ISSN 2220-3184 (online)

World Journal of *Medical Genetics*

World J Med Genet 2016 February 27; 6(1): 1-16

Published by Baishideng Publishing Group Inc

WJMG

World Journal of Medical Genetics

Contents

Quarterly Volume 6 Number 1 February 27, 2016

REVIEW

1 Gene regulatory networks in atrial fibrillation

Franco D, Lozano-Velasco E, Aranega A

World Journal of Medical Genetics
Volume 6 Number 1 February 27, 2016

Contents

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World Journal of Medical Genetics is currently no indexing/abstracting.

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

FREQUENCY
Quarterly

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PUBLICATION DATE
February 27, 2016

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World J Med Genet 2016 February 27; 6(1): 1-16
ISSN 2220-3184 (online)
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REVIEW

Gene regulatory networks in atrial fibrillation

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Author contributions: Franco D wrote the manuscript; Lozano-Velasco E and Aranega E critically read the manuscript.

Supported by A CNIC translational grant (CNIC2006/08) to Franco D; and the University of Jaén on translational biomedicine to Franco D (UJA2013/01).

Conflict-of-interest statement: The authors declare that they have not conflict of interest.

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Received: September 29, 2015
Peer-review started: October 21, 2015
First decision: November 27, 2015
Revised: January 14, 2016
Accepted: February 16, 2016
Article in press: February 18, 2016
Published online: February 27, 2016

Abstract

Atrial fibrillation (AF) is the most frequent arrhythmogenic syndrome in humans. With an estimate incidence of 1%-2% in the general population, AF raises up to almost 10%-12% in 80+ years. Thus, AF represents nowadays a highly prevalent medical problem generating a large economic burden. At the electrophysiological level, distinct mechanisms have been elucidated. Yet, despite its prevalence, the genetic and molecular culprits of this pandemic cardiac electrophysiological abnormality have remained largely obscure. Molecular genetics of AF familiar cases have demonstrated that single nucleotide mutations in distinct genes encoding for ion channels underlie the onset of AF, albeit such alterations only explain a minor subset of patients with AF. In recent years, analyses by means of genome-wide association studies have unraveled a more complex picture of the etiology of AF, pointing out to distinct cardiac-enriched transcription factors, as well as to other regulatory genes. Furthermore a new layer of regulatory mechanisms have emerged, *i.e.*, post-transcriptional regulation mediated by non-coding RNA, which have been demonstrated to exert pivotal roles in cardiac electrophysiology. In this manuscript, we aim to provide a comprehensive review of the genetic regulatory networks that if impaired exert electrophysiological abnormalities that contribute to the onset, and subsequently, on self-perpetuation of AF.

Key words: Atrial fibrillation; Genetics; MicroRNAs; Genome-wide association studies; PITX2

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Core tip: Atrial fibrillation (AF) is the most prevalent arrhythmogenic defect in the human population. Genetic factors such as mutations in distinct ion channel encoding genes have been described, yet representing less than 10% of all AF cases. Genome wide association studies have widened the genetic culprits contributing to AF. We provide herein a state-of-the art review on the genetic components underlying AF. Experimental evidences demonstrated that PITX2 plays a pivotal role regulating cellular, molecular and electrophysiological characteristics of the developing and adult heart that, if impaired, predispose to AF, leading to complex regulatory networks with transcriptional and post-transcriptional (microRNA)

regulatory mechanisms.

Franco D, Lozano-Velasco E, Aranega A. Gene regulatory networks in atrial fibrillation. *World J Med Genet* 2016; 6(1): 1-16 Available from: URL: http://www.wjgnet.com/2220-3184/full/v6/i1/1.htm DOI: http://dx.doi.org/10.5496/wjmg.v6.i1.1

BACKGROUND

Atrial fibrillation (AF) is the most frequent arrhythmogenic syndrome in humans. With an estimate incidence of 1%-2% in the general population, AF raises up to almost 10%-12% in 80+ years[1-5]. Cardiovascular risk factors such diabetes, obesity, hypertension and hyperthyroidism promote AF[6-9]. In addition, the occurrence of AF can be also triggered by preceding cardiovascular diseases such as hypertrophic cardiomyopathy and valvular heart diseases[10,11] as well as it can be boosted by cardiac surgery, obstructive sleep apnea and inflammatory processes[12-15]. Importantly, besides the global risk factors for AF, it is well-established that the debut of an AF episode triggers subsequent and more severe AF episodes, leading to electrical and structural remodeling of the diseased heart, a condition quoted as "AF begets AF"[16,17]. Thus AF *per se* is a progressive disease. Electrical remodeling involves progressive changes in the cardiac electrical properties, possibly triggered by oxidative stress imbalance, leading to EADs, DADs and/or changes in the ADP configuration[18,19], culminating thus in rotor formation[20] (Figure 1). Structural remodeling involves atrial dilation, fibrosis and/or inflammation[21,22] which then indirectly promotes the generation of rotors and thus electrical re-entry circuitries[20] (Figure 1). Moreover, suffering from AF predisposes to additional health problems, ranging from bleeding and stroke[23-25], cognitive decline and dementia[26,27], ventricular dysfunction[28,29] and even sudden death[30] (Figure 1). Thus, these data support the notion that AF is an epidemic disease with large socio-economic burden worldwide.

While large epidemiological data support the previously mentioned predictive risk factors for AF, there are also unquestionable evidences that in a subset of AF patients no concurrent previous risk factors are diagnosed and thus AF occurs in an isolated manner, a condition dubbed lone AF[31-34]. These observations, in addition to seminal observations supporting that AF can also be familial[35], strengthen the notion of a genetic component on the onset of AF. In this context, two waves of genetic information have been gained over the last decades. Seminal studies on familiar AF cases followed by genetic linkage analyses, and subsequently by genetic screening of candidate genes identified a large number of point mutations in distinct genes encoding for proteins involved in cardiac electrophysiology[36-38]. These data, along with meticulous electrophysiological mapping analyses and *in silico* data modeling, provided an important cornerstone to understand AF pathophysiology[39-43]. However, genetic identification of candidate AF genes can only explained around 10%-15% of all AF patients. Thus, new approaches to untangle the genetic bases of AF were envisioned. Genome-wide association analyses (GWAS) lightened the discovery of new genes associated to AF. Seminal worked by Gubdjartsson *et al*[44] firstly identify common risk variants highly associated to the onset of lone AF in distinct large European and Chinese patient cohorts. Subsequent GWAS studies and meta-GWAS have further identified new candidate genes for AF pathophysiology[45-49], yet the functional link between these variants and the AF pathophysiology is a rather unexplored. A tortuous route to dissect their functional roles is envisioned, given that most of these risk variants are located rather apart from gene coding sequences. Thus, a first proposal based on these findings is that risk variants might affect gene regulatory networks within the vicinity and thus underscore AF onset. In addition, a novel layer of complexity in gene regulatory networks has emerged with the discovery of non-coding RNAs that can mediate post-transcriptional regulation. In this review, we aim to elaborate on the current state-of-the-art of the gene regulatory networks involved in AF pathophysiology.

CONTRIBUTION OF THE IMPAIRED CARDIAC ACTION POTENTIAL TO THE AF PATHOPHYSIOLOGY

The cardiac action potential is driven by inward and outward flow of distinct ion currents along the cardiomyocyte membrane. In atrial and ventricular cardiomyocytes, the configuration of the action potential is initiated by the upstroke depolarization of the sodium current, followed by a repolarization mediated by distinct potassium currents until the resting membrane potential is restored. A conduction-contraction coupling is modulated within each action potential by a complex regulatory network of calcium handling[50]. Interestingly, nodal conductive cells display a distinct upstroke configuration which is modulated by cation selective currents, yet repolarization is rather similar to working cardiomyocytes. A large number of mutations in the *SCN5A* gene, coding for the pore-forming subunits of the I_{Na} current, have been associated to AF, alone[51-56] or in combination to other concurrent cardiac pathologies[57-62]. Similarly, mutations in *SCN1B*, *SCN2B*, *SCN3B* and *SCN4B* ancillary subunits, respectively, have also been linked to AF[63-67]. At present, the functional interpretation of these findings suggest that impaired I_{Na} current can promote AF onset, yet electrophysiological evidences are only available for a subset of these mutations. Importantly, mutations in *SCN5A* are also linked to other cardiac arrhythmias such as long QT and Brugada[68-70] demonstrating a pivotal role for *SNC5A* on cardiac electrophysiology and illustrating that distinct phenotype might be acquired depending on subtles differences on the mutation location as well as on plausible modulatory accompanying proteins. In addition, mutations in the *HCN4* cation channel, responsible for the cardiac

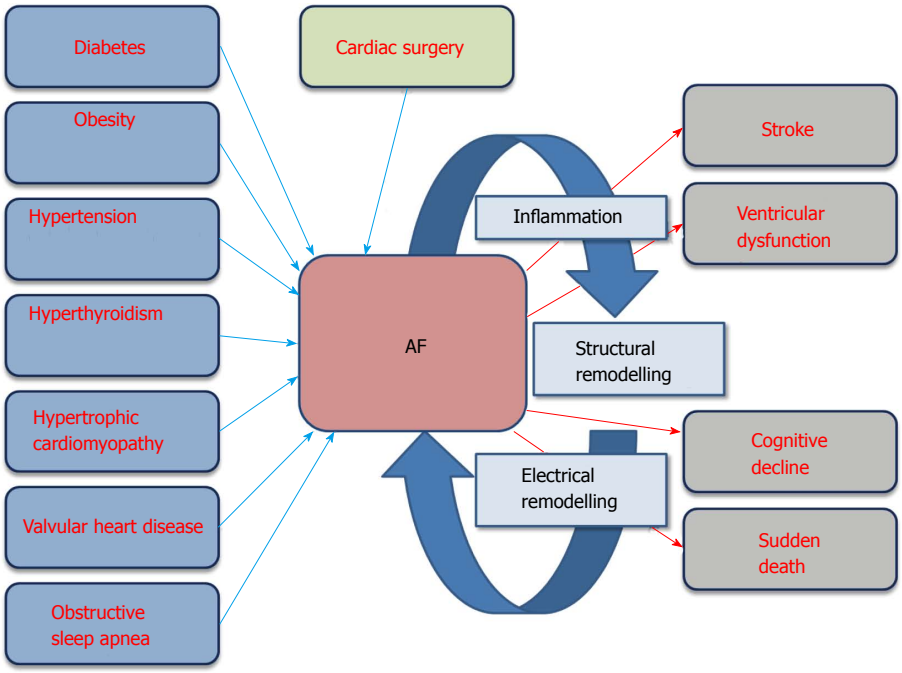

Figure 1 Schematic representation of the clinically relevant risk factors influencing the onset of atrial fibrillation. Atrial fibrillation (AF) leads thereafter to distinct cellular and molecular remodeling events, including structural and electrical remodeling, in part as caused by inflammatory process. In addition, progression of AF is highly associated with the onset of additional cardiac pathophysiologies.

action potential upstroke of conductive cells, have also been reported in AF[71].

Mutations in genes encoding for proteins controlling the repolarization phase of the cardiac action potential have also been associated to AF. In particular, *KCNQ1*[36,72-79], *KCNA5*[80] and *KCNE2*[38,81] mutations have been identified. In this setting, point mutations seem to shorten the repolarization phase providing an electrophysiological substrate for AF onset. Similarly, alteration of the resting membrane potential have been related to trigger AF onset and a large number of mutations in the gene coding for *KCNJ2* have been reported[37,38,82,83]. Similarly as for the sodium channels, mutation in potassium channels have been associated to AF and concomitantly with other arrhythmogenic syndromes such as long[72] and short[84] QT, respectively.

Multiple lines of evidences have demonstrated the essential role of impaired calcium homeostasis as a triggering factor of AF[2,85-90]. In this context, mutations in *RYR2* are associated to AF[91] and in conjunction with other cardiac pathophysiological conditions such as catecholaminergic polymorphic ventricular tachycardia[92,93]. Curiously, *RYR2* mutations can also affect sodium channel expression[94]. Importantly, murine model of *RYR2* mutation leads to AF, reinforcing the genetic evidences of impaired calcium homeostasis as a trigger of AF[95].

In addition, mutations in gap junction proteins such as *GJA1* (connexin43)[96,97] and *GJA5* (connexin40)[98-104], which are critical for the coordinated transmission of the electrical impulse among cardiomyocytes, have also been identified in association to AF. Seminal work described that mutation in connexin proteins were occurring in somatic cells (*i.e.*, cardiomyocytes)[98], whereas more recently germline mutations have also been identified[98-104] and the concept of somatic cell mutation challenged[105]. In this setting, electrophysiological analyses of connexin mutations in the AF context demonstrated that cardiomyocyte electrical transmission is impaired[106-111] and thus can result in cardiac re-entry circuitries and then on AF.

