TRANSCRIPTIONAL REGULATION OF KV4.2 GENE BY IROQUOIS FAMILY PROTEINS

by

Wenjie He

B.Eng., China Pharmaceutical University, China, 1998

M.S., University of Montana, 2004

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This dissertation was presented

by

Wenjie He

It was defended on

January 26, 2009

and approved by

Bruce Pitt, Ph.D., Professor and Chairman, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

William Walker, Ph.D., Associate Professor, Department of Cell Biology and Physiology, School of Medicine, University of Pittsburgh

Aaron Barchowsky, Ph.D., Associate Professor, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Dissertation Advisor: Koichi Takimoto, Ph.D., Associate Professor, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

TRANSCRIPTIONAL REGULATION OF KV4.2 GENE

BY IROQUOIS FAMILY PROTEINS

Wenjie He, PhD

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Normal cardiac rhythms are generated by an organized propagation of depolarization and repolarization. The voltage-gated transient potassium current (I_{to}) is a major determinant of cardiac action potential. The I_{to} is expressed in a gradient across the left ventricular wall of the hearts, which is essential for the proper repolarization sequence in the left ventricle. Altered expression of I_{to} is seen in hypertrophied and failing hearts and may contribute to the increased incidence of cardiac sudden death. Thus, elucidating mechanisms underlying the expression of I_{to} channels will provide basic knowledge essential for the prevention and treatment of cardiac diseases with high public health significance.

In the mammalian heart, I_{to} is produced by assembly of pore-forming Kv4 and accessory KChIP2 subunits. Differential expression of Kv4.2 gene underlies transmural gradient of I_{to} in the left ventricle in small rodents, whereas the size of I_{to} is correlated with different levels of KChIP2 in large animals. Recent studies have shown that atypical homeodomain Iroquois proteins are distributed in a gradient in the left ventricle and influence the expression of Kv4.2 and I_{to}. Therefore, this thesis examines the hypothesis that Irx proteins control Kv4.2 gene transcription in a cell-type specific manner and analyzes the underlying molecular mechanism. Irx3 and Irx5 are differentially expressed in a steep gradient in the left ventricle of rat hearts in an inverse pattern to Kv4.2 expression, whereas Irx4 is equally abundant in the ventricle. Irx5 activates Kv4.2 promoter in several non-myocyte cell lines, whereas the transcription factor

inhibits the promoter activity in neonatal ventricular myocytes. Moreover, Irx4 prevents Irx5 to activate the channel promoter. Structure-function studies establish that the C-terminus of Irx5 is required for its regulation of channel promoter, whereas the N-terminus of Irx4 mediates its action. Addition of histone deacetylase inhibitor relieves the inhibitory effect of Irx4. Deletion and mutation analyses demonstrate the presence of a previously unidentified Irx5-responsive element in the Kv4.2 distant promoter region. Collectively, these results indicate that the interplay between Irx4 and Irx5 contributes to the heterogeneous expression of Kv4.2 gene, and hence I_{to} density, in the left ventricle of rat hearts.

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PREFACE

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1.0 INTRODUCTION

1.1 GENERAL OVERVIEW

Electrical heterogeneity of cardiac myocytes is essential for normal cardiac rhythms. This heterogeneity is produced by different amplitudes of repolarizing potassium current expressed in each myocyte. It has long been noticed that there is a marked regional difference in the size of transient outward potassium current (I_{to}) in the hearts of many species including human, rat and mouse. For example, within the mammalian ventricles, the highest density is seen in epicardial myocytes, whereas the lowest is in endocardium.

Previous investigations have shown that I_{to} is mediated by heteromeric complexes formed by Kv4 α and KChIP2 β subunits. In small rodents such as mice and rats, differential expression of Kv4.2 is largely responsible for this transmural distribution of potassium current, whereas in larger mammals it is the distribution of KChIP2 that determines the I_{to} gradient. These observations indicate a tight control of the transmural gradient of I_{to} and its fundamental role in the maintenance of normal cardiac rhythms. However, very little is known about transcriptional mechanisms underlying the regional differences in these channel subunits in the heart.

Recent studies have shown that Iroquois (Irx) homeodomain transcription factors are expressed differentially across the left ventricular wall in an inverse expression pattern with Kv4.2 gene in the hearts of dogs, rats and mice (1, 2). Therefore, the focus of this study is to

identify the roles of Irx proteins in regulating transcription of Kv4.2 gene and the underlying molecular mechanisms. The hypothesis addressed here is that Iroquois family proteins control Kv4.2 gene transcription in a cell-type-specific manner. The overall finding of this study is that Irx4 and Irx5 cooperatively regulate transcription of Kv4.2 gene. Irx5 acts as a primary regulator of Kv4.2 gene transcription in myocytes and non-myocytes, while Irx4 is a suppressor of the Irx5 action. Structural requirement of Irx proteins for the regulation of channel promoter and identification of a *cis* element in the distal promoter suggest that Irx proteins control channel transcription by influencing the activity of other factors. These results establish the unique roles of individual Iroquois family members and the interplay between distinct Iroquois proteins in controlling gene transcription. They also suggest that the levels of distinct Iroquois proteins and their interacting partners will determine Kv4.2 gene transcription and electrophysiological phenotype of each myocyte.

1.2 MOLECULAR PHYSIOLOGY OF THE HEART

1.2.1 Cardiac Physiology

The heart is a muscular organ that supplies oxygenated blood and nutrients to the body. It is a dual circulatory system by which oxygen-poor blood is sent to the gas-exchange organ, whereas oxygen-rich blood is delivered to the entire body. Mammalian hearts contain two pumps each consisting of well-separated two chambers, atrium and ventricle. The atria act as a receiving chamber and booster pump, whereas the ventricles generate a major force to send the blood to the target organs. The right chambers return oxygen-poor blood to the lung, whereas the ones on the left send oxygen-filled blood to the entire body. The left ventricle is larger and consists of thicker muscle than the right chamber to generate strong forces necessary for the blood to reach terminal tissues.

The heart contains two functional components: the electrical conduction system and the working myocardium. Cardiac muscles are often characterized by their self-generating rhythmic beating. Indeed, all cardiac myocytes are capable of producing rhythmic action potential and beating in the early development. However, they lose this ability during development. The pace maker cells located at the sinoatrial node, the tip of upper wall of the right ventricle, retain the self-generating rhythmic electrical activity in the adulthood. In contrast, other myocardial cells, such as adult atrial and ventricular myocytes, cannot produce action potential in the absence of triggers. The electrical signals originated from the pace maker cells propagate through gap junctions between neighboring myocytes and Purkinje fibers, specialized myocytes connecting the atrioventricular node, located at the base of atria, to the ventricular walls. These specialized cells and their electrical signal-propagating connections throughout the heart are called the

electrical conduction system. The conduction system plays an essential role in coordinating beating myocytes in various heart locations in a synchronized fashion.

Ventricular myocytes constitute the working component of the heart. These cells typically generate action potentials in a notch-and-dome shape: rapid rise in membrane potential, followed by a ditch, sustained period of time at depolarized potential and smooth decline to the resting potential. The sustained period allows an increase in intracellular calcium (Ca^{2+}) concentration to induce contraction. These continuous changes in membrane potential are generated by the actions of various voltage-gated ion channels present in these cells. When cardiac myocyte is at rest, the internal and external concentrations of ions maintain in a balance, which sets a stable negative membrane potential (resting membrane potential). A rapid depolarization of an action potential occurs due to an activation of voltage-gated sodium (Na^{+}) channels and movement of Na^{+} into the cells. The influx of Na^{+} from extracellular fluid raises membrane potential. Once the threshold for the voltage-gated calcium channels is met, Ca^{2+} enters from t-tubules, invagination of plasma membrane. The increase in intracellular Ca^{2+} facilitates the release of Ca^{2+} from the sarcoplasmic reticulum, the mechanism called Ca^{2+}-induced Ca^{2+} release. The raised Ca^{2+} will cause contraction by its troponin binding and sliding movement of myofilaments. The depolarization ends because of the closure of the sodium channels at a particular membrane potential. Meanwhile, voltage-gated potassium (Kv) channels open and potassium ions move out of the cells, causing repolarization of the membrane potential to return to the resting level.

