejection rate. DPSCs are extensively used in tissue engineering and regenerative medicine, including for the treatment of sciatic nerve injury, facial nerve injury, spinal cord injury, and other neurodegenerative diseases. This article reviews research related to DPSCs and their advantages in treating PNI, aiming to summarize the therapeutic potential of DPSCs for PNI and the underlying mechanisms and providing valuable guidance and a foundation for future research.

Key Words: Dental pulp stem cells; Peripheral nerve injury; Regenerative medicine; Neural regeneration; Schwann cells; Stem cells engineering

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Core Tip: This article reviews the potential applications of dental pulp stem cells (DPSCs) and their derivatives in the field of nerve regeneration. First, this paper describes the current status of stem cell therapies for peripheral nerve injury (PNI) and discusses the advantages of DPSCs in this field. Then, the status of research on the neuroregenerative ability of DPSCs and their derivatives is reviewed. Finally, the potential of DPSCs in treating PNI and the underlying mechanism are summarized, with an aim to provide valuable guidance and a basis for future research.

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INTRODUCTION

It has been reported that approximately 2.8% of trauma patients suffer from peripheral nerve injury (PNI), resulting in permanent disabilities[1]. PNI can lead to muscle function loss, sensory impairment, and painful neuropathy[2]. While autologous nerve transplantation is a standard treatment option, its drawbacks, such as the difficulty in obtaining donor nerves, the need for surgical procedures to acquire donor nerves, secondary deformities at the donor site, and potential mismatch issues, limit its widespread use[3,4]. With advancements in biomaterials and tissue regeneration, significant progress has been made in nerve regrowth techniques[5]. The restoration of function after injury is of paramount importance, and experiments on stem cells have shown that they can accelerate nerve regeneration[6]. Compared to other types of stem cells, dental pulp stem cells (DPSCs) are more advantageous because they can be obtained *via* noninvasive operation, can be preserved at low temperature long-term, are simple to use, are associated with few ethical problems, and have low immunogenicity, so they are ideal materials for tissue engineering[7]. Human DPSCs (hDPSCs) exhibit remarkable self-renewal, multilineage differentiation, and cloning capabilities[8]. Schwann cells play a critical role in nerve regeneration, and stem cells with the same embryonic origin as Schwann cells are suitable tools for PNI treatment. DPSCs originate from neural crest cells[9] and share homology with Schwann cells[10]. Therefore, due to DPSCs' unique neural differentiation and nerve regeneration abilities, DPSCs-derived Schwann cells are viable tools for achieving nerve regeneration after PNI *in vitro*[11]. To keep abreast of the latest developments in this field, articles published in PubMed between 2010 and 2023 were screened using the following search terms: "peripheral nerve", "tooth-derived stem cells", "exosomes", and "dental stem cells". The primary aim of this review is to provide information on the recent applications of DPSCs in PNI treatment, with a specific focus on both cellular therapies and noncellular therapies involving DPSCs. Additionally, the review discusses the therapeutic effects of DPSCs and their potential future applications in treating PNI.

PNI

PNI is a prevalent clinical condition often resulting in long-term functional impairments. The effectiveness of surgical treatments is frequently unsatisfactory[12], and the restoration of nerve function is often not optimal[13]. Peripheral nerve regeneration is a complex process involving Wallerian degeneration, axon sprouting, and myelin regeneration[14]. While nerves may regenerate over relatively short distances after mild nerve injury, the outcomes of nerve regeneration are often unsatisfactory. In cases of peripheral nerve amputation, a series of molecular and cellular changes occur, known as Waller's degeneration[15,16]. Subsequently, monocytes and macrophages migrate to the nerve stump to clear axon fragments and myelin sheaths at the damaged end, while the proliferation of Schwann cells results in the formation of longitudinal cell columns, known as Bungner bands[17,18]. According to the literature, various factors, such as scaffolds for axonal migration, supporting cells (including Schwann cells and macrophages), growth factors, and the extracellular matrix, play crucial roles in regeneration after PNI[2].

Schwann cells originate from neural crest cells[19] and play a crucial role in the regeneration of peripheral nerves, influenced by neurotrophic factors (NTFs) such as Krox-20, Oct-6, and Sox-10[18]. These cells play an instrumental role in nerve regeneration by selectively promoting axonal regrowth of both motor and sensory nerves[20-22]. After nerve injury, Schwann cells may undergo dedifferentiation, a process mainly regulated by the negative regulatory factor c-Jun. This dedifferentiation is vital because it can help nerve survival and facilitate axonal regeneration[23]. Researchers have explored the effect of transplanting Schwann cells isolated from peripheral nerves into rat sciatic nerve injury models and found that these cells promote the regeneration of nerve axons, proving the potential of autologous stem cell transplantation in treating PNI[24]. However, such regenerative strategies are associated with challenges, as collecting Schwann cells is difficult and their survival rate after transplantation is low[25]. In recent studies, DPSCs-derived conditioned medium (DPSCs-CM) was shown to promote the proliferation of Schwann cells and increase the production of myelin-associated proteins[26]. This may provide insight for the development of a new method for the treatment of peripheral nerve diseases.

STEM CELL THERAPY FOR PNI

Earlier studies have revealed that allogeneic stem cell transplantation has a favorable effect on nerve regeneration and thus warrants further investigation[27]. Acellular nerve allografts (ANAs) promote axon regeneration through Schwann cell proliferation[28]. However, the therapeutic efficacy of ANAs diminishes over time after nerve injury, likely due to the limitations of Schwann cell function in the host nerve. Therefore, there is an urgent need to explore alternative or more effective therapies for Schwann cell replacement.

An increasing number of studies have demonstrated the remarkable potential of stem cells in promoting neural regeneration. Adipose-derived stem cells have been shown to differentiate into Schwann-like cells and effectively promote nerve regeneration[29-31]. Additionally, Shimizu *et al*[32] reported that human bone marrow mesenchymal stem cells (MSCs) can serve as Schwann cell substitutes, making them a viable option for nerve regeneration applications. Furthermore, muscle-derived stem/progenitor cells have shown the ability to differentiate into myelinated Schwann cells *in vivo*, thereby promoting axonal regeneration[33]. Al-Zer and Kalbouneh[11] successfully induced the differentiation of DPSCs into Schwann cells by utilizing retinoic acid, mercaptoethanol, and neuromodulin β1. Among stem cell sources, dental stem cells have garnered significant attention due to their excellent nerve regeneration capabilities and ease of availability.

Recent studies have revealed that during tooth development, glial cells related to the peripheral nervous system produce a significant number of MSCs, including dental pulp cells and odontoblasts[34]. DPSCs, stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), stem cells from the apical papilla, and dental follicle progenitor cells all fall under the category of dental stem cells[35]. These dental tissue-derived MSCs exhibit the ability of multidirectional differentiation and hold great potential in immunomodulation and tissue regeneration[36]. Moreover, MSCs derived from hair follicles, the dental pulp, and nipples show similar biological characteristics as those from the teeth of the same donor and display osteogenic, adipogenic, and chondrogenic differentiation capabilities[37].

Apical pulp-derived cells and coronal pulp cells have demonstrated the ability to differentiate into nerve cells *in vitro* and express markers associated with neural crest cells (p75, Snail, and Slug) as well as neural stem cell markers (Nestin and Musashi1)[38]. SHEDs can differentiate into many types of cells, including nerve cells[39]. Pereira *et al*[40] utilized polyglycolic acid tubes to induce SHEDs to differentiate into Schwann cells. Human PDLSCs have exhibited the ability to promote axonal regeneration after optic nerve injury, potentially through the secretion of brain-derived neurotrophic factor (BDNF)[41]. The ERK1/2 signaling pathway plays a role in the differentiation of PDLSCs into Schwann cells[42], thus making them a viable alternative source for autologous Schwann cells[43]. Ng *et al*[44] successfully induced adult PDLSCs to differentiate into retina-like cells with the biological characteristics of nerve cells. Dental embryonic stem cells have the potential to achieve nerve tissue regeneration due to their common origin with the nervous system[5,14], making them a promising source of stem cells for nerve tissue regeneration[45]. The neurological potential of DPSCs makes them a viable candidate cell type for the treatment of peripheral nerve diseases[46]. DPSCs can differentiate into neuron-like cells, and after 5 d of neuron differentiation, Tub3 is activated, accompanied by increased Nestin expression[47]. Insulin-like growth factor binding protein 5 promotes the formation of neurospheres by DPSCs. Angiogenic markers such as vascular endothelial-derived growth factor (VEGF), platelet derived growth factor subunit A, and angiopoietin-1 and neurogenic markers such as neural cell adhesion molecule, Nestin, βIII-tubulin, and tyrosine hydroxylase are upregulated in DPSCs, reflecting the vascular and neurogenic differentiation potential of DPSCs[48]. Additionally, DPSCs can secrete NTFs that support nerve cell function[49].

Compared with other stem cells, DPSCs have stronger multidifferentiation potential, self-renewal ability, and colony formation ability[8]. The Eph/ephrin interaction indicates that unlike that of other stem cells, the formation of DPSCs involves the neural crest[50,51]. Because of their spinal origin, DPSCs have the ability to differentiate into other spinal cord-related cells[52]. Unlike bone marrow MSCs (BM-MSCs), DPSCs are heterogeneous, and they also express markers of endothelial cells (vascular cell adhesion molecule 1 and MUC-18), smooth muscle (a-smooth muscle actin), bone (alkaline phosphatase, type I collagen, osteonectin, osteopontin, and osteocalcin), and fibroblasts (type III collagen and fibroblast growth factor 2)[39,53]. DPSCs express the neural precursor and glial cell markers nestin and glial fibrillary acidic protein (GFAP), which indicates that DPSCs are similar to BM-MSCs and have the potential to differentiate into neural cells[8]. Compared with BM-MSCs, DPSCs can more strongly inhibit the proliferation of PHA-stimulated T cells and exert immunosuppressive effects[54]. Compared with umbilical cord stem cells, the secretion of vascular endothelial growth factor-A and follistatin in DPSCs is more obvious, while umbilical cord stem cells tend to secrete vascular endothelial growth factor-C, which does not produce angiogenic effects. The levels of vascular endothelial growth factor-A and vascular endothelial growth factor-D secreted by DPSCs are higher than those secreted by BM-MSCs[55]. DPSCs can survive for a long time under extreme stress conditions[56]. DPSCs have the same immunomodulatory effect as BM-MSCs in the treatment of nervous system diseases. DPSCs are easier to obtain than other stem cells, have high multidifferentiation potential and a strong proliferation ability, and are not carcinogenic. They are a good substitute for stem cells in the treatment of PNI[5].

Moreover, compared with dental follicle- and papilla-derived stem cells, DPSCs have a stronger Na^+ current, indicating that they have a higher potential for neural differentiation[57]. Both DPSCs and BM-MSCs implanted in the vitreous body secrete nerve growth factor (NGF), BDNF, and neurotrophin 3 (NT-3), and the amount of NGF and BDNF secreted by DPSCs is significantly higher than that secreted by BMSCs, which support nerve survival and axonal regeneration[58]. Adipose tissue-derived stem cells also have the potential for nerve regeneration but mainly play an active role in stimulating endogenous stem cells by releasing NTFs[59]. Wang *et al*[60] successfully induced vascular endothelial cells and BM-MSCs to differentiate into neurons with tricyclodecane-9-yl xanthate. Cryopreserved DPSCs were also shown to be able to differentiate into cholinergic neurons by tricyclodecane-9-yl xanthate[61]. By comparing stem cells from

different sources, Isobe *et al*[62] found that BM-MSCs and synovial fluid-derived stem cells showed significant osteogenic effects. An increase in alkaline phosphatase and osteocalcin levels suggested that synovial fluid-derived stem cells had the highest cartilage formation ability. Reverse transcription-polymerase chain reaction showed increased expression of class III β-tubulin and microtubule-associated protein 2, suggesting that DPSCs and human deciduous tooth stem cells have the potential for neural regeneration. In addition, studies by Isobe *et al*[62] showed that DPSCs have stronger neural differentiation potential than pluripotent stem cells isolated from bone marrow and synovial fluid.

In light of these findings, DPSCs have emerged as excellent candidate stem cells for treating PNI, with stronger tissue regeneration potential than other types of stem cells (Figure 1). Consequently, the focus of this article will be on research progress related to the effectiveness of DPSCs in treating PNI.

EFFECT OF DPSCS IN THE TREATMENT OF PNI

Teeth originate from the cranial neural crest[63]. Chai *et al*[64] were the first to confirm the involvement of cranial neural crest cells in the formation of dental pulp cells. In recent years, there has been a growing interest in research on utilizing DPSCs for nerve repair[65]. Janebodin *et al*[52] demonstrated that DPSCs originate from spinal nerves and possess the potential to differentiate into tissues derived from other neural crest-derived structures. Gronthos *et al*[53] provided evidence that the dental pulp contains cells with the ability to form clones, proliferate, and regenerate tissue, classifying them as stem cells. The embryonic origin of DPSCs endows them with the potential to serve as stem cells for neural tissue engineering.

DPSCs have been shown to effectively promote axonal regeneration both *in vivo* and *in vitro*[66] (Tables 1 and 2). Typically, DPSCs exhibit several characteristics of pluripotent stem cells and display high proliferation rates, expressing CD44, CD90, and CD166[65]. Moreover, DPSCs express embryonic stem cell markers such as Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, along with several other MSC markers[67]. DPSCs have the remarkable capability to differentiate into several stromal-derived cell types (Figure 2). These cell types include osteoblasts[68-72], neurons[73], adipogenic cells[69] chondrogenic cells[69,70], smooth and skeletal muscle cells[74], dental pulp cells[75], Schwann cells [76], pancreatic cell lineage cells[70,77], blood vessel cells[78], and hepatocytes[79,80]. These findings strongly support the potential of DPSCs to be applied for tissue engineering[7].

Subsequent studies have revealed that a large number of dental MSCs originate from glial cells associated with peripheral nerves[34]. Long-term cryopreserved DPSCs retain their potential to differentiate into cholinergic nerves, indicating their suitability for prolonged preservation[61], and they have demonstrated beneficial anti-inflammatory effects in nerve injury[81]. Paes *et al*[82] conducted a pioneering comparison between monolayer and spheroid cultures of hDPSCs, demonstrating that both exhibit promising nerve regeneration potential through different mechanisms. Furthermore, hDPSCs were found to be effective in treating diabetic polyneuropathy[83]. In a groundbreaking study, Omi *et al*[84] treated neuropathy in diabetic rats through DPSCs transplantation for the first time and observed nerve function recovery that was potentially attributed to basic fibroblast growth factor (bFGF), VEGF, NGF, and NT-3 secreted by DPSCs. As an effective therapeutic tool for treating PNI, DPSCs are anticipated to be widely used in clinical settings in the future. With the deepening of research (Figure 3), it has been confirmed that DPSCs can be used to treat PNI *via* many mechanisms.

CELL THERAPEUTIC ALTERNATIVE FOR DPSCS

DPSCs can differentiate into nerve-like cells, making them be of great significance in the field of nerve regeneration. Dental pulp MSCs exhibit strong expression of nerve and glial cell markers and can be induced to adopt a nerve-like morphology[5]. DPSCs can be induced to differentiate into cells that exhibit a neuronal morphology and express neuronal-specific markers, such as the immature neuron markers Nestin and PSA-NCAM, by a neural induction regimen *in vivo* and *in vitro*[85]. Moreover, DPSCs transplantation has been shown to promote macrophage polarization toward the anti-inflammatory M2 phenotype[86]. Saez *et al*[87] demonstrated that DPSCs can effectively promote peripheral nerve regeneration when used to treat unilateral nerve crush injury in rats.

The peripheral nerve transection model is widely utilized in the study of peripheral PNI, and DPSCs have demonstrated good nerve regeneration ability in this model[88]. Stocchero *et al*[89] transplanted dental pulp cells into Wistar rats with sciatic nerve defect and observed significant promotion of nerve regeneration during the first 2 wk. Combining dental pulp cells with biodegradable materials is a promising approach to avoid secondary surgery[90]. Additionally, Sasaki *et al*[91] embedded dental pulp cells in silicone tubes to repair facial nerve defects in rats, and the results indicated the formation of blood vessels and myelin sheath tissue by dental pulp cells. DPSCs have exhibited a robust capacity to promote nerve regeneration in peripheral nerve transection models, and optimizing material combination schemes to fully unleash DPSCs' potential in nerve regeneration remains a prominent research focus in this field.

As previously reported, DPSCs, being derived from the neural ridge, possess a strong ability to differentiate into neuronal lineages. Numerous studies have explored various methods to induce the neural differentiation of DPSCs, and these cells have been applied to treat PNI[92]. After neural differentiation induction, DPSCs generate neural progenitor cells, expressing the neural markers Nestin, TuJ-1, and GFAP[93]. Additionally, inner ear neurotrophins, such as BDNF and NT-3, along with glial cell-derived neurotrophic factor (GDNF), have been found to promote the differentiation of DPSCs into spiral ganglion neuron-like cells[94]. Notably, when Schwann cells differentiated from human DPSCs were

Table 1 Progress in application of dental pulp stem cells in treating models of peripheral nerve injury

Publication year	Cell source	Induction program	Pretransplantation procedure	Material(s)	Dose/concentration	Disease model	Experiment duration	Treatment effect	Ref.
2022	Human	DMEM + 10% (FBS + P/S)	3D culture	Fibrinogen, calcium chloride, and thrombin-like protein	3×10^5 cells	Avulsion of spinal motor roots in rats	12 wk	The transcription of TNF-α, IL-1β, IL-6, and IL-17 and the expression of anti-inflammatory cytokines (TGF-β, IL-4, IL-10, and IL-13) were increased; the animals in the reimplantation + 2D group showed the best functional recovery	[82]
2021	Human	α-MEM + 20% (FBS + P/S)	Collected and resuspended in GelMA-bFGF	10% GFD in a CSM tube	1×10^6 cells/mL	15 mm defect of the sciatic nerve in rats	12 wk	Cell based therapy repaired large gap defects in peripheral nerves; the differentiation of DPSCs into nerve cells and Schwann-like nerve cells and the formation of myelinated nerve fibers were observed	[98]
2021	Human	DMEM + 10% (FBS + P/S + NEAA)	NLCs differentiated from DPSCs	-	1×10^5 NLCs		12 wk	Two weeks after transplantation, approximately 75% of the transplanted cells differentiated into platelet-derived growth factor receptor alpha + OPCs expressing p75NTRd; transplantation promoted axon growth and improved nerve function	[145]
2021	Human	α-MEM + 15% FBS	Exosome collection	-	200 μg/100 μL	Mouse model of spinal cord injury	4 wk	Inhibited the ROS-MAPK-NFκB P65 signaling pathway to reduce M1 macrophage polarization, suppress the inflammatory response, and alleviate neurological damage	[121]
2020	Human	DMEM + 20% (FBS + P/S)	Preparation of scaffold-free cell sheets by coculture with FGF2	-	2×10^6 cells/cell sheet	Rat model of facial nerve crush injury	3 wk	Cell sheets promoted axonal regeneration and functional recovery through continuous delivery of neurotrophic factors such as BDNF and GDNF	[116]
2020	Human	α-MEM + 10% (FBS + P/S + NEAA)	Induction of DPSCs differentiation into N-DPSCs; induction of DPSCs differentiation into N-DPSCs	-	-	Rat model of sciatic nerve crush injury	1 mo	Both DPSCs and N-DPSCs promoted peripheral nerve repair through the expression of neurotrophic factors such as NGF, BDNF, and GDNF; the nerve repair effect of N-DPSCs was longer lasting	[146]
2019	Human (children)	DMEM + 15% (FBS+P/S)	-	-	5×10^5 cells in 4 μL DMEM	Unilateral facial nerve crush injury in rats	6 wk	Immature DPSCs promoted nerve regeneration and the formation of new myelin; the expression of nerve growth factor and anti-inflammatory cytokines (IL-6 and IL-10) increased significantly 7 d after treatment, and there was a decrease in the levels of soluble proinflammatory factors such as IL-2, IL-4, TNF-α, and IFN-γ	[87]

2019	Human	-	Induction of DPSCs differentiation into nerve cells	A PDO-based cell carrier	7.5×10^5 cells	6 mm defect of the sciatic nerve in rats	12 wk	Multiperforated PDO tubes were effective biomaterial carriers; delivery of DPSCs impacted the inflammatory environment and promoted nerve regeneration and functional recovery	[89]
2018	Human	-	Isolation of STRO-1+/c-Kit+/CD34+ cells	Collagen scaffolds	5×10^5 cells/animal	6 mm defect of the sciatic nerve in rats	4 wk	Nerve fiber regeneration and myelination and many myelinated axons were observed; DPSCs grafted into the sciatic nerve defect expressed the typical Schwann cell marker S100B and were positive for human NeuN	[12]
2018	Human	ADMEM + 10% FBS	Differentiated into neuronal cells (DF-DPSCs)	A conduit made from a Lyoplant membrane	-	7-8 mm defect of the sciatic nerve in rats	12 wk	DPSCs relieved neuropathic pain and inhibited inflammation in rats earlier than DF-DPSCs; at 12 wk after the operation, the expression of pAMPK/SIRT1 in DF-DPSCs and DPSCs increased, the expression of proinflammatory cytokines decreased, and the expression of NFκB decreased	[81]
2018	Human	ADMEM + 10% (FBS + P/S)	Differentiation into cholinergic neurons by adding D609	Biodegradable tubule and fibrin glue	1×10^6 DF-chNs	5 mm defect of the sciatic nerve in rats	8 wk	Transplanted DF-chNs promoted motor nerve regeneration and axon growth and expressed nerve growth factor receptor (p75NGFR)	[61]
2018	Human	-	-	An absorbable hemostat filled with human DPCs containing 1% atelocollagen, fibronectin, and laminin	3×10^5 cells	Crush injury of the sciatic nerve in rats	2 wk	DPCs stimulated Schwann cell differentiation and promoted peripheral nerve regeneration	[114]
2017	Human	Standard: α-MEM + 10% (FBS + NEAA + P/S). Differentiation: Standard + forskolin + bFGF + PDGF-AA + HRG1-β	Differentiation into Schwann-like cells (d-hDPSCs)	NeuraWrap™ conduits	-	15 mm defect of the sciatic nerve in rats	8 wk	Growth of axons, myelinated nerve fibers, and blood vessels; DPSCs still exerted strong angiogenic effects after differentiating into Schwann-like cells	[102]
2017	Human	α-MEM + 15% (FBS + AA + P/S + NEAA)	-	Fibrin conduits	2×10^6/20 μL	10 mm defect of the sciatic nerve in rats	2 wk	Promoted nerve and axon regeneration; the transplanted cells expressed BDNF near the cell body, and the expression level of caspase-3 decreased	[45]
2017	Human	ADMEM + 10% FBS	Induction of DPSCs differentiation into nerve cells	Fibrin glue scaffold and collagen tubulation	1×10^6 cells	5 mm defect of the sciatic nerve in rats	12 wk	Both hDPSCs and DF-hDPSCs promoted nerve regeneration and functional recovery; they could directly differentiate into nerve cells or facilitate nerve cell differentiation	[99]
2015	Human	α-MEM + 10% (FBS +	Transfection with Olig2	-	2×10^5 cells	Mouse model of local	6 wk	Recovery of sciatic nerve function; DPSCs	[108]

		P/S/AmB)	gene *via* a tetracycline (Tet) inducible system			sciatic nerve demyelination		differentiated into oligodendrocyte progenitors, and specific markers of oligodendrocyte progenitors and oligodendrocytes were expressed	
2015	Human	DMEM + 10% FBS	G-CSF-induced stem cell mobilization (mobilized DPSCs and MDPSCs)	Collagen conduits	3.0×10^5 MDPSCs	5 mm defect of the sciatic nerve in rats	5 wk	MDPSCs secreted neurogenic/angiogenic factors and promoted peripheral nerve regeneration	[112]
2015	Human	Culture dishes containing essential medium (alpha modification) + 10% (FBS + P/S + amphotericin B)	Induction of DPSCs differentiation into OPCs by transfection with a plasmid containing the human Olig2 gene		2×10^5 cells	Sciatic nerve demyelination in mice	6 wk	DPSCs differentiated into OPCs, and transplantation promoted myelin sheath formation and peripheral nerve function recovery	[107]
2015	Human	DMEM + b-ME; DMEM + 10% (FBS + RA); DMEM + 10% (FBS + FSK + b-FGF + PDGF + HRG)	Differentiation of hDPSCs into Schwann-like cells	Cells combined with a pulsed electromagnetic field (PEMF)	1×10^6 cells/10 mL/rat	Crush injury of the peripheral nerve in rats	3 wk	Schwann-like cells derived from DPSCs exhibited the characteristics of glial cells, expressing CD104, S100, GFAP, laminin, and p75NTR; application of a PEMF promoted peripheral nerve regeneration after cell transplantation	[147]
2012	Human	DMEM + 10% FBS	-	-	1×10^6 cells	Rat spinal cord transection model	8 wk	DPSCs promoted axonal growth, differentiated into oligodendrocytes to treat spinal cord injury, and protected the nerve by inhibiting apoptosis and paracrine signaling	[105]
2018	Human	α-MEM + 10% (FBS + NEAA + P/S)	Application of fresh medium containing vitamin C cells reached approximately 80% confluence	-	-	Patients diagnosed with a traumatized permanent incisor	12 mo	HDPSCs transplantation promoted the regeneration of pulp tissue including neuronal tissue, and the neuron marker NeuN was expressed	[75]
	Human	PBS + P/S	Collection of hDPSCs aggregates	The root canals of human teeth	-	Immunocompromised mice	8 wk	Dental pulp tissue containing sensory nerves and blood vessels regenerated after HDPSCs transplantation	
	Human	α-MEM + 10% (FBS + NEAA + P/S)	-	-	3×10^5 cells	Rats injected into the dorsal root ganglion	2 mo	HDPSCs exhibited the morphology of neurons and expressed TRPV1 and TRPM8	
	Pig	PBS + P/S	Collection of hDPSCs aggregates	-	-	Permanent incisors of young female minipigs	3 mo	Pig DPSCs resulted in the 3D regeneration of dental pulp with neural function	
2015	Pig	Culture medium + 10% (FBS + L-AA -2-P + P/S)	-	Fibrin membrane	-	Porcine intercostal nerve transection model	6 mo	DPSCs alleviated nerve injury and express NSE; neuroelectrophysiological evaluation showed that neurological function was restored	[88]
2020	Rat	α-MEM + 20% FBS	-	-	1×10^6 cells/rat	Diabetic rats	4 wk	Multiple factors secreted by DPSCs increased the nerve conduction velocity and blood flow to nerves	[118]

2019	Rat	α-MEM + glucose + 20% FBS	Collection of DPSCs-CM	-	1 mL/rat	Diabetic rats	4 wk	DPSCs-CM ameliorated peripheral neuropathy by exerting neuroprotective, angiogenic, and anti-inflammatory effects	[132]
2017	Rat	α-MEM + 20% FBS	-	-	1×10^6 cells	Streptozotocin-induced diabetes rat model	4 wk	Sensory disturbance was alleviated, the thickness and area of the myelin sheath increased, the transplanted DPSCs secreted multiple factors such as angiogenic factors, neurotrophic factors, and immunosuppressive factors	[84]
2015	Rat	α-MEM + glucose + 20% FBS	-	-	1×10^6 cells	Diabetic rats	4 wk	DPSCs transplantation relieved diabetic polyneuropathy by inhibiting inflammation, exerting immunomodulatory effects, and secreting neurotrophic factors	[86]
2015	Rat	α-MEM + 20% FBS	-	-	1×10^6 cells/limb	Diabetic rats	8 wk	DPSCs increased the nerve conduction velocity and blood flow to nerves and promoted an increase in the number of nerve fibers in diabetic rats	[83]
2013	Rat	DMEM + 10% (FBS + P/S)	-	-	1.5×10^5 cells	Crush injury of the optic nerve in rats	3 wk	Transplantation of DPSCs significantly increased the survival rate of retinal ganglion cells in rats and promote axonal regeneration	[58]
2007	Rat	-	Embedded in 10 mL type I collagen gel	10-mm silicone tube	1×10^5 cells	7 mm defect of the facial nerve in rats	2 wk	Regeneration of axons, blood vessels, and Schwann cells; Tuj1-positive axons and S100-positive Schwann-like supportive cells were found in regenerated nerves	[91]
2018	Rabbit	DMEM + 10% FBS	Construction of an acellular nerve graft for nerve regeneration	Xenogenic acellular nerve matrix	6×10^5 cells per graft	10 mm defect of the sciatic nerve in rabbits	3 mo	Regeneration of nerve space showed that acellular nerve grafts containing DPSCs treated with myroilysin had a strong neural induction effect	[148]

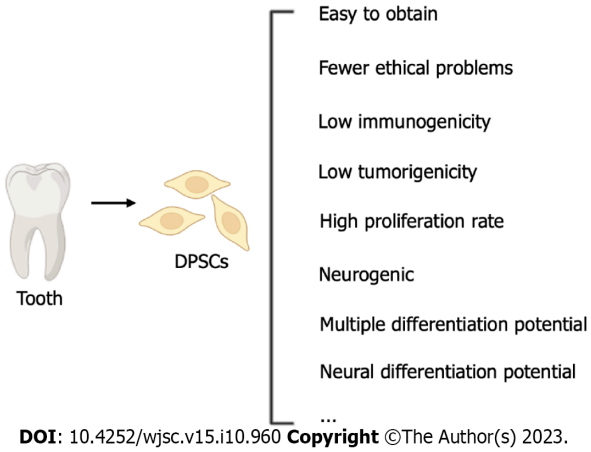
AA: Ascorbic acid; AmB: Amphotericin B; ADMEM: Advanced Dulbecco's modified Eagle medium; bFGF: Basic fibroblast growth factor; b-ME: Beta-mercaptoethanol; BG: Beta-glycerophosphate; Dex: Dexamethasone; CSM: Cellulose/soy protein isolate composite membrane; DPSCs-CM: DPSCs-conditioned medium; DMEM: Dulbecco's modified Eagle medium; DF-chNs: Differentiated cholinergic neurons; DF-DPSCs: Neuronal cells differentiated from DPSCs; FSK: Forskolin; bD609: Tricyclodecane-9-yl-xanthogenate; FBS: Fetal bovine serum; G-CSF: Granulocyte-colony stimulating factor; GFD: 10% GelMA hydrogel, recombinant human basic fibroblast growth factor and DPSCs; HRG1-β: Heregulin-β-1; L-AA-2-P: L-ascorbic acid-2-phosphate; NGF: Nerve growth factor; N-DPSCs: Neural-induced DPSCs; NSE: Neuron-specific enolase; NEAA: Nonessential amino acids; NLCs: Neural lineage cells; OPCs: Oligodendrocyte progenitor cells; P/S: Penicillin and streptomycin; PDGF-AA: Platelet-derived growth factor AA; PBS: Phosphate-buffered saline; PDGF: Platelet-derived growth factor; RA: All-trans retinoic acid; TGNCs: Trigeminal ganglion neuronal cells; IL: Interleukin; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

employed to treat a rat sciatic nerve defect model, significant regeneration of blood vessels and nerve processes was observed, highlighting the crucial role of revascularization in supporting nerve regeneration and survival[95]. Moreover, Zheng *et al*[96] demonstrated that coculture of chitosan scaffold with bFGF can enhance the neural differentiation of DPSCs. The ERK signaling pathway, a classical MAPK pathway, plays a pivotal role in this process. As a downstream effector of bFGF, DPSCs are actively engaged in neural differentiation. Numerous neural induction protocols exist for DPSCs; however, an optimal scheme that is simple, efficient, and quick is currently lacking.

Table 2 Research illustrating the ability of dental pulp stem cells to promote nerve regeneration *in vitro*

Publication year	Cell source	Induction protocol	Material(s) applied	Dose/concentration	Neurocyte type	Culture duration	Results	Ref.
2022	Human	DMEM + 20% (FBS + P/S + AA + bFGF)	Dental pulp cell sheets	3200 cells/cm^2	SH-SY5Y neuroblastoma cells	3 d	Dental pulp cell sheets provided neurotrophic support by expressing NTF; the amount of neurotrophic factors produced by dental pulp cell sheets was sufficient to induce nerve regeneration *in vitro* and promote nerve repair *in vivo*; dental pulp cell sheets improved axon guidance and reduced axon branching	[113]
2020	Rat	α-MEM + 10% (FBS + P/S + NEAA)	DPSCs-CM	-	TGNCs from rats	3 wk	DPSCs-CM was found to contain significant levels of nerve growth factor, brain-derived neurotrophic factor, neurotrophic factor-3, and glial cell line-derived neurotrophic factor; DPSCs-CM increased the survival rate of primary trigeminal ganglion neurons and promoted the growth of neurites	[130]
2020	Rat	α-MEM + 10% (FBS + P/S + NEAA)	DPSCs-CM	50% DPSCs-CM	PC12 cells	8 d	DPSCs-CM was found to contain neurotrophic factors, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor, which increased the viability and differentiation of PC12 cells and played an important role in axonal growth and survival, proving that DPSCs-CM treatment is a potential cell-free therapy for peripheral nerve repair and has a stronger effect on PC12 cells than DPSCs	[115]

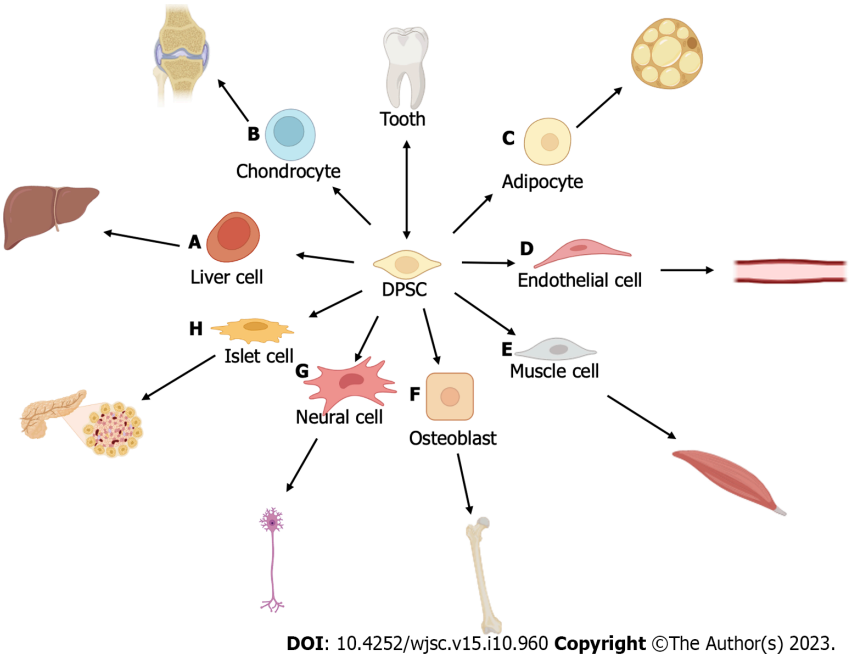
AA: Ascorbic acid; bFGF: Basic fibroblast growth factor; DPSCs-CM: DPSCs-conditioned medium; DMEM: Dulbecco's modified Eagle medium; FBS: Fetal bovine serum; NTF: Neurotrophic factor; NEAA: Nonessential amino acids; P/S: Penicillin and streptomycin; TGNC: Trigeminal ganglion neuronal cells.



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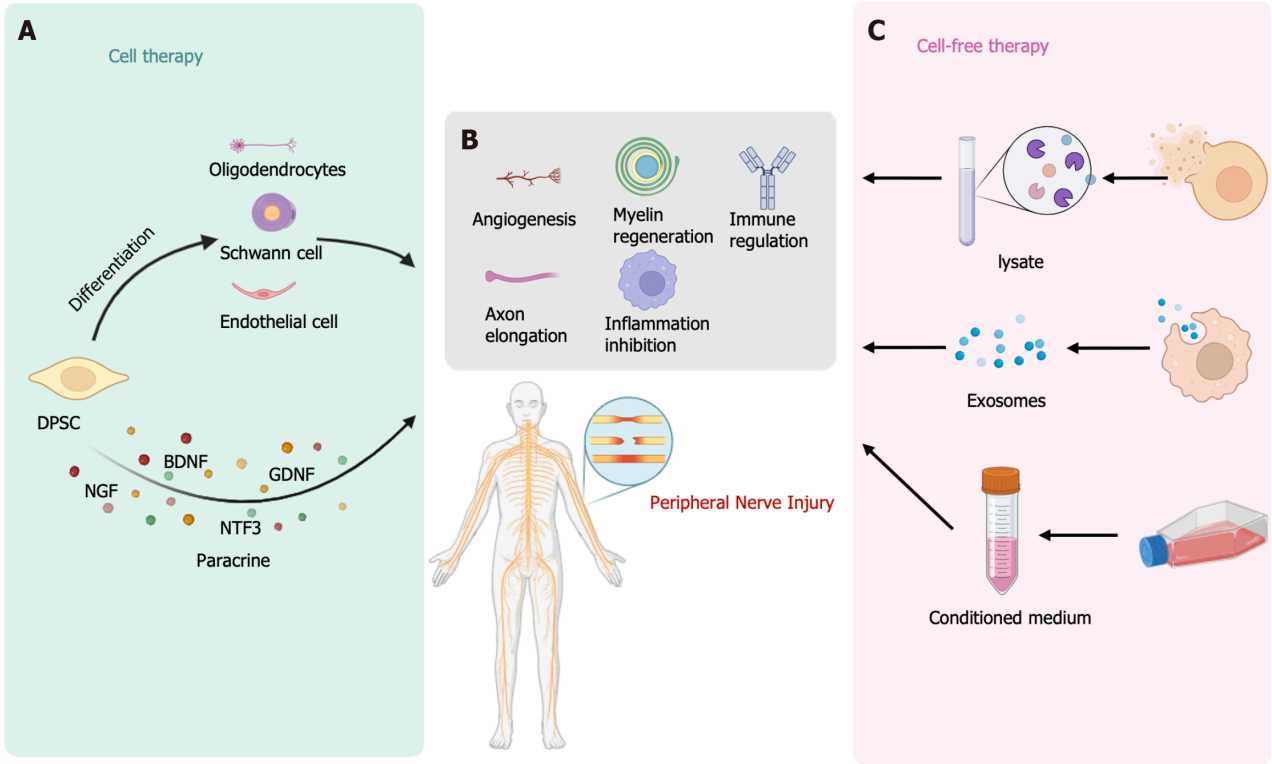
Figure 1 Advantages of dental pulp stem cells as alternative stem cells for nerve regeneration. The figure was created with BioRender.com. DPSCs: Dental pulp stem cells.

Although significant progress has been made in the tissue field, finding a nerve conduit that can match the effectiveness of autologous transplantation remains a challenge in the treatment of PNI. Das and Bellare[97] developed a uniform bead-free nanofibrous scaffold primarily composed of polycaprolactone and gelatin A, which has been shown to support DPSCs regeneration and neural differentiation. In our previous studies, we confirmed that combining DPSCs with a third-generation nerve regeneration conduit can be used to effectively repair 15 mm long defects of the sciatic nerve in rats. Remarkably, the effect of this approach is comparable to that of autotransplantation. Furthermore, we observed that nerve tissue at the repair site mainly originated from differentiating DPSCs. This promising finding offers a potential tissue engineering strategy for the treatment of PNI[98].



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Figure 2 Ability of dental pulp stem cells to differentiate into other types of cells. Dental pulp stem cells derived from dental pulp exhibit both self-proliferation ability and the capability to differentiate into multiple lineages *in vitro*. A: Liver stem cell; B: Chondrocyte; C: Adipocyte; D: Endothelial cell; E: Muscle cell; F: Osteoblast; G: Neural cell; H: Islet cell. The figure was created with BioRender.com. DPSC: Dental pulp stem cell.



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Figure 3 Treatment of peripheral nerve injury by cell/acellular therapies involving dental pulp stem cells. A: Dental pulp stem cells (DPSCs) can directly differentiate into nerve cells or endothelial cells and can also protect nerves and promote nerve regeneration through paracrine neurotrophic factors; B: DPSCs can treat peripheral nerve injury (PNI) by promoting vascular regeneration, axonal regeneration, and myelin sheath repair, regulating the immune response, and inhibiting inflammation; C: Exosomes, lysates, and conditioned media from DPSCs are also effective in treating PNI. The figure was created with BioRender.com. DPSC: Dental pulp stem cell; NGF: Nerve growth factor; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell-derived neurotrophic factor; NTF3: Neurotrophic factor 3.

Ullah *et al*[99] made an intriguing discovery that there was no significant difference in nerve regeneration ability between hDPSCs and differentiated neuronal cells derived from hDPSCs. More research on this topic is needed to clarify the underlying mechanisms and find a more effective treatment strategy.

Differentiation into Schwann cells

Previous studies have demonstrated that autologous adult Schwann cells have a remarkable capacity to support extensive peripheral nerve regeneration and can evade rejection[100]. Currently, Schwann cells remain a focal point of animal studies on PNI. Substitutive cell therapies bring renewed hope for nerve regeneration[20]. Schwann cells play a pivotal role in Wallerian degeneration, myelin regeneration, and supporting axon growth.

Martens *et al*[76] were the first to demonstrate that Schwann cells differentiated from hDPSCs can promote nerve regeneration. These differentiated Schwann cells express neural markers such as laminin, p75, GFAP, and CD104 and exhibit an increase in the expression of NTFs such as BDNF, b-NGF, NT-3, and GDNF. Medium conditioned by these differentiated Schwann cells was found to increase the survival of dorsal root ganglion cells and stimulate axon elongation. Subsequently, Al-Zer *et al*[101] induced the differentiation of DPSCs into Schwann cells by using forskolin, bFGF, platelet-derived growth factor, and recombinant human neuregulin-β1. The results revealed that the differentiated neural stem cells strongly expressed the Schwann cell marker Sox10. Additionally, Sanen *et al*[102] demonstrated that hDPSCs, after differentiating into Schwann-like cells, have the potential to stimulate endothelial cell migration and tubule formation. Neuronally differentiated DPSCs secrete higher levels of the angiogenic factor VEGF-A, suggesting that DPSCs retain their angiogenic ability even after differentiating into Schwann cells. However, the underlying mechanism by which DPSCs-derived Schwann cells promote endothelial cell proliferation requires further investigation. These findings collectively indicate the ability of DPSCs to differentiate into Schwann cells, making them potential candidates for tissue engineering-based approaches for treating nerve injuries. Lambrichts *et al*[103] showed that hDPSCs-derived Schwann cells could form myelin sheaths and dorsal root ganglia. Similarly, Carnevale *et al*[12] discovered that STRO-1+/c-Kit+/CD34+ hDPSCs, which originate from the neural ridge, promote axonal regeneration in an animal model of PNI and express S100b, a typical marker of Schwann cells. The differentiation of DPSCs into Schwann cells thus is a potential strategy for nerve regeneration, and the refinement of differentiation protocols and materials remains an important area of investigation.

Differentiation into oligodendrocytes

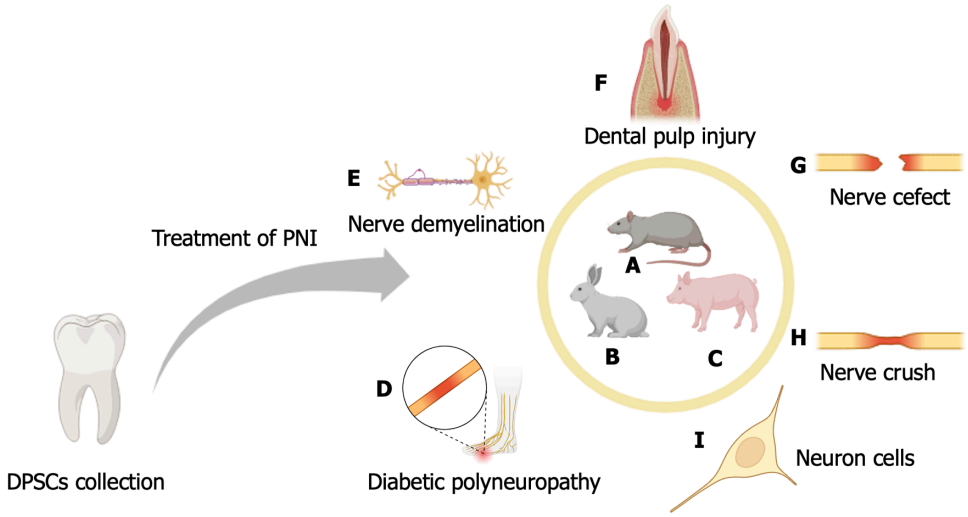
The primary function of oligodendrocytes is to form myelin, creating intricate connections between neurons in the nervous system. The transcription factor OLIG-2 and proteoglycan NG2 are markers of oligodendrocyte progenitor cells (OPCs)[104]. In a previous study, Sakai *et al*[105] transplanted SHEDs into rats with spinal cord transection and found that these cells exerted promising effects in promoting axon regeneration. This effect was achieved through the inhibition of various axon growth inhibitor signals and the differentiation of the transplanted cells into oligodendrocytes to replace damaged cells. Bagheri-Hosseinabadi *et al*[106] successfully induced DPSCs to differentiate into oligodendrocytes using cerebrospinal fluid and retinoic acid and obtained cells with a fibroblastic morphology and high adherence potential. Moreover, Askari *et al*[107] successfully induced DPSCs to differentiate into OPCs by transfecting them with a virus carrying the human Orig2 gene. The differentiated oligodendrocytes displayed a typical morphology and expressed neural markers such as GFAP, oligodendrocyte lineage transcription factor 2, and MBP. Subsequently, they applied transplanted oligodendrocytes into sciatic nerve demyelination model mice and demonstrated their effectiveness in nerve repair[108]. The nerve regeneration potential of DPSCs has been substantiated through various studies, offering hope for the treatment of peripheral nerve demyelination.

Differentiation into endothelial cells

Adequate blood supply is crucial for the survival of stem cells and nerve regeneration following injury. When MSCs differentiate into Schwann cells, they increase the secretion of angiogenic factors, including angiopoietin-1 and VEGF-A [109]. DPSCs can increase the migration of endothelial cells and promote angiogenesis *in vitro* and *in vivo*[110]. Sanen *et al* [102] also confirmed this phenomenon. Moreover, research has demonstrated that stem cells can directly differentiate into endothelial cells. In a rat facial nerve defect model, it was observed that DPSCs can directly differentiate into RECA1-positive endothelial cells, promoting nerve regeneration by increasing blood supply[91]. DPSCs were found lining the blood vessel wall of newly formed braided bone, indicating that angiogenesis occurred *in vitro*. Osteoblasts and endothelial cells were found after transplantation, and ultimately, bone-containing blood vessels were produced. Flk-1 is very important for the coupling of osteogenesis and angiogenesis. d'Aquino *et al*[69] proved that DPSCs can differentiate into Flk-1+/STRO-1+/CD44+/CD54+ endothelial progenitor cells. Sasaki *et al*[91] found that regenerated nerves contained S100-positive Schwann cells and RECA1-positive endothelial cells derived from dental pulp 14 d after DPSCs transplantation into rats with facial nerve defect. Newborn blood vessels are composed of endothelial cells from both recipient and donor sources. It has been suggested that DPSCs can differentiate into nerve cells and endothelial cells at the same time to promote nerve and vascular regeneration to treat PNI[91]. Subsequently, Maraldi *et al*[71] also proved that transplanted DPSCs can differentiate into endothelial cells *in vivo*. The ability of DPSCs to differentiate into endothelial cells further increases their potential for nerve regeneration.

Paracrine action

PNI triggers the dedifferentiation of Schwann cells and induces the formation and secretion of protogranules, which play a crucial role in nerve repair and the promotion of axonal growth[111]. Yamamoto *et al*[112] discovered that mobilized DPSCs (MDPSCs) treated with a granulocyte-colony stimulating factor (G-CSF) gradient express a variety of NTFs. These



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Figure 4 Main models used to study the effect of dental pulp stem cells in treating peripheral nerve injury at present. A: Rats; B: Rabbits; C: Pigs. D: Diabetic neuropathy; E: Nerve demyelination injury; F: Dental pulp injury; G: Nerve defect injury; H: Nerve crush injury; I: Nerve cells cultured *in vitro* are used. DPSC: Dental pulp stem cell. The figure was created with BioRender.com.

NTFs not only stimulate Schwann cells but also regulate their apoptosis and proliferation. Additionally, the differentiation of hDPSCs into Schwann cells increases the expression of glial markers and the secretion of NTFs, including BDNF, GDNF, NGF, NTF3, ANGPT1, and VEGFA[45,76]. Furthermore, linearly arranged dental pulp cell slices have been shown to guide and support axonal regeneration, and the abundant NTFs produced by the cells were found to make a significant contribution to this phenomenon[113]. Implantation of DPSCs into the vitreous body can effectively treat retinal ganglion cell injuries in adult rats, with the secretion of NTFs being a critical factor. The neuroprotective effect of DPSCs is weakened when K252a and Trk are blocked, indicating the importance of the NTFs NGF, BDNF, and NT-3 in this process[58]. In the repair of sciatic nerve crush injury in rat models, transplanted dental pulp cells may secrete NTFs [114]. In a rat model of sciatic nerve injury with a 10 mm defect, DPSCs were found to exert their effects on Schwann cells through paracrine signaling, leading to significant promotion of axonal regeneration[45]. The strong paracrine effect of DPSCs, coupled with their potential for nerve regeneration, makes them promising candidates for nerve tissue engineering.

CELL-FREE THERAPEUTIC ALTERNATIVES INVOLVING DPSCS

An increasing number of studies have demonstrated that odontogenic stem cells treat nerve injury through paracrine mechanisms[105]. NTFs, such as NGF, BDNF, and GDNF, can stimulate axonal growth, making DPSCs potential candidates for acellular therapy[115,116]. Kumar *et al*[117] showed that DPSCs secrete high levels of cytokines such as G-CSF, interferon gamma, and transforming growth factor (TGF)-β, which promote nerve differentiation and axonal growth. Unlike some other stem cell types, DPSCs demonstrate strong potential for nerve regeneration, supporting the use of their secreted factors for exocrine therapy for PNI. Kanada *et al*[118] found that both DPSCs and secreted factors from DPSCs (DPSCs-SFs) exerted therapeutic effects in a rat model of diabetic polyneuropathy. The effects included increases in sciatic nerve motor/sensory nerve conduction velocity and sciatic nerve blood flow. DPSCs-SFs were found to include angiogenic, neurotrophic, and immunomodulatory proteins. However, DPSCs transplantation may provide benefits over a longer duration of time[118].

With the continuous development of research on DPSCs-derived exosomes (DPSCs-Exos), DPSCs-Exos have been demonstrated to exert potential therapeutic effects in various diseases. They have shown promise for the treatment of periodontitis[119], Parkinson's disease[120], SCI[121], degenerative diseases[122], and cerebral ischemia[123] and in achieving pulp regeneration[124]. DPSCs-Exos have garnered significant attention due to their immunomodulatory properties[125], ability to promote angiogenesis[126], inhibitory effect on inflammation[127], and marked neuroregenerative and neuroprotective effects[128]. Studies have revealed that DPSCs-Exos can increase the formation of SH-SY5Y cell axons, leading to improved neuronal ultrastructure and increased expression of neural markers[129]. Research on acellular therapies involving DPSCs is still relatively limited, and the therapeutic potential of DPSCs-Exos and DPSCs lysates requires further experimentation and validation. Progress has been made in the techniques used to extract exosomes and analyze their composition, but there is still much work to be done before their clinical application can be realized.

CM derived from D-MSCs has been shown to promote axonal growth[45]. Sultan *et al*[130] conducted a study on the neuroprotective effect of DPSCs and DPSCs-CM on isolated TGNCs for the first time. The results demonstrated that DPSCs-CM can increase neuronal survival and promote axonal growth through the action of various NTFs, including GDNF, BDNF, NT-3, and CNTF[130]. CM from human dental pulp cells was found to contain bone morphogenetic

protein 7, FGF7, insulin-like growth factor (IGF)-1, FGF4, growth hormone, and VEGF-D, all of which are related to nerve regeneration and protection, vascular regeneration, and osteogenesis. Furthermore, the addition of B-27 to CM was observed to enhance the promotion of axon growth[131]. DPSCs-CM has shown the ability to alleviate polyneuropathy in diabetic rats and reduce the number of macrophages in diseased peripheral nerves[132]. DPSCs-CM holds significant promise in the treatment of peripheral nerve injuries. However, further research is needed to explore the underlying mechanisms of nerve regeneration.

Neurotrophin, BDNF, GDNF, IGF, NGF, and VEGF were detected near transplanted DPSCs 14 d after transplantation. The secretion of vascular endothelial growth factors supports angiogenesis, thus promoting nerve regeneration[112]. BM-MSCs can also secrete NTFs such as BDNF, b-FGF, and CNTF to promote peripheral nerve regeneration and thus treat peripheral nerve defects[133]. G-CSF-MDPSCs express higher levels of granulocyte-macrophage CSF, matrix metalloproteinase 3, VEGF, and NGF than BM-MSCs. The effects of G-CSF-MDPSCs in angiogenesis, neurite extension, and migration and their anti-apoptotic effects were found to be stronger than those of BM-MSCs in the same environment [134]. Compared with medium conditioned by CD31- cells derived from bone marrow and fat, medium conditioned by CD31- cells from pulp results in higher levels of angiogenesis/NTFs and exerts stronger angiogenic and neurogenic effects[135]. Kumar *et al*[117] also proved that DPSCs and their secreted factor may exert beneficial effects in treating neurological disorders and injuries. Exosomes derived from DPSCs can inhibit the differentiation of CD4+ T cells into helper T cells 17 (Th17), reduce the secretion of the proinflammatory factors interleukin (IL)-17 and tumor necrosis factor-alpha (TNF-α), promote the polarization of CD4+ T cells into regulatory T cells, and increase the release of the anti-inflammatory factors IL-10 and TGF-β. Compared with BM-derived exosomes, exosomes derived from DPSCs have a stronger immunoregulatory effect. In CD4+ T cells stimulated by exosomes derived from DPSCs, the expression levels of IL-10 and TGF-β mRNA were found to be the highest, while the transcription levels of IL-17 and TNF-α were found to be the lowest. Exosomes derived from DPSCs have stronger anti-inflammatory effects than exosomes derived from BM-MSCs[125]. Exosomes derived from DPSCs/BM-MSCs significantly decrease the activity of caspase3/7 and exert a significant antiapoptotic effect to play a neuroprotective role by upregulating endogenous expression of neuronal survival factors. Specifically, the cell survival-related PI3K-Bcl-2 pathway protects hippocampal neurons from excitotoxicity. Exosomes derived from DPSCs have stronger antiapoptotic and anti-visceral necrosis effects than exosomes derived from bone marrow stem cells[122]. Interestingly, the factors secreted by DPSCs are different in different environments[136]. There is no doubt that the neuroprotective effect of DPSCs-Exos is stronger than that of BM-MSC-derived exosomes. However, for clinical application, it is necessary to better determine the optimal composition, dosage, and culture conditions of exosomes[121].

CONCLUSION

PNI is a prevalent clinical issue that often leads to long-term pain in patients. Recent research has demonstrated the beneficial effects of Schwann cells on axonal regeneration and functional recovery after injury[137]. However, isolating and cultivating Schwann cells are challenging due to their limited availability and low proliferation rate[138]. Tissue engineering strategies involving nerve grafts consisting of physical scaffolds combined with supporting cells and/or growth factors or other biomolecules are promising approaches for treating PNI[139].

DPSCs may occupy the injury site and exert immunomodulatory effects, participate in paracrine signaling, and directly differentiate into relevant cell types, thus promoting the repair and regeneration of diseased and injured tissues. To promote the application of DPSCs and their products in nerve regeneration in the future, clinical strategies and various administration methods are being studied on a large scale[140]. A study revealed that inducing DPSCs to differentiate into oligoprogenitor cells is a potential treatment strategy for neurodegenerative diseases[141]. DPSCs have the multilineage differentiation potential and can differentiate into neurotrophoblasts. DPSCs-CM and DPSCs-Exos contain rich NTFs, and DPSCs-Exos can also cross the blood-brain barrier. In addition, DPSCs have the ability to reduce inflammation, promote axonal growth, and resist apoptosis. They have great potential in the treatment of PNI[142].

Previous *in vivo* studies have focused on the sciatic nerve and facial nerve of rats and analyzed the behavioral and pathological manifestations of rats with sciatic and facial nerve injury, but electrophysiological studies are lacking. Moreover, it is also necessary to compare therapeutic effects among different models to identify the optimal application of DPSCs. However, there are still some questions to be answered. Do DPSCs have the same effect on nerve crush injury and amputation injury? Do different treatment methods have different effects on different models. Which is better, cell therapy or acellular therapy? What are the key factors in the neuroprotective and regenerative abilities of acellular therapies? In addition, researchers should pay more attention to the function of differentiated nerve cells *in vitro*. Furthermore, organoids may become a hot research topic in the future. A DPSCs cell bank is also urgently needed[143]. It is believed that DPSCs will bring hope to patients with PNI soon.

The unique biological characteristics of DPSCs make them significant cell sources for the treatment of PNI. DPSCs exhibit neurogenic potential, are easily accessible, exhibit pluripotency, and can be preserved for extended periods, making them excellent candidates for tissue engineering applications. DPSCs (Figure 4) can be utilized directly or in combination with nerve conduits or hydrogels or used after differentiation into nerve-like cells to treat PNI. Moreover, DPSCs lysates, DPSCs-Exos, and DPSCs-CM all hold promise in the treatment of PNI. However, overcoming safety and ethical problems and avoiding tumor formation are still the keys for translating DPSCs for clinical use[144].

This review focused on the therapeutic potential of DPSCs in treating PNI, elaborating on the neuroprotective and regenerative capabilities of DPSCs through both cellular and acellular approaches. DPSCs hold significant promise for the treatment of PNI, and it is anticipated that they will play a crucial role in the clinical treatment of PNI in the future. As

research on the effect of DPSCs on PNI continues to progress, more efficient and expedient treatment protocols are expected to be developed. However, the safety of cell therapies and the efficacy of acellular therapies in treating long-gap injuries require thorough evaluation. The search for convenient and effective treatment strategies involving DPSCs and their products for PNI remains ongoing. Furthermore, the underlying mechanisms and key components of DPSCs in peripheral nerve treatment warrant further investigation. As acellular therapies are novel stem cell therapies, there is a need to refine the purification process of cell materials and establish a standardized and effective cell production plan. Comparative studies assessing the safety, efficacy, and production costs of various therapies are also essential.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

MicroRNA-584-5p/RUNX family transcription factor 2 axis mediates hypoxia-induced osteogenic differentiation of periosteal stem cells

Jia-Jia Lu, Xiao-Jian Shi, Qiang Fu, Yong-Chuan Li, Lei Zhu, Nan Lu

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Abstract

BACKGROUND

The hypoxic environment during bone healing is important in regulating the differentiation of periosteal stem cells (PSCs) into osteoblasts or chondrocytes; however, the underlying mechanisms remain unclear.

AIM

To determine the effect of hypoxia on PSCs, and the expression of microRNA-584-5p (miR-584-5p) and RUNX family transcription factor 2 (RUNX2) in PSCs was modulated to explore the impact of the miR-584-5p/RUNX2 axis on hypoxia-induced osteogenic differentiation of PSCs.

METHODS

In this study, we isolated primary mouse PSCs and stimulated them with hypoxia, and the characteristics and functional genes related to PSC osteogenic differentiation were assessed. Constructs expressing miR-584-5p and RUNX2 were established to determine PSC osteogenic differentiation.

RESULTS

Hypoxic stimulation induced PSC osteogenic differentiation and significantly increased calcified nodules, intracellular calcium ion levels, and alkaline phosphatase (ALP) activity in PSCs. Osteogenic differentiation-related factors such as RUNX2, bone morphogenetic protein 2, hypoxia-inducible factor 1-alpha, and ALP were upregulated; in contrast, miR-584-5p was downregulated in these cells. Furthermore, upregulation of miR-584-5p significantly inhibited RUNX2

expression and hypoxia-induced PSC osteogenic differentiation. RUNX2 was the target gene of miR-584-5p, antagonizing miR-584-5p inhibition in hypoxia-induced PSC osteogenic differentiation.

CONCLUSION
Our study showed that the interaction of miR-584-5p and RUNX2 could mediate PSC osteogenic differentiation induced by hypoxia.

Key Words: Periosteal stem cell; Osteogenic differentiation; RUNX family transcription factor 2; MiroRNA-584-5p

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Core Tip: This study simulated the hypoxic environment of periosteal stem cells (PSCs) during fracture and examined the related cellular responses to the hypoxia-induced PSC osteogenic differentiation. Importantly, the microRNA-584-5p/RUNX family transcription factor 2 axis was determined to be one of the regulating mechanisms for the hypoxia-induced PSC osteogenic differentiations.

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INTRODUCTION

The periosteum is a special connective tissue membrane that covers the surface of bone. For a long time, the types of cells that mediate osteogenic/chondrogenic differentiation at the periosteum have been unknown. The lack of generated periosteal cells following periosteal-deficient periosteal injury suggests a certain type of stem cell within the periosteum [1]. Periosteal stem cells (PSCs) were identified for the first time by Debnath *et al*[2]. PCS were extracted and identified from long bones and calvarial bones in mice, confirming the multifunctional differentiation potential of these cells in inducing the formation of tissues/organs, such as bones, cartilage, and fat. PSCs are essential for successful bone healing and provide new research ideas and directions for bone repair and maintenance since they are the main source of osteoblasts (OBs) and chondrocytes at the periosteum[3,4]. Since fracture healing is accompanied by obvious tissue hypoxia, it is worth exploring the effects of hypoxia on PSC osteogenic differentiation[5].

Noncoding RNAs are considered important biomolecules modulating cellular osteogenic differentiation[6], and microRNAs (miRNAs) are some of the most common noncoding RNAs that epigenetically modulate osteogenesis-related gene expression. Generally, aberrant miRNA expression is important in regulating stem cell differentiation into OBs/cartilage and is involved in the progression of bone diseases[7]. Based on the Gene Expression Omnibus database, a bioinformatics study[8] screened out the signature miRNAs in periodontal stem cells. MicroRNA-584-5p (miR-584-5p) was shown to be differentially expressed and inhibited during the osteogenic differentiation of periodontal stem cells. Notably, miR-584-5p might be associated with PSC osteogenic differentiation.

RUNX family transcription factor 2 (RUNX2) is a key regulatory factor for osteogenic differentiation[9]. This molecule can modulate the expression of osteogenic markers in cells by binding the functional elements of osteogenesis-related genes, regulating osteogenic differentiation[10]. RUNX2 mutation or deficiency inhibits cell differentiation into OBs, resulting in osteogenic defects[11]. Most studies have revealed the osteogenic activity of RUNX2 in bone marrow-derived mesenchymal stem cells, but its involvement in the osteogenic differentiation pathway of PSCs remains to be clarified.

This study aimed to determine the effect of hypoxia on PSCs, and the expression of miR-584-5p and RUNX2 in PSCs was modulated to explore the impact of the miR-584-5p/RUNX2 axis on hypoxia-induced osteogenic differentiation of PSCs. The findings of this study could provide new mechanistic insights into the regulation of PSC osteogenic differentiation induced by hypoxia.

MATERIALS AND METHODS

Cell isolation and experiments

PSCs were extracted from 7-d-old C57BL/6J mice (Beijing Vital River Laboratory Animal Technology). Mouse periosteal tissues were collected, chopped with a surgical blade, and digested with collagenase at 37 °C for 1 h. Then, Dulbecco's modified Eagle's medium with 2% fetal bovine serum was added to terminate digestion, and the digestive solution was centrifuged for supernatant removal. The pelleted cells were suspended in DNase solution and incubated for 5 min at 37 °C. The cells were filtered using a 70-μm cell filter, and the stem cells were induced by osteogenic differentiation medium. PSCs were incubated for 3 h under 2% O_2, 10% CO_2, and 88% N_2 to induce hypoxia; they were assigned and treated under

conditions including normoxia, hypoxia, hypoxia plus negative control (NC) mimic, miR-584-5p mimic, NC shRNA, sh-RUNX2, miR-584-5p plus mimic and pcDNA3.1, and miR-584-5p mimic plus pc-RUNX2.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity in PSCs was detected according to the ALP activity detection kit (P0321S, Beyotime, Shanghai, China). The procedure was as follows: PSCs were fully lysed with cell lysis buffer composed of 20 mmol/L Tris (pH = 7.5), 150 mmol/L NaCl, and 1% Triton X-100, and the supernatant was used for ALP activity detection after centrifugation. Fifty microliters of the supernatant samples was added to each well of 96-well plates, including blank controls, and the working solution was added for 5-10 min of cultivation at 37 °C. After incubation, 100 μL of stop buffer was added to each well to terminate the reaction. A microplate reader (Thermo Fisher, United States) was used to measure the absorbance at 405 nm, and ALP activity was calculated using the standard curve set up in the assay.

Alizarin red S staining

This study determined calcified nodule formation in cells using an Alizarin red S (ARS) staining kit (C0148S, Beyotime, Shanghai, China). The cells were carefully washed with phosphate buffered saline (PBS) solution once after osteogenic differentiation induction of PSCs, incubated with a fixative for 20 min, and then washed with PBS solution three times. After addition of ARS dye for 30 min of cultivation, the cells were thoroughly rinsed with distilled water. Calcified nodule formation was observed using an optical microscope (Olympus, Japan). ARS chelated Ca^{2+} to form orange-red complexes in this experiment.

