Iroquois Homeodomain Transcription Factors in Heart Development and Function

Kyoung-Han Kim, Anna Rosen, Benoit G. Bruneau, Chi-chung Hui, Peter H. Backx

Abstract: Numerous cardiac transcription factors play overlapping roles in both the specification and proliferation of the cardiac tissues and chambers during heart development. It has become increasingly apparent that cardiac transcription factors also play critical roles in the regulation of expression of many functional genes in the prenatal and postnatal hearts. Accordingly, mutations of cardiac transcription factors cannot only result in congenital heart defects but also alter heart function thereby predisposing to heart disease and cardiac arrhythmias. In this review, we summarize the roles of Iroquois homeobox (Irx) family of transcription factors in heart development and function. In all, 6 Irx genes are expressed with distinct and overlapping patterns in the mammalian heart. Studies in several animal models demonstrate that Irx genes are important for the establishment of ventricular chamber properties, the ventricular conduction system, as well as heterogeneity of the ventricular repolarization. The molecular mechanisms by which Irx proteins regulate gene expression and the clinical relevance of Irx functions in the heart are discussed. (Circ Res. 2012;110:1513-1524.)

Key Words: Iroquois homeobox ■ cardiac transcription factor ■ cardiac development

The heart is the first functional organ formed in the mammalian embryo in order to provide the necessary nutrients for growth and development.1 Throughout life, the contraction sequence of the heart in each beat is orchestrated by well-defined electric activation patterns designed to enable efficient mechanical pumping of blood. Electric impulses begin in the auto-rhythmic cells of the sinoatrial node and propagate to the atria, thereby initiating contraction and blood propulsion into the relaxed and compliant ventricles. The electric impulses then proceed with highly controlled delay to the ventricles via the specialized ventricular conduction system (VCS), comprising sequentially the atrioventricular node, His bundles, left and right bundle branches, and Purkinje fiber network. Electrical stimulation of the VCS ultimately results in highly ordered ventricular contractions proceeding from the apex toward the base as well as from the endomyocardium to epimyocardium,2,3 thus enabling efficient blood ejection into the systemic and pulmonary circulations. The ventricles then repolarize and relax in reverse order from epimyocardium to endomyocardium and from the base toward the apex.

Efficient heart function requires the precise and harmonious union of structure and function, which is achieved by precisely controlled patterns of gene expression within different cardiac regions at developmental stages. For example, in the early embryo, cardiac transcription factors (TFs) such as Nkx2–5,4 Gata,5 Hand,6 Nfat,7 and the T-box family members8 regulate many key aspects of heart development, including cardiac chamber septation, valve formation, and outflow tract morphogenesis. Consequently, mutations in these TFs are directly associated with several congenital heart diseases.9 On the other hand, some of these same genes control expression of key functional genes regulating contractile and electric properties of the mature heart.10–13

Recent studies have also identified and characterized the Iroquois homeobox (Irx) family of transcription factors in heart development and function of mice, rat, zebrafish, and humans.9,14–19 All 6 Irx TFs are strategically expressed in the developing mouse heart with unique and overlapping relationships reminiscent of the developmental and activation patterns of the heart. They are implicated in the establishment of cardiac chamber properties (ie, ventricle versus atrium),10,15 transmural gradients of ventricular repolarization,16,20 and the ventricular conduction system.17 This review summarizes the regulatory roles of these Irx transcription factors in heart development and function and outlines some unanswered questions about their developmental and molecular functions.
Overview of Iroquois Homeodomain TFs

Iroquois homeobox (Irx) genes were first discovered in Drosophila melanogaster, in which mutations of the Iroquois genes resulted in loss of bristle formation on the lateral notum, leaving only a band of bristles in the median part of the notum, reminiscent of the “Mohawk” common to the Iroquois tribes of Native Americans—from which the locus name is derived.\(^{21,22}\) Irx gene family members encode highly conserved homeodomain-containing TFs and play fundamental “prepatterning” roles in diverse developmental processes in both invertebrates (Drosophila) and vertebrates (Xenopus, zebrafish, chicken, and mammals).\(^{23-25}\) Unlike typical DNA-binding homeodomain (HD) transcription factors, which contain 60 amino acid residues with 3 alpha helices,\(^{26}\) Irx proteins contain an atypical HD with 3 extra amino acids between the first and second alpha helices, thus placing them in the 3-amino-acid-loop-extension (TALE) family of TF (Figure 1A).\(^{27}\) Irx TFs also have a conserved motif (Iro-box) of 13 amino acid residues in the carboxyl-terminal region, whose function has not been established.\(^{28,29}\) Although the HD and Iro-box confer DNA binding capability to the Irx proteins,\(^{23,25,28}\) its DNA binding sequence has not been completely resolved.