Furthermore, mutations in several other genes, besides those directly contributing to the electrophysiological properties of the heart, have also been identified in the AF setting. In particular, mutations in the transcription factors *TBX5*[112], *NKX2.5*[113-117] and *NKX2.6*[118] as well as in the nuclear pore component *NUP155*[119] and the atrial natriuretic factor (*NNPA*[120]). A summary of gene mutations associated to AF is provided on Figure 2. It is important to highlight in this context that mutation in *NUP155* was found in patients with AF and early sudden death[118], while TBX5 and NKX2.5 are developmental transcription factors that play determinant roles during cardiogenesis and mutations in these genes have also been reported in distinct congenital heart diseases as well as GWAS candidate genes in other cardiac electrophysiological defects[121-128].

NOVEL GENES LINKED TO AF

As previously said, classical genetic approaches provided an entry site to discover discrete genes involved in AF pathophysiology, but felt short to explain most of the diagnosed AF cases. Genome-wide association studies (GWAS) introduced a revolutionary genetic approach to understand AF, and cardiac arrhythmias at large[129,130]. Seminal work by Gudbjartsson *et al*[44] identified risk variant at 4q25 highly associated to lone AF. Soon thereafter these observations were corroborated in distinct studies worldwide[131-136], yet with some controversial findings[137,138]. Surprisingly, 4q25 risk variants are located in a gene desert, being the closest annotated gene a homeobox transcription factor, PITX2, around 150 kb downstream. Soon thereafter, experimental observations provided

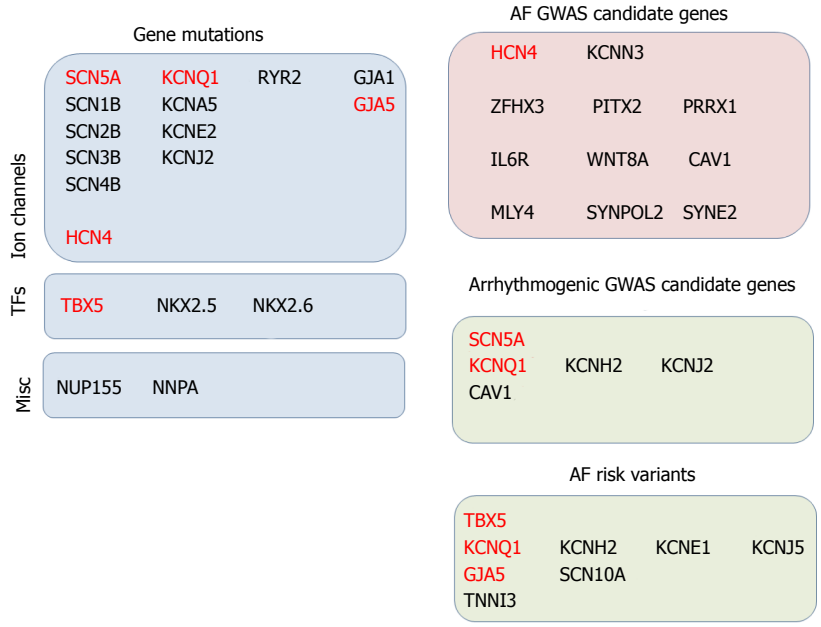

Figure 2 Schematic representation genes associated to atrial fibrillation. On the one hand, gene mutations associated to atrial fibrillation (AF) are illustrated. On the other hand, genes associated to AF by means of GWAS are also illustrated. In the latter, three subset of genes are provided: (1) those directly linked to AF GWAS analyses; (2) those related to AF, but identified in arrhythomogenic syndromes GWAS analyses; and (3) risk variants association studies in small AF cohorts by a candidate approach strategy. Genes highlighted in bold represent those genes identified in AF by multiple approaches. GWAS: Genome-wide association analyses.

evidences that impaired *Pitx2* function in animal models triggered increase atrial arrhythmias susceptibility[139,140]. However, how risk variants can influence PITX2 expression and/or function have only been partially revealed[141], since regulatory elements containing the risk variants can molecularly interact with *PITX2* but also *ENPEP* promoter elements in a tissue- and developmental-specific manner[141]. Importantly, controversial findings in humans have been reported. Chinchilla *et al*[136] firstly described decreased *PITX2* expression in AF patients, yet these claims were challenged since no correlation between risk variants and *PITX2C* expression was observed[142]. However, more recently a correlation with *PITX2A* expression has been reported, limiting their functional significance since data were obtained from right atrial biopsies[143]. Thus, further investigations are required to clarify these controversial findings.

Besides *PITX2*, additional GWAS have enlightened the putative role of other novel genes in AF pathophysiology, such as *KCNN3*[47], *ZFHX3*[45,48], *IL6R*[49] and *MLY4*[144]. In most cases, these findings have been corroborated in independent studies with distinct ethnicity cohorts as for *ZFHX3*[145,146], and *IL6R*[147], but to date no additional reports are available for *KCNN3* and *MYL4*.

While arguments for the plausible involvement in AF have been provided in all cases, experimental evidences remain largely missing. *KCNN3* loss-of-function mutant mice display no overt cardiac electrophysiological defects, yet overexpression leads to sudden death[148]. Similarly *ZFHX3* function role in the heart is scare, yet recent findings suggests that atrial arrhythmias[149] and pacing[150] leads to significant impaired *ZFHX3* expression. Importantly, risk variants associated to *KCNN3*, *ZFHX3* and *IL6R* lie within intronic regions, respectively, and thus have no predictive functional consequences. Therefore, as in the cases of 4q25 risk variants (*PITX2*), a functional role on plausible regulatory elements is hypothesized, but functional evidences are yet missing.

In addition to these GWAS studies, at least two meta-GWAS studies have digged for additional risk variants associated to AF[46,151]. In this context, six new loci have been uncovered using such approach; *CAV1, HCN4, SYNE2, SYNPOL2, PRRX1* and *WTN8A*. Replication analyses of the risk variants have been confirmed for *SYNE2*[152] and *PRRX1*[149], are controversial for *CAV1*[53,153,154] or have not been reported to date for *HCN4* and *SYNPOL2*. Importantly, a 12-SNPs risk score have been developed that can individually identify the risk of AF and stroke[155].

While this approach broadens the plausible genetic bases of AF, it is also true that their contribution to explain AF pathophysiology would be directed linked to the statistical significance associated with those findings. It is important to say in this context that risk variants associated to *PITX2* display highest significance values, *KCNN3*, *ZFHX3* and *IL6R* are moderately significant and those revealed by meta-GWAS display the lowest, yet statistical significant, values. In a simplistic way, this could be transferred as PITX2 being contributing to larger spectrum of AF pathophysiology and thus being in the upper level of a hierarchical signaling pathway while CAV1, HCN4, SYNE2, SYNPOL2, PRRX1 and WTN8A will be rather discretely contributing to AF pathophysiology in a rather discrete, small subset of AF patients, and therefore being at the bottom of such hierarchy. We have recently provided experimental evidences supporting this notion, as detailed below.

GWAS studies have also been reported in other electrophysiological parameters such as PR interval[129,156-158], heart rate[157], QRS interval[157] as well as physiopathological conditions such as Brugada syndrome[159] and long QT[160-162]. Several of these cardiac arrhythmogenic conditions are sometimes directly associated to AF, such as for example impaired PR interval or Brugada syndrome. Importantly,

such GWAS studies have identified multiple genes, some of which are shared with AF, such as *CAV1* (PR interval[129,156-158]) while other have been previously involved in AF pahthophysiology, such as *KCNQ1, KCNH2, SCN5A, KCNJ2* (long QT GWAS analyses[160-162]). In addition to GWAS studies, risk variants have been associated to AF using more discrete/small AF patient cohorts. Among them it is important to highlight risk variants at the following loci; *KCNQ1*[163-165], *KCNH2*[163], *KCNE1*[163], *KCNJ5*[166], *GJA5*[167] and *TBX5*[168]. In addition to these GWAS studies, several independent AF cohort studies have reported risk variants associated to AF in other loci, such as *TNNI3*[169] and *SCN10A*[170,171], yet the functional relevance of these findings remains unsolved. These data suggest that complex and intricate gene networks are likely to be operative between these regulatory genes resulting in AF (Figure 3) as well as other electrophysiological associated disorders.

A NOVEL AND EMERGING LAYER OF GENE REGULATORY CONTROL

Gene regulatory networks have been classically associated as hierarchical interactions between master genes, such as transcription factors, and structural genes, such as actin and myosin genes forming the sarcomere or genes encoding ion channels configuring therefore the cardiac action potential. In this setting, transcriptional regulation has been considered the master regulatory point, deciding whether to go or not to go, whereas translation and post-translational modifications are considered as minor regulatory nodes. Over the last decade, we have witnessed the discovery that large part of the so called "rubbish DNA" in fact codes for a large variety of non-coding RNAs species with highly relevant functional properties. In broad sense, non-coding RNAs can be sub-classified into long non-coding (lncRNAs) and small non-coding RNAs, exerting a distinct and variable functional roles[172]. Among small non-coding RNAs, microRNAs have emerged as the larger group, playing a pivotal role on post-transcriptional regulation of mRNA transcript expression[173]. MicroRNAs are 22-24 nt non-coding RNAs which can block translation or trigger transcript degradation by binding to complementary seed sequence in the 3'untranslated region (3'UTR) of nascent mRNA transcripts[173]. At present the hallmark of microRNA transcriptomics has been uncovered in the developing and adult heart in both normal and pathological conditions[174,175]. In the context of AF the microRNA fingerprint has been profiled in different settings[176-178], providing highly valuable biomarkers[179-183] as well as opening new avenues to dissect the functional role of these differentially expressed microRNAs in AF pathology. In addition distinct sets of microRNAs have also been reported to selectively modulate expression of distinct gene encoding for ion channels, opening thus new pathways to dissect their genetic contribution to AF.

AN INTEGRATIVE GENE REGULATORY NETWORK LINKED TO AF

As stated above, evidence on the functional role of mutiple genes have been provided by the identification and experimental testing of AF patient gene mutations. In this setting, a causative link can, in most cases, be established. However, GWAS analyses provide circumstantial evidences whereas functional linkage between the risk variants and coding genes, whether protective or deleterious, is not directly established. To date, an example of successful linkage have been reported[184] in the cardiovascular context, however in most cases, this is not yet established. Therefore, dissecting the functional role of GWAS candidate genes represents the first step for the analyses of their causative link.

Large set of evidences suggest that multiple genetic pathways can contribute to the onset of AF. Over the last years we have carefully dissected the role of the homeobox transcription factor PITX2 in this context and we have made efforts to dissect the regulatory interaction of *PITX2* with those genes previously reported to play a role in AF pathophysiology. Several studies have demonstrated that *PITX2* can influence expression of distinct ion channels in the developing and adult hearts. Wang *et al*[138] firstly demonstrate that *PITX2* haploinsufficiency is sufficient to increase AF susceptibility. These authors demonstrated that *PITX2* haploinsufficiency led to developmental impaired expression of key transcription factors (*SHOX2, TBX3*) directing sinoatrial morphogenesis and thus impacting on the normal onset of the cardiac pacemaker structures. However, in this context, no basal AF occurs and no ECG defects were observed. In this line of thinking soon thereafter Kirchoff *et al*[139] demonstrate similar findings and furthermore reported that expression of multiple ion channels were impaired as well as cell-cell interacting proteins (Figure 3). However, none of these genes were reported to play a pivotal role on cardiac action potential and thus, molecular and electrophysiological links were still on its infancy. Using a distinct experimental approach, *i.e.*, an atrial-specific *PITX2* conditional transgenic mouse line, Chinchilla *et al*[135] revealed that ECG defects were observed at rest (no triggering needed), electrophysiological measurements were impaired, *i.e.*, increased resting membrane potential and prolonged action potential duration, and furthermore using a candidate approach analyses, distinct ion channels were also impaired, particularly *SCN5A* (I_{Na}), *KCNJ2* (I_{K1}) and *KCNJ12* (I_{K1}). Thus, these data demonstrate for the first time that functional impairment of PITX2 leads to molecular defects which provoke electrophysiological alterations and thus increase rate of atrial arrhythmias. In line with these findings, Tao *et al*[185] using a *PITX2* deletion conditional approach in the adult heart also demonstrated that basal ECG defects were observed, similar to those already reported by Chinchilla *et al*[135], and moreover they