Characterization of intracellular calcium ions

The calcium ion level in PSCs was measured with a calcium-content chromogenic detection kit (S1063S, Beyotime, Shanghai, China). A lysis buffer with a volume of 100 μL was added to the cell sample for thorough lysis. Then, the lysate was centrifuged (10000 × *g*, 4 °C, 3-5 min) to obtain supernatants for calcium ion characterization. The standard curve and test working solution were prepared following the manufacturer's instructions. In a 96-well plate, the supernatant sample and 150 μL of working solution were mixed, added at 50 μL per well and incubated for 5-10 min at room temperature in the dark. The color reaction was detected at an absorbance OD of 575 nm, and the calcium ion content was calculated based on the standard curve.

Bioinformatics prediction and dual luciferase reporter gene assay

StarBase was used to predict the potential binding sites between miR-584-5p and RUNX2 mRNA, and the dual luciferase reporter (DLR) gene system was used to verify the targeting relationship between them. First, the targeted wild-type (wt) and mutant fragments of RUNX2 were cloned and inserted into pGL4 plasmids (upstream of the firefly reporter gene), yielding RUNX2-wt and RUNX2-mutant (mut), respectively, based on the predicted locus sequence information. MiR-584-5p and NC mimics were transfected into cells by the liposome method. Each constructed main reporter gene vector was then cotransfected with the Renilla reporter gene vector into cells. The firefly and Renilla luciferase activities were detected, and the relative luciferase activity (firefly/Renilla) was calculated.

Quantitative polymerase chain reaction

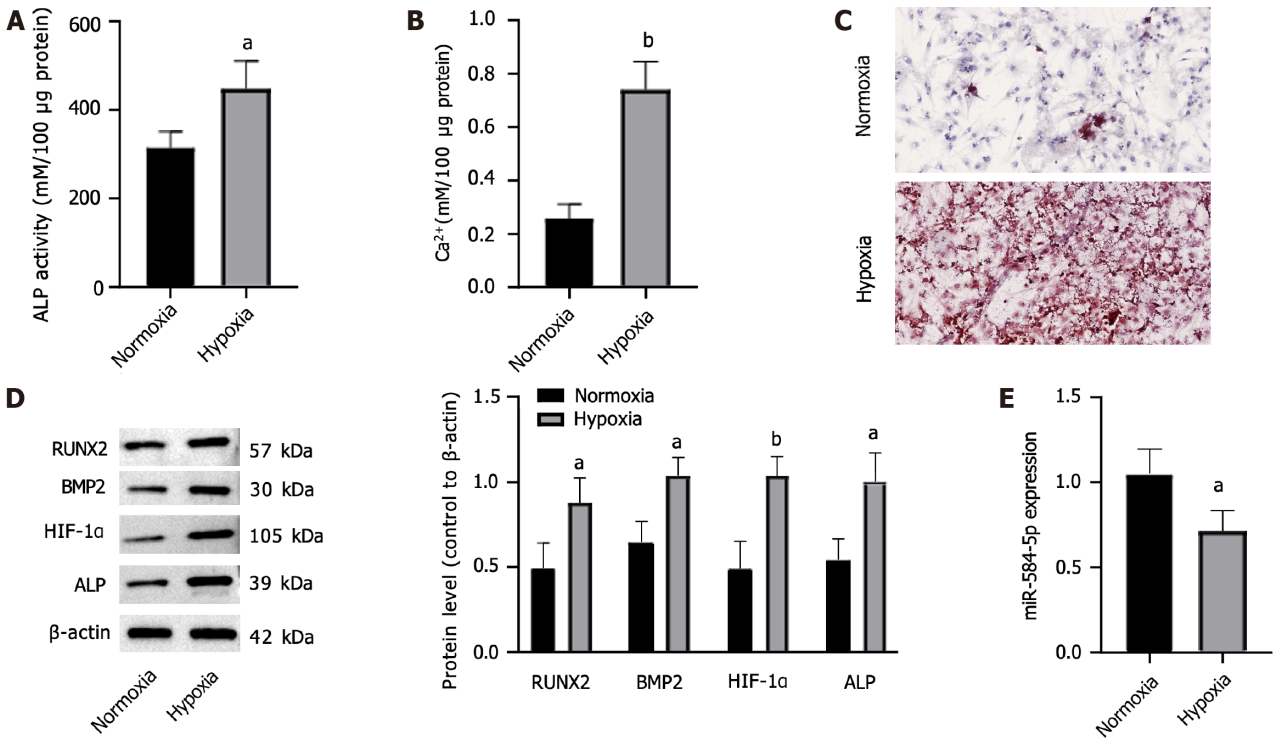
TRIzol solution-lysed cells were used to extract total RNA. RNA was reverse-transcribed into cDNA using a reverse transcription-fluorescence quantitative polymerase chain reaction (qPCR) kit (Tiangen, Beijing, China) and PCR apparatus (Applied Biosystems, United States) for real-time fluorescence quantification (qPCR) after determining the purity of the RNA samples. The miR-584-5p sense strand primer was 5′-CCGTTATGGTTTGCCTGGG-3′, and the miR-584-5p antisense strand primer was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCAGT-3′. The RUNX2 sense strand primer was 5′-AGATGGGACTGTGGTTACCG-3′, and the RUNX2 antisense strand primer was 5′-GGACCGTCCACTGTCACTTT-3′. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression, which was normalized to that of glyceraldehyde-3-phosphate dehydrogenase and U6.

Western blot

RIPA lysis buffer (Cell Signaling, United States) was used for cell lysis. After protein isolation, a BCA detection kit (Abcam, United States) was used to measure the protein concentration, and the loading amount of the protein samples was calculated. After sodium-dodecyl sulfate gel electrophoresis, the proteins were transferred to a nitrocellulose membrane (NCM; Cell Signaling, United States) with a wet membrane transfer instrument in an ice bath. Then, the NCM was sealed at room temperature with a sealing liquid containing 5% skim milk powder for 1 h. The NCM was incubated overnight at 4 °C with primary antibodies, including RUNX2 (ab236639, 1:1000, Abcam United States), bone morphogenetic protein 2 (BMP2) (ab284387, 1:1000), hypoxia-inducible factor 1-alpha (HIF-1α) (ab179483, 1:1000), ALP (ab229126, 1:2000), and b-actin antibodies (ab8226, 1:10000), and the corresponding secondary antibody (ab6721, 1:10000, Abcam, United States) was incubated at room temperature for 1 h. An enhanced chemiluminescence reagent was used to detect protein bands. Images of the protein bands were obtained by X-ray analysis, and the relative protein expression normalized to β-actin expression was calculated.

Statistics and analysis

The average of each variable was obtained, and the standard deviation was calculated after three repeated measurements. The independent sample *t* test and one-way analysis of variance were used to identify differences between groups. The statistical analysis was conducted with Statistical Package for the Social Sciences version 22.0 software, and the graph



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Figure 1 Hypoxia stimulation promotes the osteogenic differentiation of periosteal stem cells. A: Alkaline phosphatase activity; B: Intracellular calcium ion level; C: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; D: Osteogenic differentiation-related protein levels; E: MiRNA-584-5p expression in periosteal stem cells. One-way ANOVA and *t* tests were used to demonstrate differences between the hypoxia-induced and normal control groups. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; miRNA: MicroRNA.

data were processed by GraphPad version 9.0. A 95% confidence interval was used, and a *P* value of < 0.05 indicated statistical significance.

RESULTS

Hypoxia promotes PSC osteogenic differentiation

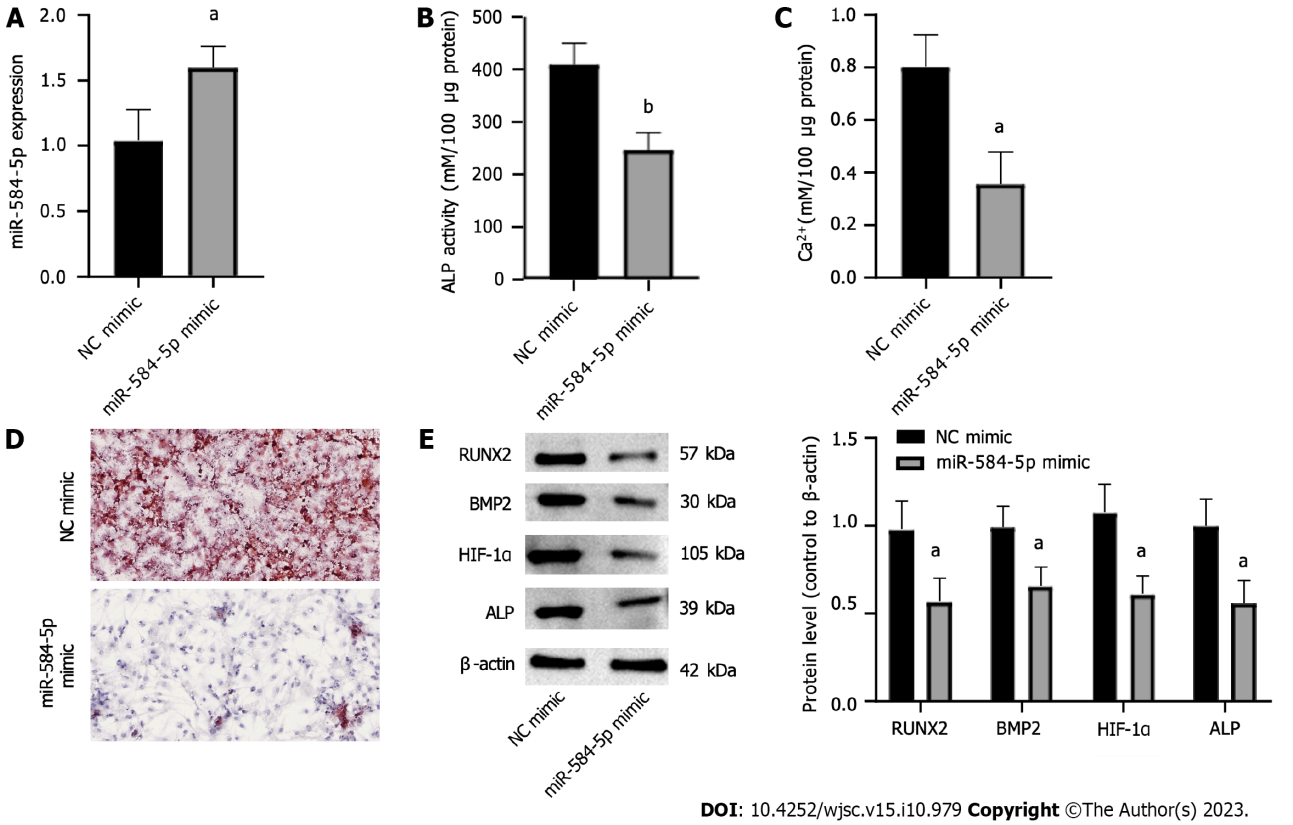
An osteogenic differentiation medium was used to induce PSCs, and normoxic/hypoxic atmospheres were created to observe the impact of hypoxia on PSC osteogenic differentiation. ALP activity, intracellular calcium levels, and the expression of osteogenic differentiation-related factors, including RUNX2, BMP2, HIF-1α, and ALP, were measured, and ARS staining of PSCs was performed. Hypoxia significantly enhanced ALP activity (Figure 1A), Ca^{2+} levels (Figure 1B), and calcified nodule formation (Figure 1C) compared with normoxia. Hypoxia treatment increased the RUNX2, BMP2, HIF-1α, and ALP protein levels (Figure 1D). In contrast, hypoxia significantly downregulated miR-584-5p in PSCs (Figure 1E). Therefore, hypoxia could promote PSC osteogenic differentiation while downregulating miR-584-5p expression.

MiR-584-5p upregulation affects hypoxia-induced PSC osteogenic differentiation

To investigate whether aberrant miR-584-5p expression was related to hypoxia-induced PSC osteogenic differentiation, we used a miR-584-5p mimic vector to upregulate miR-584-5p in PSCs, and the NC mimic was used as a control (Figure 2A). Furthermore, PSCs were divided into two groups, transfected with NC mimic and miR-584-5p mimic, and the cells were stimulated by hypoxia and induced to differentiate into OBs to observe the role of miR-584-5p in hypoxia-induced PSC osteogenic differentiation. The results showed that upregulation of miR-584-5p lowered ALP activity (Figure 2B) and intracellular calcium deposition (Figure 2C) while suppressing calcified nodule formation (Figure 2D). Moreover, miR-584-5p overexpression significantly decreased the expression of osteogenic differentiation genes, such as RUNX2, BMP2, HIF-1α, and ALP (Figure 2E). Therefore, upregulation of miR-584-5p can inhibit PSC osteoblastic differentiation induced by hypoxia.

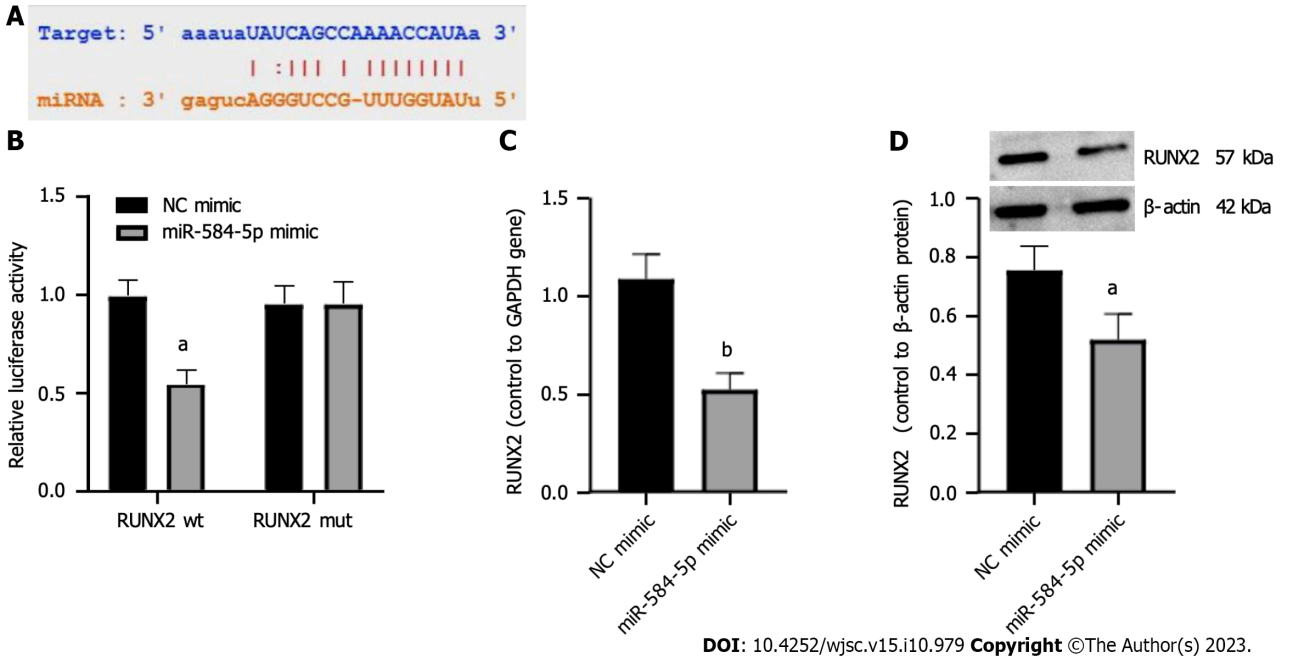
Targeted regulation of the RUNX2 axis by miR-584-5p

We hypothesized that miR-584-5p binds to a sequence fragment on the 3′ untranslated region of RUNX2 based on starBase (Figure 3A). The potential binding site was validated using a DLR gene assay. Relative luciferase activity was significantly reduced in the cells cotransfected with miR-584-5p mimic and RUNX2 wt but not in the cells with miR-584-



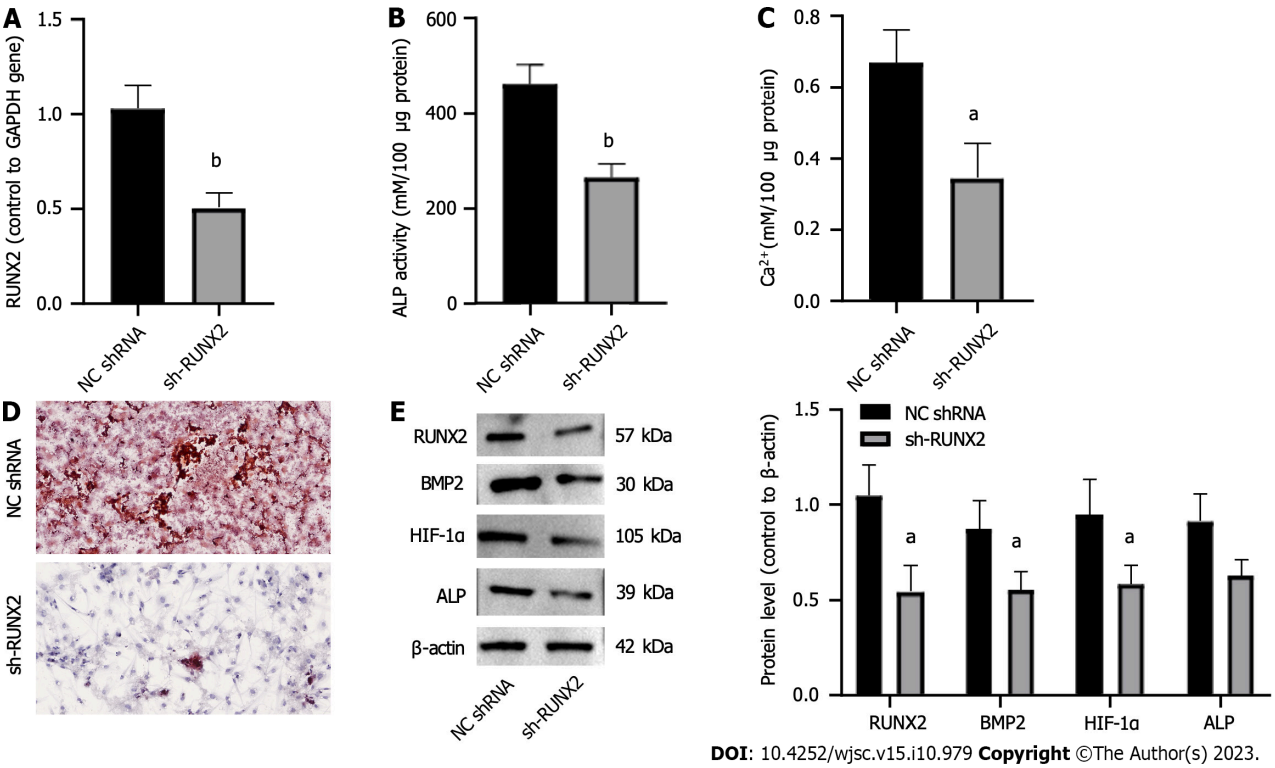
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Figure 2 Upregulation of miRNA-584-5P inhibits the osteogenic differentiation of periosteal stem cells induced by hypoxia. A: MiRNA-584-5p (miR584-5p) expression in stem cells; B: Alkaline phosphatase activity; C: Intracellular calcium ion level; D: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; E: Osteogenic differentiation-related protein expression. One-way ANOVA and *t* tests were used to demonstrate differences between the miR584-5p mimic and negative control groups. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; miRNA: MicroRNA; NC: Negative control.



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Figure 3 Prediction of the miRNA-584-5p target. A: Potential binding sites of miRNA-584-5p and the 3'-untranslated region of RUNX family transcription factor 2 (RUNX2); B: Dual luciferase reporter gene assay; C: Quantitative polymerase chain reaction determination of RUNX2 expression; D: Western blot determination of RUNX2 protein expression. One-way ANOVA and *t* tests were used to demonstrate differences in RUNX2 responses between the miR584-5p mimic and negative control groups. $^{a}P < 0.05$; $^{b}P < 0.01$. RUNX2: RUNX family transcription factor 2; NC: Negative control; wt: Wild type; mut: Mutant; miRNA: MicroRNA.



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Figure 4 Downregulation of RUNX family transcription factor 2 inhibits the osteogenic differentiation of periosteal stem cells induced by hypoxia. A: RUNX family transcription factor 2 (RUNX2) expression; B: Alkaline phosphatase activity; C: Intracellular calcium ion level; D: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; E: Osteogenic differentiation-related protein expression. One-way ANOVA and *t* tests were used to demonstrate differences between the sh-RUNX2 and normal control shRNA groups. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; shRNA: Small hairpin RNA.

5p mimic cotransfected with RUNX2-mut (Figure 3B). Furthermore, upregulation of miR-584-5 suppressed RUNX2 expression at both the RNA level (Figure 3C) and the protein level (Figure 3D). These results indicated that miR-584-5p could regulate RUNX2 through the predicted binding site.

Impact of RUNX2 on hypoxia-induced PSC osteogenic differentiation

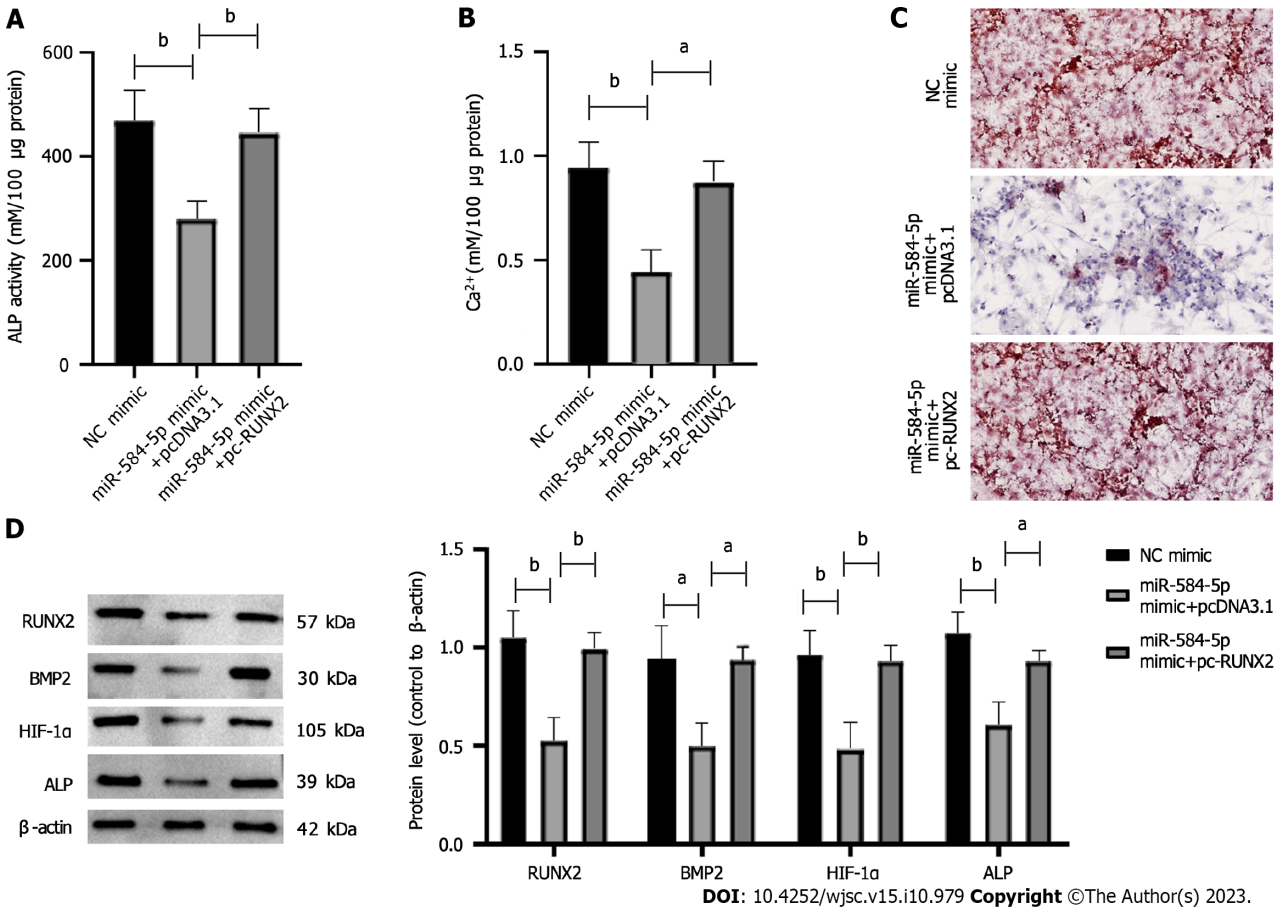
To investigate the role of miR-584-5p-induced RUNX2 downregulation in hypoxia-induced PSCs, we constructed a RUNX2 inhibitory expression vector (sh-RUNX2) and a negative control shRNA (NC shRNA) (Figure 4A). Downregulation of RUNX2 significantly decreased ALP activity (Figure 4B), calcium deposition (Figure 4C), and ARS staining of osteogenic differentiation (Figure 4D); in contrast, it suppressed RUNX2, BMP2, HIF-1α, and ALP expression (Figure 4E). Thus, downregulation of RUNX2 inhibited hypoxia-induced PSC osteogenic differentiation.

MiR-584-5p regulates hypoxia-induced PSC osteogenic differentiation by targeting RUNX2

To investigate whether miR-584-5p could mediate the RUNX2 response pathway in hypoxia-induced PSC osteogenic differentiation, we established gene constructs such as NC mimic, miR-584-5p in pcDNA3.1, and miR-584-5p mimic + pc-RUNX2 and transfected them into PSCs. The results showed that miR-584-5p significantly lowered ALP activity (Figure 5A) and intracellular calcium ion levels (Figure 5B) in hypoxia-induced PSCs, and it inhibited PSC osteogenic differentiation induced by hypoxia (Figure 5C). In contrast, upregulated RUNX2 partly rescued the inhibitory responses mediated by miR-584-5p (Figures 5A-C). Moreover, upregulation of RUNX2 significantly reversed the downregulation of osteogenic differentiation-related proteins induced by miR584-5p overexpression (Figure 5D). Thus, the upregulation of RUNX2 specifically reversed the inhibitory effect of miR-584-5p overexpression on hypoxia-induced PSC osteogenic differentiation.

DISCUSSION

PSCs have a strong differentiation potential. Bone defects can activate PSCs through a series of signal transduction pathways and induce cell migration, proliferation, and differentiation into OBs/chondrocytes at the injury site[12]. However, the mechanisms underlying PSC osteogenic differentiation are poorly understood. This study simulated the hypoxic environment of PSCs during fracture and examined the related cellular responses to hypoxia-induced PSC osteogenic differentiation. Importantly, the miR-584-5p/RUNX2 axis was determined to be one of the regulatory mechanisms for hypoxia-induced PSC osteogenic differentiation.



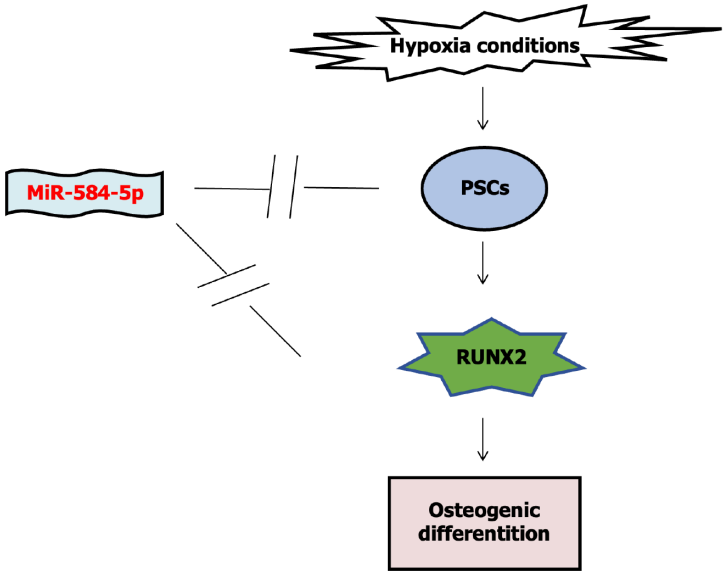
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Figure 5 MiRNA-584-5p regulates hypoxia-induced osteogenic differentiation of periosteal stem cells by targeting RUNX family transcription factor 2. A: Alkaline phosphatase activity; B: Intracellular calcium ion level; C: Alizarin red S staining chelated Ca^{2+} form an orange-red complex; D: Osteogenic differentiation-related protein expression. One-way ANOVA and *t* tests were used to demonstrate differences between the miR-581-5p mimic + pcDNA3.1-, NC mimic-, and miR-584-59 mimic + pc-RUNX family transcription factor 2-expressing constructs. $^{a}P < 0.05$; $^{b}P < 0.01$. ALP: Alkaline phosphatase; RUNX2: RUNX family transcription factor 2; BMP2: Bone morphogenetic protein 2; HIF-1α: Hypoxia-inducible factor 1; miR: MicroRNA; NC: Negative control.

Fracture healing is a complex process in which HIFs activate the osteogenic differentiation of precursor cells in response to the hypoxic environment where the fracture occurs[13]. Hypoxia is one of the main functional mechanisms of PSC osteogenic differentiation. One study emphasized that hypoxia upregulated HIF expression in PSCs, affecting the periosteal protein-mediated osteogenic differentiation pathway[14]. Similarly, we confirmed that hypoxia could activate PSC osteogenic differentiation and upregulate osteogenic differentiation-associated genes, such as RUNX2, causing cell mineralization. We also found that RUNX2 overexpression could increase osteogenic differentiation (Figure 5C). Thus, hypoxia is important for PSC osteogenic differentiation, and adjusting the oxygen concentration could induce PSC osteogenic differentiation *in vitro*.

Furthermore, we revealed that miR-584-5p was downregulated in hypoxia-induced PSCs. MiRNAs are key regulators of osteogenic differentiation and are involved in the expression of many genes in mesenchymal stem cells[15]. MiR-584-5p seems to be an important factor in bone healing and can mediate osteogenic differentiation by regulating gene expression[16-19]. Furthermore, miR-584-5p is a vital regulatory factor for osteosarcoma cell proliferation and bone homeostasis[20]. Therefore, miR-584-5p expression might be related to bone homeostasis. The significant downregulation of miR-584-5p in PSCs suggests its potential role in osteogenic differentiation. In this study, the upregulation of miR-584-5p significantly downregulated the expression of osteogenic differentiation genes such as RUNX2. This change inhibited ALP activity while lowering the degree of cell mineralization, indicating the ability of miR-584-5p upregulation to suppress PSC osteogenic differentiation. Notably, miR-584-5p plays important functions in mediating PSC osteogenic differentiation.

The biological functions of miRNAs include regulation of gene stability by binding mRNAs of downstream target genes[21]. We initially identified a sequence fragment of RUNX2 mRNA that could bind to miR-584-5p and then confirmed a binding site between miR-584-5p and RUNX2 through the DLR gene assay. We also confirmed that miR-584-5p could directly inhibit RUNX2 functions. RUNX2 is necessary for stem cell osteogenic differentiation[22]. The RUNX2 protein mediates the expression of many downstream genes, contributing to the OB phenotype[23]. RUNX2 upregulation also neutralized the regulatory effect of miR-584-5p overexpression on PSC osteogenic differentiation in our study. Therefore, miR-584-5p can affect hypoxia-induced PSC osteoblastic differentiation by regulating the RUNX2 functional pathway.



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Figure 6 The interregulation of microRNA-584-5p/RUNX family transcription factor 2 on hypoxia-induced periosteal stem cell osteogenic differentiation. The hypoxic environment induces periosteal stem cells to undergo osteogenic differentiation by activating the RUNX family transcription factor 2 (RUNX2)-related pathway; however, miR-584-5p overexpression can inhibit osteogenic differentiation by downregulating RUNX2 expression. PSC: Periosteal stem cell.

However, this study still has limitations. Skeletal reprogramming to bone regeneration requires reactivating key transcription and growth factors. The local hypoxic microenvironment can regulate cytokine synthesis. The biological performance of stem cells under hypoxia can be different from that under normoxic conditions. Thus, other key factors in the process of hypoxia-induced PSC osteogenic differentiation still need to be discovered and studied.

CONCLUSION

We revealed that miR-584-5p inhibited hypoxia-induced PSC osteogenic differentiation *via* RUNX2 (Figure 6). PSCs have good osteogenic/cartilage differentiation potential. Our findings provide a mechanism for PSC osteogenic differentiation, and modulating miR-584-5p and miR-584-5p/RUNX2 might be a new strategy for bone repair and regeneration.

ARTICLE HIGHLIGHTS

Research background

Fracture healing is accompanied by obvious tissue hypoxia, it is worth exploring the effects of hypoxia on periosteal stem cells (PSCs) osteogenic differentiation. However, the underlying mechanisms needs to be clarified.

Research motivation

RUNX family transcription factor 2 (RUNX2) is a key regulator of osteogenic differentiation, and studies have found that RUNX2 has osteogenic activity in bone marrow mesenchymal stem cells, but its role in the osteogenic differentiation pathway of PSCs is still unclear.

Research objectives

This study aimed to determine the effect of hypoxia on PSCs, and the expression of microRNA-584-5p (miR-584-5p) and RUNX family transcription factor 2 in PSCs was modulated to explore the impact of the miR-584-5p/RUNX2 axis on hypoxia-induced osteogenic differentiation of PSCs.

Research methods

PSCs were extracted from 7-d-old C57BL/6J mice. Then, PSCs were stimulated them with hypoxia, and the characteristics and functional genes related to PSC osteogenic differentiation were measured.

Research results

Hypoxia promotes PSC osteogenic differentiation. Also, miR-584-5p upregulation affects hypoxia-induced PSC osteogenic differentiation and RUNX2 axis was targeted by miR-584-59. The upregulation of RUNX2 specifically reversed

the inhibitory effect of miR-584-5p overexpression on the hypoxia-induced PSC osteogenic differentiation.

Research conclusions

MiR-584-5p inhibited the hypoxia-induced PSC osteogenic differentiation *via* RUNX2.

Research perspectives

MiR-584-5p inhibits hypoxia-induced osteogenic differentiation of PSC through RUNX2, and PSC has good osteogenic/chondrogenic differentiation potential. These results indicate that miR-584-5p/RUNX2 axis may be a key target pathway for bone repair and regeneration.

FOOTNOTES

Co-corresponding authors: Lei Zhu and Nan Lu.

Author contributions: Lu JJ, Shi XJ, Fu Q, Li YC, and Zhu L contributed equally to this work. Lu JJ, Shi XJ, Fu Q, Li YC, and Zhu L designed the research study, contributed new reagents and analytic tools, analyzed the data, and wrote the manuscript; Lu JJ, Shi XJ, Fu Q, Li YC, Zhu L, and Lu N performed the research; and all authors have read and approve the final manuscript. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper. This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Zhu L and Lu N contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study. In summary, we believe that designating Zhu L and Lu N as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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MINIREVIEWS

How to enhance the ability of mesenchymal stem cells to alleviate intervertebral disc degeneration

Qing-Xiang Zhang, Min Cui

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Abstract

Intervertebral disc (ID) degeneration (IDD) is one of the main causes of chronic low back pain, and degenerative lesions are usually caused by an imbalance between catabolic and anabolic processes in the ID. The environment in which the ID is located is harsh, with almost no vascular distribution within the disc, and the nutrient supply relies mainly on the diffusion of oxygen and nutrients from the blood vessels located under the endplate. The stability of its internal environment also plays an important role in preventing IDD. The main feature of disc degeneration is a decrease in the number of cells. Mesenchymal stem cells have been used in the treatment of disc lesions due to their ability to differentiate into nucleus pulposus cells in a nonspecific anti-inflammatory manner. The main purpose is to promote their regeneration. The current aim of stem cell therapy is to replace the aged and metamorphosed cells in the ID and to increase the content of the extracellular matrix. The treatment of disc degeneration with stem cells has achieved good efficacy, and the current challenge is how to improve this efficacy. Here, we reviewed current treatments for disc degeneration and summarize studies on stem cell vesicles, enhancement of therapeutic effects when stem cells are mixed with related substances, and improvements in the efficacy of stem cell therapy by adjuvants under adverse conditions. We reviewed the new approaches and ideas for stem cell treatment of disc degeneration in order to contribute to the development of new therapeutic approaches to meet current challenges.

Key Words: Mesenchymal stem cells; Intervertebral disc degeneration; Extracellular vesicles; Nucleus pulposus cells; Tissue regeneration

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Core Tip: Mesenchymal stem cells have a strong self-renewal capacity and multidirectional differentiation potential, and their secreted vesicles promote regeneration of myeloid cells, increase extracellular matrix production, and alleviate inflammatory status. We reviewed the current relevant targets of stem cell exosomes for the treatment of intervertebral discs and the adjuvant tools used in conjunction with stem cell therapy. This will help to improve the therapeutic efficacy of stem cells and their exosomes, which will also contribute to development of more efficient treatment strategies and approaches for the restoration of disc degeneration.

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INTRODUCTION

Intervertebral discs (IDs) have a complex structure with a unique internal environment. They contain nucleus pulposus cells, fibrous rings, and extracellular matrix (ECM)[1], Mesenchymal stem cells (MSCs), a class of pluripotent stem cells with the capacity for self-renewal and the ability to differentiate into a variety of tissues in vitro, were first mentioned in 1970 in guinea pig bone marrow[2]. Isolation and culture of MSCs from human bone marrow was first described in 1992. Since then, MSCs have been isolated and cultured from different human tissues, such as fat, amniotic membrane, gingiva, thymus, and placenta. MSCs from different sources differ in phenotype and function[3], for example, in solving the problem of ID withdrawal, umbilical cord-derived MSCs have a greater capacity for cell proliferation and osteogenesis than bone marrow-derived MSCs[4]. Stem cell therapy is designed to restore this balance of secreting exosomes and vesicles, mixing other substances to promote their differentiation into nucleus pulposus cells, regulating the content of the ECM, and when treated accordingly resisting interference by the harsh environment of the IDs[5,6]. These synergistic approaches provide new possibilities for stem cell therapy of (IDD)[7,8].

MIRNA IN EXOSOMES

For signal transduction pathways, many miRNAs play a crucial role in the growth and development of IDs. MSCs deliver miRNA-31 to the nucleus pulposus and upregulate the Wnt/β-catenin pathway to inhibit apoptosis of nucleus pulposus cells and regulate production of the ECM[9]. Similarly, under the stimulation of external effects, miRNA-21 can be transfected into mesenchymal cell exosomes, enhancing the ability of MSCs to differentiate into osteoblasts and promote vascular regeneration[10]. In other related studies, exosomes derived from bone marrow MSCs inhibited apoptosis and inflammation by upregulating autophagy through the protein kinase β/mammalian target of rapamycin signaling pathway[11]. MSCs-derived exosomes can also inhibit apoptosis of nucleus pulposus cells through miRNA-532-5p transport, demonstrating that this transport is also effective in IDD therapy[12].

Exosome-delivered exogenous miRNA-26a-5p can also be delivered to IDs, as METTL14 is highly expressed in patients with IDD, and the level of NOD-like receptor family pyrin domain containing 3 regulated by it leads to an increase in proinflammatory factors and apoptosis of nucleus pulposus cells. Exogenous miRNA-26a-5p inhibits METTL4 expression and thus treats disc degeneration[13]. It has also been found that miRNA-15a in exosomes can participate in the protein balance regulated by the phosphatidylinositol 3-kinase/protein kinase β and Wnt3a/β-catenin axes of IDD and downregulate matrix metalloproteinase-3. Therefore, type II collagen and aggrecan levels can be increased, along with differentiation of MSCs to nucleus pulposus cells[14,15]. BTB-and-CNC homologue 1 is a transcription inhibitor of heme oxygenase 1, which activates autophagy in nucleus pulposus cells. miRNA-155 in exosomes inhibits BTB-and-CNC homologue 1 expression by binding to the 3′ untranslated region of this transcription inhibitor, thereby treating IDD[16] (Table 1).

SPECIAL GENETICS AND MSCsS

Microtubules are an important part of the cytoskeleton that can regulate the assembly of proteins and the transport of substances within the cell. The stability of microtubules may extend their own life and ensure their normal basic functions. A decrease in transforming growth factor (TGF)-β1 is one of the possible factors involved in ID metamorphosis.

Table 1 Effects of mRNA in vesicles on differentiation of mesenchymal stromal cells into myeloid cells, for extracellular matrix production, and for inflammatory status

Differentiation to myeloid cells	Extracellular matrix production	Inflammatory state	RNA	Target	Ref.
Promotion			lncRNA CAHM	M1 macrophages	Li *et al*[26]
Promotion		Repression	miR-199a	GREM1	Wen *et al*[35]
Promotion			miR-140-5p	KLF5/N-cadherin/MDM2/Slug	Wang *et al*[34]
Promotion			miR-105-5p	Sirt6	Sun *et al*[33]
Promotion		Repression	miR-129-5p	p38 MAPK	Cui *et al*[31]
Promotion	Promotion		miR-17-5p	TLR4	Zhou *et al*[29]
Promotion			miR-194-5p	TRAF6	Sun *et al*[12]
Promotion			miR-21	p38 MAPK	Wang *et al*[10]
Promotion		Repression	miR-26a-5p	METTL14	Xuan *et al*[13]
Promotion			miR-532-5p	AKT-mTOR	Sun *et al*[12]
Promotion		Repression	miR-31	Wnt/β-Catenin	Wang *et al*[9]
Promotion			miR-155	BACH1	Shi *et al*[16]
	Promotion		miR-15a	PI3K/Akt	Zhang *et al*[14]
	Promotion		miR-217	EZH2	Hao *et al*[27]
Promotion	Promotion		cirRNA0050205	GPX4	Yu *et al*[25]
Promotion	Promotion		cirRNA0072464	NRF2	Yu *et al*[24]

AKT: Protein kinase B; BACH1: BTB-and-CNC homologue 1; EZH2: Enhancer of zeste homolog 2; GPX4: Glutathione peroxidase 4; GREM1: Gremlin1; KLF5: Kruppel-like factor 5; lncRNA CAHM: Long non-coding RNA colorectal adenocarcinoma hypermethylated; MAPK: Mitogen-activated protein kinase; MDM2: Murine double minute 2; mTOR: Mechanistic target of rapamycin; NRF2: Nuclear factor erythroid 2-related factor 2; PI3K: Phosphoinositide 3-kinase; TRAF6: Tumor necrosis factor receptor associated factor 6.

Stabilization of microtubules can promote the expression of collagen type 2 and SRY-box transcription factor 9 (SOX9) in nucleus pulposus cells[17], which alleviates and reduces IDD. SOX9 and TGF-β1 can be used to transfect bone marrow MSCs, and MSCs can successfully differentiate into chondrocytes, promote formation of ECM, restore ID integrity, improve the inflammatory state, and reduce pain and further IDD. Similarly, MSCs and induced cartilage progenitor cells also increase the level of SOX9 and TGF-β1 during differentiation into nucleus pulposus cells, with the purpose of regulating inflammatory states and increasing differentiation[15,18,19].

METABOLITES AND MSCsS

MSCs-derived exosomes contain martrilin-3, which regulates the content of TGF-β1 in the IDs and differentiation of nucleus pulposus cells, promotes production of ECM, and inhibits release of inflammatory mediators[20]. Urolithin A was able to hinder hydrogen peroxide in inducing the aging of nucleus pulposus cells and destroying mitochondrial function. The silent information regulator 1/peroxisome proliferator-activated receptor gamma coactivator 1-alpha signaling pathway was activated by urolithin A *in vitro*, which protected the normal physiological function of mitochondria, linked nucleus pulposus cell aging, and increased the survival time of ECM while preventing further IDD [21,22]. The compression produced by the degenerated ID can promote reactive oxygen species production by nucleus pulposus cells and cause oxidative stress, which leads to further apoptosis of nucleus pulposus cells. MSCs-derived exosomes can inhibit apoptosis of myeloid cells caused by excessive oxidative stress and ameliorate compression-induced mitochondrial damage, which relieves the pain caused by IDD[23].

GEL-LOADED MSCsS

Heat-responsive hydrogel can act as a carrier of the extracellular vesicles of MSCs. These vectors deliver vesicles in a continuous box, which contains vasorin (a type I transmembrane glycoprotein regulated by hypoxia-inducible factor 1) to the disc environment. Vasorin can regulate the expression of relevant matrix metalloenzymes in the nucleus pulposus

cells through Notch1 signaling, enhance the ability of nucleus pulposus cells to proliferate, migrate, and anabolize, and inhibit the apoptotic cells to release inflammatory mediators, preventing further exacerbation of IDD. However, whether vasorin can promote the proliferation and differentiation of MSCs has not been confirmed[21]. Therefore, the strategy of helping stem cells to treat IDD through gels is worth further study.

The combination of heat-sensitive decellularized ECM hydrogels with adipocyte-derived MSCs exosomes does not damage the therapeutic activity of MSCs. The heat-sensitive dECM@exo hydrogel system produces gelation in situ to help MSCs differentiate into nucleus pulposus cells and maintains the content of ECM. This hydrogel also creates a suitable environment for the proliferation and differentiation of nucleus pulposus cells[22]. Previous studies have demonstrated transport of MSCs with hyaluronic acid and platelet-rich hydrogels. This method enhances MSCs activity, promotes IDs to increase keratin 19 gene expression, induces differentiation of MSCs into nucleus pulposus cells, and maintains the original ID height and normal physiological activity of the ID environment[23]. This situation leads to increased expression of keratin genes, and it has been demonstrated that porcine nucleus pulposus-rich cell matrix induces differentiation into nucleus pulposus cells by stimulating porcine chordate cells[24].

CIRCULAR RNA AND EXOSOMES

Circular RNA, such as circ_0050205 and circ_0072464, is transmitted through exosomes into nucleus pulposus cells to promote cell proliferation and ECM synthesis[24,25]. Endogenous long non-coding RNA colorectal adenocarcinoma hypermethylated delivery by MSCs exosomes inhibits M1-type macrophage polarization, which reduces nucleus pulposus apoptosis, the release of inflammatory factors, and a reduction in ECM to prevent further IDD[26]. In the MSCs exosomes, miRNA-217 is transferred to the ID and binds to the forkhead box O_3 promoter by targeting enhancer of zeste homolog 2. This maintains ID homeostasis and stimulates autophagy, thereby promoting collagen II and aggrecan content and inhibiting degradation of nucleus pulposus cells[15,27,28].

In other vesicles, overexpression of miRNA-194-5p inhibits the occurrence of disc degeneration by targeting tumor necrosis factor receptor associated factor 6. Lentiviral vectors have been shown to transport the growth and differentiation factor 5 (*GDF5)* gene and integrate it into the chromosomal genome of nucleus pulposus MSCs, which then express *GDF5* along with the MSCs genes. *GDF5* can also increase the level of proteoglycan and type II collagen[12]. If vesicles are subjected to hypoxia, miRNA-17-5p regulates the proliferation and differentiation of human nucleus pulposus cells and the proliferation and synthesis of ECM through the toll-like receptor 4 pathway, alleviating progression of IDD. This is more effective than the direct injection of MSCs into the ID[29].

During disc degeneration, the release of inflammatory factors also leads to a decrease in the uptake of extracellular vesicles. Vesicles prevent excess death of nucleus pulposus cells by delivering peroxidase 2 and reversing the decline in therapeutic effectiveness of MSCs due to tumor necrosis factor-α damage[30]. miRNA-129-5p in vesicles increases MSCs proliferation and differentiation into nucleus pulposus cells by blocking the lrg1-mediated p38 mitogen-activated protein kinase pathway and polarizing macrophages to the M1 phenotype, ultimately alleviating disc degeneration[31].

The combination of electromagnetic radiation and tissue engineering can stimulate the bone morphogenetic protein/Smad and mitogen-activated protein kinase-related p38 signaling pathway of ID stem cells to regulate differentiation of MSCs and increase their osteogenic capacity for the treatment of IDD[32]. Vesicles can also transmit exogenous miRNA-105-5p to revitalized nucleus pulposus cells, and transmit miRNA-140-3p to regulate the kruppel-like factor 5/N-cadherin/Murine double minute 2/Slug axis; all of which can slow down progression of IDD and restore normal ID physiological function[33,34]. miRNA-199a also exists in MSCs exosomes, which targets gremlin1 downregulation of the TGF-β pathway to prevent apoptosis of nucleus pulposus cells and inhibit progression of IDD[35].

MSCs MIXTURE

MSCs therapy alone has been shown to be effective in IDD, but its efficacy still falls short of expectations[36]. A recent study has shown that MSCs can be mixed with cell-free bioresorbable ultra-purified alginate gel for the treatment of IDD [37]. Such mixed preparations inhibit cell necrosis in the IDs and the release of inflammatory factors and consolidate the therapeutic effect of MSCs. Similarly, the combination of MSCs with in situ bioresorbable gel (dMD-001) produced the above therapeutic effects in IDD and was used after discectomy to prevent IDD[38]. Similarly, when MSCs are combined with coenzyme Q10 for the treatment of most ID lesions, it reduces oxidative stress in the ID, inhibits degradation of nucleus pulposus cells, and steadily improves the efficacy of IDD treatment[39].

Gelatin microparticles can also be mixed with MSCs, which can regulate the release of TGF-β1 and bone morphogenetic protein-2 to promote regeneration of osteochondral tissue[38]. When bone marrow MSCs are injected into the human body, leakage and decreased viability of MSCs occur, and gelatin colloidal hydrogels using nanostructures can effectively prevent these conditions. Under the load of gelatin colloid, MSCs promote regeneration of IDs and increase the number of nucleus pulposus cells between the IDs, ECM content, and the ID height[39]. Selective cell retention technology can concentrate MSCs and then the gelatin vector described above is used, which has been shown to enhance the effect of carrier materials on MSCs in the treatment of IDD[40].

A novel amphiphilic copolymer, polyethylene glycol-PAPO, fused with lipophilic kartogenin into a complex, is an esterase-reactive micelle that can carry MSCs and maintain their activity. When this combination is injected into IDs, it protects them from oxidative stress, activates autophagy of MSCs, regulates gene expression in the ECM, promotes ECM production, and increases ID height and hydration between the IDs[41]. Previous studies have found that collagen

hydrogel can promote differentiation of MSCs[42]. A more recent study found that biocompatible earthworm gel fused with MSCs induced their differentiation into nucleus pulposus cells in a targeted manner and improved their differentiation ability and efficiency[43]. For the expression of special genes such as *SOX9*, *ACAN* and *COL2* in nucleus pulposus cells, the study found that MSCs increased the expression of appeal genes after superoxide dismutase 2 and catalase processing, thereby reducing the deterioration of inflammatory states and promoting repair of ID tissue[44]. MSCs can also fuse with connective tissue growth factor and TGF-β3, transport polydopamine nanoparticles to the corresponding positions between the nuclei of the IDs, and finally induce MSCs to differentiate into nucleus pulposus cells and fibrous ring cells, reconstructing the mechanical environment of the IDs[45] (Figure 1).

STIMULATION INDUCTION WITH MSCsS

Sustained mechanical stimulation is an innovative method of induced differentiation in MSCs, which can be continuously stimulated in a special microgel attached to them[46]. Repeated continuous stretching regulates transient receptor potential vanilloid 4 and Piezo1 channel proteins, regulates the value-added differentiation of MSCs into chondrocytes, regenerates the intercellular matrix, increases the water content in the IDs, and restores the normal structure of degenerated IDs[47]. In addition, formation of ECM has been found to correlate with tissue specificity. Therefore, the isolated nucleus pulposus cells and fiber rings are processed into corresponding hydrogels, which are specific in composition and space structure. Viscous fibrinogen-thrombin-genipin gels act specifically through the RhoA/LATS/YAP1 signaling pathway to direct differentiation of MSCs into nucleus pulposus cells or fibrous rings. This helps patients with IDD to reduce the number of nucleus pulposus cells and fibrous ring damage, which may provide a new direction for the treatment of IDD[46,48]. In the treatment of IDD and recovery of ID height by using bleomycin to induce MSCs fibrosis and by stimulating the TGFβ-SMAD2/3 signaling pathway, the gene expression of related collagen and ECM is maintained, thereby maintaining the height of the ID and increasing its ability to resist wear[49].

HARSH ENVIRONMENT OF THE ID

The treatment of IDD with MSCs has been widely applied, and the role of MSCs in delaying ID lesions has been proven [50]. However, the stability and efficacy of MSCs entering the ID are affected by the microenvironment of the ID. The IDs are located in an environment of nutritional deficiency, high tension, low pH, hypoxia, and high mechanical load[51]. In this environment with progression of IDD, most of the nucleus pulposus cells begin to die, resulting in a series of malignant chain reactions that lead to aggravation of oxidative stress, secretion of inflammatory substances, and aggravation of pain[5] (Figure 2).

Many studies have reported that the microenvironment in which cells are located has an important impact on their biological activity[52]. The low pH of IDs significantly inhibits acid-sensitive ion channels (ASICs), which are key receptors for extracellular protons in central and peripheral neurons related to IDD[53]. Due to the decreased activity of ASICs, MSCs differentiate into nucleus pulposus cells. When the number of nucleus pulposus cells decreases and production of ECM decreases, this increases the short-function peptide fragments that can recognize ASIC blockers, as ASICs can activate cell aging pathways, such as p53-p21/p27 and p16-Rb1 signaling factors, to induce apoptosis of nucleus pulposus cells[54]. ASIC blockers can significantly help stem cells overcome the acidic environment during IDD treatment, improve the ability of MSCs to proliferate and differentiate into nucleus pulposus cells, and help restore the normal state of IDs. $1,25(OH)_2D_3$-treated nucleus pulposus MSCs have better tolerance to the hypertonic and acidic microenvironment in which the IDs are located[55]. This reduces apoptosis of nucleus pulposus mesenchymal cells, restores the height between the IDs, and delays occurrence of IDD.

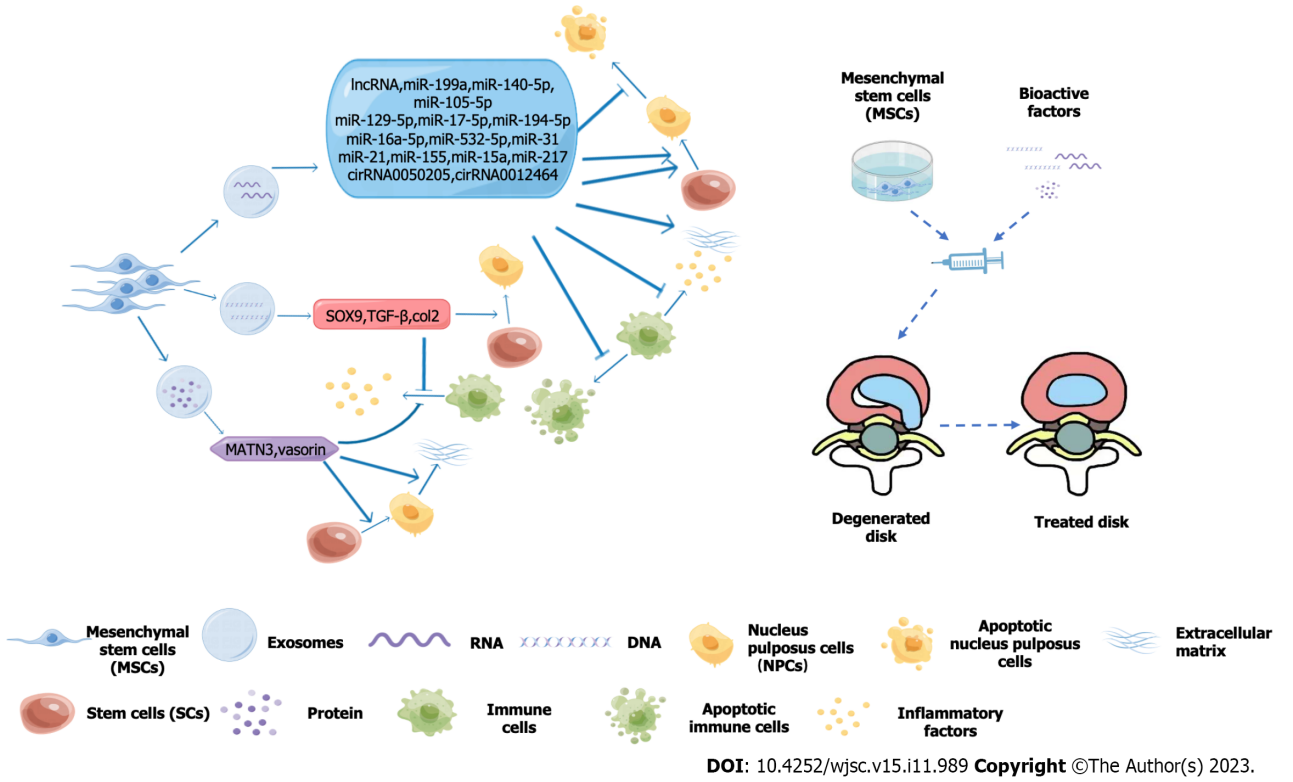
The large changes in pressure between the degenerative IDs leads to a decrease in the survival and differentiation of MSCs[56]. Medullary pulposus cell-derived hydrogels help MSCs differentiate into nucleus pulposus cells[57]. A recent study found that the current methanipine cross-linked decellularization nucleus pulposus hydrogel-like cell delivery system can transport MSCs to the IDs[58]. The changes in ID pressure ensure that MSCs differentiate into nucleus pulposus cells, repair the reduced ECM, and maintain ID height to delay IDD.

MSCs pretreated with lithium chloride can increase the adaptability of MSCs[59], as lithium chloride helps MSCs antagonize oxidative stress and protect nucleus pulposus cells by activating more extracellular signal-regulated kinase 1/2[60]. Extracellular signal-regulated kinase 1/2 plays a vital role in fighting inflammation, which is one of the main ways it works in the harsh environment of the IDs. This conduction pathway ultimately also helps to improve the ability of MSCs to reduce nucleus pulposus cell death, increase ECM production, and improve inflammatory status and IDD.

Significant progress has been made in the treatment of IDD by MSCs, but due to the harsh environment of the IDs[5], the therapeutic effect of MSCs is reduced due to the lack of oxygen in the environment[61]. However, it has been found that the differentiation of MSCs to chondrocytes under hypoxic conditions can be helped by the addition of leptin[62], which provides energy for cell differentiation through its dependent glycolysis.

FUTURE DIRECTIONS FOR STEM-CELL-BASED THERAPY

Stem cell-based therapy offers promise for disc degeneration. Related studies have applied exogenous stem cells such as



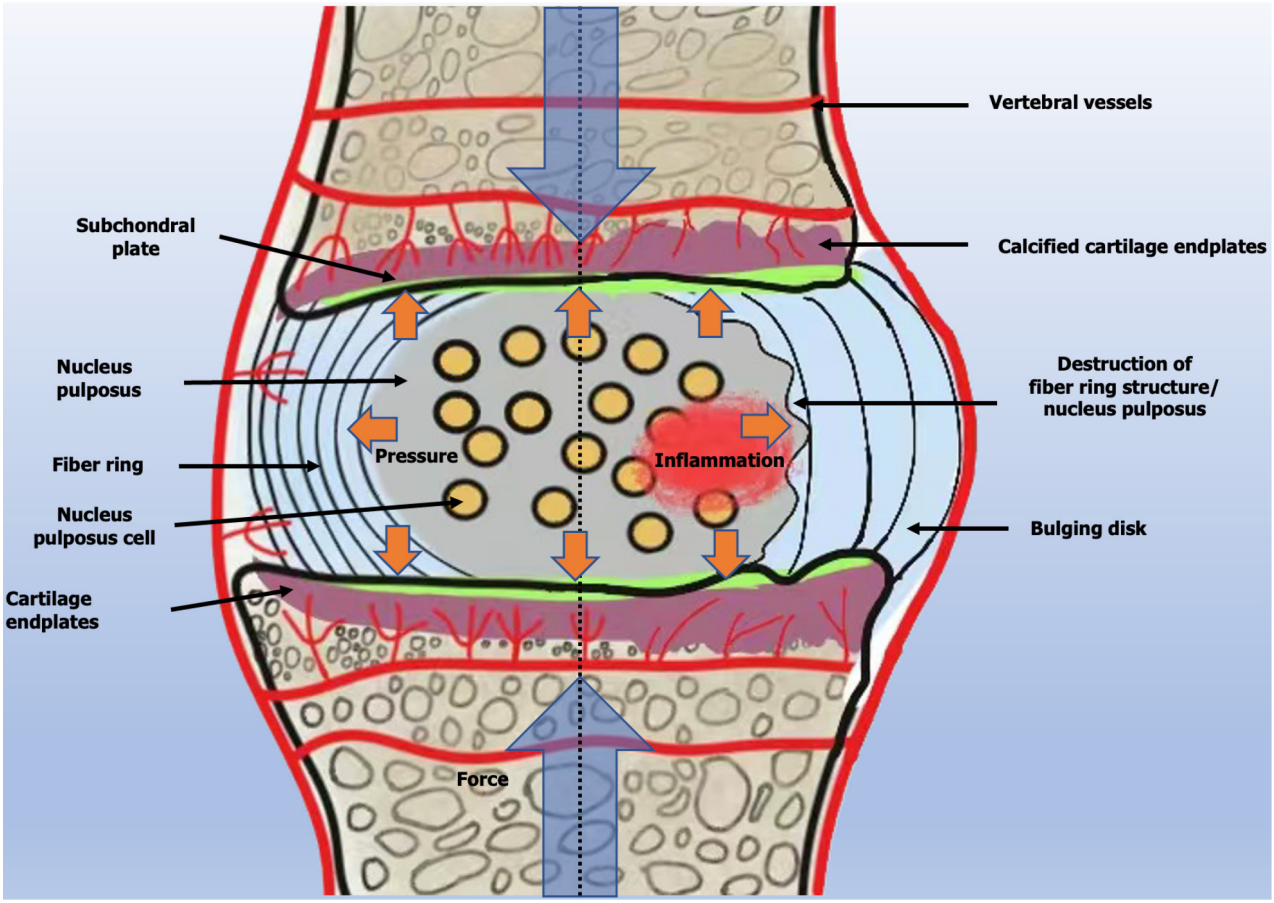
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Figure 1 Mesenchymal stromal cell exosomes can treat disc degeneration through the appropriate target. The extracellular vesicles secreted by mesenchymal stromal cells can be enriched with a variety of RNAs, DNAs, and proteins that can be targeted to treat disc degeneration. They have been shown to have a corresponding effect in promoting stem cell differentiation into myeloid cells, increasing extracellular matrix production, and reducing the release of inflammatory factors (pain reduction). MATN3: Martrilin-3; SOX9: SRY-box transcription factor 9; TGF: Transforming growth factor.

MSCs to treat disc degeneration with promising results[63]. As the field of stem cells continues to be studied[64], the histology of the IDs becomes clearer, and studies targeting the way in which stem cells restore disc structure are becoming more advanced. How to improve the efficiency of stem cell therapy for disc degeneration and how to resist the harsh disc environment for stem cell therapy are the focus of research[65]. This also includes how to help stem cells restore the normal physiological structure of the IDs under hypoxia and lack of blood supply[5]. This is a new direction that needs to be developed in the field of stem cells.

Sometimes it is not the stem cell therapy that is ineffective, as the way MSCs are administered similarly affects their behavior once inside the body. Overall, factors such as injection point site, syringe, carrier material, and buffer can affect the therapeutic efficacy of stem cells. Different injection sites may lead to variations in reflux of the cellular injection fluid [66], and the syringe (needle size/shape) may lead to variations in the shear rate and shear stress of the cellular injection fluid, which can affect the viability of the injected cells. There are various challenges in the clinical application of stem cells, both for local administration and circulatory system administration[65]. Therefore, does the appropriate route of administration always guarantee the clinical outcome of MSCs? Obviously not. While performing stem cell therapy, we not only need to choose the appropriate delivery method but also need to consider the individualization of the patient at the same time. We need to consider all these factors together in order to make the most appropriate stem cell treatment plan, thus improving the efficiency of MSCs treatment[67].

Different sources of MSCs have their own advantages and disadvantages in terms of therapeutic efficacy, and one of the major dilemmas that needs to be explored further is the production of stable MSCs at the production site[68]. Current research allows us to understand the current potential of MSCs for cell transplantation, tissue engineering, and cell-based therapies to improve the lives of those affected by disc injuries[65]. This requires us to deepen our understanding of MSCs, refine therapeutic approaches, and address the challenges of translating research findings into clinical practice. We can do this by further optimizing the sources of MSCs[69], delivery methods and timing of interventions, as well as standardized protocols for isolation, expansion, and characterization. Conducting well-designed clinical trials will help evaluate the safety, efficacy, and long-term outcomes of MSCs-based therapies[70]. As the clinical application of MSCs continues to be studied globally, stem cell therapeutic drugs are gradually being introduced, and more research teams and medical institutions are involved, this will gradually deepen the clinical application of stem cell therapy and bring hope to the majority of lumbar disc patients.



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Figure 2 The harsh environment in which the intervertebral discs are located. There is almost no vascular distribution in the intervertebral discs, and the nutrient supply relies mainly on the diffusion of oxygen and nutrients in the subendothelial vessels. The closer to the center of the nucleus pulposus, the lower the partial pressure of oxygen and the concentration of proteoglycans, which results in an extreme microenvironment, such as nutrient deficiency, high osmotic pressure, and acidic PH.

