Review

Iroquois Homeodomain transcription factors in ventricular conduction system and arrhythmia

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Abstract

Iroquois homeobox genes, *Irx*, encode cardiac transcription factors, Irx1-6 in most mammals. These six transcription factors are expressed in different patterns mainly in the ventricular part of the heart. Existing researches show that *Irx* genes play key roles in the differentiation and development of ventricular conduction system and the establishment and maintenance of gradient expression of potassium channels, Kv4.2. Our main focus of this review is on the recent advances in the discovery of above-mentioned genes and the function of the encoding products, how *Irx* genes establish ventricular conduction system and regulate ventricular repolarization, how the individual and complementary functions can be verified to complement our cognition and leads to novel therapeutic approaches.

Key words: Arrhythmia, Ionic channels, *Iroquois homeobox*, Ventricular conduction system, Ventricular repolarization

Introduction

The conduction of electrocardiosignals and the synchronous contraction between ventricles with rhythmicity are essentially important for forming effective cardiac beating cycles and thus ensuring unidirectional blood flow circulation in whole bodies. As an indispensable part of the heart, ventricular conduction system (VCS) plays a vital role in coordinating the heartbeat to activate contraction from the apex and maximize efficiency of expulsion of the blood through aorta (systemic circulation) and pulmonary artery (pulmonary circulation) at the base of the heart. VCS is majorly composed of the following parts: atrioventricular bundle (AVB), left and right bundle branches (BB), and Purkinje fiber (PF) network. The deficiency or damage of this system and genetic loci involved in the pathogenesis will lead to slowed conduction velocities, abnormal activation patterns of the ventricles, different kinds of ventri-cular arrhythmia and even sudden cardiac death [1-3].

Homeobox genes encode various kinds of transcription factors containing homeodomains, and mutations of these genes or transcription factors can lead to dysembryoplasia, or even early embryonic death. Among which, recent studies have revealed *Iroquois homeobox* (*Irx*) family of cardiac transcription factors are important for the development of VCS[4] and heterogeneity of the ventricular repolarization of rodents and humans[5, 6]. This review focuses on the regulatory effects of these *Irx* transcription factors in VCS and grants a summarization of some unanswered questions about their developmental and molecular functions.

Iroquois Homeodomain transcription factors

Three homeobox genes of the *Irx* family,

caupolican, *araucan* and *mirror* were first discovered in *Drosophila* which play a critical role in establishing an organizing center that is essential for formation of follicle[7], head[8], eyes[9, 10], muscle[11], meso-thorax[12], lateral notum[13], bristles on the thorax[14] and heart development[15] and specification of the veins in the wing and associated sensory organs[16-19]. *Irx* gene family consists of *Irx1*, *Irx2* and *Irx4* in the *IrxA* cluster, and *Irx3*, *Irx5* and *Irx6* in the *IrxB* cluster in mouse and human[20] (Figure 1), 11 genes, *Ziro1a, 2a, 4a* of *IrxAa*, *Ziro1b, 4b* of *IrxAb*, *Ziro3a, 5a, 6a* of *IrxBa*, *Ziro3b, 5b* of *IrxBb* and *Ziro7* in *Zebrafish[21-24]*. In vertebrates, *Irx* gene family members encode highly conserved homeodomain-containing transcription factors of a 13 amino-acid domain (the Iro box) and the 3-amino-acid-loop-extension (TALE) family[25] (Figure 2), which are required for the opportune patterning and formation of the nervous system[26-31] and the heart[32], specification of the membranous labyrinth[33] and nephron segment fate during kidney development [34-36], lung development and maturation[37], pancreatic, retinal and microvascular endothelial cell development and function[38-40], female gonad[41] and early limb development[42] and also give an advantage to morbid obesity, type 2 diabetes mellitus [43], fused toes[44] and tumor cells[45]. In the heart, all the Irx transcription factors are expressed in unique and overlapping patterns, abnormality or absence of encoding *Irx* may cause congenital structural heart disease[46], cardiomyopathy[47, 48] and a variety of arrhythmia[49]. The present article focuses on their roles in VCS development, ionic channels maintenance and thus involvement in arrhythmia as below (Table 1).