With the exception of Caenorhabditis elegans, which has only 1 ancestral Iroquois gene,\(^{30}\) Irx genes are generally found in genomic clusters. As shown in Figure 1B, D melanogaster expresses 3 closely related proteins, araucan (ara), caupolican (caup), and mirror (mirr), organized in a gene cluster called the Iroquois complex (Iro-C),\(^{21,31}\) whereas mammals have 6 Irx genes clustered in two 3-gene groups; the IrxA cluster on mouse chromosome 13 (human chromosome 5) contains Irx1, Irx2, and Irx4, and the IrxB cluster on mouse chromosome 8 (human chromosome 16) consists of Irx3, Irx5, and Irx6 (Figure 1C).\(^{28,30,32}\) Interestingly, zebrafish and pufferfish express 11 Irx genes, organized into 4 genomic clusters: IrxAa (Ziro1a, 2a, 4a), IrxAB (Ziro1b, 4b), IrxAb (Ziro3a, 5a, 6a), and IrxBB (Ziro5b, 5b) and 1 divergent member, called Ziro7,\(^{33-36}\) suggesting that additional round of genomic duplication has probably occurred in the fish.\(^{37}\)

Irx genes have early regulatory functions in embryonic patterning and specification and play a later role in tissue differentiation and function by establishing proper spatial and temporal patterns of target gene expression.\(^{33}\) In Drosophila, Irx genes control specification of large territories by establishing planar polarity in the eye and wing disc and by directing dorsal-ventral axis patterning in the ovary.\(^{21,29,31,38,39}\) In vertebrates, Irx genes also participate in developmental processes in several tissues. For instance, they are required for the proper patterning and formation of the nervous system\(^{35,40,41,25}\) as well as specification of nephron segment fate during kidney development.\(^{42-44}\) In addition, lung development and maturation,\(^{45}\) pancreatic cell development and function,\(^{46}\) retinal cell development in the eyes,\(^{40}\) female gonad development during sex determination,\(^{47}\) and early limb development\(^{48}\) are regulated by Irx genes.

Expression and Functions of Irx Genes in the Heart

Recent studies have established that all six Irx genes (Irx1-Irx6) are expressed in distinct and overlapping patterns in the developing and mature heart (Figure 2). These expression patterns are linked to their roles in structural specification and functional regulation in the heart as discussed in further detail below.

Irx1 and Irx2

The expression pattern of Irx1 and Irx2 is almost identical in the developing heart with expression being first detected around E10.5 in the trabecular cardiomyocytes of the ventricular septum.\(^{49,50}\) By E11.5, Irx1 and Irx2 expression is restricted to the interventricular septum, and from E14.5 onward, it expands to include the developing regions of the VCS, specifically the His-bundle and bundle branches. This expression pattern suggests that Irx1 and Irx2 could play a role in the development of the interventricular septum and ventricular conduction system. Only in zebrafish has the role of Irx1 in the heart been studied. Specifically, zebrafish irx1b, which is highly homologous to mouse Irx1, is expressed in the trabeculae of the ventricle as well as in the compact working myocardium, and knockdown of Irx1b results in slower heart rates via a mechanism that has not been studied.\(^{19}\) Since recent studies show that Irx1 has been implicated in other organ development and cancer,\(^{51-54}\) it would be of interest to examine the effects of Irx1 knockout (KO) in mouse hearts. Despite its strong expression in heart, Irx2 KO mice are viable and fertile, and display no notable phenotype in the developing heart.\(^{55}\) Moreover, hemodynamic and ECG assessments in the adult heart revealed no difference in cardio-