CONCLUSION

IDs have a complex structure with a unique internal environment. They contain nucleus pulposus cells, fibrous rings, and ECM, which are in a dynamic balance of self-renewal. Stem cells therapy is designed to restore this balance of secreting exosomes and vesicles, mixing other substances to promote their differentiation into nucleus pulposus cells, regulating the content of the ECM, and when treated accordingly resisting interference by the harsh environment of the IDs. These synergistic approaches provide new possibilities for stem cell therapy of IDD. They contain nucleus pulposus cells, annulus fibrosus, and ECM, and as the nucleus pulposus cells age and the ECM is lost, among other things, the disc becomes less stable. Stem cell therapy aims to restore the structure of the disc by secreting exosomes and vesicles, it mixes other substances to promote their differentiation into nucleus pulposus cells, regulates the content of the ECM and resists interference from the hostile environment of the IDs when treated accordingly. These synergistic approaches offer new possibilities for stem cell therapy for IDD. In the future, as people continue to explore the field of stem cells in disc therapy, stem cells will bring more hope to disc degeneration.

FOOTNOTES

Author contributions: Zhang QX wrote the manuscript and collected the data; Cui M revised and approved the manuscript; all authors read and approved the final manuscript.

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ORIGINAL ARTICLE

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Hypoxia and inflammatory factor preconditioning enhances the immunosuppressive properties of human umbilical cord mesenchymal stem cells

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have great potential for the treatment of various immune diseases due to their unique immunomodulatory properties. However, MSCs exposed to the harsh inflammatory environment of damaged tissue after intravenous transplantation cannot exert their biological effects, and therefore, their therapeutic efficacy is reduced. In this challenging context, an *in vitro* preconditioning method is necessary for the development of MSC-based therapies with increased immunomodulatory capacity and transplantation efficacy.

AIM

To determine whether hypoxia and inflammatory factor preconditioning increases the immunosuppressive properties of MSCs without affecting their biological characteristics.

METHODS

Umbilical cord MSCs (UC-MSCs) were pretreated with hypoxia (2% O_2) exposure and inflammatory factors (interleukin-1β, tumor necrosis factor-α, interferon-γ) for 24 h. Flow cytometry, polymerase chain reaction, enzyme-linked immunosorbent assay and other experimental methods were used to evaluate the biological characteristics of pretreated UC-MSCs and to determine whether pretreatment

affected the immunosuppressive ability of UC-MSCs in coculture with immune cells.

RESULTS

Pretreatment with hypoxia and inflammatory factors caused UC-MSCs to be elongated but did not affect their viability, proliferation or size. In addition, pretreatment significantly decreased the expression of coagulation-related tissue factors but did not affect the expression of other surface markers. Similarly, mitochondrial function and integrity were retained. Although pretreatment promoted UC-MSC apoptosis and senescence, it increased the expression of genes and proteins related to immune regulation. Pretreatment increased peripheral blood mononuclear cell and natural killer (NK) cell proliferation rates and inhibited NK cell-induced toxicity to varying degrees.

CONCLUSION

In summary, hypoxia and inflammatory factor preconditioning led to higher immunosuppressive effects of MSCs without damaging their biological characteristics.

Key Words: Mesenchymal stem cells; Umbilical cord; Preconditioning; Hypoxia, Inflammatory factors; Immune regulation

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Core Tip: Mesenchymal stem cells (MSCs) are potential candidates for treating many immune diseases due to their unique immunomodulatory abilities, but low survival rates and weakened function after venous transplantation reduces their treatment potential. Therefore, our study reveals a combination pretreatment method based on *in vitro* hypoxia exposure and inflammatory factor treatment that simulates the harsh *in vivo* environment to protect MSCs from injury after intravenous transfusion and promote high immunosuppressive effects of MSCs.

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INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) have been shown to have a variety of biological properties, such as self-renewal[1], multilineage differentiation[2,3], immunomodulation functions[4,5], tissue repair effects[6], anti-aging and regeneration activity[7]. Furthermore, due to the abundance and ready availability of MSCs[8], they do not lead to rejection after allografting[9]; thus, MSCs have great economic value, importance in the cell therapy field and broad application prospects. Although many questions remain unanswered, the immunomodulatory effects of MSCs make them candidates for cell-based tissue repair and disease treatment[10-12]. For example, MSCs have great potential in treating graft-*versus*-host disease[13], cardiovascular disease[14,15], liver disease[16,17], systemic lupus erythematosus [18], autoimmune encephalomyelitis[19] and spinal cord injury[20]. Studies have shown that the immunomodulatory ability of MSCs is primarily regulated by cytokines and other soluble factors, including prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α-stimulated gene protein-6 (TSG-6), inducible indoleamine 2,3-dioxygenase (IDO), interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1)[21]. They contribute to MSC-mediated immunosuppression by inhibiting natural killer (NK) cell proliferation, cytotoxicity and cytokine secretion[22], inhibiting T-cell proliferation, promoting regulatory T cell production[5,23,24], and inhibiting B-cell proliferation and immunoglobulin release[25,26].

It has been shown that when MSCs are injected into the body and migrate to damaged tissues or organs, the activation of innate immune cells leads to increased chemokine and cytokine release (such as TNF-α, IL-1α and IL-1β)[27] and is accompanied by the development of a harsh environment caused by hypoxic stress[28], leading to decreased MSC activity. Therefore, *in vitro* preconditioning culture developed through hypoxia exposure and inflammatory factor supplementation can effectively mimic the *in vivo* microenvironment. Preconditioning MSCs *in vitro* activates various signaling pathways, influencing the biological activities of MSCs *in vitro* and *in vivo*, thereby preparing them to survive the harsh environment to which they are subjected during *in vivo* administration and protecting them from damage[29, 30]. In addition, preconditioning can induce MSCs to secrete immunosuppressive molecules to target innate and adaptive immune cells, thereby mediating immune regulation by releasing water-soluble factors[31,32]. Therefore, pretreatment is an adaptive strategy to enhance the efficacy of MSC therapy, which can not only prolong MSC survival and function after transplantation but also endows them with higher immunomodulatory activity levels. Studies have shown that preconditioning with inflammatory factors such as TNF-α and interferon (IFN)-γ can increase MSC immunoregulatory ability[33, 34]. Hypoxia preconditioning is often used to enhance the therapeutic effect of MSCs by inducing the expression of survival genes, chemokines, growth factors, and angiogenic factors (such as vascular endothelial growth factor), enabling the MSCs to adapt to the harsh environment of damaged tissue[35,36].

Although different preconditioning methods can enhance cell properties and increase their function, many are accompanied by safety concerns. In contrast to the aforementioned positive outcomes of pretreatment, improper pretreatment can negatively impact cell morphology and function, damage mitochondrial function[37], and affect MSC surface marker expression. For example, previous studies have shown that tissue factor (TF/CD142) binds to coagulation factor VII/VIIB to initiate the exogenous coagulation system. The relative cell surface expression of TF is strongly correlated with procoagulant activity[38]. MSCs expressing surface TF show high coagulant activity and promote blood and plasma coagulation[39]. Therefore, high TF expression on MSCs increases the risk of thrombosis after intravenous injection[40-43].

We found that most studies have been focused on bone marrow- or adipose-derived MSCs (AD-MSCs), but human umbilical cord-derived MSCs (hUC-MSCs) are more suitable for clinical research and large-scale use because they are not associated with ethical problems, are abundant and highly proliferative. Therefore, we chose hUC-MSCs to study. To our knowledge, studies using a combination of hypoxia (2% O_2) exposure and inflammatory factor (IL-1β, TNF-α, IFN-γ) treatment to precondition hUC-MSCs are rare, and our study supports the current theory. Our aim was to simulate the injury-induced environment *in vitro* using a combination of hypoxia and inflammatory factor preconditioning to determine whether preconditioning increases the immunosuppressive properties of MSCs without affecting their biological characteristics.

MATERIALS AND METHODS

UC-MSC extraction and culture

Umbilical cord and cord blood were obtained with the approval of the Medical Ethics Committee of Shanxi Medical University and the consent of the donors, and experiments were performed in accordance with the Declaration of Helsinki.

Umbilical cord tissue was provided by one hospital, and the donors gave informed consent. After obtaining the umbilical cords, two veins and one artery were removed, and Wharton's jelly was cut into pieces and placed into a 10 cm^2 petri dish (Nice) with 5 mL of DMEM/F12 culture medium (Thermo Fisher Scientific, Waltham, MA, United States) containing 2.5% serum substitute (Shanxi Yinshi Cell Technology, Xian, China). Approximately 14 d later, the UC-MSCs were passaged, and P4 generation cells were used for experiments. UC-MSCs were cultured in a carbon dioxide incubator (Thermo Fisher Scientific) at standard oxygen tension with 5% carbon dioxide, 95% air, and 37 °C (*i.e.* normoxia). When UC-MSCs were 70%-80% confluent, a mixture of IFN-γ (R&D), TNF-α (R&D Systems, Minneapolis, MN, United States) and IL-1β (PeproTech, Cranbury, NJ, United States) was added to the medium. The cells were then immediately placed into a three-gas incubator (Panasonic, Osaka, Japan) with 2% O_2, 5% CO_2, and 93% N_2 at 37 °C (*i.e.* hypoxia). After 24 h, primed UC-MSCs (PUC-MSCs) were obtained. Therefore, our experiments were divided into two groups: Control (UC-MSCs) with no treatment and an experimental group (PUC-MSCs) in which cells were exposed to hypoxia and inflammatory factor supplementation.

Extraction and culture of peripheral blood mononuclear cells

Umbilical cord blood was obtained and centrifuged at 700 × *g* for 10 min and the plasma was extracted and placed into a water bath at 56 °C for 30 min for heat inactivation. The plasma was removed from the water bath, centrifuged at 850 × *g* for 10 min, and placed in a refrigerator at 4 °C for later use. The blood cells were resuspended in phosphate buffered saline (PBS) and mixed. The cell suspension was slowly added into a centrifuge tube containing Ficoll human peripheral blood lymphocyte isolation medium (Tianjin Haoyang Biological Products Technology Co., Ltd., Tianjin, China). After centrifugation at 400 × *g* for 30 min, white cells were extracted and washed twice with PBS. The cells [(1-2) × 10^6 cells/mL] were inoculated in peripheral blood mononuclear cell (PBMC) medium containing 10% of the heat-inactivated autologous plasma (Shanxi Yin Cell Technology Co., Ltd., Hebei, China) in the presence or absence of UC-MSCs or PUC-MSCs. The PBMCs were treated with 100 U/mL IL-2 (PeproTech). For coculture experiments, PBMCs were inoculated with UC-MSCs or PUC-MSCs at a PBMC/MSC ratio of 3:1 so that the cells were in direct contact, which is the appropriate proportion of cells to obtain MSC-mediated inhibition of PBMC proliferation.

NK cell extraction and culture

Umbilical cord blood was obtained and centrifuged at 700 × *g* for 10 min, and the plasma was placed into a water bath at 56 °C for 30 min for heat inactivation. The plasma was removed, centrifuged at 850 × *g* for 10 min, and placed into a refrigerator at 4 °C for later use. The blood cells were resuspended in PBS, mixed, and incubated at room temperature for 20 min with RosetteSepT NK Enrichment Cocktail. The cell suspension was slowly added into a centrifuge tube containing Ficoll human peripheral blood lymphocyte isolation medium (Tianjin Haoyang Biological Products Technology Co., Ltd.). After centrifugation at 400 × *g* for 30 min, white cells were extracted and washed twice with PBS. The cells [(1-2) × 10^6 cells/mL] were inoculated into NK medium containing 10% heat-inactivated autologous plasma (Shanxi Yin Cell Technology Co., Ltd.). NK cells were cultured with or without UC-MSCs or PUC-MSCs and treated with 100 U/mL IL-2 (PeproTech).

Cell survival rate and cell size detection

Double fluorescence acridine orange/propidium iodide (AO/PI) cell viability counting was performed. The AO/PI reagent consists of the DNA-binding dye AO, which fluoresces green, and the DNA-binding dye PI, which fluoresces red.

AO can pass through a complete cell membrane and enter the nuclei of all cells (living and dead cells), emitting green fluorescence. PI can only pass through an incomplete cell membrane and enter the nuclei of dead cells, resulting in red fluorescence. After mixing 10 μL of cell suspension with 10 μL of AOPI reagent, the viability and size of the cells in suspension were measured by a Countstar Rigel S2 (Shanghai Rui Yu Biotech, Shanghai, China).

Monoclonal antibodies and cytofluorometric analysis

Flow cytometry analysis of UC-MSCs and PUC-MSCs was performed. The cells were resuspended in staining buffer after centrifugation, and the corresponding antibodies were added to the buffer. Cells were incubated for 30 min, neutralized and washed 1-2 times. The UC-MSC or PUC-MSC antigen markers (BD Biosciences, San Jose, CA, United States) were CD105-APC, CD90-FITC, CD73-PE, CD142-PC5.5, CD29-PE, CD34-PE, CD14-PE, CD45-APC, human leukocyte antigen (HLA)-ABC-APC, CD47-APC, CD166-PE, CD44-PE, and CD31-APC.

Reactive oxygen species measurement

Mitochondrial reactive oxygen species (ROS) production was measured by flow cytometry (Beckman Coulter Life Sciences, Brea, CA, United States) using a ROS detection kit (Biyuntian, Shanghai, China). 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was diluted in serum-free medium at 1:1000 to a final concentration of 10 μmol/L. After collection, 1 million to 20 million/mL cells were suspended in diluted DCFH-DA and incubated in an infrared carbon dioxide incubator (Thermo Fisher Scientific) at 37 °C for 20 min. The cells were turned over and mixed every 3-5 min to ensure that the probe was in full contact with the cells. The cells were washed with serum-free cell culture solution three times to fully remove excess DCFH-DA. Finally, the results were detected by flow cytometry.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was detected using MMP detection reagent JC-1 (Beyotime, Jiangsu, China). The culture medium was removed and the cells were washed twice with PBS. JC-1 staining solution (1 mL) was added, mixed thoroughly, and incubated with cells in an infrared carbon dioxide incubator (Thermo Fisher Scientific) at 37 °C for 20 min. During the incubation period, JC-1 staining buffer (1×) was prepared by adding approximately 4 mL distilled water to approximately 1 mL JC-1 staining buffer (5×), and the buffer was placed in an ice bath. After incubation at 37 °C, the supernatant was removed, and the cells were washed twice with JC-1 staining buffer (1×). Finally, 2 mL of the cell culture solution was added. Images were obtained with an inverted fluorescence microscope (CKX53; Olympus, Tokyo, Japan).

Real time-polymerase chain reaction

TRIzol (Invitrogen, Waltham, MA, United States) was used to lyse the cells, total RNA was extracted, and an ultratrace nucleic acid protein detector (Shanghai Jiapeng Materials, Shanghai, China) was used to determine RNA concentration. mRNA expression levels were quantitatively analyzed on a real-time polymerase chain reaction (RT-PCR) instrument (Bio-Rad, Hercules, CA, United States) using a One Step TB Green PrimeScript PLUS RT□PCR Kit (Takara Bio Inc., Shiga, Japan) to measure the levels of the following transcripts: Catalase, Istanniocalcin-1 (STC1), hemeoxygenase-1 (HOMX1), B-cell lymphoma 2-associated protein (Bax), B-cell lymphoma 2 (Bcl2), silencing information regulator 2-related enzyme 1 (SIRT1), P53, P16, P21, PGE2, kynurenine (KYN), IDO, IL-1 receptor antagonist (IL-1ra), cyclooxygenaese-2 (COX2), IL-10, TGF-β1, HLA-G5, TSG-6, ligands for programmed cell death 1 (PD-L1). All primer sequences for qRT-PCR are listed in Table 1.

Enzyme-linked immunosorbent assays

UC-MSCs and PUC-MSCs supernatants were collected and centrifuged at 210 × *g* for 5 min, and the suspended cells were removed and stored at -80 °C. The protein levels of PGE2, TSG-6, IDO, IL-10 and TGF-β1 secreted by UC-MSCs and PUC-MSCs were measured by enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Eliret Biotechnology Co, Ltd., Wuhan, China). Finally, the optical density (OD) value of each well was measured on the basis of enzyme label (Thermo Fisher Scientific) absorption at 450 nm.

Annexin V-FITC apoptosis detection

UC-MSC and PUC-MSC suspensions were obtained and washed twice with PBS. Annexin V-FITC apoptosis reagent (Biyuntian) was added and incubated with the cells at room temperature for 10-20 min in the dark, and flow cytometry was performed. Annexin V-FITC emits green fluorescence, and PI emits red fluorescence.

β-galactosidase (SA-β-gal) activity detection

The cell culture medium was removed, the cells were washed once with PBS, and 1 mL/well β-galactosidase (SA-β-gal) staining fixation solution (Solarbio, Beijing, China) was added and incubated at room temperature for 15 min. Fixative was removed, and the cells were washed 3 times with PBS for 3 min each. The PBS was removed, and 1 mL of staining solution was added to each well. The plate was incubated at 37°C overnight and sealed with plastic wrap to prevent evaporation. The positive expression of blue particles was observed under an inverted microscope (CKX53; Olympus), and the number of positive cells per 100 cells was quantified. Each experiment was repeated three times.

Proliferation experiment

NK cells were cultured in the presence or absence of UC-MSCs or PUC-MSCs for 3 d at a 3:1 ratio. The cells were prelabelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye (BioLegend, San Diego, CA, United States)

Table 1 Primer used to amplify the human transcripts during real-time quantitative polymerase chain reaction

Gene	Sequence, 5' to 3'	Application
Catalae (human)	Upper: TTCTGTTGAAGATGCGGCGAGAC	qRT-PCR
	Lower: GGGGTACTTTCCTGTGGCAATGG	
STC-1 (human)	Upper: CCATGAGGCGGAGCAGAATGAC	qRT-PCR
	Lower: GCCGACCTGTAGAGCACTGTTG	
HMOX1 (human)	Upper: TGCCAGTGCCACCAAGTTCAAG	qRT-PCR
	Lower: TGTTGAGCAGGAACGCAGTCTTG	
Bax (human)	Upper: ACCAAGAAGCTGAGCGAGTGTC	qRT-PCR
	Lower: TGTCCACGGCGGCAATCATC	
Bcl2 (human)	Upper: TGAGTTCGGTGGGGTCATGT	qRT-PCR
	Lower: TTCCACAAAGGCATCCCAGC	
SIRT1 (human)	Upper: CCTGGACAATTCCAGCCATCT	qRT-PCR
	Lower: TGCAAAGGAACCATGACACTGA	
P53 (human)	Upper: GCCCATCCTCACCATCATCACAC	qRT-PCR
	Lower: GCACAAACACGCACCTCAAAGC	
P16 (human)	Upper: CCGTGGACCTGGCTGAGGAG	qRT-PCR
	Lower: CGGGGATGTCTGAGGGACCTTC	
P21 (human)	Upper: CTTGTACCCTTGTGCCTCGC	qRT-PCR
	Lower: AGAAGATCAGCCGGCGTTTG	
β-actin (human)	Upper: AGTGTGACGTGGACATCCGCA	qRT-PCR
	Lower: ATCCACATCTGCTGGAAGGTGGAC	
PGE2 (human)	Upper: ATTCTCCTGGCTATCATGAC	qRT-PCR
	Lower: GAACAGGAGGCCTAAGGATG	
IDO (human)	Upper: CTCTGCCAAATCCACAGGAAA	qRT-PCR
	Lower: ATGACCTTTGCCCCACACAT	
KYN (human)	Upper: CAAGCGAAGGGTTGTTATGTTGGC	qRT-PCR
	Lower: GGAACACCAGCAGGCAAAATCAAC	
COX2 (human)	Upper: AATCTGGCTGCGGGAACACAAC	qRT-PCR
	Lower: TGTCTGGAACAACTGCTCATCACC	
IL-1ra (human)	Upper: GTGCCTGTCCTGTGTCAAGTCTG	qRT-PCR
	Lower: GCCACTGTCTGAGCGGATGAAG	
IL-10 (human)	Upper: GCCAAGCCTTGTCTGAGATGATCC	qRT-PCR
	Lower: GCCTTGATGTCTGGGTCTTGGTTC	
TGF-β1 (human)	Upper: AGCAACAATTCCTGGCGATACCTC	qRT-PCR
	Lower: TCAACCACTGCCGCACAACTC	
HLA-G5 (human)	Upper: AGAGGAGACACGGAACACCAAGG	qRT-PCR
	Lower: CAGGTCGCAGCCAATCATCCAC	
TSG-6 (human)	Upper: AGAGAAGCACGGTCTGGCAAATAC	qRT-PCR
	Lower: GCCATCCATCCAGCAGCACAG	
PD-L1 (human)	Upper: TGACCTACTGGCATTTGCTGAACG	qRT-PCR
	Lower: CACTGCTTGTCCAGATGACTTCGG	

Bax: B-cell lymphoma 2-associated protein; Bcl2: B-cell lymphoma 2; COX2: Cyclooxygenaese-2; HLA-G5: Human leukocyte antigen-G5; HOMX1: Hemeoxygenase-1; IDO: Idoleamine-2,3-dioxygenase; IL-1ra: Interleukin-1 receptor antagonist; IL-10: Interleukin-10; KYN: Kynurenine; PD-L1: Ligands for programmed cell death 1; PGE2: Prostaglandin E2; qRT-PCR: Quantitative real-time polymerase chain reaction; SIRT1: Silencing information regulator 2-related enzyme 1; STC1: Stanniocalcin-1; TGF-β1: Transforming growth factor-beta 1; TSG-6: Tumor necrosis factor-α-induced protein-6.

and the fluorescence intensity of CFSE was measured by flow cytometry.

Cell growth and proliferation

UC-MSCs and PUC-MSCs were collected for DNA extraction and DNA concentration was determined by an ultramicro nucleic acid protein detector (Shanghai Jiapeng Materials) to evaluate the cell proliferation rate. PBMCs were cultured in the presence or absence of UC-MSCs or PUC-MSCs at a ratio of 3:1. The number of cells (Countstar Rigel S2; Shanghai Rui Yu Biotech) was counted by a cell imaging analyzer for 5 consecutive days and the growth curve was plotted.

Cytotoxicity of K562 target cells

NK cells were cultured alone or in direct contact with MSCs (NK: UC-MSCs or PUC-MSCs = 3:1 ratio) for 72 h. NK cells were then collected and cocultured with CFSE-stained (Biolegend) K562 target cells for 4 h at the effective target ratio of 1:5. All cells were collected and washed twice with PBS. K562 cell apoptosis was analyzed by PI staining (Biyuntian), and the fluorescence intensity was measured by flow cytometry.

Statistical analysis

SPSS 19.0.0 and GraphPad Prism 7 (GraphPad Prism, La Jolla, CA, United States) statistical software were used for data processing. All experimental data are expressed as the mean ± SD. A *t* test was performed to compare two datasets, and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of hypoxia and inflammatory factor pretreatment on UC-MSC morphology, size, proliferation and viability

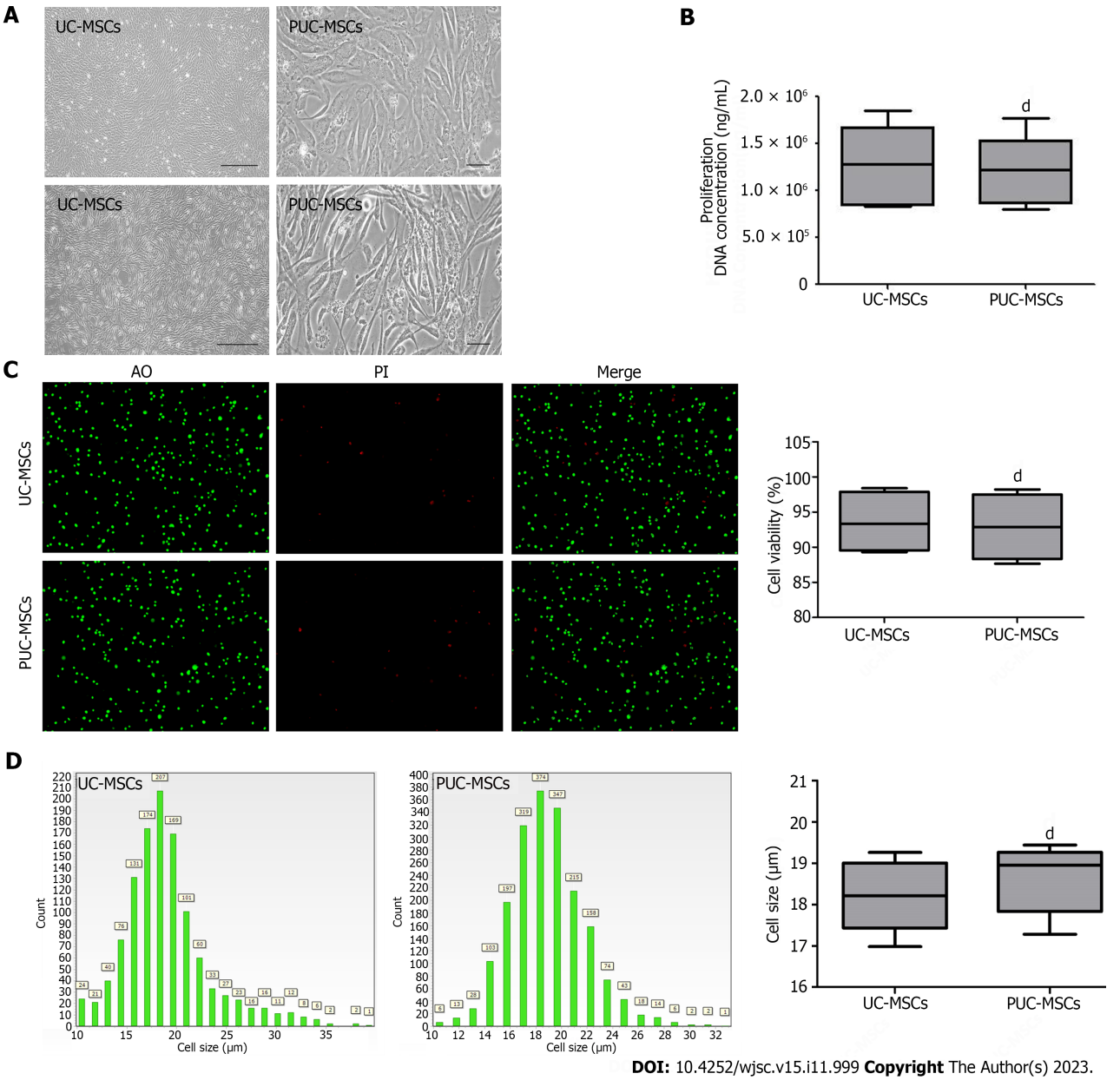
We evaluated the effects of hypoxia and inflammatory factor pretreatment on UC-MSC morphology, vitality and size. The morphological differences between UC-MSCs and PUC-MSCs were compared under a microscope when cell confluency reached 80% to 90%. Untreated UC-MSCs appeared as either short rods or long spindles in an adherent state and were arranged in a spiral pattern. After 24 h of exposure to hypoxia and inflammatory factors, cell morphology changed from short and rod-like to thin and elongated (Figure 1A), but the proliferation rate did not change (Figure 1B). We further evaluated the effects of pretreatment on UC-MSC viability and size using AOPI staining and a cell imaging analyzer. UC-MSC viability (Figure 1C) in suspension was 93.59% ± 3.87% ($n = 5$) and the mean diameter (Figure 1D) was 18.216 ± 0.78 μm ($n = 5$), while PUC-MSC viability (Figure 1C) was 92.89% ± 4.13% ($n = 5$) and the mean diameter (Figure 1D) was 18.628 ± 0.76 μm ($n = 5$). These results showed that pretreated MSCs became elongated, the measured viability slightly decreased ($P = 0.84$), and the cells were slightly enlarged ($P = 0.47$), but these differences were not statistically significant.

MSC surface marker expression

We further evaluated whether preconditioning altered the cellular phenotype by analyzing MSC surface marker expression by flow cytometry. UC-MSCs (Figure 2A) and PUC-MSCs (Figure 2B) from three donors were positive for CD105, CD90, CD73, CD29, CD166, CD47 and HLA-ABC surface expression and negative for CD31, CD45, CD14 and CD34. The results showed that surface marker expression was consistent between PUC-MSCs and UC-MSCs. Interestingly, CD142 expression (59.3%) (Figure 2B) was significantly lower in PUC-MSCs compared to UC-MSCs (99.6%) (Figure 2A).

Effect of pretreatment on UC-MSC mitochondrial function

ROS play key roles in the proapoptotic signaling cascade, and excess ROS attacks the mitochondrial membrane and loss of MMP, leading to MSC apoptosis[44]. Therefore, we used flow cytometry to examine the effect of MSC pretreatment on ROS production. Intracellular ROS levels showed a three-fold increase in DCFH-DA fluorescence intensity in PUC-MSCs compared to untreated UC-MSCs (Figure 3A), but all the effects were within the range of values obtained in positive controls. STC1, catalase and HOMX1 expression levels increased (Figure 3B), indicating that the antioxidant capacity of the cells was increased and that mitochondrial ROS removal was increased, enhancing antioxidant defense effects and preventing ROS-induced DNA damage. Maintaining a stable MMP (Ψm) is essential to ensure efficient ROS clearance and prevent apoptosis or other stress-related events caused by excessive ROS[45]. Therefore, we further determined whether hypoxia and inflammatory factor preconditioning induced MMP dysfunction in UC-MSCs. We used the sensitive fluorescent probe JC-1 to evaluate the effect of pretreatment. We determined whether the membrane potential changed in UC-MSCs by measuring a change in JC-1 red fluorescence to green fluorescence. Microscopically, untreated UC-MSCs showed a normal MMP, as shown by red fluorescence staining with JC-1 (Figure 3C). Neither the red and green fluorescence nor the MMP was significantly different between PUC-MSCs and UC-MSCs. We further measured the



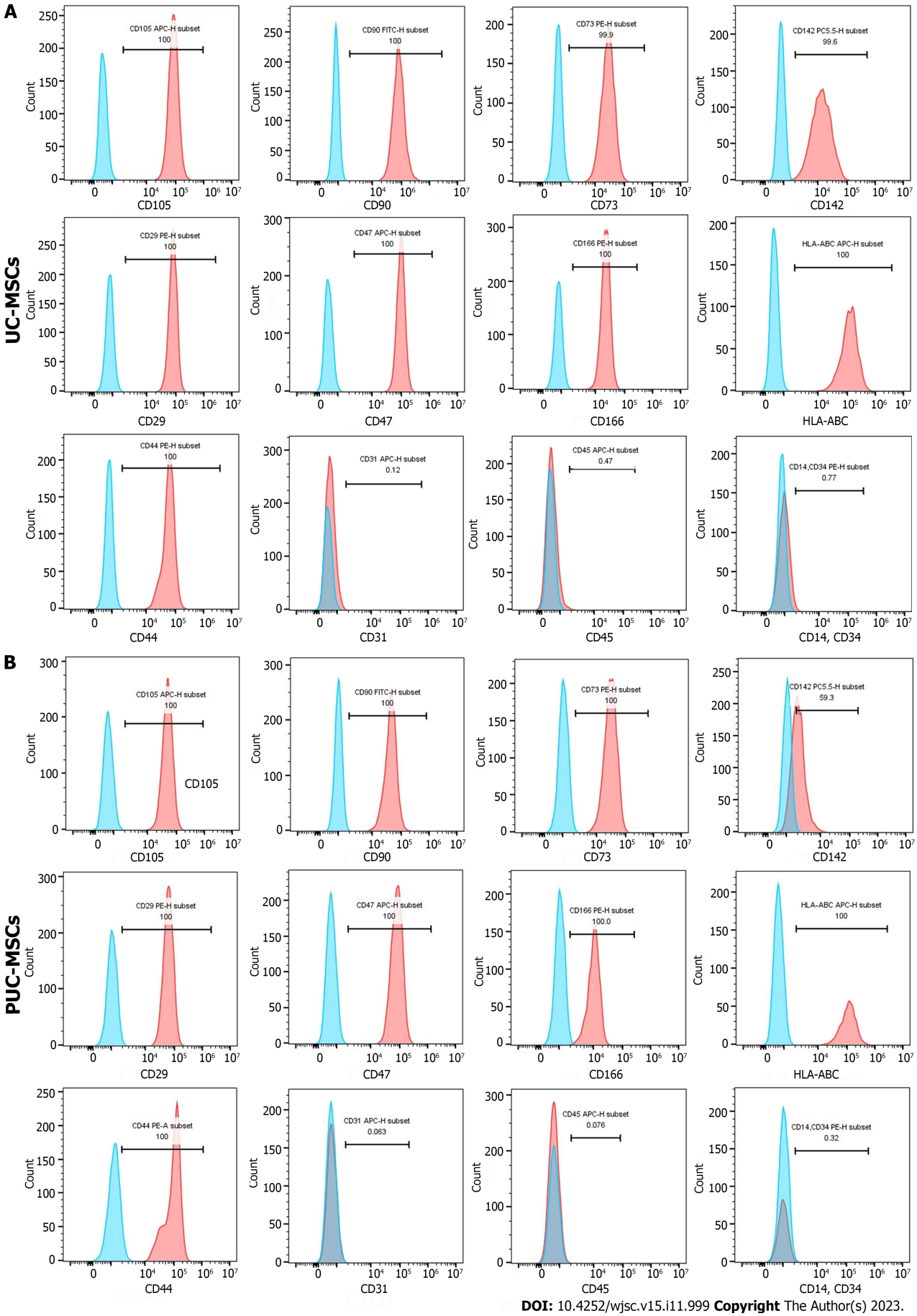
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Figure 1 Morphology, viability, proliferation, and size of mesenchymal stem cells. A: Representative micrographs showing umbilical cord mesenchymal stem cells (UC-MSCs) and primed umbilical cord mesenchymal stem cells (PUC-MSCs). Microscopy observation of cell morphology revealed that UC-MSCs were elongated after hypoxia and inflammatory factor pretreatment (Bar = 200 μm); B: The DNA concentration was measured by an ultramicro nucleic acid protein detector. The DNA concentration of PUC-MSCs was not significantly different from that of UC-MSCs, and pretreatment had no effect on their proliferation; C: Cell viability was determined by an acridine orange (AO) propidium iodide (PI) staining cell image analyzer, and no significant difference between UC-MSCs and PUC-MSCs was observed; D: Cell size was determined with a cell imaging analyzer, and the sizes of the UC-MSCs and PUC-MSCs were comparable. $^{d}P > 0.05$.

JC-1 red/green fluorescence ratio by flow cytometry and confirmed our microscopy results (Figure 3D). These results showed that mitochondrial function in UC-MSCs was not damaged by hypoxia and inflammatory factor pretreatment.

Hypoxia and inflammatory factor preconditioning induces UC-MSC apoptosis and senescence

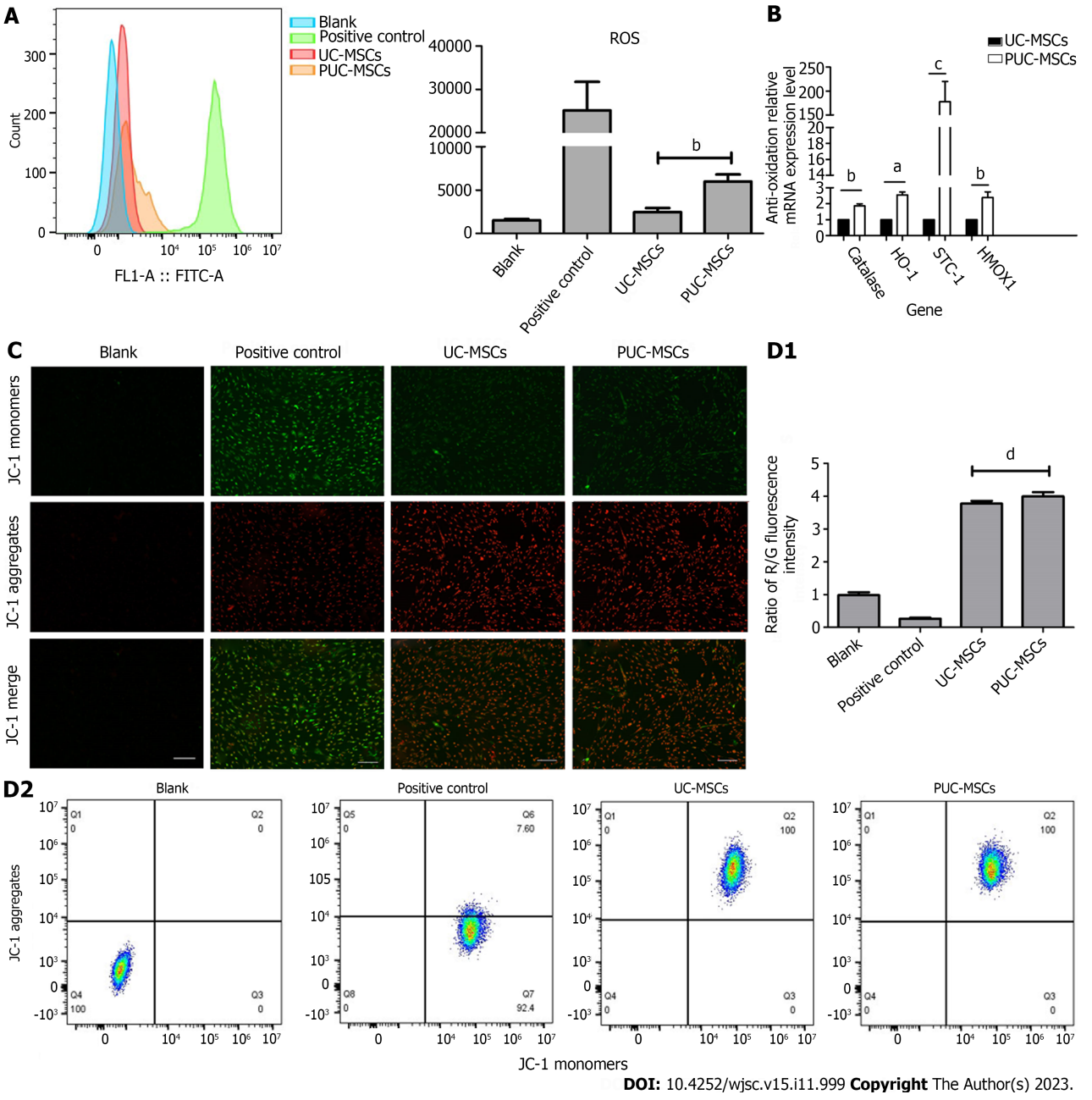
Bax expression decreased and Bcl2 and SIRT1 expression increased after pretreatment, suggesting that the anti-apoptotic capacity increased after pretreatment (Figure 4A). The expression of the senescence-related genes P53, P16 and P21 was upregulated by pretreatment (Figure 4B). We analyzed apoptosis by Annexin V-PI staining and flow cytometry. We compared the proportion of cells that were positive for both Annexin V-FITC and PI (necrotic cells), and the apoptotic index of PUC-MSCs (15.08% ± 4.11%) significantly increased (Figure 4C) compared to UC-MSCs (6.22% ± 3.03%). Next, we investigated the effect of preconditioning on cell senescence. SA-β-gal has been the most widely accepted senescence marker since Dimri first published its use in 1995[46], so we performed SA-β-gal staining to evaluate cell senescence ratios. SA-β-gal levels increased in PUC-MSCs (14.83% ± 1.57%) compared to UC-MSCs (4.83% ± 1.34%) (Figure 4D). These results suggest that hypoxia and inflammatory factor preconditioning not only induces UC-MSC apoptosis and senescence but also increases anti-apoptotic factor levels.



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Figure 2 Phenotypes of mesenchymal stem cells were detected by flow cytometry. A: Cell surface markers of umbilical cord mesenchymal stem cells

(UC-MSCs); B: Cell surface markers of primed umbilical cord mesenchymal stem cells (PUC-MSCs). The signals from the unstained control cells are shown in blue in the histogram, and the signals from stained cells are shown in red in the histogram. HLA: Human leukocyte antigen.

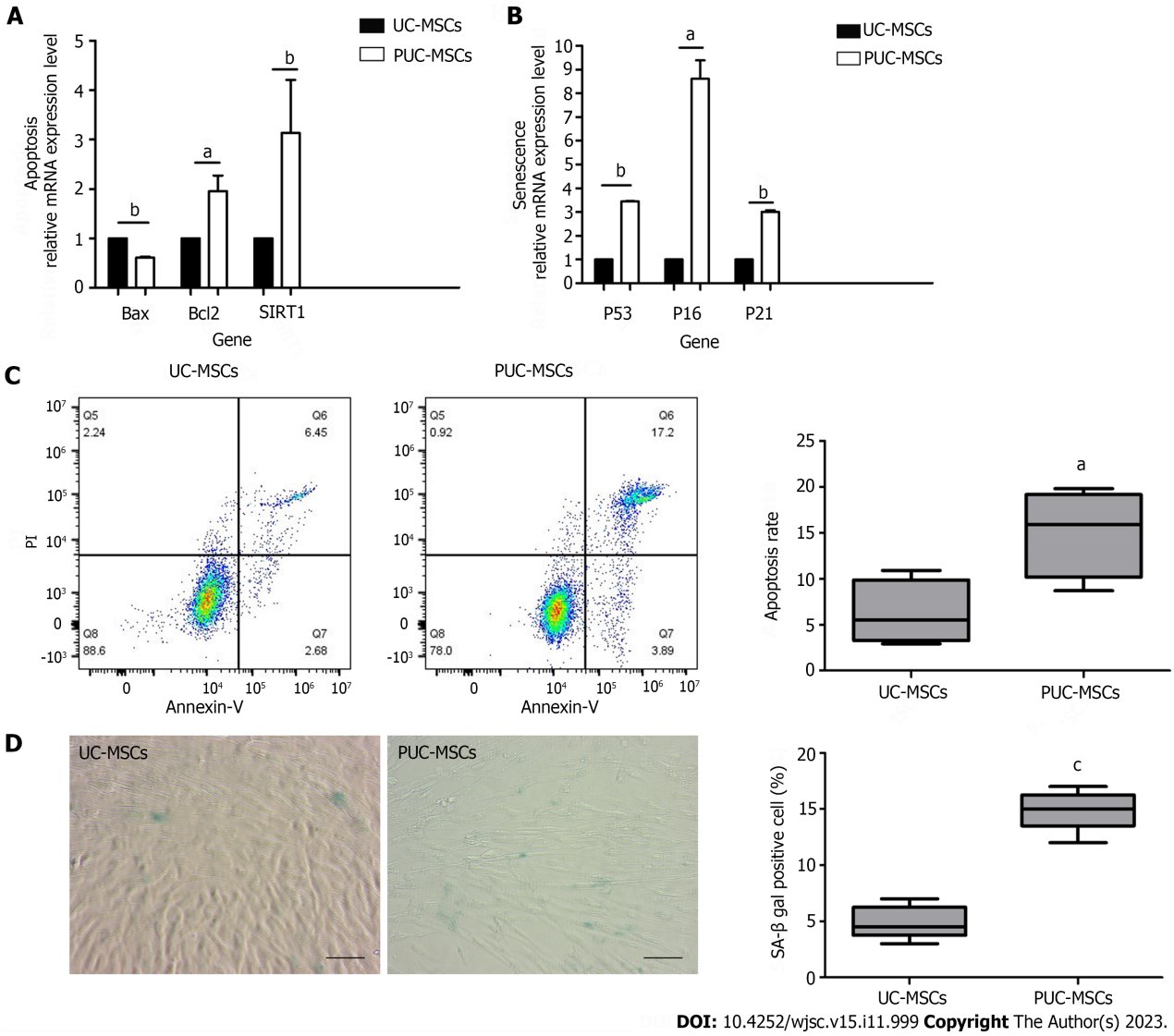


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Figure 3 Mitochondrial function. A: Reactive oxygen species (ROS) levels in umbilical cord mesenchymal stem cells (UC-MSCs) and primed UC-MSCs (PUC-MSCs) were analyzed by flow cytometry. The fluorescence intensity of 2,7-dichlorodihydrofluorescein (DCFH-DA) in PUC-MSCs increased by 3-fold compared to UC-MSCs; B: The expression of the antioxidant-related genes catalase, stanniocalcin-1 (STC1), and hemeoxygenase-1 (HOMX1) was increased; C: Representative image showing the mitochondrial membrane potential (MMP) of mesenchymal stem cells stained with JC-1. The red fluorescence of potential-dependent JC-1 aggregation in each group and the green fluorescence of the JC-1 monomer in the cytoplasm after depolarization of the mitochondrial membrane were observed. Fluorescence microscopy showed no significant difference in red and green fluorescence between UC-MSCs and PU-MSCs; Bar = 200 μm; D: Flow cytometry was used to detect the red and green fluorescence of the JC-1 probe in UC-MSCs and PUC-MSCs. There was no difference in the ratios of red and green fluorescence. [a] $P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]$P > 0.05$.

Hypoxia and inflammatory factor preconditioning enhances UC-MSC immunosuppressive properties

MSCs are pluripotent stem cells that have been shown to hold promise in tissue regeneration due to their ability to self-renew and differentiate and their broad immunomodulatory properties[1-5]. We evaluated whether UC-MSC immunomodulatory activities increased after pretreatment. qRT-PCR analysis revealed that the expression of PGE2, IDO, KYN, COX2, IL-10, TGF-β1, TSG-6, HLA-G5, and PD-L1 increased in PUC-MSCs compared to untreated UC-MSCs. IL-1ra expression was slightly elevated, but the difference was not statistically significant (Figure 5A). Because paracrine activity



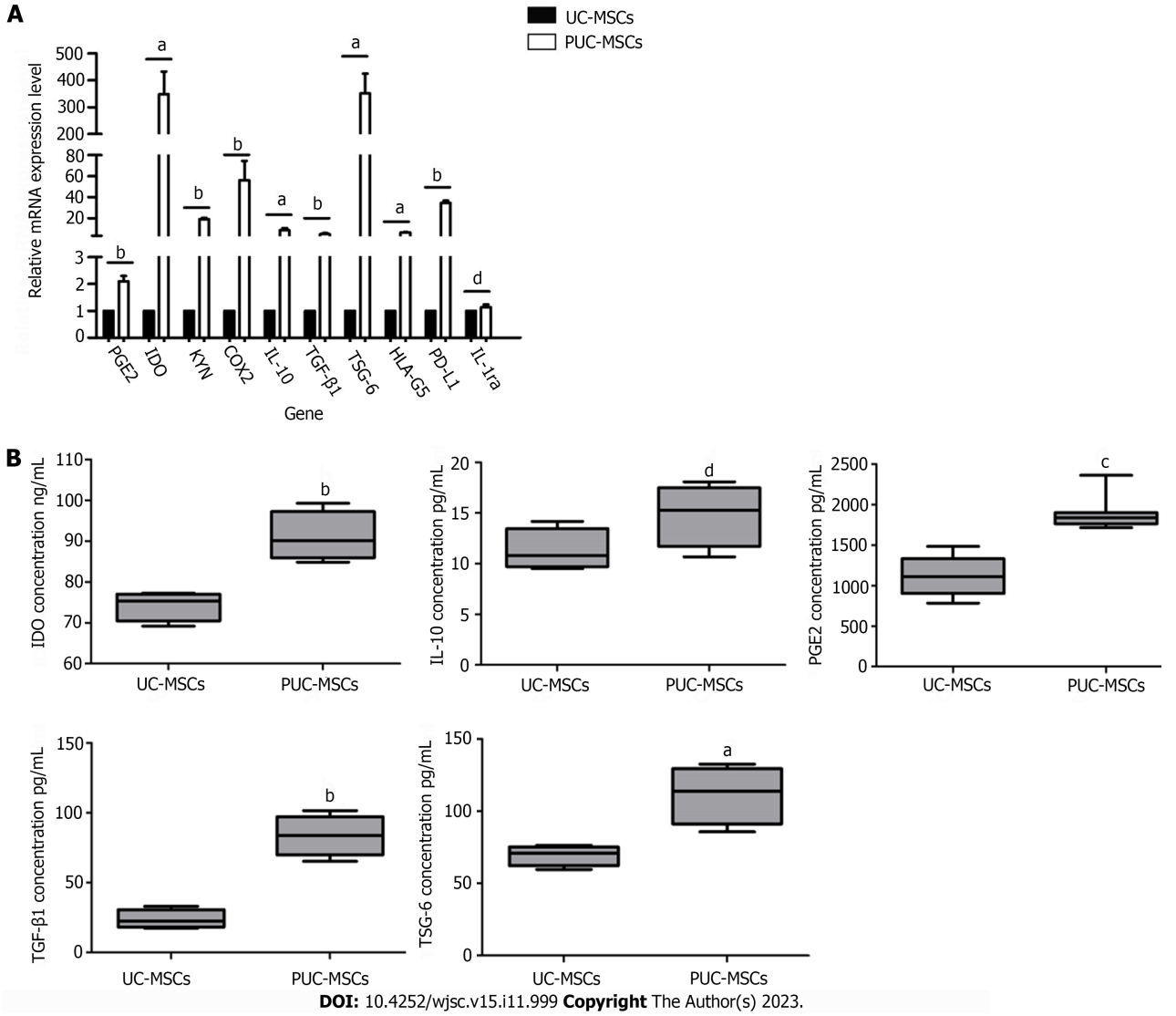
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Figure 4 Effects of pretreatment on apoptosis and senescence in mesenchymal stem cells. A: Apoptosis-related gene analysis showed that the expression of B-cell lymphoma 2 (BCL-2)-associated protein (Bax) was decreased and the expression of Bcl2 and silencing information regulator 2-related enzyme 1 (SIRT1) was increased after hypoxia and inflammatory factor pretreatment; B: Expression of P53, P16 and P21 was upregulated after hypoxia and inflammatory factor pretreatment; C: Umbilical cord mesenchymal stem cells (UC-MSCs) and primed UC-MSCs (PUC-MSCs) were stained with Annexin V-FITC apoptosis assay kit reagents and apoptotic cells were detected by flow cytometry. The results showed that the apoptosis index of PUC-MSCs increased; D: Representative images of β-galactosidase (SA-β-gal) staining and quantitative analysis of positive SA-β-gal staining. Compared with that of the UC-MSCs, the number of SA-β-gal-positive PUC-MSCs was significantly increased; Bar = 100 μm. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

is a key mechanism underlying MSC effects, we compared the bioactive factor secretion levels in PUC-MSCs and untreated UC-MSCs. We assessed MSC paracrine function by measuring the protein levels of immunosuppression-related soluble factors released into the culture supernatant, including IDO, PGE2, TGF-β1, TSG-6, and IL-10 by ELISAs. IDO, PGE2, TGF-β1 and TSG-6 levels significantly increased in the PUC-MSC supernatant, while IL-10 levels slightly increased but did not reach statistical significance (Figure 5B). In summary, these data strongly suggest that UC-MSC preconditioning with hypoxia and inflammatory factors upregulates the expression of soluble immunomodulatory factors and enhances their immunomodulatory activity.

Hypoxia and inflammatory factor preconditioning increases UC-MSC immunosuppressive properties

Considering the high expression of immunomodulatory molecules in PUC-MSCs, we evaluated their immunosuppressive capacity. We compared UC-MSC and PUC-MSC immunosuppressive capacity using PBMC and NK cell proliferation assays by analyzing growth dynamics of PBMCs cultured alone, with UC-MSCs or with PUC-MSCs (Figure 6A). Both UC-MSCs and PUC-MSCs significantly inhibited PBMC proliferation compared to PBMCs cultured alone. However, PUC-MSCs had a stronger inhibitory effect on PBMC proliferation, further indicating that pretreatment enhanced UC-MSC immunosuppressive abilities. We further investigated MSC-mediated inhibition of NK cell proliferation in the presence of the two MSC populations. We cultured CFSE-stained NK cells in the presence of IL-2 alone or with UC-MSCs or PUC-MSCs and analyzed CFSE fluorescence intensity by flow cytometry after 3 d. The proliferation rate of NK cells was reduced in cocultures with UC-MSCs or PUC-MSCs compared to NK cells cultured alone, but the PUC-MSC group



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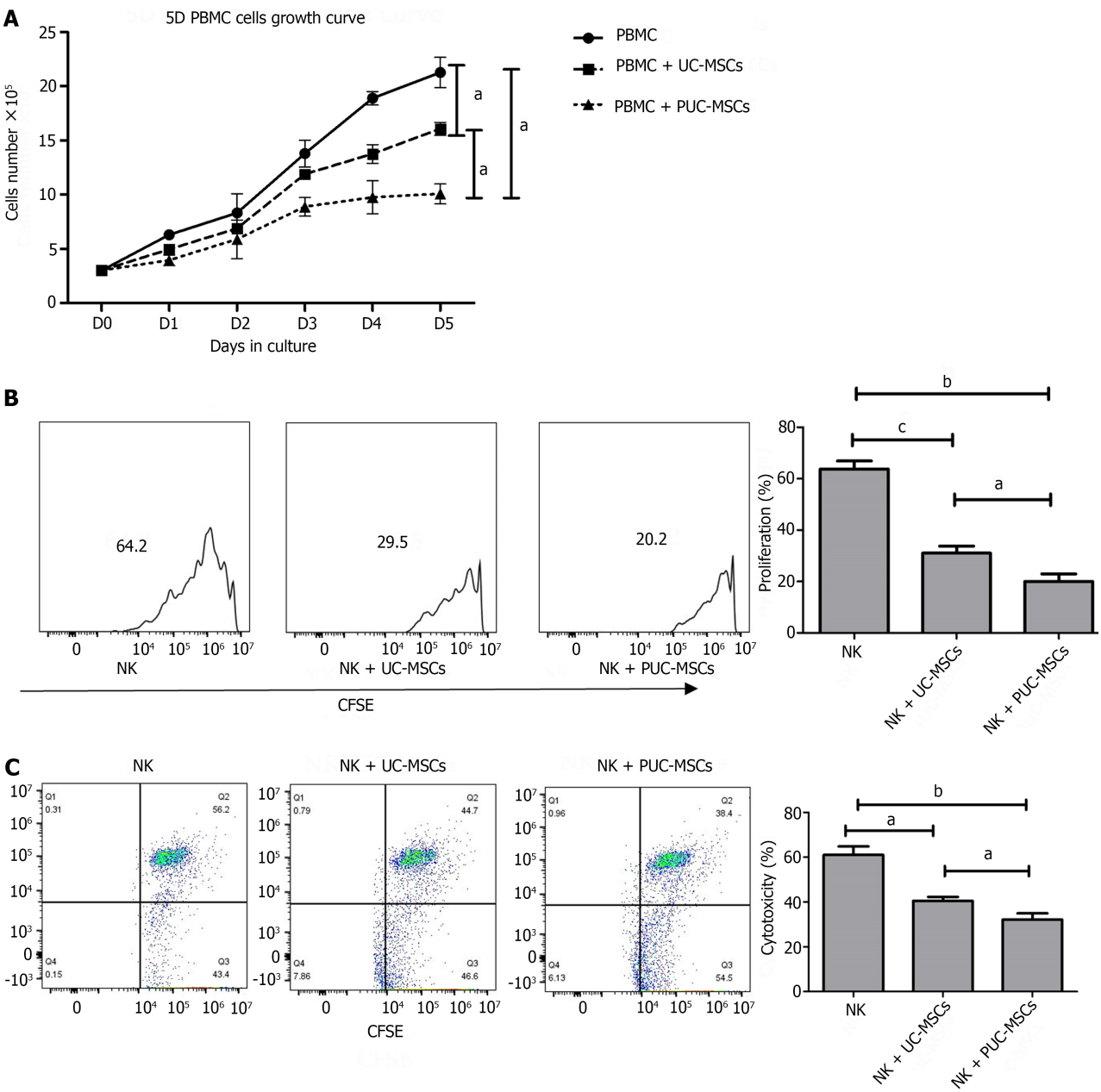
Figure 5 Relative expression of immunomodulatory genes and proteins in umbilical cord mesenchymal stem cells after pretreatment. A: Gene expression levels of umbilical cord mesenchymal stem cells (UC-MSCs) and primed UC-MSCs (PUC-MSCs) were analyzed by quantitative real-time polymerase chain reaction. Expression levels of prostaglandin E2 (PGE2), kynurenine, idoleamine-2,3-dioxygenase (IDO), cyclooxygenaese-2 (COX2), interleukin-10 (IL-10), transforming growth factor-beta 1 (TGF-β1), human leukocyte antigen-G5 (HLA-G5), tumor necrosis factor-α-induced protein-6 (TSG-6) and ligands for programmed cell death 1 (PD-L1) were all increased after hypoxia and inflammatory factor preconditioning. IL-1 receptor antagonist (IL-1ra) was not significantly different between the two groups; B: Levels of immunoregulatory proteins in UC-MSCs and PUC-MSCs were detected by enzyme-linked immunosorbent assay. After hypoxia and inflammatory factor pretreatment, the protein levels of IDO, PGE2, TGF-β1 and TSG-6 were increased, while the expression of IL-10 was not significantly different, as determined *via* three independent experiments. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P > 0.05$.

exerted stronger inhibitory effects on NK cells compared to UC-MSCs (Figure 6B).

Finally, we evaluated the effect of UC-MSC pretreatment with hypoxia exposure and inflammatory factor treatment on NK cell cytotoxic activity. NK cells were cultured alone or with UC-MSCs/PUC-MSCs at a 3:1 ratio for 72 h, then collected and incubated with CFSE-stained K562 target cells at an effective target ratio (E:T = 1:5) for 4 h. The cells were collected, and K562 cell apoptosis was analyzed by PI staining. The results showed that NK cells cocultured with UC-MSCs or PUC-MSCs had lower cytolytic activity, and the inhibition of NK cell-mediated cytotoxic activity was higher with PUC-MSCs than UC-MSCs (Figure 6C).

DISCUSSION

Intravenous injection of MSCs has been increasingly used in clinical research and has shown great potential in the treatment of various diseases. Because of their immunomodulatory functions, MSCs have become a promising alternative treatment for inflammatory diseases. MSCs come from a wide range of sources. Currently, the most studied MSCs are derived from bone marrow and adipose tissue, followed by those from umbilical cord tissue[47]. Compared with those from other sources, umbilical cord-derived MSCs are abundant, easy to collect, genetically stable, and do not readily



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Figure 6 Detection of the immunomodulatory function of umbilical cord mesenchymal stem cells after preconditioning. A: Peripheral blood mononuclear cells (PBMCs) alone or directly exposed to umbilical cord mesenchymal stem cells (UC-MSCs/primed UC-MSCs) for 5 d in a 3:1 ratio. The number of PBMCs in three groups was measured daily and the growth curve was plotted; B: Carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained natural killer (NK) cells were cultured alone or cocultured with UC-MSCs/PUC-MSCs at a 3:1 ratio for 72 h. NK cells were harvested, and the fluorescence intensity of CFSE was measured by flow cytometry to measure the proliferation rate of NK cells; C: NK cells were collected and incubated with CFSE-stained K562 target cells for 4 h at an effector ratio (E:T = 1:5). Effector cell (NK cell)-mediated cytotoxicity of K562 cells was analyzed by flow cytometry. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; $^{d}P > 0.05$.

mutate. Therefore, umbilical cord MSCs have broad application prospects in the cell therapy field[48-50]. Therefore, we focused on UC-MSCs in this study.

In addition to the effects of their origin, MSCs exhibit decreased biological performance when subjected to vein transplantation and exposure to the harsh inflammatory environment of damaged tissue[29]. Studies have shown that preconditioning cells by exposing them to the external environment can enhance their therapeutic effects by preparing them for the harsh conditions they encounter when injected into the body. Antebi *et al*[51] showed that hypoxic preconditioning of bone marrow MSCs enhanced the therapeutic function of these cells. Gorgun *et al*[34] analyzed the effects of hypoxia and inflammatory factor (TNF-α, IL-1α) pretreatment on AD-MSC angiogenic potential. In addition, Rodriguez *et al*[52] pretreated AD-MSCs with a mixture of inflammatory factors under anoxic culture conditions, which significantly enhanced their functional characteristics and immunosuppressive and immunoregulatory functions.

In this study, we developed a combination strategy of hypoxia plus a mixture of TNF-α, IL-1β, and IFN-γ preconditioning to enhance hUC-MSC immunomodulatory capacity. To our knowledge, this is a previously unanalyzed combination pretreatment. We found that this preconditioning approach successfully mimicked the harsh inflammatory

environment and effectively enhanced the immunosuppressive function of MSCs. To simulate hypoxia, we cultured UC-MSCs in a special three-gas incubator containing 2% O_2, 5% CO_2 and 93% N_2 at 37°C for 24 h. We had previously evaluated these gas conditions and found them to be most suitable for preserving the functional characteristics of MSCs. To simulate the harsh inflammatory environment, we independently developed and utilized a mixture of IL-1β, IFN-γ and TNF-α. After pretreatment, we found that the cells became elongated, but their proliferation, viability and size did not significantly change. Notably, Klinker *et al*[53] recently demonstrated that the morphological characteristics of MSCs were significantly correlated with their immunosuppressive ability and can be used to predict their overall immunosuppressive effects[53]. The results of the study by Klinker *et al*[53] showed that pretreated UC-MSCs became elongated and had stronger immunosuppressive ability, consistent with the results of our study.

Although different forms of preconditioning have been shown to successfully enhance cell properties and increase cell function, there are still noteworthy safety concerns. Because pretreatment may have negative effects on cell function, we used flow cytometry to characterize MSC phenotypes. The expression of specific MSC surface markers can be used as indicators of cell differentiation potential, lineage commitment, aging, and therapeutic function[54]. The International Society for Cell and Gene Therapy defines minimum standards for MSC characterization, and most experiments are conducted on this basis[55]. However, we examined additional MSC surface markers. We found that UC-MSCs and PUC-MSCs from three donors retained high expression levels of CD105, CD90, CD73, CD29, CD166, CD47 and HLA-ABC and were negative for CD31, CD45, CD14, and CD34 expression. These findings indicated that UC-MSCs retained MSC properties after pretreatment. Interestingly, we found that CD142 expression significantly decreased after pretreatment. Oeller *et al*[39] found that UC-MSCs showed higher procoagulant activity than adipose-derived MSCs, and UC-MSCs showed extensive TF (CD142) expression and long-lasting clotting; a higher cell number significantly increased clot formation, which was partially dependent on coagulation factors[43]. Our study showed that CD142 expression in UC-MSCs without treatment exceeded 99% and CD142 expression significantly decreased after pretreatment (59.3%). Our experiment showed significant decreases in TF expression, which has been shown to be closely related to procoagulant activity[38]. However, Rodriguez *et al*[52] demonstrated that bone marrow-derived MSCs (BM-MSCs) and AD-MSCs that were pretreated with a combination of hypoxia and inflammatory factors showed increased CD142 expression, which was inconsistent with previous studies[53]. We determined that these discrepancies may be due to different origins of MSCs, MSC heterogeneity and/or differences between culture systems. However, we found pretreatment significantly reduced CD142 expression. In addition, studies have suggested the use of anticoagulants or genetic methods to inhibit TF activity for the clinical application of MSCs to maximize clinical benefits for patients, which also underscores the importance of insights into the mechanisms underlying safety issues related to nonhematopoietic cell transplantation[43].

The efficacy of MSCs depends on the full function of their mitochondria, which can be damaged after cell exposure to harmful environments[56]. Therefore, we examined the effects of hypoxia and inflammatory factor pretreatment on mitochondrial function. ROS levels were increased after pretreatment but were within the range of those in the positive controls. In addition, the increased expression of antioxidants such as STC1, catalase and HOMX1 can eliminate ROS to maintain cell redox homeostasis[57]. Previous studies have shown that both HMOX1 and catalase play key roles in protecting cells from ROS-induced damage[58,59]. Therefore, although ROS levels increased after pretreatment, the antioxidant capacity of the cells also correspondingly increased and the ROS removal rate increased. In addition, the MMP (Δψm) plays a key role in important mitochondrial functions, and its dissipation is an indicator of mitochondrial dysfunction[60]. Maintaining a stable MMP (Ψm) is essential for ensuring efficient ROS clearance and preventing apoptosis or other stress-related events caused by excessive ROS. Therefore, we examined the MMP and found that pretreatment had no effect on MMP. In summary, although ROS levels increased after pretreatment, the antioxidant capacity also increased, and the MMP did not change, indicating that there was no damage to mitochondrial function after pretreatment.

We next examined the effect of pretreatment on MSC apoptosis and senescence and found that the apoptosis index of UC-MSCs increased to a certain extent after pretreatment. Galleu *et al*[61] showed that all patients can receive apoptotic MSCs for *in vivo* injection and that these cells can induce receptor-mediated immune regulation. MSC apoptosis of is critical to their therapeutic functions[62]. BCL-2, BAX and SIRT1 genes play important roles in the apoptosis pathway. In this study, we found increased expression of the SIRT1 gene, which protected cells from apoptosis by activating autophagy[63]. The ratio of BAX/BCL-2 is closely related to the apoptosis potential of cells[64]. In this study, an inverse proportional relationship between BAX and BCL-2 indicated that MSCs exerted stronger anti-apoptosis effects after pretreatment. This may indicate that the cells were protecting themselves from the harmful inflammatory environment. In addition, MSCs showed increased SA-β-gal activity and p53, P16 and p21 expression after hypoxia and inflammatory factor pretreatment, suggesting that MSCs underwent senescence after pretreatment. Salminen *et al*[65] found that aging MSCs exert stronger immunosuppressive effects. The experimental results showed that even when some of the pretreated UC-MSCs underwent senescence and apoptosis, these effects did not reduce their immunoregulatory abilities, and even apoptotic and senescent MSCs showed increased immunosuppressive abilities.