1.2.2 Diversity of Voltage-gated Potassium Channels

While one type of voltage-gated sodium and calcium currents dominates in ventricular myocytes, these cells contain various voltage-gated potassium currents with distinct time- and voltage-dependent properties and pharmacological sensitivities. Voltage-gated potassium currents were classically divided into two types: transient outward potassium current (I_{to}) and delayed, outwardly rectifying potassium current (I_k). The I_{to} is activated at subthreshold of action potential and rapidly inactivates. It is the major determinant of the early repolarization. The delayed rectifier does not show inactivation and is mainly responsible for the late repolarization to the resting potential.

The two types of voltage-gated potassium currents are further classified into several types. The delayed rectifier appears to consist of at least three distinct components: I_{kr}, I_{ks} and I_{kur}. The first current is very slowly activated without any detectable inactivation, whereas the second exhibits complex activation and inactivation kinetics that significantly contributes to the late repolarization. Inherited abnormalities in these currents and drugs affecting these currents are known to cause arrhythmias. The transient outward current is also comprised of two components: $I_{to,\,fast}$ ($I_{to,\,f}$) and $I_{to,\,slow}$ ($I_{to,\,s}$). Based on the kinetics of current inactivation and recovery from steady-state inactivation, as well as by the sensitivities of channels to the spider potassium channel toxins, heteropodatoxins, the two types of transient outward potassium currents have been identified in a number of cardiac cell types. Cardiac $I_{to,\,f}$ recovers rapidly from steady-state inactivation and is sensitive to heteropodatoxins. In contrast, the $I_{to,s}$ requires seconds to restore full activity from inactivation and is insensitive to the toxin. The $I_{to,\,f}$ predominates in cardiac I_{to} in many species, whereas the $I_{to,s}$ may increase under pathological conditions.

1.2.3 Molecular Correlates of Cardiac Voltage-gated Potassium Currents

Voltage-gated potassium channels are formed by tetramerization of pore-forming subunits. In addition, some channels are associated with ancillary subunits that influence gating properties and physiological regulation. For example, the I_{kr}, channel appears to be formed by the pore-forming KvLQT1 (KCQ1) and auxiliary minK (KCNE1) subunits. Likewise, ERG (KCNH1) constitutes the pore for the I_{ks} channel. Kv1.5 (KCNA5) and Kv2.1 (KCNB1) are considered to be major pore-forming subunits for the I_{kur} channel.

The cloning and expression studies have also identified several molecular candidates for the cardiac I_{to} channel. The first candidates are channels formed by the pore-forming subunit Kv1.4 (KCNA4). Kv1.4 contains a long-stretched amino-terminal peptide that acts as a ball-and-chain to produce rapid inactivation. Since Kv1.4 is less abundant in ventricular tissues in many species, Kv1.4 may be present as heteromeric channels with other Kv1-family members. Very slow recovery from inactivation of Kv1.4 homomeric and heteromeric channels suggests that these channels carry the cardiac $I_{to,s}$, a minor component of the I_{to}. Other candidates for the I_{to} channels are the ones formed by pore-forming subunits in Kv4 or KCND family. Kv4.2 (KCND2), a potassium channel gene, was originally cloned from rat heart (3). Functional studies in *Xenopus* oocytes expression system have revealed its induction of voltage-dependent outward potassium current with rapid inactivation (3, 4). The use of a dominant-negative Kv4.2 construct demonstrated that adenovirus-mediated overexpression of a truncated Kv4.2 suppresses I_{to} in adult rat ventricular myocytes (5). A similar reduction in I_{to} was later seen in mouse ventricular myocytes using a dominant-negative subunit with a missense mutation in the pore region (6). These results established the concept that Kv4.2 gene underlies I_{to} in mouse and rat ventricles. However, in canine and human hearts, Kv4.2 is less significantly expressed. Instead,

Kv4.3 (KCND3), another Kv4-family member, is abundant in these tissues (7, 8). Thus, Kv4-family pore-forming subunits are responsible for the major component of the cardiac I_{to}. These findings also indicate the species difference in the utilization of Kv4 members: Kv4.2 is the main subunit in rodent hearts, whereas Kv4.3 plays the major role in the generation of the I_{to} channels in larger mammals including human.

Heterologous expression of Kv4.2 or Kv4.3 indicated that these channels exhibit faster recovery from inactivation than Kv1.4-containing channels. Yet, the observed recovery was substantially slower than the native $I_{to,f}$ in cardiac myocytes. The auxiliary subunits that interact with Kv4 subunits were discovered with the use of cytoplasmic amino-terminal peptide of Kv4.2 as a "bait" in a yeast two-hybrid screening, and are named Kv channel interacting proteins (KChIPs) (9). Coexpression of Kv4 channels with KChIPs appeared to generate outward currents with rapid recovery from inactivation similar to the native $I_{to,f}$. KChIPs are generated from four genes (KChIP1-4). All KChIPs are highly abundant in the brain, whereas only KChIP2 is significantly expressed in the heart (9). Structural analysis has revealed that Kv4 pore-forming and KChIP accessory subunits assemble in a 4:4 stoichiometry to form a functional channel (10, 11). Therefore, Kv4-KChIP2 complexes underlies $I_{to,f}$, the major component of transient outward potassium current in ventricular myocytes.

1.2.4 The Heterogeneity of Cardiac Action Potential

The cardiac action potential differs significantly in different portions of the heart. These differences are essential for the proper sequence of cardiac rhythms and beating. While the initiation of action potential is triggered by the electrical signal propagated by the conduction system, the duration and shape of action potential are determined by the intrinsic electrical properties of individual myocytes. Earlier studies revealed that the heterogeneity of action potential is attributable to the size difference in the transient outward potassium current, I_{to}. For example, the I_{to} is significant in epicardium and midmyocardium in the canine ventricular wall (12), which gives rise to a notched appearance of the action potential. In contrast, myocytes in endocardium lack I_{to}, resulting in action potential with long duration without a notch. Similar regional differences in I_{to} have later been found in feline, rabbit, rat, mouse and human ventricular myocytes (13-16). The transmural gradient in the amplitude of I_{to} underlies the proper repolarization sequence in the left ventricular wall from epicardial to endocardial region (17), which has been shown to contribute to the J and T waves of the ECG (18, 19).

In most species, including human, the differential expression of I_{to} is the primary determinant of action potential heterogeneity and is not exclusive to left ventricular walls. For example, I_{to} densities are significantly higher in atrial myocytes than ventricular cells in human and rat hearts (16, 20). In contrast, mouse ventricular myocytes contain a larger I_{to} density than atrial cells (21). In canine and mouse hearts, I_{to} density is higher in the right ventricle (RV) than left ventricle (LV), and lower in the base of the LV than the LV apex. It is hypothesized that these intrinsic electrophysiological heterogeneities are responsible for a normal sequence of repolarization in healthy hearts. Loss of the repolarization heterogeneity can be proarrhythmic (22). The alteration may occur due to drug actions, inherited mutations, or acquired disease.

However, the exact functional importance of the heterogeneity of cardiac action potential remains largely unknown. Also the molecular mechanisms that establish and maintain the cardiac heterogeneity during normal development, as well as in adult hearts are unclear.

It appears that Kv4.2 mRNA is differentially expressed in a gradient across the left ventricular wall of rodent hearts, with expression eight times higher in epicardium than endocardium (7, 23). In contrast, Kv4.3 message is equally expressed in this tissue. This transmural gradient of Kv4.2 across the left ventricular wall parallels with the gradient of I_{to} density. However, in larger mammals including human, KChIP2 is differentially distributed across the left ventricular wall. Also KChIP2 protein in canine ventricle parallels with KChIP2 message, leading to further support to the hypothesis that KChIP2, not Kv4.3, underlies the transmural gradient of I_{to} in canine and human ventricles. In rat and mouse hearts, KChIP2 mRNA and protein are equally abundant across the left ventricular wall.