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<tr>
<th>Non-standard Abbreviations and Acronyms</th>
<th>Description</th>
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<tr>
<td>AMHC1</td>
<td>atrial myosin heavy chain-1</td>
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<tr>
<td>CNE</td>
<td>conserved noncoding element</td>
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<tr>
<td>ENDO</td>
<td>subendocardial myocardium</td>
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<td>EPI</td>
<td>subepicardial myocardium</td>
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<td>HD</td>
<td>homeodomain</td>
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<td>HDAC</td>
<td>histone deacetylase</td>
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<td>Iro-C</td>
<td>Drosophila Iroquois complex</td>
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<td>Irx</td>
<td>Iroquois homeobox</td>
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<tr>
<td>I_{hsf}</td>
<td>fast, transient outward K+ current</td>
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<td>KChIP</td>
<td>K+ channel interacting protein</td>
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<td>KO</td>
<td>knockout</td>
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<td>NKE</td>
<td>Nkx2-5 binding motif</td>
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<td>RBBB</td>
<td>right bundle-branch block</td>
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<td>RXR</td>
<td>retinoic X receptor</td>
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<td>SMHC3</td>
<td>slow myosin heavy chain 3</td>
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<td>TALE</td>
<td>3-amino acid-loop-extension</td>
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<tr>
<td>TF</td>
<td>transcription factor</td>
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<td>VCS</td>
<td>ventricular conduction system</td>
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<td>VDRE</td>
<td>vitamin D response element</td>
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<td>ventricular myosin heavy chain-1</td>
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<td>WT</td>
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vascular function between Irx2 KO mice and littermate controls. This suggests that Irx2 function is dispensable, possibly as a result of functional redundancy with other Irx genes.

Irx3

In embryonic hearts, Irx3 is expressed in regions contributing substantially to the VCS, such as the crest of the ventricular septum and the trabecular region of the ventricle, but is not expressed in the compact myocardial outer layer, the core of the ventricular septum, and the endocardial cells lining the trabeculae (Figure 2). In the adult heart, Irx3 expression is overlapped with Cx40, a functional marker of the His-bundle, bundle branches, and Purkinje fibers of the VCS, and accurately delineates the mature His-Purkinje system (which is far more extensive in left ventricular myocardium than in the right ventricular septum of mice and murine hearts)56. Adult mice lacking Irx3 exhibit abnormal electrocardiographic characteristics, such as prolonged QRS duration, R notches, and prolonged conduction times between the His- bundle and ventricle but not between atria and His-bundle (Figure 3A). Furthermore, whereas normal hearts show synchronous electric activation of the left and right ventricles, the activation pattern in Irx3 KO mouse hearts is consistent with right bundle-branch block (RBBB), which necessitates the spread of depolarization from the left ventricle to activate the right ventricle, as also documented previously in dog58 and mouse studies59 (Figure 3B). Block was associated with slowed conduction in the VCS as well as decreased expression of Cx40, the major connection required for rapid electric propagation between myocytes within the VCS. Interestingly, ectopic expression of Cx43 was also observed in the proximal VCS with clear evidence of an inappropriate direct coupling to the septal working myocardium (Figure 3C). Consistent with these observations, Irx3 activates Cx40 gene expression (Gja5) while repressing Cx43 gene expression (Gja1). Moreover, the Engrailed (EnR) repressor mutant of Irx3 increased Cx40 mRNA levels but decreased Cx43 mRNA levels. These results suggest that Irx3 indirectly activates Cx40 expression by repressing an unidentified repressor, whereas Cx43 expression appears to be directly suppressed by Irx3 since Irx3 binds to the Cx43 promoter and antagonizes Nkx2.5 dependent activation (Figure 3D). Together, these results suggest that Irx3 is required to maintain rapid electric conduction through the VCS as required for proper ventricular activation, via antithetical regulation of Cx40 and Cx43 expression in the VCS. It is, however, unclear whether the mild changes in gap junction expression in the VCS of Irx3 KO mice can fully account for the changes seen in mice lacking Irx3. Although it has been observed that His-bundle is ensheathed in a fibrous matrix in the adult mouse heart, which should provide a physical barrier to coupling of the VCS to the working myocardium, similar to the heart of large animals56,58 and rat,67 a previous study concluded that this fibrotic sheet may be lacking in mice resulting in a base-to-apex activation pattern within the interventricular septum.68 Clearly, additional experiments will be required to better understand the mechanism for the altered electric activation of interventricular septum in Irx3 KO mice and whether subtle structural alterations also occur with Irx3 ablation.

