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Review

Shox2: The Role in Differentiation and Development of Cardiac Conduction System

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The formation and conduction of electrocardiosignals and the synchronous contraction of atria and ventricles with rhythmicity are both triggered and regulated by the cardiac conduction system (CCS). Defect of this system will lead to various types of cardiac arrhythmias. In recent years, the research progress of molecular genetics and developmental biology revealed a clearer understanding of differentiation and development of the cardiac conduction system and their regulatory mechanisms. Short stature homeobox 2 (Shox2) transcription factor, encoded by *Shox2* gene in the mouse, is crucial in the formation and differentiation of the sinoatrial node (SAN). *Shox2* drives embryonic development processes and is widely expressed in the appendicular skeleton, palate, temporomandibular joints, and heart. Mutations of *Shox2* can lead to dysembryoplasia and abnormal phenotypes, including bradycardiac arrhythmia. In this review, we provide a summary of the latest research progress on the regulatory effects of the *Shox2* gene in differentiation and development processes of the cardiac conduction system, hoping to deepen the knowledge and understanding of this systematic process based on the cardiac conduction system. Overall, the *Shox2* gene is intimately involved in the differentiation and development of cardiac conduction system, especially sinoatrial node. We also summarize the current information about human SHOX2. This review article provides a new direction in biological pacemaker therapies.

Keywords: arrhythmia; cardiac conduction system; differentiation; heart evelopment; *Shox2*
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Introduction

The generation and propagation of electrical impulses are important for formation of effective cardiac beating cycles ensuring systemic unidirectional blood flow (circulation). The cardiac conduction system (CCS) is mainly composed of the slowly propagating atrial nodes, including sinoatrial node (SAN) and atrioventricular node (AVN), and the rapidly propagating ventricular conduction system, including atrioventricular bundle (AVB), left and right bundle branches, and Purkinje fiber network. SAN plays a major role as the pacemaker. The rhythmic impulses arise in SAN depolarize neighboring atrial cardiomyocytes, then propagate to atria and ventricles for stimulation of the working myocardium to generate cardiac contractions. Malignant cardiac arrhythmia caused by a genetic defect, a common cause of cardiac sudden death, severely threatens human health (Arking et al. 2004; Gheeraert et al. 2006; Marsman et al. 2014). After years of exploration and practice, there is still room for improvement of symptomatic treatment methods including pharmacotherapy, percutaneous catheter ablation, or pacemaker implantation (Nattel and Carlsson 2006; Carlsson et al. 2010; Yamada and Kay 2012; Vernooy et al. 2014). Many factors can disrupt cardiac rhythms, including myocardial ischemia, prescription drugs, electrolyte disorders, smoking, alcohol, coffee, physical exercise, mental stimulation, ion channel abnormalities, or genetic defects (Kathiresan and Srivastava 2012; Peyronnet et al. 2016; Weisbrod et al. 2016; Chrysant 2017; Du et al. 2017; Goyal et al. 2017). Undoubtedly, defects of cardiac pacemaker cells and the corresponding tissues also disrupt cardiac rhythms (Yazawa et al. 2011; Vedantham 2015). As for the CCS, many unsolved mysteries regarding the differentiation and development of SAN tissue specifically need to be studied and resolved urgently.

The development of cardiac conduction system

At early stage of embryogenesis, when the heart is still a tube structure, all cardiomyocytes can automatically initiate impulses. What interests us is that the cells in the medial extremity have the fastest automatic frequency and control the frequency of cardiac beating. This regional

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electric activity even appears before contraction ability of cardiomyocytes during cardiac development (Moorman and Christoffels 2003). Cardiac pacemaker signals can be recorded in the venous pole after the primitive cardiac tube forms at E8.5. The impulse current is transmitted slowly in the myocardium, forming sinusoid electrocardiographic waves and peristaltic cardiac contractions (Van Mierop 1967; Paff et al. 1968; Anderson et al. 2006). After the formation of primitive cardiac tube (Fig. 1), mesenchymal cardiac progenitor cells gradually approach to form the sinus horn, sinus venous (SV), and finally, SAN (Van Mierop and Gessner 1970; Viragh and Challice 1980; Christoffels et al. 2006; Mommersteeg et al. 2007; Wiese et al. 2009).

During the differentiation and development of mouse SV and SAN, sinus horn-forming cardiomyocytes in the venous pole start to express Tbx3, a member of the T-box family identifies the developing central conduction system (Hoogaars et al. 2004, 2007), and Hcn4, which marks distinct precursors or components of the CCS at distinct times during development (Liang et al. 2013). Part of myocardium persistently expressing Nkx2.5, which is essential for the development of atrial, ventricular, and conotruncal septation, atrioventricular (AV) valve formation, and maintenance of AV conduction in primitive cardiac tube (Zhang et al. 2014) can activate Cx40, which forms gap junction channels with high conductance and is expressed in all compartments of the fast conduction system, including the AVB, left and right bundle branches, and Purkinje fiber network (Gros et al. 2004), while inhibiting *Tbx3* and *Hcn4* expression to promote differentiation toward atria. Shortly after the formation of atria, the SAN can differentially form from a part of SV tissue at E9.0-9.5 (Viragh and Challice 1980; Hoogaars et al. 2004; Anderson et al. 2006; Mommersteeg et al. 2007; Christoffels et al. 2010). Afterwards, the rest of sinus horn finally develops into the SV of the right atrium, a part of superior and inferior vena cava, and the coronary sinus.