These findings indicate two important differences regarding I_{to} channels between rodents and large mammals including human. First, I_{to} channels reflect the heteromeric assembly of Kv4.2/Kv4.3, and KChIP2 in rat and mouse, and Kv4.3 and KChIP2 in large animals. Second, the difference in Kv4.2 expression underlies the regional difference in I_{to} density in rodents, whereas KChIP2 appears to be the primary determinant of the observed regional difference in I_{to} density in large animals.

1.3 IROQUOIS HOMEOBOX FAMILY GENES

The Iroquois homeobox (Irx) family genes encode homeodomain-containing transcription factors that play fundamental roles in many developmental processes. The homeodomain is a ~60-amino acid peptide consisting of three alpha helices and binds to DNA. Irx proteins contain an atypical homeodomain with the insertion of three amino acids between the first and second alpha helices. Proteins containing this atypical homeodomain are found in plants, fungi and animals and constitute the three-amino acid-loop-extension (TALE) superclass. Little is known about the structure or other characteristics of this atypical homeodomain except that it binds to DNA in a sequence-selective manner *in vitro*. In addition to the atypical homeodomain, Irx proteins share a 13-amino acid domain in the carboxyl-terminal region, called the Iro box, although no function has been assigned to this domain. Analysis of the human and mouse genomes revealed that there are six Irx-family genes clustered on two loci (24, 25). In mouse, Irx1, Irx2 and Irx4 are on the chromosome 13 to form the IrxA cluster, while Irx3, Irx5 and Irx6 are on the chromosome 8 as the IrxB cluster (25). The *Drosophila* genome contains only three Irx genes as a cluster. Furthermore, the sequence similarity between Irx members indicates that the two Irx gene clusters in mammalian genomes may be generated by duplication during evolution.

1.3.1 Irx4

Irx4 is the earliest known marker of the ventricular precursors and its cardiac expression is restricted to the ventricles during all stages of cardiac development (26, 27). Mice, frogs, birds and fishes have all show ventricle-specific expression of Irx4, suggesting that Irx4 has an evolutionarily conserved role in heart development (28). An ectopic expression experiment in chicken embryo has shown that a dominant-negative Irx4 molecule disrupts the chamber-specific expression of cardiac myosin heavy chain genes (27). Moreover, Irx4 deficiency in mouse causes an inappropriate expression of atrial genes in the ventricle resulting in adult-onset cardiac hypertrophy (26). However, the mechanism by which Irx4 regulates the expression of these cardiac genes is uncertain. A report suggested that Irx4 represses transcription of the atrium-type myosin heavy chain in the ventricle by forming an inhibitory complex with retinoic X receptors, instead of interacting with the promoter directly (29). Therefore, Irx4 at least in part, indirectly exerts its effect on gene transcription by interacting with other transcription factors.

1.3.2 Irx5

The Iroquois homeodomain protein 5 (Irx5) was first identified in mouse embryo with a similarity to other previously identified Irx members (30). However, the functional role of Irx5 is largely unknown. Very recent studies have shown that Irx5, along with another Iroquois homeobox gene Irx3, is differentially expressed across the left ventricular wall in a gradient in rat, mouse and canine hearts (1, 2). These factors are expressed at higher levels in the endocardium than epicardium. Moreover, Irx5-deficient mouse is proarrhythmic due to the loss of normal transmural repolarization sequence and Kv4.2 gradient across the left ventricular wall (2). These studies indicate that Irx5 is essential for the generation of transmural gradient of I_{to} across the left ventricular wall likely through regulating Kv4.2 gene transcription. However, the mechanism by which Irx5 controls transcription of Kv4.2 gene remains obscure. Moreover, the roles of other Irx members in the regulation of Kv4.2 gene are unknown.

1.4 SUMMARY

Normal cardiac repolarization is orchestrated by the assortment of various potassium currents. The transient potassium current is the primary determinant of early repolarization and influences the time course of repolarization. It has long been noticed that there is a transmural gradient of I_{to} across the left ventricular wall of the heart in almost all mammalian species. The heterogeneity of I_{to} is essential for synchronizing cardiac repolarization thereby contributing to the generation of normal cardiac rhythms. An alteration in the I_{to} gradient due to drug actions, inherited mutations (31), or acquired disease (32) can trigger arrhythmia which is the leading cause of sudden death. Despite the pivotal role of the region-selective I_{to} expression in cardiac pathophysiology, very little is known about the mechanism by which this heterogeneity is generated during heart development and maintained in adult hearts.

In the past few years, some progresses have been made in understanding the molecular mechanism underlying the generating of transmural gradient of $I_{to.}$ It has been found that the two Iroquois-family genes (Irx3 and Irx5) are distributed in a gradient across the left ventricular wall of adult rat, mouse and canine hearts, with highest expression in endocardium (1). The expression pattern of these transcription factors has made them potential candidates to control the regional heterogeneity of Kv4.2 and/or KChIP2 in the ventricle. Another study has shown that deletion of Irx5 gene in mouse abolishes the differential expression of I_{to} across the left ventricular wall, resulting in an increased susceptibility to arrhythmia (2).

Meanwhile, another Iroquois family member, Irx4, has gained much interest in controlling chamber-specific genes expression. Irx4 deficiency in mouse caused no obvious cardiac malformation, but resulting in an inappropriate expression of atrial genes, such as *Anf* and *α-skeletal actin* in the ventricle, leading to adult-onset cardiomyopathy. The relative mild

phenotype of Irx4-deficient mouse may result from the functional redundancy of other Iroquois family members. A similar functional redundancy has been seen with GATA4, GATA5, GATA6 and other transcription factors important for heart development. Irx4 coordinates both up- and down-regulation of various genes that fulfills ventricle-specific functional requirement. Therefore, Irx4 may also play important roles in regulating the region-selective Kv channel gene expression. A molecular study revealed that Irx4 forms an inhibitory heteromeric complex with retinoid X receptor α (RXR α) and vitamin D receptor (VDR) to repress the promoter activity of the *smyHC3*, a gene that is specifically expressed in the atria (29). It is unclear whether this ventricle-restricted repression mechanism by Irx4 found for the myosin heavy chain gene applies to other genes. Taken together, these recent studies all implied important roles of Iroquois transcription factors in controlling the chamber- and region-selective transcription of genes.

Therefore, the current study focuses on the potential roles of Iroquois family members in the regulation of Kv4.2 gene transcription. This study is also aimed at elucidating the cellular context in which these factors influence channel gene transcription.

2.0 THE EFFECTS OF IROQUOIS PROTEINS ON TRANSCRIPTION OF THE RAT KV4.2 GENE

2.1 ABSTRACT

It is well established that there is a gradient of Kv4.2 gene expression across the left ventricular wall in the hearts of small rodents (23). This differential expression evidently parallels with the transmural gradient of I_{to}. However, a fundamental question about how this potassium channel gene is transcriptionally regulated remains to be answered. Recent studies showed a regional difference in Iroquois homeodomain protein 5 (Irx5) in the left ventricle of mouse and rat hearts (1, 2). Irx5 belongs to a family of transcription factors that are essential for cellular speciation and morphogenesis during development. Therefore, the mRNA levels for all Iroquois family members and potassium channel pore-forming subunits in various regions of rat heart were first examined in this study. Irx3 and Irx5 were expressed in a steep gradient across the left ventricular wall in an inverse pattern to the expression of Kv4.2 gene. In contrast, Irx4 was equally abundant throughout the ventricular tissue. Other Irx members were either undetectable or very low without any region selectivity. Luciferase reporter gene assays were then used to test the effects of differentially-expressed Irx3 and Irx5, as well as abundant Irx4, on Kv4.2 promoter. Irx5 influenced the promoter activity of rat Kv4.2 gene in a cell-type specific manner: it decreased the promoter activity in neonatal ventricular myocytes and increased the

activity in 10T1/2 cells (a murine embryonic fibroblast cell line). Irx4 appeared to suppress the Irx5-induced enhancement of promoter activity in fibroblast cells. The dose relationship between Irx4 and Irx5 indicated that the ratio of Irx5/Irx4 might determine the level of Kv4.2 gene transcription. Furthermore, Irx4 siRNA was employed to reduce the endogenous expression of Irx4 in neonatal myocytes. The siRNA-mediated suppression abolished the inhibitory activity of Irx5 and further increased Kv4.2 promoter activity in response to Irx5. Thus, Irx5 acts as a primary regulator of Kv4.2 gene transcription with opposite effects in myocytes and non-myocytes, whereas Irx4 suppresses the stimulatory activity of Irx5. Given the abundant expression in the rat ventricle, Irx4 might constitutively prevent the stimulatory effect of Irx5 and, at least partially, be responsible for, the inverse relationship between Irx5 and Kv4.2 levels across the left ventricular wall.