The function of Irx3 in the heart appears to be evolutionarily conserved. Expression of Ziro3a (homolog of mammalian Irx3) is detected in the zebrafish ventricle at 48 hours after fertilization. Optical mapping using live whole zebrafish expressing the in vivo calcium transient reporter Tg(cmlc2:gCaMP)s878 showed that knockdown of Ziro3a resulted in slowed and asynchronous impulse conduction. Although no histological evidence has been found for the existence of a cardiac conduction system in the embryonic zebrafish heart, these results suggest that Irx3 is required for proper ventricular activation.
**Irx4**

Irx4 is considered the most divergent member among the vertebrate Iroquois homeobox family. In early embryonic development, at E7.5, Irx4 transcripts are found in the anterior domain of the cardiac primordial, whereas at E9.5, it is expressed in ventricles, atrioventricular canal, inner curvature, and proximal outflow tract. At E10.5 to 11.5, Irx4 is still expressed in the myocardial cells of the atrioventricular canal but not in nonmyocardial cushion cells. After E11.5, Irx4 is exclusively expressed in the ventricular myocardium while absent from both atria and the outflow tract. Irx4 positively regulate chamber-specific gene expression by activating ventricular myosin heavy chain-1 (VMHC1) while suppressing atrial myosin heavy chain-1 (AMHC1) expression in the chick (Figure 4A). Although it is not known what determines the activator versus repressor function of Irx4, forced expression of Irx4 also activates VMHC1 and represses AMHC1 in the atria. On the other hand, the repressor form of Irx4 in the heart decreases VMHC1 expression while elevating AMHC1 expression in the ventricle, suggesting that Irx4 directly activates VMHC1, while indirectly suppressing AMHC1, possibly by activating a negative transcriptional regulator of the AMHC1 promoter, in the heart (Figure 4B). In addition, the vitamin D response element (VDRE) is essential for Irx4-mediated inhibition of the expression of SMHC3 (slow myosin heavy chain 3, a quail homologue of chick AMHC1), even though Irx4 does not directly bind to the VDRE. Rather, Irx4 interacts with retinoic X receptor α (RXRα), a major binding partner of the vitamin D receptor (VDR), which binds to VDRE. The repressive role of Irx4 in the ventricular expression of AMHC1 was further confirmed in vivo, as loss of Irx4 in mice leads to ectopic ventricular induction of SMHC3 reporter gene expression. Although Irx4 KO mice showed no morphological and histological defects, expression of ANF, an atrial-specific marker after birth, was observed in the ventricle of 10-day-old Irx4 KO mice. These data suggest that a major role of Irx4 in the heart is the suppression of atrial gene expression while possibly activating ventricular gene expression. Interestingly, Irx4-deficient adult heart exhibits increased expression of well-known heart diseases markers, such as BNP, α-skeletal actin, and β-MHC, at 6 week of age. Moreover, Irx4 KO mice develop spontaneous cardiomyopathy with impaired systolic function and cardiac hypertrophy around 6 month of age. These results suggest that loss of Irx4 in the heart disrupts ventricular properties, leading to cardiac decompensation. Thus, Irx4 is an important regulator of ventricular differentiation by defining the ventricular chamber and maintaining cardiac function.

**Irx5**

Cardiac expression of Irx5 is detected as early as E9 at the ventral side of the looping tubular heart. Of electric impulse propagation, respectively. D, Schematic model depicting mechanisms of Irx3 regulating gap junction gene expression shows that Irx3 antagonizes Nkx2.5-dependent activation of Cx43 gene expression while activating Cx40 gene expression by repressing a potential repressor of Cx40.
expression is found in the endocardial lining of the atrial and ventricular chamber myocardium but is absent from the endocardium, which lines the atroventricular canal, inner curvature, and outflow track (Figure 2). In the adult heart of mice, rat, canine, and human, Irx5 is expressed in the ventricle with transmural, subendocardial (ENDO) to subepicardial (EPI) gradients.16,20,71,72 Despite the distinct expression of the Irx5 in developing and adult heart myocardium, Irx5 KO mice are viable and fertile, with no noticeable morphological defects, suggesting that either Irx5 is not required for cardiac development or that other Irx genes can compensate for loss of Irx5. On the other hand, like the Irx3-deficient mice, mice lacking Irx5 exhibited abnormal ECGs characterized by T-wave alterations (Figure 5A), consistent with accelerated repolarization and reduced ventricular refractoriness.