Part of the atrioventricular junction (AVJ) constitutes the AVN and the right AV ring bundle distributed around the tricuspid annulus (Wessels et al. 1996). Most investigators hold the idea that the above-mentioned structure evolves from the atrioventricular canal (AVC) at the embryonic stage (Davis et al. 2001; Moorman and Christoffels 2003). The mature AVC and cardiac outflow tract (OFT) preserve characteristic of slow-conduction, similar to the primitive cardiac tube. While the enhanced expression of Cx40 and Cx43 cause the mature working cardiomyocytes in atria and ventricles to gradually acquire the traits of fast conduction in order to rapidly transmit the electrical impulse governing ventricular contraction (Van Mierop 1967; Paff et al. 1968). With the pacemaker impulses initiated by the venous pole being rapidly transmitted inside the ventricles after the AVC delay, the slow contraction mode of the primitive cardiac tube is replaced by the rapid sequential contraction mode of atria and ventricles. This observation indicates that the divides of cardiac chambers and retention of the delayed conduction trait of AVC and AVN tissues perform indispensable functions in the establishment of normal functions of the heart.

Before the heart developments maturely, the electric signals generated by CCS can slowly transmit through ventral and dorsal parts of the AVC between atria and ventricles (Valderrábano et al. 2006). After the cardiac chambers being divided, the fibrotic insulated tissues form from the atrioventricular cushion and epicardial mesenchymal tissues generate electric insulation between atria and ventricles, with the only retained atrioventricular electric channel being the AVB. The latter is a part of the atrioventricular conduction system, originating from interventricular septum crista and connecting with the AVC and AVN. With further development of the interventricular septum, the left and right bundle branches gradually develop and form from AVB branches under the endocardium (Miquerol et al. 2011). Cells forming AVB and bundle branches retain more characteristics of primitive cardiomyocytes and have gene expression patterns strongly resembling those of the AVN. They can substitute the pacemaker tissues above them to emit rhythmic electric impulses to form junctional rhythms during atrioventricular block. In contrast to the AVN, Cx40 is upregulated between the cells from AVB and bundle branches, leading to relatively fast conduction velocity.

Within the fully developed heart, distal bundle branches and the peripheral ventricular conduction system (PVCS) are the tissues containing only several layers of cells, and they constitute the rapid intraventricular conduction system with the formation of intracardiac trabecula (Rentschler et al. 2001; Tallini et al. 2006; Valderrábano et al. 2006). Even in lower vertebrates without PVCS development, similar conduction traits can also be observed with the formation of intraventricular trabecula (Sedmera et al. 2003). With the development of embryonic ventricles, the special expression of Cx40, expressed in the peripheral ventricular conduction system and bundle branches, Nppa, derived from the ventricular chambers (Houweling et al. 2002), and *Kcnk3* that encodes TASK-1 channel in the developing ventricular conduction system (Graham et al. 2006) are gradually restricted within the trabecula tissues while the simultaneous formation of PVCS and cardiac pericardium-linking layer do not express the aforementioned molecular markers. With the cardiac transcription factor Nkx2.5 regulating the cardiac developmental maturation in the mouse, the trabecular region of the myocardium finally forms the PVCS tissue. Consequently, the trabecular region of the myocardium is the tissue of origin for the PVCS in terms of structure and function (Fig. 1).

The *Shox2* gene and distribution of its expression

The human *SHOX* gene is located in the pseudoautosomal region of the X chromosome. The related homolog *SHOX2* gene is localized on chromosome 3. *SHOX* and *SHOX2* compose the short stature homeobox gene family in humans. The amino acid sequence and common homology

domains in the proteins encoded by human *SHOX* and *SHOX2* genes share 99% and 83% similarity (Blaschke et al. 1998; Semina et al. 1998). During the development of limbs, the *SHOX* expression is distributed in the relatively distal regions of the skeleton and matches the lesions of skeletal dysplasia caused by the above-mentioned diseases. *SHOX2* is expressed not only in the skeleton but also in branchial arches, the nasal cavity, heart, central nervous system, and human embryonic reproductive nodules (Clement-Jones et al. 2000). The mutation or deficiency of human *SHOX* can lead to genetic skeleton developmental diseases with short stature as a major clinical phenotype, including Turner syndrome, Leri-Weill dyschondrosteosis, and Langer mesomelic dysplasia (Ellison et al. 1997; Rao et al. 1997; Shears et al. 1998; Zinn et al. 2002). *SHOX2* was not considered linked to any known syndromes, until recently, but a p.H283Q missense mutation of *SHOX2* has been found related to the early-onset atrial fibrillation (AF) in humans (Hoffmann et al. 2016).