2.2 INTRODUCTION

In the mammalian heart, there is a marked regional difference in the shape and duration of action potential. It is hypothesized that this spatial heterogeneity of action potential is responsible for the normal spread of excitation and the proper dispersion of repolarization in the heart. One such example is the pronounced transmural gradient of repolarization current (I_{to}) across the left ventricular wall. I_{to} is voltage-dependent transient outward potassium current that contributes to the early stage of repolarization and influences the time course of action potential. The cardiac I_{to} channels are produced by assembly of pore-forming Kv4.2 and/or Kv4.3 and accessory KChIP2 subunits. In small rodents, differential expression of Kv4.2 gene is the molecular basis of the I_{to} gradient across the left ventricle wall. Despite the fact that the transmural gradient of Kv4.2 has been documented for a long time (23), the mechanism underlying this region-selective expression remains largely unknown. In addition, the downregulation of Kv4.2 gene expression has been seen in hypertrophied and failing hearts in various animal studies (33-35). Yet, little studies have examined the transcriptional regulatory mechanism controlling Kv4.2 gene. Our laboratory has isolated rat Kv4.2 promoter and characterized the basal promoter lacking TATA box (36). Identification of this promoter promoted us to elucidate the molecular mechanism underlying the region-selective Kv4.2 gene transcription.

The *Iroquois* (*Irx*) atypical homeobox genes encode a family of transcription factors implicated in key developmental processes in both vertebrates and invertebrates. They are characterized by the presence of a highly conserved 63-amino acid homeodomain with a 3-amino acid extension, which places them into the three-amino acid-loop-extension (TALE) superclass (37). Outside the atypical homeodomain, Irx proteins share little sequence similarity, but all

contain a highly conserved 13-amino acid motif, called the Iro box. Yet, functional roles of these domains and other diverse regions largely remain unclear.

The *Iroquois* homeobox genes, *ara* and *caup*, were first identified in *Drosophila* and control proneural genes expression (38, 39). The vertebrate *Iroquois* homeobox genes show localized expression patterns and encode transcription factors that are essential for organogenesis and regionalization during embryonic development of various organs, such as kidney, brain, lung and heart (40-43).

Earlier studies have shown that the cardiac expression of Irx4 is restricted to the ventricle to control the expression of chamber-specific genes (26, 27). For instance, Irx4 suppresses the atrial myosin heavy chain-1 gene expression (*MyHC-1*) and stimulates the ventricular *MyHC-1* expression in the ventricle of embryonic chicken (27). Similarly, deletion of the Irx4 gene in mouse caused aberrant ventricular gene expression including decreased expression of *eHand* and inappropriate postnatal ventricular expression of *ANF*, *α-skeletal actin*, and *βMHC*, therefore resulting in adult-onset cardiac hypertrophy (26). The role of Irx4 in directing chamber-specific gene expression has further been confirmed by experiments of mating Irx4-deficient mice to transgenic mice expressing a reporter gene under the control of the atrium-restricted *slow myosin heavy chain 3* (*sMyHC3*) promoter. The lack of Irx4 caused the appearance of the atrial specific promoter-driven reporter in the left ventricle (26). A detailed *in vitro* functional study showed that Irx4 indirectly influences the *sMyHC3* gene expression by forming heteromeric complexes with VDR and RXRα that bind to the vitamin D response element (VDRE) in the *slow MyHC3* gene promoter (29).

Other Iroquois members that may play important roles in controlling region-selective genes expression in the heart include Irx5. Irx5 cDNA was initially isolated from a cardiac

library using the conserved homeobox region of the chicken *Irx4* as a probe (44). Recent studies suggested the importance of Irx5 in controlling the region-selective expression of Kv4.2 gene in the heart. A microarray analysis and RNase protection assays have shown a transmural gradient of Irx3 and Irx5 expression across the left ventricular wall of rat and canine hearts in an inverse pattern to that of the Kv4.2 gene (1). Costantini *et al*. (2005) made a more intriguing observation that Irx5-deficient mice lack the gradient of cardiac repolarization due to increased expression of Kv4.2 gene in the endocardium, resulting in an increased susceptibility to arrhythmia. They also showed that Irx5 represses Kv4.2 promoter activity by recruitment of the muscle transcription repressor mBOP. Taken together, these studies suggested pivotal roles of Iroquois proteins in determining the region-selective gene expression patterns in the heart. However, the molecular framework by various Iroquois members in controlling transmural gradient of Kv4.2 remains unclear. Therefore, we first performed a full examination of the expression of Iroquois family members and Kv channel pore-forming subunits in various regions of rat heart. In addition, the effects of various Iroquois members on the promoter activity of Kv4.2 gene were examined. The data presented in this chapter clearly indicate that Irx5 regulates Kv4.2 gene transcription in a cell-type specific manner. Also the relative ratio of Irx5/Irx4 determines the promoter activity of Kv4.2 gene. These findings imply that the cooperation of Iroquois family members is essential for establishing the transmural gradient of Kv4.2 gene in rodent hearts.

2.3 METHODS AND MATERIALS

2.3.1 Cell Culture and Reagents

2.3.1.1 10T1/2 Cells

Murine C3H 10T1/2 fibroblast cell line was originally obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in the laboratory. It is derived from early mouse embryo and exhibits less differentiated fibroblast properties. These cells were grown at 37ºC under 5% CO_2 atmosphere in Basal Medium Eagle (BME) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% $_L$-glutamine (Invitrogen, Carlsbad, CA). The cells were passed just before confluence.

2.3.1.2 Neonatal Ventricular Myocyte

Primary cultures of neonatal cardiac myocytes were prepared from the ventricles of 1-day-old Sprague-Dawley rat pups as described previously (45). Neonatal ventricular myocytes were plated on 60-mm plastic dishes with Minimal Essential Medium (Invitrogen, Carlsbad, CA) supplemented with 5% calf serum and antibiotics overnight in a humidified incubator with 1% CO_2 at 37ºC. The medium was then changed to the one without serum for further culture. Myocytes were maintained in serum-free medium for the entire experimental period.

2.3.1.3 HEK293 Cells

HEK293 human embryonic kidney epithelial cells were originally obtained from ATCC American Type Culture Collection (ATCC, Manassas, VA) and maintained in our laboratory.

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS at 37ºC under 5% CO_2 atmosphere.

2.3.1.4 Reagents

Anti-Myc and anti-Flag monoclonal antibodies were purchased from Sigma (St. Louis, MO). Anti-GFP was from MBL (Nagoya, Japan). Secondary antibodies conjugated with horseradish peroxidase were from Jackson Laboratories (West Grove, PA). Chemiluminescent reagents were from Pierce (Thermo Fisher Scientific *Inc*., Rockford, IL). Synthetic oligonucleotides were from Integrated DNA Technologies (Coralville, IA) unless otherwise specified.

2.3.2 RT-PCR Analysis

2.3.2.1 Animal Care and Tissue Isolation

The animal investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was performed under the animal protocol approved by the University of Pittsburgh Animal Committee. Hearts were obtained from 12-weeks old male Sprague-Dawley rats obtained from Taconic (Hudson, NY). Various heart regions were dissected using fine scissors and forceps, rapidly frozen on dry ice, and stored in liquid nitrogen (-70ºC).