The pleiotropic effect of MSCs is mostly mediated by soluble paracrine factors, and active paracrine factors produced by these cells regulate cellular immunity when they come into contact with the host[66]. Therefore, we investigated the response of paracrine factors involved in the immune regulation of MSCs to preconditioning. Many bioactive molecules produced by MSCs, such as IDO, PGE2, IL-10, TSG-6, and TGF-β1, effectively regulate innate and adaptive immunity and play a key role in the immunosuppressive effect of MSCs[67]. The detection of immunomodulation-related genes showed that the expression levels of PGE2, KYN, IDO, COX2, IL-10, TGF-β1, TSG-6, HLA-G5 and PD-L1 were significantly increased in addition to that of IL-1ra. In addition, PGE2, TSG-6, TGF-β1 and IDO protein levels in the supernatant were significantly increased. IL-10 levels were slightly increased, but the difference between the pretreatment and control groups was not significant. These results showed that preconditioning can promote the production of immunomodulatory paracrine factors in UC-MSCs and increase their immunomodulatory effect.

Finally, we studied the immunosuppressive effects of MSCs on PBMCs and NK cells. We found that UC-MSCs and PUC-MSCs inhibited PBMCs and NK cell proliferation, but PUC-MSCs exerted a stronger inhibitory effect. Some work has been done to characterize the interaction between BM-MSCs and NK cells[22,68,69]. However, the interaction of UC-MSCs with NK cells has been largely unexplored. Our results add to our understanding of the immunosuppressive effects of UC-MSCs[70,71]. In addition, we found that PUC-MSCs exerted a stronger inhibitory effect on NK cell-mediated cytotoxic activity. Reportedly, IDO and PGE2 or TGF-β1 produced by MSCs are critical for inhibiting NK cell cytotoxicity[22,72]. PUC-MSCs may exert stronger inhibitory effects on NK cell-mediated toxicity due to increased IDO, TGF-β1 and PGE2 expression.

We acknowledge that this study has limitations. Whether the combination of hypoxia (2% O_2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) is superior to other preconditioning methods is unclear. However, our study shows a preconditioning strategy that adds to the existing experimental options to use in further research. In addition, whether these cells exhibit superior immunomodulatory functions under harsh *in vivo* inflammatory conditions remains to be demonstrated and is the focus of our future work. Moreover, although TF expression is reduced by preconditioning, the mechanism is unclear. Thromboembolism may still have significant clinical consequences for patients who receive MSCs after preconditioning. Therefore, for whole-body cell administration, it is still necessary to use anticoagulants during the clinical application of MSCs to inhibit TF activity and increase the clinical benefits to patients.

CONCLUSION

In conclusion, we successfully developed an *in vitro* preconditioning method that mimics the impaired environment through a combination of hypoxia (2% O_2) and inflammatory factors (IL-1β, TNF-α, IFN-γ) to enhance UC-MSC immunosuppressive ability without compromising their biological characteristics. Most notably, this approach greatly reduced the expression of the clotting-related TF in MSCs, which was a surprising result.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have great potential in the treatment of a variety of immune-related diseases due to their unique immunomodulatory and anti-inflammatory abilities. However, after intravenous transplantation, MSCs cannot effectively exert their biological effects when they encounter a harsh environment *in vivo*, which reduces the efficacy of cell therapy. To increase transplantation efficacy, appropriate pretreatment methods are particularly important.

Research motivation

Although a variety of pretreatment methods are used to increase MSC transplantation efficacy, suitable and effective *in vitro* pretreatment methods are still worth studying.

Research objectives

To evaluate whether umbilical cord MSCs (UC-MSCs) pretreated with hypoxia exposure and inflammatory factors show enhanced immunosuppressive effects without affecting cell biological characteristics.

Research methods

In this study, we used a combination of hypoxia (2% O_2) and inflammatory factors (interleukin-1β, tumor necrosis factor-α , interferon-γ) to pretreat UC-MSCs for 24 h to simulate the *in vivo* injury environment. We then comprehensively evaluated the biological properties of pretreated UC-MSCs and investigated their immunosuppressive properties.

Research results

Our results showed that compared to UC-MSCs, pretreated UC-MSCs were morphologically elongated, but their viability, proliferation and size were not affected, the expression of coagulation-related tissue factors was significantly reduced, and mitochondria maintained their function and integrity. Although some cells underwent apoptosis or senescence, polymerase chain reactions and enzyme-linked immunosorbent assays revealed a significant increase in the levels of immunomodulation-related factors. Coculture with peripheral blood mononuclear cell and natural killer cells exerted a stronger immunosuppressive effect.

Research conclusions

The combined pretreatment of hypoxia exposure and inflammatory factors enhanced the immunosuppressive ability of MSCs but did not affect the biological characteristics of these cells.

Research perspectives

Our study provides new strategies for the preconditioning of UC-MSCs.

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FOOTNOTES

Author contributions: Li H, Ji XQ, Zhang SM, and Bi RH designed and coordinated the study; Li H performed experiments and wrote the manuscript; Ji XQ acquired and analyzed the data; Bi RH contributed to ideas, supervision, review and editing; All authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Dissecting molecular mechanisms underlying ferroptosis in human umbilical cord mesenchymal stem cells: Role of cystathionine γ-lyase/hydrogen sulfide pathway

Bin Hu, Xiang-Xi Zhang, Tao Zhang, Wan-Cheng Yu

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Abstract

BACKGROUND

Ferroptosis can induce low retention and engraftment after mesenchymal stem cell (MSC) delivery, which is considered a major challenge to the effectiveness of MSC-based pulmonary arterial hypertension (PAH) therapy. Interestingly, the cystathionine γ-lyase (CSE)/hydrogen sulfide (H_2S) pathway may contribute to mediating ferroptosis. However, the influence of the CSE/H_2S pathway on ferroptosis in human umbilical cord MSCs (HUCMSCs) remains unclear.

AIM

To clarify whether the effect of HUCMSCs on vascular remodelling in PAH mice is affected by CSE/H_2S pathway-mediated ferroptosis, and to investigate the functions of the CSE/H_2S pathway in ferroptosis in HUCMSCs and the underlying mechanisms.

METHODS

Erastin and ferrostatin-1 (Fer-1) were used to induce and inhibit ferroptosis, respectively. HUCMSCs were transfected with a vector to overexpress or inhibit expression of CSE. A PAH mouse model was established using 4-wk-old male BALB/c nude mice under hypoxic conditions, and pulmonary pressure and vascular remodelling were measured. The survival of HUCMSCs after delivery was observed by *in vivo* bioluminescence imaging. Cell viability, iron accumulation, reactive oxygen species production, cystine uptake, and lipid peroxidation in HUCMSCs were tested. Ferroptosis-related proteins and S-sulfhydrated Kelch-like ECH-associating protein 1 (Keap1) were detected by western blot analysis.

RESULTS

In vivo, CSE overexpression improved cell survival after erastin-treated HUCMSC delivery in mice with hypoxia-induced PAH. *In vitro*, CSE overexpression improved H_2S production and ferroptosis-related indexes, such as cell viability, iron level, reactive oxygen species production, cystine uptake, lipid peroxidation, mitochondrial membrane density, and ferroptosis-related protein expression, in erastin-treated HUCMSCs. In contrast, *in vivo*, CSE inhibition decreased cell survival after Fer-1-treated HUCMSC delivery and aggravated vascular remodelling in PAH mice. *In vitro*, CSE inhibition decreased H_2S levels and restored ferroptosis in Fer-1-treated HUCMSCs. Interestingly, upregulation of the CSE/H_2S pathway induced Keap1 S-sulfhydration, which contributed to the inhibition of ferroptosis.

CONCLUSION

Regulation of the CSE/H_2S pathway in HUCMSCs contributes to the inhibition of ferroptosis and improves the suppressive effect on vascular remodelling in mice with hypoxia-induced PAH. Moreover, the protective effect of the CSE/H_2S pathway against ferroptosis in HUCMSCs is mediated *via* S-sulfhydrated Keap1/nuclear factor erythroid 2-related factor 2 signalling. The present study may provide a novel therapeutic avenue for improving the protective capacity of transplanted MSCs in PAH.

Key Words: Human umbilical cord mesenchymal stem cells; Cystathionine γ-lyase/hydrogen sulfide pathway; Ferroptosis; Pulmonary arterial hypertension; S-sulfhydration

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Core Tip: Regulation of the cystathionine γ-lyase (CSE)/hydrogen sulfide (H_2S) pathway in human umbilical cord mesenchymal stem cells (HUCMSCs) contributes to the inhibition of ferroptosis and improves the suppressive effect of HUCMSCs on vascular remodelling in hypoxia-induced pulmonary arterial hypertension (PAH) mice. Moreover, the protective effect of the CSE/H_2S pathway against ferroptosis in HUCMSCs was mediated *via* S-sulfhydrated Kelch-like ECH-associating protein 1/nuclear factor erythroid 2-related factor 2 signalling. The present study may provide a novel therapeutic avenue for improving the protective capacity of transplanted MSCs in PAH.

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INTRODUCTION

Mesenchymal stem cells (MSCs), derived from placenta, bone marrow, adipose, or other tissues, have emerged as a new regenerative therapy for pulmonary arterial hypertension (PAH) over the last decade[1]. MSC delivery significantly inhibits pulmonary artery remodelling, resulting in the reduced progression of PAH. However, low cell retention and engraftment after cell delivery are considered major challenges to the effectiveness of MSC-based therapy[2]. Interestingly, increasing evidence indicates that programmed cell death (PCD) is closely linked to the low engraftment and survival rates of transplanted MSCs[3].

Ferroptosis is a novel type of PCD with distinct properties. Ferroptosis essentially consists of polyunsaturated fatty acid-containing phospholipid synthesis and peroxidation, iron metabolism, and mitochondrial metabolism, which result in oxidative stress and lipid peroxidation, triggering cell death[4]. Studies have shown a relationship between ferroptosis and MSCs; ferroptosis can be induced by erastin in MSCs[5,6]. Inhibiting ferroptosis maintained the viability and differentiation of MSCs[7,8]. Moreover, suppressing ferroptosis worsened MSC fate and enhanced the exposure and efficacy of transplanted MSCs in live disease[9]. However, few studies have directly evaluated the effect of ferroptosis on MSC delivery in PAH.

Decreased antioxidative stress can cause ferroptosis[10]. In addition, the mediation of antioxidative stress-related proteins, such as nuclear factor erythroid 2-related factor 2 (Nrf2), can inhibit ferroptosis[11]. Thus, understanding the signalling pathways that maintain redox balance will provide new approaches for inhibiting ferroptosis. The novel gaseous mediator hydrogen sulfide (H_2S) has been shown to exert protective effects, including its antioxidant properties [12]. Therefore, the impact of H_2S on the contribution of antioxidative stress to decreased ferroptosis is worth exploring. In mammalian cells, cystathionine γ-lyase (CSE) is predominantly responsible for endogenous H_2S production in the cardiovascular system[13]. However, the effect of CSE on the ferroptosis of MSCs remains unclear.

Hence, the present study aimed to clarify whether ferroptosis is involved in the therapeutic effect of human umbilical cord MSCs (HUCMSCs) in PAH mice. Furthermore, we investigated the role of the CSE/H_2S pathway in ferroptosis in HUCMSCs.

MATERIALS AND METHODS

Cell culture

Primary HUCMSCs were purchased from ScienCell Research Laboratories, Inc. HUCMSCs were cultured in MSC medium (7501, ScienCell Research Laboratories, United States) containing 5% foetal bovine serum, 1% penicillin/streptomycin, and 1% MSC growth supplement at 37 °C in a 5% CO_2 atmosphere. HUCMSCs at passages three to five were used in this study.

Cell transfection

HUCMSCs were transduced with a lentiviral vector expressing firefly luciferase (HBLV-LUC-BSD, Hanbio, China) in serum-free medium containing 3 μg/mL blasticidin (SBR00022, Sigma-Aldrich, United States) at a multiplicity of infection of 100. Transduction was analysed by luciferase imaging 72 h after transfection. The expression of LUC was confirmed by measuring luciferase activity [*in vivo* imaging system (IVIS) Lumina imaging station, Caliper Life Sciences]. Surface markers and the differentiation of HUCMSCs-LUC into osteoblasts and adipocytes were evaluated as previously described[14]. The antibodies that were used are shown in Supplementary Table 1.

Short hairpin RNAs targeting CSE were designed, cloned, and inserted into a modified pLKO.1-TRC vector by GenePharma Co. Ltd. (Shanghai, CHN). To genetically engineer cells to overexpress CSE, the open reading frame of human CSE was cloned and inserted into the vector pLVX-IRES-Puro by GenePharma Co. Ltd. (Shanghai, CHN). HUCMSCs-LUC were transfected with the PLKO.1-sh-CSE vector (HUCMSC/sh-CSE), negative control vector (HUCMSCs/sh-NC), PLVX-CSE-Puro vector (HUCMSCs/CSE), or PLVX-empty-Puro vector (HUCMSCs/NC) using Lipofectamine 2000 (11668019, Invitrogen, United States). After 48 h, the transfection efficacy was determined using western blot and real-time polymerase chain reaction (RT-PCR) (Supplementary Figure 1).

Drug treatments

Erastin (S7242), ferrostatin-1 (Fer-1, S7243), and the Nrf2 inhibitor brusatol (S7956, 10 nM, 6 h) were purchased from Selleck Chemicals (Houston, United States) and initially dissolved in DMSO before being diluted in the culture medium to their final concentration.

Quantification of cell viability

Cell Counting Kit-8 (CCK8, 96992, Sigma-Aldrich, United States) was used to examine cell viability according to the manufacturer's instructions. Briefly, cells were seeded at a density of 5000 cells/well in 96-well plates in three replicates and incubated overnight. At the end of the different treatments, CCK8 reagent was added to each well and incubated for 2 h. The optical density (OD) values at 450 nm were measured using a plate reader (Bio-Rad).

Lactase dehydrogenase release assay

Lactate dehydrogenase (LDH) release was measured using an LDH cytotoxicity detection kit (CK12, Dojindo Laboratory, JPN) in accordance with the manufacturer's instructions to evaluate cell membrane integrity. In brief, cells were seeded at a density of 5000 cells/well in 96-well plates. At the end of the different treatments, 100 μL of fresh reaction mixture was added to each well and incubated for 30 min, and the OD values at 490 nm were measured using a microplate reader.

Animal experiments

Animal procedures were performed in compliance with the Institutional Animal Care and Use Committee of Wuhan Servicebio Technology Co., Ltd., China (No. 2022231). Four-week-old male mice (BALB/c nude mice) were used to establish a hypoxia-induced PAH mouse model. The mice were obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China) and cared for in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. All animals were housed in the same specific pathogen-free room under a constant temperature and humidity on a 12-h light and 12-h dark cycle, and the animals were fed sterile food and water *ad libitum*. Each animal was considered one experimental unit. Researchers who analysed the data were blinded to the group allocation until all the statistical results were finally obtained.

The mice (totally 42) were numbered and divided into the following seven groups based on a table of random numbers: Control group: The mice received phosphate buffered saline (PBS) (n = 6); hypoxia group: The mice were exposed to hypoxia under 10% O_2 for 4 wk (n = 6); HUCMSCs group: After 2 wk of exposure to hypoxia, the mice were injected with 1.5 × 10^5 HUCMSCs-LUC per mouse *via* the tail vein and then housed under hypoxia for 2 wk (n = 6); HUCMSCs + erastin group: After 2 wk of exposure to hypoxia, the mice were infected with 1.5 × 10^5 HUCMSCs-LUC that were treated with erastin (4 μM, 6 h) per mouse *via* the tail vein and then housed under hypoxia for 2 wk (n = 6); HUCMSCs/CSE + erastin group: After 2 wk of exposure to hypoxia, the mice were injected with 1.5 × 10^5 HUCMSCs-LUC/CSE that were treated with erastin (4 μM, 6 h) per mouse *via* the tail vein and then housed under hypoxia for 2 wk (n = 6); HUCMSCs + Fer-1 group: After 2 wk of exposure to hypoxia, the mice were injected with 1.5 × 10^5 HUCMSCs-LUC that were treated with Fer-1 (4 μM, 6 h) per mouse *via* the tail vein and then housed under hypoxia for 2 wk (n = 6); and HUCMSCs/sh-CSE + Fer-1 group: After 2 wk of exposure to hypoxia, the mice were injected with 1.5 × 10^5 HUCMSCs-LUC/sh-CSE that were treated with Fer-1 (4 μM, 6 h) per mouse *via* the tail vein and then housed under hypoxia for 2 wk (n = 6).

One day and seven days after intravenous injection *via* the tail, mice were anaesthetized using isoflurane and placed in an IVIS Lumina imaging station. Images of regions of interest were collected 10 min after the intraperitoneal injection of D-luciferin (L6152, Sigma-Aldrich, United States), the luciferase substrate, at 3.75 mg per 25 g mouse weight.

Measurement of pulmonary artery pressure and right ventricular hypertrophy

After 4 wk, the mice were anaesthetized using isoflurane for haemodynamic assessment. Briefly, a Miler catheter was used to obtain the right ventricular systolic pressure (RVSP) *via* insertion into the right external jugular vein. After exsanguination and collection of the blood, the cardiopulmonary tissues were removed. The right ventricle (RV) free wall and the left ventricle plus septum (LV + S) were weighed separately. The degree of right ventricular hypertrophy was determined by the ratio RV/(LV + S).

Assessment of pulmonary vascular morphometry

After the lung tissue was embedded in paraffin and cut into slices with a thickness of 5 μm, the slices were dewaxed, rehydrated, and stained with haematoxylin and eosin staining. Then, the ratio of the vascular medial cross-sectional area to the total arterial cross-sectional area was calculated as a measurement of distant small pulmonary artery (50-100 μm) remodelling.

For immunofluorescence staining of the lung tissue, after deparaffinization and washing with PBST, lung tissue slides were heated for 20 min at 95 °C in a Tris-EdTA solution for antigen retrieval and then blocked with 3% H_2O_2 for 15 min to block endogenous peroxidase activity and 5% goat serum for 1 h to eliminate nonspecific staining. These slides were incubated with VEcadherin rabbit polyclonal antibody (1:100, ab205336, Abcam) and alpha-smooth muscle actin rabbit monoclonal antibody (1:100, 19245S, CST) at 4 °C overnight and then incubated with the secondary antibody: Antirabbit immunoglobulin G (IgG) (1:500, 4413s, CST) or antirabbit IgG (1:500, 4412S, CST). Nuclei were counterstained with DAPI (D9542, Sigma-Aldrich, United States) at room temperature for another 2 min. Immunofluorescence was visualized under a microscope.

Analysis of cell apoptosis

After treatment, the cells were washed, trypsinized, resuspended, and stained with 5 μL of Annexin V/FITC (331200, Invitrogen, United States) and 5 μL of propidium iodide (PI, P1304MP, Invitrogen, United States) at room temperature for 15 min. Then, cell apoptosis was evaluated by flow cytometry.

Iron assays

Two different methods were used to measure the levels of intracellular ferrous iron. For a colorimetric method using the Iron Assay Kit (ab83366, Abcam, United Kingdom), cells were collected, homogenized (in cold iron assay buffer), and centrifuged. After adding 5 μL of iron reducer to each sample, the supernatant was incubated for 30 min at 37 °C. Next, 100 μL of iron probe was added to each sample and incubated for 60 min at 37 °C in the dark. The absorbance at 593 nm was measured using a microplate reader. For FeRhoNox-1 staining, cells were incubated away from light in a 37 °C, 5% CO_2 incubator for 40 min after the addition of 5 μM FeRhoNox-1 (MX4558, MKbio, CHN). Then, the cells were washed with PBS three times and imaged by fluorescence microscopy.

Reactive oxygen species assay

Intracellular reactive oxygen species (ROS) levels were measured by applying DCFH-DA (S0033, Beyotime, CHN). Briefly, after treatment, the cells were washed with PBS three times and treated with 10 μM DCFH-DA for 30 min away from light. Then, the cells were washed with PBS and observed under a fluorescence microscope.

Lipid peroxide measurement

Cells were labelled with BODIPY™ 581/591 C11 (D3861, Thermo Fisher Scientific, United States) at a final concentration of 2 μM for 20 min at 37 °C. After trypsinization and resuspension in PBS, lipid peroxidation was analysed using a flow cytometer. A lipid peroxidation [malonaldehyde (MDA)] assay (ab118970, Abcam, United Kingdom) was used according to the manufacturer's protocol to quantify the MDA concentration. The results were quantified colorimetrically (OD = 532 nm). The reduced glutathione/oxidized glutathione disulfide (GSH/GSSG) ratio in cells was determined using the GSH/GSSG Ratio Detection Assay Kit (ab205811, Abcam, United Kingdom) following the manufacturer's instructions.

Cystine uptake

Cystine uptake was measured with BioTracker Cystine-FITC Live Cell Dye (SCT047, Sigma-Aldrich, United States). The pretreated cells were incubated with complete medium containing 5 μM cystine-FITC in the dark for 30 min. Then, the cells were washed with PBS and harvested by trypsinization. The cells were resuspended in PBS and assessed by flow cytometry.

Transmission electron microscopy

Cells were fixed, postfixed, dehydrated, cut, and stained as previously described[15]. The cells were viewed under a transmission electron microscope (JEM1200, Tokyo, Japan), and images were taken.

Measurement of the H_2S concentration

The dye 7-azido-4-methylcoumarin (AzMC) was used to measure the H_2S concentration *in vitro*. Cells were plated at a density of 10000 cells per well in 96-well plates. After treatment, cells were loaded with the fluorogenic dye AzMC (802409, Sigma-Aldrich, United States) at 10 μM for 30 min. Then, the cells were washed three times with PBS and measured on a SpectraMax M5 plate reader.

Nrf2 activity assay

Nuclear lysates were isolated following extraction using NE-PER Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Fisher Scientific, United States) according to the manufacturer's instructions. Nrf2 transcriptional activity was then measured by ELISA using an Nrf2 Transcription Factor Assay Kit (ab207223, Abcam, United Kingdom) in accordance with the manufacturer's guidelines.

S-sulfhydration assay

Kelch-like ECH-associating protein 1 (Keap1) S-sulfhydration was detected with standard procedures using the "tag-switch" method[16]. The Keap1 protein was pulled down by immunoprecipitation. The proteins were dissolved in solution buffer, purified with streptavidin-agarose beads, and subjected to western blotting with anti-Keap1 (sc-365626, Santa Cruz Biotechnology, United States). Cells were treated with 1 mmol/L 1,4-dithio-DL-threitol (DTT, 43815, Sigma-Aldrich, United States) for 3 h as a negative control group.

Quantitative real-time polymerase chain reaction

qPCR was performed as previously described[15,17]. Human GAPDH expression was used for normalization. Fold changes were calculated using the comparative Ct method (ΔΔCt). The sequences of the primers used are as follows: CSE: Forward: GGCCTGGTGTCTGTTAATTGT and reverse: GCCATTCCGTTTTTGAAATGCT; GAPDH: forward: GACATCAAGAAGGTGGTGAAGCAGG and reverse: GTGTCGCTGTTGAAGTCAGAGGAGA.

Western blot analysis

Whole-cell, cytosolic, and nuclear proteins were extracted using RIPA Lysis and Extraction Buffer (89900, Thermo Fisher Scientific, United States) or NE-PER Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Fisher Scientific, United States). The extractions were performed as previously described[17]. Primary antibodies against the following were used: Glutathione peroxidase 4 (GPX4, 1:1000, 59735S, CST), intracellular ferritin heavy chain 1 (FTH1, 1:1000, 4393S, CST), nuclear receptor coactivator 4 (NOCA4, 1:1000, 66849S, CST), Fe^{3+}-bound transferrin receptor 1 (TFRC1, 1:1000, ab214039, Abcam), 4-hydroxynonenal (4-HNE, 1:1000, ab46545, Abcam), SLC7A11 (1:1000, ab175186, Abcam), Nrf2 (1:1000, ab62352, Abcam), Keap1 (sc-365626, Santa Cruz Biotechnology), CSE (1:1000, 19689S, CST), GAPDH (1:1000, 5174S, CST), and Histone H3 (1:1000, ab1791, Abcam). The intensity of the resulting bands was analysed with ImageJ software.

Statistical analysis

All experimental data are presented as the mean ± SEM. Statistical significance was analysed with GraphPad Prism version 5.01 (San Diego, CA, United States) using one-way analysis of variance (ANOVA). Differences at $P < 0.05$ were considered statistically significant. All experiments were performed with at least three independent replicates.

RESULTS

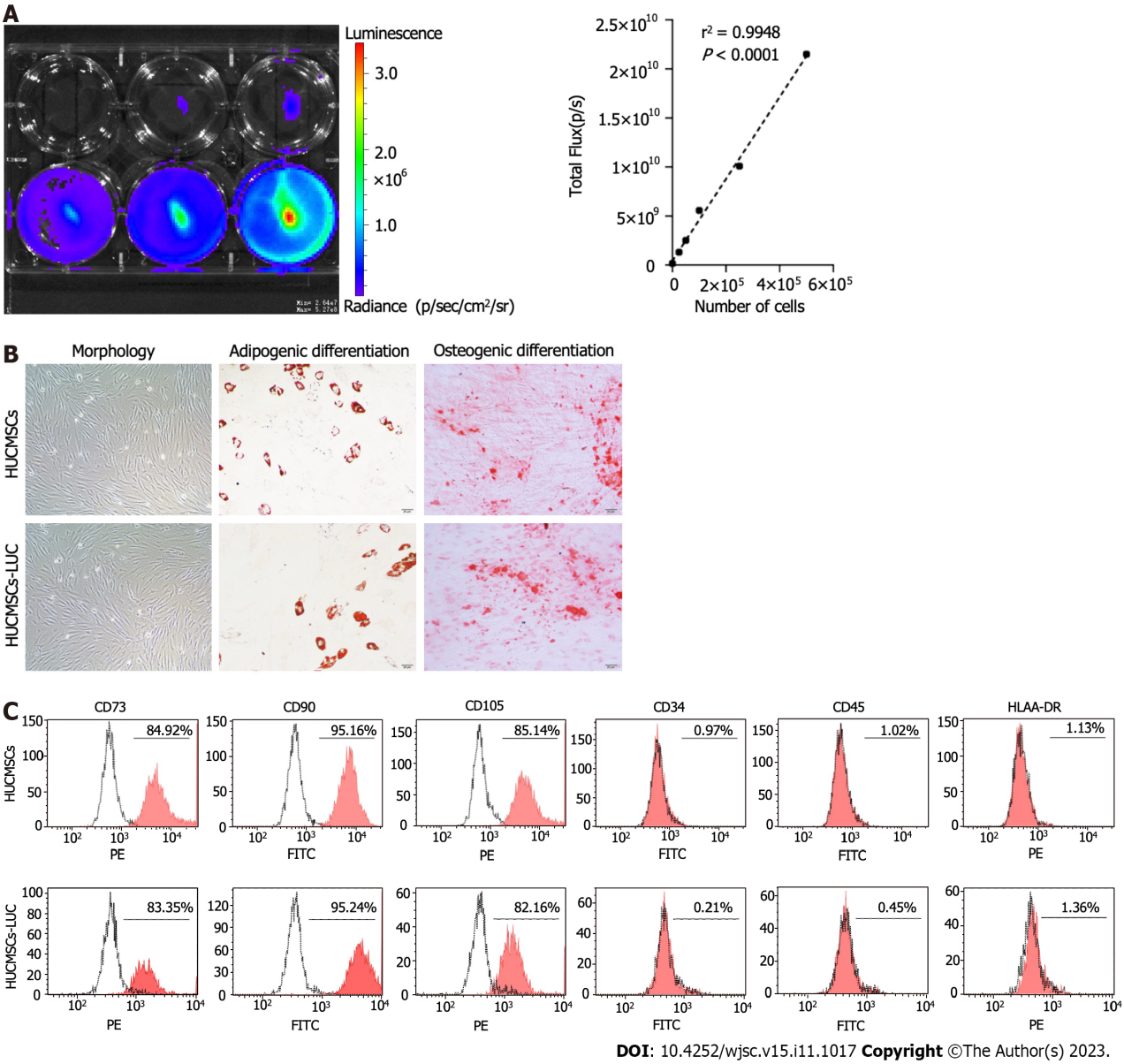
In vitro assessment of HUCMSCs

HUCMSCs-LUC were analysed *in vitro* by bioluminescence imaging (BLI), cell morphology assessment, flow cytometry, and differentiation potential determination to confirm the properties typical of HUCMSCs. A linear relationship between the number of cells and the bioluminescent signal was observed (Figure 1A). Cells displayed a spindle-like shape and were arranged in radial concentric circles (Figure 1B). Adipogenic and osteogenic differentiation confirmed the multipotentiality and differentiation capacity of the HUCMSCs-LUC (Figure 1B). Flow cytometry confirmed the presence of the following stem cell-specific molecules on the cell surface: CD90 in 94.25% of the cells, CD73 in 83.35%, CD105 in 90.18%, CD44 in 82.16%, CD34 in 0.21%, CD45 in 0.45%, and HLA-DR in 1.36% (Figure 1C).

Ferroptosis could be induced in HUCMSCs

To verify whether ferroptosis could be induced in HUCMSCs and to ascertain the optimal concentration and time, HUCMSCs were exposed to increasing doses (1 μM, 2 μM, 3 μM, 4 μM, and 5 μM) of erastin, a ferroptosis inducer, for three different durations (12 h, 24 h, and 48 h). As shown in Figures 2A-C, the CCK-8 assay illustrated that compared to that in untreated cells, cellular viability was notably reduced to 50% by erastin treatment at concentrations greater than 3 μM for less than 24 h of exposure. In addition, because cell membrane structures can be destroyed by ferroptosis, which leads to the release of LDH from the cytoplasm into the culture medium[18], the levels of LDH in the supernatant were monitored after treatment with increasing erastin concentrations for 12 h, 24 h, and 48 h (Figures 2E-G). The results showed that LDH release increased in a time- and dose-dependent manner. Similarly, treatment with erastin at a concentration greater than 3 μM for 24 h significantly increased LDH release to about 50% compared with that of the untreated HUCMSCs. Thus, HUCMSCs were treated with erastin at a concentration of 3 μM for 24 h in subsequent experiments.

Fer-1 was used to determine whether ferroptosis inhibition could protect HUCMSCs from erastin-induced cell death. Erastin-treated HUCMSCs were cotreated with Fer-1 at concentrations of 0, 2, 4, 6, and 8 μM for 24 h. As shown in Figures 2D and H, Fer-1 above 4 μM had a significant protective effect over treatment without Fer-1.



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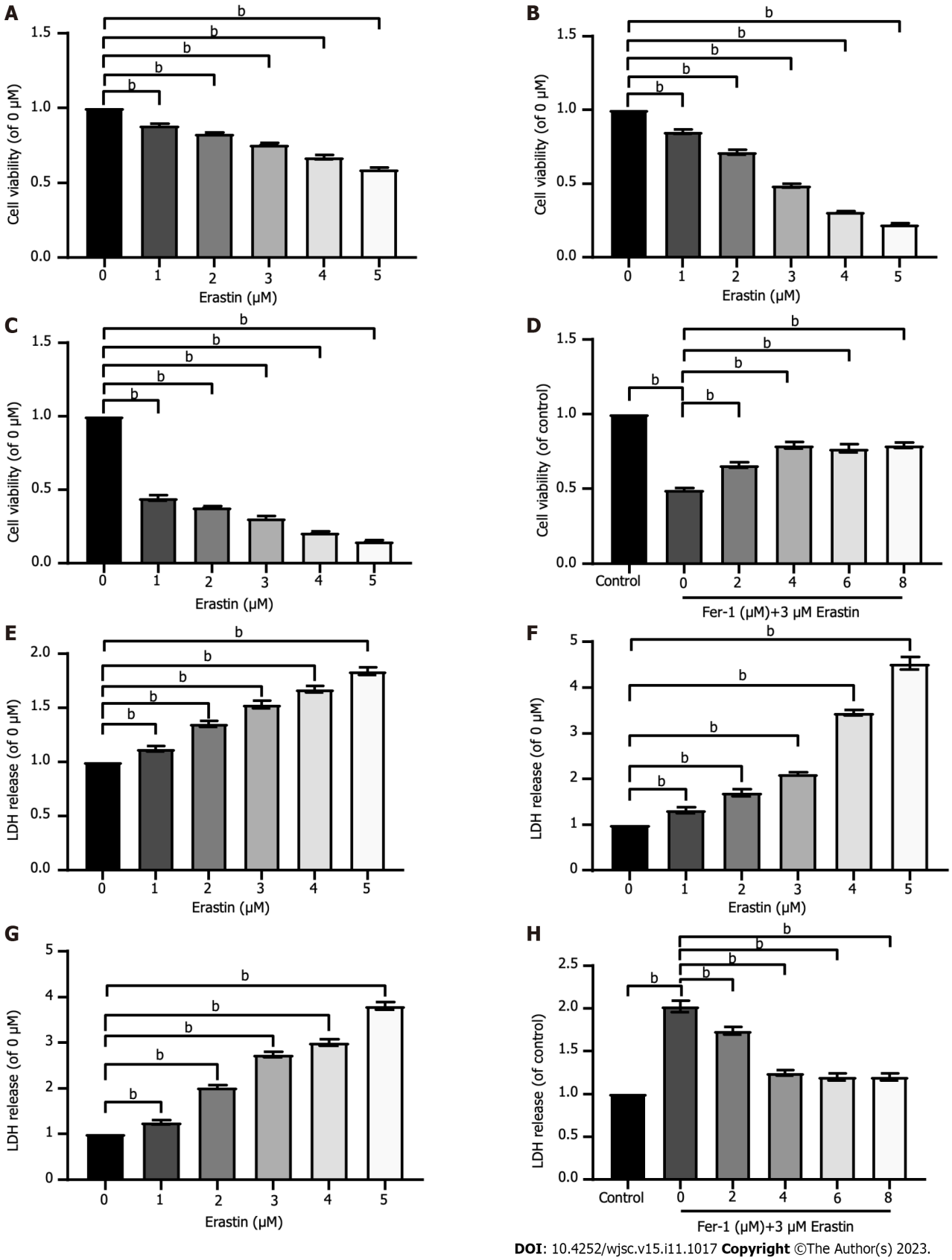
Figure 1 Characteristics of human umbilical cord mesenchymal stem cells expressing firefly luciferase gene. A: Luciferase activity of human umbilical cord mesenchymal stem cells expressing firefly luciferase gene (HUCMSCs-LUC). Representative luciferase imaging is shown using *in vivo* imaging system; B: Morphologies of HUCMSCs-LUC. HUCMSCs-LUC differentiated into adipocytes (stained with oil red O for lipid droplets) and osteocytes (stained with alizaline red for mineral deposition); C: Surface marker expression of HUCMSCs-LUC in flow cytometry analysis. HUCMSCS: Human umbilical cord mesenchymal stem cells; HUCMSCs-LUC: Human umbilical cord mesenchymal stem cells expressing firefly luciferase gene; PE: Phycoerythrin; FITC: Fluorescein isothiocyanate.

Longitudinal in vivo BLI of HUCMSC grafts

After 1.5 × 10^5 HUCMSCs-LUC were grafted in each mouse, longitudinal *in vivo* BLI was performed on day 1 post-implantation. For quantitative analysis of the observed BLI signals, cell graft-specific BLI signals from fixed regions on the mouse chest and the mean background BLI signals from fixed control regions on the mouse head were plotted *vs* time post-implantation (Figure 3A). The BLI signal was decreased in the HUCMSCs + erastin group and increased in the HUCMSCs + Fer-1 group compared to the HUCMSCs group (Figure 3B). In addition, the signal was further increased in the HUCMSCs/CSE + erastin group but decreased in the HUCMSCs/sh-CSE + Fer-1 group (Figure 3B).

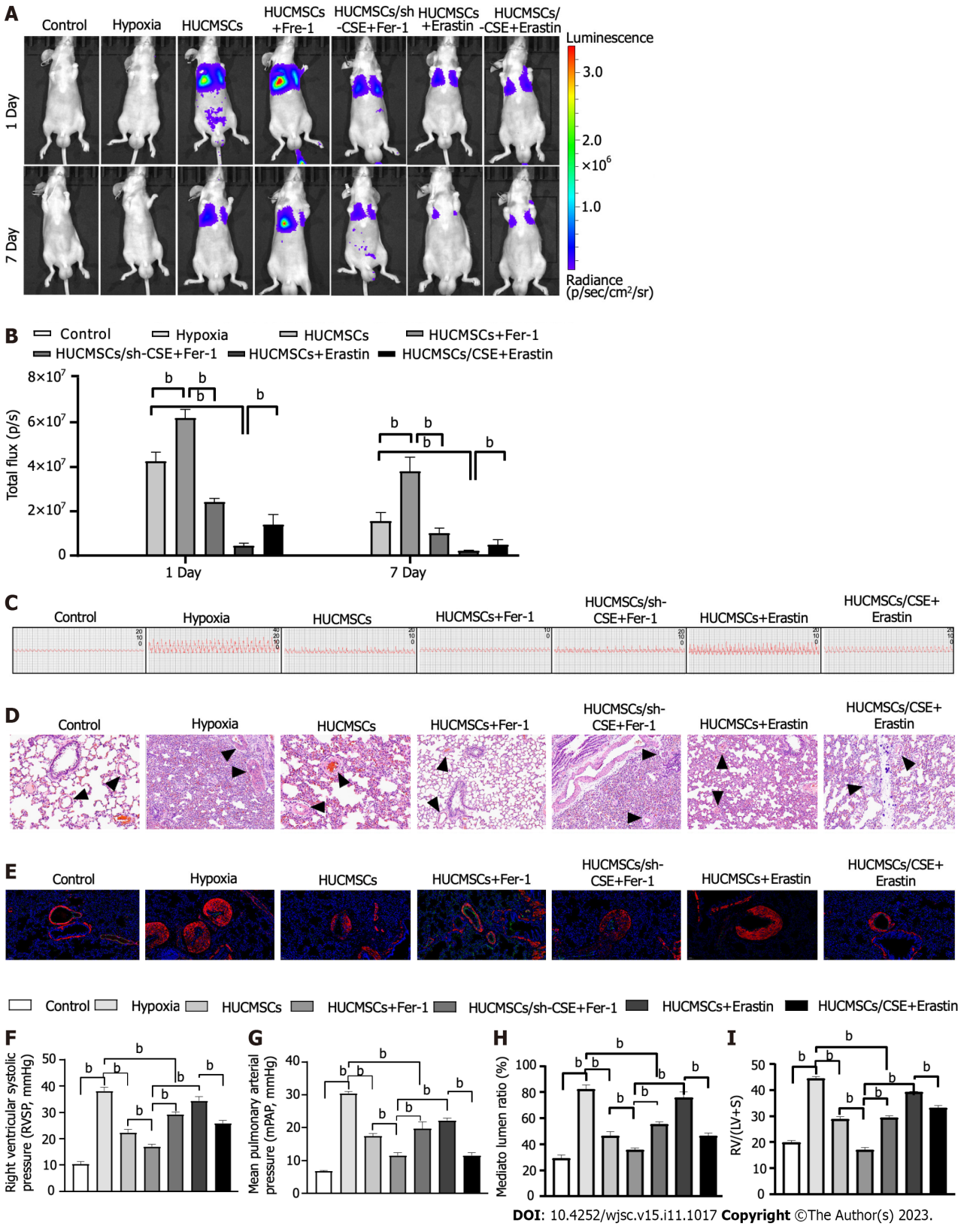
Pulmonary artery remodelling in each group

In PAH, pulmonary artery remodelling results in progressively elevated pulmonary arterial pressure, which ultimately leads to right heart failure[19]. As shown in Figures 3C, F, G, and I, the administration of HUCMSCs or HUCMSCs + Fer-1 decreased the RVSP and RV/(LV + S) ratio in mice with hypoxia-induced PAH ($P < 0.01$). Interestingly, compared to those in the HUCMSCs group, the RVSP and RV/(LV + S) decreased to a greater extent in the HUCMSCs + Fer-1 group (17.000 ± 2.215 *vs* 22.280 ± 3.115, 23.376 ± 0.537 *vs* 28.781 ± 2.311, both $P < 0.01$) and increased in the HUCMSCs + erastin group (34.363 ± 4.065 *vs* 22.280 ± 3.115, 39.158 ± 0.809 *vs* 28.781 ± 2.311, both $P < 0.01$). Moreover, compared to those in the HUCMSCs + erastin group, the RVSP and RV/(LV + S) were decreased in the HUCMSCs/CSE + erastin group (25.817 ± 2.843 *vs* 34.363 ± 4.065, 33.082 ± 2.405 *vs* 39.158 ± 0.809, both $P < 0.01$). Furthermore, the RVSP and RV/(LV + S) were



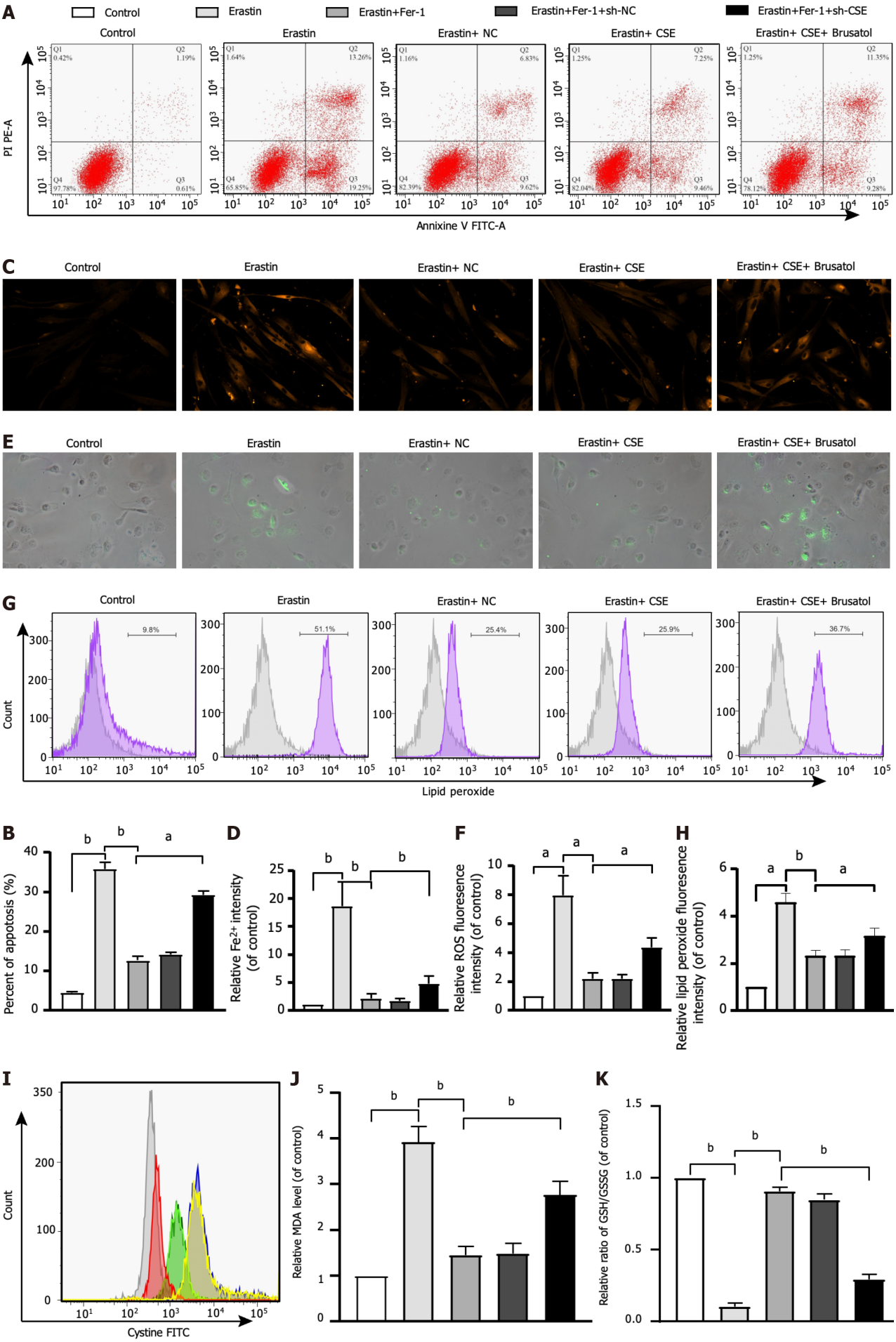
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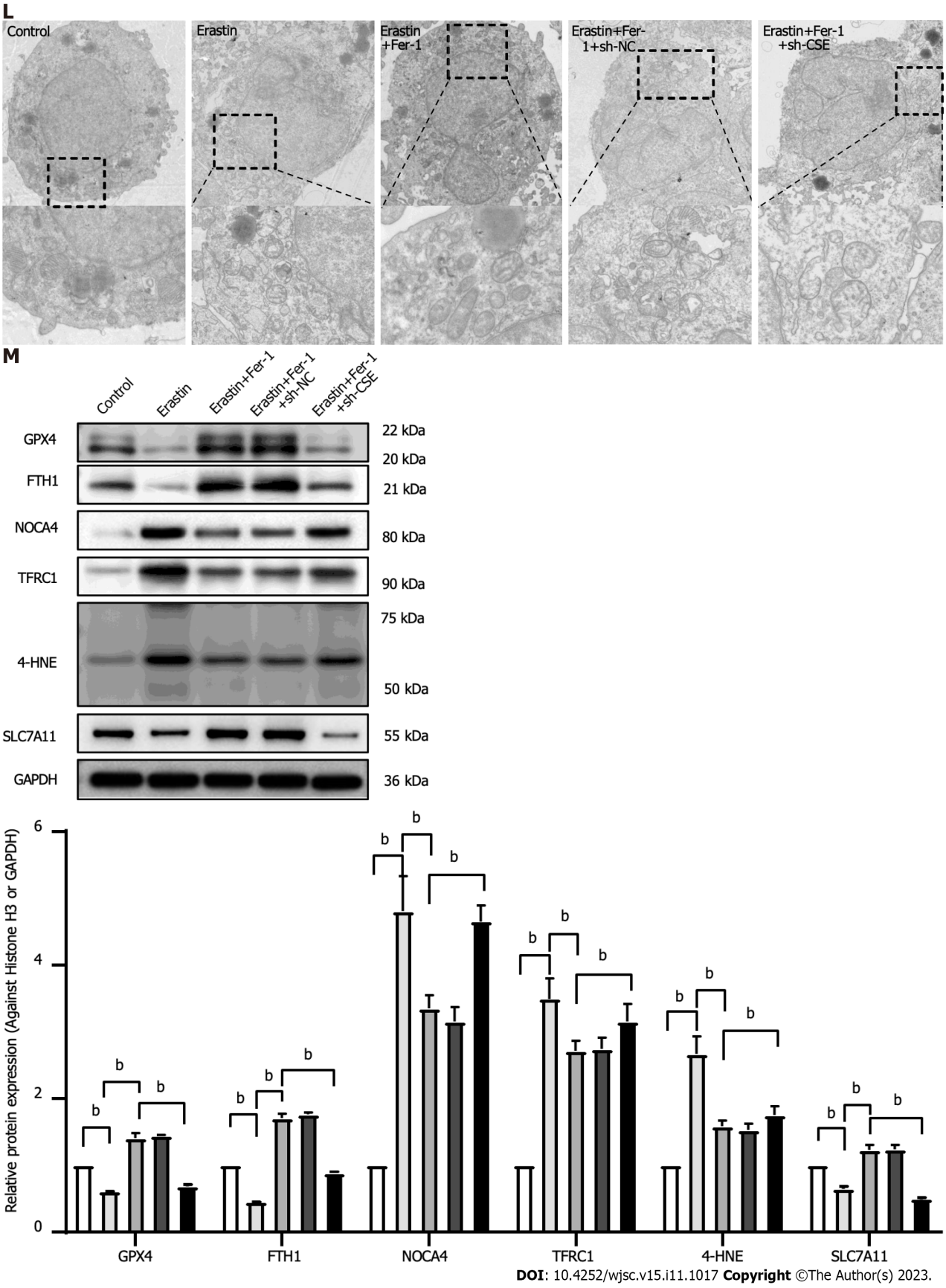
Figure 2 Ferroptosis exists in human umbilical cord mesenchymal stem cells. A-C: Cell viability was assessed after exposure to different concentrations of erastin for different times (12 h, 24 h, and 48 h); D: Cell viability was assessed after exposure to 3 μM erastin and different concentrations of ferrostatin-1 (Fer-1). Cell viability was measured with Cell Counting Kit-8 kit; E-G: Lactic dehydrogenase (LDH) release was assessed after exposure to different concentrations of erastin for different times (12 h, 24 h, and 48 h); H: LDH release was assessed after exposure to 3 μM erastin and different concentrations of Fer-1. LDH release was measured with LDH cytotoxicity detection kit. The data were from at least three independent experiments. Data were quantified for cells subjected to erastin (0 μM), and values are represented as the mean ± SD. Fer-1: Ferrostatin-1; LDH: Lactic dehydrogenase. $^{b}P < 0.01$.



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Figure 3 Change of pulmonary arterial remodeling. A: *In vivo* bioluminescence imaging (BLI). Representative time course image showing *in vivo* BLI of mice grafted with living human umbilical cord mesenchymal stem cells expressing firefly luciferase gene in the lungs. Images were acquired at 24 h and 7 d post-implantation. Regions of interest are drawn on the mouse lung and on the mouse shoulder, considered as background signal; B: *In vivo* BLI. Quantitative analysis of *in vivo* BLI at days 1 and 7 post-implantation; C-H: Representing pulmonary arterial pressure (C, F, and G). Representative haematoxylin and eosin-stained lung sections and quantification of the ratio of the medial wall area to the total vessel cross sectional area of the distal pulmonary artery sections (D and H). Immunofluorescent staining (E). Green fluorescence represents VEcadherin, red fluorescence represents alpha-smooth muscle actin, and blue fluorescence indicates 4',6-diamidino-2-phenylindole nuclear staining; I: Right ventricle/left ventricle plus septum. Data are expressed as the mean ± SEM (n = 6/group). HUCMSCS: Human umbilical cord mesenchymal stem cells; Fer-1: Ferrostatin-1; CSE: Cystathionine γ-lyase; RV/(LV + S): Right ventricle/(left ventricle plus septum). $^{b}P < 0.01$.


A
Control
Erastin
Erastin+Fer-1
Erastin+Fer-1+sh-NC
Erastin+Fer-1+sh-CSE
Control
Erastin
Erastin+ NC
Erastin+ CSE
Erastin+ CSE+ Brusatol
PI PE-A
Annixine V FITC-A
C
Control
Erastin
Erastin+ NC
Erastin+ CSE
Erastin+ CSE+ Brusatol
E
Control
Erastin
Erastin+ NC
Erastin+ CSE
Erastin+ CSE+ Brusatol
G
Control
Erastin
Erastin+ NC
Erastin+ CSE
Erastin+ CSE+ Brusatol
9.8%
51.1%
25.4%
25.9%
36.7%
Count
Lipid peroxide
B
Percent of appotosis (%)
D
Relative Fe2+ intensity (of control)
F
Relative ROS fluoresence intensity (of control)
H
Relative lipid peroxide fluoresence intensity (of control)
I
Count
Cystine FITC
J
Relative MDA level (of control)
K
Relative ratio of GSH/GSSG (of control)



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Figure 4 Effect of cystathionine γ-lyase overexpression on ferroptosis in human umbilical cord mesenchymal stem cells. A and B: Cell apoptosis was analyzed by Annexin V-fluorescein isothiocyanate (FITC)/propidine iodide staining using flow cytometry analysis (n = 3), and quantified on the basis of apoptosis rate (n = 3); C and D: Iron level was detected by FeRhoNox-1 staining (C) and iron assay kit (D); E and F: Immunofluorescent staining of total reactive oxygen species; G and H: Level of lipid peroxidation detected by flow cytometry analysis after staining with C11-BODIPY; I: Intracellular cystine-FITC levels measured by flow cytometry. Results represent three independent experiments; J: Malonaldehyde level; K: Ratio of reduced glutathione/oxidized glutathione disulfide;

L: Mitochondrial morphological changes detected by transmission electron microscopy; M: Western blot analysis of expression of the glutathione-dependent antioxidant enzyme glutathione peroxidase, ferritin heavy chain 1, nuclear receptor coactivator 4, Fe^{3+}-bound transferrin receptor 1, 4-hydroxynonenal, and SLC7A11 protein. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization for protein (n = 3). Fer-1: Ferrostatin-1; Annexin V-FITC/PI: Annexin V-fluorescein isothiocyanate/propidine iodide; ROS: Reactive oxygen species; sh-CSE: Short hairpin RNA targeting cystathionine γ-lyase; MDA: Malonaldehyde; GSH/GSSG: Reduced glutathione/oxidized glutathione disulfide; GPX4: Glutathione-dependent antioxidant enzyme glutathione peroxidase; FTH1: Ferritin heavy chain 1; NCOA4: Nuclear receptor coactivator 4; TFRC1: Fe^{3+}-bound transferrin receptor 1; 4-HNE: 4-hydroxynonenal; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. $^{a}P < 0.05$, $^{b}P < 0.01$.

increased in the HUCMSCs/sh-CSE + Fer-1 group compared with those in the HUCMSCs + Fer-1 group (29.232 ± 2.333 *vs* 17.000 ± 2.215, 29.820 ± 1.526 *vs* 23.376 ± 0.537, both $P < 0.01$).

Endothelial to mesenchymal transition (EnMT) has been causally linked to pulmonary arterial remodelling in PAH[20, 21]. EnMT is a form of cellular plasticity described as a phenotypic conversion whereby endothelial cells lose endothelial characteristics (CD31 expression) and acquire mesenchymal characteristics (VE-cadherin expression)[22]. Here, as shown in Figures 3D, E and H, histological and immunofluorescence staining showed slight pulmonary artery stenosis and EnMT in the groups exposed to hypoxia, and these effects were largely attenuated by treatment with HUCMSCs and HUCMSCs + Fer-1 ($P < 0.01$). Interestingly, the inhibitory effect of HUCMSCs + Fer-1 on small pulmonary artery stenosis and EnMT was stronger than that of HUCMSCs. In addition, compared to those in the HUCMSCs + erastin group, small pulmonary artery stenosis and EnMT were alleviated in the HUCMSCs/CSE + erastin group ($P < 0.01$). Meanwhile, small pulmonary artery stenosis and EnMT were aggravated in the HUCMSCs/sh-CSE group compared with the HUCMSCs/Fer-1 group ($P < 0.05$).

CSE inhibition exacerbates Fer-1-mediated suppression of ferroptosis in HUCMSCs

We observed ferroptosis-related changes in erastin-treated HUCMSCs. As shown in Figure 4, cell apoptosis; the levels of Fe^{2+}, ROS, lipid peroxidation, and MDA; and the expression of 4-HNE, TFRC1, and NOCA4 were significantly increased, while the GSH/GSSG ratio; cystine uptake; and GPX4, FTH1, and SLC7A11 expression were decreased in the erastin group compared with the control group (all $P < 0.05$). Consistent with these results, transmission electron microscopy (TEM) also showed an increased mitochondrial membrane density and shorter mitochondrial cristae in the erastin group (Figure 4L). Moreover, the above changes in erastin-treated HUCMSCs were reversed by Fer-1 administration (all $P < 0.05$). Interestingly, the inhibitory effect of Fer-1 on ferroptosis was aggravated by CSE inhibition (all $P < 0.05$).

CSE overexpression negatively regulates erastin-induced ferroptosis in HUCMSCs

As shown in Figure 5, there was a significant decrease in cell apoptosis; the levels of Fe^{2+}, ROS, lipid peroxidation, and MDA; and the expression of 4-HNE, TFRC1, and NOCA4, whereas there was an increase in the GSH/GSSG ratio; cystine uptake; and the expression of GPX4, FTH1, and SLC7A11 in the erastin + CSE group compared to the erastin group (all $P < 0.05$). Consistent with these results, TEM also showed that the mitochondrial membrane was intact and that the mitochondria were narrower with an increased number of cristae in the erastin + CSE group (Figure 5L). In addition, the inhibitory effect of CSE overexpression on ferroptosis was abolished by brusatol, an Nrf2 inhibitor (all $P < 0.05$).

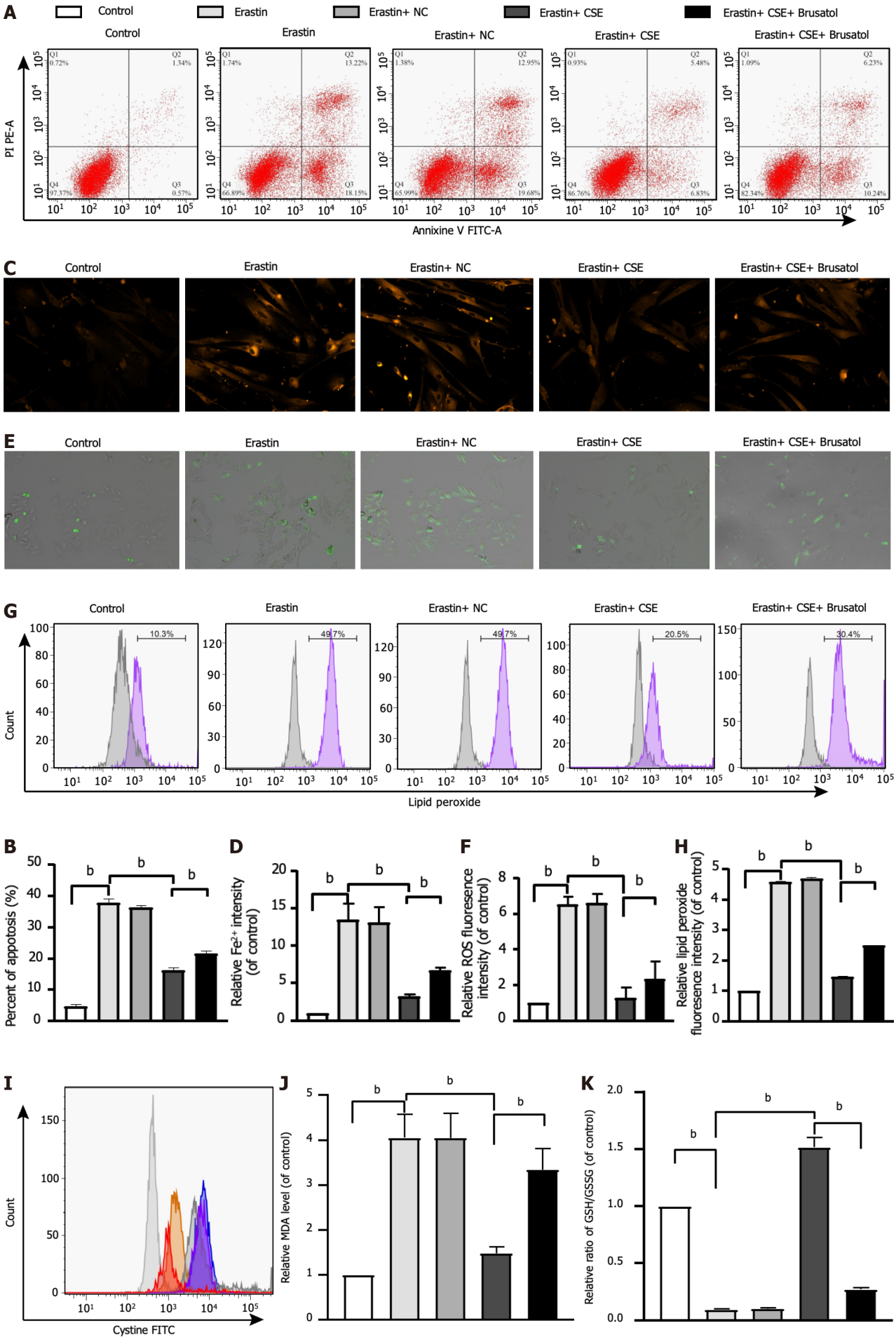
The CSE/H_2S pathway induces Keap1 S-sulfhydration and nuclear translocation of Nrf2

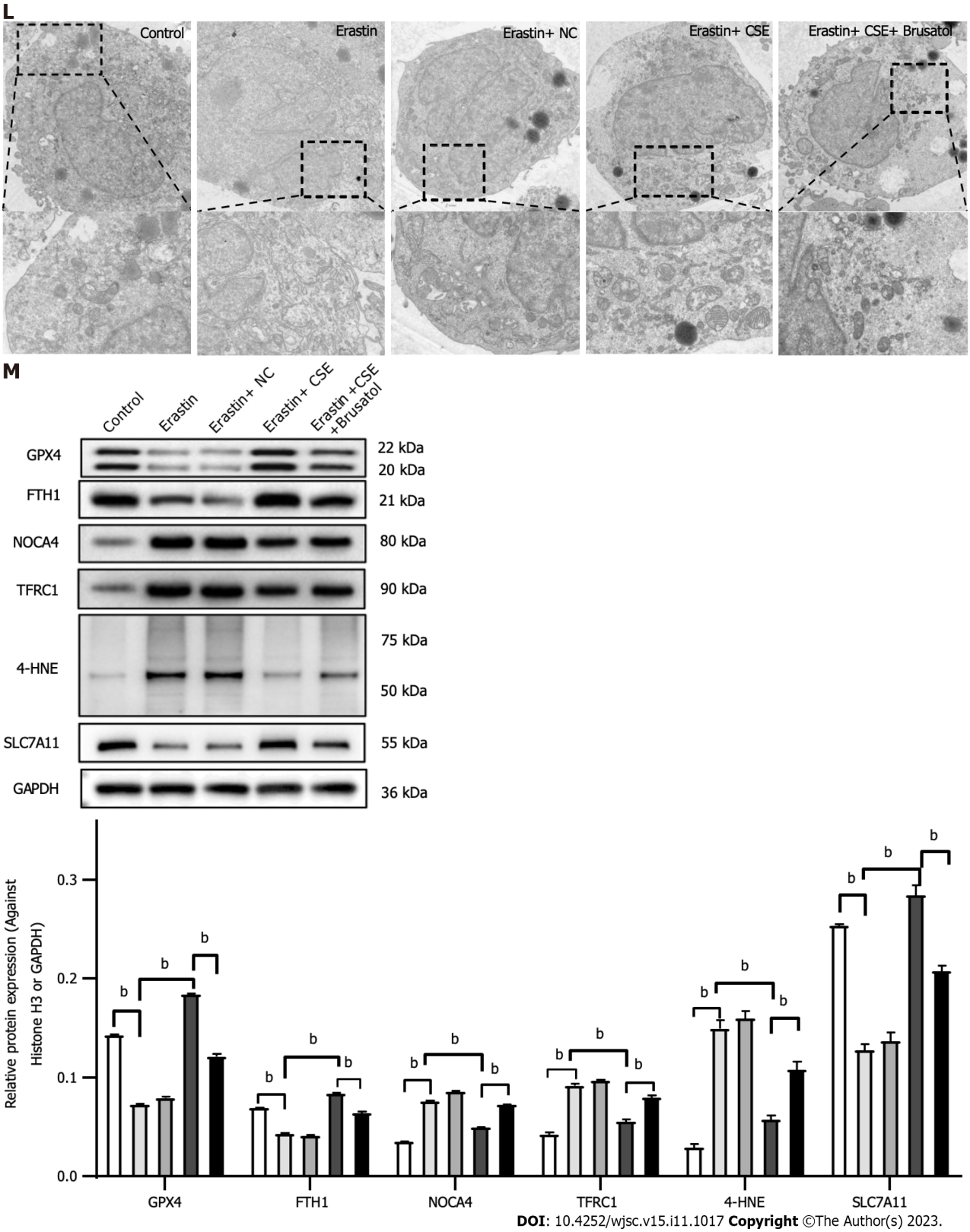
We examined whether Nrf2 is involved in the protective effect of the CSE/H_2S pathway against the ferroptosis of HUCMSCs. First, we found that CSE inhibition decreased H_2S production (Figure 6A), while CSE overexpression increased H_2S levels in HUCMSCs (Figure 6B). In addition, CSE inhibition further downregulated Fer-1-induced Nrf2 activation (Figure 6C), while CSE overexpression further upregulated erastin-induced Nrf2 inactivation (Figure 6D).

Then, the expression of Nrf2 was examined. Western blot analysis revealed that erastin treatment significantly inhibited Nrf2 nuclear translocation, and this effect was markedly enhanced when erastin treatment was combined with Fer-1 treatment (Figure 6E). Moreover, CSE inhibition upregulated Nrf2 expression in the cytoplasm and downregulated Nrf2 expression in the nucleus (Figure 6E). In contrast, CSE overexpression increased nuclear Nrf2 expression and decreased cytoplasmic Nrf2 expression (Figure 6F).

Next, we found that CSE overexpression-induced ferroptosis inhibition was successfully abolished by treatment with 10 nM brusatol, an Nrf2 inhibitor, for 6 h in HUCSMCs (Figure 6F). We also found no difference in the expression of Keap1 among the groups (Figures 6E and F).