Formation of ventricular conduction system during cardiac development

Before heart becomes developmentally mature, the electric signals generated from pacemaker tissue can be slowly transmitted through the ventral and dorsal parts of atrioventricular canal (AVC) between embryonic atrium and ventricle[50]. After the cardiac chambers have been divided, the fibrotic nonconductors consist mainly of atrioventricular cushion and extraepicardial mesenchymal tissues shield electric conduction between atrium and ventricle, with the exclusive atrioventricular electric channel, AVB. AVB is part of the VCS, deriving from interventricular septum crista and physically connecting with AVC and atrioventricular node (AVN). With the further development of interventricular septum, both left and right BB develop inch by inch and form from AVB branches under endocardium[51]. The cells forming AVB and BB continue to feature in primitive

cardiomyocytes and are more like the genetic and electrophysiological phenotypes of AVN. This specific lineage of cardiomyocytes can substitute for the upper positioned pacemaker tissues to discharge rhythmic electric impulses, junctional rhythms, during atrioventricular block. Different than AVN, Cx40 is enriched between the cells from these tissues, leading to relatively fast conduction velocity.

Within the maturely developed heart, the distal BB and PF network are the tissues containing only several layers of cells, and they constitute the rapid intraventricular conduction system with the maturation of endocardial trabecula[52]. Even in the lower vertebrates, similar conduction characteristics can also be recorded[53]. During the developmental process of embryonic ventricles, the regions specifically expressing Cx40, Nppa and TASK-1 in the heart are gradually restricted within the trabecula tissues, and form peripheral ventricular conduction system simultaneously with the cardiac pericardium linking layer not expressing the above-mentioned ventricular chamber and conduction system specific molecular markers. With the Nkx2.5 regulating the cardiac developmental maturation, the trabecular region of myocardium finally composes the peripheral ventricular conduction system in terms of structure and function.
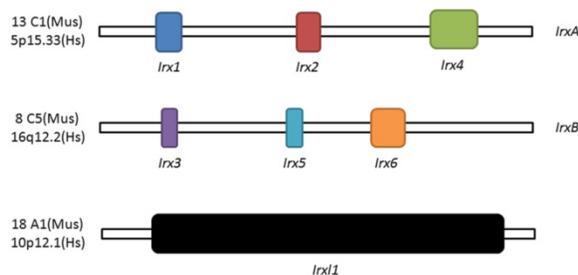

Figure 1. Schematic illustration of Irx and Irxl1 genes. Location and organization of Irx and Irxl1 genes in mouse (Mus) and human (Hs).
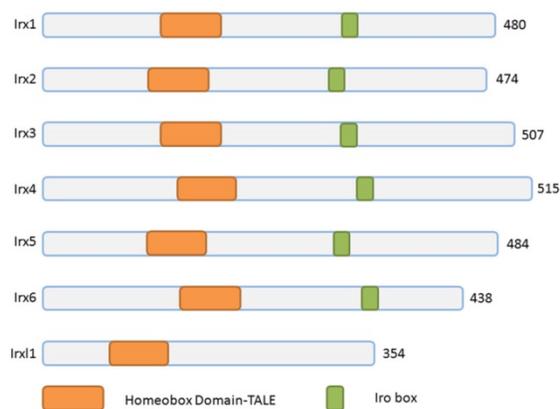

Figure 2. Schematic representation of Irx and Irxl1 proteins. The Irx, Irxl1 proteins and homeobox domains are depicted according to the size. TALE, 3-amino acid-loop-extension.

Table 1. Expression, function and associated phenotype of Irx genes in conduction system.