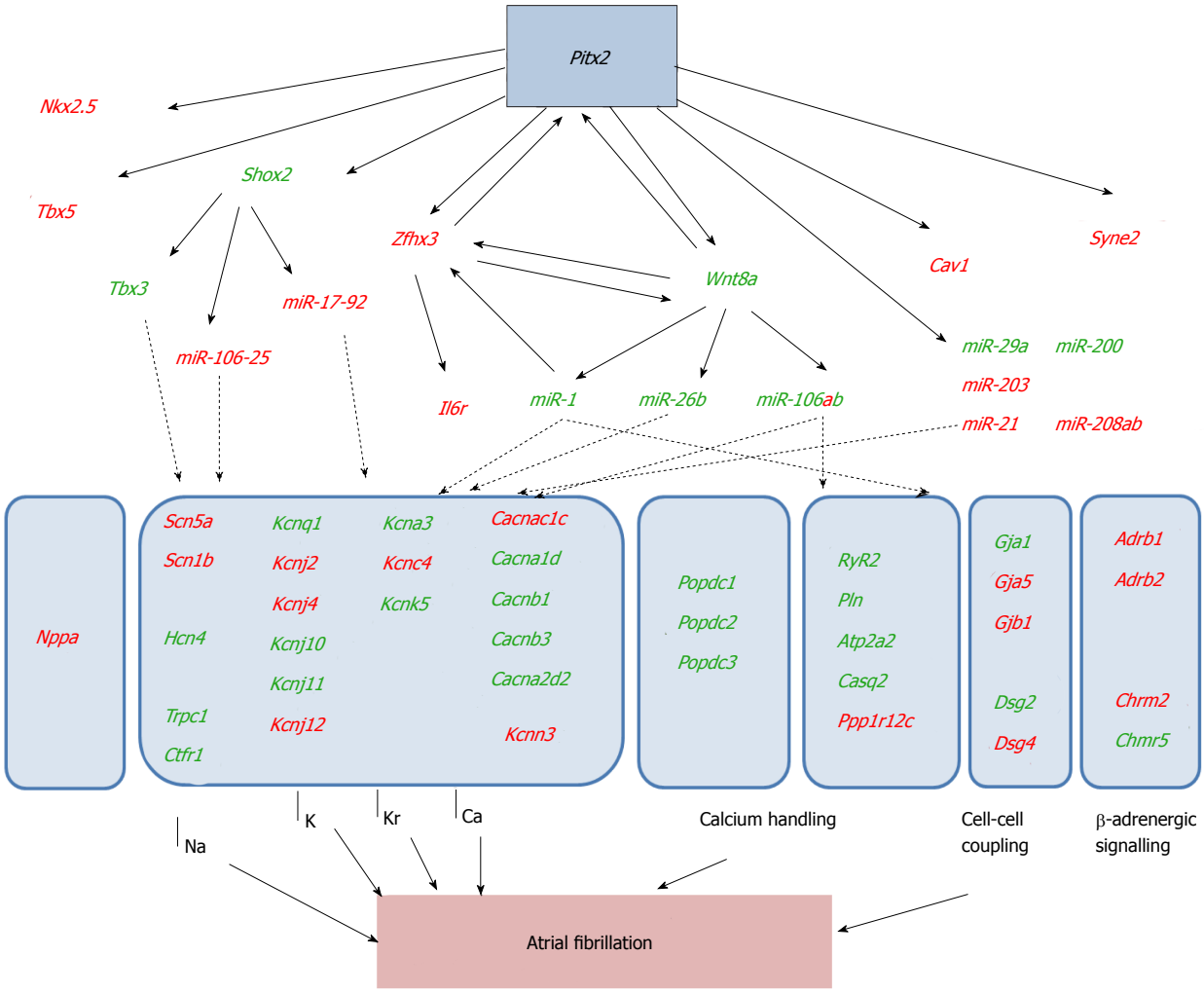

Figure 3 Schematic representation of the Pitx2 downstream pathways involved in pro-arrhythogenic events leading to atrial fibrillation. Multiple lines of evidences demonstrate that PITX2 exerts a pivotal role regulating expression of distinct ion channels, cell-cell coupling, blood pressure controlers and beta-adrenergic stimulation. Most of these pathways are modulated by microRNAs which are under control of PITX2 action. Red denotes down-regulation and green upregulation.

demonstrated that key determinants of the cardiac action potential were impaired, such as *KCNQ1* (I_{Ks}), *KCNH2* (I_{Kr}), *CACNA1C* (I_{Ca}), among others. Overall these data demonstrated that *Pitx2* insufficiency can both promote and trigger molecular and electrophysiological alterations, namely affecting the depolarization, repolarization and resting membrane potential characteristics of the cardiac action potential. They also illustrate that alteration of a single transcription factor can influence the functional role of multiple ion channels previously reported to be associated to AF. Therefore it can be inferred that PITX2 is upstream of this hierarchical signaling pathway and its impairment would explain a large fraction of AF patients.

In addition to *PITX2*, other GWAS studies have provided evidence on the plausible role of distinct gene in AF pathophysiology, yet as previously said, their functional implication has been poorly documented. We investigated whether these genes would be under control of PITX2. Using a series of gain and loss-of-function approaches, Lozano-Velasco *et al*[149] demonstrated that *PITX2* can modulate expression of *ZFHX3*, *KCNN3* and *IL6R*. The role of ZFHX3 in atrial arrhythmias is basically unexplored. However, given its transcriptional capacity if might be plausible that it can modulate multiple genes previously related to AF. The role of KCNN3 in atrial arrhythmias is under debate, its plausible contribution to the configuration of the cardiac action potential, if any, seems to be residual. On the other hand, the involvement of IL6R could be related to inflammatory processes that, if impaired, are associated to AF as previously mentioned. We have noticed that *IL6R* expression in the atrial chambers is impaired in *PITX2* deficient mice yet plasma levels of soluble IL6 and TFNα ligands are not altered. While these data support a tissue-specific involvement of IL6R, its links to AF remain to be elucidated.

We also investigated if PITX2 can influence the role of those genes related to AF by meta-GWAS analyses. Among them, previous evidences support that PITX2 can modulate expression of *HCN4*[138]. More recently, Lozano-Velasco *et al*[151] demonstrated that also *CAV1, SYNE2* and *WNT8A* are modulated by PITX2. It is important nonetheless to highlight that risk variants associated

to *CAV1* and *SYNE2* have also been reported in other cardiac electrophysiological disorders yet their functional role remains poorly characterized. Importantly, WNT signaling has been largely documented to play pivotal role during cardiac development and homeostasis[186,187], however WNT8 has not been reported to play fundamental roles therein[188].

Given the potential signaling properties of WNT8A and those transcriptional capacity exerted by ZFHX3 we explored in any of those pathways might be involved on the regulation of distinct ion channels previously reported to be modulated by PITX2 and related to AF. Gain- and loss-of-function approaches demonstrated that *WNT8A* but not *ZFHX3* can modulate expression of calcium handling proteins[151]. Importantly, impaired expression of *WNT8A* is observed in *PITX2* insufficiency models with basal ECG alterations but not in those with normal ECG recordings[151], supporting the notion that WNT signaling plays a fundamental role conferring susceptibility *vs* triggering capacity to the atrial chambers during AF onset.

In addition to gene regulatory mechanisms driven by *PITX2*, several lines of evidence support that *PITX2*-regulated microRNAs also contribute to AF pathophysiology. Chinchilla *et al*[135] already demonstrated that impaired miR-1 expression in *PITX2* deficient mice underlie abnormal resting membrane potential configuration (by modulating *KCNJ2* and *KCNJ12* post-transcriptional regulation). Huang *et al*[153] has confirmed that *PITX2* inhibits miR-1 expression, which negatively regulates *ZFHX3*. Wang *et al*[189] demonstrated that two distinct genomic microRNA clusters, namely *miR-17-92* and *miR-106b-25*, as well as *miR-335* and *miR-423*, are under control of *PITX2*. Genetic deletion of *miR-17-92* and *miR-106b-25* microRNA clusters, respectively display normal baseline electrophysiological parameters but were susceptible to pacing-induced AF. Cardiac-specific deletion (Nkx2.5Cre) of *miR-17-92* cluster resulted in prolonged PR interval. Compound double mutant mice using conditional Nkx2.5Cre mediated deletion resulted in sinus node dysfunction, a condition reminiscent of sick sinus syndrome in humans. Molecular analyses revealed that *SHOX2* and *TBX3* expression were impaired. Furthermore, RYR2-mediated calcium-leak was reported in *miR-106b-25* deficient mice[190] since several members of this cluster, namely miR-106b, miR-93 and miR-25 directly target the 3´UTR of Ryr2. More recently, Lozano-Velasco *et al*[149] demonstrate that a large number of microRNAs, previously reported to be associated to AF in patients, are also modulated by *PITX2*. In particular, *miR-1, miR-26b, miR-29a, miR-30e, miR-106b, miR-133* and *miR-200* were up-regulated in absence of *PITX2* whereas *miR-21, miR-106a, miR-203, miR-208a* and *miR-208b* were down-regulated. Several of these microRNAs, such as *miR-1, miR-133, miR-21, miR-106b* and *miR-26* have been previously reported to regulate calcium (*CACNA1C*[191]; *RYR2*[190]), sodium (*SCN5A*[192]), potassium (*KCNJ2*[193], *KCNE1*[194], *KCNB2*[194]), cation (*HCN4*[195]) channel subunits, respectively. Overall, these data demonstrate a highly complex gene regulatory networks leading to AF as summarized in Figure 3.

CONCLUSION

In this review we have highlighted the complexity of risk factors influencing the onset of AF, both clinically and genetically. Several lines of evidences demonstrated that *PITX2* exerts a pivotal role on the genetic determinants of AF[136,139,140,149,185]. While it has been robustly demonstrated that *PITX2* insufficiency predisposes to atrial arrhythmia genesis in experimental mouse models[136,139,140,149,185], discrepancies remain as whether *PITX2* is impaired in AF risk variants human patient carriers[136,142,143]. Importantly some discrepancies also exist as whether predisposes or triggers AF[136,139]. Our recent findings suggest the notion that *PITX2* insufficiency in the embryo predisposes whereas *PITX2* insufficiency in the adult atrial chamber triggers AF. In this context, we have provided evidences that such switching mechanisms seems to be regulated by WNT signaling and the downstream activation/repression of key microRNAs[149,165], yet future experiments are needed to provide additional evidences on this front. In the last decade, our understanding of the downstream pathways controlled by *PITX2* has advanced at a quick pace. Several studies have demonstrated that *PITX2* controls signaling pathways regulation sinus node formation[189,196], including therein the pivotal role of a microRNA cluster[189,190]. These data shed light thus on the concurrence of AF with other cardiac physiopathological conditions such as sick sinus syndrome[190]. Similarly we and others have demonstrated the key role exerted by *PITX2* regulating microRNAs impacting on calcium handling and thus on profibrillatory risk factors[136,153,189]. It remains nonetheless to be established if *PITX2* also leads to impaired regulation of inflammatory processes and/or redox signaling which might impact on structural remodeling of the atrial chambers. To date, no evidences of atrial fibrosis have been reported in any of the *PITX2* deficient mouse models[136,139,140,149,185], while some incipient evidences have been reported on the inflammatory link[149] but not on redox signaling. Thus, future lines of research should be envisioned to clarify this point.

In addition, whereas our understanding of the *PITX2* downstream signaling pathways in the context of AF have progressively increased, scarce insights are currently available on the impact of AF clinically related risk factors on *PITX2*. A link between 4q25 risk variant carriers and increase left atrial volume has been recently reported[197] in AF patients, however it remains to be established this is modulated by *PITX2*, as suggested by previous evidence in *PITX2* deficient mice in which atrial volume is already increased at fetal developmental stages[136]. A recent study reported that aging and hypertension, two well-established risk factors for AF, severely decreased *PITX2* expression in a rat experimental model when both risk factors were combined[198]. We recently reported that *Pitx2* is severely impaired in dilated cardiomyopathy patients as well as in an experimental heart failure pig model, shedding light

into a possible connection between ventricular disfunction and AF[199,200]. Yet, it remains to be established if other clinically relevant AF risk factors, such as diabetes, obesity, hyperthyroidism, valvular heart disease and/or obstructive sleep apnea are also impairing *PITX2* function and thus predisposing to AF. Overall these data demonstrate the pivotal role of PITX2 regulating multiple aspect that if impaired are pro-arrhythogenic and they also open new pathways to explore therapeutical approaches that could eventually lead to minime the burden of AF in the human population.

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ISSN 2220-3184 (online)

World Journal of *Medical Genetics*

World J Med Genet 2016 May 27; 6(2): 17-21

Published by Baishideng Publishing Group Inc

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World Journal of Medical Genetics

Contents

Quarterly Volume 6 Number 2 May 27, 2016

CASE REPORT

17 Mutation in *TNXB* gene causes moderate to severe Ehlers-Danlos syndrome

Kaufman CS, Butler MG

World Journal of Medical Genetics
Volume 6 Number 2 May 27, 2016

Contents

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

FREQUENCY
Quarterly

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PUBLICATION DATE
May 27, 2016

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World J Med Genet 2016 May 27; 6(2): 17-21
ISSN 2220-3184 (online)
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CASE REPORT

Mutation in *TNXB* gene causes moderate to severe Ehlers-Danlos syndrome

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Author contributions: Kaufman CS and Butler MG designed the report and wrote the manuscript.