Finally, with the "tag-switch" assay, the S-sulfhydration of Keap1 was tested. S-sulfhydration is a novel posttranslational modification involving H_2S. However, S-sulfhydration, a covalent modification, can be reversed by DDT. Increased S-sulfhydration of Keap1 was found in the HUCMSCs/CSE + erastin group than in the HUCMSCs + erastin group, and decreased S-sulfhydration of Keap1 was detected in the HUCMSCs/sh-CSE + Fer-1 group than in the HUCMSCs + Fer-1 group (Figure 6G). Moreover, the S-sulfhydration of Keap1 was further decreased after treatment with DTT, which indicated that Keap1 is S-sulfhydrated (Figure 6G). As shown in Figure 6H, S- of Keap1 led to decreased nuclear Nrf2 expression, which indicated that the activation of Nrf2 by the CSE/H_2S pathway is dependent on Keap1 S-sulfhydration in HUCMSCs.


A
Control
Erastin
Erastin+ NC
Erastin+ CSE
Erastin+ CSE+ Brusatol
PI PE-A
Annixine V FITC-A
C
E
G
Count
Lipid peroxide
B
Percent of appotosis (%)
D
Relative Fe2+ intensity (of control)
F
Relative ROS fluoresence intensity (of control)
H
Relative lipid peroxide fluoresence intensity (of control)
I
Cystine FITC
J
Relative MDA level (of control)
K
Relative ratio of GSH/GSSG (of control)



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Figure 5 Effect of cystathionine γ-lyase inhibition on ferroptosis in human umbilical cord mesenchymal stem cells. A and B: Cell apoptosis was analyzed by Annexin V-fluorescein isothiocyanate (FITC)/propidine iodide staining using FACS (n = 3), and quantified on the basis of apoptosis rate (n = 3); C and D: Iron level detected by FeRhoNox-1 staining (C) and iron assay kit (D); E and F: Immunofluorescent staining of total reactive oxygen species; G and H: Level of lipid peroxidation detected by flow cytometry after staining with C11-BODIPY; I: Intracellular cystine-FITC levels measured by flow cytometry. Results represent three independent experiments; J: Malonaldehyde level; K: Ratio of reduced glutathione/oxidized glutathione disulfide; L: Mitochondrial morphological changes detected by transmission electron microscopy; M: Western blot analysis of expression of glutathione-dependent antioxidant enzyme glutathione peroxidase, ferritin heavy chain 1, nuclear receptor coactivator 4, Fe^{3+}-bound transferrin receptor 1, 4-hydroxynonenal, and SLC7A11 protein. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization for protein (n = 3). Fer-1: Ferrostatin-1; Annexin V-FITC/PI: Annexin V-fluorescein isothiocyanate/propidine iodide; ROS: Reactive oxygen species;

MDA: Malonaldehyde; CSE: Cystathionine γ-lyase; GSH/GSSG: Reduced glutathione/oxidized glutathione disulfide; GPX4: Glutathione-dependent antioxidant enzyme glutathione peroxidase; FTH1: Ferritin heavy chain 1; NCOA4: Nuclear receptor coactivator 4; TFRC1: Fe^{3+}-bound transferrin receptor 1; 4-HNE: 4-hydroxynonenal; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. [b]$P < 0.01$.

DISCUSSION

Ferroptosis has been reported as a form of cell death; however, to date, understanding the potential effects of ferroptosis on MSC delivery requires further investigation. In the present study, we showed that ferroptosis occurred during HUCMSC delivery in mice with hypoxia-induced PAH. Moreover, mediation of the CSE/H_2S pathway suppressed ferroptosis in HUCMSCs.

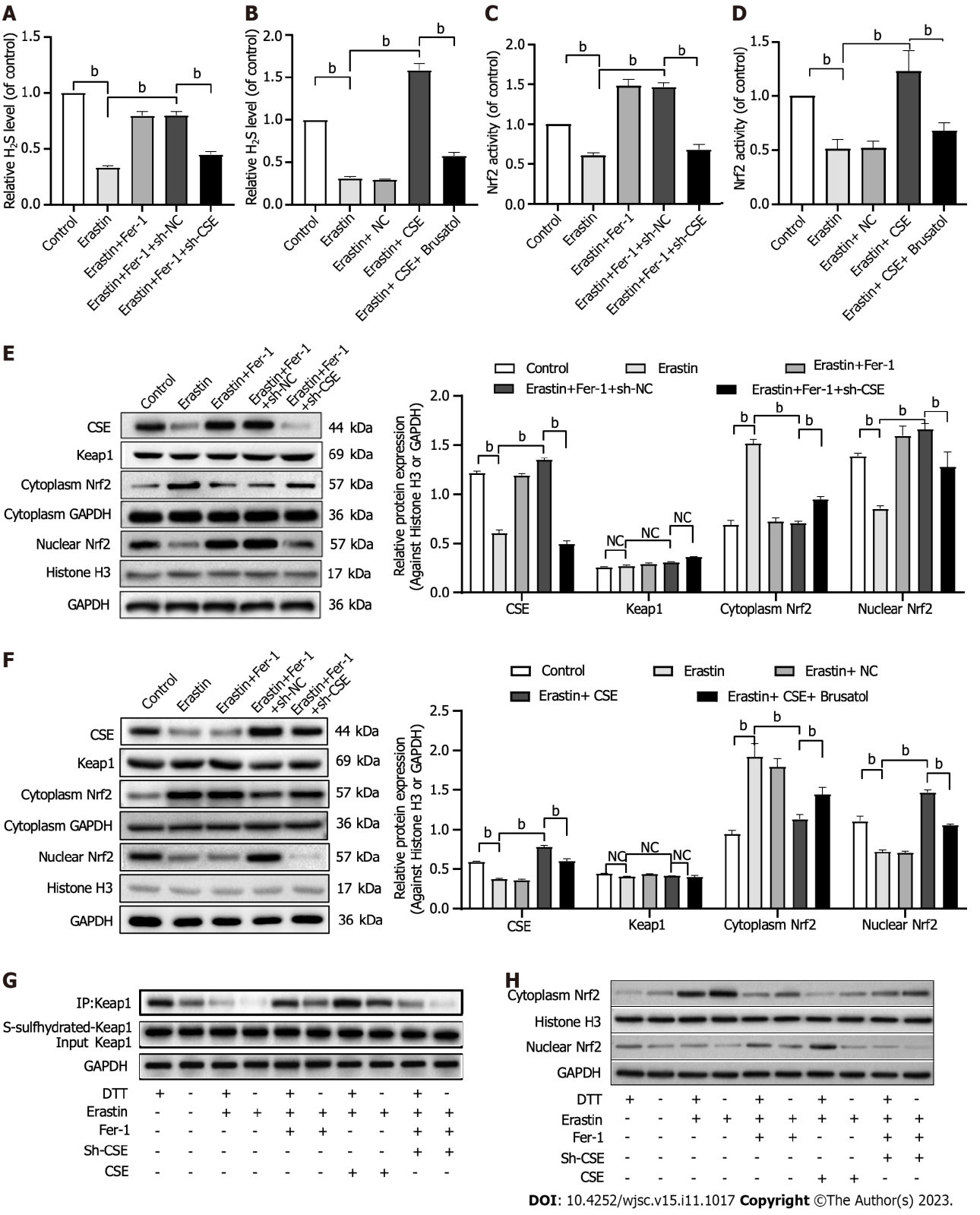
A desirable advantage of MSCs is their ability to home to damaged tissue sites. Evidence has demonstrated that transplanted MSCs can accumulate in lung tissue, inhibit vascular remodelling, and effectively attenuate pulmonary hypertension. In the present study, HUCMSCs were chosen because of their many characteristics, which include their ability to be extracted *via* a noninvasive procedure and high differentiation potential, the ease of their *in vitro* expansion, and their prominent repair properties. To detect transplanted HUCMSCs *in vivo*, HUCMSCs were labelled with firefly luciferase by transduction with a lentiviral vector. Furthermore, we determined that the surface markers and differentiation potential of the HUCMSCs were not disrupted after transduction. In this study, we monitored the survival of HUCMSCs in lung tissue 1 d and 7 d after intravenous injection and found that the HUCMSCs homed to injured lung tissue. Moreover, our data indicated the protective roles of HUCMSCs against pulmonary artery remodelling in mice under hypoxia.

Although MSCs can reach impaired sites due to their homing capacity, cell survival in these places is rare. At present, many potential methods to reduce MSC fate *in vivo* have been identified, including drug pretreatment, genetic modification, and preconditioning. However, our study indicated that pretreatment with Fer-1, an inhibitor of ferroptosis, increased HUCMSC survival in the lung, and combined treatment of HUCMSCs with Fer-1 further decreased vascular remodelling in mice with PAH. Conversely, worse cell survival and decreased therapeutic effects were detected in PAH mice after the delivery of HUCMSCs pretreated with erastin, which induces ferroptosis. Thus, the results showed that ferroptosis may have been linked to HUCMSCs survival in mice with hypoxia-induced PAH.

Researchers have indicated that ferroptosis can be activated by iron overload or the inactivation of GPX4[23-25]. During ferroptosis, following its import through TFRC1, Fe^{3+} is catalysed and converted to Fe^{2+} in the cytoplasm, which then participates in the Fenton reaction, causing ferroptosis. Moreover, Fe^{2+} also binds intracellular FTH1, which can be transported to lysosomes by NCOA4, leading to intracellular iron overload. However, GPX4 is the major endogenous factor that reduces lipid peroxidation[26]. Cystine is converted to cysteine by the cystine/glutamate antiporter system (Xc-), which generates GSH, a cofactor for GPX4[27,28]. In addition, the inhibition of SLC7A11, a key component of system Xc^-, initiates ferroptosis[29], and MDA and 4-HNE, which are byproducts of lipid peroxidation, represent stable markers of oxidative stress that are also associated with ferroptosis[30]. In the present *in vitro* experiments, after Fer-1 administration, we detected decreased cell death, the rebalancing of intracellular iron homeostasis, and reduced levels of lipid peroxidation products, indicating that ferroptosis was suppressed by Fer-1 in erastin-treated HUCMSCs. This is consistent with the ability of Fer-1 and erastin to induce and inhibit ferroptosis, respectively[31].

On the basis of our *in vitro* and *in vivo* experimental results, we suggest that the CSE/H_2S pathway mediates ferroptosis in HUCMSCs. Synthesized by CSE in mammalian cells, H_2S has been shown to directly scavenge ROS[32], facilitate GSH production[33], inhibit GPX4 inactivation[34], and enhance the activity of system Xc[35], mitigating ferroptosis. Indeed, in our study, CSE inhibition decreased endogenous H_2S levels and exacerbated ferroptosis in HUCMSCs, while CSE overexpression increased intracellular H_2S production and inhibited ferroptosis in HUCMSCs. Although emerging evidence has shown that Nrf2 is among the major cellular defences against ferroptosis as it promotes the translation of antioxidant genes, such as FTH1[36], GPX4[37], and Xc[38], the effects of the CSE/H_2S pathway on Nrf2 activation in HUCMSCs have not been described. In our investigation, it was apparent that upregulation of the CSE/H_2S pathway promoted the translocation of Nrf2 into the nucleus, which indicated that Nrf2 was activated. In addition, using an Nrf2 inhibitor, we demonstrated that the inhibitory effects of the CSE/H_2S pathway against ferroptosis were mediated through the activation of Nrf2. Interestingly, we also found that the inactivation of Nrf2, in turn, led to the downregulation of CSE, which may have been caused by excessive oxidative stress-induced oxidation of CSE.

Keap1 is known to bind Nrf2 in the cytoplasm and inhibit it, preventing Nrf2 translocation to the nucleus. Under oxidative stress, the dissociation of Keap1 and Nrf2 results in the entrance of Nrf2 into the nucleus, where it initiates gene transcription[39]. In this study, we found that the CSE/H_2S pathway had little effect on total Keap1 protein expression in HUCMSCs. Thus, to further explore the mechanism underlying the effects of the CSE/H_2S pathway on Nrf2 activation, Keap1 S-sulfhydration was investigated in HUCMSCs. S-sulfhydration is a posttranslational modification induced by H_2S that leads to the attachment of sulfhydryl groups to selective proteins, yielding a hydropersulfide moiety (-SSH)[40]. However, the covalent modification that occurs in sulfhydration is reversable by DTT[40]. Recent publications have revealed that H_2S induces the S-sulfhydration of transcription factors, such as Keap1[41]. In this study, we found that Keap1 S-sulfhydration was inhibited upon ferroptosis. By comparing the difference in Keap1 S-sulfhydration between the HUCMSCs/CSE and HUCMSCs/sh-CSE groups, we demonstrated the significant role of Keap1 S-sulfhydration in the protective effects of the CSE/H_2S pathway against ferroptosis in HUCMSCs.



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Figure 6 Effect of cystathionine γ-lyase/hydrogen sulfide pathway on nuclear factor erythroid 2-related factor 2 activation and ferroptosis in human umbilical cord mesenchymal stem cells. A and B: Exogenous hydrogen sulfide (H_2S) production; C and D: Nuclear factor erythroid 2-related factor 2 (Nrf2) activity; E and F: Western blot analysis and quantification of cystathionine γ-lyase (CSE) protein, Kelch-like ECH-associating protein 1 (Keap1), and cytoplasmic and nuclear Nrf2 protein; G: Change of Keap1 S-sulfhydration. 1,4-Dithio-DL-threitol (DTT, 1 mmol/L, 3 h) served as a negative control; H: Effect of DTT on cytoplasmic and nuclear Nrf2 expression. Histone H3 was used for normalization for nuclear proteins, and glyceraldehyde-3-phosphate dehydrogenase was used for normalization for whole and cytoplasmic protein (n = 3). CSE: Cystathionine γ-lyase; H_2S: Hydrogen sulfide; Nrf2: Nuclear factor erythroid 2-related factor 2; HUCMSCs: Human umbilical cord mesenchymal stem cells; Fer-1: Ferrostatin-1; sh-CSE: Short hairpin RNA targeting cystathionine γ-lyase; Keap1: Kelch-like ECH-associating protein 1; DTT: 1,4-Dithio-DL-threitol; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. $^bP < 0.01$.

CONCLUSION

In conclusion, the present data provide evidence for the first time that inhibiting ferroptosis improves the survival of HUCMSC transplanted in mice with hypoxia-induced PAH. Moreover, ferroptosis can be suppressed by regulating the CSE/H_2S pathway *via* S-sulfhydrated Keap1/Nrf2 signalling in HUCMSCs. Collectively, the ability of CSE to inhibit ferroptosis suggests that genetic approaches to manipulate CSE expression and H_2S production may provide a novel therapeutic avenue for improving the protective capacity of transplanted MSCs in PAH.

ARTICLE HIGHLIGHTS

Research background

Ferroptosis can decrease retention and engraftment after mesenchymal stem cell (MSC) delivery, which is considered a major challenge to the effectiveness of MSC-based therapy for pulmonary arterial hypertension (PAH). Interestingly, the cystathionine γ-lyase (CSE)/hydrogen sulfide (H_2S) pathway may contribute to mediating ferroptosis.

Research motivation

We aimed to investigate the influence of the CSE/H_2S pathway on ferroptosis in human umbilical cord MSCs (HUCMSCs).

Research objectives

We aimed to clarify whether the effect of HUCMSCs on vascular remodelling in mice with PAH is by CSE/H_2S pathway-mediated ferroptosis. Furthermore, the effect of the CSE/H_2S pathway on ferroptosis in HUCMSCs and the underlying mechanisms were investigated.

Research methods

Erastin and ferrostatin-1 (Fer-1) were used to induce and inhibit ferroptosis, respectively. HUCMSCs were transfected with a vector that overexpressed or inhibited CSE. A PAH mouse model was established using 4-wk-old male BALB/c nude mice under hypoxic conditions, and pulmonary pressure and vascular remodelling were measured. The survival of HUCMSCs after delivery was observed by *in vivo* bioluminescence imaging. Cell viability, iron accumulation, reactive oxygen species (ROS) production, cystine uptake, and lipid peroxidation in HUCMSCs were tested. Ferroptosis-related proteins and S-sulfhydrated Kelch-like ECH-associating protein 1 (Keap1) were detected by western blot analysis.

Research results

In vivo, CSE overexpression improved cell survival after erastin-treated HUCMSCs were delivered to mice with hypoxia-induced PAH. *In vitro*, CSE overexpression improved H_2S production and ferroptosis-related indexes in erastin-treated HUCMSCs, such as cell viability, the iron level, ROS production, cystine uptake, lipid peroxidation, mitochondrial membrane density, and ferroptosis-related protein expression. In contrast, *in vivo*, CSE inhibition decreased cell survival after Fer-1-treated HUCMSC delivery and aggravated vascular remodelling in the PAH mice. *In vitro*, CSE inhibition decreased H_2S levels and restored ferroptosis in Fer-1-treated HUCMSCs. Interestingly, we found that upregulation of the CSE/H_2S pathway induced the S-sulfhydration of Keap1, which contributed to the inhibition of ferroptosis.

Research conclusions

Regulation of the CSE/H_2S pathway in HUCMSCs contributes to the inhibition of ferroptosis and improves the effect of vascular remodelling suppression in hypoxia-induced PAH mice. Moreover, the protective effect of the CSE/H_2S pathway on ferroptosis in HUCMSCs is mediated *via* S-sulfhydrated Keap1/nuclear factor erythroid 2-related factor 2 (Nrf2) signalling. The present study may provide a novel therapeutic avenue for improving the protective capacity of transplanted MSCs in PAH.

Research perspectives

Regulation of the CSE/H_2S pathway in HUCMSCs contributes to the inhibition of ferroptosis and improves the ability of HUCMSCs to suppress vascular remodelling in mice with hypoxia-induced PAH. Moreover, the protective effect of the CSE/H_2S pathway on ferroptosis in HUCMSCs is mediated *via* S-sulfhydrated Keap1/Nrf2 signalling. The present study may provide a novel therapeutic avenue for improving the protective capacity of transplanted MSCs in PAH.

FOOTNOTES

Co-first authors: Bin Hu and Xiang-Xi Zhang.

Author contributions: Hu B and Zhang XX contributed equally to this work; Yu WC conceived the research; Hu B and Yu WC participated in the design of the study, performed the statistical analysis, and helped to draft the manuscript; Hu B, Zhang XX, Zhang T, and Yu WC performed the experiments; and all authors participated in discussing and revising the manuscript, and approving the final manuscript.

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AIMS AND SCOPE

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REVIEW

Stem cells and pain

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Abstract

Pain can be defined as an unpleasant sensory and emotional experience caused by either actual or potential tissue damage or even resemble that unpleasant experience. For years, science has sought to find treatment alternatives, with minimal side effects, to relieve pain. However, the currently available pharmacological options on the market show significant adverse events. Therefore, the search for a safer and highly efficient analgesic treatment has become a priority. Stem cells (SCs) are non-specialized cells with a high capacity for replication, self-renewal, and a wide range of differentiation possibilities. In this review, we provide evidence that the immune and neuromodulatory properties of SCs can be a valuable tool in the search for ideal treatment strategies for different types of pain. With the advantage of multiple administration routes and dosages, therapies based on SCs for pain relief have demonstrated meaningful results with few downsides. Nonetheless, there are still more questions than answers when it comes to the mechanisms and pathways of pain targeted by SCs. Thus, this is an evolving field that merits further investigation towards the development of SC-based analgesic therapies, and this review will approach all of these aspects.

Key Words: Inflammation; Neuropathy; Nociceptive; Pain; Pain treatment; Stem cells

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Core Tip: Since the discovery of stem cells (SCs), they have emerged from a distant dream into a routine therapeutic approach depending on the field. Nowadays, the use of SCs in pain management is mainly based on their anti-inflammatory capacities, releasing neurotrophic factors and providing cellular support to replace damaged neural cells. Evidence supports that SCs can influence nociceptor neuron sensitization building a foundation for the application of these versatile cells in the treatment of neuropathic and inflammatory pain.

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INTRODUCTION

Pain is a major cause of suffering and disability, and can be characterized as a distressing experience that usually signals the presence of injury or disease, generating complex physiologic and emotional responses[1]. According to the Global Pain Index Study by GlaxoSmithKline released in 2020, interviews with 19000 individuals around the world indicate that 34% of the globe's population is in pain every day[2]. Consistently, United States's National Center of Health and Statistics revealed that 20.4% of adults suffered from chronic pain in 2019, and indicated the three major consequences of the condition were: Decreased quality of life, opioid dependence, and poor mental health[3]. This alarming scenario highlights the urgency to pinpoint the physiopathological mechanisms underlying pain and how they interconnect with other systems, which are essential to developing and improving the availability of therapeutic approaches. In this review, we highlight the stem cell (SC)-based therapies aiming to reduce pain. Despite the existence of review articles on SCs and specific types of pain, we observed that there is a gap in the literature regarding comprehensive review articles in this topic approaching various types of pain, the mechanisms of action of SC-based analgesic therapies, and pre-clinical and clinical articles. Therefore, the present review article aims to fill this gap. Data supports that SC-based approaches will revolutionize the field of pain treatment of varied etiologies as we will discuss.

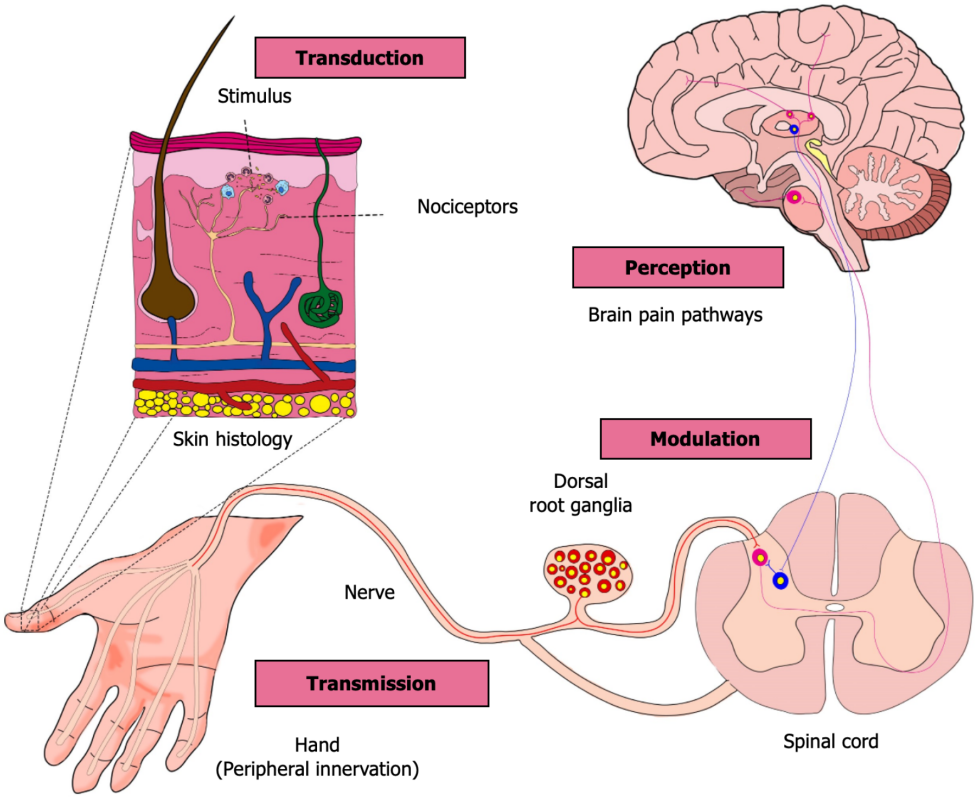
GENERAL VIEW OF PAIN MECHANISMS AND DEFINITIONS

According to the International Association for the Study of Pain (IASP), pain can be defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"[4]. The mechanisms underlying the physiology of pain are extremely complex, involving at least two types of neurons; one whose cellular bodies are in the dorsal root ganglia (DRG) and axons projecting to peripheral tissues and the spinal cord, which are specialized in the perception of potentially harmful stimuli, and then neurons which are present in the spinal cord and in the cortex of the brain, responsible for interpreting the harmful stimuli[5] (Figure 1).

To fully comprehend the pain-related states that can affect the human body, it is imperative to define their source or the causal initiators. The present review will divide pain scenarios as: (1) Inflammatory pain, which involves the presence of inflammation as the primary cause of pain and is responsible for nociceptor neuron activation and plasticity to induce chronic pain[4]; (2) Pathogen-induced pain, a painful state caused by microbial pathogens that directly activate pain-related receptors, which also involves inflammation, but with the presence of a microorganism initiating the process[6]; and (3) Neuropathic pain (NP), a consequence of damage to the nervous system and extensive tissue repair, leading to residual nerve-healing pain[7].

Usually, pain begins with the recognition of possible damage or potentially harmful molecules. When facing a noxious stimulus, our body is able to respond, at cellular and molecular levels, through the immune and nervous system in an attempt to neutralize and repair the damage caused by such stimulus[8]. The immune system and nervous system are responsible for mediating the inflammatory process, generating edema, heat, redness, pain, and loss of function depending on the intensity of those cardinal signs of inflammation[9,10].

For an inflammatory response to occur, the harmful agent must cause tissue damage in the host, or possess molecules that are recognized by immune cells or neurons to trigger either a pro-inflammatory cascade or neurogenic inflammation, respectively[9]. After recognition, a complex cell signaling process begins, inducing vascular alterations to recruit leukocytes that will reach the primary inflammatory foci by diapedesis[10]. Several molecules secreted by immune cells (*e.g.*, cytokines, chemokines, prostanoids) as well as receptors present in their cell membranes and molecules expressed by the pathogen itself are capable of activating nociceptors[9,11]. Nociceptors consist of a subset of sensory neurons, which innervate peripheral tissues (*e.g.*, joints, skin, respiratory and gastrointestinal tract) and have a role in sensing nociceptive stimuli that will be interpreted in the cortex as pain with all its affective and cultural aspects[12].



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Figure 1 Pain mechanism. Representative diagram of anatomical levels involved in the steps of pain from detection of stimulus up to its interpretation and modulation. Starting from the left to the right sides of the figure. The initiation of pain processing starts with the sensing of a noxius stimulus on the skin. This stimulus is detected by nociceptors (whose cellular bodies are in dorsal root ganglia and axons that project to the peripheral tissues and spinal cord) and then relayed to the spinal cord before ultimately reaching the brain (depicted by the ascending pathways in pink). In the brain, the stimulus is interpreted and converted into the sensation of pain. Subsequently, descending pathways (represented in blue) become active, limiting nociceptive input at the spinal cord level. It is crucial to emphasize that glial cells play a significant role in the transmission and modulation of pain. They are activated by neuropeptides released by neurons as well as inflammatory molecules released by immune cells. The recognition of these molecules, whether they are pro-nociceptive or anti-nociceptive, can stimulate glial cells to alter their behavior and contribute to the persistence of the stimulus by releasing nociceptive molecules. Nociceptive neurons can also interact with immune cells that release molecules capable of activating and sensitizing these neurons as well as cause neurogenic inflammation. Varied pathogens (*e.g.*, bacteria, parasites, virus) present virulence factors that can activate nociceptor neurons. For a further in-depth understanding of pain mechanisms in varied conditions we recommend the following review articles[125,174-177].

These stimuli (*e.g.*, inflammatory molecules, pathogen virulence factors) can activate receptors present in nociceptors triggering the phosphorylation of ion channels controlled by ligands [transient receptor potential (TRP) channels] or modify sodium channels controlled by voltage [Voltage-gated sodium (Nav) channels]. Those stimuli, therefore, cause changes in the ion channels facilitating and/or inducing nociceptive neuron depolarization resulting in their sensitization to mechanical and thermal stimulation as well as neuronal firing to transduce the nociceptive information, respectively[9,13]. It is important to stress that the expression of these channels can also be increased due to chronic stimulus, so that neurons that initially express low levels of an ion channel or cytokine receptor start to express them at higher levels[14].

Another important fact, as touched on above, is that the receptor/ion channel activation and pattern of expression can lead to pain sensitization. According to IASP, pain sensitization is defined as increased responsiveness of nociceptors to their normal or subthreshold afferent, and can be divided as hyperalgesia, characterized by increased pain due to a noxious stimulus, or allodynia, a painful response to normally innocuous mechanical or thermal stimuli[15]. Thus, the modifications of what is expressed by nociceptive neurons, and modulating their activation state and responsiveness to stimuli are plastic changes potentially leading to chronic pain.

The functions of the TRP ion channels are related to thermal and mechanical perception[16]. For example the TRP cation channel subfamily V member 1 (TRPV1), involved in heat hypersensitivity and activated by capsaicin; TRP melastatin 8 (TRPM8), involved in cold hypersensitivity; and TRP ankyrin 1 (TRPA1), involved in hypersensitivity to chemical and mechanical stimulation[17]. Furthermore, Nav channels (Nav1.7, Nav1.8 and Nav1.9) are directly responsible for neuronal depolarization, and their expression and activation state can also be modulated during inflammation, infection, and nerve lesions leading to acute and chronic pain[18].

HOW CAN PAIN BE QUANTIFIED IN ANIMAL MODELS?

As this review will approach in great part pre-clinical data using animals, it is important to briefly discuss how "pain" is quantified in animal models. The usage of the term pain to define the quantification of nociceptive behavior in animal models is not widely accepted, because pain assessment involves an emotional component that is often lost in the evaluation of animal responses in most tests. On the other hand, simplifying the terminology by using the word pain, facilitates the understanding by the non-specialized reader about what is under discussion. As an essential physiological mechanism that helps to guarantee the integrity of the organism, pain triggers behavioral responses, for example, moving the hand or paw from a noxious stimulus that can cause tissue damage[19]. In fact, there are subjective components associated with the painful sensation that can only be assessed in humans, since there are variations in the quality and intensity of pain experienced by different individuals for similar injuries, influenced by culture, sex, age, personal experiences, comorbidities and genetic factors[20].

There are different methods to analyze the presence of pain, as well as methods to quantitate it. Most laboratory studies use experimental rodent models, thus, the methodologies are focused on the evaluation of behaviors that can be quantified, such as paw withdraw, paw flinching, paw licking, and abdominal contortions[21], with or without the combination of other methodologies (*i.e.,* place preferences to temperatures or even self-administration of treatment).

Nociceptive assessment methods can be divided into stimulus-evoked and non-stimulus-evoked (spontaneous) behaviors. Spontaneous pain occurs regardless of the presence of an additional evoking stimulus and can be assessed using grimace scales, burrowing assays, gait analysis and weight-bearing methods[21]. Pain evoked by a stimulus can be described as hyperalgesia or allodynia[22]. This parameter is evaluated according to the type of stimulus, being subdivided into mechanical, heat and cold stimuli. Methods that assess pain evoked by mechanical stimuli seek to assess nociceptive sensitivity to a mechanical stimulus (*i.e.,* mechanical pressure on the paw), normally using the von Frey filament method (allodynia indicator), electronic pressure meter test (hyperalgesia indicator), and Randall & Sellito tests (which use increasing pressure or constant pressure over time, assessing hyperalgesia). The analysis of pain evoked by temperature stimuli seeks to assess thermal nociception, either by a heat source (*i.e.,* hot plate, Hargreaves test, tail flick), cold (*i.e.,* cold plate, cold plantar assay and acetone application/evaporation test) or both (*i.e.,* temperature place preference)[21].

Furthermore, behavioral tests are usually analyzed in conjunction with data obtained from cellular and molecular experimental approaches. For instance, neuronal function, activity, and phenotype can be assessed *in vivo* and *in vitro* using electrophysiology studies, intracellular calcium levels, immune staining of neuronal populations and their markers of activation and/or function, release of neuropeptides, cytokines and neurotransmitters, patterns of mRNA, protein and lipid profiles, and optogenetics. Additionally, it is also possible to study the contribution of non-neuronal cells in the nociceptive processes by staining glial immune and parenchymal cells, phenotype markers, and quantitating their production of mediators and functions. Thus, behavioral assays can be accompanied by a great variety of non-behavioral methods to demonstrate a specific hypothesis[23,24].

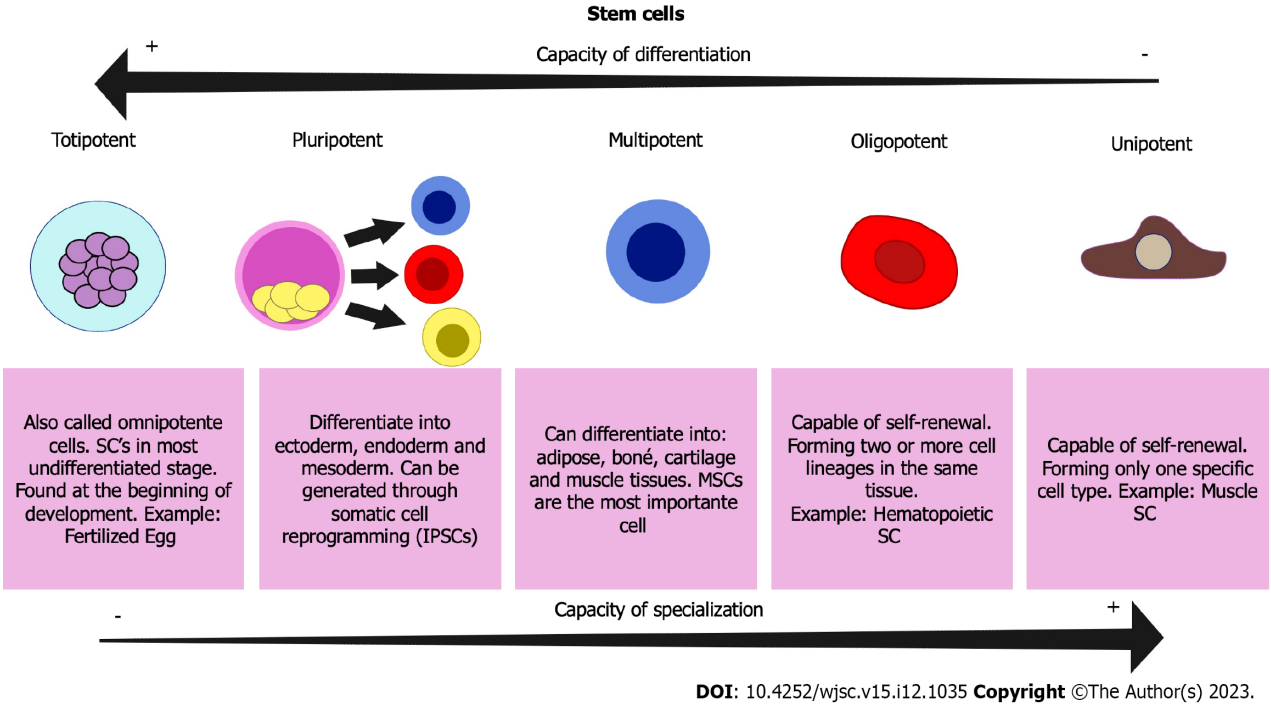
SCS: TYPES AND SOURCES

SCs are non-specialized cells with a high capacity for replication and self-renewal that have a wide range of differentiation possibilities[25]. These cells are present in all stages of life (embryonic, fetal, and adult), they give rise to differentiated cells in organs[26] and are involved in the development, maintenance, repair and renewal of tissues[27].

SC can be categorically divided into five distinct groups (totipotent, pluripotent, multipotent, oligopotent, and unipotent) according to their ability to differentiate, which varies according to the origin and derivation of the cell[26]. Totipotent SC, also called omnipotent, are the cells in the most undifferentiated stage and are found at the beginning of development (*i.e.,* fertilized oocyte)[28]. Pluripotent SC are cells that differentiate from the three germ layers (ectoderm, endoderm and mesoderm)[29]. These cells can be generated through somatic cell reprogramming and are called induced pluripotent SC (iPSC)[26]. Multipotent SC are found in most tissues and have the ability to differentiate into varied tissues, such as adipose, bone, cartilage, and muscle[30]. Within this group, mesenchymal SC (MSC) are the most important cells as will be discussed[26]. Oligopotent SC (*i.e.,* hematopoietic SC) are cells capable of self-renewal, forming two or more cell lineages in the same tissue[31]. Finally, unipotent SC are cells capable of self-renewal and differentiation into only one specific cell type[26] (Figure 2).

Although it might seem that specialization could mean restriction in some tissue types, totipotent SC have virtually no boundaries for differentiation, whereas pluripotent SC demonstrate some degree of specialization. As for the multipotent subgroup, MSC have the advantage of originating in different tissues. MSC have a differentiation rate compatible with the potential application as a pain treatment, particularly for inflammatory and autoimmune diseases[25,32,33].

In addition to classification according to their ability to differentiate, SC can also be classified according to their origin, forming four distinct groups (embryonic, fetal, adult and induced). Embryonic SC (ESC) are pluripotent SC derived from the blastocyst after fertilization (5 to 6 d)[34]. Similar to pluripotent SC, ESC differentiate into the three germ layers or remain in an undifferentiated stage[29,35]. Fetal SC are cells that remain in tissues in a quiescent state until local stimulus induces their proliferation and differentiation into specific cells of the tissues in which they are located[26]. Adult SC are cells derived from the three germ layers and the placenta[26] and depend on specific signals to enter cell division[36]. These cells are important resources in the cell repair and healing processes, as they help to maintain tissue homeostasis by replacing senescent or damaged cells[36]. Finally, iPSC are pluripotent SC produced from the genetic reprogramming of adult somatic cells[26], developing a state similar to ESC, both in morphology, proliferation and gene expression[37].



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Figure 2 Types of stem cells: Different types and characteristics. Representative scheme of the different subtypes of stem cells and their main characteristics. SC: Stem cells; MSC: Mesenchymal stem cell; iPSC: Induced pluripotent stem cell.

Currently, MSC are the main subset of SC used in therapeutic approaches, as they can be isolated from any source of human tissue and reprogrammed. MSC have the advantage of being derived from the patient and, therefore, are adequate for the donor/patient, minimizing the possible ethical issues that could arise from the use of cells from a third-party donor[38,39].

In this review, we discuss recent studies that illustrate the advantages and disadvantages of SC technology to treat painful conditions. Although SC studies have become a popular field of research, there are still a lot of unanswered questions regarding the possible application of SC in pain treatment as well as data supporting their therapeutic benefit.

HOW CAN SC TREATMENT BE USED IN PAIN MANAGEMENT?

Currently, analgesic treatment strategies include acetaminophen, nonsteroidal anti-inflammatory drugs, antidepressants, antiepileptics, local anesthetics, and opioids; and their use is closely linked to significant side effects such as: High renal and hepatic toxicity, headaches, mood swings, constipation, nausea, weight gain and even dependence[40]. Furthermore, these drugs have limited efficacy (Table 1). The statistical measurement for this effectiveness is the number needed to treat (NNT), which is the number of people who must be treated with a given drug for the desired effect to be observed in one person. Thus, the closer the NNT is to 1 the higher the treatment efficacy is[41]. Along with concerning adverse effects, the present scenario highlights the need of additional options to control and treat painful conditions[40].

In this context, the use of SC as a therapeutic approach for pain treatment has great potential due to their unique properties. In general, the use of SC for pain treatment is based on their ability to: (1) Modulate the inflammatory process, switching the pro-inflammatory profile into a pro-resolving state; (2) Interacting directly on the peripheral nervous system, promoting changes on neuronal excitability of primary afferent nociceptor neurons; and (3) Acting on the central nervous system (CNS), *via* alteration of neuronal excitability in the spinal cord and brain (Figure 3). These mechanisms will be discussed in detail throughout the following paragraphs.

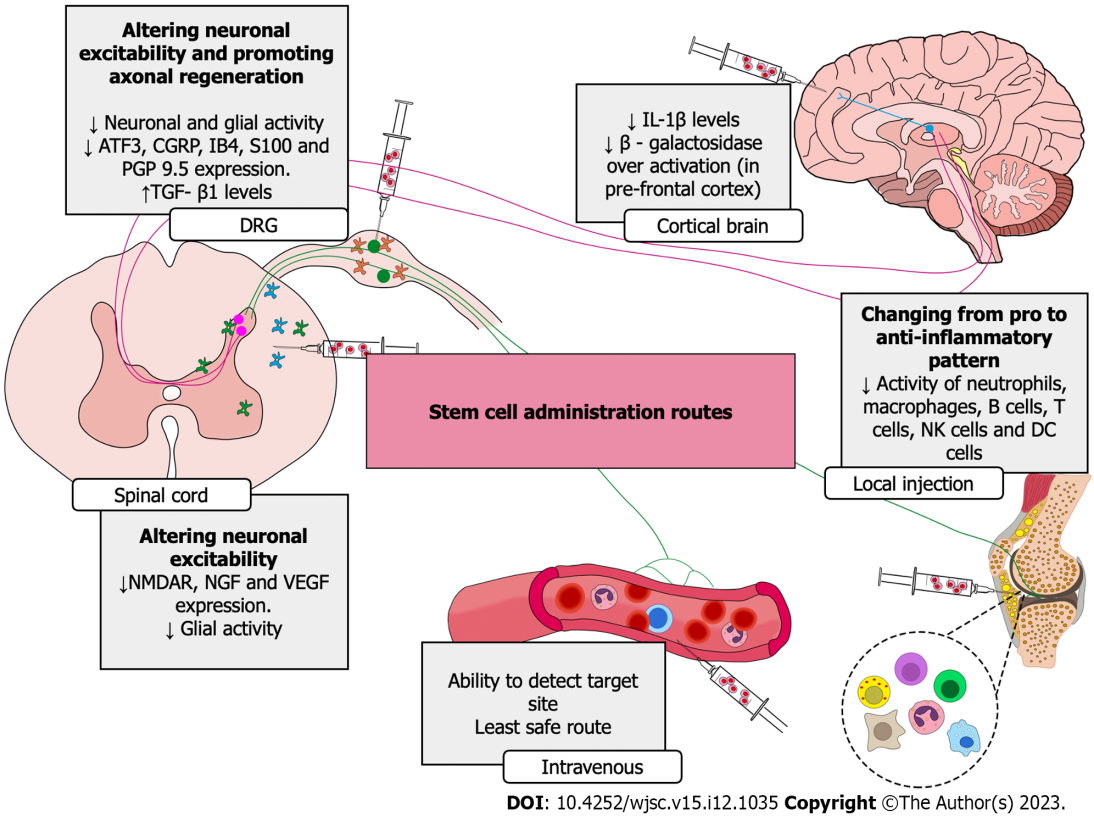
The first mechanism of action of SC to control pain is to modulate the inflammatory process. SC can shape the activity of neutrophils, macrophages, B cells, T cells, natural killer (NK) cells and dendritic cells[42] (Figure 3). In innate immunity, SC regulatory action is based on their ability to produce soluble human leukocyte antigen G5 [capable of inhibiting NK cell-mediated cytolysis and interferon-gamma (IFN-γ) secretion][43]. Another important point is that SC are able to increase the production of interleukin-10 (IL-10), an important cytokine involved in the polarization of M2 macrophages, inducing tissue repair effects[44]. In adaptive immunity, the effects of SC actions involve increased nitric oxide (NO) production (suppressing T cell activity)[45], reduced prostaglandin E_2 (PGE_2) levels[46] and increased indoleamine 2,3-dioxygenase (IDO) activity[47]. Furthermore, it has also been described that treatment with SC increase the expression of IL-4 (by type 2 helper cells)[46] and IL-10[44].

On the other hand, SC can also produce soluble mediators to shape the inflammatory response. SC can be stimulated by inflammatory cytokines such as IFN-γ, IL-1α, IL-1β and tumor necrosis factor alpha (TNF-α)[45,48]. However, in response to those inflammatory cytokines, SC produce transforming growth factor-beta (TGF-β) and IL-10, which are

Table 1 Painkillers and numbers needed to treat

Drug	Drug class	NNT	Ref.
Acetaminophen 650 mg + oxycodone 10 mg	NSAID + opioid	2.7	Gaskell *et al*[178], 2009
Acetaminophen 500 mg + ibuprofen 200 mg	NSAID combination	1.6	Moore and Hersh[179], 2013
Aspirin 1200 mg	NSAID	2.4	Bandolier Extra[180], 2003
Codeine 60 mg	Opioid	16.7	Maxwell and Bateman[181], 2007
Diclofenac 100 mg	NSAID	1.8	Gaskell *et al*[178], 2009
Ibuprofen 400 mg	NSAID	2.5	Lyngstad *et al*[182], 2021
Morphine 10 mg (intramuscular)	Opioid	2.9	Bandolier Extra[180], 2003
Naproxen 500 mg	NSAID	2.7	Derry *et al*[183], 2009
Oxycodone 15 mg	Opioid	4.6	Gaskell *et al*[178], 2009

NSAIDs: Nonsteroidal anti-inflammatory drugs; NNT: Number needed to treat.



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Figure 3 Analgesic mechanisms of stem cells depending on the route of administration and targets/tissues. This scheme summarizes the mechanistic changes caused by stem cell (SC) treatment that resulted in analgesia. The explanation of analgesic mechanisms of SC treatments in the cerebral cortex, spinal cord, dorsal root ganglia, intravenous and local treatment (intra articulary) (indicated by the syringes) can be observed by up and down arrows plus the changed parameter. ATF3: Activating transcription factor 3; CGRP: Calcitonin gene-related peptide; IB4: Isolectin B4; NMDAR: N-methyl-D-aspartate receptor; NGF: Nerve growth factor; PGP9.5: Protein gene product 9.5; VEGF: Vascular endothelial growth factor; TGF: Transforming growth factor; IL: Interleukin.

anti-inflammatory and analgesic cytokines[49,50].

Added to their anti-inflammatory capabilities, SC become promising potential candidates to reduce peripheral sensitization of afferent sensory neurons[51] (Figure 3). SC can decrease the production of cytokines (IFN-γ[46], IL-2[46], IL-17[52] and TNF-α[46]) capable of sensitizing nociceptors and leading to hyperalgesia. SC can also increase the level of cytokines capable of decreasing nociception such as IL-4[46] and IL-10[53,54]. On the other hand, treatment with SC can increase the level of molecules indirectly linked to the promotion of hyperalgesia, such as NO (has a dual role in pain)[45,55], PGE_2[46,56] and IDO[47,57].

These data provide evidence that the applicability of treatment in inflammatory conditions directly depends on the inflammatory microenvironment, which can be positively influenced by SC both by decreasing molecules linked to nociceptive sensitization and by increasing molecules indirectly linked to reducing pain (Figure 3). However, specific cellular targets and the source of soluble mediators were not fully investigated in the experimental settings. We envisage that the field would present a huge evolution if the studies focused not only in quantitating the modulation of soluble mediators, but also investigating the cellular targets and interactions explaining the activity of SC.

In addition to their action on the neuroimmune axis (DRGs and the immune system), an interesting fact that has not been explored much is that SC have a key structural similarity with primary afferent nociceptive sensory neurons. Primary afferent nociceptive sensory neurons and bone marrow-derived mesenchymal stromal cells express the TRPM8 receptor. Recent studies demonstrate that the influence of this receptor on neurons involves the detection of cold temperatures (18-23 °C), and that TRPM8 inhibition reduces pain[58]. In addition, this channel is also capable of modulating cell differentiation in SC, as its activation increases osteogenic differentiation in human bone marrow MSC[59].

The use of SC in pain treatment is also based on the ability of these cells to act on the CNS since they have the ability to desensitize the CNS by inhibiting glutamate-related pathways (reduction of NMDAR expression and TGF-β1 secretion) [60]. Furthermore, SC can decrease central sensitization, *via* reduced glial cell activity, once again contributing to the attenuation of hyperalgesia[61] (Figure 3), these points will be discussed in further details in the following topics.

SC IN THE TREATMENT OF INFLAMMATORY DISEASES AND INFLAMMATORY PAIN

The number of articles investigating the relationship between pain and SC is still small, so this section was divided into two parts: (1) The use of SC to treat inflammatory diseases. This part is of interest in terms of the perspective of application to inflammatory pain since inflammatory mediators can induce nociceptor sensitization mechanisms; and (2) Articles that analyze the analgesic activity of SC treatment.

SC in the treatment of inflammatory diseases without the assessment of pain

In this topic we will present the mechanisms behind the ability of SC to modulate the inflammatory response, and the most recent discoveries involving SC and inflammation. Reducing pain is a potential outcome since inflammation was reduced in these studies, however, this specific disease symptom was not tested.

As previously discussed, the inflammatory process is composed of a series of signals. Through the release of inflammatory molecules (cytokines, leukotrienes, and prostanoids), which are used for cellular communication, tissue resident immune cells start the inflammatory response process. Examples of these inflammatory molecules include, for instance, IL-1β, IL-5, IL-6, IL-17A, TNF-α, nerve growth factor (NGF), LTB_4, 5-HT and PGE_2. They have a role in the recruitment and activation of leukocytes and some can also activate receptors expressed by the primary afferent nociceptor sensory neurons inducing the activation (causing depolarization) or sensitization (causing an enhancement of response upon other chemical, mechanical or thermal stimulation) of these neurons. This neuro-immune interaction is relevant to pain and inflammation[62] and we will discuss how SC can interfere with it.

Neutrophils are the most abundant cell type in the blood and large numbers are recruited in acute inflammation[63]. SC activity on neutrophils may present two distinct patterns[64]. MSC can suppress hydrogen peroxide production in activated neutrophils in vitro. On the other hand, tissue resident glandular MSC seem to play an early role in lipopolysaccharide (LPS)-triggered inflammation by producing cytokines and chemokines to recruit neutrophils. These polymorphonuclear leukocytes presented an increase in their lifespan, chemokine production and response to LPS stimulation[65]. This MSC-dependent response is protective in the sense that LPS stimulation represents part of an infection. On the other hand, neutrophils can participate in the induction of pain by producing LTB_4 and PGE_2, which activate and sensitize nociceptor neurons[66,67]. Reactive oxygen species can also activate nociceptor neurons[9].

One of the most important anti-inflammatory mechanisms of SC is to induce a class switch in the pattern of macrophages from M1 phenotype to M2 phenotype. This ability was observed in models of osteoarthritis using the treatment with exosomes from iPSC and MSC. The M2 macrophage phenotype is involved in tissue repair as well as in the resolution phase of inflammation[68]. Upon inducing this macrophage phenotype switch, there is a decrease in the production of pro-inflammatory cytokines IL-1β and TNF-α produced by M1 macrophages[68,69]. Interestingly, this activity seems to be related to SC-derived PGE_2. Contrasting with the hyperalgesic role of PGE_2 in inflammation by sensitizing primary nociceptive sensory neurons[70], this prostanoid also has other regulatory functions. For instance, when dendritic cells are stimulated to produce PGE_2 and IL-10, these antigen presenting cells reduce the expression of major histocompatibility complex (MHC)-II and CD86, thus, reducing their function of presenting antigens. As a result, there is a reduction of lymphocyte proliferation and adaptive immune response[71]. This evidence points to specific roles of PGE_2 and how somewhat opposing effects can be triggered by this prostanoid depending on the site of production and cellular target as well as explain anti-inflammatory activities of SC. Furthermore, PGE_2 can induce the production of TSG-6[72], which is capable of converting the macrophage phenotype from pro-inflammatory to anti-inflammatory[73]. Finally, MSC can recruit monocytes and macrophages through the production of chemokines such as C-C motif chemokine ligand (CCL)2, CCL3 and CCL12 in inflamed tissue, which contributes to the tissue repair[72,74].

Another characteristic of SC is their ability to interfere with the lymphocytic pattern. In Crohn's disease patients, it was observed that treatment using MSC was able to decrease lymphocyte proliferation, the proportion of CD4+ T cells, and decreases the levels of TNF-α and IL-6. The authors also demonstrated an increase in regulatory T cells (Tregs) and IL-10 production, thus, suggesting that an MSC would shift the T cell population towards an increase of Tregs that produce IL-10 to limit inflammation and reducing CD4+ T cells[75]. It has also been reported that SC are able to inhibit the prolif-

eration of B lymphocytes[76]. The mechanism by which SC can affect these changes in both T and B lymphocytes is not fully elucidated. However, Lin *et al*[77] demonstrated that SC express adhesion molecules vascular cellular adhesion molecule-1 and intracellular adhesion molecule 1, which lead to adhesion to lymphocytes indicating a possible SC contact dependent regulation of lymphocyte function[78]. In addition, SC release NO, PGE_2 and hepatocyte growth factor[79], as well as activate the programmed cell death 1 death receptor[80], suggesting that SC could reduce lymphocyte proliferation and survival[81-83].

In addition to its influence on the immune system, it is also interesting to note the opposite. One of the reasons why most studies use MSC is due to their ability to evade the immune system explained by their lack of HLA class I and II surface markers. Both molecules are necessary for recognition by immune cells, thus, lacking such molecules is an essential characteristic to avoid the rejection of the transplanted SC and also leaves open the possibility of transplanting cells from one donor to a patient, and not solely autologous transplantation[77]. Despite this characteristic, most human studies involving SC and inflammation use MSC in a non-randomized manner. It is also noteworthy that MSC are believed to be the only SC with immunoregulatory and regenerative capabilities, in addition to presenting almost all of the effects mentioned above in this topic[77].

Application of SC in the treatment of inflammatory pain

Inflammatory pain occurs when afferent sensory neurons detect specific molecules that are able to sensitize or activate these neurons, such as cytokines, peptides, and other molecules[84]. Most of these molecules are secreted by immune and glial cells, which normally communicate in a controlled and homeostatic manner. In inflammatory diseases, there is an imbalance between pro and anti-inflammatory molecules, leading to the activation of membrane receptors in nociceptive neurons and the consequent activity of sodium channels and TRP channels resulting in neuronal sensitization and activation[9]. Inflammation also leads to neuronal plasticity in which they express higher levels of ion channels, additional receptors, and present enhanced activity and response when compared to a non-sensitized neuron. These plastic alterations cause the transition from an acute to a chronic pain state[85].

Table 2 summarizes the current literature in which SC treatment was applied to reduce pain; taking as a principle that these cells can reduce the production of pro-hyperalgesic molecules. It is noteworthy that studies on the analgesic activity of SC in inflammatory pain have been increasing in the last 10 years. Before that, the articles were mainly focused on the mechanisms of inflammation control using SC, but not necessarily on pain. Evidence supports that the mechanism by which SC reduce thermal and mechanical hypersensitivity is based on three perspectives: (1) Targeting the inflammatory response: By having the ability to reduce the secretion of pro-inflammatory cytokines (capable of sensitizing nociceptors) such as IL-6, TNF-α and IL-17[69,86,87] as well as increasing IL-4 levels, a cytokine capable of mediating analgesia[88]; (2) Modifying cell phenotype: the effects of these cytokines (mentioned in the perspective 1) are also based on the ability of SC to induce a change in the macrophages phenotype from M1 to M2 (non-phlogistic macrophage)[88], in addition to decreasing CCL2, CCL5 and CXC10 (macrophage recruiting factors)[87]. Additionally, SC participate in the reduction of mast cell degranulation, which would result in the secretion of varied molecules with nociceptive activities such as 5-HT, histamine, LTB_4 and cytokines[89]; and (3) Neuronal and glial effects: The treatment with SC can decrease the expression in the spinal cord of calcitonin gene-related peptide (CGRP)[90] and ionized calcium-binding adaptor molecule 1 (IBA-1) (marker of glial activation, related to central sensitization)[91] and decrease the activity of immune cells. The current understanding is that SC activity occurs through the secretion of TSG-6[92], a soluble chemokine-binding protein. In turn, TSG-6 acts through inhibiting the expression of protein kinase C-γ[87] and suppressing the Toll-like receptor 2 (TLR2)/myeloid differentiation factor-88 adaptor protein (MyD88)/nuclear factor kappa B (NF-κB)[91] signaling pathway. This TLR2/MyD88/NF-κB cascade occurs in glial cells in the spinal cord and its activation leads to the production of pro-inflammatory/hyperalgesic cytokines.

The studies that address treatment in humans do not address possible signaling mechanisms of SC. However, SC treatment decreases pain for 6 mo [autologous bone marrow concentrate, one treatment, (0.5-1) × 10^6 cells][93] to 1 year (autologous adipose-derived stromal vascular cells, one treatment, 14 × 10^6 cells)[90] in knee osteoarthritis patients and for 2 years for discogenic back pain patients[93]. Thus, although the analgesic mechanisms of SC in humans remains elusive, the data supports the analgesic effect of clinical SC treatment.

SC TREATMENT OF PATHOGEN-INDUCED PAIN

In general, treatment using SC has great analgesic and anti-inflammatory potential. Similarly to sterile inflammatory diseases, infections also cause inflammation, however, there is a dual role in which inflammation involves the immune response against the pathogen as well as being responsible for tissue damage. Finding the balance between these two effects is difficult[94]. Studies involving SC and infections caused by bacteria and viruses are restricted only to the inflammatory context of infections. On this topic we will present data that demonstrate a potential analgesic effect in diseases caused by SC in bacterial and viral infections.

Bacteria-induced pain

Bacterial infections can commonly cause discomfort and pain[95]. It is believed that pain caused by bacteria and their bacterial components can occur in two different manners: The first and most classic one occurs through the activation of immune cells, production of pro-inflammatory cytokines, and the consequent nociceptor sensitization by neuronal effects of inflammatory molecules[96,97]. The second occurs through the direct activation of nociceptor neurons by bacterial virulence components, such as α-hemolysin, capable of forming pores (as its primary activity), thus activating both

Table 2 Articles that used stem cells to treat inflammatory pain (stem cell-based treatment of inflammatory pain or in models where pain is certainly involved, but was not investigated)

Ref.	Stem cell therapy design	Key findings	Pain-related highlights	Route of administration	Number of cells or amount of extracellular vesicles and exosomes
Hsueh *et al* [69], 2023	iPSC-derived EVs for the treatment of rabbit articular cartilage OA in an *in vivo* model and an *in vitro* interleukin (IL)-1 β-induced model	Improvement in both *in vivo* and *in vitro* models of OA by stimulation of chondrocytes proliferation and decreasing senescence were accompanied by: Decreasing of TNF-α); IL-6; protein 21 (p21); MMP 13; ADAMTS5; and increasing of collagen II	Indirect: Specific pain receptors/pathways weren't investigated	i.a.	100 μg iPSC-EV
Gao *et al* [184], 2022	Small EVs from iPSC-derived mesenchymal stem-cells ameliorate tendinopathy pain by inhibiting mast cell activation	The treatment was able to decrease acute pain in tendinopathy, as well as inhibit infiltration of activated mast cells and interactions with nerve fibers *in vivo*. In the *in vitro* experiments, the treatment decreased mast cell degranulation and the expression of pro-inflammatory cytokines and genes involved in the hypoxia inducible factor-1 signaling pathway	Pain behavior was measured by the von Frey method. And the weight distribution on the knees by SWB; immunofluorescence staining of tendon sections for tryptase (mast cell marker) and PGP9.5 (nerve fiber marker) was performed to assess the number of activated mast cells and the anatomical interaction between mast cells and nerve fibers. In addition, the SWB and CatWalk test was also. carried out	Local injection (quadriceps tendon)	1×10^9 particles
Yu *et al* [185], 2022	Intravital imaging and single cell transcriptomic analysis for engraftment of mesenchymal stem-cells in an animal model of interstitial cystitis/bladder pain syndrome	The transplanted cells formed a perivascular-like structure. They were also shown to express cyclin-dependent FOSe kinase-1 which played a key role in modulating the migration, engraftment and anti-inflammatory functions of multipotent MSCs, which determined their therapeutic potency *in vivo*	*In vivo* two-photon intravital microscopy and single-cell transcriptome analysis were used to assess the effects of stem cell treatment on interstitial cystitis/bladder pain syndrome	Injected into the outer layer of the anterior wall and dome of the bladder	10^6
Zhang *et al* [186], 2022	EVs derived from MSCs alleviate neuroinflammation and mechanical allodynia in interstitial cystitis rats by inhibiting NLRP3 inflammasome activation	SC treatment decreased suprapubic mechanical allodynia and frequent urination in rats with interstitial cystitis. It also decreased glial cell activity as well as neuroinflammation in the spinal cord. Furthermore, the treatment was able to decrease the activation of NLRP3 inflammasomes and the TLR4/NF-κB signaling pathway	Behavioral test (von Frey) was performed to measure allodynia and western blot and immunofluorescence for protein related to inflammation and central sensitization analysis: CD9, CD63, CD81, ALIX, TNF-α, IL-1β, IL-6, IBA-1, GFAP, NLRP3, Caspase-1, IL-18, TLR4, p65 NK-κB, phospho-p65 NK-κB (western blot). NLRP3, neuron-specific nuclear protein, GFAP and OX-42 labeling (immunofluorescence)	i.t.	20 μg
González-Cubero *et al* [86], 2022	EV and soluble fractions of adipose tissue-derived MSCs secretome induce inflammatory cytokines modulation in an *in vitro* model of discogenic pain	There was a decrease in the expression of IL-6, IL-8 and IL-17	Indirect method: The authors measured the regulatory capacity of EVs on the inflammatory molecules IL-1α, IL-1β, IL-6, IL-8, IL-17, nerve growth factor, brain-derived neurotrophic factor, IFN-γ, NF-κB and TNF and MMP-1, MMP-2, MMP-3, MMP-13 and ADAMTS-5	*In vitro* model	1×10^6
Yang *et al* [91], 2020	Anti-inflammatory protein TSG-6 secreted by bone marrow MSCs attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia	Stem cells are capable of secreting TSG-6. This article demonstrated that i.t. administration of this protein leads to a decrease in mechanical allodynia and heat hyperalgesia. In addition to inhibiting neuroinflammation in the spinal cord (IBA-1), the protein administration inhibited the activation of the TLR2/MyD88/NF-κB pathway in the dorsal horn of the ipsilateral spinal cord by the secretion of TSG-	The activation of the TLR2/MyD88/NF-κB signaling pathway was evaluated by western blot and by immunofluorescence. Mechanical allodynia and heat hyperalgesia were observed by behavioral tests	i.t.	5×10^6

		6 and reduced the production levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α			
Zhang *et al* [187], 2019	MSCs exosomes alleviate temporomandibular joint OA by attenuating inflammation and restoring matrix homeostasis	It was observed that the treatment led to repair of the temporo-mandibular joint, along with a reduction in inflammation and pain. Treatment increased IL-1β-impaired sulfated glycosa-minoglycan synthesis and suppressed IL-1β-induced nitric oxide and MMP13 production. These effects were partially abrogated by inhibitors of adenosine receptor, protein kinase B, ERK and adenosine monophosphate activated protein kinase phosphorylation	Mechanical hyper-nociception was assessed using the von Frey microfilament. The expression of inflammatory mediators and other components was measured using quantitative polymerase chain reaction	i.a.	100 μg
Ebbinghaus *et al*[88], 2018	A promising new approach for the treatment of inflam-matory pain: Transfer of stem cell-derived tyrosine hydroxylase-positive cells (mouse model)	It has been demonstrated that the administration of endogenous tyrosine hydroxylase positive cells (iTH+) cells, prior to the induction of antigen-induced arthritis, was not sufficient to suppress the disease. However, the treatment was able to decrease pain behavior evoked by inflammation, largely due to the production of IL-4 induced by iTH+ cells. Furthermore, the treatment was able to reduce the levels of pro-inflammatory molecules, in addition to increasing the number of M2 macrophages in dorsal root ganglia	Inflammatory molecules were quantified, such as: IFN-γ, IL-2, IL-4, IL-6, IL-10, CCL3, CCL5, CXCL1, CXCL2, CXCL10, and CXCL12. Additionally, pain-related behavior tests and IBA-1 and arginase 1 labeling in the dorsal root ganglion *via* immunofluorescence was performed	i.v.	10^6
Ichiseki *et al* [92], 2018	I.a.-injected MSC stimulate anti-inflam-matory molecules and inhibits pain related protein and chondrolytic enzymes in a monoiodo-acetate-induced rat arthritis model	The treatment was able to inhibit central pain sensitization (decreased expression of CGRP in the spinal cord) and increase the secretion of TSG-6 by stem cells, an anti-inflammatory factor and cartilage protector	For the evaluation of central sensit-ization, CGRP staining was performed by immunofluorescence. And the histochemical technique was also used for the evidence in the joint of ADAMTS5 and TSG-6	i.a.	5.0×10^6
Fodor and Paulseth [90], 2016	Adipose derived stromal cell injections for pain management of OA in the human knee joint	After 3 mo of treatment, patients showed improvement in WOMAC and VAS scores, which were maintained for 1 yr. ROM and TUG improved until the third month. All patients achieved full activity with decreased knee pain and no infections or adverse effects reported	Patients were evaluated by following scores on the WOMAC, VAS pain scale, ROM, TUG, and magnetic resonance imaging	i.a.	14.1×10^6 nucleated stromal vascular fraction cells per knee
Pettine *et al* [93], 2016	Treatment of discogenic back pain with autologous bone marrow concentrate injection with minimum two-year follow-up (humans)	Stem cell treatment reduced visual analog scale and Initial Oswestry Disability Index scores. In addition to reducing pain in patients. The treatment proved to be effective for up to 2 yr after the injection	Pain was assessed using scores provided by patients	Intradiscal injection	$(0.5\text{-}1) \times 10^6$
Durand *et al* [89], 2015	Persistent visceral allodynia in rats exposed to colorectal irradiation is reversed by MSC treatment	Induced a time-dependent reversion of the visceral allodynia and a reduction of the number of anatomical interactions between mast cells and PGP9.51 nerve fibers	Spinal sensitization (was available for labeling of phospho-ERK neurons), colonic neuroplasticity (as increased density of substance P1 nerve fibers); s, visceral sensitivity was evaluated by studying the contraction of the abdominal muscles in response to colorectal distension	i.v.	1.5×10^6
Watanabe *et al*[87], 2015	Early transplantation of MSC after SCI relieves pain hypersensitivity through suppression of pain-related signaling cascades and reduced inflammatory cell	The treatment was able to decrease thermal and mechanical hypersens-itivity. Improvements in pain were mediated by suppression of PKC-γ and p-CREB expression in dorsal horn neurons. The authors also reported a decrease in the levels of	Mechanical allodynia and thermal sensitivity were recorded. In addition, immunofluorescence was performed on spinal cord sections, labeling for: PKC-γ or p-CREB, GFAP, cD11B and phospho-protein 38. For immunoblot analysis,	Injection into the middle of the contusion site, identified as the middle point of the laminectomy area	2.0×10^5

	recruitment	pro-inflammatory cytokines (TNF-α, IL-6), mediators of early secondary vascular pathogenesis (MMP9) and macrophage recruitment factors (CCL2, CCL5 and CXCL10). All in addition to increased levels of a microglial stimulating factor GM-CSF)	components of the mitogen-activated protein kinase family, inflammatory mediators (TNF-α, IL-6, MMP-9), macrophage recruiting factors (CCL2, CCL5, and CXCL10) and GM-CSF (a microglial stimulating factor) were analyzed		
Emadedin *et al*[188], 2012	Intra-articular injection of autologous MSC in six patients with knee OA	The treatment was able to improve scores related to pain, the functional status of the knee and the distance covered up to six months after the injection	VAS which is a subjective assessment that represents the patient's perception of the current pain state with a higher score reflecting more severe pain. Functional status of the knee was assessed by WOMAC OA index. This index evaluates pain, joint stiffness, physical and social function of patients with OA of the knee	i.a.	(20-24) × 10^6

iPSC: Induced pluripotent stem cells; OA: Osteoarthritis; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; p21: Protein 21; MMP: matrix metalloproteinase; ADAMTS5: A disintegrin and metalloproteinase with thrombospondin motifs 5; i.a.: Intra-articular; EV: Extracellular vesicle; SWB: Static weight bearing; PGP9.5: Protein gene product 9.5; CDK1: Cyclin-dependent FOSe kinase 1; MSC: Mesenchymal stem cells; NLRP3: NOD-like receptor protein 3; i.t.: Intrathecal; TLR: Toll-like receptor; NF-κB: Nuclear factor kappa B; IBA-1: Ionized calcium-binding adapter molecule 1; GFAP: Glial fibrillary acidic protein; CD: Cluster of differentiation; IFN-γ: interferon-gamma; TSG-6: Tumor necrosis factor alpha-stimulated gene 6; MyD88: Myeloid differentiation primary response 88; ERK: Extracellular signal-regulated kinase 1; iTH+: Tyrosine hydroxylase positive cells; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; DRG: Dorsal root ganglion; i.v.: Intravenous; CGRP: Calcitonin gene-related peptide; WOMAC: Western Ontario and McMaster Universities Arthritis Index; VAS: Visual Analogue Scale; ROM: Range of motion; TUG: Timed ascent and descent; PKC-γ: Protein kinase C gamma; GM-CSF: Granulocyte-macrophage colony stimulating factor; p-CREB: Phospho cyclic AMP response element binding protein; p-p38: Phospho-protein 38.