Gene	Cluster	Chromosomal position	Expression ranges	Physiological function	Associated phenotype
Irx1	A	13 C1(Mus) 5p15.33(Hs)	E10.5, interventricular septum trabecular E11.5, ventricular septum E14.5, regions of the developing VCS, including AVB and BB	Potentially involved the development of VCS	*Irx1b*-MO in zebrafish, bradycardia
Irx2	A	13 C1(Mus) 5p15.33(Hs)	identical to *Irx1*	Not been verified	None
Irx3	B	8 C5(Mus) 16q12.2(Hs)	From E9.5, trabeculated component of the ventricles	Establish fast conduction in the VCS	*Irx3*-KO mice, prolonged QRS, notched R waves and RBBB on ECG
Irx4	A	13 C1(Mus) 5p15.33(Hs)	E7.5, anterior domain of the linear heart; E9.5, atrioventricular canal, inner curvature, and proximal outflow tract; E11.5, ventricular myocardium.	Regulate chamber-specific gene expression, act as a multifunctional co-factor	No defect in cardiacelectricalactivity
Irx5	B	8 C5(Mus) 16q12.2(Hs)	E9, heart tube ventricle; E9.5, endocardial chamber myocardium (atrioventricular canal, inner curvature, and outflow track); E14.5, ventricular trabeculae, AVB and BB.	Establish and maintain the gradients of $I_{to,f}$ in ventricles	*Irx5*-/- mice, T-wave alterations
Irx6	B	8 C5(Mus) 16q12.2(Hs)	E10.5, endocardial lining of atrial and ventricular chambers	Not been verified	None
Irxl1		18 A1(Mus) 10p12.1(Hs)	No expression found in the heart	Not been verified	No defect in cardiacelectricalactivity

Expression of Irx and roles in the VCS

During the development process of heart, Irx1 expression could be detected as early as E10.5 in the ventricular septal trabecular myocardium, which is restricted to the ventricular septum at E11.5, and expands to the developing VCS regions from E14.5 onward, including the AVB and BB [54] (Figure 3), which implies that Irx1 may be involved in the development of VCS. *Zebrafish*, after injection of a morpholino oligonucleotide designed to knockdown *Ziro1b*, a gene homologous to *Irx1*, exhibits profound dose-dependent bradycardia, with slower heart rate reduced by 35% of the normal control[55]. Further studies should be carried out to evaluate the functions of *Irx1* knockout (KO) in conduction system.
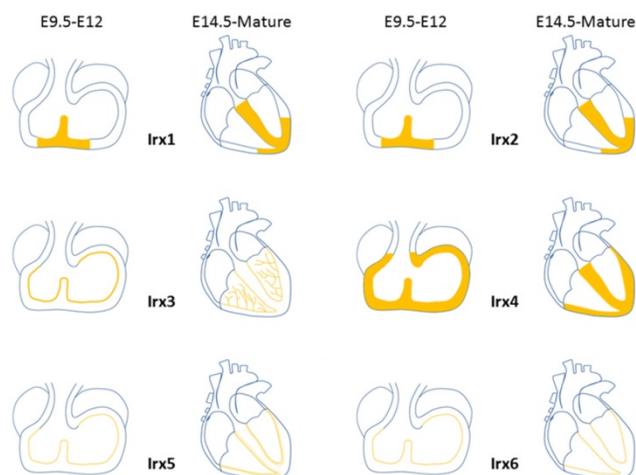

Figure 3. Expression distribution of Irx transcription factors in the heart. Schematic illustration of Irx transcription factors expression in the embryonic and mature hearts.

Expression pattern of Irx2 is nearly identical to that of Irx1 [54] (Figure 3). Despite the high expression in the developing heart, *Irx2* KO mice are fertile and well-grown, and show no phenotype. And Electrocardiographic analysis of 8- to 10-week-old mice revealed no difference between *Irx2*-/- mice and wildtype controls[56], implying that Irx2 is unessential to the cardiac conduction system, probably as a result of functional redundancy of other *Irx* genes.