Supported by The National Institute of Child Health and Human Development (NICHD), No. HD02528.

Institutional review board statement: University of Kansas Medical Center Institutional Review Board.

Informed consent statement: Verbal consent was obtained from the patient. There are no identifying images included in the manuscript.

Conflict-of-interest statement: There are no conflicts of interest.

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Received: April 16, 2016
Peer-review started: April 19, 2016
First decision: May 10, 2016
Revised: May 15, 2016
Accepted: May 18, 2016
Article in press: May 20, 2016
Published online: May 27, 2016

Abstract

We report a 28-year-old female who presented with severe joint pain, chronic muscle weakness, Raynaud's phenomenon, and hypermobility. She was found to have a 6074A > T nucleotide transition in the *TNXB* gene causing an amino acid protein change at Asp2025Val classified as likely pathogenic. We add this clinical report to the literature and classical human disease gene catalogs to identify this specific mutation as disease-causing. This gene variant was reported previously in a different 36-year-old patient who shared our patient's symptoms of joint hypermobility, skeletal and joint pain, skin elasticity and musculoskeletal problems, thereby causing a more severe presentation than seen in the hypermobility type of Ehlers-Danlos syndrome (EDS). At the time of writing, a few mutations in the *TNXB* gene have been recognized as pathogenic causing EDS due to tenascin-X deficiency, but the variant identified in our patient has not been recognized as pathogenic in online genetic databases. Our case study in combination with peer-reviewed literature suggests that the 6074A > T nucleotide transition in the *TNXB* gene may be classified as disease-causing for EDS due to tenascin-X deficiency.

Key words: Ehlers-Danlos syndrome; Genetic variants; Mutations; Hypermobility; Joint pain; Muscle weakness; Raynaud's phenomenon; Tenascin-X; *TNXB*; Ehlers-Danlos syndrome due to tenascin-X deficiency

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Core tip: Various types of Ehlers-Danlos syndrome (EDS) have unique phenotypic features and genetic causes that are under investigation. This case report presents a gene variant (6074A > T nucleotide transition in the *TNXB* gene) not previously classified as disease-causing which we propose should be classified as pathogenic. This variant appears to produce joint hypermobility, skeletal pain, and musculoskeletal problems and should be classified as causing EDS due to tenascin-X deficiency.

Kaufman CS, Butler MG. Mutation in *TNXB* gene causes moderate to severe Ehlers-Danlos syndrome. *World J Med Genet* 2016; 6(2): 17-21 Available from: URL: http://www.wjgnet.com/2220-3184/full/v6/i2/17.htm DOI: http://dx.doi.org/10.5496/wjmg.v6.i2.17

INTRODUCTION

Ehlers-Danlos syndrome (EDS) encompasses a heterogeneous collection of connective tissue disorders with joint, skin and vascular involvement. Patients diagnosed with EDS have diverse clinical findings, which may be better understood and classified by identifying the genetic contribution to symptoms[1,2]. Although this condition has been recognized since 1901[3] and is now classified into nine types, delineation of the types and relationships to causative genes and their variants are underway, impacted by advances in genetic technology and next generation (exome) sequencing[4,5]. Here, we assert that mutations in the *TNXB* gene, which encodes the glycoprotein tenascin-XB and related to the benign hypermobility form of EDS, may cause a more severe form or presentation of EDS.

CASE REPORT

Our 28-year-old patient showed no developmental delays as a child. She did undergo a right knee arthroscopic surgery not related to an injury at 18 years of age and breast augmentation at 21 years of age. She has had two pregnancies and was anemic during both pregnancies, due to decreased red blood cell count. The pregnancies were full-term and labor and delivery were normal. She experienced increased joint hypermobility during both of her pregnancies. Previous lab work showed normal thyroid and hepatic function. The patient had generalized joint pain since childhood with mild scoliosis. Anti-inflammatory agents did not relieve her pain. She has experienced instability of the right shoulder, pelvis and both hip joints with more problems with advancing age. During the clinic visit, we completed an assessment of the patient's joint mobility using the Beighton scale[6], which results in a numerical score from 0 to 9 with one point assigned for the ability to perform each of the following actions: Passive dorsiflexion of the little finger beyond 90 degrees; passive apposition of the thumb to the flexor aspects of the forearm; hyperextension of the elbow beyond 10 degrees; hyperextension of the knee beyond 10 degrees; and forward flexion of the trunk, with knees straight, so that palms of hands rest easily on the floor. Our patient's Beighton score was 6 out of 9, including easy placement of palms to the floor (one point), hyperextension of both knees (two points), hyperextension of one elbow (one point), and passive apposition of both thumbs to the forearms (two points) as shown in Figure 1.

Furthermore, our patient also experienced Raynaud's phenomenon, manifested as paresthesia with decreased blood flow in her fingers, lip, ears, and nose (Figure 1). This clinical presentation led to rheumatology evaluation including antibody studies and SED rate to diagnose a mixed connective tissue disorder such as lupus or rheumatoid arthritis. The results were normal. The patient's back pain continues to worsen and she experiences weakness throughout much of her body. She is unable to participate in mildly strenuous manual labor or movement related activities. Our patient identifies chronic pain and generalized muscle weakness as the most significant medical problems detrimental to daily functioning and quality of life.

The family history was limited due to little to no contact with her siblings and parents, all of whom live in Israel. Our patient has 100% Ashkenazi Jewish ancestry but has not had any Jewish specific genetic disease testing. She has a son who is 4 years of age and a daughter who is 10 years of age, both currently without relevant medical concerns or health problems.

We ordered next generation DNA sequencing panel and deletion/duplication analysis of 33 genes related to connective tissue disorders (*ABCC6, ACTA2, ATP7A, CBS, CHST14, COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL9A1, COL9A2, COL9A3, COL11A1, COL11A2, ELN, FBN1, FBN2, FKBP14, FLNA, MYH11, MYLK, PLOD1, SKI, SLC2A10, SLC39A13, SMAD3, SMAD4, TGFB2, TGFBR1, TGFBR2,* and *TNXB)* performed commercially at the University of Nebraska Medical Center (Omaha, NE) using a blood sample from our patient. The only gene variant identified was a 6074A > T transition in the *TNXB* gene causing a Asp2025Val amino acid change at the protein level, classified as a variant of uncertain clinical significance. No known pathogenic deletions or duplications were detected within these genes.

DISCUSSION

Classification for EDS began in the late 1960s[4] and continues to evolve and expand over time, as diagnoses move beyond focusing solely on clinical symptoms to a more genomics-driven approach. A popular simplified classification based on the Villenfranche nosology outlined the following six major types of EDS[4]: Classical (Type Ⅰ and Type Ⅱ) with defect in type 5 collagen; Hypermobile (Type Ⅲ) with unknown genetic causation; Vascular (Type Ⅳ) with abnormal type 3 collagen; Kyphoscoliosis (Type Ⅵ) with deficiency of lysyl hydroxylase (LH); Arthrochalasia (Type Ⅶ) with deficiency of chains in type Ⅰ collagen; and Dermatosparaxis (Type Ⅶ) with deficiency of procollagen N-proteinase enzyme in type I collagen.

In a more recent review[5], this classification was expanded based on current evidence, and a total of nine types of EDS were outlined: Classical (caused by defects in collagen *COL5A1* and *COL5A2* genes) involving skin laxity, joint hypermobility, widened scars, and easy bruising; Hypermobile (unknown cause) with velvet

Figure 1 Patient displays extreme hyperflexibility as represented by bilateral positive thumb to wrist signs on the Beighton hyperflexibility scale[6]. Raynaud's phenomenon of the fingers with cool temperature and decreased blood flow.

skin and laxity, joint dislocations and hypermobility; Vascular (*COL3A1* gene) with arterial rupture, hypermobility, varicose veins, thin translucent skin, and easy bruising; Kyphoscoliosis (LH 1 - *PLOD1* gene) with muscle hypotonia in infants, scoliosis from birth, joint laxity, gross motor delay, easy bruising, and fragility of sclerae; Arthrochalasia (collagen 1 - *COL1A1*, *COL1A2* genes) with severe joint hypermobility, congenital bilateral hip dislocations, skin elasticity, easy bruising, kyphoscoliosis, and osteopenia; Dermatosparaxis (procollagen N-proteinase - *ADAMTS2* gene) with sagging redundant skin, blue sclerae, short stature, and umbilical hernia; EDS with scoliosis, myopathy, hearing impairment (*FKBP14* gene); Musculocontractural EDS (*CHST14* gene) with progressive kyphoscioliosis, adducted thumbs in infancy, joint hypermobility, clubfoot, arachnodactyly, elastic skin, and poor wound healing; and finally Tenascin-X deficient (*TNXB* gene). Those with Tenascin-X (*TNXB*) deficient EDS involve features of marked skin laxity, pronounced joint hypermobility and severe bruising[5]. We extend the description of those with mutations in the *TNXB* gene to include significant joint pain and Raynaud's phenomenon, thus resulting in a more severe form of EDS than previously classified. Whether the severity depends on the type or location of the gene variant (mutation) is unknown.

The *TNXB* gene (sometimes known by other names such as *HXBL, TNX, TENX*, *XB*) belongs to a family of genes called fibronectin type Ⅲ domain containing and localizes to the major histocompatibility complex (MHC) class Ⅲ region on chromosome 6, at cytogenetic location 6p21.3. The *TNXB* gene encodes for a protein called tenascin-X, which may regulate assembly and production of specific types of collagen. Tenascin-X helps organize and maintain the structure of tissues supporting the joints, organs, skin, connective tissues, and muscles[7].

Tenascin-X is an extracellular matrix glycoprotein, thought to function in matrix maturation during wound healing[7] and became the first *EDS* gene that does not encode a collagen-modifying enzyme or a fibrillar collagen[8]. Mice knockouts ($Tnxb^{-/-}$) showed skin hyperextensibility, reflecting the human phenotype of EDS, and the skin collagen content was significantly reduced compared to wildtype. Additionally, the density of fibrils in the skin of knockout ($Tnxb^{-/-}$) mice was reduced compared to wildtype, suggesting tenascin-X plays a significant regulatory role in collagen deposition by dermal fibroblasts[8]. Hence, tenascin-X appears to be involved in the regulation of the structure and stability of elastic fibers, which give elasticity and flexibility to connective tissues.

Alterations in the *TNXB* gene and effects on protein levels have also been studied[9]. The researchers measured tenascin-X in serum from 21 healthy subjects and 151 patients diagnosed with EDS. They reported that 146 of the 151 patients with EDS had detectable tenascin-X levels in their serum. A closer examination of the 5 patients diagnosed with EDS and lacking serum tenascin-X revealed that all had hyperelastic skin and

hypermobility, and a subset had joint pain and multiple subluxations. One patient was homozygous and another heterozygous for a 30-kb deletion in the *TNXB* gene resulting in a nonfunctional fusion gene. Two patients were homozygous for a 2-bp deletion in exon 8 of the *TNXB* gene, which altered the open reading frame coding for amino acids 1184 through 1230 before causing a premature stop codon. The fifth patient was homozygous for a 2-bp insertion in exon 3 which caused a stop codon at position 707. These genetic variations are now classified as causing EDS specifically due to tenascin-X protein deficiency.

Furthermore, a different study[10] identified three missense mutations in the *TNXB* gene, one in each of three patients diagnosed with the hypermobility type EDS but with normal tenascin-X serum levels: A 3582A > G transition (Val1195Met), an 85C > T transition (Arg29Trp), and a 12097C > A transition (Leu4033Ile). These wild type amino acids and locations are strongly conserved in this protein. The researchers then compared skin biopsies from these patients with other EDS hypermobility type patients who did not have gene variants in the *TNXB* gene and determined that elastic fiber abnormalities in hypermobility type EDS are specific for TNX-haploinsufficient individuals and confirm an important role for TNX in regulating elastic fiber integrity in humans[10].

As demonstrated in the literature by examples described, a variety of mutations, deletions, and insertions in the *TNXB* gene may cause features of EDS. Our patient's gene change was classified as a variant of unknown significance and not as yet classified as disease-causing. This missense mutation in exon 17 of the *TNXB* gene in our patient is moderately conserved with an in silico prediction of trending damaging. This mutation causes a significant amino acid change from an aspartic acid which is negatively charged to a valine which is non-polar; this transition could theoretically impact protein structure due to such a dramatic change in the amino acid classification type. Based on our patient's clinical symptoms and DNA findings with evidence from the literature, we propose that the 6074A > T nucleotide transition (p.Asp2025Val) identified in our patient causes a moderate to severe form of EDS that includes significant joint pain, muscle weakness, hypermobility and Raynaud's phenomenon.