Nav1.8+ and TRPV1+ neurons[98,99]; or by LPS, which is capable of activating TLR4 expressed by neurons or be sensed by TRPA1 (at lower doses) and even TRPV1 (at higher doses)[100,101]. Thus, bacteria can induce nociceptor sensory neuron sensitization indirectly by activating immune cells that will produce nociceptor sensitization molecules or directly by activating neuronal receptors and triggering nociceptor depolarization by forming membrane pores[96].

As discussed in the topic "SC in the treatment of inflammatory diseases and inflammatory pain", the effects of SC activity on bacterial infections have two main characteristics. The first is that treatment with SC can increase phagocytic activity and neutrophil survival in bacterial infections[102,103]. Second, SC treatment can attenuate exacerbated immune responses, as seen in a murine model of endotoxemia induced by LPS, the administration of MSC by the intraperitoneal route is capable of reducing the severity of the disease, mainly by reducing the levels of IL-1β, IL-6, IL-8 and TNF-α and increasing IL-10 in the plasma, as well as reducing the recruitment of neutrophils in the liver[104].

Despite demonstrating great analgesic potential by reducing the levels of cytokines causing hypersensitivity and increasing bacterial clearance, no articles were found that investigated a possible decrease in pain in models that use bacteria and SC treatment. This fact highlights a gap in the literature and potential field to be explored.

Viral infection-induced pain

In general, viral infections can cause pain. Depending on the type of virus and site of infection, an inflammatory process begins, characterized by the high release of inflammatory mediators. The detection of these mediators can occur through the central or peripheral nervous system[11]. A recent study demonstrated that most of the symptoms of intranasal H1N1 infection (reduction in food intake, water intake, and mobility during early-stage infection and improved survival) come from the detection of PGE_2. This prostanoid activates EP3 receptors, which are expressed in both the hypothalamus and circumventricular organs, as well as in nerve endings in the nasopharynx[105]. It has also been demonstrated that the herpes simplex virus 1 can also infect the neurons present in the DRG, and the persistence of the virus leads to the recruitment of leukocytes to the region, TNF-α secretion and neuronal hypersensitivity[106]. While the pain caused by Chikungunya virus depends on its envelope protein E2 activation of TRPV1+ nociceptor sensory neurons[107].

As discussed earlier, SC have a high anti-inflammatory capacity. This characteristic is also observed against viruses since treatment with SC can decrease the levels of cytokines[108] that sensitize nociceptors. An interesting fact about SC is that these cells have IFN-responsive genetic machinery. Therefore, upon detecting the presence of the signal produced in viral infections (flavivirus, dengue and Chikungunya virus), they express genes related to IFN-induced transmembrane proteins, which prevents the contamination of these cells[109]. In murine models, treatment with MSC reduces the levels of IL-1α, IL-6, TNF-α and IFN-γ in response to H9N2 infection[110]. It has also been described that the administration of extracellular vesicles derived from MSC was able to reduce the viral load in lung epithelial cells of pigs infected with H1N1/H7N2/H9N5[111]. Recent evidence demonstrates that extracellular cells derived from MSC have the *in vitro* capacity to inhibit the replication of the Influenza virus and severe acute respiratory syndrome coronavirus 2[112]. It is also important to report that recently a number of clinical studies have been carried out in humans using MSC treatment in coronavirus disease (results not yet reported)[113-117]. Despite the potential of SC treatment to reduce pain and the fact that viral infections can cause pain, there is a gap in the literature of studies investigating the analgesic effectiveness of SC in the treatment of viral infection-triggered pain.

SC-BASED TREATMENTS FOR NP

Studies involving inflammation and pain are still somewhat restricted when compared to the volume of articles that study NP and treatment using SC. On this topic, we will present an overview of the mechanisms involved in the development of NP and then explain the mechanisms underpinning SC therapy.

What mechanisms are involved in the development of NP?

To fully comprehend the mechanisms that modulate NP, it is essential to understand how our body processes the external and internal stimuli that can lead to nociceptive alterations. Pain sensation is the product of higher brain center perception and can be influenced by a number of factors like attention, affective dimensions, autonomic variables, immune variables, and hormones[118].

In normal tissue, pain is triggered by intense or noxious stimuli that leads to the activation of high threshold transmembrane ion channels, a process defined as nociceptive pain. These ion channels present on nociceptor neurons convert mechanical, thermal or chemical stimuli - that can vary from pinpricks and light touch to vibrations, indentations, gravity and sound waves - into biochemical regulated electrical signals that are directed to the brain through the generation and conduction of action potentials, characterizing mechanotransduction[119].

NP, on the other hand, occurs when a pathological process leads to nerve damage, and the healing process of the nervous system results in maladaptation observed by the lower unbalanced threshold of neuronal activation that can involve numerous pain-related processes, from the detection by the nociceptor neuron to the acknowledgement of nociceptive signaling by the brain[120]. NP comprises peripheral neuropathy, postherpetic neuralgia, trigeminal neuralgia, nerve root pain, and phantom limb pain; and can be caused by lesions or diseases involving the primary afferent sensory neurons of the somatosensory nervous system, including peripheral fibers (Aβ, Aδ and C fibers) and CNS neurons[121-123].

The extensive modulatory possibilities that can unravel during the healing process of nervous tissue make evident the importance of neuroplasticity, a phenomenon that can be defined as the ability of the nervous system to adapt its responses according to intrinsic or extrinsic stimuli by reorganizing its structure, functions, or connections after injuries. Thus, neuroplasticity is a key factor in the development of NP[124].

NP typically arises from an incorrect healing process due to an imbalance between neuroimmune interactions, glial cells, and neurotrophic factors. Briefly, a nervous system lesion triggers inflammatory and repair responses that are not always successful. Unsuccessful nerve repair will lead to plastic changes causing the sensitization of nociceptive neurons, sympathetic sprouting forming basket structures that explain sympathetic maintained chronic pain, incorrect formation of novel synapses causing the stimulation of second order neurons upon touch (causing allodynia). Maladaptive tissue repair can also involve the activation of glial cells that further stimulate nociceptive neurons causing retrograde sensitization and boosting second order nociceptive signaling to the brain when a nervous fiber is sectioned. Cellular events occur at the site of injury and in the neuronal soma corresponding to the area in the DRG. These cellular events include, for instance, local immune cell signaling *via* purinergic P2 receptors-ATP signaling[125]. Nerve damage is, therefore, an initiating event, but it is not the sole orchestrating factor. Neuronal plastic changes include the alteration of ion channel properties, affecting spinal and brain sensory signaling, shifting pain perception so that normal innocuous stimuli can result in pain by facilitating neuronal depolarization upon thermal and mechanical stimulation as a result of nociceptor neuron sensitization owing to increased membrane excitability. Spontaneous neuronal firing can also be observed[119,125,126].

The regeneration process can be divided into five steps: (1) Fluid phase; (2) Matrix phase; (3) Cellular migration phase; (4) Axonal phase; and (5) Myelination phase[127]. After injury, neuronal genetic expression is altered to induce the release of neurotrophic and angiogenic factors like NGF, brain-derived neurotrophic factor, glial cell-derived neurotrophic factor (GDNF), vascular endothelial growth factor-A and angiopoietin-1[128], and to upregulate the expression of their corresponding receptors. These growth factors support the axonal lengthening of the injured nerve from its proximal fragment, as the damaged axons in the distal nerve fragments undergo degeneration, a process known as Wallerian degeneration[127]. Infiltrating macrophages and Schwann cells also take part by clearing myelin debris and retro feeding the secretion of neurotrophic and pro-angiogenic factors, enabling the formation of connective tissue bridging the nerve clefts, as Schwann cells create an endoneurial tube that guides the axonal regeneration process starting from a growth cone located at the Ranvier's node[129].

The accurate balance between compensatory and decompensatory reactions of the nervous system when facing neural damage is of the utmost importance, because many of the changes that occur in response to neural injury are potentially adaptive, such as the removal of cell and myelin debris, regulation of receptors that counterbalance the loss of input, and appropriate signaling in order to dampen ion fluxes and metabolic stress after the acute injury[130]. Among the adaptive modifications, we can also cite anti-apoptotic signaling to prevent neuronal cell death, induction of axonal growth and sprouting, synaptic remodeling, and remyelination[131].

A defining characteristic that is often present in NP is the absence of an identifiable stimulus upon spontaneous pain. Such abnormal sensitization can be generated at any anatomical level related to the nociceptive sensory experience: (1) The site of the injury that induced the NP in the first place and underwent maladaptive healing - or neuroma - where the regenerated axon can get misdirected and become unable to reach the desired target[132]; (2) Cell death of the corresponding DRG neurons; (3) Alterations in gene regulation and expression of the surrounding intact afferent fibers; (4) Central sensitization and altered connectivity in the spinal cord of low-threshold large myelinated afferents *via* synaptic facilitation and loss of inhibition at multiple levels of the neuraxis; and (5) Voltage-gated channel-related generation of spontaneous ectopic activity in nociceptors, a mechanism whose importance is supported by the effectiveness of nonselective sodium channel blockers as local anesthetics[133]. Increased synaptic strength enables previously sub-

threshold inputs to activate nociceptive neurons, reducing their threshold and enhancing their responsiveness, which results in the expansion of their receptive fields. In addition, phenomena like conduction slowing or blocking, reduced inhibition, inappropriate connectivity, altered processing of both nociceptive and innocuous afferent input, abortive growth, neuronal loss, and glial scarring can be decisive factors underlying the pathogenesis of NP and the onset of spontaneous pain[126,134].

It is important to note that once NP is generated, the sensory hypersensitivity tends to persist for prolonged periods, even though the original initiator factor may have long since disappeared[126]. The complexity and abundance of factors involved in the central and peripheral nervous system modifications, make the treatment of NP challenging and expensive[51].

The use of SC as a therapy for NP

Given the fact that current treatments for NP are not fully effective, there is a need for improving NP treatment. Among the characteristics that stand out in the use of SC for NP is the ability of these cells to migrate to the injured site and repair damaged cells, even when administered systemically[135]. Most literature reports address SC treatment in the context of NP models, in which strategies are based on replacing damaged nerve cells and induce the production of neurotrophic factors. In addition, SC inhibit apoptosis and degeneration processes, and increase the survival of both injured and uninjured nerves[136] (Table 3). Furthermore, the mechanisms of action of SC modulating NP vary according to the stimulus in question (disease model/condition), since each disorder presents its own alterations that can be classified as peripheral and/or central, we will follow this rationale in the next sections.

Peripheral nervous system-related analgesic mechanisms of SC

The peripheral actions of SC focus on their anti-neuro-inflammatory capacity and their neuroprotective potential and ability to promote growth. SC anti-neuro-inflammatory capacity (described in the previous topics) includes the ability to reduce the levels of pro-inflammatory cytokines IL-1β, IL-6[137] and TNF-α[87]. These cytokines are extremely important in peripheral sensitization, because they are capable of sensitizing nociceptors and increase the expression of TRP and Na channels, consequently leading to mechanical and thermal sensitization[9]. In addition to reducing the production of hyperalgesic cytokines, SC can increase IL-10 levels in NP models[137], which also explains the decrease in the pro-inflammatory cytokine production[138] and induction of the class switch of macrophages from an M1 to an M2 profile. M1 macrophages are responsible for proinflammatory responses, overexpress CD80, CD86, and CD16/32 which are essential for activating lymphocytes and thus adaptive immunity. Moreover, M1 phenotype macrophages are capable of secreting pro-inflammatory cytokines[139]. In contrast, M2 macrophages express chemokines CCL17 and CCL22 that mediate the control of Treg cell biology[140], mannose receptor (CD206) that induces endocytosis[141], as well as anti-inflammatory responses and contribute to the repair of damaged tissues by the phagocytosis of debris[138]. Moreover, M2 macrophages can be manipulated *in vitro* to produce an opioid-mediated analgesic effect, demonstrated by its complete blockade by the opioid receptor antagonist naloxone methiodide in an *in vivo* model of chronic constriction injury (CCI) of the sciatic nerve induced NP[142]. Although there is no data on SC and microglia polarization, considering the activity of SC to polarize macrophages towards a M2 profile, if this activity is also true for microglia polarization to an M2 profile, this would be an important analgesic mechanism in NP. M1 microglia are actively involved in NP by producing mediators that sensitize nociceptors[143].

SC are also able to directly interact with the DRG neurons through GDNF production[144]. GDNF is of great importance for studies where there is neuronal damage, such as in sciatic nerve injury[136]. Since the administration of GDNF is capable of decreasing pain-related behaviors, due to its inhibitory action on the molecules such as activating transcription factor 3 - marker of neuronal injury and IB4 (a noceptive neuron marker of a neuronal population that do not express CGRP, and that downregulate NGF and receptor tyrosine kinase)[145,146].

The actions of SC on DRG neurons also affect the production of neuropeptides related to pain signaling. In models of CCI-induced NP, SC treatment was able to decrease nociceptive behavior by releasing TGF-β1, which activates neuronal TGF-β1R[147], as well as reducing the production of hyperalgesic cytokines IL-1β and TNF-α[148].

CNS-RELATED ANALGESIC MECHANISMS OF SC

The actions of SC treatment targeting mechanisms in the CNS environment are based on three hypotheses: (1) Desensitization of the CNS; (2) Inhibition of glial cells; and (3) Reduction in apoptosis and autophagy[149].

The SC mechanisms that are dependent on: (1) Desensitizing the CNS in NP mainly involve the downregulation of glutamate neurotransmission. After the damage of peripheral neurons, glutamate is released in the spinal cord as well as N-methyl-d-aspartate (NMDA) and an increase of its receptor (NMDAR) expression is seen. This mechanism aims to maintain the transmission of the captured noxious signal to the cerebral cortex, where it will be interpreted as pain[150]. Treatment with SC decreases the CNS expression of NMDAR, interrupting the maintenance of nociceptive signaling [150]. In addition, SC can secrete TGF-β1, which inhibits the signaling carried out by glutamate that would otherwise stimulate nociceptive signaling by the activation and proliferation of microglia and astrocytes in NP. TGF-β1 also reduces the expression of proinflammatory cytokines in the CNS in NP, thus, reducing neuroinflammation and nociceptor neuron sensitization[147].

Second, glial cells play a key role in central sensitization. As previously explained, after the detection of noxious stimuli by nociceptors, primary sensory afferent neurons secrete neuropeptides in the spinal cord dorsal horn, where central signaling takes place. If the noxious stimulus persists, glial cells can be activated by these neuropeptides (CGRP and

Table 3 Articles that used stem cells for neuropathic pain treatment (stem cell-based treatment of neuropathic pain)

Ref.	Stem cell therapy design	Key findings	Pain-related highlights	Delivery route	Number of cells or amount of extracellular vesicles and exosomes
Gao *et al* [189], 2023	Huc-MSCs-derived exosomes attenuate neuropathic pain by inhibiting activation of the TLR2/MyD88/NF-κB signaling pathway in the spinal microglia by targeting radical S-adenosyl methionine domain containing 2	Huc-MSCs-derived decreased protein levels of TLR2, MyD88, and p-p65 that were significantly upregulated in the CCI group in model rats	The protein levels of TLR2, MyD88, p65, and p-p65 were examined by western blotting	i.t.	5 μg
Miyano *et al* [190], 2022	I.v. administration of human MSCs derived from adipose tissue and umbilical cord improves neuropathic pain *via* suppression of neuronal damage and anti-inflammatory actions in rats	Both the mechanical threshold and the differences in weight bearing of the right and left hind paws improved significantly. In addition, the authors also reported a decrease in the ATF-3 and IBA-1 in DRG. The authors also reported that the treatment significantly improved the partial sciatic nerve ligation-induced decrease in the level of myelin basic protein in the sciatic nerve	Was performed by von Frey and dynamic weight bearing. Also, the authors did I against ATF-3, IBA-1, myelin basic protein, NeuN, neurofilament (NF) 200	i.v.	5×10^6
González-Cubero *et al* [191], 2022	Application of adipose-derived MSCs in an *in vivo* model of peripheral nerve damage	Rat sciatic nerve damage models both *ex vivo*, on TNF-induced Schwann cells, and *in vivo* using biomaterial implants containing TNF. Upregulation of c-Jun and downregulation of early growth response protein 2 myelin-associated transcription factors were induced by TNF-related damage, but the addition of ASCs or ASC-conditioned medium (secretome) were able to revert the profile	qPCR, western blot, and confocal microscopy were chosen to quantify nerve healing-related protein expression and production *in vivo* and *ex vivo*. The sciatic functional index was calculated to assess nerve regeneration, but no pain-specific mechanisms were investigated	Sciatic nerve	*ex vivo* 0.5×10^6 cells; *in vivo* 4×10^6 ASCs
An *et al* [192], 2022	Immortalized bone MSCs with inducible galanin expression produce controllable pain relief in neuropathic rats	hTERT-BMSCs/Tet-on/GAL cells were able to induce controllable pain relief by spared nerve injury of sciatic nerve under the transcriptional control of doxycycline	To determine the analgesic efficacy acted through GalR1, GalR2, and phospho-protein kinase Mζ expression levels in spinal dorsal horn were analyzed by western blot assay	Subarachnoid space	10^6
Lee *et al* [193], 2022	MSCs spheroids alleviate neuropathic pain by modulating chronic inflammatory response genes	The authors report a decrease in mechanical allodynia, related to a decrease in TNF-α and IFN-γ levels. In addition to a smaller number of cells marked with cluster of differentiation (CD) 68 in the region	The von Frey test was performed to assess mechanical allodynia, while immunofluorescence was used to observe changes in CD68 and IBA-1 levels. TNF-α and IFN-γ levels were assessed by the ELISA assay	Intramuscular	10^6
Chen *et al* [194], 2022	Synergic Effect of early administration of probiotics and adipose-derived MSCs on alleviating inflammation-induced chronic neuropathic pain in rodents	The authors demonstrate that treatment with stem cells alone can reduce thermal hyperalgesia and mechanical allodynia, with the potentiated effects after combined treatment with probiotics. Interestingly, they found a reverse correlation between protein expressions of inflammatory (phospho-NF-κB, IL-1β, TNF-α and MMP-9), apoptotic (cleaved-caspase-3, cleaved-PARP), oxidative-stress (NOX-1, NOX-2), deoxyribonucleic acid (DNA)-damaged (γ-H2AX) and MAPK-family (p-P38, p-JNK, p-ERK 1/2) biomarkers as	To observe pain-related behavior alterations, Hargreaves and von Frey tests were applied. Immunofluorescence was performed for p-p38; NF200; peripherin, 53BP1, β3 Tubulin analysis. Western blot was chosen to identify alteration of p-NF-kB, IL-1ß, TNF-α, MMP-9, NOX-1, NOX-2, caspase 3, cleaved-PARP, γ-H2AX, p-ERK1/2, p-JNK, p-p38, Nav.1.3, Nav.1.8, Nav.1.9 and immunoglobulin G	i.v.	3.0×10^5

		well as the protein levels of voltage-gated sodium channels (Nav.) 1.3, Nav.1.8, and Nav.1.9 in L4-L5 in DRG to the pain-behavior results obtained by thermal hyperalgesia and mechanical allodynia testing, characterizing a set of "pain-connived cells" presenting the following profiles: Nav1.8+/peripherin+, p-ERK+/peripherin+, p-p38+/peripherin+ and p-p38+/NF200+. Mainly by suppressing inflammation and oxidative stress, the combination of probiotic and ASCs therapy was found superior for alleviating CCI-induced neuropathic pain			
Zhang *et al* [195], 2021	Lncenc1 is identified as a novel regulator in neuropathic pain by interacting with EZH2 and downregulating the expression of Bai1 in mouse microglia	Virgin embryonic stem cells express Lncenc1, which can activate microglia in DRG and induce the production of TNF-α, IL-1β, and MCP-1. Lncenc1 silencing reduced mechanical and thermal hyperalgesia, as well as lower levels of pro-inflammatory cytokines	The mechanical withdrawal threshold was measured by von Frey filaments and thermal hyperalgesia *via* hot plate assay. Immunofluorescence was performed to analyze OX-42, western blot to assess EZH2, suppressor of zeste 12, embryonic ectoderm development, BAI1, tri-methylation of histone 3 lysine 27 (H3K27me3), H3K27ac, total histone H3, glyceraldehyde-3-phosphate dehydro-genase and OX-42. RT-qPCR was performed to identify expression alterations on Lncenc1, EZH2, BAI1, OX-42, inflammatory factors TNF-α, IL-1β and chemokine MCP-1. TNF-α, IL-1β and MCP-1 protein changes were assessed by ELISA	Not informed	Not informed
Masoodifar *et al*[161], 2021	Effect of the conditioned medium of MSCs on the expression levels of P2X4 and P2X7 purinergic receptors in the spinal cord of rats with neuropathic pain	Animals treated with the conditioned medium (stem cells secretome) showed a reduction in mechanical and thermal hyperalgesia. A decrease in the expression of P2X4 and P2X7 receptors was related to the interaction of neurons and glial cells in neuropathic pain	The von Frey and hot plate tests were applied to measure mechanical and thermal hyperalgesia, respectively. In addition, qPCR was performed to measure the expression of P2X4 and P2X7 receptors	i.p.	1×10^5
Kotb *et al* [196], 2021	Preemptive stem cells ameliorate neuropathic pain in rats: A central component of preemptive analgesia	MSCs-treatment increased allodynia, mechanical hyperalgesia, and thermal hyperalgesia thresholds. Stem cells were able to reach the cerebral cortex, as the CCI group had few stem cells expressing PCNA, CD117 and nestin in the cerebral cortex. The treated group had numerous CD117-, nestin-, PCNA-positive stem cells recently proliferated in the cerebral cortex. Together, the results indicate a potential central analgesic effect of i.v. MSC-treatment	To evaluate pain behavior, von Frey, Randall and Selitto, and hot plate tests were performed. Immunohistochemical analyses of GFAP, PCNA and nestin were also performed	i.v.	1×10^6
Zhang *et al* [197], 2021	Therapeutic effects of peripherally administered neural crest stem cells on pain and spinal cord changes after sciatic nerve transection	The treatment was able to induce thermal and mechanical analgesia, possibly by decreasing the expression of TRPV1, cFOS, p-ERK, ERK, iNOS and NF-κB, p65 and increasing BDNF and GAP-43 in the spinal cord	To assess mechanical allodynia, the authors used the BEM-404 device (similar to the von Frey). For thermal withdrawal latency, they use Hargreaves. In the western blot, they had the following targets: BDNF, cFOS, GAP-43, p-ERK, ERK 1/2, TRPV1 and iNOS. Immunofluor-escence: IBA-1, GFAP and CGRP were assessed	Local injection	2×10^6
Yang *et al* [91], 2020	Anti-inflammatory protein TSG-6 secreted by BMSCs attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB	I.t. administration of TSG-6 secreted from stem cells decreases mechanical allodynia and thermal hyperalgesia, inhibiting IBA-1 and the activation of the	The activation of the TLR2/MyD88/NF-κB signaling pathway was evaluated by western blot and immunofluorescence, while allodynia and hyperalgesia were assessed by the behavioral tests Dynamic Plantar Aesthesiometer, Hargreaves and	i.t.	5×10^6

	signaling pathway in spinal microglia	TLR2/MyD88/NF-κB pathway in the dorsal horn of the ipsilateral spinal cord. Levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, were also reduced	rotarod system		
Jwa *et al* [198], 2020	ASCs alleviate cold allodynia in a rat spinal nerve ligation model of neuropathic pain	ASCs or ASC-derived culture medium decreased neuropathic pain behaviors in a rat model with L5 spinal nerve ligation	Mechanical and cold allodynia were assessed by von Frey filaments and acetone assay, respectively. Mechanisms were not assessed	Intrathecal or injection into the right retro-orbital sinus	10^6
Gama *et al* [164], 2018	Conditioned medium of BMSCs as a therapeutic approach to neuropathic pain: A preclinical evaluation	The animals showed improvement in thermal hyperalgesia and mechanical allodynia. They also showed reduced levels of IL-1β, TNF-α and IL-6 and increased IL-10 in the spinal cord and sciatic nerve	To evaluate thermal hyperalgesia, the Hargreaves test was performed, and von Frey mechanical allodynia. To evaluate the motor function test, the rotarod test was performed. Using the ELISA method, TNF-α, IL-1β, IL-6 and IL-10 were quantified	i.v.	10^6
Lin *et al* [165], 2017	Autologous ASCs reduce burn-induced neuropathic pain in a rat model	There was no difference between the groups regarding thermal hyperalgesia, whereas in mechanical allodynia, the treated group presented analgesia from the 3rd wk of the first treatment. Western blot analyses revealed a decrease in p-Akt/Akt and Bax/Bcl-2 and levels of LC3B-II and Beclin 1 in the spinal cord, suggesting that the treatment also decreased apoptosis and autophagy. This effect was accompanied by a reduction in COX-2, iNOS and nNOS. The treated group also showed lower expression of p-JNK (an inflammatory marker), TUNEL (apoptosis marker), phospho-NFκB (inflammatory marker) and increased p-IκB (an inhibitor of NFκB activation)	Immunofluorescence were performed to analyze p-IκB; NeuN, GFAP, phospho-NFκB and p-JNK; and western blot for COX-2, iNOS, nNOS, Akt/protein kinase B, p-Akt, B-cell lymphoma 2, Bcl-2-associated X protein, β-actin, LC3B and Beclin 1	Subcutaneous into the scar tissue of the right hind paw	10^6
Vaquero *et al*[199], 2018	I.t. administration of autologous bone marrow stromal cells improves neuropathic pain in patients with SCI	Treatment with mesenchymal stromal cells for human chronic SCI: Pain scores demonstrated a continuous decrease in neuropathic pain from the first month until the 10th	Intensity of neuropathic pain was evaluated by standard numerical rating scale (visual analogue scale) from 0 to 10. Mechanisms were not assessed	i.t.	10^6
Sun *et al* [200], 2017	I.t. administration of hBMSCs genetically modified with human proenkephalin gene decrease nociceptive pain in neuropathic rats	hBMSCs engineered with human proenkephalin gene were used on sciatic nerve (CCI)-induced model to reduce neuropathic pain in rats	Mechanical withdrawal threshold (von Frey filaments) and paw thermal withdrawal assays were used to assess the changes in pain-related behavior. Levels of Leu-enkephalin, a neurotransmitter that activates opioid receptors and is released by hBMSCs were found augmented *via* ELISA assay in genetically modified BMSCs compared to secretions released by naıve BMSCs	i.t.	6×10^6
Fischer *et al* [201], 2017	Inhibition of neuropathic hyperalgesia by i.t. BMSCs is associated with alteration of multiple soluble factors in cerebrospinal fluid	BMSCs decrease the levels of intracellular adhesion molecule 1, IL-1β, hepatocyte growth factor), IL-10, and Nope protein relacionated by Tibial nerve injury	Antibody array analysis was performed and the levels of cytokines and other soluble factors in cerebrospinal fluid samples was measured	i.t.	2.5×10^5
Xie *et al* [202], 2017	Active nerve regeneration with failed target reinnervation drives persistent neuropathic pain	Semaphorin 3A, an inhibitory axonal guidance molecule, reduces functional regeneration, spontaneous activity, and pain behaviors when applied to the injury site in vivo. Silencing of the upregulated GAP43 with interfering RNA injected into the axotomized sensory ganglion reduced pain behaviors	Behavior assays: von Frey filaments acetone cold sensitivity, dynamic tactile allodynia with a wisp of cotton across the plantar surface of the hindpaws, and spontaneous guarding behavior score. Immunohistochemistry for GAP43 tracer methods to assess anatomical nerve regeneration	Injury site	

Brini *et al* [203], 2017	Therapeutic effect of human ASCs and their secretome in experimental diabetic pain	Treatments with both human ASC and their secretome were able to reverse mechanical, thermal allodynia and thermal hyperalgesia inducing high IL-1β, IL-6 and TNF-α and low IL-10 levels, restoring cytokine balance, Th1/Th2 balance and preventing skin innervation loss in neuropathic STZ-diabetic mice model	Mechanical allodynia was tested using the Dynamic Plantar aesthesiometer, a drop (50 μL) of acetone was placed in the middle of the plantar surface of the hind paw to evaluate cold allodynia and the hot-plate test was used to assess thermal hyperalgesia. Immunohistochemistry and ELISA were performed for cytokines assessment	i.v.	1×10^6
Watanabe *et al*[87], 2015	Early transplantation of MSCs after SCI relieves pain hypersensitivity through suppression of pain-related signaling cascades and reduced inflammatory cell recruitment	BMSC improved SCI model *via*: Down of protein kinase C-γ and phosphocyclic AMP response element binding protein on DRG neurons, both of which are upregulated in association with at-level allodynia after contusion spinal cord. Decreased activation of MAPK signaling in injured spinal cord by p-p38 and p-ERK1/2 decrease. Decreasing macrophage recruitment through. Down TNF-α, IL-6, MMP-9, CCL2, CCL5, and C-X-C motif chemokine ligand 10. Decreased microglia stimulation factor, granulocyte-macrophage colony stimulating factor, platelet-derived growth factor receptor α	For behavioral and sensory testing, the Basso Mouse Locomotor Scale, the Dynamic Plantar Aesthesiometer (allodynia), and the Plantar Test Apparatus (thermal sensitivity) were assessed. immunohistochemistry, flow cytometry and immunoblot assays were performed to determine protein levels	BMSCs were injected into the middle of the contusion site, identified as the middle point of the laminectomy area	2×10^5
Zhang *et al* [204], 2014	I.t. administration of MSCs reduces the ROS and pain behavior in neuropathic rats	I.t. rat MSCs injection reduced pain response and ROS production in the dorsal horn of neuropathic rats induced by spinal nerve L5 ligation model	Mechanical sensitivity was assessed using von Frey filaments and production of ROS *via* dihydroethidium fluorescent staining	i.t.	10^5
Liu *et al* [205], 2014	MSCs inhibit lipopolysaccharide-induced inflammatory responses of BV2 microglial cells through TSG-6	Anti-inflammatory effects of MSCs and TSG-6 in an *in vitro* LPS-induced BV2 microglial activation model inhibiting NF-κB and MAPK pathways. MSCs can modulate microglia activation through TSG-6 and TSG-6 attenuates the inflammatory cascade in activated microglia	RT-qPCR, western blot, electrophoretic mobility shift assay, immunofluorescence and laser-scanning confocal microscopy techniques were used	*In vitro*	1.0×10^5 LPS-activated MSCs
Vicker *et al* [206], 2014	A preliminary report on stem cell therapy for neuropathic pain in humans	Treatment led to a reduction in stem cell treatment pain intensity scores in 7/9 patients (two with marginal improvement and five subjects with good to excellent pain reduction). Five of these positive responders also reduced their need for gabapentin medication	Patients were assessed for: Change in pain intensity and the secondary outcome was any reduction in daily consumption of anti-neuropathic medication	Perineural, directly in the center or source of pain, and in the adjacent pain field of the affected branches of the trigeminal nerve	Number of cells not reported, but extracted from 100-200 g of patient tissue
Tao *et al* [154], 2013	Role of NRG1/ErbB signaling in stem cell therapy for SCI-induced chronic neuropathic pain	The treatment induces remyelination in the injured spinal cord and reduces SCI-injury-induced chronic neuropathic pain. In addition to increasing levels of NRG1 and ErbB4 slightly reduced by SCI. Also, the author related that Stem cells differentiated into oligodendrocytes	To evaluate mechanical allodynia, the von Frey filament test was applied. Immunofluorescence for NG2, APC-CC1, GFAP, NeuN, and western blot for NRG1 and ErbB4 levels assessment	i.t.	10^6
Xu *et al* [207], 2013	I.t. transplantation of NSCs appears to alleviate neuropathic pain in rats through release of GDNF	The treatment was able to cause thermal and mechanical analgesia. Accompanied by an increase in GDNF in the DRG and spinal cord. The authors also suspected that these changes occurred due to the transformation of stem cells into astrocytes in the spinal cord	To evaluate the mechanical withdrawal threshold, the Electric von Frey test was used. For thermal withdrawal latency, a method with a high-intensity projection lamp bulb was used. For immunofluorescence: Nestin; βIII-tubulin; GFAP. For ELISA: BDNF and GDNF	i.t.	10^6

Choi *et al* [208], 2013	Core-shell nanoparticle controlled human adipose tissue-derived stem cells neurogenesis for neuropathic pain therapy	Treatment activated biochemical functions of Dicer, Oct4, Sox2, Nanog, and glutathione peroxidase 3 improving stem cells self-renewal and differentiation abilities	von Frey and Hargreaves behavior tests were performed to assess mechanical and thermal hyperalgesia changes, respectively. Immunofluorescence, western blot and RT-qPCR techniques were used to study alterations in protein production/expression/localization	i.t.	Unspecified
Franchi *et al* [137], 2012	I.v. NSCs abolish nociceptive hypersensitivity and trigger nerve regeneration in experimental neuropathy	NSCs administration in CCI mouse model significantly decreased proinflammatory (IL-1 β, IL-6), activated anti inflammatory (IL-10) cytokines in the sciatic nerve, and reduced spinal cord Fos expression in laminae I-VI	Thermal hyperalgesia was tested according to the Hargreaves using a Plantar Test Apparatus, while mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer. Immunohistochemistry, immunofluorescence, and qPCR plus ELISA assays were performed for Fos and GFPI; substance P and CGRP; and IL-1β, IL-6 and IL-10, respectively	i.v.	10^6
Sacerdote *et al*[209], 2013	Systemic aAdministration of human ASCs reverts nociceptive hypersensitivity in an experimental model of neuropathy	Human ASCs were able to completely revert neuropathic pain symptoms in a murine CCI model by: IL-1β decreased and IL-10 increased in the lesioned nerve. Restored normal iNOS expression	Thermal hyperalgesia was tested according to the Hargreaves, while mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (von Frey filament)	i.v.	1×10^6, 3×10^6 and 6×10^6
Choi *et al* [73], 2011	Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages	TSG-6 interacts through the CD44 receptor on resident macrophages to decrease zymosan/TLR2-mediated nuclear translocation of the NF-κB	RT-qPCR, ELISA, NF-κB translocation assays and isolation of resident macrophage RNA was performed	i.p.	1.6×10^6
Siniscalco *et al*[210], 2011	Long-lasting effects of human MSCs systemic administration on pain-like behaviors, cellular, and biomolecular modifications in neuropathic mice	The treatment was able to reduce pain-like behaviors such as mechanical allodynia and thermal hyperalgesia. In addition to reducing IL-1β and IL-17 levels and increasing IL-10 in the spinal cord and reducing labeling for alternatively activated macrophages (CD106)	For behavior analysis, the following tests were applied: von Frey filaments, Rotarod and Hargreaves. Immunofluorescence: CD73; IL-1β; IL-17; CD4; GFAP; IBA-1; western blot: IL-1β, IL-17, IL-10 e CD106	i.v.	2×10^6

Huc-MSCs: Human umbilical cord mesenchymal stem cells; TLR2: Toll-like receptor 2; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappa B; Rsad2: Radical S-adenosyl methionine domain containing 2; p-p65: Phospho-protein 65; CCI: Chronic constriction injury; i.t.: Intrathecal; i.v.: Intravenous; MSCs: Mesenchymal stem cells; ATF-3: Activating transcription factor 3; IBA-1: Ionized calcium-binding adapter molecule 1; DRG: Dorsal root ganglion; MBP: Myelin basic protein; NeuN: Neuron-specific nuclear protein; NF: Neurofilament; TNF: Tumor necrosis factor; ASCs: Adipose tissue derived-mesenchymal stem cells; RT-qPCR: Real time quantitative polymerase chain reaction; hTERT-BMSCs/Tet-on/GAL: Rafted human telomerase reverse transcriptase-immortalized bone marrow mesenchymal stromal cells with inducible galanin expression; GalR: Galanin receptor; SDH: Spinal dorsal horn; IFN-γ: Interferon gamma; CD: Cluster of differentiation; ELISA: Enzyme-linked immunosorbent assay; i.m.: Intramuscular; IL: Interleukin; MMP: Matrix metalloproteinase; PARP: Poly ADP-ribose polymerase; NOX: NADPH oxidase; MAPK: Mitogen-activated protein kinase; p-JNK: Phosphorylated Jun N-terminal kinase, p-ERK: Phospho-extracellular signal-regulated kinase; Nav: Voltage-gated sodium channels; LncRNA: Long-chain noncoding ribonucleic acid; Lncenc1: Long-chain noncoding RNA embryonic stem cells expressed 1; EZH2: Enhancer of zeste homologue 2; MCP-1: Monocyte chemoattractant protein-1; SUZ12: Suppressor of zeste 12; EED: Embryonic ectoderm development; BAI1: Brain-specific angiogenesis inhibitor 1; H3K27me3: Tri-methylation of histone 3 lysine 27; P2X4: P2X purinoceptor 4; i.p.: Intraperitoneal; PCNA: Proliferating cell nuclear antigen; GFAP: Glial fibrillary acidic protein; TRPV1: Transient receptor potential cation channel subfamily vanilloid 1; iNOS: Inducible nitric oxide synthase; p65: Protein 65; BDNF: Brain-derived neurotrophic factor; GAP-43: Growth-associated protein 43; CGRP: Calcitonin gene-related peptide; TSG-6: Tumor necrosis factor-α-stimulated gene 6 protein; BMSC: Bone marrow mesenchymal stem cells; p-Akt: Phospho protein kinase B; LC3B: Light chain-3B; COX-2: Cyclooxygenase 2; nNOS: Neural nitric oxide synthase; hBMSCs: Human bone marrow stem cells; CSF: Cerebrospinal fluid; Th1: Type 1 helper; SCI: Spinal cord injury; p-CREB: Phosphocyclic AMP response element binding protein; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; GM-CSF: Granulocyte-macropgahe colony stimulating factor; PDGFR-α: Platelet-derived growth factor receptor α; ROS: Reactive oxygen species; LPS: Lipopolysaccharides; NRG1: Neuregulin-1; GDNF: Glial cell derived neurotrophic factor; NSC: Neural stem cells.

substance P)[151]. When activated, glial cells' function will be related to the maintenance and enhancement of the interaction between the peripheral and CNSs, secreting, for instance, cytokines that will activate spinal cord neurons [152]. Interestingly, this glial activation can be detected by the expression of some targets, such as IBA-1 for microglia and glial fibrillary acidic protein (GFAP) for astrocytes[61]. These glial cells are responsible for the subsequent release of cytokines inducing a series of cellular responses, such as upregulation of glucocorticoids and glutamate receptors, leading to spinal cord excitation and neuroplasticity[136].

It has been reported that in experimental conditions involving spinal cord injury and spinal cord treatment with SC, embryonic stem (ES) cells differentiate into oligodendrocytes using positive selection and mechanical enrichment[153],

promoting functional recovery after a spinal cord injury producing myelination[154]. Spinal cord SC treatment also decreases GFAP[155] and IBA1[156] indicating the down-regulation of astrocyte and microglia activation, respectively. SC express high levels of CXCL12[157]. CXCL12 can reduce the activation of astrocytes and microglia in spinal nerve ligation models, thus, potentially contributing to the activity of SC in NP[158]. There are other potential repercussions of glial inhibition. For instance, the decrease of IBA1 also occurs by inhibiting gasdermin D-induced microglia pyroptosis, thus, promoting autophagy[159]. In fact, impaired autophagic flux aggravates NP by increasing neuroinflammation[160]. Moreover, the mitogen-activated protein kinase signal pathway is activated after microglial activation, which promotes long-term potentiation and central sensitization in pain[136].

Another mechanism of action of SC that explains their analgesic activity by down-modulating glial cell activation is the capability they have at reducing the expression of purinergic P2X purinoceptor 4 (P2X4) and P2X7 receptors in a rat model of NP induced by CCI of the sciatic nerve[161]. P2X4 and P2X7 receptor activation mainly by ATP (coming from nerve damage) leads to the activation of glial cells and the release of IL-1β, TNF-α and IL-6, capable of sensitizing nociceptors and perpetuating NP[162,163].

In a different perspective, it has also been shown that SC are capable of increasing IL-10 levels in the spinal cord[164], in addition to secreting TSG-6 in the spinal cord[91], and decreasing IBA-1 activity by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. These activities reduce nociceptor neuron activation and neuronal plasticity.

The third analgesic mechanism of SC in the CNS involves the inhibition of neuronal death in the CNS. SC treatment can reduce the levels of p-Akt/Akt and Bax/Bcl-2, LC3B-II, Beclin 1 and TUNEL (markers of cellular death) in the dorsal horn of lumbar spinal cords in burn-induced NP. Thus, suggesting that SC can reduce the levels of apoptosis, necrosis and autophagy related to inflammation in spinal cord neurons of dorsal horn cells[165].

SC TECHNOLOGY AS A TOOL FOR INVESTIGATING PAIN-RELATED MECHANISMS

Although most of the efforts in scientific research regarding SC and pain are guided towards finding suitable painkillers, it is worth mentioning that SC technology can also be proven valuable to create different experimental models, which can contribute to understanding pathophysiological mechanism of pain and thus, evolving towards therapy. As an example, Kaneski *et al*[166] developed a human ESC (hESC)-based model to study the poorly understood pathophysiology of pain in Fabry disease. This X-linked glycolipid storage disorder that results in a deficiency in the lysosomal enzyme alpha galactosidase A (AGA) can cause recurrent attacks of excruciating pain ("Fabry pain crisis") that occur spontaneously or in response to extreme temperatures, fever, fatigue, stress, overheating, or exercise. The group generated two AGA-deficient hESC clones using CRISPR-Cas9 gene editing techniques and demonstrated that AGA-deficient human SC could be differentiated into peripheral neurons with nociceptor properties, offering a tool for the investigation of cellular mechanisms for this and other peripheral neuropathies[166].

Some studies utilized dental pulp SC to investigate the possible effects of sirtuin 6 (SIRT6) - an NAD-dependent protein deacetylase known for its role as a differentiation regulator - as a modulation factor using a model of LPS-induced pulpitis (inflammation in the dental pulp). LPS is the major virulence component of gram-negative bacteria cell walls and is widely recognized as a potent activator of inflammation[167]. Their results demonstrated that injection of lentiviral vector-expression SIRT6 leads to SIRT6 overexpression in rats, reduced LPS-induced neutrophils infiltration, a marked decrease in proinflammatory cytokines (IL-6, IL-1β, and TNF-α) and deactivation of the NF-κB pathway. LPS-induced pulpitis in turn, upregulated TRPV1 expression and activity, by downregulating SIRT6. Interestingly, CGRP release was induced by pulpitis while the overexpression of SIRT6 inhibited TRPV1 expression and CGRP release. The expression of inflammatory cytokines, dentin matrix acidic phosphoprotein 1, and NF-κB activation were upregulated after the addition of capsaicin, a TRPV1 channel agonist. Taken together, their results suggest that SIRT6 may be both a negative regulator of pulpitis and an inflammatory pain modulator[168].

DOWNSIDES AND POSSIBLE TREATMENT SIDE-EFFECTS

The biggest concern surrounding SC treatment is the onset of tumors as a result of the therapy, particularly *via* systemic administration routes. This concern is due to the proliferation potential that these cells have, thus, raising the possibility of tumor development if they continue to proliferate after transplantation[169]. The literature lacks *in vivo* studies evaluating the tumorigenic effect of SC treatment. However, it is currently acknowledged that tumorigenicity can develop through three different manners. Firstly, the presence of undifferentiated SC anchored in other already differentiated lineages can lead to tumor formation, due to their high replication rate[169]. Second, reprogramming factors may remain active in transplanted SC, promoting the transformation of these cells and, consequently, tumorigenic transplantation[169]. Finally, tumorigenicity can also develop during *in vitro* culture through genetic mutations[169]. Studies demonstrate that a significant rate of SC derived from bone marrow undergo spontaneous transformations towards a malignant profile in long-term cultures (5 to 106 wk), showing an increase in the rate of proliferation and morphological and phenotypic changes[170].

Another disadvantage is the possible development of cellular rejections. This can occur when the individual's immune system recognizes the SC as foreign antigens and develops an immune response against them[25,169]. Polymorphic molecules of the MHC are an example of immunologically recognized molecules that induce rejection[171]. In fact, studies have already demonstrated that the administration of ESC in the myocardium of allogeneic mice results in the development of an immune response with significant infiltration of T lymphocytes and dendritic cells[172]. Likewise,

abnormal gene expression in cells differentiated from iPSC can induce a T cell-dependent immune response[173]. Finally, even though there are studies that demonstrate the effects of systemic administration of SC, there is a lack of data in the literature that assess their distribution and actions in the body after treatment.

CONCLUSION

In this review, we provide evidence on the therapeutic potential of the use of SC in pain treatment. Analgesia was observed with SC administration as well as SC extracellular vesicle administration. This analgesic effect was found to be achieved with a variety of routes, such as intra-articular, intravenous, intrathecal, intramuscular, intraganglionar, perineural, subcutaneous, and local injection. Additionally, the SC load for these administrations varied between 1.0×10^5 to 24×10^6 for SC, and 1×10^6 to 1×10^9 for SC extracellular vesicle. The specific conditions of each experiment can be appreciated in the Tables 2 and 3.

This review discussed that the analgesic mechanism of action of SC treatment can be indirect by acting on inflammation, changing the pattern from pro-inflammatory to anti-inflammatory mediators. SC can decrease the levels of cytokines that have a role in mechanical and thermal hyperalgesia in addition to promoting the secretion of cytokines with analgesic roles. These anti-inflammatory analgesic mechanisms have been demonstrated in peripheral tissue. In the CNS, SC can cause analgesia by inhibiting the effects of glutamate on spinal cord neurons and by its ability to decrease glial activity and therefore central sensitization.

There are still gaps in the elucidation of the mechanism of action of SC, since most of the articles in the literature aim mainly at the treatment of NP. Among the literature hiatus we can cite the lack of studies on the analgesic mechanisms of SC in models using microorganisms as stimuli. Confirmatory studies on SC therapy long-term safety are also missing. SC rejection is also a potential drawback and there are no studies comparing the success of therapy using autologous cells to the patient, and cells of exogenous origin. On the other hand, much of the success of therapy using SC comes from the use of MSC, which have a lower possibility of rejection, as well as greater effectiveness in the treatment.

Even with all the limitations and shortcomings, there are already clinical studies using SC both in the treatment of inflammatory diseases and in NP. In all reports, treatments were able to decrease pain scores and restore mobility-related functions. Therefore, SC treatment is a potential approach for pain relief and to achieve such biotechnological advancements, there is a need to fill the current knowledge gaps in order to develop efficient and safe therapies based on SC.

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ORIGINAL ARTICLE

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Mid-term outcomes of microfragmented adipose tissue plus arthroscopic surgery for knee osteoarthritis: A randomized, active-control, multicenter clinical trial

Cong-Zi Wu, Zhen-Yu Shi, Zhen Wu, Wen-Jun Lin, Wei-Bo Chen, Xue-Wen Jia, Si-Cheng Xiang, Hui-Hui Xu, Qin-Wen Ge, Kai-Ao Zou, Xu Wang, Jia-Li Chen, Ping-Er Wang, Wen-Hua Yuan, Hong-Ting Jin, Pei-Jian Tong

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Abstract

BACKGROUND

Osteoarthritis (OA) is the most prevalent form of degenerative whole-joint disease. Before the final option of knee replacement, arthroscopic surgery was the most widely used joint-preserving surgical treatment. Emerging regenerative therapies, such as those involving platelet-rich plasma, mesenchymal stem cells, and microfragmented adipose tissue (MFAT), have been pushed to the forefront of treatment to prevent the progression of OA. Currently, MFAT has been

successfully applied to treat different types of orthopedic diseases.

AIM
To assess the efficacy and safety of MFAT with arthroscopic surgery in patients with knee OA (KOA).

METHODS
A randomized, multicenter study was conducted between June 2017 and November 2022 in 10 hospitals in Zhejiang, China. Overall, 302 patients diagnosed with KOA (Kellgren-Lawrence grades 2-3) were randomized to the MFAT group (n = 151, were administered MFAT following arthroscopic surgery), or the control group (n = 151, were administered hyaluronic acid following arthroscopic surgery). The study outcomes were changes in the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score, the visual analog scale (VAS) score, the Lequesne index score, the Whole-Organ Magnetic Resonance Imaging Score (WORMS), and safety over a 24-mo period from baseline.

RESULTS
The changes in the WOMAC score (including the three subscale scores), VAS pain score, and Lequesne index score at the 24-mo mark were significantly different in the MFAT and control groups, as well as when comparing values at the posttreatment visit and those at baseline (P < 0.001). The MFAT group consistently demonstrated significant decreases in the WOMAC pain scores and VAS scores at all follow-ups compared to the control group (P < 0.05). Furthermore, the WOMAC stiffness score, WOMAC function score, and Lequesne index score differed significantly between the groups at 12 and 24 mo (P < 0.05). However, no significant between-group differences were observed in the WORMS at 24 mo (P = 0.367). No serious adverse events occurred in both groups.

CONCLUSION
The MFAT injection combined with arthroscopic surgery treatment group showed better mid-term clinical outcomes compared to the control group, suggesting its efficacy as a therapeutic approach for patients with KOA.

Key Words: Osteoarthritis; Microfragmented adipose tissue; Lipogems; Arthroscopic surgery; Knee

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Core Tip: Microfragmented adipose tissue (MFAT) has been successfully applied to treat different types of orthopedic diseases. To assess the efficacy and safety of MFAT with arthroscopic surgery in patients with knee osteoarthritis (OA). In this study, patients' own MFAT combined with arthroscopic surgery was used to promote recovery from OA. Our findings provide evidence supporting the safety and feasibility of this approach in treating knee OA.

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INTRODUCTION

Osteoarthritis (OA) is the most prevalent form of degenerative whole-joint disease, resulting in joint pain, stiffness, and decreased function. It is also a leading cause of disability in adults, with an enormous and ever-increasing burden on society[1,2]. Although several nonsurgical treatments are available for OA, such as physical therapy and medication, current mainstream medicine mainly focuses on relieving symptoms and cannot prevent the disease from progressing to the late stages of arthritis that require knee replacement[3]. Before this final option of knee replacement, arthroscopic surgery was the most widely used joint-preserving surgical treatment. Available evidence suggests that arthroscopic knee surgery temporarily improves pain or function in patients with mild arthritis; however, its long-term efficacy in treating moderate-to-severe knee OA (KOA) has not been generally accepted[4-7].

In contrast to limited symptom relief therapy, emerging regenerative therapies, such as those involving platelet-rich plasma (PRP), mesenchymal stem cells (MSCs), and microfragmented adipose tissue (MFAT), have been pushed to the forefront of treatment to prevent the progression of OA[8-10]. MFAT is obtained with a Lipogems (Lipogems Int Spa, Milan, Italy) device, which concentrates extracted autologous adipose tissue into small fragments using mild mechanical forces[11]. This one-step procedure is simple, time-saving, cost-effective, and minimally invasive; hence, it eliminates the need for complicated and time-consuming cell culture procedures. MFAT retains an intact stromal vascular niche harboring abundant functional cells, including adipose-derived stem cells (ADSCs: CD90+CD45-, CD90+CD45-, CD73+CD45-, CD73+CD45-, CD105+CD45-, and CD105+CD45-) and pericytes (CD146+CD34-), and secretes various

functional molecules, such as growth factors, cytokines, and exosomes[12,13]. MFAT can match and sometimes even outperform ADSCs due to its functional advantages over ADSCs; notably, the use of MFAT preserves the microenvironment and the most primitive state of cells, MFAT can be conveniently and easily acquired, there are a wide range of sources of MFAT, and autologous tissues are safe to use[14].

MFAT was initially used in the field of plastic surgery and then expanded to orthopedic procedures due to its antibacterial, anti-inflammatory, antiapoptotic, angiogenesis-promoting, and tissue repair functions[15-17]. Currently, MFAT has been successfully applied to treat different types of orthopedic diseases, such as diabetic foot minor amputations, spinal cord injury, cartilage defects, and articular disease[18-22]. Previous animal studies have successfully shown the benefit of intra-articular MFAT therapy for OA in pain relief and functional improvement[23,24]. Moreover, several clinical trials have reported an overall positive result for pain relief and functional improvement in patients with symptomatic KOA following MFAT therapy[25-27].

However, these successful single-center clinical trials are insufficient to demonstrate widespread therapeutic utility. Hence, well-controlled, high-quality scientific evidence to support the efficacy of MFAT is needed. This multicenter, prospective, randomized controlled trial aimed to evaluate the efficacy and safety of a single intra-articular injection of MFAT during arthroscopic surgery in a larger and more heterogeneous population of patients with KOA.

MATERIALS AND METHODS

Study design and participants

This was a prospective, randomized, controlled, single-blind (blind observer), multicenter trial performed in 10 hospitals in Zhejiang, China, including the First Affiliated Hospital of Zhejiang Chinese Medical University, the First Affiliated Hospital of Wenzhou Medical University, Ningbo First Hospital, Ruian Hospital of Traditional Chinese Medicine, Taizhou Hospital of Zhejiang Province, Hangzhou Red Cross Hospital, Huzhou First Hospital, the First People's Hospital of Hangzhou Linan District, Xiaoshan Hospital of Zhejiang, and Zhuji First Hospital (Table 1). A 24-mo follow-up was performed between June 2017 and November 2022. The inclusion criteria were participants aged 30-80 years with KOA symptoms for over 6 mo, including mechanical (locking and grinding) or meniscal (pain with pivoting) symptoms; Kellgren-Lawrence grades 2-3; and being unresponsive to conservative treatment. The exclusion criteria included local infection of the knee joint; systemic diseases such as blood disorders or diabetes, rheumatoid arthritis, gout, autoimmune disease, or malignancy in the past 5 years; prior injection or use of oral steroids within 3 wk before screening; and knee surgery within 6 mo before screening. The trial was registered at chictr.org.cn (registration number ChiCTR2200055124). All participants provided informed consent, and this study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhejiang Chinese Medical University.

Interventions

Enrolled patients were randomly assigned to the MFAT or control group at a 1:1 ratio using a computer-generated method, stratified by hospital and patient age (30-55 and 56-80 years). The control group received arthroscopic and hyaluronic acid (HA) treatment, while the MFAT group received arthroscopic surgery and MFAT therapy.

All participants underwent one or more of the following knee arthroscopic treatments: Debridement; abrasion or microfracture of chondral defects; trimming of degenerative meniscal tears, fragments of articular cartilage, or chondral flaps; and trimming of osteophytes that blocked full extension. Patients randomized to the MFAT group received an MFAT injection during the same arthroscopic procedure. After subcutaneous infiltration with a solution (a total of 250 mL of a saline solution mixed with 0.5 mL adrenalin 1:1000 and 25 mL lidocaine 0.02%) and resting for at least 15 min, liposuction was performed to obtain abdominal fat. We then introduced the harvested fat into Lipogems® (Lipogems International SpA, Milan, Italy) to produce MFAT according to the manufacturer's instructions[12]. Finally, the processed final product was transferred to 10 mL syringes and injected into the knee after the arthroscopic procedure. Patients in the control group received three injections of HA at a dosage of 5 mL once every month, as previously mentioned.

Outcomes

All patients were clinically evaluated before surgery and during follow-up visits at 6, 12, and 24 mo. The primary outcomes were the change in the total Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score from baseline to 24 mo. Secondary outcome measures included the WOMAC pain, stiffness, and function score from baseline to 24 mo, the visual analog scale (VAS) score for pain, the Lequesne index score, and the Whole-Organ Magnetic Resonance Imaging Score (WORMS).

Safety assessment

The safety assessment was based on adverse events reported by the patients and physical findings by the evaluators at each follow-up. Mild adverse events were defined as the presence of infection, significant pain, or swelling. A serious adverse event was defined as one that posed a life-threatening risk, was permanently disabling, or required hospitalization.

Statistical analysis

The sample size was based on the endpoint of the total WOMAC score at 24 mo. According to a previous pilot study, the standard deviation of the total WOMAC score at 24 mo was 12.8 points. Considering a 2-sided error alpha of 0.05, a

Table 1 Baseline characteristics of the patients

Characteristics	MFAT group	Control group	*P* value
No. of knees	146	146	
Age, yr	56.4 ± 10.6	54.8 ± 10.1	0.183
Sex, *n* (%)			0.455
Female	95 (65.1)	101 (69.2)	
Male	51 (34.9)	45 (30.8)	
BMI, kg/m^2	22.6 ± 3.4	23.1 ± 3.1	0.246
Kellgren-Lawrence grade, *n* (%)			0.833
2	80 (54.8)	93 (63.7)	
3	66 (45.2)	53 (36.3)	
Osteoarthritis site, *n* (%)			0.815
Left	70 (47.9)	72 (49.3)	
Right	76 (52.1)	74 (50.7)	
VAS pain	5.3 ± 1.4	5.2 ± 1.3	0.637
WOMAC score			
Pain 0-20	9.8 ± 2.7	9.5 ± 2.6	0.264
Stiffness 0-8	2.3 ± 1.4	2.2 ± 1.3	0.519
Function 0-68	35.3 ± 8.4	33.6 ± 10.2	0.115
Total 0-96	47.5 ± 9.6	45.3 ± 11.0	0.071
Lequesne index	10.7 ± 2.8	10.3 ± 2.6	0.215
WORMS	59.1 ± 9.2	60.8 ± 7.0	0.648

WORMS: Whole-Organ Magnetic Resonance Imaging Score; VAS: Visual analog scale; BMI: Body mass index; MFAT: Microfragmented adipose tissue.

minimum power of at least 0.9, a margin of 5.3 points on the total WOMAC, and a 15% drop-out rate, the final sample size was 290 knees (145 for each group).

SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, United States) was used for statistical analyses. The χ^2 test or Fisher's exact probability test, was used to compare count data. The normal distribution of the data was evaluated using a QQ plot and histogram. Different variables are described as the mean ± SD. When comparing the means between groups, a one-way ANOVA or Student's *t* test was used. Changes in primary and secondary outcome measures among the baseline, 6-, 12-, and 24-mo follow-up evaluations were assessed using repeated-measures one-way ANOVA and the Bonferroni post hoc test. A *P*-value < 0.05 indicated statistical significance.

Outcomes were assessed in an intention-to-treat analysis. The intention-to-treat population comprised all patients who had received the injection and had undergone at least one postbaseline assessment; the last-observation-carried-forward method was used to account for missing data.

RESULTS

A total of 322 patients were assessed for eligibility. Fifteen patients were not eligible, and five declined to participate; hence, a total of 302 participants were randomized to either the MFAT group (*n* = 151) or the control group (*n* = 151). Four patients (two in the MFAT group and two in the control group) withdrew consent, and six patients (four in the MFAT group and two in the control group) were lost to follow-up after randomization. It was decided that two patients assigned to the MFAT treatment group should not receive the procedure; according to the intention-to-treat principle, the results from these patients were evaluated with the results of the MFAT group. Thirty-seven patients did not return for follow-up visits; hence, 255 participants (87.3%) completed follow-up after 24 mo (Figure 1). The baseline characteristics of the two groups were similar (Table 1).

Primary and secondary outcomes

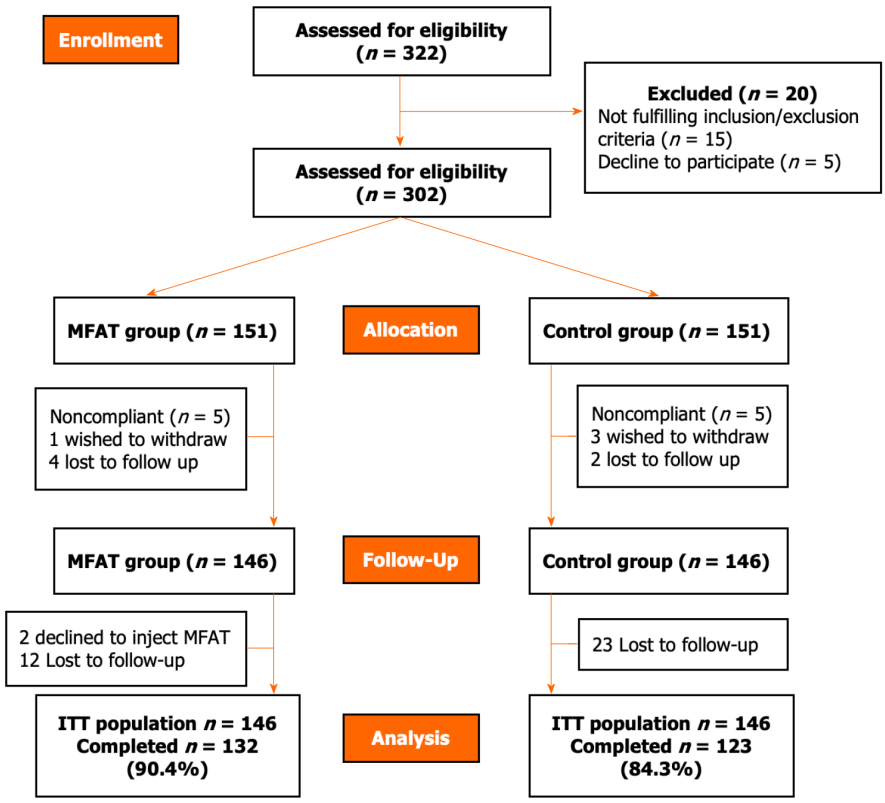
A comparison of the WOMAC scores, VAS scores, and Lequesne index scores between the MFAT group and the control group before and after treatment is shown in Table 2, Figures 2 and 3. In both groups, the WOMAC total score was

Table 2 Primary and second outcomes of the trial from baseline to all follow-ups

	MFAT group (*n* = 146)	Control group (*n* = 146)	*F* value	*P* value
WOMAC total score				
Baseline	48.3 ± 12.9	46.5 ± 13.4	0.406[1]	0.233[1]
6 mo	23.5 ± 8.6	29.8 ± 10.9	20.002[1]	< 0.001[1]
12 mo	22.0 ± 8.1	29.8 ± 10.7	24.479[1]	< 0.001[1]
24 mo	22.8 ± 8.2	31.7 ± 12.6	26.763[1]	< 0.001[1]
F value	379.38[1]	141.916[1]	23.468	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
WOMAC pain score				
Baseline	9.8 ± 2.7	9.5 ± 2.6	1.544[1]	0.264[1]
6 mo	4.3 ± 2.0	5.7 ± 2.7	18.373[1]	< 0.001[1]
12 mo	4.0 ± 2.0	5.7 ± 2.2	32.046[1]	< 0.001[1]
24 mo	4.0 ± 2.0	5.9 ± 2.3	45.115[1]	< 0.001[1]
F value	676.741[1]	240.46[1]	36.69	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
WOMAC stiffness score				
Baseline	3.2 ± 1.7	3.3 ± 2.0	0.003[1]	0.924[1]
6 mo	2.0 ± 1.4	2.4 ± 1.6	9.505[1]	0.054[1]
12 mo	1.7 ± 1.3	2.3 ± 1.4	13.737[1]	< 0.001[1]
24 mo	1.6 ± 1.2	2.2 ± 1.4	13.548[1]	< 0.001[1]
F value	155.117[1]	29.140[1]	9.605	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
WOMAC function score				
Baseline	35.3 ± 12.3	33.8 ± 12.8	0.406[1]	0.304[1]
6 mo	17.1 ± 8.3	21.7 ± 10.2	20.002[1]	< 0.001[1]
12 mo	16.3 ± 7.6	21.9 ± 10.2	24.479[1]	< 0.001[1]
24 mo	17.2 ± 7.8	23.6 ± 11.8	26.763[1]	< 0.001[1]
F value	379.38[1]	141.915[1]	23.468	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
VAS pain				
Baseline	5.3 ± 1.4	5.2 ± 1.3	0.257[1]	0.607[1]
6 mo	2.9 ± 1.3	3.3 ± 1.6	5.355[1]	0.008[1]
12 mo	2.2 ± 1.3	2.7 ± 1.5	5.937[1]	0.007[1]
24 mo	2.2 ± 1.4	2.7 ± 1.6	7.835[1]	0.006[1]
F value	581.206[1]	527.797[1]	11.921	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
Lequesne index				
Baseline	10.7 ± 2.8	10.3 ± 2.7	2.327[1]	0.217[1]
6 mo	7.7 ± 3.2	8.0 ± 3.0	0[1]	0.458[1]
12 mo	7.2 ± 3.2	8.2 ± 3.3	6.684[1]	0.012[1]
24 mo	7.2 ± 2.5	8.2 ± 3.0	8.633[1]	0.004[1]
F value	216.554[1]	45.136[1]	16.293	

P value	< 0.001[1]	< 0.001[1]	< 0.001[2]

[1]*F* statistic and *P* value of the main effect.
[2]*F* statistic and *P* value of the crossover effect.
WORMS: Whole-Organ Magnetic Resonance Imaging Score; VAS: Visual analog scale; MFAT: Microfragmented adipose tissue.