T-wave deflections originate from heterogeneity of ventricular repolarization which in mice is attributed primarily to gradients of the fast transient outward K⁺ currents, Ito,f (i.e., EPI-ENDO).73,74 Ito,f in the rodent heart is encoded by 2 pore-forming α-subunits, Kᵥ4.2 and Kᵥ4.3, along with the accessory subunit, KChIP2.75 The Ito,f gradient in rodent ventricles is linked with gradients in Kᵥ4.2 expression across the ventricular wall,16,20,76,77 which are abolished in Irx5 KO hearts as a result of selective increases in Ito,f and Kᵥ4.2 along with action potential duration abbreviation in ENDO myocardium (Figure 5A).16,78 The inverse relationship between Irx5 and Kᵥ4.2 expression suggest that Irx5 determines the repolarization properties in mice by acting as a repressor of Kᵥ4.2 expression. Notably, similar to other Irx proteins,21,25,79 Irx5 can act as either an activator or repressor in a context-dependent manner. For example, Irx5 dose-dependently represses Kcnd2 promoter activity in cardiomyocytes while activating it in COS-7 cells (fibroblast-like kidney cells). This antithetical role of Irx5 in cardiomyocytes and noncardiomyocytes as a repressor and activator, respectively, is achieved by recruitment of a cardiac corepressor, mBop (Smyd1)80 (Figure 5B). Coexpression of mBop in noncardiomyocytes causes Irx5 to act as a repressor on the Kcnd2 promoter, whereas silencing mBop by RNA interference in cardiomyocytes eliminates Irx5-mediated repression on Kcnd2 promoter activity. In addition, Irx5 physically interacts with mBop, which is known to recruit histone deacetylase (HDAC),80,81 and HDAC inhibition relieves the inhibition of Irx5 activity by mBop. Additionally, it has been demonstrated that Irx5 physically binds to Irx4, and that Irx4 is required to suppress Kcnd2 promoter activation mediated by Irx5 in noncardiomyocytes.82 The degree of Kcnd2 promoter activation appears to be determined by the ratio of Irx5 and Irx4 expression, since siRNA against Irx4 alleviates Irx5-mediated repression of Kcnd2 promoter in cardiomyocytes. On the other hand, He et al.82 reported that coexpression of mBop does not affect Irx5-mediated Kcnd2 promoter activation in noncardiomyocyte and that Irx5 and mBop may not interact. Further studies will be necessary to examine how Irx5, Irx4 and mBop are interacting to regulate Kᵥ4.2 gene expression.

Although Irx5 KO mice have no detectable structural abnormalities, they are susceptible to ventricular tachyarrhythmia induced by intracardiac programmed stimulation. Although these findings are consistent with alterations in ventricular repolarization,76,83–85 it is unclear whether altered transmural repolarization gradients are sufficient in small mouse hearts, as they are in larger species to induce arrhythmia. It is conceivable that the elimination of the regional differences in Ito,f between septal apex and base, which is also observed in Irx5 KO mouse hearts,16 are the major cause of enhanced arrhythmia susceptibility when Irx5 is abolished, even though no differences in Irx5 expression are detected in between left ventricular apex and base.16 Alternatively, the enhanced
arrhythmias may simply arise from the global abbreviation of ventricular refractoriness in Irx5 KO hearts. It will clearly be of interest to further explore the cause of the increased cardiac arrhythmogenesis in the Irx5 KO mice. In addition to arrhythmia, we have also observed reduced contractility of both ex vivo Langendorff-perfused and in vivo Irx5 KO hearts, along with reduced Ca\textsuperscript{2+} transient amplitudes in ENDO, but not EPI, myocytes (unpublished data). These findings are consistent with previous studies showing a strong ENDO to EPI gradient in cardiomyocyte contractility in rodents\textsuperscript{86,87} as well as the observation that I\textsubscript{to,f} is a key regulator of cardiac excitation-contraction coupling by its ability to modulate early action potential repolarization.\textsuperscript{86} Indeed, we have also found using mice lacking I\textsubscript{to,f} that the effects of Irx5 on cardiac contractility are entirely mediated by alteration in I\textsubscript{to,f}. Although the effects of Irx5 on contractility are I\textsubscript{to,f-dependent}, Irx5 is also a critical determinant of proper adaptive responses to biomechanical stress that occurs independent of I\textsubscript{to,f}, suggesting that the functional effects of Irx5’s actions are not limited to modulation of electric repolarization.

\textbf{Irx6}

Irx6 expression is relatively weak compared with other Irx genes and is detected first at E10.5 in the heart. Expression pattern of Irx6 in the developing heart is similar to Irx5, showing expression in the endocardial lining of atrial and ventricular chambers while absent in the endocardial lining of the endocardial cushion.\textsuperscript{49,88} The function of Irx6 has not been examined yet.