The homeodomain transcription factor Shox2 is a protein encoded by *Shox2* and participates in the development of multiple tissues and organs during mouse embryo formation. Currently, this gene family is found only in vertebrates, and named *Shox2* in rodents (Clement-Jones et al. 2000), which plays an important role in the development of skeleton and related tissues.

The mouse Shox2 protein shows 99% identity in its amino acid sequence of SHOX2, and its expression pattern at the embryonic stage is also similar to that of humans (Rovescalli et al. 1996; Blaschke et al. 1998; Semina et al. 1998; Clement-Jones et al. 2000). It was named as *Og12x* before, as *Prx3* in rats, and *SHOT* in humans. *SHOX2* expression is first detected in the upper limb at Carnegie stage (CS) 13 (mouse E10), as well as craniofacial, brain and heart since then (Rovescalli et al. 1996; van Schaick et al. 1997; Blaschke et al. 1998). Unlike *SHOX,* restricted *SHOX2* expression is also detected in the SAN of a human embryo of 7 weeks post-conception (Liu et al. 2011). Nucleic acid sequencing analysis revealed that there are two subtypes: Shox2a the longer and Shox2b the shorter, each of which contains the homologous domains, an SH3-binding domain, a P-loop cyclic nucleotide-binding site and the OAR domain (Furukawa et al. 1997). Both of the Shox2a and Shox2b function as transcription factors in a tissue-specific manner, during embryogenesis, including the SAN (Liu et al. 2011). In humans, SHOX2a and SHOX2b contain the homeodomain, an SH3-binding domain, and the OAR domain (Rovescalli et al. 1996; Blaschke et al. 1998) (Fig. 2), but the function of these isoforms is not clear. The mRNA of *Shox2* first appears in mouse E8.0 mesoderm, and is enriched in the nasal cavity, primitive palate, eye lids, peripheral tissue around optic nerve, developing heart, and limbs' cartilage (Blaschke et al. 1998).

In the mouse heart, *Shox2* gene is expressed earliest at dorsal primitive heart tube from E8.5, and on E9.5 its expression is limited to the cardiac inflow tract (IFT) region and the mesenchymal tissues of the transition zone between SV and common atria (Fig. 3). In sharp contrast to its expression in atria on E10.5, *Shox2* is expressed definitely in two SV-originating venous valves, left superior vena cava, and the pulmonary vein side of the cardiac proximal part of the connecting region between pulmonary vein and atria. Until E11.5, the *Shox2* gene-expressing region expands to two parallel fascicular zones crossing the atrium vertical axis formed by SAN and venous valves, and these regions will constitute an indivisible part of CCS later. Simultaneously, *Shox2* is expressed in the mouse AVJ and the upper part of ventricles, forming the left and right bundle branches (Sun et al. 2015) (Fig. 3).

The function of the *Shox2* gene in SAN differentiation and development

The *Shox2* gene is first expressed in the posterior part of the IFT region of the primitive cardiac tube. This gene is not only essential for IFT development and pacemaker cell differentiation but can also distinguish SAN tissue from atria in its expression status. By inhibiting *Nkx2.5* expression and activating the gene programs of pacemaker cells (Espinoza-Lewis et al. 2011), including *Hcn4* and *Islet1* (Hoffmann et al. 2013), Shox2 mostly plays a negative regulatory role in IFT development and in the cardiomyocyte gene program. Between E11.5 and E17.5, $Shox2^{-/-}$ mice die from SV and SAN hypoplasia and bradycardia induced by a reduction in pacemaker cell proliferation (Blaschke et al. 2007; Espinoza-Lewis et al. 2009). Mice with a *Shox2* hypomorphic allele similarly develop bradycardia and die within days after birth (Liu et al. 2011). In the $Shox2^{-/-}$ SAN region of mice, downregulation of genes *Tbx3*, *Hcn4*, and *Islet1*, as well as ectopic expression of *Nkx2.5*, *Nppa*, and *Cx40* can be observed with the promotion of pacemaker cell gene programs and the reduction derepression of atrial myocardium development cause (Blaschke et al. 2007; Espinoza-Lewis et al. 2009), while the *Tbx18* gene expression is unchanged in the SAN. Overexpression of the *Shox2* gene in African *Xenopus laevis* embryos causes a decrease in numbers of cardiac progenitor cells, downregulation of *Nkx2.5* expression, cardiac tube dysplasia and bradycardiac arrhythmia, similar to those found in $Nkx2.5^{-/-}$ animals (Espinoza-Lewis et al. 2009).