In embryonic hearts, Irx3 can be detected in regions contributing to the VCS, such as the ventricular septum and trabeculae at E9.5 [54, 57]. Irx3 expression is overlapped in the mature heart with Cx40, a major functional gap junction protein of the AVB, BB, and PF of the VCS[51, 58-60], and accurately labels the mature VCS[4] (Figure 3). *Irx3*-null mice show QRS complex duration prolongation, notched R waves (R') on an electrocardiogram and an increase of HV interval (the conduction time between the AVB and ventricles) in electrophysiological examination, all indicative of prolonged conduction times in the VCS, by 2 weeks of age[4]. And telemetry ECG displays right axis deviation in 70% of *Irx3* KO mice, consistent with right bundle-branch block (RBBB) or abnormal impulse conduction in the right ventricular free wall as published in dog and mouse studies previously[59-61]. The impaired conduction in the VCS is identified with decreased expression of Cx40, which forms gap junction channels with a high conductance and is expressed in all compartments of the fast conduction system, from the AVB, left and right BB, to the whole PF network[60, 62]. Meanwhile, ectopic expression of Cx43, characterize the nodal

conduction structures with slow velocity[63], was also verified in the proximal VCS with a direct coupling to the septal myocardium. In accordance with the results that Irx3 activates Cx40 expression while inhibits Cx43. These results suggest that transcription of Cx40 is activated by Irx3 repressing an unidentified repressor indirectly, nevertheless, expression of Cx43 is likely to be suppressed by Irx3 directly through binding to the Cx43 promoter and antagonizing Nkx2.5 dependent activation, which is required for the postnatal differentiation of VCS[64, 65]. Thus, Irx3 plays a key role for maintaining rapid electric conduction through the VCS as well as proper ventricular activation. It is still unclear that whether deficiency in the VCS lacking Irx3 would induce the changes of the expression of gap junction expression located in the VCS. Additionally, optical mapping utilizing intact *Zebrafish* expressing the *in vivo* cardiac-specific fluorescent calcium indicator *Tg(cmlc2:gCaMP)^{s878}* showed that knockdown of *Ziro3a* led to low conduction velocity and asynchronization[66, 67]. Aforementioned results illuminate that *Irx3* is required for normal ventricular activation and impulse conduction.

In early stage of heart development at E7.5, Irx4 starts to be found in the anterior cardiac primordia, and in atrioventricular canal, inner curvature, ventricles, and proximal outflow tract at E9.5. Irx4 does not show any expression until E11.5 in the myocardial but not in non-myocardial cushion cells located in the atrioventricular canal. After E11.5, expression of Irx4 is restricted to the ventricular myocardium while absent from both atria and the outflow tract [54] (Figure 3). Unlike its involvement in establishing chamber-specific gene expression and differentiation of atrial and ventricular myocytes in the developmental heart [68-70], there is no direct evidence that Irx4 participates in the differentiation and development of VCS.

Irx5 expression was first detected at E9 at the ventral side of the looping heart tube. At E9.5, Irx5 started to be observed in the endocardial lining of the atrial and ventricular chamber myocardium, conversely, it is absent from the endocardium of the atrioventricular canal, inflow tract, inner curvature, outflow tract, and endothelium of the aortic arch arteries [54]. By E14.5, expression region of Irx5 in ventricles is confined to the ventricular trabeculae, AVB and BB[57] (Figure 3). The pattern of expression remained unchanged during expansion of the chambers [54]. Irx5 expresses in the region of immature AVB and BB, which opens the possibility of the sealed regulatory effect of the developing VCS.

Irx6's level of expression is much lower compared to the other five *Irx* genes and is detected

first at E10.5 in the heart[71]. Expression pattern of Irx6 is restricted to the endocardial lining of atrial and ventricular chambers, similar to Irx5 (Figure 3). The function of Irx6 in VCS has not been verified.

Actually, there is another divergent *Irx* family transcription factor gene *Irxl1, Iroquois homeobox-like 1,* or *Mkx, Mohawk,* which also belongs to the TALE (three-amino-acid loop extension) superclass of the atypical homeobox gene family[72] (Figure 1). *Irxl1/Mkx* encodes transcription factor Irxl1 (Figure 2) that regulates the expression of matrix molecule genes and differentiation in developing tendons[73-76], including type 1 collagen and Tenomodulin, volar plates[77], homeostasis of the periodontal ligament [78], MyoD expression and myoblast differentiation [79], brain and pharyngeal arch morphogenesis[80] and participates in some pathological process, such as, diabetic tendinopathy[81], anterior cruciate ligament degradation[82], serous ovarian cancer[83], and Rett Syndrome[84]. Mouse *Mkx* shares 56% homology with *Irx2* over the entire homeodomain, though, no expression found in the heart.