\textbf{Regulation of \textit{Irx} Gene Expression}

Little is known about how Irx gene expression is regulated and patterned in the heart. Irx4 expression is severely reduced in Nkx2–5 KO embryos but is unaffected in embryos lacking Me\textsubscript{2c} or RXR\alpha.\textsuperscript{69} In addition, whereas Irx4 expression is activated in dHand KO embryos at E9.5, it is not maintained and completely lost at E10.5. These data suggest that Nkx2–5 and dHand are upstream regulators of Irx4 in the heart. It remains unknown whether other Irx genes are affected by loss of Nkx2–5 or dHand. On the other hand, overexpression of Irx3 or Irx5 in cardiomyocytes does not affect Nkx2–5 expression levels (unpublished data; Kyoung-Han Kim and Peter H. Backx). Downregulation of Irx4 was also observed in mouse embryos lacking mBop (Smyd1) or Smyd1-interacting transcription factor, skNASDAQ, both of which showed similar heart defects with ventricular hypoplasia.\textsuperscript{80,89} In addition, Irx3 expression was expanded in the heart of Foxp1 mutant embryos, which show various congenital heart de-
fects, such as outflow tract septation and cushion defects, ventricular septal defect, and a thin ventricular myocardial compact zone with cardiomyocyte maturation and proliferation defect. These observations suggest that Irx expression is affected by other cardiac TF during heart development. Further studies are required to investigate whether altered Irx expressions are directly controlled by these cardiac TF, or whether Irx3 expression changes represent compensatory responses to developmental defects induced by loss of these cardiac TF.

Irx gene expression is also regulated by miRNAs in the heart. The 3′UTR of Irx5 has a well-conserved miR-1 binding site, and Irx5 as well as Irx4 expression are increased in the heart of mice lacking miR-1–2, which exhibit developmental, electrophysiological, and cell cycle defects. Consistent with the observations in Irx5 KO mice, a decrease in K_v4.2 gene expression along with increased Irx5 was observed in the miR-1–2 knockout heart. Moreover, cardiac specific overexpression of miR-1 in mice leads to downregulation of Irx4, Irx5, and Irx6 in the heart. Since miR-1 plays multiple roles in the heart and its expression is altered in the diseased heart, future studies will be necessary to examine if miR-1 mediates these functions via regulating Irx expression in the heart.

Another important aspect in the regulation of Irx gene expression is the concerted transcriptional control of paired Irx genes by conserved noncoding elements (CNE). Patterns of Irx1 and Irx2 expression in the developing heart are almost identical, while the Irx3 and Irx5 expression patterns also being very similar in the heart. A likely possibility is the existence of cis-regulatory elements that controls the transcription of these clustered Irx genes. Indeed, sequence comparison of the IrxB clusters between different vertebrates demonstrated that there are multiple highly conserved noncoding regions located on large intergenic regions (denoted as gene deserts) between Irx genes. Using transgenic Xenopus and zebrafish embryos, these highly conserved noncoding regions were shown to be responsible for driving specific Irx expression domains. A recent study has further identified an evolutionarily conserved 3-dimensional architecture that enables interactions between the enhancers and promoters of Irx through the formation of DNA loop via the CCCTC-binding factor CTCF. As previously discussed, elucidating an enhancer specifically involved in the regulation and patterning of heart expression of Irx genes will be of importance to further comprehend the role of Irx genes in the normal and diseased heart.

Relationship of Irx Genes to Cardiovascular Disease

Because Irx genes play important roles in heart development as well as postdevelopment function, the loss of Irx genes or their mutation have many potential clinical implications. For example, two Irx4 mutations have been recently identified in patients with congenital heart diseases. Molecular analysis of these mutations revealed that the interaction between IRX4 and RXRα was significantly altered, suggesting this transcriptional complex play an important role in heart development. On the other hand, Irx3 KO mice develop conduction disturbances characterized by ECG alterations (QRS prolongation and notched R waves), RBBB, prolonged HV durations, slowed conduction velocities in the Purkinje fibers of the VCS, and an abnormal activation pattern of the ventricles. These conduction defects occur commonly in humans, particularly in the setting of heart disease, wherein ECG abnormalities correlate strongly with morbidity and mortality. Indeed, left BBB by itself can induce cardiac hypertrophy and disease, whereas both the development of left BBB and RBBB are strongly linked to heart disease progression and poor disease outcomes, including sudden cardiac death. Clearly, future studies are needed to determine whether mutations or expression changes in human Irx3 gene contribute to ventricular conduction disturbances in heart disease. It is important to appreciate that ECG abnormalities, particularly in association with BBB, are key criteria in selecting patients for ventricular resynchronization and defibrillation therapy, which, while alleviating and reversing disease symptoms, dismally affect long-term disease outcome in advanced heart disease. These observations emphasize the need to better understand the mechanisms underlying abnormal conduction in the setting of advanced heart disease.