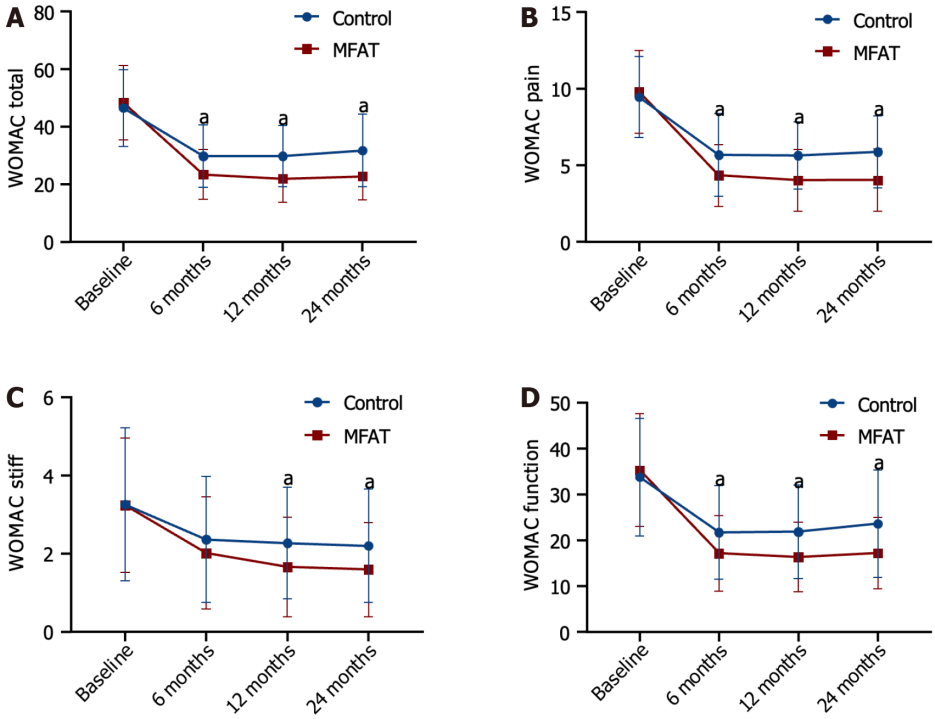
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Figure 1 Participant enrollment flow diagram. ITT: Intention-to-treat; MFAT: Microfragmented adipose tissue.

significantly different between time before and after treatment, with separate effect analyses in the MFAT group (F = 379.38) and control group (F = 141.916) (all P < 0.001). The WOMAC total score in the MFAT group was lowest after 12 mo and then increased slightly but remained lower than the values at baseline. The WOMAC total score in the control group decreased, with a maximum decline observed at 6 mo after treatment, and slightly increased at 24 mo. The overall WOMAC total score in the MFAT group was significantly lower than that in the control group after treatment (F = 838.277, P < 0.001). There was also a crossover effect between time and treatment (F = 23.468, P < 0.001). We performed an analysis of the separate effects for each time point. The WOMAC total score in the MFAT group was significantly lower than that in the control group at the same time points during all follow-ups (P < 0.001) (Figure 2A, Table 2). These results indicated the therapeutic benefit of MFAT injection combined with arthroscopy in patients with KOA.

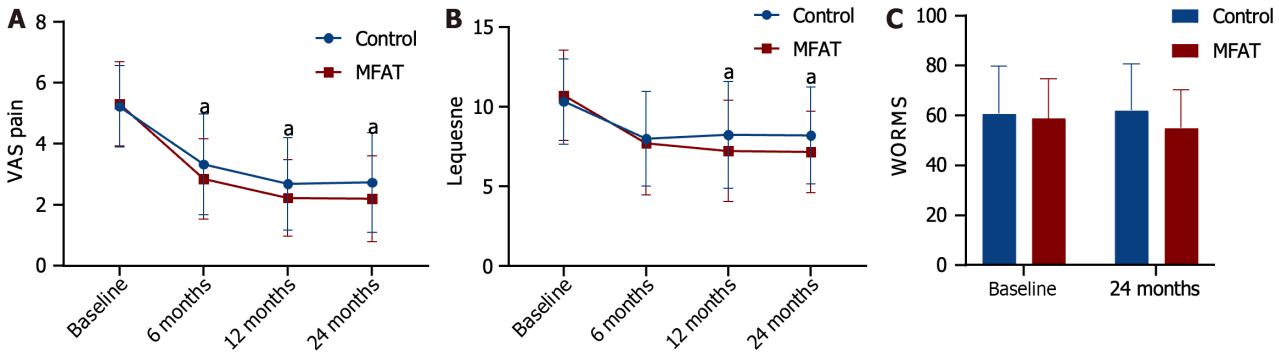
Both groups had a significant reduction in WOMAC pain scores and VAS scores from baseline to posttreatment at 6 to 24 mo ($F_{WOMAC\ pain}$ = 676.741, F_{VAS} = 581.206 for the MFAT group; $F_{WOMAC\ pain}$ = 240.46, F_{VAS} = 527.797 for the control group; all P < 0.001). The most significant decreases in the WOMAC pain score and VAS score from baseline to posttreatment were observed at months 12 and 24 in the MFAT group and at month 12 in the control group. A significant difference in the mean WOMAC pain score and VAS score was observed at all follow-ups in the MFAT group in comparison with the control group (WOMAC pain: All P < 0.001, VAS: All P < 0.01) (Figures 2B and 3A, Table 2). These results indicated that MFAT injection combined with arthroscopy significantly improved pain in patients with KOA.

The WOMAC stiffness score, WOMAC function score, and Lequesne index score at all follow-ups progressively decreased compared with their respective baseline values in both the MFAT group ($F_{WOMAC\ stiffness}$ = 155,117, $F_{WOMAC\ function}$ = 379.38, $F_{Lequesne}$ = 216.554, all P < 0.001) and the control group ($F_{WOMAC\ stiffness}$ = 29.140, $F_{WOMAC\ function}$ = 141.915, $F_{Lequesne}$ = 45.136, all P < 0.001). The WOMAC stiffness score and Lequesne index score in the MFAT group maintained a downward trend up to 24 mo after treatment. Additionally, the WOMAC function score decreased most significantly at 12 mo and slightly increased at 24 mo. The WOMAC function score and Lequesne index score in the control group were lowest after 6 mo and then increased monthly, while the WOMAC stiffness score was lowest at 24 mo. The WOMAC stiffness score, WOMAC function score, and Lequesne index score in both groups were lower than the values at baseline. However, the WOMAC function score of patients in the MFAT group was still lower than that of the control group at all follow-ups (all P < 0.001) (Figure 2D, Table 2). Similar patterns were observed in the WOMAC stiffness score and Lequesne index score



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Figure 2 Trends of Western Ontario and McMaster Universities Osteoarthritis Index scores during the 24-mo follow-up for both groups. A: Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) total score trends; B: WOMAC pain score trends; C: WOMAC stiffness score trends; D: WOMAC function score trends. Statistically significant differences have been reported as $^{a}P < 0.05$ (microfragmented adipose tissue *vs* control). Results are presented as the means ± SD. WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; MFAT: Microfragmented adipose tissue.



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Figure 3 Trends of the visual analog scale pain score, Lequesne index score, and Whole-Organ Magnetic Resonance Imaging Score during the 24-mo follow-up for both groups. A: Visual analog scale pain score trends; B: Lequesne index score trends; C: Whole-Organ Magnetic Resonance Imaging Score change (n = 10 for each group). Statistically significant differences have been reported as $^{a}P < 0.05$ (microfragmented adipose tissue *vs* control). Results are presented as the means ± SD. WORMS: Whole-Organ Magnetic Resonance Imaging Score; VAS: Visual analog scale; MFAT: Microfragmented adipose tissue.

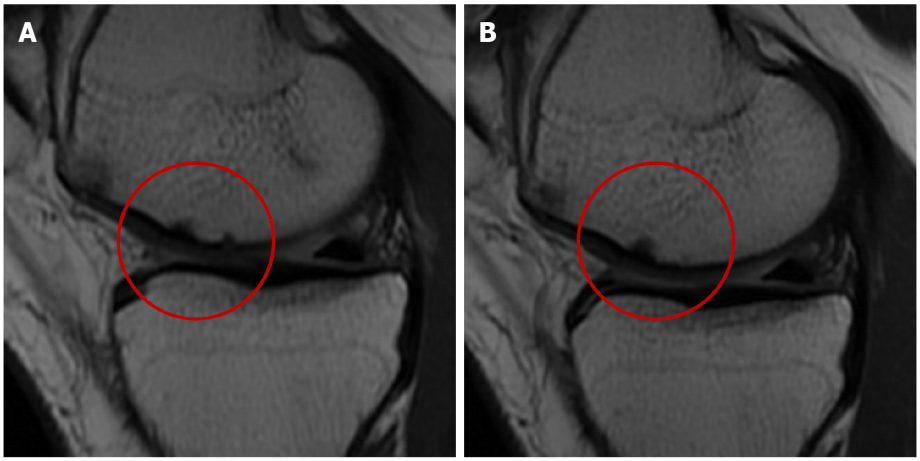
of the two groups at 12 and 24 mo (all $P < 0.001$), while there was no significant difference between the two groups at 6 mo ($P_{WOMAC\ stiffness\ score} = 0.054$, $P_{Lequesne\ index\ score} = 0.458$) (Figures 2C and 3B, Table 2). The WOMAC stiffness score, WOMAC function score, and Lequesne index score showed that MFAT injection combined with arthroscopy effectively improved function and quality of life in patients with KOA.

The WORMS as an indicator of radiological changes at 24 mo is documented in Table 3. The mean WORMS at 24 mo had significantly greater improvements than at baseline in the MFAT group ($P = 0.020$), while no statistically significant difference was observed compared to the baseline score in the control group ($P = 0.850$). There were no significant between-group differences in the WORMS at 24 mo (55.0 ± 15.5 *vs* 62.1 ± 18.7, $P = 0.367$) (Figure 3C, Table 2). However, a higher proportion of patients in the MAFT group had reduced femoral and tibia bone marrow lesions (BML), repaired meniscus, and improved osteochondral defect compared with the control group (Figures 4 and 5).

Table 3 Comparison of the Whole-Organ Magnetic Resonance Imaging Score before and after treatment in the microfragmented adipose tissue and the control group

	MFAT group	Control group	*t* value	*P* value
Baseline	58.9 ± 15.9	60.8 ± 19.0	0.243	0.811
24 mo	55.0 ± 15.5	62.1 ± 18.7	0.924	0.367
t value	2.814	-0.195		
P value	0.0203	0.85		

WORMS: Whole-Organ Magnetic Resonance Imaging Score; MFAT: Microfragmented adipose tissue.



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Figure 4 Magnetic resonance imaging evaluation of osteochondral defect changes at 24 mo. A: Sagittal images of the medial femur and tibia before injection of microfragmented adipose tissue (MFAT); B: Sagittal images of the medial femur and tibia 24 mo after MFAT injections. The osteochondral defect in the circled area was reduced, indicating articular cartilage regeneration at the site of the osteochondral defect.

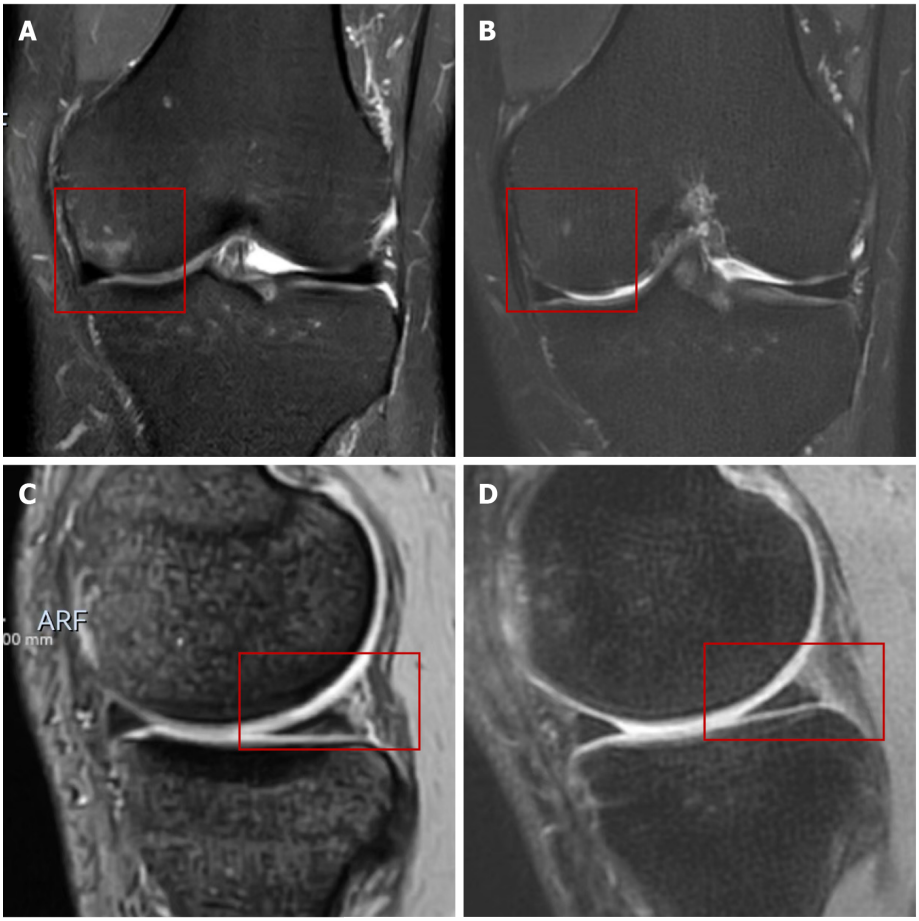
Safety outcomes

No serious adverse events occurred in the two groups. A total of 17 non-serious adverse events were observed: In eight knees (5.4%) in the MFAT group and nine knees (6.2%) in the control group, joint effusion, pain, or swelling were reported for over 5 d. None of these adverse events led to trial suspension in either group.

DISCUSSION

This multicenter study prospectively analyzed the safety and potential benefits of using MFAT as an adjuvant in the arthroscopic treatment of KOA. The results showed that over a 24-mo follow-up period, the injection of MFAT plus arthroscopic surgery was superior to HA plus arthroscopic surgery across multiple outcome measures, including reductions in the total WOMAC score and the scores for each subscale, VAS pain score, Lequesne index score, and WORMS.

Comprehensive studies and characterization of the injected MFAT have been performed *in vitro*[11-13]. MFAT has been shown to contains large amounts of cells; moreover, various bioactive molecules are released by these cells through a paracrine mechanism to prime and preserve angiogenic, antifibrotic, antiapoptotic, antimicrobial, and immunomodulatory responses in the target tissue. Therefore, MFAT might be a novel source of cell therapy for KOA. Striano *et al*[28] first showed that MFAT transplantation significantly improved the VAS score and expanded the joint space in a 59-year-old male patient with severe knee pain. PRP is an established regeneration therapy for KOA treatment. Recently, a study demonstrated that a single intra-articular injection of MFAT did not differ from an injection of PRP in terms of clinical outcomes at up to 24 mo[29]. This study indirectly reflected the efficacy of MFAT in KOA. Several studies have also confirmed the efficacy and safety of MFAT in the treatment of KOA, including four studies that used MFAT associated with arthroscopic surgery for the treatment of KOA in humans[25,30-39]. The first clinical trial showed that a combination of arthroscopy and a single intra-articular injection of MFAT improved the International Knee Documentation Committee (IKDC) subjective score and total knee injury and OA outcome score (KOOS) in 30 patients affected by diffuse degenerative chondral lesions at 12 mo posttreatment (70% and 67% of the patients improved by at least 10 points for the IKDC subjective score and total KOOS, respectively)[32]. In another study, Cattaneo *et al*[37] reported on 38 patients



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Figure 5 Magnetic resonance imaging evaluation of bone marrow lesions and meniscus tear changes at 24 mo. A and C: Coronal and sagittal images of the medial femur and tibia before injection of microfragmented adipose tissue (MFAT). The bone marrow lesions (BML) and meniscus tears can be observed in the rectangle; B and D: Coronal and sagittal images of the medial femur and tibia 24 mo after MFAT injections. The BML in the rectangular area was reduced, and the meniscus injury was repaired. ARF: Anterior right inferior.

treated with an arthroscopic procedure combined with an injection of MFAT. The results showed that all the patients were satisfied with the treatment and that 92% of them had improved KOOSs on direct physical examination at the final follow-up. A steady and statistically significant improvement in all KOOSs from the preoperative evaluation to the 1-, 3-, and 6-mo follow-ups was observed. However, the KOOS slightly, but not statistically significantly, decreased at 12 mo. The latest published trial in 2023 included two groups of 78 patients with severe KOA (Kellgren-Lawrence grade 3-4). The study results indicated that compared to arthroscopic debridement (AD) alone, injection of MFAT along with AD was more effective in improving the KOOS score, VAS score, WOMAC score, and KSS score at the 24-mo follow-up. Additionally, magnetic resonance imaging (MRI) T2-mapping scores significantly improved in the medial and lateral condyle compartments[39]. These studies preliminarily confirmed the efficacy of MFAT for treating KOA. However, the results of efficacy trends at different time points following treatment were not consistent. In this multicenter, randomized, placebo-controlled study, patients in the MFAT combined with arthroscopy group achieved better primary and secondary endpoints at 24 mo following treatment than those in the control group. Furthermore, clinical symptoms improved following MFAT treatment in a time-dependent manner, which suggested the efficacy of MFAT treatment for patients with KOA. However, the improvement in clinical outcomes in patients in our MFAT group was smaller than those reported in other studies, which might be explained by the heterogeneity of the enrolled participants.

In our study, arthroscopic surgery in the control group had a favorable mid-term outcome at 6 mo, but this efficacy was not long-lasting, which was consistent with the findings of earlier trials[40]. Interestingly, injecting MFAT could prolong the improving trend in most of the indicators to 24 mo. Regarding the mechanism of MFAT for KOA treatment, we speculate that MFAT may first act through differentiation or paracrine action of the different cells it contains. There is evidence that MFAT itself, not only its derived human MSCs, can directly differentiate into chondrocytes in vitro[20]. Other differentiated cells, such as pericytes in MFAT, may also play important roles since MFAT releases many more growth factors and cytokines involved in tissue repair and regeneration than the enzymatically derived stromal vascular fraction[41]. Second, matrix metalloproteinases and proinflammatory mediators, such as interleukin-1β and tumor necrosis factor-α cause cartilage degradation and synovial swelling, stimulating synovial proliferative and inflammatory responses of both resident synoviocytes and macrophages[42]. After co-cultivation of MFAT and inflamed synoviocytes, the levels of macrophage-specific chemokines [C-C motif ligand 2 (CCL2)/monocyte chemoattractant protein-1 and CCL3/macrophage inflammatory protein-1α) and the degradative marker matrix metalloproteinase-9 were downreg-

ulated, and the mRNA levels of inflammatory factors were reduced, which was partially dependent on Toll-like receptor 4 and nuclear factor-kappaB signaling[43,44]. Additionally, other secreted molecules in MFAT may play anti-inflammatory roles. Ragni *et al*[42] identified extracellular vesicle-shuttled microRNAs (miRNAs) (miR-24-3p, miR-222-3p, and miR-193b-3p) and soluble factors [tissue inhibitor of metalloproteinases-1 (TIMP1), TIMP2, PLG, and CTSS) as both cartilage-protective factors and factors that trigger the switch from M1 to M2 macrophages in the secretome of adipose-derived stem cells cultivated *in vitro* under inflammatory conditions. Third, preserving cartilage function and integrity is a feasible mechanism. Chen *et al*[23] demonstrated that MFAT attenuated pain symptoms and protected chondrocytes and cartilage extracellular matrix from damage in a monoiodoacetate-induced OA rat model. Another clinical trial also indicated that the contents of cartilage glycosaminoglycans significantly increased in specific areas of the treated knee joint by delayed gadolinium-enhanced MRI of cartilage[45]. Finally, MFAT may act as a natural scaffold and display interesting mechanical properties, such as the infrapatellar fat pad[20,46].

MRI can provide a more accurate and reliable basis for assessing factors, such as the lesion location and morphological organization, in patients. A 12-mo follow-up of 64 patients undergoing MFAT therapy revealed that BML was negatively correlated with the therapeutic response rate at 12 mo[47]. Similarly, another study showed that BML was significantly deceased 12 mo after surgery in the treatment group. However, the cartilage scores were nonsignificant between the treatment and placebo groups, although the cartilage layer was thicker in the treatment group at 12 mo[48]. We compared the MRI results of the MFAT group to those of the control group using WORMS, which revealed no significant differences between these two groups at 24 mo. However, similar to the findings of a study by Nguyen *et al*[48], we found that the WORMS at the 24-mo follow-up in the MFAT group had significantly reduced, while it was relatively increased in the control group. Furthermore, MRI of a higher percentage of patients in the MFAT group showed signs of repaired cartilage damage, meniscus repair, and reduced BML, supporting the therapeutic potential of MFAT from the perspective of MRI findings. Additionally, the insignificant results at 24 mo might be partially explained by the limited number of postoperative MRI results in this study due to economic reasons (n = 10 for each group). Although it is well known that BML is closely related to pain in the arthritic knee joint, the current conclusions about the imaging changes in MFAT in the treatment of KOA and the association of imaging with clinical results are inconsistent[49]. Therefore, well-controlled studies with larger sample sizes are needed.

In the current study, we only found a handful of mild adverse events with pain or swelling in the liposuction or injection area, without severe adverse events or complications. This result is consistent with those of a previous study in which only 5.8% of patients who were administered MFAT injections had a transitory hematoma of the abdominal region that had no impact on the knee[50]. Likewise, Bisicchia *et al*[38] reported no adverse events related to MFAT injection at the 12-mo follow-up, and there was only one case of knee effusion 3 d postoperatively in the microfracture control group, demonstrating the safety of MFAT for clinical use.

A major strength of this study is that, as the first multicenter prospective randomized trial in a Chinese population, the study's sample size was relatively large. Moreover, the primary and secondary outcomes included both patient-reported indexes and imaging technologies, and most patients completed the final 24-mo follow-up. However, our study has some limitations. First, we only recruited patients with Kellgren-Lawrence grades of 2-3. We excluded a Kellgren-Lawrence grade of 4 because it indicated marked joint-space narrowing and definite deformity, which may not be suitable for arthroscopic treatment; however, other studies have demonstrated the efficacy of MFAT in patients with severe OA[33,39]. Second, the patients and the surgeons who performed the surgeries could not be blinded to the patients' groups owing to the extra liposuction in the MFAT group. Nevertheless, the operator who processed the data was unaware of the patients' groups. Third, the follow-up time was short, and differences in the volume of MFAT were not examined. Moreover, the differences in the intervention in the control and study groups may have influenced the results. In addition, we should also consider cost-effectiveness since the cost of MFAT might be prohibitive for some patients. Finally, this study was conducted in only Zhejiang, China, as a representative sample of the Chinese population. Long-term studies on the potential dose-dependent effect, including more districts, should be conducted in the future.

CONCLUSION

In conclusion, our study demonstrates that MFAT injection combined with arthroscopic surgery is a safe and effective approach for improving function and alleviating pain in patients with KOA. MFAT therapy produced longer-lasting, statistically significant durations of efficacy at 12 and 24 mo in comparison to those of the control (HA) group. Based on these results, MFAT injection combined with arthroscopic surgery may be considered a potential therapeutic option for KOA.

ARTICLE HIGHLIGHTS

Research background

The most common kind of degenerative whole-joint disease is osteoarthritis (OA). Before the ultimate choice of knee replacement, the most common joint-preserving surgical procedure was arthroscopic surgery. Mesenchymal stem cells, platelet-rich plasma, and microfragmented adipose tissue (MFAT) are examples of emerging regenerative medicines that have been thrust into the forefront of treatment to stop the progression of OA. MFAT is now being used to treat various orthopedic diseases with effectiveness.

Research motivation
Current mainstream medicine mainly focuses on relieving symptoms and cannot prevent the disease from progressing to the late stages of arthritis that require knee replacement. MFAT as a novel way of treatment may be of great significance in alleviating knee OA (KOA).

Research objectives
The present study aimed to assess the efficacy and safety of MFAT with arthroscopic surgery in patients with KOA.

Research methods
Patients diagnosed with KOA (Kellgren-Lawrence grades 2-3) were included in a multicenter, prospective, single-blind randomized trial. In this trial, 302 patients were randomized into the MFAT group (n = 151, were administered MFAT following arthroscopic surgery), or the control group (n = 151, were administered hyaluronic acid following arthroscopic surgery). The study outcomes included changes in the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score, the visual analog scale (VAS) score, the Lequesne index score, the Whole-Organ Magnetic Resonance Imaging Score (WORMS), and safety, from baseline to 24 mo.

Research results
The changes in the WOMAC score (including the 3 subscale scores), VAS pain score, and Lequesne index score at 24 mo showed significant differences in both groups between the posttreatment visit and baseline ($P < 0.001$). The MFAT group exhibited significant decreases in the WOMAC pain scores and VAS scores at all follow-ups, suggesting the pain-relieving potential of MFAT combined with arthroscopy compared to arthroscopy alone HA treatment ($P < 0.05$). The significant differences in the WOMAC stiffness score, WOMAC function score, and Lequesne index score at 12 and 24 mo after MFAT administration and surgery suggested the potential of MFAT combined with arthroscopy compared to arthroscopy alone control group to improve function and quality of life ($P < 0.05$). There were no significant between-group differences in the WORMS at 24 mo ($P = 0.367$). No serious adverse events occurred in the two groups.

Research conclusions
In summary, our study demonstrates that MFAT injection combined with arthroscopic surgery is a safe and effective approach for improving function and alleviating pain in patients with KOA. Based on these results, MFAT injection combined with arthroscopic surgery may be considered a potential therapeutic option for KOA.

Research perspectives
We demonstrated that MFAT injection combined with arthroscopic surgery had better clinical efficacy than control group for treating KOA at a mid-term follow-up and could be a potential therapeutic approach for patients with KOA.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

ADSC-Exos outperform BMSC-Exos in alleviating hydrostatic pressure-induced injury to retinal ganglion cells by upregulating nerve growth factors

Zhi-Kun Zheng, Lei Kong, Min Dai, Yi-Dan Chen, Yan-Hua Chen

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have protective effects on the cornea, lacrimal gland, retina, and photoreceptor cell damage, which may be mediated by exosomes (exos) released by MSCs.

AIM

To investigate the ameliorating effect of exos derived from different MSCs on retinal ganglion cell (RGC) injury induced by hydrostatic pressure.

METHODS

The RGC injury model was constructed by RGC damage under different hydrostatic pressures (40, 80, 120 mmHg). Then RGCs were cultured with adipose-derived stem cell (ADSC)-Exos and bone marrow-derived stem cell (BMSC)-Exos. Cell Counting Kit-8, transmission electron microscopy, flow cytometry, immunofluorescence, real-time quantitative polymerase chain reaction, and western blotting were performed to detect the ameliorating effect of exos on pressure-induced RGC injury.

RESULTS

ADSC-Exos and BMSC-Exos were successfully isolated and obtained. The gibbosity of RGCs was lower, the cells were irregularly ellipsoidal under pressure, and the addition of ADSC-Exos and BMSC-Exos significantly restored RGC morphology. Furthermore, the proliferative activity of RGCs was increased and the apoptosis of RGCs was inhibited. Moreover, the levels of lactate dehydrogenase and apoptosis-related proteins were increased, and the concentrations of antiapoptotic proteins and neurotrophic factors were decreased in damaged RGCs. However, the above indicators were significantly improved after ADSC-

Exos and BMSC-Exos treatment.

CONCLUSION
These findings indicated that ADSC-Exos and BMSC-Exos could ameliorate RGC injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors.

Key Words: Adipose-derived stem cells; Bone marrow-derived stem cells; Exosomes; Glaucoma

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Core Tip: We discovered for the first time that adipose-derived stem cell-exosomes (ADSC-Exos) significantly ameliorate retinal ganglion cell (RGC) injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors. ADSC-Exos manifested better ameliorating effects than bone marrow-derived stem cell (BMSC)-Exos in ameliorating the RGC injury induced by hydrostatic pressure. BMSC-Exos ameliorate optic nerve injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors.

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INTRODUCTION

Glaucoma is the main cause of irreversible blindness around the world and is a chronic degenerative optic nerve disease [1]. The early pathological changes of glaucoma include reduced axonal transport, axon loss, and dendrite reconstruction, leading to retinal ganglion cell (RGC) apoptosis and thinning of the retinal nerve fiber layer, eventually resulting in visual field defects and loss of vision[2,3]. Apoptosis of RGCs in glaucoma has been demonstrated *in vivo* in several animal models and in humans[4,5]. The mechanism of RGC apoptosis in glaucoma, including oxidative injury[6], inflammatory response[4], and glutamate toxicity[7], particularly how elevated intraocular pressure (IOP) leads to RGC apoptosis, is yet to be fully elucidated. There are three treatment methods for glaucoma caused by high IOP, including using drugs to reduce the production of aqueous humor, surgically aqueous humor drainage, and laser stimulation for cell proliferation and differentiation to repair damaged eye tissue[8]. However, existing IOP-lowering treatments cannot prevent the reduction of RGCs[9], and there is the limitation of repeated treatment, even worse.

The only hope for glaucoma patients with RGC loss to regain vision is preventing RGC apoptosis[10]. Current research is focused on developing neuroprotective measures, particularly the use of stem cell therapy to protect RGCs[11]. Stem cells produce many molecules responsible for cell signaling, such as cytokines, growth factors, morphogens, chemokines, and extracellular vesicles (EVs), improving various cellular mechanisms[12-15]. However, the direct transplantation of stem cells has some risks such as safety, tumorigenesis, and immune rejection[16]. Recent studies have shown that stem cells play a therapeutic role in a paracrine manner, with stem cell-derived exosomes (exos) being the major contributors to stem cell function, carrying and transporting cytokines, proteins, and nucleic acids from stem cells[17]. It also has stronger target specificity and lower carcinogenic and immune risks[18], which can avoid the problems existing in the direct use of stem cell therapy, and increasing the use of stem cell-derived exos is the focus of research to enhance the efficacy of stem cell therapy.

Mesenchymal stem cells (MSCs) are fibroblast-like, self-renewing cells that are present in almost all postpartum tissues and organs, including bone marrow, adipose tissue, blood, umbilical cord, amniotic fluid, and dental pulp and are responsible for regulating the normal development and maintenance of mesenchymal tissue[19]. Bone marrow, adipose tissue, and human MSCs of the umbilical cord have protective effects on cornea, lacrimal gland, retina, and photoreceptor cell damage[20]. This ameliorating effect may be mediated at least in part by exos. MSC-Exos can maintain activity for at least 4 wk after vitreous injection and can integrate into tissues more quickly to provide nutritional factors for RGCs due to their smaller diameter[15]. Bone marrow stem cell (BMSC)-derived exos are also more effective than directly transplanted BMSCs in the treatment of glaucoma because BMSCs have a poor ability to integrate into the retina, remain in the vitreous after injection, and cannot deliver neurotrophic factors to RGCs[21]. Mead *et al*[22] found that the neural function of RGCs was significantly improved after intravitreal injection of BMSC-Exos in a glaucoma rat model. A similar result was reported in the hereditary DBA/2J mouse model of glaucoma[23]. Moreover, studies have shown that BMSC-Exos injected in an optic nerve compression rat model by intravitreal injection can integrate into the cell bodies and axons of retinal neurons and promote axon regeneration by activating the Akt pathway dependent on microRNA (miRNA)[24]. These findings indicate that BMSC-Exos have an ameliorative effect on glaucoma. Moreover, Sheykhhasan *et al*[25] demonstrated that adipose-derived stem cell (ADSC)-Exo and CoQ10 administration could ameliorate memory deficits by modulating SOX2 and brain-derived neurotrophic factor (BDNF) expression in a rat model of Alzheimer's disease. Numerous preclinical studies have confirmed the therapeutic potential of ADSC-Exos in neurodegenerative diseases[26,

27]. Glaucoma is a neurodegenerative disease in which the role of ADSC-Exos has not been studied yet. Therefore, this study aimed to investigate the protective effect of BMSC-Exos and ADSC-Exos on RGC injury, which is the main feature of glaucoma, induced by hydrostatic pressures.

MATERIALS AND METHODS

Extraction of ADSC-Exos and BMSC-Exos

ADSCs and BMSCs were a kind gift from Kunming Cell Bank of Chinese Academy of Sciences (Kunming, China). Cells in the third generation with good logarithmic phase growth were selected. The cell culture medium was discarded, and the cells were washed 3 times with phosphate-buffered solution (PBS) and replaced with serum-free medium. After 48 h in culture, the cells were adjusted to a concentration of 1×10^5 cells/mL. The cells were then centrifuged at 3000 r/min for 10 min at room temperature to collect the supernatant. The supernatant was centrifuged at 20000 r/min for 20 min at 4 °C to remove dead cells and debris, and the supernatant was then filtered through a sterile, 0.22 μm pore size syringe filter to obtain the crude extract of exos. Then the crude extract was centrifuged at 50000 r/min at 4 °C for 1 h. The white precipitate could be seen at the bottom of the tube after centrifugation, and the supernatant was removed. The white precipitate was cleaned with 0.9% sodium chloride injection several times, and the resulting suspension was collected and centrifuged at 4 °C for 10 min at 3000 r/min. Exo protein quantification was performed using a BCA protein assay kit (Beyotime, Shanghai, China). Dynamic light scattering was used to detect the size distribution of the exos. The structure of exos was observed by transmission electron microscopy (TEM) (HITACHI, Japan).

Primary RGCs extraction, culture[28,29], and grouping

Mice 1-3 d old were sacrificed by intraperitoneal injection of 4.3% chloral hydrate, and the eyeballs were removed under aseptic conditions and rinsed three times. Under the microscope, the retinal neuroepithelial layer tissue was isolated. The tissue was rinsed 3 times and digested in 0.5 g/L trypsin for 30 min at 37 °C. The digestion was terminated by the addition of DMEM containing 10% foetal bovine serum (FBS) and filtered through a 40-μm filter. Centrifuge at room temperature for 5 min, discard the supernatant, and add complete DMEM. After adjusting the cell density, the cells were inoculated into cell culture flasks. The cells were placed in a 5% CO_2 incubator at 37 °C for 7-10 d. The obtained cells were taken and identified by immunofluorescence staining using a Thy1.1 antibody. The project was approved by the Yunnan University Ethics Committee (approval number YUN20230463).

Then, the cells were grouped into different experiments as follows: (1) Normal control (NC) group, 40 mmHg group, 40 mmHg + ADSC-exo group, and 40 mmHg + BMSC-exo group; (2) NC group, 80 mmHg group, 80 mmHg + ADSC-exo group, and 80 mmHg + BMSC-exo group; and (3) NC group, 120 mmHg group, 120 mmHg + ADSC-exo group, and 120 mmHg + BMSC-exo group.

Hydrostatic pressure damage model preparation

RGC culture dishes were placed within the pressure chamber[30,31], and the incubating gas mix was pressurized. The cells were exposed to conditions of elevated ambient hydrostatic pressure (40 mmHg, 80 mmHg, 120 mmHg) over and above atmospheric pressure for a period of 2 h. The pressure conditions were then restored to atmospheric, and the culture dishes were removed from the chamber. Under normal cell culture conditions (37 °C, 5% CO_2), the culture was continued for 24 h.

Coculture of ADSC-Exos or BMSC-Exos and RGCs

The concentrations of ADSC-Exos and BMSC-Exos were measured by a BCA kit (P0010, Beyotime). P3-generation SCs with good digestive state were taken from each group to make a uniform cell suspension and put 100 μL per well in 96-well cell culture plates. ADSC-Exos (20 μg/mL)[32] were added after the stem cells were completely attached to the wall. BMSC-Exos (20 μg/mL)[33] were cultured for 24 h, and the cells of each group were collected for subsequent experiments. Then, cell morphology was observed under an inverted phase contrast microscope (Olympus).

Ultrastructure observation of RGCs by transmission electron microscopy

After the cells were treated for 24 h according to the above groups, the cells were digested and collected with Trypsin-EDTA Solution (Beyotime, Shanghai, China), centrifuged in a 1.5 mL EP tube, and the supernatant was discarded. Then, 2.5% glutaraldehyde fixing solution was quickly added to completely cover the cell specimens, which were refrigerated at 4 °C overnight. The fixing solution was poured away, and the samples were rinsed with PBS first and then fixed with 1% osmic acid solution. The samples were subjected to gradient dehydration with an ethanol solution and finally treated with pure acetone for 20 min. The samples were treated with a mixture of equal volume embedding medium and acetone for 1 h. Then, the samples were treated with a mixture of embedding medium and acetone (V/V = 3/1) for 3 h. Next, the embedding medium was used to treat the sample overnight. The samples with osmotic treatment were embedded and heated at 70 °C overnight to prepare the needed samples. The samples were sliced in a Leica EM UC7 ultramicrotome, and slices of 70-90 nm were obtained, double-dyed with lead citrate solution and 50% ethanol saturated solution of uranyl acetate, dried and observed under a transmission electron microscope (Tecnai G2 spirit).

RGCs were labeled by immunofluorescence with β-III tubulin

RGCs were collected in each group, washed twice with PBS, fixed at room temperature for 30 min with 4% paraformal-

dehyde, permeated with 0.1% Triton X-100 for 10 min, washed with PBS 3 times, and sealed with 10% goat serum at room temperature for 1 h. The blocking buffer was removed, and β-III tubulin primary antibody (ab78078, Abcam) was added and incubated overnight at 4 °C. After washing with PBS 3 times, sheep anti-rabbit IgG fluorescent secondary antibody (P0186, Beyotime) was added to the dark, and incubated at room temperature for 2 h. After 3 washes with PBS, 5-10 nonrepetitive visual fields were randomly taken under a fluorescence microscope (Nikon fluorescence microscope), and the number of retinal ganglion cells was quantified by ImageJ software (ImageJ, RRID:SCR_003070).

Detection of cell activity by Cell Counting Kit-8 assay

The cells of each group were digested with Trypsin-EDTA Solution, and the cells were suspended in 10% FBS medium. Each well was inoculated with 1×10^5 cells in 96-well plates. The 96-well plates were cultured in a constant temperature incubator containing 5% CO_2 at 37 °C for 0, 24, 48, 72, and 96 h. At the corresponding time, 10 μL Cell Counting Kit-8 (CCK-8) reagent (Beyotime) was added, and the absorption value at 450 nm was detected by a microplate reader after continuous culture for 2 h.

Detection of the cell apoptosis rate by Annexin V-FITC kit flow cytometry

Cells in each group were digested with trypsin-EDTA solution, collected, washed with PBS, resuspended in 300 μL of binding buffer, and incubated with 10 μL of Annexin V-FITC at room temperature for 15 min in the dark. Before the operation, 5 μL PI was added and then 200 μL binding buffer was added. The apoptosis rate of cells was detected by a flow cytometer.

The localization of CREB and p-CREB detected by immunofluorescence chemical staining

The cells were inoculated into 24-well plates with 5×10^4 cells per well, and each well was cultured with 500 μL medium for 12 h. Then, the cells were fixed with 4% paraformaldehyde at room temperature. PBS containing 0.1% Triton X-100 was added, and permeabilization was performed at room temperature for 10 min. The cells were sealed with 5% BSA at room temperature for 30 min. The liquid was removed and rabbit CREB antibody (Beyotime) and rabbit p-CREB antibody (Beyotime) diluted with 1% BSA were added. Next, the cells were incubated at 4 °C overnight and washed with PBS. The goat anti-rabbit secondary antibody was incubated at room temperature for 1 h and washed with PBS. Finally, the samples were dyed with DAPI solution at room temperature for 5 min and washed with PBS. A fluorescence microscope was used to observe and photograph the cells.

The degree of RGC neuron damage evaluated by the lactate dehydrogenase release test

One hundred microliters of the cell supernatant in each group was collected for the lactate dehydrogenase (LDH) concentration test according to the LDH kit (Keygen, Nanjing, China) instructions. Then, the reaction mixture was added and incubated in the dark for 30 min at room temperature. The LDH concentration was quantified by measuring the absorbance at 490 nm.

Detection of the expression of Bcl-2, Bax, Caspase-3/9, TRkA, TrkB, CREB, and p-CREB by western blotting

The cells of each group were collected and lysed with RIPA lysis buffer containing a protease inhibitor, and the protein concentration was determined with a BCA protein concentration detection kit. The sample was loaded at 30-50 μg/well, separated by 10% sodium-dodecyl sulfate gel electrophoresis for 1 h, transferred to a polyvinylidene fluoride membrane, sealed with PBS buffer containing 5% skim milk powder for 2 h, added to the corresponding primary antibody, and incubated overnight at 4 °C. Then, the cells were rinsed with PBST buffer 3 times for 5 min each, the corresponding secondary antibody was added, and the cells were incubated at 37 °C for 4 h. Next, PBST buffer was used to rinse the cells 3 times. Color development was achieved by using the ECL Western Blot Detection Kit (Thermo Scientific). β-Actin was used as the internal reference. All antibodies were purchased from Abcam Company in Shanghai, China. Image J was used to analyze the gray value of the target protein.

Detection of the relative expression of CNTF, BDNF, NGF, TrkB, TrkA, and CREB mRNA by real-time quantitative polymerase chain reaction

TRIzol reagent (TRIzol Invitrogen) was employed to extract total RNA from cells, which were then treated with DNA enzymes for reverse transcription using a reverse transcription kit (Takara Biotechnology). Two microliters of cDNA product was placed into an EP tube and amplified by a SYBR Green Master Mix reaction system. The Ct value of the template was determined using GAPDH as the internal parameter, and the relative expression level of the target mRNA was quantified by the cycle counting method ($2^{-\Delta\Delta Ct}$). The primer sequences are shown in Table 1.

Statistical analyses

All data in this study were analyzed using SPSS 22.0 statistical software. Data are shown as the means ± SE (the number of samples was 3 per group, and each sample was repeated three times). Comparisons between the two groups were made using independent *t* tests, and comparisons between multiple groups were made using one-way ANOVA. $P < 0.05$ was considered to be a statistically significant difference.

Table 1 Primer sequences

Genes	Primer sequences
GAPDH-F	TGTGACAGTGACTTGGGACA
GAPDH-R	AGGTGGAAGAGTGGGAGTTG
Bcl2-F	TCGCAGAGATGTCCAGTCAG
Bcl2-R	ATCTCCCTGTTGACGCTCTC
Caspase-3-F	AAGGAGCAGCTTTGTGTGTG
Caspase-3-R	TGTCTCAATGCCACAGTCCA
Bax-F	TGCAGAGGATGATTGCTGAC
Bax-F	GATCAGCTCGGGCACTTTAG
CNTF-F	GGTGACTTCCATCAGGCAAT
CNTF-R	TGACACGGAGGTCATGGATA
BDNF-F	TAATGCAGCATGATGGGAAA
BDNF-R	TCACAGTGAAAGCACCTTGC
NGF-F	CATGGGGGAGTTCTCAGTGT
NGF-R	GCACCCACTCTCAACAGGAT
TrkA-F	GTCTGGTGGGTCAGGGACTA
TrkA-R	AAAGCTCCACACATCGCTCT
TrkB-F	ACTCGCTTCTGGCATTGTCT
TrkB-R	TGTTAGTTGTGGTGGGCAAA
CREB-F	TTTTACCCAGGTGCCACTTC
CREB-R	TGGGGCATTATAACCGATGT
Caspase 9-F	TGCCCTTGCCTCTGAGTAGT
Caspase 9-R	AACAAAGAAACGCCCACAAC

Bcl2: B-cell lymphoma 2; Trk: Tropomyosin receptor kinase; BDNF: Brain-derived neurotrophic factor; CREB: Cyclic adenosine monophosphate response element-binding protein; CNTF: Ciliary neurotrophic factor; NGF: Nerve growth factor.

RESULTS

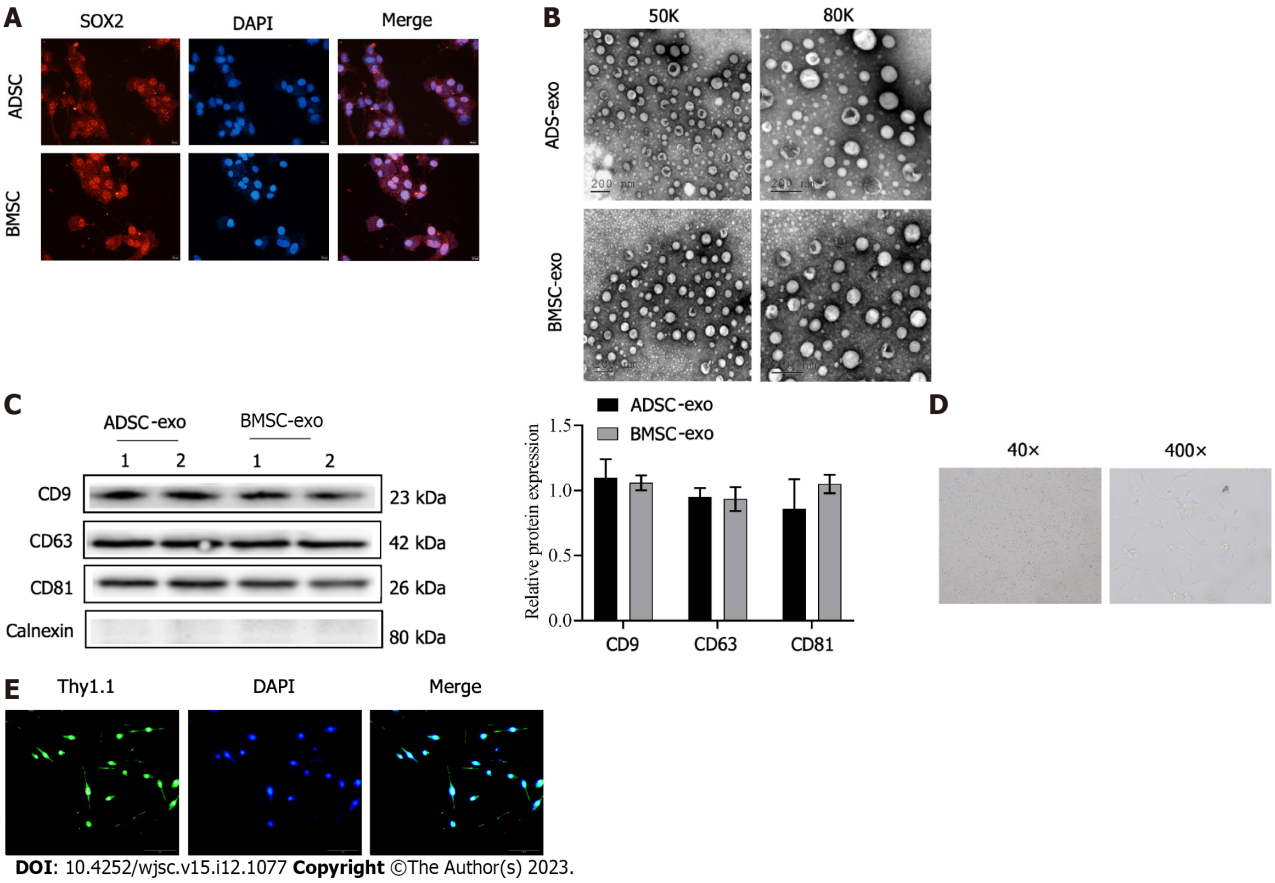
Characterization of ADSC- and BMSC-Exos and RGCs

The immunofluorescence labeled SOX2, which is the marker protein of stem cells was highly positive in the two kinds of stem cells (Figure 1A). Then, exos were further isolated from the stem cell solution, and suborbicular vesicles in the form of complex exo were observed by electron microscopy (Figure 1B). The exo marker molecules CD9, CD63, and CD81 detected by western blot were positive (Figure 1C), which proved that ADSC- and BMSC-Exos were successfully isolated and obtained. Finally, primary RGCs were extracted. Figure 1D shows light microscope images of RGCs. The isolated RGCs were examined by Thy1.1 immunofluorescence (Figure 1E).

ADSC-Exos and BMSC-Exos ameliorate RGCs

RGC growth observation: The RGC damage models under 40, 80, and 120 mmHg pressure were constructed, and cultured with ADSC-Exos (20 μg/mL) and BMSC-Exos (20 μg/mL) for 24 h, and then the morphology of RGCs was observed. The results of phase contrast microscopy showed that the gibbosity of RGCs was lower and that the cells were irregularly ellipsoidal under pressure. The addition of ADSC-Exos significantly restored the morphology of RGCs, but BMSC-Exos failed to restore the morphology of RGCs in the 120 mmHg pressure group (Figure 2A). After pressure was applied, transmission electron microscopy observations showed that RGCs had nuclear swelling, structural disorder, atrophy, malformation, and nucleolar shrinkage or disappearance. The addition of ADSC-Exos and BMSC-Exos significantly restored RGC morphology (Figure 2B).

The number of RGCs: To further explore the effect of ADSC-Exos and BMSC-Exos on RGCs, we performed neuron-specific β III-tubulin expression by immunofluorescence. The results showed that the number of RGCs was significantly reduced after pressure was applied, while the number of RGCs was markedly increased after ADSC-Exos and BMSC-Exos were administered (Figures 3A-C).



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Figure 1 Adipose-derived stem cell and bone marrow-derived stem cell-exosomes, and retinal ganglion cells were isolated and identified. A: The immunofluorescence staining of stem cell markers SOX2 (Scale bar: 20 μm); B: The morphology of adipose-derived stem cell-exosomes (ADSC-Exos) and bone marrow-derived stem cell (BMSC)-Exos was observed by electron microscopy (scale bar: 200 nm); C: The ADSC-Exos and BMSC-Exos marker molecules (CD9, CD63, and CD81) were detected by western blotting; D: The light microscope images of retinal ganglion cells (RGCs) (magnification: 40 × and 400 ×); E: The isolated RGCs was examined by Thy1.1 immunofluorescence (scale bar: 50 μm). ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome.

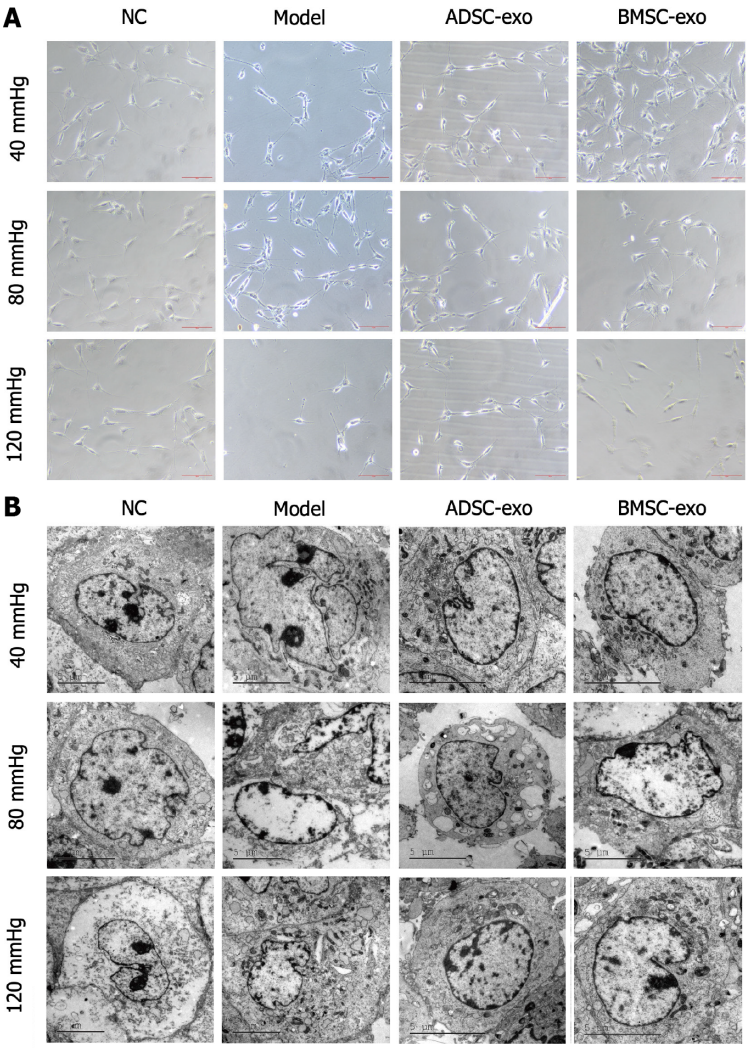
RGC proliferation and apoptosis: The CCK-8 results showed that RGC proliferation activity in the pressure injury group was significantly lower than that in the NC group, while proliferation activity was elevated after ADSC- and BMSC-Exos treatment (Figure 4A). The flow cytometry results indicated that compared with that in the NC group, the apoptosis rate in the pressure injury group was significantly increased, while the apoptosis rate was significantly decreased after ADSC- and BMSC-Exos treatment (Figure 4B).

The expression of CREB and p-CREB in RGCs

CREB inhibits apoptosis by upregulating the expression of the antiapoptotic genes Bcl-2 and Bcl-xL through a transcription-dependent mechanism. Therefore, this study further explored the effects of exos from different sources on the apoptosis-related protein CREB in RGCs. As shown in Figures 5A-C, the ratio of p-CREB/CREB in the pressure injury group was significantly reduced compared with that in the NC group, while the ratio of p-CREB/CREB was significantly increased after ADSC- and BMSC-Exos treatment.

The expression of LDH, neurotrophic factor and apoptosis-related mRNA and protein

LDH is present in all cells. When RGCs are damaged, they are released rapidly. When LDH is elevated, RGCs are damaged or dysfunctional, which leads to apoptosis. Therefore, this study further explored the effects of exos from different sources on LDH in RGCs. The LDH test kit results showed that compared with that in the NC group, the LDH level was significantly increased in the pressure injury group, while the LDH level was significantly decreased after ADSC-Exos and BMSC-Exos treatment (Figure 6A). western blot and real-time quantitative polymerase chain reaction results indicated that compared with the NC group, the anti-apoptotic protein (Bcl-2) and neurotrophic factor (BDNF, NGF, CNTF, TRKA, TRKB) were significantly decreased and the pro-apoptotic protein (Bax, caspase3, caspase9, CREB) was significantly increased in the pressure injury group, while the above indices could be reversed after ADSC-Exos and BMSC-Exos treatment (Figures 6B and C).



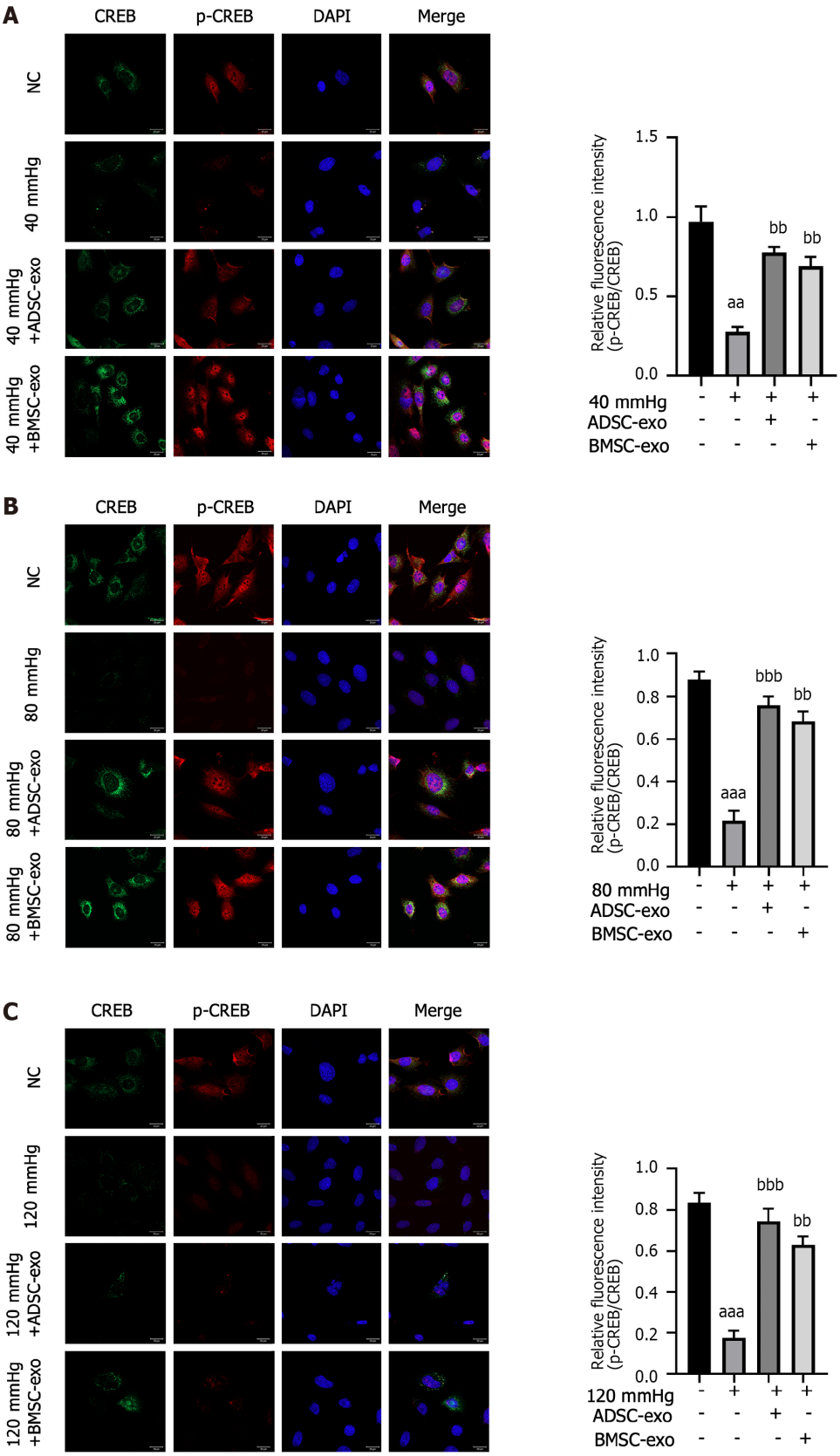
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Figure 2 The growth of retinal ganglion cells. A: The observation of morphology and growth of retinal ganglion cells (RGCs) by phase contrast microscope (Scale bar: 50 μm); B: The observation of RGCs morphology by transmission electron microscope (Scale bar: 5 μm). ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control.

DISCUSSION

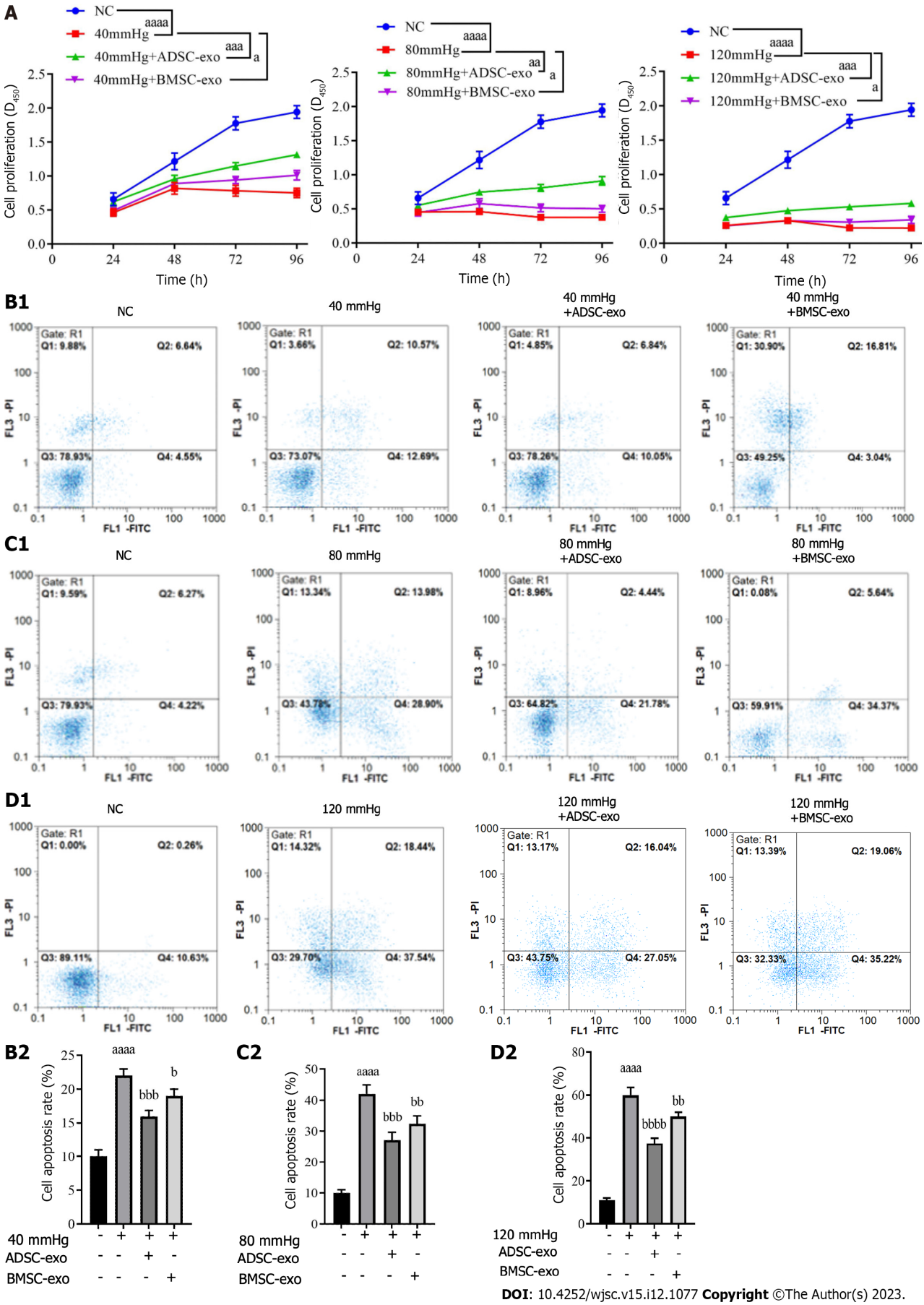
Hydrostatic pressure is known to impact various aspects of cellular anatomy and physiology. Morphological changes in cell shape, alignment and processes and cytoskeletal actin redistribution have been demonstrated in rat RGCs[34,35]. Agar *et al*[36] showed that neurons may undergo apoptosis in direct response to increased pressures at clinically relevant levels. Cell cultures were subjected to elevated hydrostatic pressures in an *in vitro* system based on established pressure chamber models[37,38]. Experimental pressure conditions were selected to be relevant to the IOPs seen in clinical settings, with levels of 100 mmHg analogous to acute glaucoma, 30 mmHg for chronic glaucoma and 15 mmHg for the so-called 'normal' IOP. Therefore, hydrostatic pressures of 40, 80, and 120 mmHg were chosen for this study and the intervention of ADSC- and BMSC-Exos could improve RGC damage by inhibiting RGC apoptosis and promoting neural restoration of RGCs.