In the trailing edge region of IFT, SAN and within pulmonary vein, the antagonizing expression pattern of *Shox2* and *Nkx2.5* plays a critical role in determining the differentiation of cardiac progenitor cells into pacemaker cells or working cardiomyocytes. Disruption of this balance will change the direction of cellular differentiation (Ye et al. 2015a, b). A conditional knockout (cKO) of the *Shox2* gene in *Nkx2.5-Cre* cells can severely inhibit the expression of pacemaker program genes (*Tbx3*, *Hcn4*, *Islet1*) and promote ectopic expression of *Cx40*. Variations in the expression of the above-mentioned genes in adult mice can contribute the symptoms of sick sinus syndromes

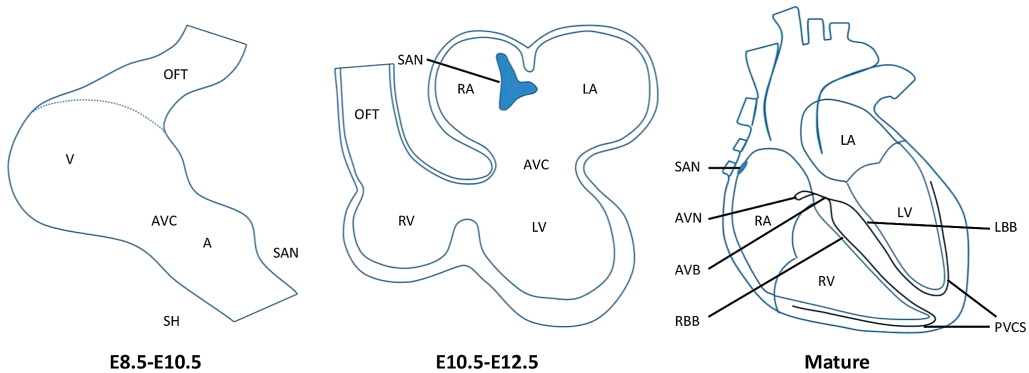

Fig. 1. Schematic representation of cardiac conduction system development.
After the formation of the primitive cardiac tube, mesenchymal cardiac progenitor cells gradually approach the cardiac tube to form the sinus horn, and sinus horn myocardium gives rise to the SAN, atrioventricular canal myocardium to the AVN. AVB is originating from interventricular septum crista and connecting with the AVC and AVN, the trabecular region of the myocardium forms distal bundle branches and the peripheral ventricular conduction system.
OFT, outflow tract; SAN, sinoatrial node; SH, sinus horn; A, atrium; V, ventricle; AVC, atrioventricular canal; L/RA, left/right atrium; L/RV, left/right ventricle; AVN, atrioventricular node; AVB, atrioventricular bundle; L/RBB, left/right bundle branch; PVCS, peripheral ventricular conduction system.

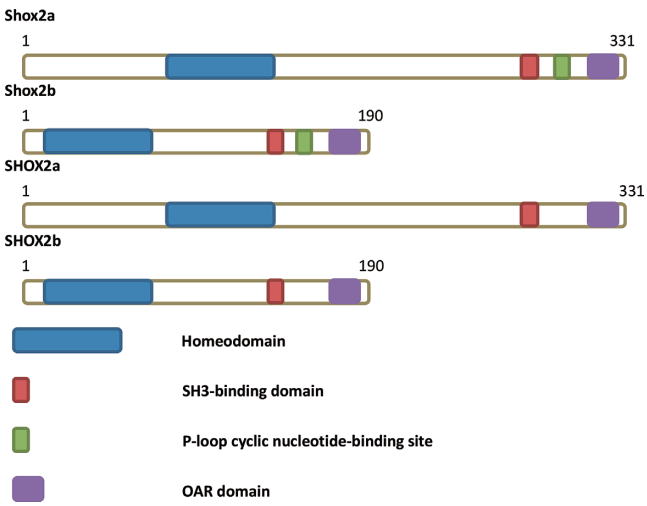

Fig. 2. Schematic representation of Shox2 and SHOX2 proteins.
The domains and motifs of Shox2 and SHOX2 are depicted according to the size.