A limitation of our report is the lack of contact by the patient with her siblings or parents, all of whom live in Israel. Therefore, it is not possible for us to reach members of her family to identify the presence or absence of the gene variant and to better characterize the genotype-phenotype relationship. Furthermore, our patient lives at a considerable distance from the medical center making travel difficult for her and her immediate family (*e.g.,* younger children). It is unlikely that arrangements could be made in a timely fashion to undertake skin biopsy and assessment of elastic fibers in the patient or her living children without evidence of disease in which an invasive procedure is not warranted even if accessible.

Our patient is also of Ashkenazi Jewish ancestry. A 36-year-old patient with this same amino acid change at the protein level reported to be likely pathogenic[2,11-14] and that patient was diagnosed clinically with the hypermobility type of EDS. The patient's symptoms included hypermobility, skeletal pain, skin elasticity, gastrointestinal and musculoskeletal problems, all consistent with EDS. The status of Jewish ancestry with this reported patient is unknown. We propose that both of these patients may have a form of EDS due to their specific mutation in the *TNXB* gene, causing more severe symptoms sufficiently significant to interfere with daily functioning and quality of life. More research is needed to conclude that this gene variant is causative and authors encourage the reporting of other patients with variants of the *TNXB* gene, clinical features and genotype-phenotype correlations.

ACKNOWLEDGMENTS

We thank our patient.

COMMENTS

Case characteristics

A 28-year-old female presented with joint pain, muscle weakness, hypermobility, and Raynaud's phenomenon. She was referred to our clinic for evaluation for a connective tissue disorder.

Clinical diagnosis

Severe joint pain, chronic muscle weakness, Raynaud's phenomenon, hypermobility with Beighton hypermobility score of 6 out of 9, mild scoliosis.

Differential diagnosis

Ehlers-Danlos syndrome.

Laboratory diagnosis

Previous rheumatology evaluation ruled out lupus or rheumatoid arthritis through antibody studies and SED rate. Testing through the University of Nebraska Medical Center (Omaha, NE) showed a nucleotide 6074A > T transition in the *TNXB* gene using a blood sample.

Treatment

Reduce daily stress on joints by minimizing mildly strenuous activity. Avoid hyper-extending joints.

Related report

Lee H, Deignan JL, Dorrani N, Strom SP, Kantarci S, Quintero-Rivera F, Das K, Toy T, Harry B, Yourshaw M, Fox M, Fogel BL, Martinez-Agosto JA, Wong DA, Chang VY, Shieh PB, Palmer CG, Dipple KM, Grody WW, Vilain E, Nelson SF. Clinical exome sequencing for genetic identification of rare Mendelian disorders. *JAMA* 2014; 312: 1880-1887 [PMID: 25326637 DOI: 10.1001/jama.2014.14604].

Term explanation

Ehlers-Danlos syndrome includes a heterogeneous collection of connective tissue disorders with vascular, skeletal and skin involvement.

Experiences and lesson

As technology advances, authors are able to order more sophisticated genetic testing in the clinical setting, such as the sequencing and deletion/duplication panel ordered in this case report. However, the meaning of the results is not always clear, as more often than not they identify "variants of unknown clinical

significance", meaning the change at the DNA level does not have a clear, recognized outcome for the phenotype. In these cases, they use computer simulation to predict whether or not the change may harmful to protein structure or function. They also review literature for cases involving the variant, with the hope of associating genotype with phenotype. In the case of the patient reported in this manuscript, they concluded through in-depth examination of the variant and a comprehensive literature review that the 6074A > T transition in the *TNXB* gene seen in our patient is likely pathogenic and causes a moderate to severe form of Ehlers-Danlos syndrome.

Peer-review

The authors report a case of Ehlers-Danlos syndrome with a mutation in *TNXB* gene. The report is interesting.

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ISSN 2220-3184 (online)

World Journal of *Medical Genetics*

World J Med Genet 2016 August 27; 6(3): 22-33

Published by Baishideng Publishing Group Inc

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World Journal of Medical Genetics

Contents

Quarterly Volume 6 Number 3 August 27, 2016

REVIEW

22 Chitosan DNA nanoparticles for oral gene delivery

Patel BJ, Vignesh NK, Hortelano G

World Journal of Medical Genetics
Volume 6 Number 3 August 27, 2016

Contents

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

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Quarterly

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PUBLICATION DATE
August 27, 2016

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DOI: 10.5496/wjmg.v6.i3.22

World J Med Genet 2016 August 27; 6(3): 22-33
ISSN 2220-3184 (online)
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REVIEW

Chitosan DNA nanoparticles for oral gene delivery

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Author contributions: All authors contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: Non declared.

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Manuscript source: Unsolicited manuscript

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Received: January 17, 2016
Peer-review started: January 18, 2016
First decision: February 29, 2016
Revised: July 20, 2016
Accepted: August 6, 2016
Article in press: August 8, 2016
Published online: August 27, 2016

Abstract

Gene therapy is a promising technology with potential applications in the treatment of medical conditions, both congenital and acquired. Despite its label as breakthrough technology for the 21st century, the simple concept of gene therapy - the introduction of a functional copy of desired genes in affected individuals - is proving to be more challenging than expected. Oral gene delivery has shown intriguing results and warrants further exploration. In particular, oral administration of chitosan DNA nanoparticles, one the most commonly used formulations of therapeutic DNA, has repeatedly demonstrated successful *in vitro* and *in vivo* gene transfection. While oral gene therapy has shown immense promise as treatment options in a variety of diseases, there are still significant barriers to overcome before it can be considered for clinical applications. In this review we provide an overview of the physiologic challenges facing the use of chitosan DNA nanoparticles for oral gene delivery at both the extracellular and intracellular level. From administration at the oral cavity, chitosan nanoparticles must traverse the gastrointestinal tract and protect its DNA contents from significant jumps in pH levels, various intestinal digestive enzymes, thick mucus layers with high turnover, and a proteinaceous glycocalyx meshwork. Once these extracellular barriers are overcome, chitosan DNA nanoparticles must enter intestinal cells, escape endolysosomes, and disassociate from genetic material at the appropriate time allowing transport of genetic material into the nucleus to deliver a therapeutic effect. The properties of chitosan nanoparticles and modified nanoparticles are discussed in this review. An understanding of the barriers to oral gene delivery and how to overcome them would be invaluable for future gene therapy development.

Key words: Gene therapy; Oral gene delivery; Chitosan nanoparticles; Glycocalyx; pH; Enzymes; Endolysosomal escape; Intracellular transport; Nuclear transport; Mucus

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Core tip: Gene therapy is facing considerable challenges to realize its promise, the most important which is arguably achieving effective delivery of the therapeutic gene to the target cells. Therefore, developing alternative delivery strategies is crucial. This manuscript reviews the oral administration of chitosan DNA nanoparticles as a novel gene therapy delivery strategy.

Patel BJ, Vignesh NK, Hortelano G. Chitosan DNA nanoparticles for oral gene delivery. *World J Med Genet* 2016; 6(3): 22-33 Available from: URL: http://www.wjgnet.com/2220-3184/full/v6/i3/22.htm DOI: http://dx.doi.org/10.5496/wjmg.v6.i3.22

INTRODUCTION

Gene therapy, broadly understood as the delivery of nucleic acids for therapeutic purposes, has therapeutic potential for a number of medical conditions. Since its first clinical trials in 1990, gene therapy research has made a number of advancements[1]. Despite its tribulations there has been some success, such as the treatment of immunodeficiencies through retroviral and lentiviral transduction of stem cells[2], X-linked adrenoleukodystrophy through lentiviral transduction[3], hemophilia through Adeno-associated virus delivery[4], as well as the use of transduced T cells as a treatment for leukemia[5]. Due to low cell survival upon transplantation and logistics inherent to *ex vivo* cell therapy, nucleic acids (including DNA, antisense oligonucleotides, and RNA interference systems) are typically delivered using viral vectors because of the high expression they induce in target cells[6]. However, the ability of viral vectors to transduce cells and induce transgene expression may also trigger immune responses, and the potential activation of cancer-promoting genes in the case of integrating viral vectors[7]. The safety of current gene therapy delivery systems, particularly that of integrating vectors is still not fully studied[8]. Non-viral gene delivery lags behind viral vectors in targeting efficacy and transgene expression, which can be enhanced using mechanical techniques (*e.g.,* use of electroporation or ultrasound) or chemical carriers (*e.g.,* use of lipid, peptides, or polymers that protect the genetic material)[9]. The use of non-viral DNA formulations for oral gene delivery is an area of active research interest[10].

Oral gene delivery *via* chemical carriers has the potential to treat a number of inherited and acquired diseases[11]. The gastrointestinal (GI) tract's large surface area provides a massive number of cells that can be potentially targeted. Successful delivery of therapeutic material to the GI is not restricted to the treatment of intestinal diseases such as inflammatory bowel disease, intestinal cancers, and intestinal symptoms of cystic fibrosis, but could also be potentially used as a treatment for systemic diseases (such as hemophilia) using the extensive capillary network for efficient distribution of the transgenes[6]. Additionally, effective exogenous DNA targeting of the intestinal stem cells in the crypts of Lieberkuhn in a stable manner could lead to potential long-term therapeutic gene expression[6].

Despite such therapeutic potential, oral gene delivery must first overcome formidable extracellular and intracellular barriers in the GI tract. These include stomach acidity, glycocalyx, mucus, cell-uptake, endolysosomal escape, and nuclear import. The coupling of nucleic acids to polymers may assist in some of these challenges. Chitosan, a deacetylated form of chitin (Figure 1), which is derived from crustacean, has been shown to be an appropriate chemical polymer for overcoming such barriers. In addition to biodegradable and biocompatible properties[12], this α(1-4)2-amino 2-deoxy β-glycan can efficiently compact DNA and has mucoadhesive and absorptive properties, buffering capacity, and membrane perturbing properties that make it a promising vehicle for oral gene delivery[13]. This review discusses the various extracellular and intracellular barriers of oral gene delivery and how they can be overcome using chitosan and chitosan-modified nanoparticles.

EXTRACELLULAR BARRIERS

Free DNA is quickly denatured by the acidic pH in the stomach and intestinal and cellular enzymes, so it would have no therapeutic effect[6]. However, chitosan-coupled therapeutic DNA sequences formulated as chitosan nanoparticles are effectively protected from various physiological and cellular barriers.

Acidity

pH varies greatly in the gastrointestinal tract, ranging from the extremely acidic stomach (mean pH 1.7) to the slightly alkaline ileum (mean pH 7.5) (Figure 2A)[14-16]. Chitosan nanoparticles have been shown to protect enclosed nucleic acids across a wide range of pH. In the harsh stomach acid[17,18] increased electrostatic interactions between positively charged chitosan and negatively charged DNA creates a more stable nanoparticle (Figure 2B). It is chitosan's amino-group with a pKa of 6.5 (Figure 1B) that accounts for protonation of the polymer at pHs below 6.5, and the resulting binding affinity between cationic chitosan and anionic DNA[19].

As nanoparticles transit towards the small intestine, pH increases drastically from 1.7 in the stomach to 6.1 and 6.6 in the duodenum and jejunum respectively[14,15]. During this shift, chitosan's amino groups are deprotonated, decreasing chitosan's binding affinity for DNA[19,20]. Nanoparticle stability is slightly reduced, achieving an effective balance between DNA association and dissociation. It is at pH 6.8 and 7.0 that transfection efficiency of chondrocytes peaked[21]. Interestingly however, Roy *et al*[17] reported transfection of stomach tissue by chitosan nanoparticles.

As the pH continues to climb distally in the intestine,

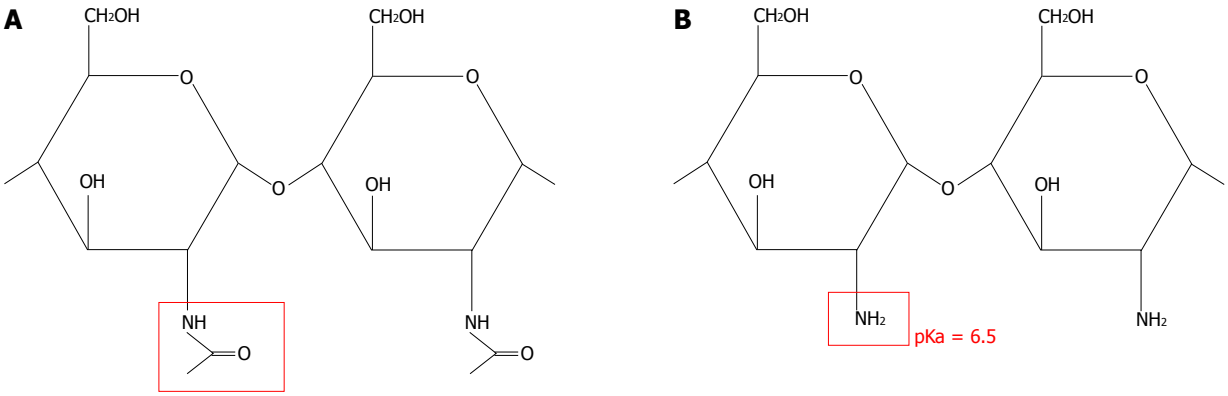

Figure 1 Chitin and chitosan molecular structure. The molecular structure of chitin (A) differs from chitosan (B) through its deacetylation. The resulting amino group on chitosan has a pKa of 6.5[12].