Because transmural gradients of repolarization are major factors influencing vulnerability to cardiac arrhythmias, proper establishment and maintenance of electrical gradients within the ventricular wall are clearly of great interest and importance. In particular, enhanced dispersion of repolarization is associated with several forms of life-threatening reentrant and torsade-related ventricular arrhythmias, as occur in long-QT, short-QT, and Brugada syndromes, as well as catecholaminergic polymorphic ventricular tachycardia. In addition, heart disease is accompanied by electric remodeling, which is usually characterized by elevated heterogeneity of repolarization and increased susceptibility to arrhythmia. Interestingly, reduced transmural repolarization gradients can also occur in some heart failure patients, which may explain the increased susceptibility of Irx5 KO mice to arrhythmias, although these mice also show reduced refractoriness, a condition strongly linked to arrhythmia vulnerability. Thus, it is conceivable that changes in expression or mutations of Irx5 may be linked to cardiac arrhythmia in humans. Whereas Irx5 ablation increases K_v4.2-dependent I_{no,f} and abolish transmural repolarization gradients, Irx5 also negatively regulates the expression of KChIP2 (unpublished data; J.N. Wylie and B.G. Brunseau), which is responsible for creating the transmural gradients of I_{no,f} in humans and large animals. These observations suggest that Irx5 could help establish the I_{no,f} gradient in human hearts. Since I_{no,f} levels are critical determinants of the magnitude and kinetics of excitation-contraction coupling as a result of their influence on early repolarization (and the action potential notch in large mammals), Irx5 is also able to modulate regional differences in cardiac contractility (unpublished data; Kyoung-Han Kim and Peter Backx). Moreover, given that I_{no,f} is invariably reduced in heart disease and that Irx5 levels are found to be elevated in end-stage heart failure patients with dilated cardiomyopathy as well as in mice with heart disease,
Irx5 may act as an essential coordinator of the electric remodeling and impaired contractility observed in the diseased heart.

Discussion

In this review, we summarize the known functions and regulatory mechanisms of Irx genes in the mammalian heart. Although we now have a better comprehension of the importance of Irx TF in heart development and function, the molecular details underlying their actions and regulations are largely unknown and remain to be investigated.

Unlike many cardiac TF whose losses frequently lead to both morphological and functional defects in the heart, loss of single Irx gene does not affect heart morphology. These observations might suggest that the functions of Irx may be restricted to the establishment of the regional and/or electrophysiological as well as contractile properties in the heart. On the other hand, because cells often express multiple Irx genes, the absence of morphological defects in single Irx KO mice may arise from functional redundancy of the Irx family. Interestingly, although loss of Irx3 or Irx5 in mice develops distinct phenotypes, it appears that Irx3 and Irx5 have similar activities in vitro. For example, overexpression studies in cardiomyocytes revealed that, like Irx5, Irx3 is able to repress K_+4.2 gene expression and that, similar to Irx3, Irx5 regulates gap junction expression (Kyoung-Han Kim, Anna Rosen, Chi-chung Hui, Peter Backx; unpublished data). These data suggest that Irx3 and Irx5 could conceivably compensate for one another, although incompletely. Functional redundancy of Irx genes has been previously noted in other organisms. For example, in Drosophila, a deletion of at least 2 of the 3 Irx genes is required to cause a morphological defect, whereas the deletion of all three genes leads to more profound abnormalities. Moreover, fused-toes homozygous mice generated by a deletion of 6 genes including the entire IrxB cluster showed embryonic lethality with heart malformation. Therefore, it will be of interest to investigate the overlapping role of Irx genes in the heart using compound Irx mutant mice. However, we still lack insight into their redundant functions. Is it simply a reflection of functional redundancy or is it due to a more complicated TF complex formation among the Irx family? Or is it resulting from changes in expression level from embryo to adult? In addition, it is unclear what makes the difference between the Irx family and other cardiac TF families such as T-box family. For example, although T-box genes also often function in a combinational and hierarchical manner, unlike normal heterozygous Irx mice, haplo-insufficiency of most T-box members is associated with human congenital diseases. Further studies will be needed to study whether the redundant function of Irx genes is a reflection of a more recent evolution created by genomic duplication that could contribute to increased complexity and diversity as well as reduced risk of extinction.