Stem cells are a kind of cell population with the potential for self-renewal, high proliferation and differentiation, and multidirectional differentiation. MSCs are derived from connective tissue and can be differentiated into osteoblasts, chondrocytes, fat cells, and so on. In neurodegenerative diseases, MSC secretes a variety of neurotrophic factors that directly promote the survival and growth of nerve cells and act on the microenvironment of nerve tissue[39,40]. MSCs isolated from rat femoral bone marrow injected into a vitreous body can integrate into the retina, survive for at least 5 wk, significantly reduce IOP and have a protective effect on RGCs[41]. Liu *et al*[42] found that BMSC transplantation can significantly reduce photoreceptor cell death and preserve retinal structure in retinal detachment models. Hu *et al*[43] reported that BMSCs promoted the survival of RGCs in the transplanted eye compared with the control eye. MSCs play a role in neuroprotection mainly by regulating the intraocular microenvironment. This suggests that BMSCs have an ameliorating effect. However, the structural integration of MSCs into retinal tissue is complex and difficult due to the barrier action of the inner boundary membrane of the retina and the intercellular junctions[44]. In addition, transplanting MSCs in tissue reconstruction carries risks of tumorigenesis and immunological rejection. Transplanting MSC-Exos may overcome these obstacles in future applications. For example, MSC-Exos have an ameliorating effect on RGCs in some



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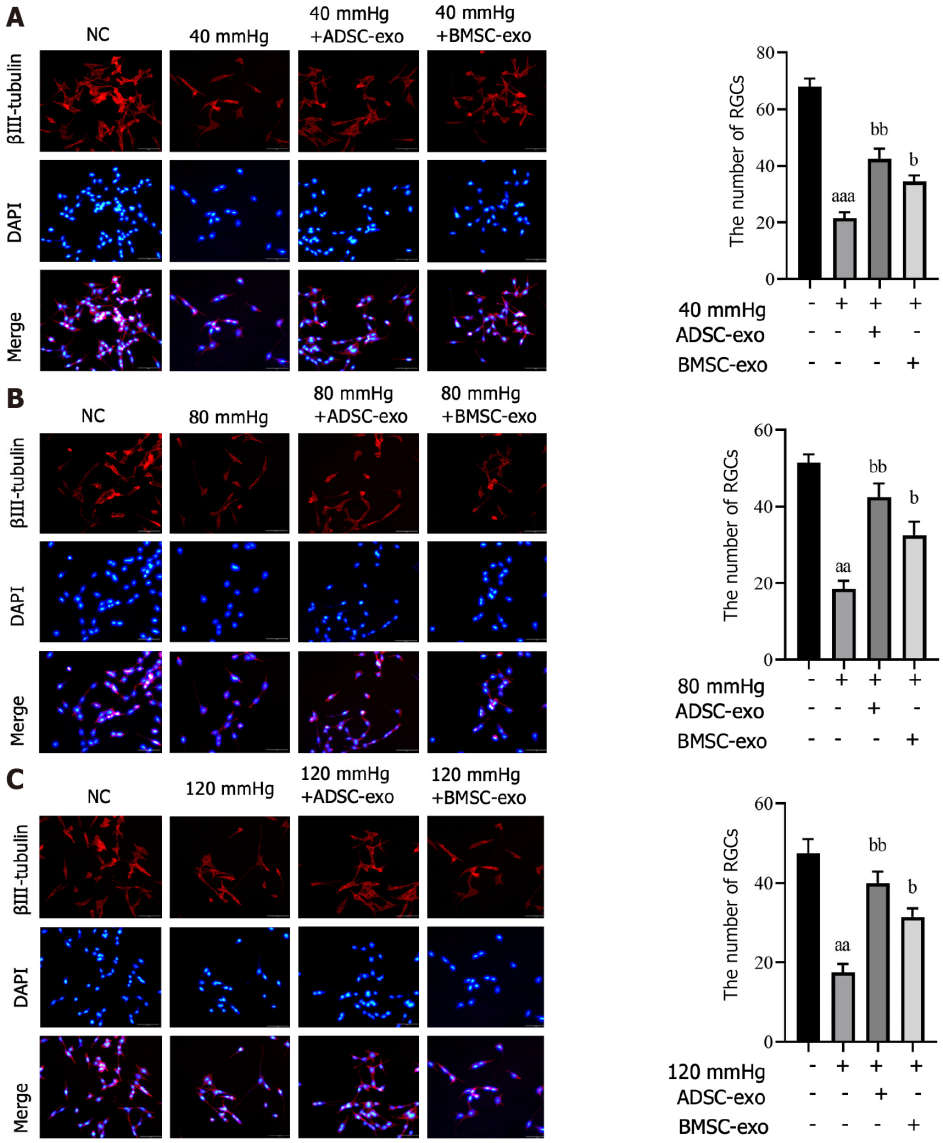
Figure 3 Immunofluorescence detection of β-III tubulin. A-C: Immunofluorescence staining of β-III tubulin (red) and nuclear staining (DAPI, blue) (left panel) and the number of β-III tubulin-positive retinal ganglion cells (right panel) in different groups after exposure to 40 mmHg (A), 80 mmHg (B), and 120 mmHg (C). [a]*P* < 0.05, compared with the normal control group (scale bar: 50 μm); [b]*P* < 0.05, compared with model (40, 80, and 120 mmHg) group. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control; CREB: Cyclic adenosine monophosphate response element-binding protein.



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Figure 4 Retinal ganglion cells proliferation and apoptosis. A: Cell proliferation was detected by using the Cell Counting Kit-8 proliferation reagent; B-D: Cell apoptosis was detected by flow cytometry for apoptosis detection, and representative flow cytometry density plots (left) and statistical bar chart (right). $^{a}P < 0.05$,

compared with the normal control group; [b]*P* < 0.05, compared with model (40, 80, and 120 mmHg) group. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control.

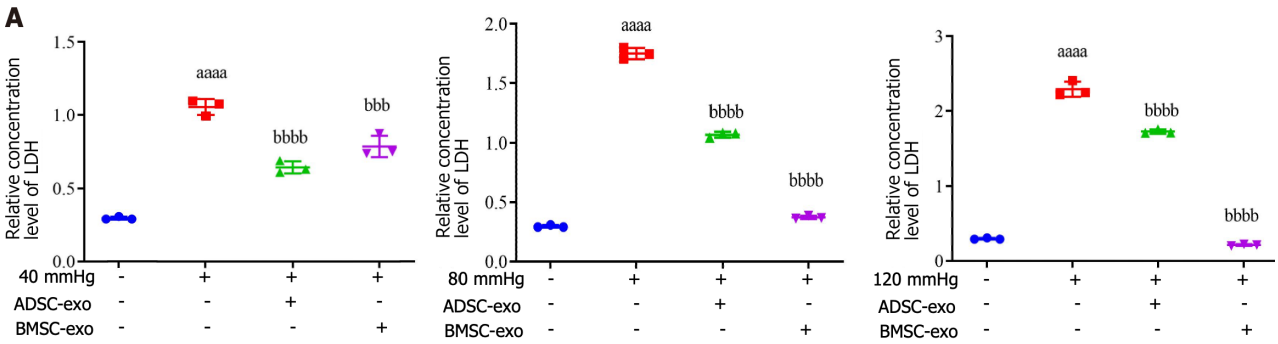


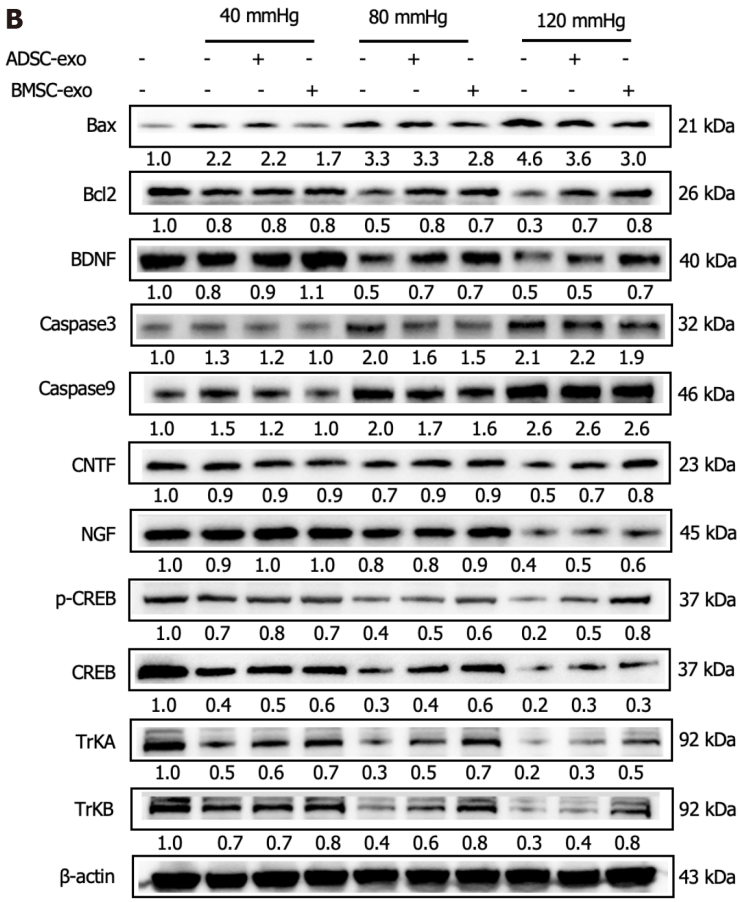
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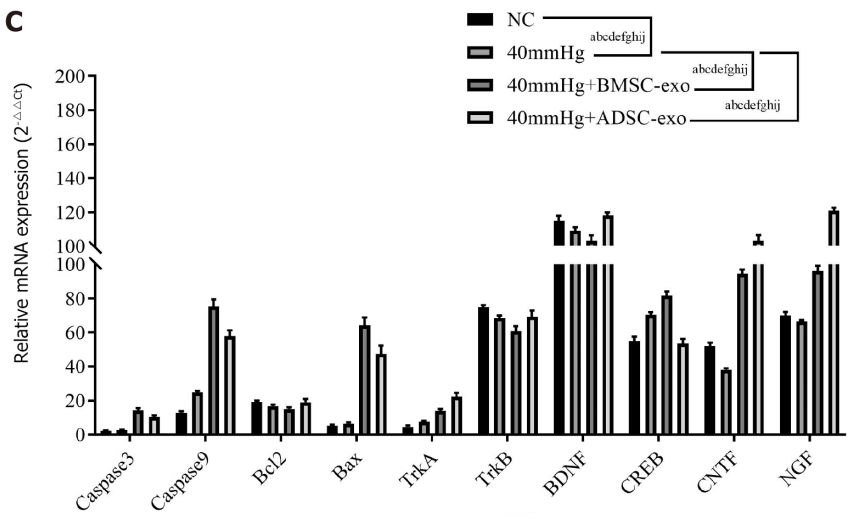
Figure 5 Immunofluorescence detection of p-CREB and CREB. A-C: Immunofluorescence staining of CREB (green), p-CREB (red) and nuclear staining (DAPI, blue) (left panel) and quantification of the ratio of p-CREB/CREB (right panel) in different groups after exposure to (A) 40 mmHg, (B) 80 mmHg, and (C) 120 mmHg (Scale bar: 20 μm). [a]*P* < 0.05, compared with the normal control group; [b]*P* < 0.05, compared with the model (40, 80, and 120 mmHg) group. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control.

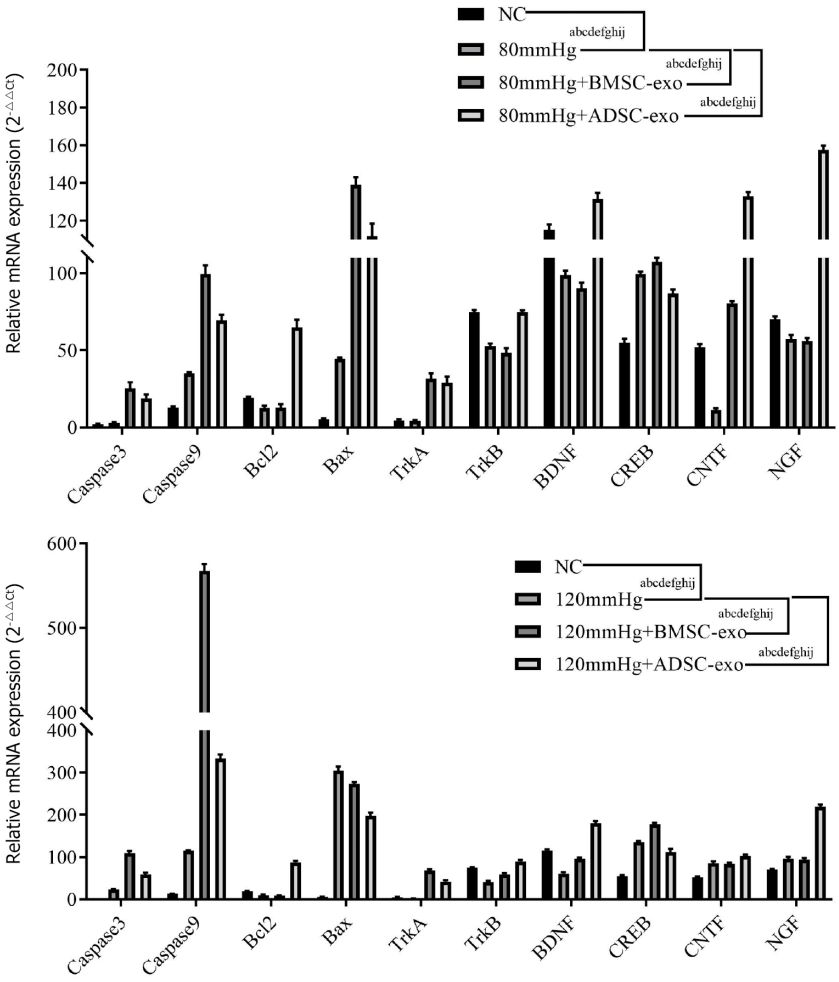
optic neuropathy rat models[45,46]. Therefore, exos derived from stem cells are emerging as a promising method for glaucoma treatment.

This study elucidated that BMSC-Exos significantly reversed RGC damage induced by hydrostatic pressure in a RGC injury model *in vitro*. The protective effect was mainly reflected in the fact that ADSC-Exos and BMSC-Exos promoted the proliferation of RGCs, the regeneration of RGC axons, and the secretion of RGC neurotrophic factors while inhibiting RGC apoptosis. We emphasized its potential in the prevention or treatment of glaucomatous optic neuropathy. Our study confirmed previous findings that MSC-Exos have an ameliorating effect against RGC damage in some optic neuropathies. BMSC-Exos have been found to increase RGC survival and promote axon regeneration. This ameliorating effect of BMSC-Exos was also observed in a genetic modification DBA/2J glaucoma mouse model[23]. Furthermore, in rats with optic nerve compression, Pan *et al*[47] found that umbilical cord-MSC-Exos enhanced RGC survival. Yu *et al*[48] confirmed that MSC-derived EVs can help alleviate optic nerve injury caused by chronic ocular hypertension, and this effect is achieved by inhibiting cell apoptosis. In addition, we discovered for the first time that ADSC-Exos significantly reversed high-pressure-induced RGC damage. It was reported that after ADSCs and ADSC-the trabecular meshwork (ADSC-TM) were transplanted into the eyes of infantile mice, the cells integrated into TM tissue expressed TM cell markers, and maintained


A
Relative concentration level of LDH
aaaa
bbbb
bbb
40 mmHg
ADSC-exo
BMSC-exo
80 mmHg
120 mmHg


B
40 mmHg
80 mmHg
120 mmHg
ADSC-exo
BMSC-exo
Bax 21 kDa
Bcl2 26 kDa
BDNF 40 kDa
Caspase3 32 kDa
Caspase9 46 kDa
CNTF 23 kDa
NGF 45 kDa
p-CREB 37 kDa
CREB 37 kDa
TrKA 92 kDa
TrKB 92 kDa
β-actin 43 kDa


C
NC
40mmHg
40mmHg+BMSC-exo
40mmHg+ADSC-exo
Relative mRNA expression (2-△△Ct)
Caspase3
Caspase9
Bcl2
Bax
TrkA
TrkB
BDNF
CREB
CNTF
NGF



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Figure 6 The expression of lactate dehydrogenase, neurotrophic factor and apoptosis-related protein and mRNA. A: Lactate dehydrogenase (LDH) expression level in each group was measured by the LDH test kit; B: The expression of apoptosis-related proteins and neurotrophic factors were detected by western blotting; C: The mRNA expression of apoptosis-related proteins and neurotrophic factors were detected by real-time quantitative polymerase chain reaction. ^{a}P, ^{b}P, ^{c}P, ^{d}P, ^{e}P, ^{f}P, ^{g}P, ^{h}P, ^{i}P, and $^{j}P < 0.05$, represent the comparison of caspase3, caspase9, B-cell lymphoma 2, Bax, tropomyosin receptor kinase (TrK)A, TrKB, brain-derived neurotrophic factor, cyclic adenosine monophosphate response element-binding protein, ciliary neurotrophic factor, and nerve growth factor proteins between the two groups. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control; LDH: Lactate dehydrogenase; Bcl2: polyvinylidene fluoride; Trk: Tropomyosin receptor kinase; BDNF: Brain-derived neurotrophic factor; CREB: Cyclic adenosine monophosphate response element-binding protein; CNTF: Ciliary neurotrophic factor; NGF: Nerve growth factor.

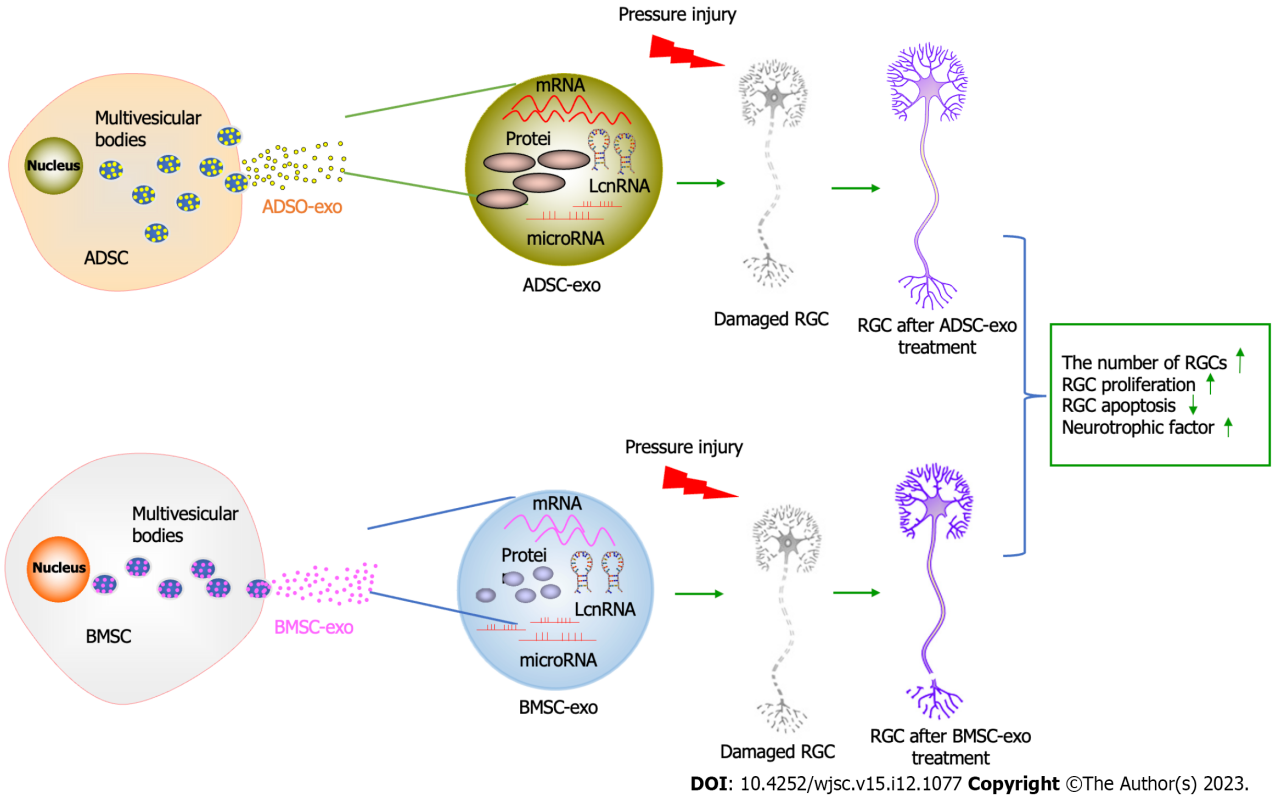
normal IOP, outflow facility, and extracellular matrix[49]. The results demonstrated the possibility of applying autologous or allogeneic ADSC and ADSC-TM cells as a potential therapeutic approach to restore the structure and function of the TM in glaucoma. Therefore, combined with literature reports and our study, ADSC-Exos are expected to be an adjunct therapeutic agent for glaucoma in the future.

Surprisingly, ADSC-Exos manifested better protective effects than BMSC-Exos in the present study. There is evidence that ADSCs are most conducive to clinical utilization. In addition, adipose tissue is relatively abundant in the human body compared with other tissues. ADSCs can be isolated from adipose tissue. In addition, Kern *et al*[50], through comparative analysis of MSCs from bone marrow, umbilical cord blood, or adipose tissue, concluded that 500 times more stem cells were obtained from adipose tissue than from the same amount of bone marrow. Moreover, ADSCs are easier to obtain from adipose tissue due to their subcutaneous location than BMSCs. Patients tend to choose less traumatic sites for collecting tissue. Significant discrepancies are observed in cellular exos from different sources, including the type and content of mRNAs, miRNAs, and proteins[51]. Importantly, ADSCs have a higher proliferation capacity than BMSCs[52]. Moreover, ADSC-Exos possess the advantages of ADSCs. Hence, ADSC-Exos seem to be more favorable than BMSC-Exos because they are easily obtained and have a high proliferation capacity. ADSC-Exos could also be considered for the treatment of glaucoma. The graphic abstract is shown in Figure 7.

Some limitations should be stated. First, we lacked the comparison of neurotrophic factors and miRNAs between ADSC-Exos and BMSC-Exos by bioinformatics and other basic biological methods. Second, the effects of ADSC-Exos and BMSC-Exos on RGCs should be further validated by an *in vivo* experiment. Last but not least, more *in vivo* and *in vitro* experiments are needed to provide theoretical evidence for that MSC-Exos protect RGCs.

CONCLUSION

These findings indicated that ADSC-Exos and BMSC-Exos could ameliorate RGC injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors.



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Figure 7 Graphical abstract. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; LncRNA: Long non-coding RNA; RGC: Retinal ganglion cell; Exo: Eexosome.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have a protective effect against damage to the cornea, lacrimal gland, retina and photoreceptor cells, and this protective effect can be mediated through exosomes (exos) released by MSCs.

Research motivation

Experimental pressure conditions were selected to be relevant to intraocular pressures seen in clinical settings, with levels of 100 mmHg analogous to acute glaucoma, 30 mmHg for chronic glaucoma and 15 mmHg for the so-called 'normal' intraocular pressure. Bone marrow-derived stem cell (BMSC)-Exos have an ameliorative effect on glaucoma. However, the effect of adipose-derived stem cell (ADSC)-Exos which is the ideal source of exos on optic neural degeneration has not been studied yet.

Research objectives

This study investigated the ameliorating effect of exos derived from different MSCs on retinal ganglion cell (RGC) injury induced by hydrostatic pressure.

Research methods

The RGC injury model was constructed by RGC damage under different hydrostatic pressures (40, 80, 120 mmHg). Then RGCs were cultured with ADSC-Exos and BMSC-Exos. Cell Counting Kit-8, transmission electron microscopy, flow cytometry, immunofluorescence, real-time quantitative polymerase chain reaction, and western blotting were performed to detect the ameliorating effect of exos on pressure-induced RGC injury.

Research results

ADSC-Exos and BMSC-Exos were successfully separated. RGCs were in the shape of irregular ellipsoids, and the addition of ADSC-Exos and BMSC-Exos significantly restored the morphology of RGCs. In addition, the proliferative activity of

RGCs was increased and cell apoptosis was inhibited. Increased levels of lactate dehydrogenase and apoptosis-related proteins, and increased concentrations of anti-apoptotic proteins and neurotrophic substances.

Research conclusions

The results indicate that ADSC-Exos and BMSC-Exos can improve hydrostatic pressure-induced RGC damage by inhibiting apoptosis and increasing neurotrophic factor secretion.

Research perspectives

The results of this study will be verified by the experiment *in vivo* in the future.

FOOTNOTES

Co-first authors: Zhi-Kun Zheng and Lei Kong.

Author contributions: Zheng ZK and Kong L designed the study; Zheng ZK, Kong L, and Dai M acquired and analyzed data; Dai M performed the experiments; Chen YD interpreted the data; Zheng ZK, Kong L, and Chen YH wrote the manuscript; and all authors approved the final version of the article.

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ORIGINAL ARTICLE

Basic Study

Human mesenchymal stem cells exhibit altered mitochondrial dynamics and poor survival in high glucose microenvironment

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) are a type of stem cells that possess relevant regenerative abilities and can be used to treat many chronic diseases. Diabetes mellitus (DM) is a frequently diagnosed chronic disease characterized by hyperglycemia which initiates many multisystem complications in the long-run. DM patients can benefit from MSCs transplantation to curb down the pathological consequences associated with hyperglycemia persistence and restore the function of damaged tissues. MSCs therapeutic outcomes are found to last for short period of time and ultimately these regenerative cells are eradicated and died in DM disease model.

AIM

To investigate the impact of high glucose or hyperglycemia on the cellular and molecular characteristics of MSCs.

METHODS

Human adipose tissue-derived MSCs (hAD-MSCs) were seeded in low (5.6 mmol/L of glucose) and high glucose (25 mmol/L of glucose) for 7 d. Cytotoxicity, viability, mitochondrial dynamics, and apoptosis were deplored using specific kits. Western blotting was performed to measure the protein

expression of phosphatidylinositol 3-kinase (PI3K), TSC1, and mammalian target of rapamycin (mTOR) in these cells.

RESULTS

hAD-MSCs cultured in high glucose for 7 d demonstrated marked decrease in their viability, as shown by a significant increase in lactate dehydrogenase ($P < 0.01$) and a significant decrease in Trypan blue ($P < 0.05$) in these cells compared to low glucose control. Mitochondrial membrane potential, indicated by tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity, and nicotinamide adenine dinucleotide (NAD+)/NADH ratio were significantly dropped ($P < 0.05$ for TMRE and $P < 0.01$ for NAD+/NADH) in high glucose exposed hAD-MSCs, indicating disturbed mitochondrial function. PI3K protein expression significantly decreased in high glucose culture MSCs ($P < 0.05$ compared to low glucose) and it was coupled with significant upregulation in TSC1 ($P < 0.05$) and downregulation in mTOR protein expression ($P < 0.05$). Mitochondrial complexes I, IV, and V were downregulated profoundly in high glucose ($P < 0.05$ compared to low glucose). Apoptosis was induced as a result of mitochondrial impairment and explained the poor survival of MSCs in high glucose.

CONCLUSION

High glucose impaired the mitochondrial dynamics and regulatory proteins in hAD-MSCs ensuing their poor survival and high apoptosis rate in hyperglycemic microenvironment.

Key Words: Mesenchymal stem cells; High glucose; Mitochondrial dynamics; Apoptosis; Poor survival; Phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway

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Core Tip: Mesenchymal stem cells (MSCs) have significant regenerative properties that make them a potential treatment for many chronic diseases. Among these, diabetes mellitus (DM) was found to benefit from MSCs transplantation in which they restored the damaged tissues and prevented hyperglycemia-related complications. However, these therapeutic are short-lived hindering the clinical use of MSCs in the treatment of DM. This study aims to elucidate the mechanisms of hyperglycemia-induced effects on MSCs which will help in improving the therapeutic functions of these cells in this stress environment.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are the most commonly studied forms of stem cells as they possess powerful regenerative and immunomodulatory abilities[1]. MSCs have unique molecular machinery that empowers them to modulate many cellular signaling pathways and restore the functionality of damaged tissues rendering them suitable to treat many acute and chronic diseases[1]. Despite their unprecedented abilities to adapt to many stress conditions, they can still behave unexpectedly under certain stress microenvironments. Many preclinical and clinical studies not only demonstrated the positive outcomes after transplanting MSCs to injured sites[2,3], but also reported the short-lived therapeutic results in hostile microenvironments which need to be figured out[4]. When MSCs are undifferentiated, they rely mainly on glycolysis to support their proliferation, expansion, and immunomodulation[5]. When MSCs are programmed to differentiate, they switch to oxidative phosphorylation (OxPhos)[6], thereby, intact mitochondria are also important to maintain their multi-lineage differentiation capacities[6].

In the last decades, diabetes mellitus (DM) incidence has increased rapidly, and it is deemed among top five chronic diseases[7]. DM is associated with serious multisystem complications that impair the life quality of many patients[8]. DM is characterized mainly by chronic hyperglycemic state that causes many devastating, long-term complications[8]. DM patients can benefit remarkably from MSCs therapy to repair and regenerate damaged tissues, restore their functionality, and reduce the severity of DM-related pathological complications[7]. Several studies reported a significant improvement in glycemic parameters and cardiovascular complications after transplanting MSCs in DM patients[7,9,10]; however, other studies reported that DM can accelerate the decline in MSCs quantity and therapeutic quality, and impair their regenerative capabilities[11]. When MSCs turn into a dysfunctioning cells, they may increase the severity of DM-related complications[11]. Although previous studies had addressed the effects of hyperglycemia on the molecular and cellular characteristics of transplanted MSCs, many aspects still need to be elucidated. Thus, it is important to investigate the effect of high glucose on MSCs biological dynamics, which are essential to understand their fate and performance in

diabetic microenvironment, and provide new avenues on possible molecular targets to modulate their survival and therapeutic outcomes in DM patients.

MATERIALS AND METHODS

Human MSCs

Human adipose tissue-derived MSCs (hAD-MSCs) were commercially purchased from Lonza (Cat# PT5006, Lot# 21TL138912) and expanded using Dulbecco's Modified Eagle's Medium Low Glucose (DMEM-Low Glucose, Euroclone) which contained 5.6 mmol/L glucose and were supplemented with 10% fetal bovine serum (FBS, Gibco)[12], 0.1 mg/mL streptomycin and 100 units/mL penicillin G in standard cell culture incubators (5% CO_2/95% air; 37 °C). Medium was changed every 72 h, and cells were sub-cultured when confluence exceeded 60%[13]. For high glucose conditions, cells were cultured in Dulbecco's Modified Eagle's Medium High Glucose (DMEM-High Glucose, Euroclone) which contained 25 mmol/L glucose[14] and were supplemented with 10% FBS, 0.1 mg/mL streptomycin and 100 units/mL penicillin G for 3, 7, and 14 d in standard cell culture incubators (5% CO_2/95% air; 37 °C). High glucose complete medium was changed every 72 h. MSCs that were cultured in low glucose were considered as our study control. MSCs of passage 5 and 6 were used to perform the experiments. AD-MSCs characterization markers can be found in (Table 1).

Cytotoxicity assays

To measure the level of cytotoxicity in human MSCs after being cultured in low and high glucose, we seeded 5 × 10^4 cells/well in 96 well plate and we measured the lactate dehydrogenase (LDH) which was released from the damaged MSCs (LDH Assay Kit, Abcam, Cat# ab102526) and the absorbance values were obtained using Cytation 5 (BioTek, United States). The viability of MSCs in low and high glucose were also assessed using 0.4% Trypan blue and the percentage of viable cells as well as representative fluorescent images were obtained using Corning® CytoSmart Cell Counter.

Tetramethylrhodamine ethyl ester (MtMP measurement)

Mitochondrial membrane potential was assessed using tetramethylrhodamine ethyl ester (TMRE)-Mitochondrial Membrane Potential Assay Kit (Abcam, Cat # ab113852). Briefly, human AD-MSCs were placed in 24-well plate at 1 × 10^4 cells per well and allowed to adhere overnight. After that, cells were cultured either in low glucose or high glucose for 7 d. Then, media were aspirated, and cells were stained using 400 nM TMRE in culture media for 30 min in the incubator, and then media were replaced with 200 µL phosphate buffered saline (PBS) per well. Fluorescence intensity was detected using Cytation 5 (BioTek, United States). (λex = 549 nm, λem = 575 nm), and TMRE fluorescent images were captured at Texas red filter using Cytation 5 (BioTek, United States).

Nicotinamide adenine dinucleotide /NADH assay

Nicotinamide adenine dinucleotide (NAD+)/NADH ratio in AD-MSCs that were cultured in low and high glucose media was assessed using NAD/NADH-Glo™ assay kit (Promega, Cat# G9071). Briefly, cells were cultured in low and high glucose media for 7 d. After that, cells were detached and placed in 96 well plate at 5 × 10^5 in 50 µL of either low or high glucose media and were allowed to adhere overnight. 50 µL of NAD/NADH-Glo™ Detection Reagent was added to each well and the plate was gently shaken to mix and lyse the cells and was incubated with the added reagent for 60 min at room temperature. The luminescence values were recorded using Cytation 5 (BioTek, United States) which were corresponding to the total amount of NAD+ produced from NADH.

Western blotting

The protein levels for phosphatidylinositol 3-kinase (PI3K) (Santa Cruz Biotechnology Cat # sc-1637), TSC1 (Santa Cruz Biotechnology Cat # sc-377386), and mammalian target of rapamycin (mTOR) (Santa Cruz Biotechnology Cat # sc-293133), NDUFB8 (Abcam, Cat # ab192878), SDHB (Santa Cruz Biotechnology Cat # sc-271548), UQCRC2 (Santa Cruz Biotechnology Cat # sc-390378), COX5a (Santa Cruz Biotechnology Cat # sc-376907), ATP5A (Santa Cruz Biotechnology Cat # sc-136178), and β-actin (Santa Cruz Biotechnology Cat # sc-47778 HRP) were measured by western blot. Briefly, total protein levels were measured using NanoDrop™ Lite Spectrophotometer, and 40 µg of protein was loaded onto sodium-dodecyl sulfate gel electrophoresis. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membrane and were incubated with appropriate primary and secondary antibodies. The membranes were visualized using VILBER FUSION Gel Documentation System, and bands were quantified using ImageJ for densitometry.

Apoptosis assay

To detect apoptosis in hAD-MSCs after being cultured in low and high glucose, we used RealTime-Glo™ Annexin V Apoptosis live assay (Promega, Cat# JA1011, Lot# 0000400486 following the manufacturer's guidelines. Briefly, cells were seeded 1 × 10^4 cells/well in 24 well plate and then cultured in low and high glucose for 7 d. The media were aspirated and each well was washed with PBS followed by the addition of 100 µL of fresh medium having Annexin V-LgBiT, Annexin V-SmBiT, CaCl2, and Annexin V NanoBiT Substrate to each well. After 1 h incubation, the fluorescent images of green color which represented cells undergoing apoptosis were detected at GFP filter using Cytation 5 (BioTek, United States).

Table 1 Cell surface characterization of human adipose tissue-derived mesenchymal stem cells based on the company analysis report

Positive markers	Negative markers
CD13	CD14
CD29	CD31
CD44	CD45
CD73	HLA-DR
CD90	
CD105	
CD166	

Statistical analysis

Data were reported as mean ± SD. Comparison of data between multiple groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, and analysis between two groups was made using Student's *t*-test (two-tailed). Statistical significance is determined as $P < 0.05$. Each figure represents one of at least three independent quantifiable experiments.

RESULTS

Culturing MSCs in high glucose triggers massive cytotoxicity and poor survival

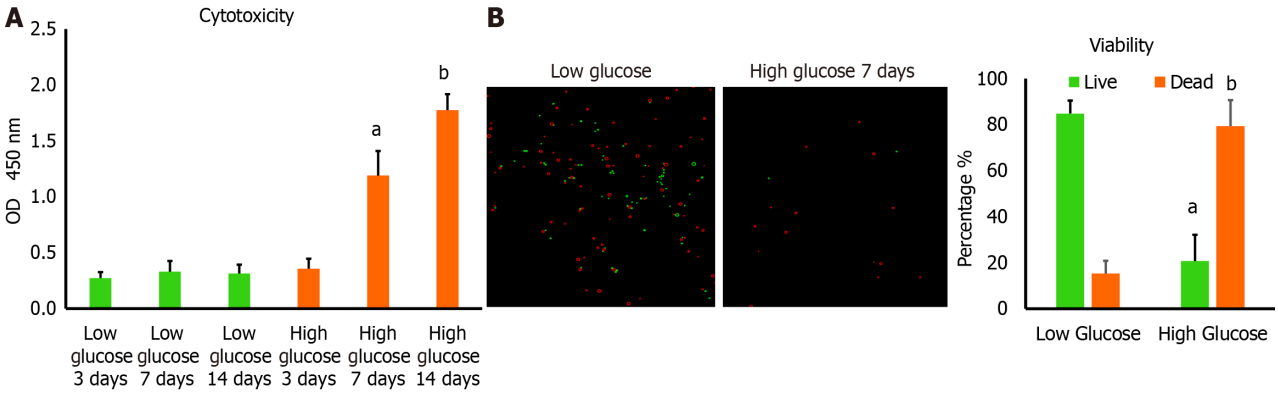
MSCs are known to survive well in low glucose medium which is a physiological requirement to preserve their stemness features. We want to determine the viability of these cells in high glucose microenvironment. hAD-MSCs were cultured in high glucose medium containing 25 mmol/L of glucose for 3, 7, and 14 d. The cytotoxicity was measured by determining the amount of LDH released and was found to be significantly greater ($P < 0.01$) in hAD-MSCs cultured in high glucose for 7 and 14 d compared with hAD-MSCs cultured in normal low glucose medium containing 5.6 mmol/L of glucose (Figure 1A). To confirm these findings, the viability of hAD-MSCs was measured using 0.4% Trypan blue and found to be remarkably low ($P < 0.05$) in hAD-MSCs that were cultured in high glucose for 7 d compared to hAD-MSCs cultured in a low glucose medium (Figure 1B).

The mitochondrial dynamics of MSCs impaired by high glucose

Mitochondria are integral intracellular organelles promoting cells survival and viability. The variance of mitochondrial dynamics depends on the type of microenvironment where cells exist. Previous reports demonstrated that high glucose can induce the fission and fragmentation of mitochondria in glomeruli podocytes[15], but the knowledge surrounding the impact of high glucose on the mitochondrial dynamics of MSCs is still limited. The mitochondrial membrane potential (ΔΨm) generated by proton pumps (complexes I, III and IV) is imperative to ensure proper ATP production by mitochondrial OxPhos. Abnormalities in the mitochondrial membrane potential (ΔΨm) can significantly elicit a decline in cells viability and trigger unwanted signaling pathways. We measured the mitochondrial membrane potential (ΔΨm) in hAD-MSCs after being grown in a high glucose medium for 7 d using TMRE which is sequestered by active mitochondria. Our results illustrated that the fluorescent intensity of TMRE was significantly low ($P < 0.05$) in hAD-MSCs cultured in a high glucose medium compared to hAD-MSCs cultured in a normal low glucose medium indicating an impairment in the mitochondrial membrane potential in these cells (Figures 2A and B). NAD+ and its reduced form (NADH) regulate many metabolic pathways, including mitochondrial OxPhos. Maintaining NAD+/NADH pool is required to ensure that the influx of electrons is coupled with the translocation of protons to the intermembrane space to generate the required membrane potential (ΔΨm) across the inner mitochondrial membrane to be harnessed by complex V to produce ATP[16]. To investigate if the decrease in the mitochondrial membrane potential (ΔΨm) is correlated with abnormalities in NAD+/NADH pool, we measure the NAD+/NADH ratio in hAD-MSCs cultured in low and high glucose media (Figure 3). The findings revealed a considerable drop ($P < 0.01$) in NAD+/NADH ratio when hAD-MSCs were cultured in high glucose. This may explain the disturbance in the inner mitochondrial potential detected by TMRE assay (Figures 2A and B).

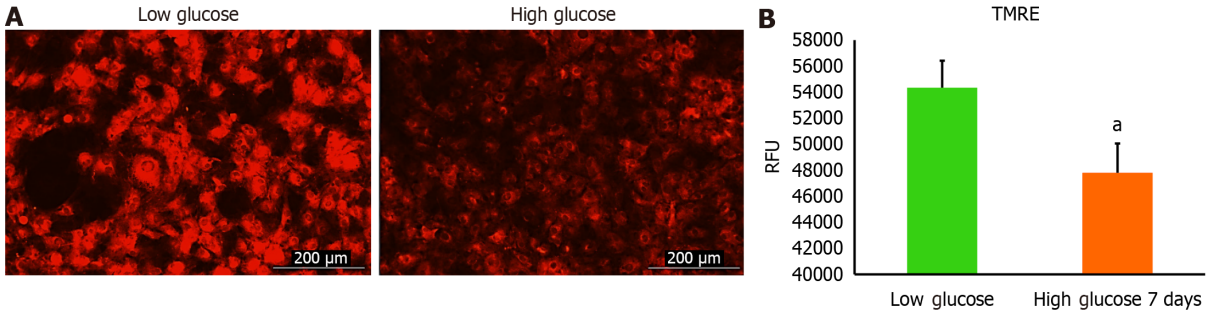
Disturbance of MSCs mitochondria dynamics parameters in high glucose engenders their apoptosis

Apoptosis is a programmed cell death that can be physiological or pathological depending on the triggering factors that enkindle it. Mitochondrial dysfunction is a notable inciting factor which is able to fire up debilitating cellular apoptosis and impact the survival of numerous cells. The noticeable deterioration in the mitochondrial parameters detected in hAD-MSCs after being cultured in high glucose prompted us to examine the level of apoptosis in these cells. To achieve that, we used RealTime-Glo™ Annexin V Apoptosis kit to unveil the presence of apoptosis or not. The luminescence signal which is proportionally linked to apoptosis intensity was considerably higher in hAD-MSCs that were cultured in high glucose (Figure 4). Fluorescent microscopic images showed that apoptosis (marked by green stained cells) was more in



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Figure 1 Culturing human adipose tissue-derived mesenchymal stem cells in high glucose reduced their viability. A: Lactate dehydrogenase cytotoxicity assay revealed higher level of cytotoxicity in human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) cultured in high glucose for 7 and 14 d compared to cells cultured in low glucose. n = 5, ^{a}P < 0.01 compared to low glucose 7 d, ^{b}P < 0.01 compared to low glucose 14 d; B: The percentage of cells viability detected by 0.4% Trypan blue showed significant reduction in the number of viable hAD-MSCs after being cultured in high glucose for 7 d. Fluorescent images of live and dead cells were obtained using Corning® CytoSmart Cell Counter. n = 5. n = 5, ^{a}P < 0.01 compared to live cells in low glucose 7 d, ^{b}P < 0.01 compared to dead cells in low glucose 7 d.



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Figure 2 High glucose decreased the mitochondrial membrane potential level of human adipose tissue-derived mesenchymal stem cells. The tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity values of human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) cultured in high glucose for 7 d were substantially declined compared to fluorescent intensity values of cells cultured in low glucose. Live fluorescent images showed a weak signal of TMRE stain in hAD-MSCs after being placed in high glucose for 7 d compared to strong TMRE signal in low glucose cultured cells. n = 4, ^{a}P < 0.05 compared to low glucose control. Fluroscnet images were captured at Texas Red filter using Cytation 5 (BioTek, United States). TMRE: Tetramethylrhodamine ethyl ester; RFU: Relative fluorescence units.

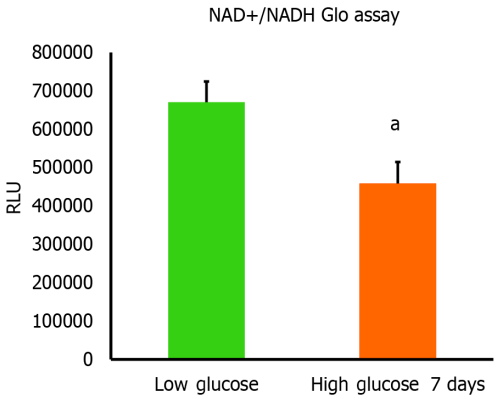
high glucose cultured hAD-MSCs (Figure 4).

High glucose affects the level of PI3K and mTOR in MSCs

PI3K orchestrates many vital signaling pathways and targets downstream factors that are required to maintain the vivacity of cells. PI3K controls integral proteins that are necessary to preserve mitochondrial dynamics[17,18]. PI3K is a known activator of mTOR which is a central regulator of cell survival and metabolism. It has been reported that mTOR is important to preserve mitochondrial dynamics and generate the required mitochondrial potential to produce ATP. PI3K is required to remove the inhibitory effect of tuberous sclerosis tumor suppressor (TSC1) which binds mTOR and inactivates it[19]. We performed western blotting analysis to measure the expression levels of PI3K, TSC1, and mTOR in high glucose treated hAD-MSCs. Western blotting results demonstrated the loss of PI3K and mTOR in high glucose treated hAD-MSCs (P < 0.05 compared to low glucose), while TSC1 is significantly increased in these cells (P < 0.05 compared to low glucose) (Figure 5). Those findings indicated impaired PI3K/mTOR axis which may explain the disruption in mitochondrial parameters in high glucose cultured MSCs. Taking together, our findings highlight a new mechanism that can be released by high glucose causing the poor survival of MSCs in diabetic microenvironment.

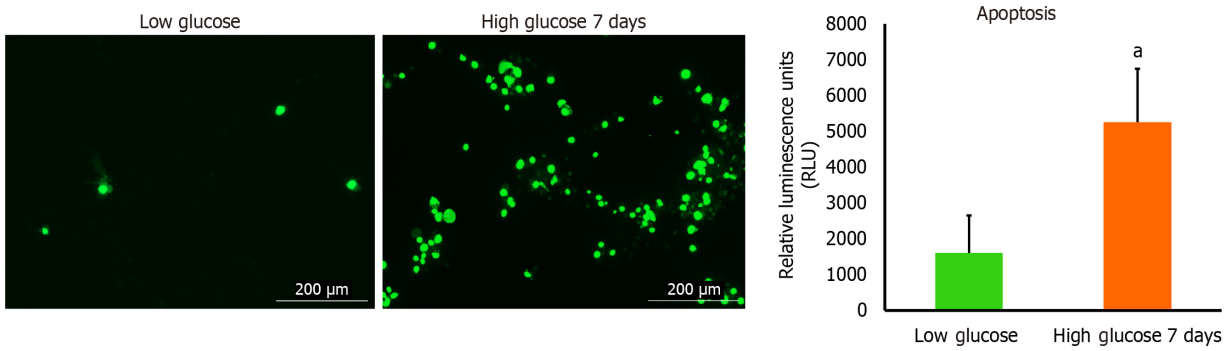
High glucose disturbs major mitochondrial complexes in MSCs

The reduction in mTOR which is essential for inducing the biogenesis of mitochondrial complexes, most importantly complex I and V[20] prompted us to investigate the expression of mitochondrial complexes in low and high glucose-cultured hAD-MSCs. The results demonstrated that hAD-MSCs that were cultured in high glucose exhibited significant downregulation (P < 0.05) in the level of complex I, IV, and V comparing to hAD-MSCs that were cultured in low glucose (Figure 6), while the expression of complex II and III showed no significant changes (P = 0.85) in both culturing



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Figure 3 Nicotinamide adenine dinucleotide and NADH amounts were declined in high glucose cultured human adipose tissue-derived mesenchymal stem cells. The amount of nicotinamide adenine dinucleotide (NAD+) produced from NADH was measured using NAD+/NADH Glo assay. Luminescence intensity was significantly less in human adipose tissue-derived mesenchymal stem cells cultured in high glucose for 7 d compared to cells cultured in low glucose. n = 4, ^{a}P < 0.01 compared to low glucose control. Luminescence values were measured using Cytation 5 (BioTek, United States). RFU: Relative fluorescence units.



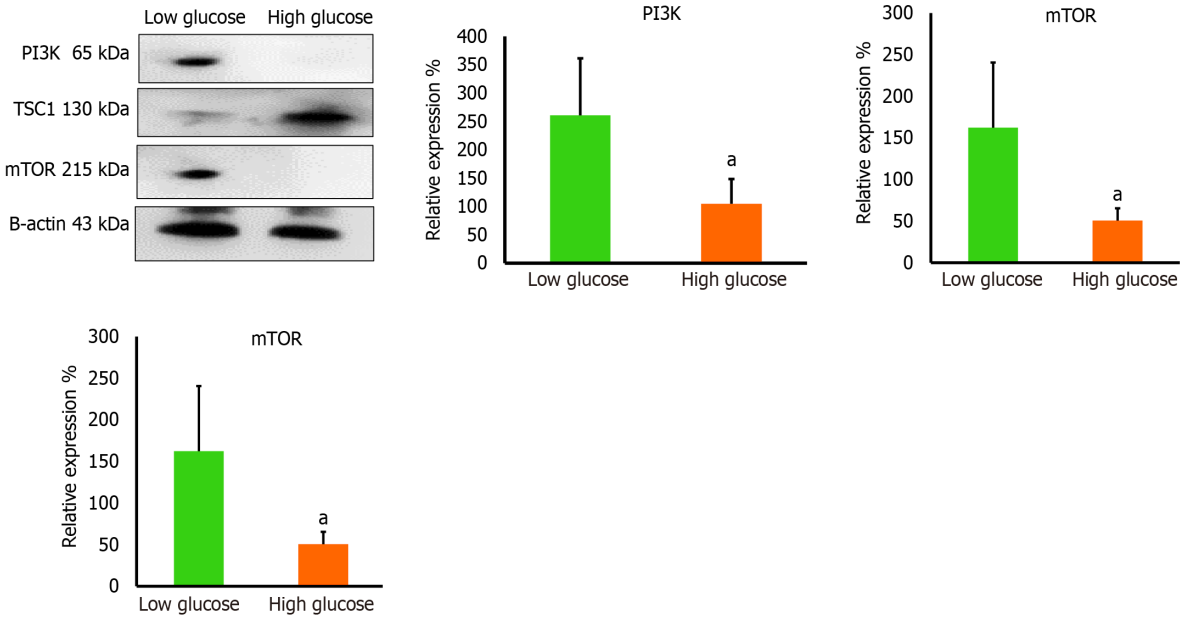
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Figure 4 The level of apoptosis was higher in human adipose tissue-derived mesenchymal stem cells cultured in high glucose. RealTime-Glo™ Annexin V Apoptosis live assay was used. The level of apoptosis was remarkably increased in human adipose tissue-derived mesenchymal stem cells cultured in high glucose for 7 d. Green fluorescence intensity is corresponding to the number of cells undergoing apoptosis. Fluorescent images were captured at GFP filter using Cytation 5 (BioTek, United States). n = 3, ^{a}P < 0.05 compared to low glucose control.

conditions (Figure 6). The disturbance in the level of these major complexes can impact the mitochondrial OxPhos and trigger the mitophagy of mitochondria[21].

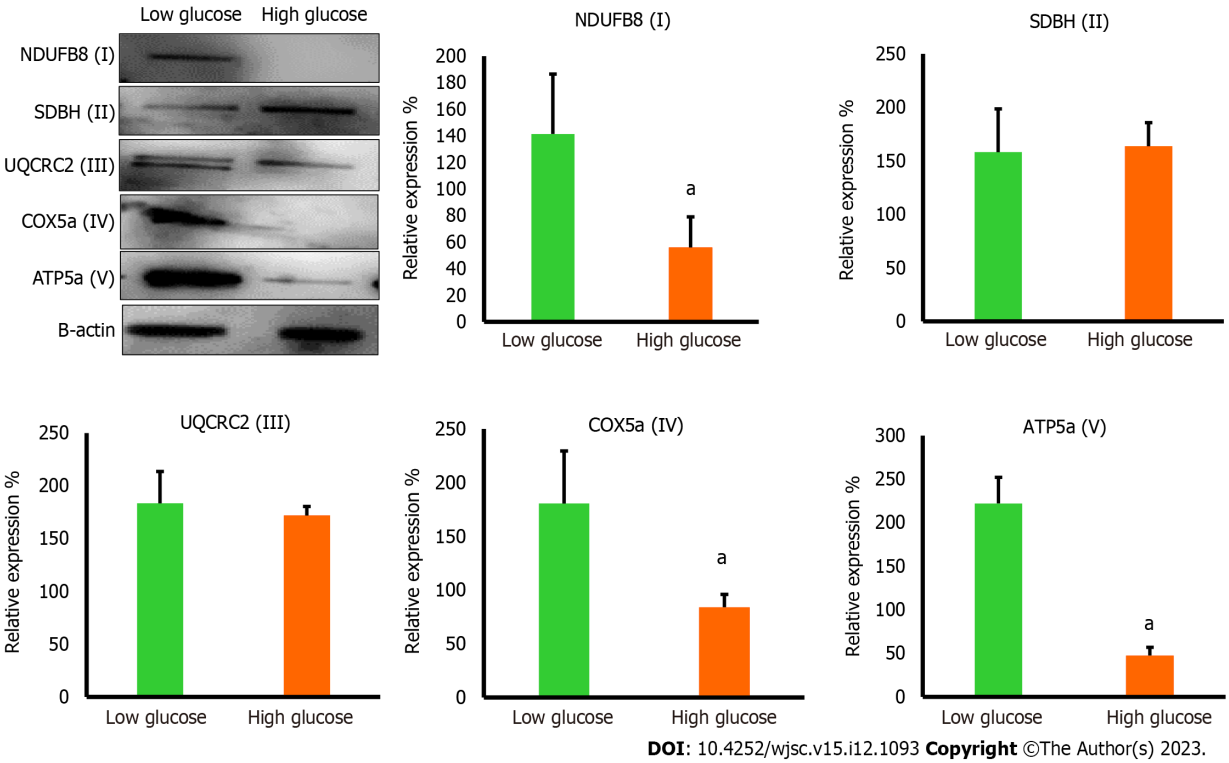
DISCUSSION

MSCs are the most promising type of stem cells for regenerating damaged tissues and restoring their normal functions which can improve the quality of life of many patients who suffer from debilitating disorders[1]. However, the remarkable improvements in pathological markers reported after transplanting MSCs[22,23] were hindered by the short-lived outcomes and poor survival rate, which need extensive investigation[24,25]. Many pathologies are characterized by stress microenvironments, including hypoxia, inflammation, and nutritional imbalances, which can impact the expected outcomes of transplanting MSCs[26-29], by either inhibiting or activating cellular signaling pathways. DM is a frequent chronic disease that leads to significant multi-system complications[8]. The major hallmark of DM is glucotoxicity, which is featured by the presence of high glucose levels and impaired insulin secretion and function[8]. This glucotoxicity produces massive molecular changes in the residing cells and cause the appearance of DM-related complications, such as cardiovascular and neurological complications[8]. Both animal and clinical studies have demonstrated the great therapeutic efficacy of MSCs transplantation in alleviating chronic hyperglycemia by reversing insulin resistance, improving insulin sensitivity, controlling inflammation, relieving metabolic syndrome symptoms, and ameliorating β-cell destructive abilities[10,30]. Nevertheless, studies also revealed that MSCs exhibited short-lived outcomes due to their poor survival after being transplanted in DM disease model[4]. Several studies have asserted the importance of investigating the mechanisms leading to the poor survival of MSCs in hyperglycemia which is the major insult to DM patients [24,31]. Up to date little is known about the molecular and biological changes in MSCs under hyperglycemia. MSCs are



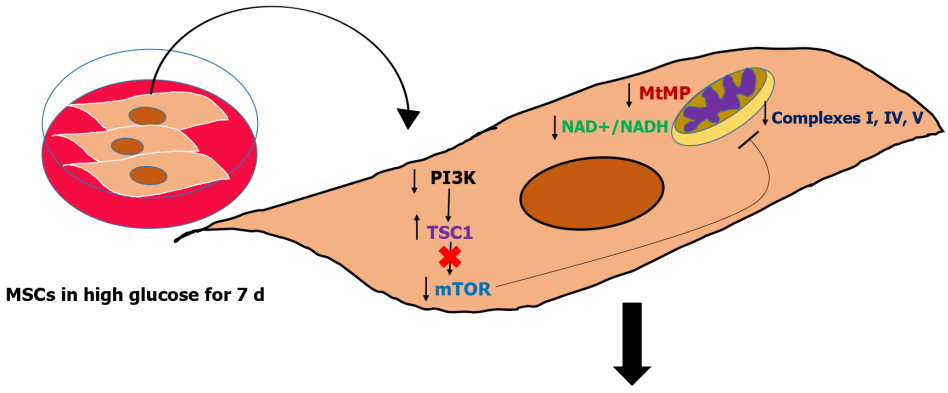
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Figure 5 The protein levels of mitochondrial regulators were reduced in high glucose cultured human adipose tissue-derived mesenchymal stem cells. Western blot protein analysis showed significant reduction in the protein levels of phosphatidylinositol 3-kinase and mammalian target of rapamycin (mTOR) in high glucose cultured cells, while the protein level of mTOR inhibitory protein; TSC1, was elevated in high glucose cultured cells. These proteins are important to maintain proper mitochondrial dynamics. $n = 3$, $^{a}P < 0.05$ compared to low glucose control. PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin.



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Figure 6 The protein levels of mitochondrial complexes were reduced in high glucose cultured human adipose tissue-derived mesenchymal stem cells. Western blot protein analysis showed significant reduction in the protein levels of NDUF6B (complex I), COX5a (complex IV), and ATP5a (complex V) in high glucose cultured human adipose tissue-derived mesenchymal stem cells comparing to low glucose cultured cells, while the protein levels of SDBH (complex II) and UQCRC2 (complex III) were not significantly changed in both culturing conditions. $n = 3$, $^{a}P < 0.05$ compared to low glucose control.



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Figure 7 Graphical summary of the study findings. High glucose impaired the mitochondrial function in mesenchymal stem cells (MSCs) by perturbing many mitochondrial regulatory dynamics and factors, particularly mitochondrial membrane potential, nicotinamide adenine dinucleotide (NAD+)/NADH pool, and mammalian target of rapamycin protein. This mitochondrial dysfunction is associated with triggering apoptosis in MSCs, which mechanistically explained the poor survival of MSCs in hyperglycemia. MSCs: Mesenchymal stem cells; PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin; NAD+: Nicotinamide adenine dinucleotide.

capable of switching their metabolism on to support their regenerative and immunomodulation abilities[32]. MSCs rely primarily on glycolysis when they are undifferentiated as it is important to preserve their stemness and proliferation[33]. Several reports illustrated that glycolysis is required to preserve the immunosuppressive characteristics of MSCs[34]. Upregulation in glycolysis has been found to increase the immunomodulatory abilities of MSCs and promote their capabilities to reprogram immune cells[35]. MSCs use mitochondrial OxPhos when they are induced to differentiate, thus the multi-lineage differentiation capacities of MSCs require intact mitochondria[6]. Maintaining intact mitochondrial dynamics is not only required to support the differentiation capacities of MSCs, but it is also crucial for redox regulation and prevention of excessive production of reactive oxygen species[36]. Recently, it has been proposed by many studies that MSCs are capable of transferring mitochondrial DNA to recipient cells and restoring the normal mitochondrial functions in these cells[37,38]. MSCs-mediated mitochondrial transfer further supports the necessity to conserve normal mitochondrial parameters[38]. Previous reports revealed that the MSCs proliferation decreased in high glucose medium [13,14]. Furthermore, other studies showed that MSCs differentiation capacities might vary when they are cultured in high glucose conditions[39,40]. Our study illustrated that high glucose can reduce the survival rate of MSCs. The reduction in MSCs viability reported in our study is correlated with an impairment in the inner mitochondrial membrane potential (ΔΨm) which is an important parameter and the energy generated from this potential gradient will be utilized by complex V to produce ATP molecules. The maintenance of mitochondrial membrane potential depends on the presence of a plentiful amount of NAD+ molecules[41]. NAD+ serves as an oxidoreductase cofactor that controls many vital energy production pathways. Maintaining normal NAD+/NADH ratio is integral to providing the required electron flux that will be used to generate proper mitochondrial membrane potential (ΔΨm)[41]. High glucose insult causing a substantial drop in NAD+/NADH ratio in hAD-MSCs indicates a needed disturbance in the oxidization power to produce NAD+ and donating electrons that will keep the functionality of mitochondrial complexes. Another important complex that is required to preserve the mitochondrial dynamics is mTOR complex[42]. mTOR is a known regulator of many cellular processes, including the biogenesis of mitochondrial complexes, most importantly complex I and V and physiological mitophagy[42]. The downregulation in the level of mTOR in MSCs cultured in high glucose was associated with significant disturbances in mitochondrial complexes I, IV, and V which can induce mitochondrial dysfunction, mitophagy and massive oxidative stress. The effect of high glucose on modulating mitochondrial functions by targeting mTOR is barely investigated and with controversial results[43]. While other studies found that hyperglycemia activate mTOR pathway in cardiomyocytes[44,45], other evidence showed that insulin can activate mTOR pathway in DM patients and protect cells from developing mitochondrial dysfunction[46], indicating that high glucose can deactivate mTOR and cause pathological consequences[46]. These discrepancies regarding the effect of high glucose on mTOR level and activity seem to differ depending on the type of cells and the upstream molecular regulators. Based on that, the role of high glucose in modulating the level of mTOR needs further investigation in different cell types. In our study, the upstream regulatory protein PI3K decreased remarkably in hAD-MSCs exposed to high glucose. A plentiful number of reports corroborated that PI3K-AKT pathway is required to activate mTOR by removing the inhibitory effect produced by TSC1 or Hamartin protein. We reported in this study that the reduction in PI3K protein level was coupled with marked elevation in TSC1 and downregulation in mTOR in hAD-MSCs cultured in high glucose. Our findings provide a mechanistic explanation of poor survival of MSCs in hyperglycemic microenvironment and suggests new targets that can be modified to enhance the survival rate of MSCs in DM patients. Moreover, our results highlighted the importance of maintaining the desirable pre-transplantation culturing conditions that preserves the salutary properties of MSCs by controlling the level of glucose in the culture media. Schematic summary of the study findings was illustrated in (Figure 7) which highlighted that high glucose microenvironment reduced the level of PI3K and removed its inhibitory effect on TSC1 and caused its upregulation. The increase in the level of TSC1 caused a reduction in mTOR which

disturbed many mitochondrial dynamics, mainly MtMB, NAD+/NADH ratio, and mitochondrial complexes that triggered the apoptosis of hAD-MSCs.

CONCLUSION

MSCs have the potential to regenerate damaged tissues and restore their functions. DM patients can hugely benefit from MSCs transplantation to abrogate the pathological consequences and improve their quality of life. The poor survival and short-lived positive outcomes following MSCs transplantation in DM patients are considered stumbling blocks. High glucose impaired mitochondrial function in MSCs by perturbing mitochondrial regulatory factors, particularly mitochondrial membrane potential, NAD+/NADH pool, and mTOR protein. This mitochondrial dysfunction is associated with triggering apoptosis in MSCs. Preserving these factors may help in improving the survival rate of MSCs in diabetic microenvironment and initiate long-lasting therapeutic outcomes. Future studies should focus on providing new strategies to overcome the poor survival of MSCs in high glucose using genetic modification or biomaterials and nanoparticles. Moreover, the impact of other stressors existed in DM patients other than hyperglycemia should be studied.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have the ability to cure many chronic diseases, including diabetes mellitus (DM) and its related multisystem complications.

Research motivation

The therapeutic outcomes of MSCs transplantation in DM are short-lived which require thorough investigation.

Research objectives

This study aimed to determine the effects of hyperglycemia microenvironment on various mitochondrial-related parameters in MSCs to better understand their fate in DM patients.

Research methods

Adipose tissue-derived MSCs were exposed to low and high glucose media and mitochondrial dynamics and regulators were measured and analyzed.

Research results

High glucose induces the apoptosis of adipose tissue-derived MSCs by disturbing many mitochondrial parameters, including mitochondrial membrane potential, nicotinamide adenine dinucleotide (NAD+)/NADH pool, mammalian target of rapamycin, and mitochondrial complexes I, IV, and V.

Research conclusions

Hyperglycemia decreases the survival of MSCs by triggering mitochondrial dysfunction in these cells causing their short-lived therapeutic outcomes.

Research perspectives

New strategies to improve the survival rate of MSCs in hyperglycemia should be the focus of future studies which can help in increasing the chances of using these cells clinically for the treatment of DM.

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FOOTNOTES

Author contributions: Abu-El-Rub E conceptualized the study; Abu-El-Rub E, Khasawneh RR, Almazari R, Sanajleh A, Magableh H, Shlool H, Mazari M, Bader NS, and Al-Momani J carried out the experiments; Abu-El-Rub E and Almahasneh F analyzed the data; Abu-El-Rub E, Almahasneh F, Alzu'bi A, and Ghorab D drafted and wrote the manuscript; Abu-El-Rub E and Almahasneh F revised and formatted the content of the manuscript and verified spelling, punctuation and grammatical errors; and all authors have read and

approve the final manuscript.

Institutional review board statement: The ethical approval was not needed in this study. The human cell lines used for this study were commercially purchased.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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