2.3.2.2 Total RNA Isolation from Tissue Samples

Total RNA was isolated by one step extraction with acid phenol-chloroform guanidium thiocyanate (46). Briefly, frozen tissues were crashed into powder-like pieces in liquid nitrogen. The crashed tissues were homogenized in denaturing solution (solution D: 4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-mercaptoethanol) using a polytron homogenizer (PowerGene 700, Fisher) at speed 20. The homogenate was mixed vigorously with 0.1 volume of sodium acetate (pH 5), one volume of water-saturated phenol and 0.2 volume of chloroform-isoamylalcohol (49:1). The mixture was centrifuged at ~10,000 *g* for 30 minutes at 4ºC. The obtained aqueous layer was mixed with one volume of isopropanol, and RNA was precipitated by centrifugation. The isolated crude RNA was further purified using a column-based method (RNeasy Mini Kit, Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically using the absorbance at 260 nm = 40 μg. RNA samples were stored at -80ºC until used.

2.3.2.3 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

First strand cDNA was synthesized with 2.5 μg of total RNA in a 20-μl reaction, using oligo(dT)20 primer and ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA) at 50ºC for 1 hour. For a standard PCR reaction, 0.5 μl of the synthesized cDNA was used in a 25-μl PCR reaction containing 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U Taq DNA polymerase (Genechoice, Frederick, MD), and 0.5 μM forward and reverse primers (Table 1). The standard amplification protocol was 1 minute denaturation at 94°C and 14-35 cycles consisting of 94°C for 10 seconds, 58-62°C for 10 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 4 minutes. Different cycle numbers and amounts of cDNAs were used to test the linearity of PCR reactions. The level of mRNA was estimated from the data that were in the linear range with

respect to the amount of cDNA used. cDNA produced without reverse transcriptase was used as negative control. These negative controls yielded no detectable signals at the expected size in the standard PCR at 35 cycles. PCR products were separated on a 1.8% agarose gel and visualized by ethidium bromide staining. Stained gel images were captured, and band intensities were analyzed using a charge-coupled device camera-based system (BioChem II; UVP, Upland, CA).

Table 1. Primers Used for RT-PCR Analysis

Name	Sequence	Position	Size	GeneBank#
Irx1	AACGAAGACGAGGAGGACAA	724-743	386	XM_343741.1
	TTGCCTATGTGGCAGGTGTA	1109-1090		
Irx2	TACGCACCTGTCACTCAAGG	1333-1352	362	XM_225077.2
	TCATCGTCCTCCTCATCCTC	1694-1675		
Irx3	AGCCCAAGATCTGGTCACTG	1019-1119	386	XM_226322.2
	TTCTCCACTTCCAAGGCACT	1484-1465		
Irx4	TGCTGATGGAGCTGTTATGG	939-958	333	XM_225068.2
	TGGTGCCTATCCAGAGTTCC	1271-1252		
Irx5	CCACAGAAGCCCGAGGACA	607-625	352	DQ109812
	GTGGTGAGTGGATGACCGAG	958-939		
Irx6	GCTGTCAAAGCTGTGCATGT	1144-1163	262	XM_226359.2
	CTTGGAAAAGCATCCGTAGC	1405-1386		
Hand1	ACGAACCCTTCCTCTTTGGT	80-98	379	NM_021592.1
	AGCACGTCCATCAAGTAGGC	458-439		
Hand2	CGAGGAGAACCCCTATTTCC	139-159	351	NM_022696.1
	GATCCATGAGGTAGGCGATG	489-470		
GADPH	GCCATCACTGCCACTCAG	1381-1397	148	NM_017008
	GTGAGCTTCCCGTTCAGC	1528-1512		
mBOP	GGCCAAACTGCACTGTCATA	968-987	217 (Long)	XM_216172
	TCTGGCAGTGTTCACAGGAG	1183-1164	177 (Short)	

2.3.2.4 Real-time Polymerase Chain Reaction

Taqman-based real-time PCR for Kv channel and Irx mRNA was performed with primers probes listed in Table 2. The PCR reaction was done with 200 nM primers and 100 nM 5' 6-FAM/ 3'TAMRA-labeled probe (Integrated DNA Technology, Coralville, IA) on an ABI Prizm-7000 machine. A commercially available rat β-glucuronidase kit was used as a normalization control (Applied Biosciences, Foster City, CA). The same brain RNA was used as an internal control for all experiments, and Kv channel mRNA levels were determined with a threshold cycle (CT) with the comparative CT method using the value obtained with the brain RNA. Irx4 and Irx5 mRNA levels were estimated by real-time analysis with SYBR green dye using a commercially available master mix (Applied Biosystems, Foster City, CA) on an Opticon DNA engine equipped with a continuous fluorescence detection system (MJ Research, Waltham, MA). Primers used for semi-quantitative assays in Table 1 were used for this analysis. A series of dilution of a targeted gene cDNA were used as a positive control to estimate the copy number of the transcript using CT.

2.3.3 Constructions

2.3.3.1 Irx3, Irx4 and Irx5

Full-length rat Irx3, Irx4 and Irx5 cDNAs were obtained by multiple RT-PCR with primers (Table 1) that were designed based on the sequences of rat and mouse cDNAs using high-fidelity DNA polymerase (Phusion, Finnzymes, Espoo, Finland). Obtained cDNA sequences were verified by direct sequencing with the established cDNA and genomic sequences. Identified misincorporations were fixed using two primer-based mutagenesis (Quickchange XL II, Stratagene, La Jolla, CA). Irx cDNAs were then cloned into

cytomegalovirus-based mammalian expression vector (pcDNA3) (Invitrogen, Carlsbad, CA). These cDNAs were also cloned into the two vectors that are designed to produce epitope-tagged target proteins: p3x FLAG-CMV-10 expression vector for three Flag tags at the N-terminus of a target protein (Sigma-Aldrich, St. Louis, MO) and pCS2+MT for six Myc tags at the N-terminus of a target proteins (a gift from Dr. Angeles B. Ribera, University of Colorado Health Center).

2.3.3.2 mBOP

Full-length cDNAs for rat mBOP splicing variants and a partial cDNA for mouse skeletal nascent polypeptide-associated complex (skNAC) were obtained by RT-PCR. The latter cDNA corresponded to the amino acid 1141-2187. mBOP cDNAs were cloned into pcDNA3 or N-terminally-tagged in pCS+MT, whereas skNAC peptide cDNA was in Flag10.

2.3.3.3 Small Interfering RNAs

Two siRNAs targeted to rat Irx4 were synthesized (Table 7) in which two thymidines were added at the 3'end of the target sequence. As a control, a commercially available negative control (Silencer® Negative Control #1 siRNA, Ambion, Austin, TX) was used.

Table 2. Irx4 RNA Interference Used For This Study

siRNA	Sense strand	Anti-sense strand
Irx4 siRNA-1	5'-CUCCCUGAGCACGUGCUG Ctt-3'	5'GCAGCACGUGCUCAGGG AGtt-3'
Irx4 siRNA-2	5'-CUCAAUUCUGCCGCCGCG Ctt-3'	5'GCGCGGCGGCAGAAUUG AGtt-3'

2.3.4 Reporter Gene Assays

2.3.4.1 Transfection of Plasmid DNAs into 10T1/2 Cells

A luciferase reporter gene (pGL3-basic) containing the region between -1094 and +592 of the rat Kv4.2 gene was generated as previously described (36). 10T1/2 cells were seeded on 6-well plates with 50% confluence. Kv4.2 promoter (-1094~ +592) -reporter gene, pRL-tk containing the HSV thymidine kinase promoter linked to Renilla luciferase (Promega, Madison, WI), and pcDNA3 for rIrx3, rIrx4 or rIrx5, were transfected into 10T1/2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at 1 μg DNA: 2 μl transfection reagent ratio for 3 hours in the absence of serum. To test the promoter activity by mBOP, 10T1/2 cells were transfected with various amounts of expression vector for mBOP (0.1, 0.2, 0.5 μg) with the presence of expression constructs for Irx. Total amount of expression vector was kept constant by supplementing empty vector. The transfected cells were cultured in the complete medium for one more day before harvesting for luciferase assay.