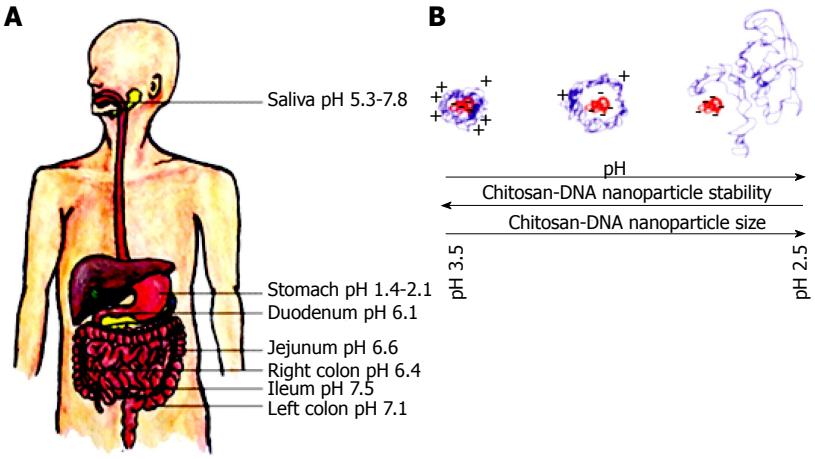

Figure 2 Effect of gastrointestinal pH on chitosan-DNA nanoparticles. A: pH varies drastically throughout the human gastrointestinal tract from acidic stomach to slightly basic distal bowel[14,15]. This in turn influences how chitosan-DNA polymers behave intraluminally; B: The conceptual interaction between negatively charged DNA and positively charged chitosan that becomes protonated at acidic pHs. This allows chitosan-DNA nanoparticles to remain stable at lower pHs.

chitosan deprotonates further until it is neutral. Neutral chitosan nanoparticles are quite unstable as they easily dissociate from DNA and begin to aggregate with themselves, no longer inhibited by positive-positive repulsive forces (Figure 2B)[21,22]. Transfection efficiency also decreases at these high pH levels as has been demonstrated in human-lung carcinoma A549 cells (transfection at pH 7.6 < transfection at pH 6.9)[23] and HEK 293 cells (transfection at pH 7.6 < transfection at pH 6.5)[20].

Therefore, chitosan (pKa 6.5) is able to withstand a wide pH range from 1.5 (highly stabilized) to 6.8 (intermediate stability - optimal for transfection), before becoming destabilized and aggregating. In support of this model, *ex vivo* experiments on gastrointestinal tissue internalization of chitosan nanoparticles found internalization to be higher for the jejunum (pH 6.6) than the ileum (pH 7.5 - too basic) and duodenum (pH 6.1 - too acidic)[24].

Mucus

The gastrointestinal mucus, composed of mucin fibers linked to various proteoglycans[25], is constantly produced, secreted and shed. This rapid turnover combined with the thickness of the mucus layer (greatest in the colon at 830 μm) must be overcome by chitosan nanoparticles for effective oral gene delivery[26]. There are three main mechanisms that have been explored.

For a long time mucoadhesion was thought to improve absorption by extending nanoparticle residence time in the GI tract and increasing bioavailability of nanoparticles for passive paracellular uptake[12,27]. Therefore, researchers sought to improve mucoadhesion by formulating nanoparticles capable of greater interactions with mucus through electrostatic bonds, hydrogen bonds, hydrophobic interactions, and van der Waals forces[28]. Chitosan is reported to have strong mucoadhesive properties[27]. However mucoadhesion, on its own, will not solve this barrier to oral gene delivery, and may in fact impede drug delivery. This is because despite optimal mucoadhesion, nanoparticle delivery is limited by the rapid turnover time of mucus (50 to 270 min)[29] during which the luminal layer of mucus is sloughed off taking the adherent nanoparticles with it (Figure 3)[27].

Mucopenetration, or the ability to traverse the mucus

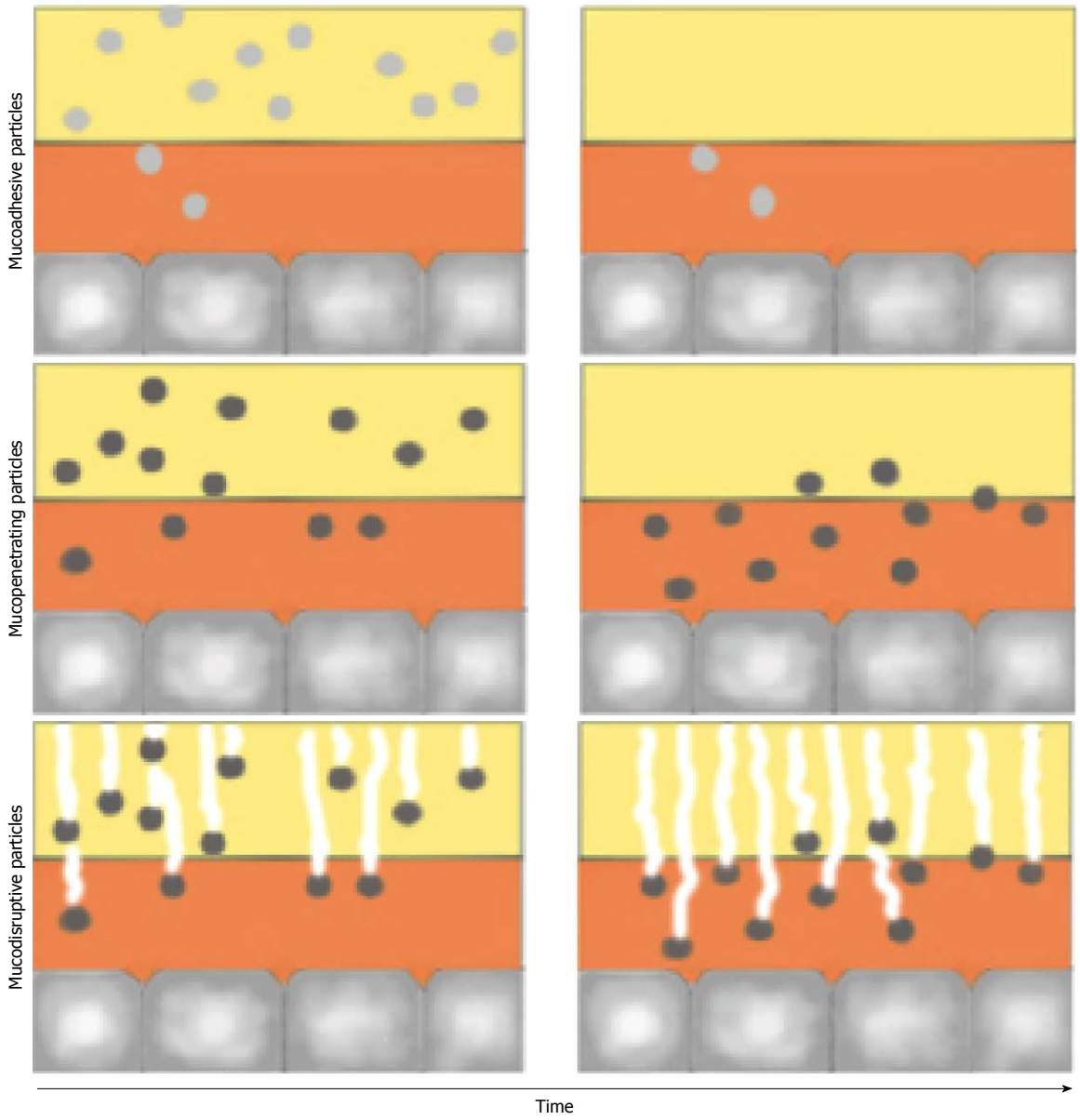

Figure 3 Gastrointestinal mucus as a barrier to chitosan-DNA nanoparticles transfection. Overcoming the mucus layer for oral gene delivery can be accomplished through three key mechanisms. Mucoadhesive nanoparticles adhere to mucus through chemical interactions. Bioavailability is limited by time, because intestinal mucus has a rapid turnover and adhered nanoparticles would be sloughed off with the luminal layer of mucus[27]. Mucopenetration involves nanoparticles that are able to traverse intestinal mucus due to chemical properties. These nanoparticles do improve bioavailability even with time[27]. Mucodisruptive nanoparticles cross the mucus layer by disrupting mucus structure. This can be accomplished by various mucolytic molecules and enzymes. Bioavailability increases with this mechanism, however intestinal mucus may be affected in the long-term[27].

layer (Figure 3), has become the focus of overcoming the mucosal barrier in the last few years. Mucopenetration is achieved by creating a "slippery" nanoparticle that, in contrast to mucoadhesion, has decreased interactions with mucus[30]. Chitosan was combined with hydrophilic 5 kDa polyethylene glycol (PEG) which would have decreased interactions with hydrophobic mucus and would not be sterically hindered[30]. Nanoparticles with greater PEGylation permeated fresh mucus 5-fold compared with non-PEGylated nanoparticles (Figure 4A)[30]. Pereira de Sousa *et al*[31] combined chitosan with chondroitin sulphate to create a densely charged but overall neutral nanoparticle surface that would theoretically have minimal interactions with mucus. Chitosan chondroitin sulphate nanoparticles permeated to a greater depth (4-6 mm) of fresh undiluted porcine intestinal mucus compared with PDLG "purasorb" chitosan nanoparticles (Figure 4B)[31]. Importantly, mucopenetration research remains in its early stages and further studies are required. To our knowledge no studies evaluating the effect of pDNA on the performance of chitosan nanoparticles modulated for mucopenetration have been conducted. This would be important to assess in the future.

Finally disruption of the mucosal layer can be achieved by combining nanoparticles with mucolytic agents (Figure 3). Studies have shown enhanced mucodisruption ability

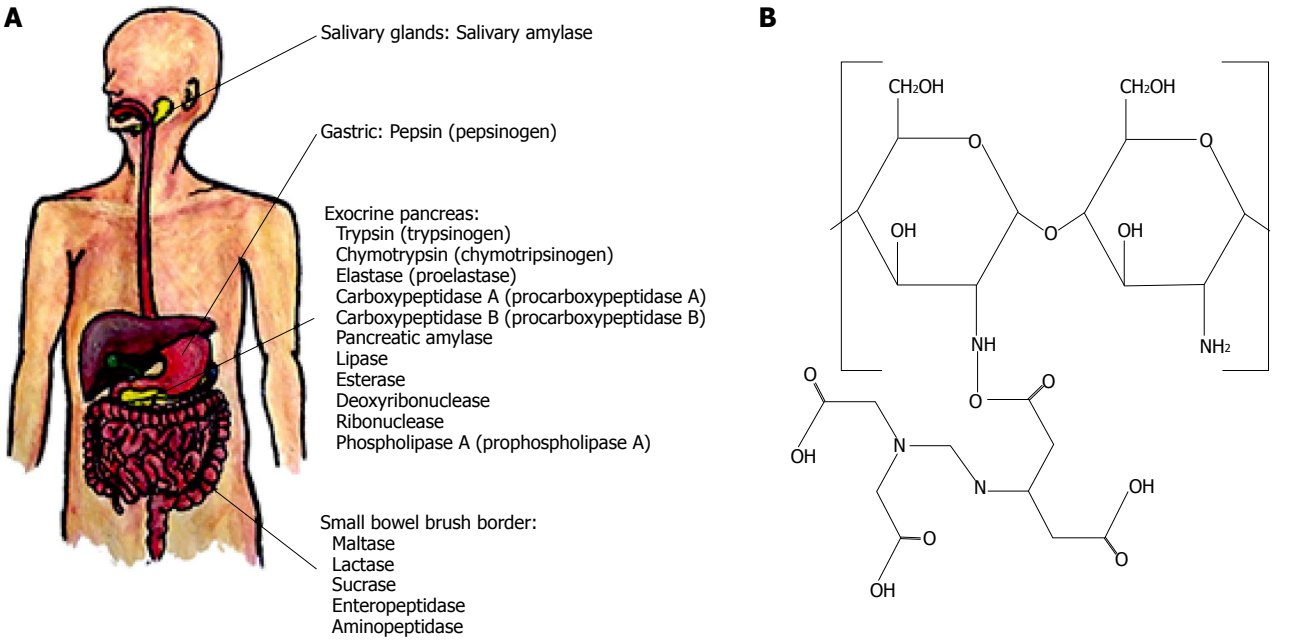

Figure 4 Effect of gastrointestinal enzymes on chitosan-DNA nanoparticles. A: Various digestive enzymes are secreted throughout the gastrointestinal tract. The genetic material within chitosan nanoparticles is most threatened by pancreatic nucleases such as deoxyribonuclease and ribonuclease[34]; B: Conjugation of chitosan polymer with ethylenediaminetetraacetic acid through a covalent bond at the amino group[37].

of nanoparticles when combined with mucolytic molecules (N-acetylcystein, L-cysteine, sulfhydryl compounds)[32] and mucolytic enzymes (papain, bromelain, trypsin)[33]. These studies, however were not conducted with chitosan. With the mucodisruption technique, reversibility of mucolysis must continue to be assessed in order to ensure that the intestinal mucus is not completely damaged and that it retains its protective function against viruses and pathogens[32].