Network and interaction of cardiac TF are critical for regulating gene expression during heart development, whereas very little is known about an interaction of Irx with other cardiac TF. Our studies revealed that Irx3 interacts with Nkx2–5 for Cx43 gene regulation, and Irx5 forms a complex with mBop (Smyd1) for regulating K_+4.2 gene expression. Are these interactions specific to Irx members? Indeed, our studies revealed that both Irx3 and Irx5 can interact with Nkx2.5, and it has been discussed that both Irx4 and Irx5 can act as an interacting partner of mBop, suggesting that Irx members probably share a domain for a TF complex formation. Thus, it will be of interest to examine whether all Irx members can function by interacting with these TFs such as Nkx2.5, mBop, and RxRα, and to study the molecular and structural domain of Irx proteins to reveal commonality and specificity of these protein-protein interactions. Moreover, previous studies showing the roles of the cardiac TF in the VCS or ventricle as well as transgenic mice exhibiting similar phenotypes to Irx3- or Irx5-deficient mice suggest potential interaction of Irx with other cardiac TF. For example, Cx40 expression in the VCS is regulated by Nkx2–5, Tbx5, Id2, Hopx, and HF-1b. Tbx3, expressed in the proximal region of the VCS, is also known to repress Cx40 and Cx43. Furthermore, K_+4.2 encoding Igf is regulated by several cardiac TF, such as Gata-Fog2, NF-kb, and calcineurin-Nfat pathway. In particular, it is notable that mice lacking Nfat3 exhibit an identical phenotype to Irx5 KO mice with loss of Transmural gradient in the heart without a change in Irx5 expression. Therefore, further studies will be necessary to examine whether Irx proteins interact with these cardiac TF to control gap junctions and ion channels expression in the heart. In addition, Irx is the least-known member of TALE class TF, which include Pbx and Meis. Pbx/Meis functions as cofactors to Hox and other TFs (ie, Engrailed, Pdx, and basic-helix–loop-helix proteins), thereby controlling various cellular mechanisms during embryonic development and organogenesis. Loss of Pbx/Meis members result in various forms of congenital heart defect. It has also been suggested that they act as “pioneer TF” that penetrate condensed chromatin and mark specific genes for activation as well as a bridging protein within the transcriptional complex. These suggest Irx may mediate transcriptional regulation similar to Pbx/Meis. Therefore, identifying interacting proteins with Irx is important to further understand molecular mechanism of Irx in gene regulation. For example, revealing the composition of Irx TF complex will help us to comprehend how Irx positively or negatively regulates gene expression in context-dependent manner.

Identification of the conserved DNA binding site of Irx has been of major interest. Although we have recently shown that Irx3 binds to the conserved Irx3/Nkx2–5 element “GGTAAATTG” to negatively regulate Cx43 transcription, it is not known whether all Irx members in the heart bind to this site to synergize or antagonize with Nkx2–5. A previous study has demonstrated that Drosophila Mrr2 preferably binds to a unique sequence “ACAnntTG,” which is also recognized by vertebrate Irx4. In addition, a prediction of homeodomain binding sites has revealed that Irx members bind to an indistinguishable 8-mer sequence “TACATGTA,” and they all share most of common amino acids among the DNA contacting residues. This uniform binding sequence and high sequence homology of all Irx members suggests that a binding partner of Irx may determine the
functional characteristics and diversities of Irx proteins. In addition, the palindromic sequence suggests that Irx binds DNA as a dimer,33 consistent with potential Irx multimeric complex formation,82 suggesting that Irx factors may function in homomeric or heteromeric Irx complexes. Clearly, further investigations to find binding sites and partner as well as new downstream candidate genes will be important to decipher the mechanism of transcriptional regulation of Irx in the heart.

Paradoxically, genetic duplication of the Irx cluster, overlapping expression of Irx genes as well as lack of detrimental phenotype in single Irx KO mice emphasizes the importance of their sophisticated roles in life. The fine-tuning role of Irx in regulating gene expression also implies a potential regulatory role in the differentiation of stem cells to generate specifically differentiated cardiomyocytes. As we are approaching the age of regenerative medicine, progress in our understanding of the regulatory roles and molecular mechanisms underlying Irx actions in the heart will provide important insights to develop better treatment option for congenital and acquired heart disease patients in the future.

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