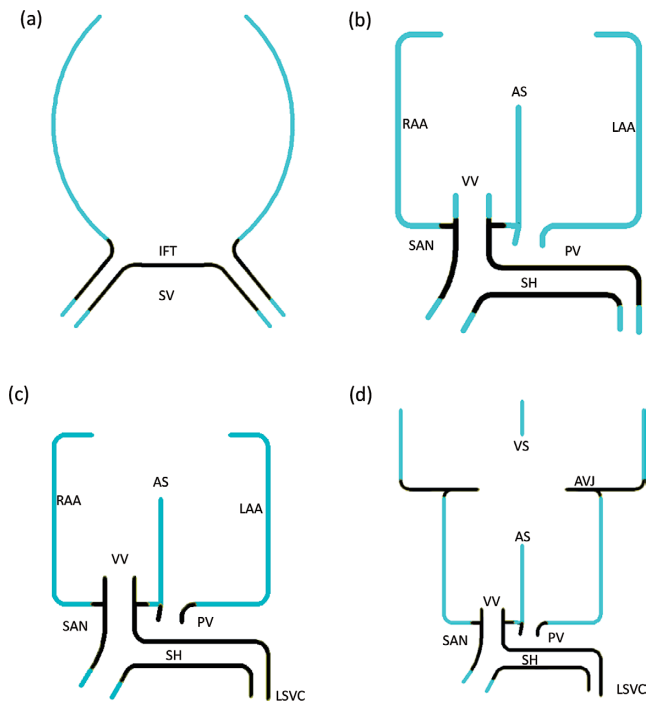

Fig. 3. Distribution of *Shox2* expression in embryonic heart.
(a) *Shox2* gene is expressed earliest at dorsal primitive heart tube from E8.5 (in black). (b) Expression of *Shox2* is limited to the inflow tract region and the mesenchymal tissues between SV and atria from E9.5. (c) On E10.5, *Shox2* is expressed in venous valves, left superior vena cava, and the connecting region between pulmonary vein and atria. (d) Until E11.5, the expressing region expands to two parallel fascicular zones crossing the atrium vertical axis, AVJ and the upper part of ventricles. IFT, inflow tract; SV, sinus venosus; SAN, sinoatrial node; SH, sinus horn; LAA, left atrial appendage; RAA, right atrial appendage; AS, atrial septum; LSVC, left superior vena cava; PV, pulmonary vein; VV, venous valves; AVJ, atrioventricular junction; VS, ventricular septum.

including severe sinus bradycardia, cardiac arrhythmia, and sinoatrial block. Hypomorphic variation of the *Nkx2.5* gene based on this phenomenon can lead to certain recovery of the expression of *Tbx3*, *Hcn4*, *Islet1*, and *Cx40* in the SAN trailing edge region. The pure *Nkx2.5* hypomorphic variation can cause the myocardium inside pulmonary vein to acquire the phenotype $Hcn4^{+}Cx40^{-}$ similar to that of pacemaker cells, whereas after a knockout of *Shox2*, it recovers to the working cardiomyocyte phenotype ($Cx40^{+}Hcn4^{-}$). Ectopic activation of the *Shox2* gene can downregulate *Nkx2.5* and lead to cardiac anomalies, whereas ectopic expression of *Nkx2.5* can lead to the phenotype similar to *Shox2* gene deficiency such as SAN dysplasia (Espinoza-Lewis et al. 2011). The above research confirmed that *Shox2* serves to activate the gene programs

of pacemaker cells by inhibiting *Nkx2.5* expression. Previous studies have pointed out that when there is a deficiency in *Nkx2.5*, gene *Shox2* is not required for the expression of *Tbx3*, *Hcn4*, and *Islet1*, whereas ectopic *Shox2* expression does not promote ectopic expression of *Tbx3* and *Hcn4* (Espinoza-Lewis et al. 2011). Recent research showed that in *Shox2*-deficient mice, the expression of *Islet1* in the SAN is also down-regulated; nonetheless, *Shox2* plays its regulatory role through binding to the enhancers of *Islet1* and overexpression of this gene can ameliorate bradycardia symptoms caused by *Shox2* gene deficiency (Hoffmann et al. 2013). Knocking out *Islet1* gene in mice can lead to similar effects (Liang et al. 2015), indicating that *Shox2* gene regulates SAN differentiation and development through a direct effect on *Islet1*. When *Shox2* and *Islet1* are expressed simultaneously, *Tbx3* is also upregulated and co-regulates the gene programs for the SAN (Hoogaars et al. 2007). All in all, the specific gene expression including *Shox2*, *Islet1*, *Hcn4*, and *Tbx3* can be suppressed by *Nkx2.5*; conversely, *Nkx2.5* expression can be inhibited by *Shox2* and *Tbx3* (Ye et al. 2015a).

During the development of IFT, *Tbx5* activates and upregulates *Shox2* gene expression. In the heterozygous *Tbx5*-mutant mice and *Tbx5*-deficient zebra fish IFT tissues, *Shox2* and *Bmp4* expression significantly decreases, while *Bmp4* gene expression is totally lost in *Shox2*-mutant murine IFT tissues (Garrity et al. 2002; Puskaric et al. 2010). Via collective effects, *Nkx2.5* and *Tbx5* can regulate the expression of *Shox2*, and further regulate *Bmp4* expression in the cardiac IFT at the embryonic stage (Puskaric et al. 2010). Exogenous *Shox2* can stimulate the *Bmp4* expression in African *Xenopus laevis* embryos through binding to the *Bmp4* promoter, while silencing of the *Shox2* gene inside cardiomyocytes can lead to reduced *Bmp4* expression (Puskaric et al. 2010). As a major regulatory factor in the BMP signaling pathway, *Smad4* also participates in this regulatory process.

The *Pitx2c* gene, expressed in the left compartment of the heart at embryonic stage, and in the left atrium, pulmonary vein, and right ventricle after birth, can inhibit *Shox2* expression through direct interaction with its promoter (Ai et al. 2006; Nowotschin et al. 2006; Wang et al. 2010, 2014; Ammirabile et al. 2012) and indirectly regulates *Shox2* expression through the inhibitory effects of microRNAs (miR-1792 and miR-106b-25) (Viereck and Thum 2017). The latter phenomenon is due to the expression of *Pitx2c* in the left heart compartment; thus, the *Shox2* expression is restricted in SAN tissue of the right atrium, and the SAN differentiation and development are activated via *Shox2*-inhibited *Nkx2.5* expression. Development of the SAN proceeds under the influence of this series of complicated interacting networks (Fig. 4).