To test the promoter activity by mBOP, 10T1/2 cells were transfected with various amounts of expression vector for mBOP (0.1, 0.2, 0.5 μg), Kv4.2 promoter (-1094~ +592)-pGL3 basic, and/or 0.2 μg Irx5. Total amount of expression vector was kept constant by supplementing empty vector.

2.3.4.2 Transfection of Plasmid DNAs into Neonatal Myocytes

Two days after preparation, neonatal myocytes on 60-mm plastic dishes were transfected with Kv4.2 promoter (-1094~ +592)-pGL3 basic, expression constructs for rIrx3, rIrx4 or rIrx5 and pRL-tk at 1 μg DNA: 2 μl transfection reagent ratio. Total amount of expression vector was kept constant by supplementing empty vector. The transfected cells were maintained in 5% calf

serum-containing medium overnight, and then cultured in the serum-free medium for an additional day before luciferase assay.

2.3.4.3 Evaluation of Effectiveness of siRNA and Transfection of siRNA into Neonatal Myocytes

To evaluate the efficacy of siRNAs to reduce Irx4 protein, siRNA (10 or 50 nM), Flag-Irx4 cDNA (2 μg) and EGFP-C1 (0.5 μg, BD Bioscience, San Jose, CA) were transfected into HEK293 cells on a 60-mm dish using Lipofectamine 2000 at 1 μg DNA: 2 μl transfection reagent ratio. Two day after transfection, anti-Flag and anti-GFP immunoreactivities were measured by immunoblot analysis.

Neonatal ventricular myocytes on 60-mm plastic dishes were transfected with 0.4 μg Kv4.2 promoter luciferase construct, 0.2 μg and 50 nM siRNA using Lipofectamine 2000 at 1 μg DNA: 2 μl transfection reagent ratio for 3 hours. Two days after transfection, cells were harvested for dual luciferase assays.

2.3.4.4 Luciferase Assay

Transfected cells on plastic dishes were washed with ice-cold phosphate-buffered saline (PBS) and harvested by scraping off from plastic surface using a plastic lifter in a small volume of PBS. The cell pellets were collected by centrifugation at 2000 rpm for 1 minute, followed by washing with ice-cold PBS. The cells were lysed with 1x Passive Lysis buffer provided with the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly and Renilla luciferases were continuously detected according to manufacture's instruction using Turner Luminometer 20-20 (Sunny Vale, CA).

Irx5, as well as Irx4, influenced activity of the thymidine kinase promoter in neonatal myocytes and 10T1/2 cells. However, all firefly readouts used in this study were normalized to Renilla luciferase levels since this normalization provided more consistent results without altering the direction or statistical significance of changes.

2.3.5 mBOP Immunoprecipitation

2.3.5.1 Transfection

10T1/2 cells in a 100-mm plastic dish were transfected with 5 μg of Myc-mBOP (long splicing variant), and 5 μg of Flag-Irx, Flag-skNAC peptide or empty vector using the method described above.

2.3.5.2 Immunoprecipitation

One day after transfection, cells were collected with ice-cold PBS. Cell lysate was prepared by suspending the collected cell pellet in 400 μl of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.2 M NaCl and 1 mM EDTA supplemented with protease inhibitor cocktails (Complete, Roche, Indianapolis, IN). After centrifugation at 10,000 x*g* for 10 minutes, the obtained supernatant was referred as to cell extract. Cell extract was incubated with protein-G agarose (Sigma-Aldrich, St. Louis, MO), monoclonal anti-Flag M2 or anti-Myc antibody (2 μg) for 2 hours at 4 ºC on a rotating plate for immunocomplex formation. The bound materials were collected by centrifugation and washed four times with the same ice-cold lysis solution. The bound proteins were then eluted by mixing with 2 x SDS sample buffer at room temperature and analyzed by immunoblot analysis.

2.3.5.3 Immunoblot Analysis

Samples were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 1x PBST (PBS supplemented with 0.1% Tween 20) overnight at 4 ºC. The coated membrane was probed with monoclonal anti-Flag or polyclonal anti-Myc antibodies at 4 ºC for 2 hours. The membrane was washed with 1x PBST for 3 times, followed by incubation with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. The membrane was washed for 4 times with 1x PBST. The immunoreactive proteins were detected with chemiluminescent reagents (Supersignal WestPico, Pierece, Thermo Fisher Scientific Inc. Rockford, IL). Chemiluminescence images were captured and analyzed using charged-coupled device camera-based system (BioChem II; UVP, Upland, CA).

2.3.6 Statistical Analysis

Each luciferase value was obtained as the average of duplicate assays. Luciferase values obtained from at least three independent experiments were used for statistical analysis. Statistical comparison was performed by one-way ANOVA, followed by Bonferroni's posthoc test, using Prism 5.0 (GraphPad Software, San Diego, CA). The threshold for statistical significance was set at the *p* value less than 0.05.

2.4 RESULTS

2.4.1 Irx Members Are Differentially Expressed in Various Heart Regions

Previous studies revealed that Irx3 and Irx5 mRNAs are expressed in a gradient across the left ventricular wall of rodent and canine hearts (1). Since Iroquois transcription factors are known to control heart morphogenesis and chamber-specific gene expression (44, 47, 48), the expression of all Irx members and some related factors in various regions of rat hearts were examined using semi-quantitative assays (Fig. 1A and B). Irx3 and Irx5 mRNA levels appeared to be ~5-times and more than ~15-times higher, respectively, in the endocardial layer than the epicardial region. Using the same samples, it was found that Kv4.2 mRNA level was ~5-times more abundant in the epicardial layer than the endocardial portion. Real-time analyses revealed similar differential expression of Irx5 and Kv4.2 mRNAs across the left ventricular wall (Fig. 2A and B): Kv4.2 mRNA level was 5.1 ± 0.7-fold higher in the epicardial region than the endocardial portion (n=4). Irx5 mRNA level was 18.6 ± 7.9-fold more abundant in the endocardial layer than the epicardial region (n=3). In contrast, Irx4 was equally abundant throughout the rat ventricular tissue (Fig. 1A and B for Irx4). Likewise, Kv4.3 mRNA was constantly expressed across the left ventricular wall. Thus, the expression of Irx3 and Irx5 mRNAs is inversely related to that of Kv4.2 message across the left ventricular wall of adult rat heart.