Irx in cardiacelectricalactivity and ionic channel

In mammalian mature heart, both Irx3 and Irx5 are expressed in the ventricles with transmural, endocardium to epicardium gradients [5, 85-87]. *Irx5^{-/-}* mice exhibit abnormal ECG characterized by T-wave alterations, whereas heart rate, atrioventricular (PR interval) and intraventricular conduction (QRS duration) are not affected, consistent with accelerated repolarization and reduced ventricular refractoriness [5]. Depression of T-wave originated from ventricular repolarization abnormity which in mice is attributed mainly to gradients of the fast transient outward K$^+$ currents, I$_{to,f}$[88-91]. I$_{to,f}$ in the rodent heart is initiated mainly by two voltage-gated potassium (Kv) channels, Kv4.2 and Kv4.3[92], along with KChIP2, an accessory subunit [93-95], and cardiovascular disease-related[96, 97]. In *Irx5* KO mice, the I$_{to,f}$ gradient in ventricular walls flattens as a result of selective increases in I$_{to,f}$ and Kv4.2 along with shorter action potential duration in endocardium[5, 87]. The inverse relationship between Irx5 and Kv4.2 expression suggests that an Irx5 repressor gradient negatively regulates Kv4.2 gene expression, forms an opposite gradient of I$_{to,f}$ and coordinates repolarization in the heart. Also, we should note that Irx5 can act as either an activator or repressor in a cell-type-dependent manner. For instance, the C-terminal portion of Irx5 physically binds to the N-terminal region of Irx4, through which Irx4 could suppress Kv4.2 gene promoter activation mediated by Irx5 in non-cardiomyocytes, like 10T1/2 fibroblastic

and PC12 cells. Further tests demonstrate endogenous Irx4 inhibits the function of transfected Irx5 to increase Kv4.2 promoter activity which degree may be determined by the expression ratio of Irx5/Irx4 [6]. This contradictorily activated or repressive impact of Irx5 in cardiomyocytes or non-cardiomyocytes is gained from recruitment of mBop (*Smyd1*), which has been verified as a cardiac corepressor essential for cardiomyocyte differentiation and cardiac morphogenesis[98, 99]. However, physical interaction between Irx5 and mBop might not exist, nor affect Irx5-mediated *Kcnd2* promoter activation [6], this is just contrary to previous conclusion that Irx5 represses Kv4.2 expression through mBop recruitment [5]. Therefore, further studies will be necessary to examine whether and how Irx5, Irx4 and mBop are interacting to regulate Kv4.2 expression.

Recent studies have revealed crucial roles of miRNAs as regulators of the development and function of the heart [100-103]. *Irx* gene expression is also regulated by miRNAs in the heart, including miR-1,which is highly conserved and enriched in heart and encoded by two separate genes, *miR-1-1* and *miR-1-2[104, 105],* and has important pathophysiological functions (regulates cardiac arrhythmogenic potential) in the heart[106]. There is a miR-1 binding site in the 3`UTR of *Irx5* , and in *miR-1–2⁻ᐟ⁻* mice, Irx4 and Irx5 expression increase in the heart, which exhibit lower heart rate, prolonged PR interval and QRS complex, consistent with the observations in *Irx5* KO mice, a decrease in *Kcnd2* along with increased Irx5 expression[107].

Irx and arrhythmia

As the *Irx* genes are critical for efficient conduction in the VCS development, defects of *Irx* genes may be associated with arrhythmia. Loss of *Irx3* leads to demolition of the rapid concerted spread of excitation in ventricles, prolonged QRS, notched R waves (R') and an increase of HV interval, right bundle branch block to be specific, which is considered to be associated with increase of mortality in patients with acute myocardial infarction[108], and a reasonable candidate of cardiac resynchronization therapy for heart failure patients with evidence of either electrical or mechanical left-sided delay[109].