The effect of the *Shox2* gene in the atrioventricular conduction system

During the development of the venous pole, with embryo stage mesenchymal cells aggregating to IFT, the *Shox2* gene starts to express in the proximal cardiac part of the connecting region between pulmonary vein and atria, whereas the emergence of the ectopic pacemaker in abovementioned tissues and in the myocardial sleeves of systemic venous return is believed to be one of the major pathological mechanisms of AF (Dobrev et al. 2012; Lip et al. 2016; Nattel and Dobrev 2016). The expression of *Nkx2.5* in pulmonary vein can facilitate maintenance of the traits similar to those of the myocardium within atria, while *Shox2* can change the differentiation direction by inhibiting the expression of *Nkx2.5* within the myocardium of pulmonary vein. Explanted *Shox2*$^+$ cells from the embryonic pulmonary vein show electrophysiological characteristics similar to those of sinoatrial node tissues and the traits of pacemaker cells, leading to pacing of surrounding *Shox2*-expressing cells *in vitro*; *Shox2* gene deficiency in the myocardium of the cardiac proximal part of pulmonary vein can turn off *Hcn4*, whereas hypomorphic variants of the *Nkx2.5* gene can reverse above differentiation toward a working myocardium (Espinoza-Lewis et al. 2011). A chromatin immunoprecipitation sequencing assay (ChIP-seq) showed that Pitx2c directly inhibits *Shox2* gene expression by binding to the target elements in intron 2 of *Shox2* gene expressed in the mouse left atrium (Wang et al. 2014) and can indirectly inhibit *Shox2* gene expression through the regulation by miR-17-92 and miR-106b-25 (Liu and Olson 2010; Espinoza-Lewis et al. 2011; Hoffmann et al. 2016). *Pitx2c* deficiency can lead to various atrial arrhythmias in adult mice, and during this process, the expression range and levels of *Shox2* in the left atrium are both increased (Wang et al. 2014). Thus, *Shox2* performs an important function in the development of the atrial conduction system and atrial arrhythmia.

AVJ is located in the integration region between atria and ventricles and actively participates in cardiac chamber division, CCS development, and facilitation of rhythmic atrioventricular contraction (Christoffels et al. 2010). AVJ develops from the dorsal mesenchymal protrusion, cushion septum tissues and the AVC-originating myocardium, including AVN progenitor cells (Munshi 2012; Ionta et al. 2015). In contrast to the previous opinion that *Shox2* expression is specifically distributed in the SAN and its precursor tissues, *Shox2* is also expressed in the dorsal mesenchymal protrusion tissues and AVJ during embryonic development (Sun et al. 2015). Pacemaker cell-specific expression levels of *Hcn4* and *Tbx3* genes and SAN-like electrophysiological characteristics have been identified in the *Shox2*$^+$ dorsal mesenchymal protrusion tissues and gradually disappear with the maturation of the AVN. *Shox2*-deficient mice present with dorsal mesenchymal protrusion dysplasia, and this phenomenon is related to