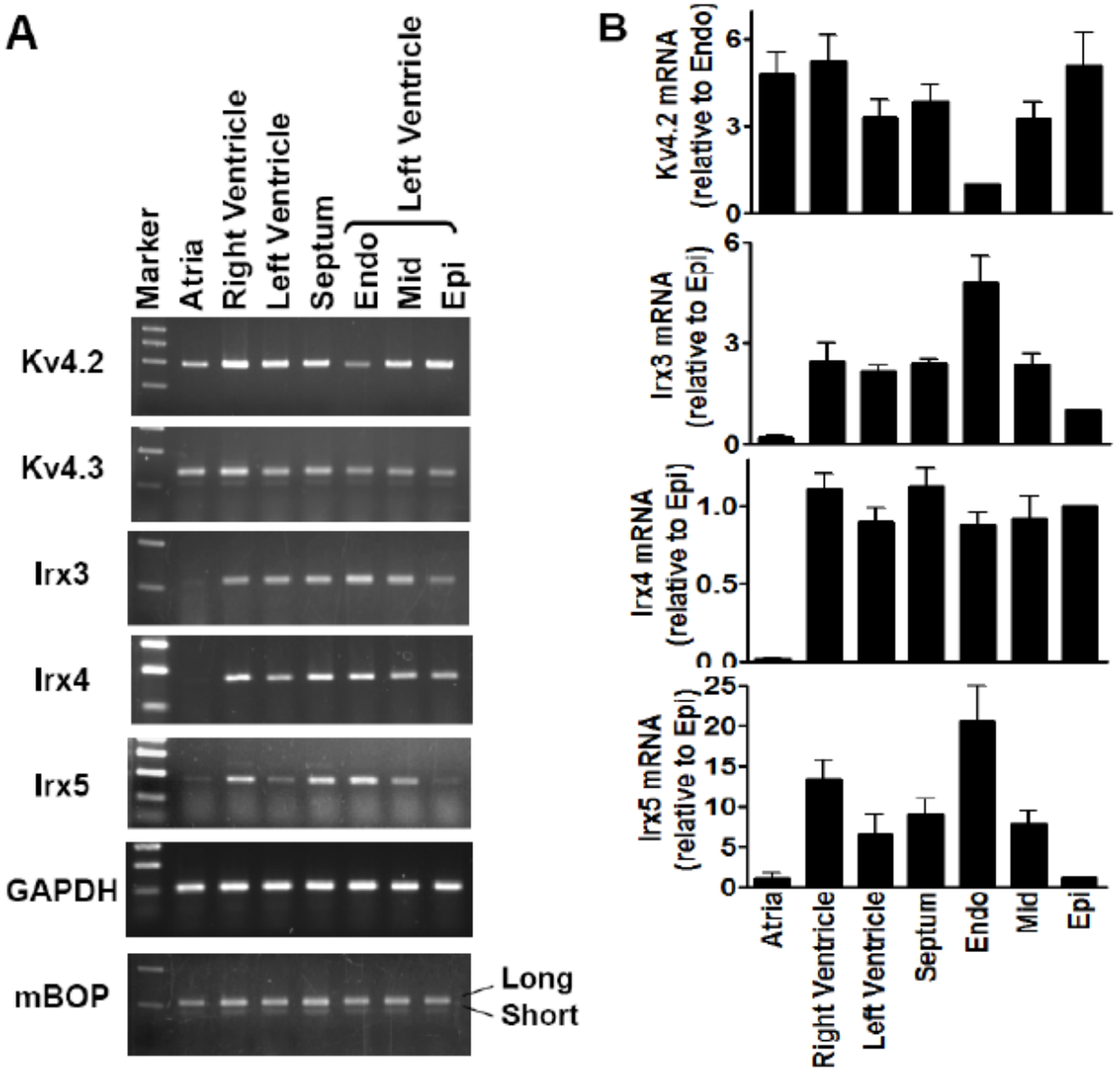

Figure 1. Irx3 and Irx5 mRNAs are differentially expressed across the left ventricular wall of rat heart by semi-quantitative PCR.

(A) RT-PCR analysis was performed with total RNAs isolated from indicated regions of adult rat heart. Left ventricular free wall (L.V.) was dissected into three layers with equal thickness: endocardial (Endo), midlayer (Mid), and epicardial (Epi) layers. (B) Levels of Kv4.2, Kv4.3, Irx4 and Irx5 mRNAs were determined with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as a control. Points and error bars represent the mean and S.E.M., respectively (n=4 for each point).

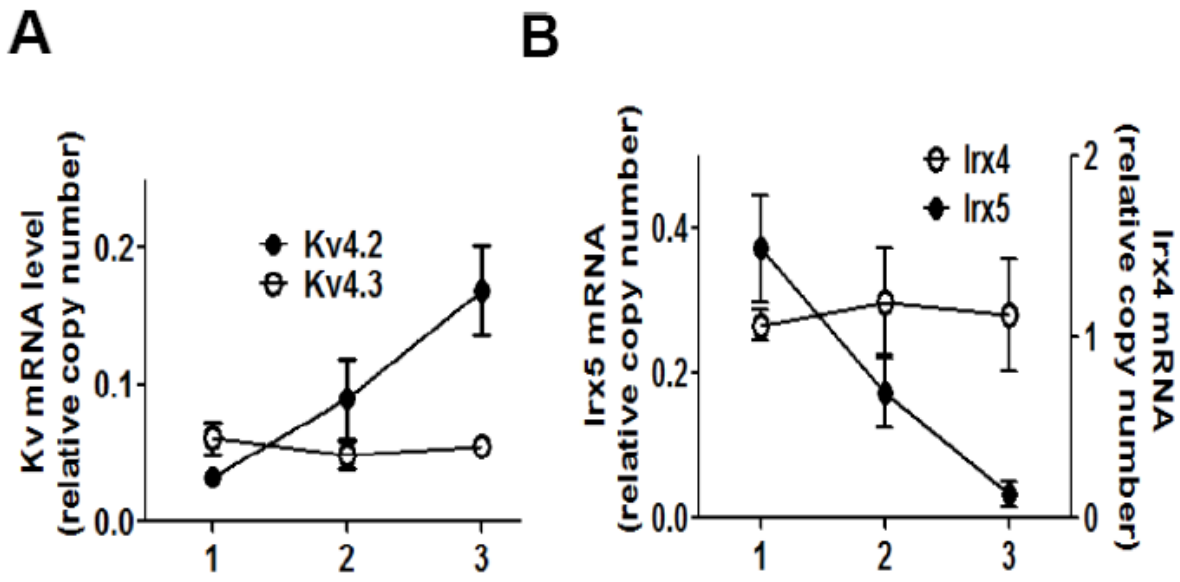

Figure 2. Irx5 mRNAs are differentially expressed across the left ventricular wall of rat heart in an inverse pattern to that of the Kv4.2 gene by real time PCR.

The levels of Kv channel and Irx factor mRNAs were measured using real-time analyses, as described in Materials and Methods. (A) Kv4.2 and Kv4.3 mRNA levels were plotted on the same scale, whereas (B) Irx4 and Irx5 messages were on different scales. Points and error bars represent the mean + S.E.M. (n=4 for Kv4.2 and Kv4.3, and n=3 for Irx4 and Irx5).

2.4.2 Irx5 Influences Kv4.2 Promoter in a Cell Type-Specific Manner

Irx5 has been reported to inhibit and activate rat Kv4.2 gene promoter in neonatal mouse cardiomyocytes and 10T1/2 fibroblastic cells, respectively (2). Luciferase reporter gene assays were used to test if differentially expressed Irx3 and abundant Irx4 might similarly influence rat Kv4.2 gene promoter. Because the region upstream of -1094 strongly inhibits channel promoter activity, the luciferase gene fused with the region between -1094 and +592 of the rat Kv4.2 gene was used (36). As expected, Irx5 decreased and increased channel promoter activity in neonatal ventricular myocytes and 10T1/2 cells, respectively (Fig. 3). In cardiac myocytes, the reduction in promoter activity was evident with lower doses of Irx5 and no apparent effect at higher doses (Fig. 3A). The promoter activation by Irx5 showed no reversal with increasing amounts of the transcription factor in fibroblastic cells (Fig. 3B). Unlike Irx5, Irx3 or Irx4 caused no significant change in channel promoter activity in any cell types. In parallel transfection, the expression of Flag-tagged Irx members in 10T1/2 cells showed comparable levels (Fig. 4C), indicating that the observed specificity is not due to the difference in expression of transfected genes. Therefore, Irx5 specifically inhibits Kv4.2 gene transcription in neonatal ventricular myocytes, whereas the transcription factor activates the channel promoter in fibroblasts.