Enzymes

Administered genes are exposed to the harsh environment of the GI lumen, filled with resident degrading enzymes, those secreted into the lumen and those membrane bound[34]. Unavoidably, therapeutic nanoparticles are also subject to the degradation processes of these enzymes and thus, pose a barrier that must be dealt with to improve the efficacy of oral gene delivery[34].

Conjugation of enzyme inhibitors with carrier vehicles was proposed a long time ago[35] and has been explored using a variety of enzyme inhibitors[36]. These inhibitor conjugated chitosan nanoparticles have been shown to protect a variety of drug molecules (including pDNA[34,37], insulin[38-40], and calcitonin[41-44]) and thereby promote therapeutic effect (Table 1). For oral gene delivery, inhibiting nuclease destruction of genetic material is most important and can be accomplished using the following inhibitors (ranked by efficacy): Ethylendiaminetetraacetic acid (EDTA) > sodium dodecyl sulphate (SDS) > aurintricarboxylic acid (ATA) > and poly(acrylic acid) (PAA)[37]. Loretz *et al*[37] demonstrated that 50% of pDNA was protected from nuclease destruction when complexed with EDTA-conjugated chitosan. This is compared to almost complete destruction of unprotected pDNA[37]. EDTA-chitosan nanoparticles also have 35% improved transfection efficiency in Caco-2 cells compared with unmodified chitosan nanoparticles[45]. Similarly, Martien *et al*[34] demonstrated almost complete protection of pDNA from lysozyme and deoxyribonuclease when packaged in ATA-chitosan conjugated nanoparticles compared with unmodified chitosan nanoparticles (complete destruction with both lysozyme and deoxyribonuclease). Conjugation of enzyme inhibitors to chitosan involves the formation of a covalent bond between a carboxylic acid group on the inhibitor and free amino acids on chitosan. Notably, the covalent conjugation ensures that the inhibitor is not orally absorbed[37] and reduces the toxicity and side effects. This is supported by LDH-release cytotoxicity tests on Caco-2 cells that showed significantly less toxicity (< 1% over 4 h) for chitosan-EDTA particles and polymers compared with unmodified chitosan particles and polymers[45]. It is important to note that prolonged administration of enzyme inhibitors has severe side effects including pancreatic hyperplasia and carcinoma and thus chronic effects of inhibitor-conjugated chitosan nanoparticles must be explored[46].

Glycocalyx

The fibrous brush border glycocalyx (FBBG) is a 400-500 nm thick glycoprotein network that covers the apical surface of intestinal epithelial cells, particularly enterocytes (Figure 5). As visualised through high resolution scanning electron microscopy, the FBBG consists of mucin-like glycoproteins[47] that originate from the tips of tightly packed microvilli and anastomose with neighbouring glycoproteins[48]. The resulting intricate mesh has the

Table 1 Chitosan-enzyme inhibitor conjugates[1]

	Enzyme inhibition	Drug protection
Chitosan-ATA	DNAase Lysozyme	pDNA[34]
Chitosan-EDTA	DNAase Trypsin Chymotrypsin	pDNA[37] Insulin[39] Trypsin and bromelain[93]
Chitosan-aprotinin	Trypsin Chymotrypsin	Insulin[38] Calcitonin[41]
Chitosan-BBI	Trypsin Chymotrypsin	Calcitonin[42] Insulin[40]
Chitosan-pepstatin	Pepsin	Calcitonin[43]
Chitosan-elastinal	Elastase	Calcitonin[42] Insulin[40]
Chitosan-phenylboronic acid	Trypsin Elastase	Calcitonin[44]
Chitosan-bacitracin		Hepatitis B surface antigen[94]

[1]Summary of the various enzyme inhibitors that chitosan has been conjugated with in order to decrease degradation and maximize delivery of drug product. All references of original studies are included. Chitosan-ATA and chitosan-EDTA conjugates are most relevant to gene therapy advancements, as they have been shown to protect pDNA and increase transfection efficiency. EDTA: Ethylenediaminetetraacetic acid; ATA: Aurintricarboxylic acid; BBI: Bowman-birk inhibitor.

potential to affect transfection efficiency of chitosan nanoparticles. The mechanism by which this would occur is unclear as existing data is mixed.

Originally, the glycocalyx was theorized to act as a size-selective diffusion barrier, with pores between 7.4 and 28.8 nm wide, allowing only appropriately sized particles to pass through and access transmembrane receptors located on microvilli[49]. More recent studies however have demonstrated that the glycocalyx does not have a diffusion effect on drug delivery (in particular insulin) but rather has a digestive effect on the therapeutic molecule due increased concentration of digestive enzymes at the FBBG[50]. More research is required to better understand this potential barrier to oral gene delivery.

The data on how to overcome the intestinal glycocalyx barrier for improved drug delivery is also mixed. Some studies promote the theory that increased interaction with glycocalyx layer, similar to that of bacteria and viruses, promotes effective drug delivery due to increased bioavailability of drugs at the apical membrane[49]. In contrast, other research demonstrates that electrostatic interactions between anionic glycocalyx and cationic nanoparticles would decrease nanoparticle charge, stimulate premature released of DNA, and cause DNA relaxation[51]. Overall, more research is required on the glycocalyx and its impact on transfection efficiency of nanoparticles.

INTRACELLULAR BARRIERS

Transport and cellular uptake

The process of cellular uptake of the nanoparticle is critical, such that its genetic component is internalized and functionally available to elicit the desired biological effect. In addition to the key effect of pH on transfection[52] nanoparticles are internalized *via* a receptor-mediated endocytosis[53,54]. However, it is important to note that nanoparticles of different formulations - and sizes - may not necessarily share the same mechanism for cell entry[55]. Thus, chitosan may have the ability to interact with the cell membrane *via* electrostatic forces leading to an energy dependent adsorptive endocytosis process. Additionally, Nam *et al*[55] showed that the uptake process of chitosan nanoparticles involved multiple distinct pathways, including the clathrin-mediated endocytosis, macropinocytosis and caveolae-mediated endocytosis. Further, they showed that each pathway was independent from the other.

In addition to delivering therapeutic nucleic acids to intestinal cells, ingested chitosan nanoparticles may also reach more distal sites by crossing the intestinal epithelium and entering the circulation system. This may be achieved through paracellular and/or transcellular transport of nanoparticles[56].

Paracellular transport is improved by chitosan's permeation enhancing properties. Positively charged chitosan interacts with tight junction proteins (occludin, ZO-1, and F-actin) and destabilizes plasma membranes to decrease the trans-epithelial electrical resistance (TEER) of cell monolayers[54]. Increased paracellular transport of macromolecules such as insulin[57] has been shown to consistently occur when chitosan is in solution. The permeability influence of chitosan-nanoparticles is less conclusive, but a study by Vllasaliu *et al*[58] showed that both chitosan and chitosan nanoparticles are effective at opening tight-junctions. Chitosan permeability depends on electrostatic interactions between chitosan and tight junction proteins, and thus, the pore-opening process may be affected by the degree of deacetylation and surface area of charge exposed on chitosan nanoparticles[58]. Further, nanoparticle size may inhibit paracellular transport due to maximal widening of tight-junction pores[58]. Thus far, few safety issues have been identified with chitosan's ability to traverse across the intestinal epithelium. This is because chitosan's ability to open tight junctions is reversible[58], and because common negatively charged GI endotoxins (such as lipopolysaccharide) are unable to cross the intestinal epithelium along with chitosan nanoparticles[59].

Transcellular transport may occur through transcytosis[60], which involves cellular uptake (pinocytosis, clatharin-mediated endocytosis, discussed earlier) at the apical membrane, transport across the cell interior, and release at the basolateral pole[56]. Although M-cells make up only 1% of the total intestinal surface, it is believed that most transcytosis occurs with these cells due to higher rates of endocytosis[56]. Transcytosis efficiency decreases with nanoparticle size[56] and increases with nanoparticle stability[61].

Endolysosome escape

DNA escape from the endolysosomal pathway is a key barrier to chitosan gene delivery. It is theorized that

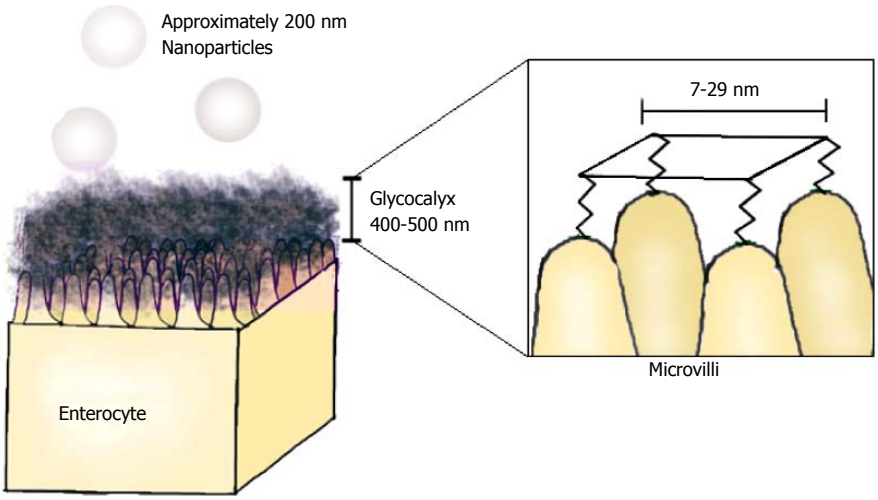

Figure 5 Glycocalyx effect on nanoparticles. A thick mesh like structure of glycocalyx proteins lines the apical membrane of the small bowel with pores 7-29 nm in size[49]. It is unclear whether the glycocalyx has a size effect on diffusion, as existing literature contains mixed results.

chitosan nanoparticles may escape from endosomes through a "Proton Sponge Effect"[62,63]. According to this theory, endocytosed chitosan nanoparticles become protonated upon fusion with acidic lysosomes. This is followed by an influx of cytosolic H^+, Cl^- and H_2O, to maintain the endosomal pH, charge, and osmolarity, respectively[64]. Such influx causes the lysosome to swell and rupture, thereby releasing nanoparticles into the cell cytosol (Figure 6)[64]. Although the proton sponge effect is supported by cationic polymers with strong buffering capacities (*e.g.,* PEI) chitosan has a relatively low buffering capacity over a narrow pH range (pH 5-7)[65]. This makes it a poor "proton-sponge" at endosomal pH, only having a limited influx of protons[65]. This is reflected in its delayed endosomal escape and transfection, observed in HEK 293 cells treated with chitosan and PEI[65]. Chitosan endosomal rupture occurred at 72 h-post-treatment, compared with PEI's escape within 24 h post-treatment[65], a delay perhaps required for a sufficient amount of chitosan to accumulate to induce a proton-sponge rupture.

Proton sponge endosomal escape can be enhanced through the addition of high buffering-capacity groups. For example, studies have examined the effect of the biocompatible and buffering imidazole ring (pKa 6.15) on the release of chitosan nanoparticles into the cytoplasm[66]. In separate studies, increased expression was observed for chitosan-graft-histadine[67] and chitosan-graft-urocanic acid[66], both of which bear the imidazole ring. Although expression was improved relative to chitosan, improvement was cell-specific and neither complex was able to reach the expression-levels of lipofectamine[67,68]. Confocal microscopy and treatment with bafilomycin A (proton pump inhibitor) confirmed that histadine's effect occurs due to improved endosomal escape of complexes[67]. Multiple experiments have also been conducted on PEI-chitosan hybrids including chitosan/PEI mixes and chitosan-graft-PEI systems. PEI is an effective non-viral vector due to its high buffering capacity (multiple amino groups) and enhanced endosomal escape properties[68]. However, PEI's effect is limited *in vivo*, due to its cytotoxicity in multiple cell lines[69]. Thus, combining PEI with chitosan, known for its biocompatibility and biodegradability, has shown to be particularly effective with synergistic results[68]. Compared with PEI, chitosan-graft-PEI has higher transfection efficiency *in vitro* and *in vivo*[70], and has decreased cytotoxicity across three cell lines (293 T, HeLa and HepG2)[71]. Transfection efficiency even reached that of lipofectamine in the human HEK293 cell line[71].