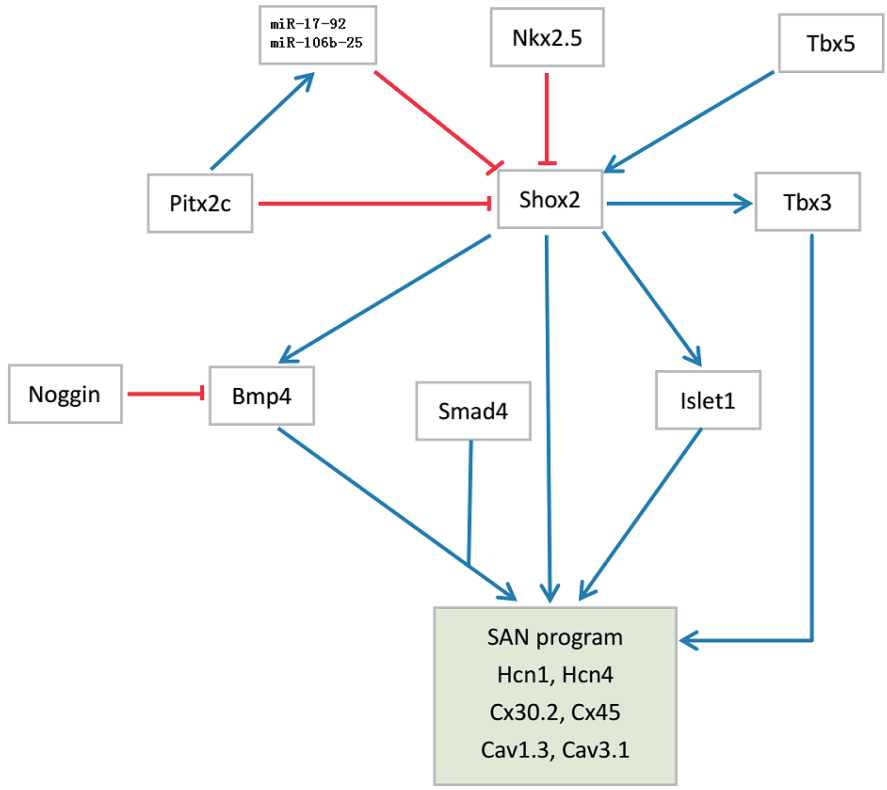

Fig. 4. Gene network of *Shox2* in SAN.
The schematic illustration of a cross regulatory network of *Shox2* that controls the SAN development.
Pitx2c, pituitary homeobox 2c; Nkx2.5, NK2 homeobox 5; Tbx3, T-box transcription factor 3; Tbx5, T-box transcription factor 5; Shox2, short stature homeobox 2; Bmp4, bone morphogenetic protein 4; Smad4, mother against decapentaplegic homolog 4; Islet1, insulin gene enhancer protein 1; Hcn1, hyperpolarization-activated, cyclic nucleotide-gated cation channel 1; Hcn4, hyperpolarization-activated, cyclic nucleotide-gated cation channel 4; Cx30.2, connexin 30.2; Cx45, connexin 45; $Ca_v1.3$, voltage-gated L-type calcium channel 1.3; $Ca_v3.1$, voltage-gated L-type calcium channel 3.1.

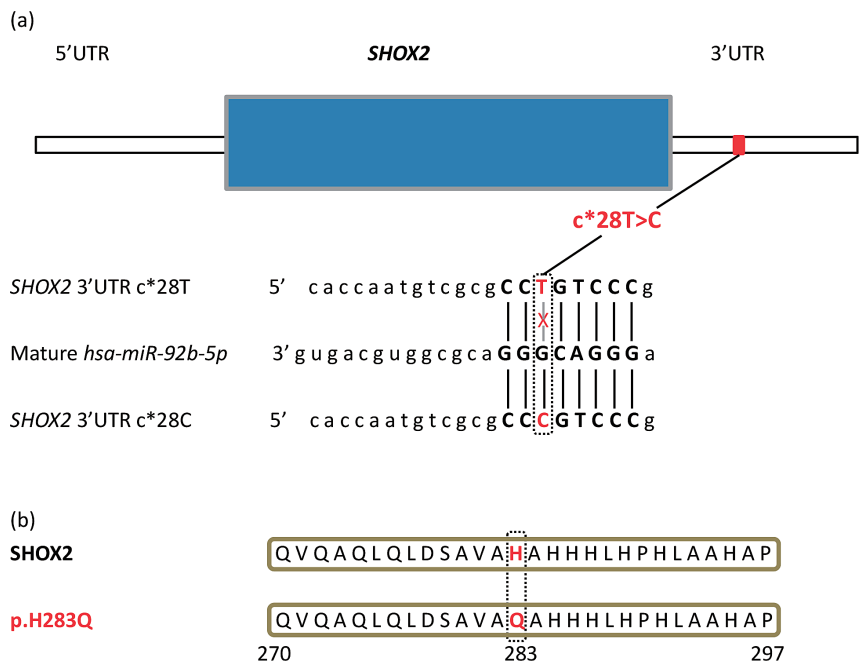

Fig. 5. p.H283Q missense mutation and c*28T>C mutation of *SHOX2* in AF patients.
(a) The position of the 3' UTR c*28T>C variant of the *SHOX2* gene and the binding site of has-*miR-92b-5p*. (b) A substitution of histidine by glutamine at position 283 of SHOX2a (p.H283Q).

increased apoptosis, decreased expression of *Bmp4* and *Hcn4*, ectopic activation of *Cx40*, and an abnormal action potential mode. A conditional knockout of the *Bmp4* gene or inhibition of BMP signaling pathway by Noggin can lead to similar dorsal mesenchymal protrusion dysplasia and decrease *Hcn4* expression, whereas *Bmp4*-activating alleles in *Shox2*$^{-/-}$ mice can alleviate the above-mentioned defects. Knocking out *Smad4* in the *Shox2*-Cre background leads to a direct binding of *Hcn4* and phospho-Smad1/5/8, causing *Hcn4* underexpression, thereby confirming the regulatory function of the Shox2-BMP pathway in dorsal mesenchymal protrusion development. Accordingly, the *Shox2* gene regulates dorsal mesenchymal protrusion differentiation and development through *Bmp4* and thus affects the development of the atrioventricular junction.

Although a conditional knockout of *Shox2* in mice can lead to thinness of the ventricular wall, dysplasia of atrium trabecular muscles, spherical heart and/or thickening in the left ventricular dense area and/or thinning in the right ventricular dense area because of the lack of the apex, its impact on the ventricular conduction system is still unclear (Espinoza-Lewis et al. 2011).