Among the many factors can impact cardiac rhythms and cause arrhythmias, ion channel abnormality and defect in repolarization could contribute [2]. Establishment and maintenance of electric transmural gradients play a key role in ventricular repolarization. Irregular myocardium repolarization is associated with many kinds of malignant arrhythmia, including torsades de pointes (in long-QT, short-QT and Brugada syndromes),

ventricular tachycardia and fibrillation. The $I_{to,f}$ gradient in ventricular walls reduces and ventricular tachyarrhythmia could be reproducibly induced by intracardiac programmed stimulation in *Irx5* KO mice, consistent with alterations in ventricular repolarization to induce arrhythmia, so there is a fair speculation that defects of *IRX5* may be related to cardiac arrhythmia in human. [5] These findings suggest that loss of the ventricular transmural gradient of $I_{to,f}$ in *Irx5⁻ᐟ⁻* hearts is the major cause of enhanced arrhythmia susceptibility, or simply the shorten of ventricular refractory period. It will clearly be of interest to further explore the increased pathogenesis of arrhythmias in the *Irx5* KO mice.

Irx as new therapy for clinical applications

Recently, Ban et al. developed a method of profound significance to isolate pure ventricular cardiomyocytes by targeting the mRNA of Irx4, with high specificity using molecular beacons from differentiating human and mouse pluripotent stem cells[110]. This approach of producing a pure population of working cardiomyocytes offers a new option for cell therapy of heart failure and arrhythmia caused by it, and can be used to model the abnormal functional phenotype of genetic or idiopathic cardiac diseases, such as long QT syndrome, and to identify potential new therapeutic agents. For ischemic heart disease, the proangiogenic function of Irx3 in human microvascular endothelial cells opens new avenues for basic research and clinical applications[40].

Discussion

The six *Irx* genes of mammals are mainly distributed in the ventricles during embryonic development, including the parts that develop into the conduction system. Although only *Irx3* has been confirmed to participate in the establishment of the ventricular conduction system, other *Irx* genes have not been shown any abnormal phenotypes in the conduction system after the individual knockout (normal ECG characteristics), given the overlap and functional redundancy of these six genes, more often function as the form of two clusters, A and B[111], wherein *Irx* genes are closely related to each other, and regulate certain physiological and pathological processes in contradictory ways, so it's very likely that all these six genes play certain roles in the development of the VCS. Even if it is difficult to identify individual genes alone, it is still necessary to conduct further experiments through multiple gene editing methods to find specific effects of some or certain cluster of *Irx* genes.

In the same way, like the previous experiment,

Irx3 indirectly promotes Cx40 expression by combining with an unknown factor in the development of conduction system, Irx4 plays an important role in the process of cardiac compartmentation by combination with Vitamin D, the interaction between Irx4, Irx5 and mBop has yet to be clarified, and in addition, in recent years, the understanding of the three-dimensional structure of *Irx* gene clusters has been further deepened. In the process of verifying the function of individual *Irx* genes, it also needs to verify the function of the complexes and their structures.

In an interesting study, the gender-dependent effects of *Irx* genes on ionic channels have been verified[86], it may be possible to explain that female gender is associated with a higher risk of torsades de pointes than the opposite sex[112]. And further research in such gender difference could blaze new trails on the basis of that *Irx3* gene was confirmed to be involved in the development of the female sex gland[41] and several sex hormones, including progesterone, estradiol and testosterone, rained influence on ionic currents, ventricular action potential and QTc interval duration[112, 113].

Irx genes play critical roles in the differentiation development of VCS and maintenance of normal cardiacelectricalactivity. With the in depth development of genome-editing and stem cell technologies, people will gain in-depth understanding of the development process of VCS, the mechanisms of various cardiac arrhythmia and regulation pathways involved, the clinical application of *Irx* gene family as a genomic target of arrhythmia will develop ideas for initiating new therapeutic methods.

Abbreviations

VCS: ventricular conduction system; AVB: atrioventricular bundle; BB: bundle branches; PF: Purkinje fiber; Irx: Iroquois homeobox; TALE: 3-amino-acid-loop-extension; AVC: atrioventricular canal; AVN: atrioventricular node; KO: knockout; RBBB: right bundle-branch block; Kv: voltage-gated potassium channel.

Acknowledgments

Competing Interests

The authors have declared that no competing interest exists.

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