In addition to the described proton-sponge effect, there are other means by which nanoparticles escape from endosomes. Membrane destabilization by chitosan is supported by experiments tracking endocytosed nanoparticles[72,73]. In these experiments, some DNA is released from early endosomes[72] before the proton-sponge effect can be triggered by acidic lysosomes. Modifications to improve chitosan's membrane perturbant properties include addition of chloroquine[74], poly(propylacrylic acid)[75], and synthetic peptides[76]. Synthetic peptides are pH sensitive, changing conformation from random coils (at physiological pH) to alpha helices (at endosomal acidity) that interact with and destabilize the membrane through pore-formation[77]. Second, free chitosan polymer may also aid endosomal escape. Unlike protonated nanoparticles (stabilized by DNA) that decrease in volume, free chitosan expands from its coiled form due to repulsive forces[78]. This directly increases osmotic pressure and leads to endosomal rupture[78]. Thibault *et al*[79] added free chitosan at different time-points to HEK 293 cells already treated with inefficient chitosan nanoparticles (< 5 N:P levels)[79]. Addition of free chitosan at 4 and 8 h post-nanoparticle treatment resulted in a near 100% rescue of transfection, reaching similar levels to the optimal N:P of 5[79].

DNA-release

In addition to the protective role of chitosan discussed above it is also important for chitosan to have a timely release of DNA. Nanoparticles of intermediate stability, associated with intermediate molecular weight and degree of deacytylation appropriately release DNA in synchrony with lysosomal escape and achieve efficient transfection[80]. This is in contrast to the inefficient transfection ability of

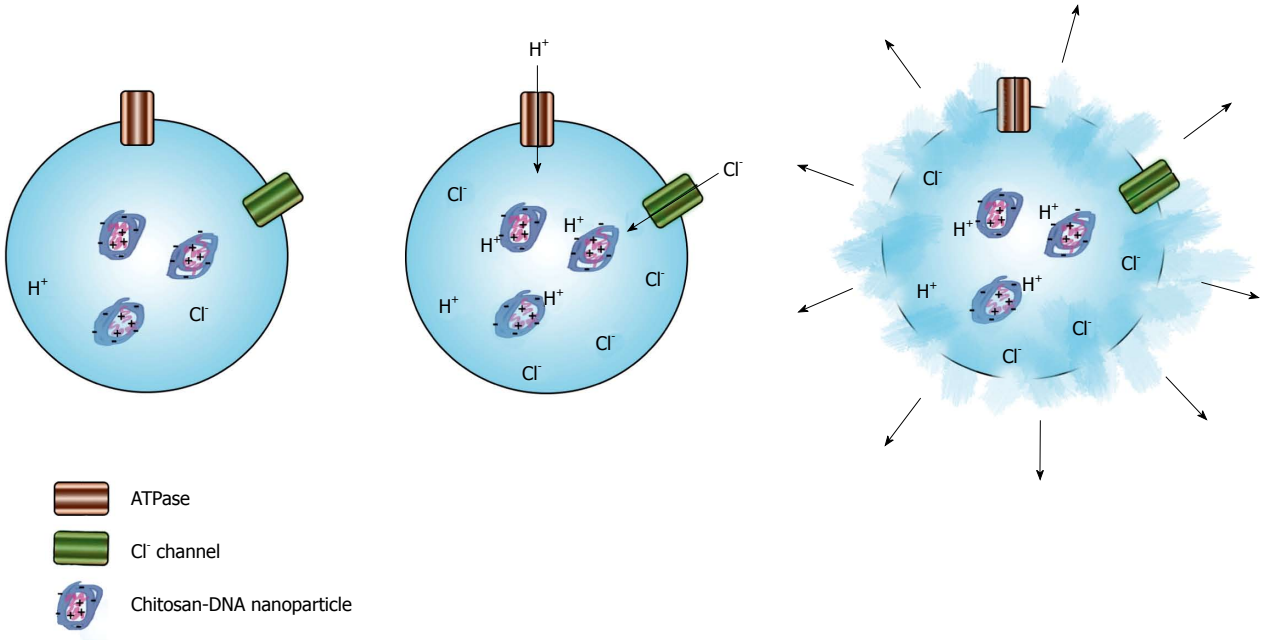

Figure 6 Proton sponge effect. Conceptual image of how chitosan-DNA nanoparticles may escape from lysosomes once endocytosed. H^+, Cl^-, and H_2O influx into the lysosome in order to maintain pH, charge, and osmolarity. This movement of molecules causes swelling of the lysosome, eventual bursting, and escape from lysosomal digestion[77].

hyperstabilised and hypostabilized nanoparticles. Up to 80% of hyperstabilized nanoparticles (high molecular weight and degree of deacetylation) remained associated after lysosomal escape, and were too large to enter the nucleus[80]. Hypostabilized nanoparticles (low molecular weight and/or degree of deacetylation) dissociated prematurely before endolysosomal escape and were degraded[80]. Thus, chitosan nanoparticles of intermediate stability are optimal for timely release of DNA and efficient transfection.

Nuclear import

In order to have a therapeutic effect, DNA must then enter the nucleus and be accessible to transcriptional enzymes, while siRNA - but not DNA coding for siRNA- exerts its inhibitory function in the cytoplasm. Considering that the majority of GI cells (*e.g.,* enterocytes, goblet cells, paneth cells) are post-mitotic, genetic material must pass through nuclear pores, as opposed to waiting for the nuclear envelope to disintegrate during cell division. Import of material (> 40 kDa) through the nuclear pore occurs through indirect active transport[81]. It involves interactions between importins and proteins containing nuclear localization signal (NLS) (Figure 7A).

Therefore, enhanced nuclear import of pDNA is an important goal. Sequences where NLS-containing proteins bind are often added to therapeutic pDNA. The 72 bp SV40 enhancer sequence upon which many mammalian transcription factors (AP1, AP2, NF-κB, Oct1, TEF1, each containing NLS sequences) bind has been used to enhance the import of perinuclear localized pDNA into the nucleus through interaction with importin proteins[80,82]. Opanasopit *et al*[83] showed increased transfection efficiency of nanoparticles containing DNA and NLS-containing peptides. Additionally, it is possible to enhance gene expression by covalently complex NLS-containing proteins onto the pDNA strand itself[83]. However, it is important for the protein's interaction with pDNA to be spatially distinct from its NLS-binding sequence so that importins are not sterically hindered from binding (Figure 7B).

Interestingly, some evidence exists for nuclear import of entire chitosan/DNA complexes. Zhao *et al*[84] found increased localization of complexes when a synthetic protein, containing SV40 NLS signal, was covalently linked to chitosan. Further addition of kinases promoted intra-nuclear disassociation and improved transfection efficacy beyond that of lipofectamine[84]. Chitosan localized in the nucleus did not interfere with gene expression[73].

Immune response

Chitosan is a biodegradable polysaccharide that has been administered to individuals without any adverse side effects[85]. Chitosan is also naturally degraded through lysosome digestion according to its molecular weight and degree of deacetylation. These characteristics of chitosan have triggered the utilization of chitosan as an adjuvant in vaccines[86]. The viscous nature of chitosan promotes the formation of an antigen depot. Injection of antigen with chitosan resulted in 60% of the antigen remaining at the injection site for 7 d whereas less than 9% of the antigen remained after 8 h when it was administered with saline[86]. In combination with antigens, chitosan has been demonstrated to induce activation of NK cells, macrophages, inducing cytokines, stimulating antibody

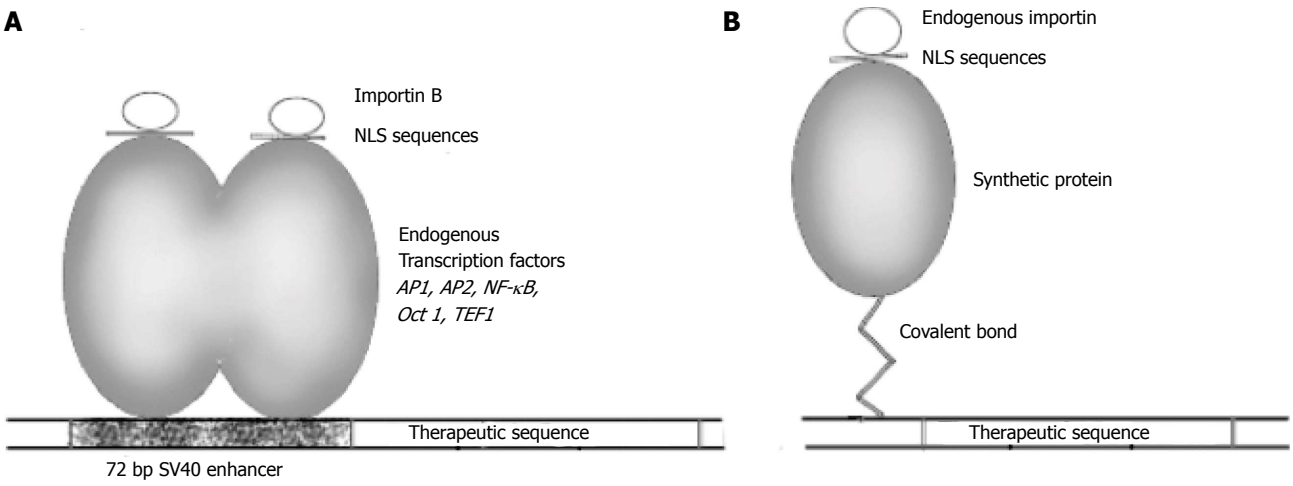

Figure 7 Model of DNA combined with importin/nuclear localization signal complex to enhance importation of DNA through nucleopores. A: Plasmid DNA bound to importins/nuclear localization signal (NLS) complex through transcription factors[79,81]; B: Plasmid DNA bound to importins/NLS complex through a synthetic protein[83].

production and cytotoxic T lymphocyte responses[87]. Chitosan has been shown to elicit a combination TH1/TH2 response, thus promoting both humoral and cell mediated immunity. The increased immune response has been reported to produce a high titer antibody response to the antigen[88].

When dealing with oral vaccination, the uptake by the Peyer's patch is a crucial step in stimulation an immune response. Studies have developed chitosan particles that have the optimal size and zeta potential to be targeted to the M-cells of the Peyer's patch[89]. Particles that are < 10 μm are taken up by M-cells while those < 5 μm can be transported to the spleen or local lymph nodes to stimulate IgM and IgG production[89]. As a result, chitosan promises to serve as a carrier system of antigens and/or antigen-coding genetic material to act as an adjuvant in vaccinations to enhance both cell mediated as well as humoral immune responses. However, chitosan's immunomodulatory effects have also been shown in studies that utilize the nasal administration of chitosan to induce the immune system[90]. Chitosan is also a viable vehicle to be used in oral administration due to its adhesive and transport properties[17]. Its stability when complexed with DNA make it suitable for the delivery of the cargo to the M-cells of the GI-tract, allowing for the generation of both mucosal as well as systemic immunity[17]. The immunity of chitosan nanoparticles has also been exploited to induce oral tolerance. Goldmann *et al*[91] fed mice with oral nanoparticles complexed to ovalbumin (OVA) DNA. Despite OVA being a very good antigen, the nanoparticle treatment suppressed a humoral response after subsequent exposure to OVA protein. The authors noticed a shift from TH_1 to a TH_2/TH_3 together with the induction of OVA-specific regulatory T cells[91]. The same group took this work a step further by protecting mice against abdominal aortic transplantation after feeding mice with chitosan nanoparticles containing DNA coding for k(b), one of the MHC-I molecules of the donor[92]. These results open new applications for oral chitosan nanoparticles.

CONCLUSION

Oral delivery of genes specifically is an intriguing technique to treat GI pathologies as well as other chronic systemic diseases such as haemophilia[12,18]. However, it should be noted that hormone related disorders are much more challenging to treat due to the strict regulation the protein expression and the systemic affect that many hormones exhibit. While oral gene therapy has shown immense promise as treatment options in a variety of diseases, there are still significant barriers to overcome before it can be considered for clinical applications. By modulating the physical and chemical characteristics of the nanoparticle both extracellular and intracellular barriers may be circumvented. By judicious design of the nanoparticles therapeutic potential for the targeting of many systemic diseases may be possible. Further research in this field is required in order to develop in the future chitosan nanoparticles of viable clinical use.

ACKNOWLEDGMENTS

The authors thank the Canadian Hemophilia Society Bayer Inc. and Nazarbayev University ORAU for their supporting to this work.

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P- Reviewer: Fernandes JC, Zhao ST **S- Editor**: Kong JX
L- Editor: A **E- Editor**: Lu YJ

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