SHOX and *SHOX2* in human cardiac conduction system

The association of human *SHOX* and *SHOX2* genes with CCS remains unclear, since no direct evidence of such genes has been verified involving the development process of human CCS. However, replacement of *Shox2* with *SHOX* demonstrated a fully developed SAN and normal pacemaking function in *Shox2* deficient mice (Liu et al. 2011). In addition, overexpression of *SHOX2* during differentiation of embryonic stem cells upregulated the pacemaker gene program, enhanced automaticity and induced biological pacing upon transplantation *in vivo* (Ionta et al. 2015). According to a study on 378 early-onset AF patients (Hoffmann et al. 2016), the 3'-untranslated region (UTR) variant c*28T>C of the *SHOX2* gene is related to AF and leads to pulmonary vein interval elongation during sinus rhythm in patients (Fig. 5a). The aforementioned mutated version interacts with hsa-*miR-92b-5p* through newly generated functional sites, leading to a noticeable change in the expression of hsa-*miR-92b-5p* in the blood of patients with atrial fibrillation (Hoffmann et al. 2016) (Fig. 5a). Besides, the obvious decrease in *SHOX2* gene expression inside right atrial appendage in such patients is also one of the mechanisms behind the elevated incidence of AF. Transactivation research indicates that p.H283Q missense mutation severely affects the function of *SHOX2*$^{+}$ pacemaker cells (Fig. 5b), while the p.G81E variant of SHOX2 still has activity similar to the wild type (Hoffmann et al. 2016). Functional redundancy between two homologous genes may be a natural gift to prevent a single genetic defect from causing adverse consequences, but also brings challenges to our understanding. Multiple gene editing studies of human *SHOX* and *SHOX2* will be needed to solve the mystery.

The progress in *Shox2* gene research in the field of biological pacemakers

Among the therapeutic methods against cardiac arrhythmia, the advances in electronic heart pacemakers cannot be overlooked. Nonetheless, the implantation of pacemakers itself arises various problems including a deficiency in *in vivo* hormone responses, envelop infection, limited battery life, and inadaptability to the gradually enlarged cardiac chambers in pediatric patients. Recent studies on biological pacemakers will probably provide a complete solution to this series of issues in the near future (Kapoor et al. 2013; Vatta and Zipes 2013; Hu et al. 2014; Saito et al. 2015; Protze et al. 2017). The biological pacemaker is formed by electroactive cells, not only with the corresponding morphological and electrophysiological characteristics, but also with the dynamic gene expression program of autonomic response and pacemaker cell specificity (Ma et al. 2011; Vedantham 2015). Recently, with the maturation of experimental techniques for various pluripotent stem cells (Braam et al. 2009; Bernstein and Srivastava 2012; Burridge et al. 2012; Priori et al. 2013; Karakikes et al. 2015; Broughton and Sussman 2016; Atmanli and Domian 2017; Ratajczak et al. 2017), the research on induction of stem cells to directionally differentiate into pacemaker cells to form biological pacemakers has shown a breakthrough and thus should promote the CCS regeneration and *in vivo* transplantation therapies (Davis et al. 2011; Bernstein and Srivastava 2012; Xin et al. 2013; Broughton and Sussman 2016; Kishore and Khan 2016). Exogenous overexpression of human *SHOX2* in mouse embryonic stem cells can enhance the expression of Cx45, Hcn4, and endogenous *Shox2* and inhibit the expression of Nkx2.5 and Cx43 (Ionta et al. 2015). An embryoid body (EB) overexpressing the *SHOX2* gene can show higher automatic rhythmicity compared to pacemaker cells as well as the function of biological pacemakers after a transplant into the rat heart (Hashem et al. 2013). The neomycin gene driven by the *Shox2* gene promoter has been used to identify and separate embryonic stem cell-originating SAN and AVN cells, and these starlike cells have intercellular calcium-loading properties similar to those of pacemaker cells and show specific upregulation of *Hcn4*, *Cx45*, *Cx30.2*, *Tbx2*, and *Tbx3* (Hashem and Claycomb 2013). The contraction frequency of the EB with *Shox2* gene knockout obviously decreases, while the change in the gene expression pattern of the EB is reflected in the downregulation of *Hcn4*, *Cx45*, *Tbx2*, *Tbx3*, and *Bmp4* and upregulation of *Cx40*, *Cx43*, *Nkx2.5*, and *Tbx5* (Feng et al. 2016). In a recent study, lentivirus was used to infect canine mesenchymal stem cells to cause *Shox2* overexpression to enhance these cells' pacemaker function. Thus, *Shox2* has a substantial value in the field of biological pacemakers (Feng et al. 2016), which can promote CCS regeneration and may advance *in vivo* transplantation therapies via the development of the technology for

pluripotent stem cell-directed differentiation induction.

Conclusion

The characteristics of CCS differentiation and development are different from those of common cardiomyocytes. Currently, we know little about the corresponding regulatory genes involved in CCS differentiation and developmental processes. On the basis of the complexity of CCS differentiation and development, academia holds different opinions about which factors determine the origin, differentiation and development mechanisms. In order to elucidate the pathogenesis of cardiac arrhythmias induced by native or acquired abnormality of the CCS and for designing new therapeutic methods, further exploration of the origin of pacemaker cells and the functions of certain key genes during CCS differentiation and development is important.

The *Shox2* gene is intimately involved in the differentiation and development of many parts of the CCS, especially the sinoatrial node. With the in-depth advances in stem cell technologies, researchers will gain a better understanding of development of the CCS and the mechanisms of various cardiac arrhythmias. Clinical application of the *Shox2* gene in the field of biological pacemakers should foster ideas about new therapeutic methods.

Conflict of Interest

The authors declare no conflict of interest.

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