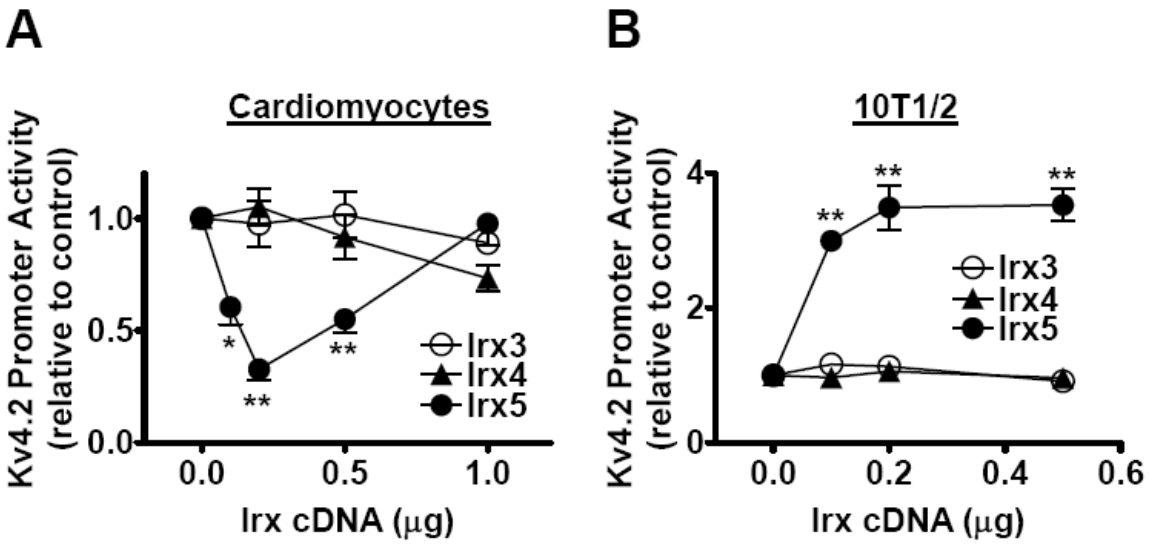

Figure 3. Irx5 decreases and increases Kv4.2 promoter activity in neonatal ventricular myocytes and 10T1/2 cells, respectively.

Neonatal ventricular myocytes (A) and 10T1/2 cells (B) were transfected with indicated amounts of expression vector for Irx3, Irx4 or Irx5, Kv4.2 promoter-luciferase construct and pRL-tk. Total amount of expression vector was kept constant by supplementing empty vector. *$p<0.05$ and ** $p<0.01$ compared to control (no Irx cDNA) ($n>5$ for each condition).

2.4.3 mBOP Does Not Affect the Induction of Promoter Activity by Irx5

A previous study has shown that the muscle-specific transcription factor, mBOP, physically interacts with Irx5 and suppresses the Irx5-induced activation of the channel promoter in 10T1/2 cells (2). RT-PCR detected long and short splicing isoforms of mBOP in the rat heart (Fig. 4A). Total mBOP mRNA levels, as well as the ratio of the two variants, were nearly identical throughout the heart. Reporter gene assays were then used to examine if long- or short-splicing variants of mBOP might affect Kv4.2 promoter activity or its regulation by Irx5 in 10T1/2 cells (Fig. 4B). The long or short variants of mBOP by itself produced no significant effects on channel promoter. In the presence of Irx5, mBOP caused a slightly higher promoter activity compared to Irx5 alone. Therefore, mBOP appeared to lack any regulatory activity on Kv4.2 promoter, unlike the previous observation.

Immunoprecipitation was used to test if mBOP might physically interact with Irx proteins. The skeletal isoform of nascent polypeptide-associated complex alpha subunit (skNAC) is known to associate specifically with mBOP (49). Therefore, a portion of skNAC containing the identified association site was used as a positive control. As expected, skNAC peptide appeared to interact with mBOP. In contrast, Irx4 or Irx5 were unable to exhibit any significant association with mBOP (Fig. 4C with the long splicing variant). Therefore, an alternative mechanism was sought for the cell type-specific regulation of the channel promoter by Irx5.

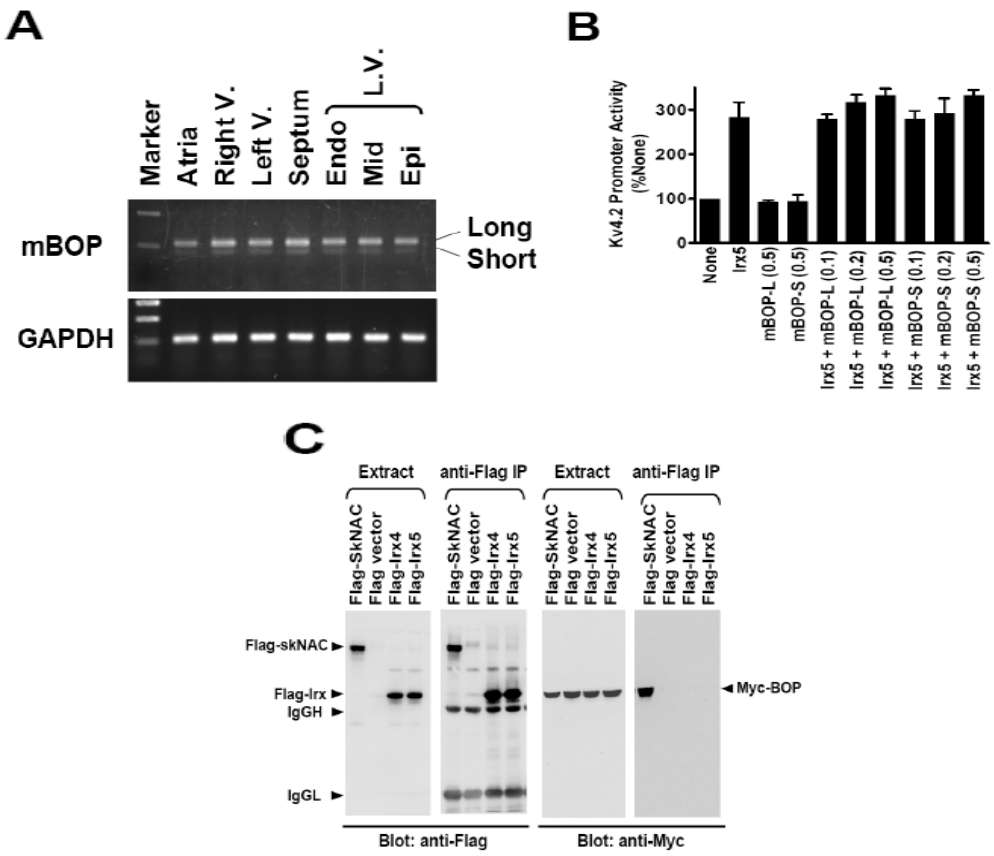

Figure 4. mBOP doesn't affect the induction of promoter activity by Irx5.

(A) Semi-quantitative PCR analysis was performed with total RNAs isolated from indicated regions of adult rat heart. Strong upper (216-bp) and weak lower (177-bp) bands correspond to the long and short splicing variants of mBOP. (B) 10T1/2 cells were transfected with indicated amounts of expression vector for mBOP, 0.2 μg Irx5 and Kv4.2 promoter-luciferase construct. Total amount of expression vector was kept constant by supplementing empty vector. (C) 10T1/2 cells were transfected with Myc-mBOP (long splicing variant) and indicated Flag-Irx, Flag-skNAC peptide or empty vector. Extract was prepared with 1% Triton X-100 and immunoprecipitation was performed with anti-Flag antibody. Extract and immunoprecipitate (anti-Flag IP) were subjected to immunoblot analysis. Arrow heads indicate the position of indicated proteins. IgGH and IgGL indicate IgG heavy and light chains, respectively.

2.4.4 Irx4 Specifically Represses the Induction of Promoter Activity by Irx5

Because Irx members may form homomeric and heteromeric dimmers (50), co-expression of different Irx members might affect the Irx5-induced promoter activity of Kv4.2 gene. Specifically, RT-PCR analysis in this study detected abundant expression of Irx4 in adult heart (Fig. 1). Thus, it was tested if co-expression of Irx4 might alter the outcome of the Irx5-induced regulation of Kv4.2 promoter. Co-expression of Irx4 appeared to suppress the Irx5-induced increase in promoter activity in 10T1/2 cells (Fig. 5A). This suppression occurred in an Irx4 dose-dependent fashion at the constant amount of Irx5, and higher doses of Irx4 were required when Irx5 was present at a higher level. In contrast, Irx3 produced no significant effect on the Irx5-induced enhancement (Fig. 5B). Furthermore, when a constant level of Irx4 was present, more Irx5 was required to induce activation of the channel promoter (Fig. 5C). Again, with greater levels of Irx4, more Irx5 were necessary to cause promoter activation. Thus, Irx4 specifically suppresses the Irx5-induced activation of Kv4.2 promoter in fibroblastic cells. The observed dose relationship suggests that the ratio of Irx5/Irx4 determines the degree of promoter activation.

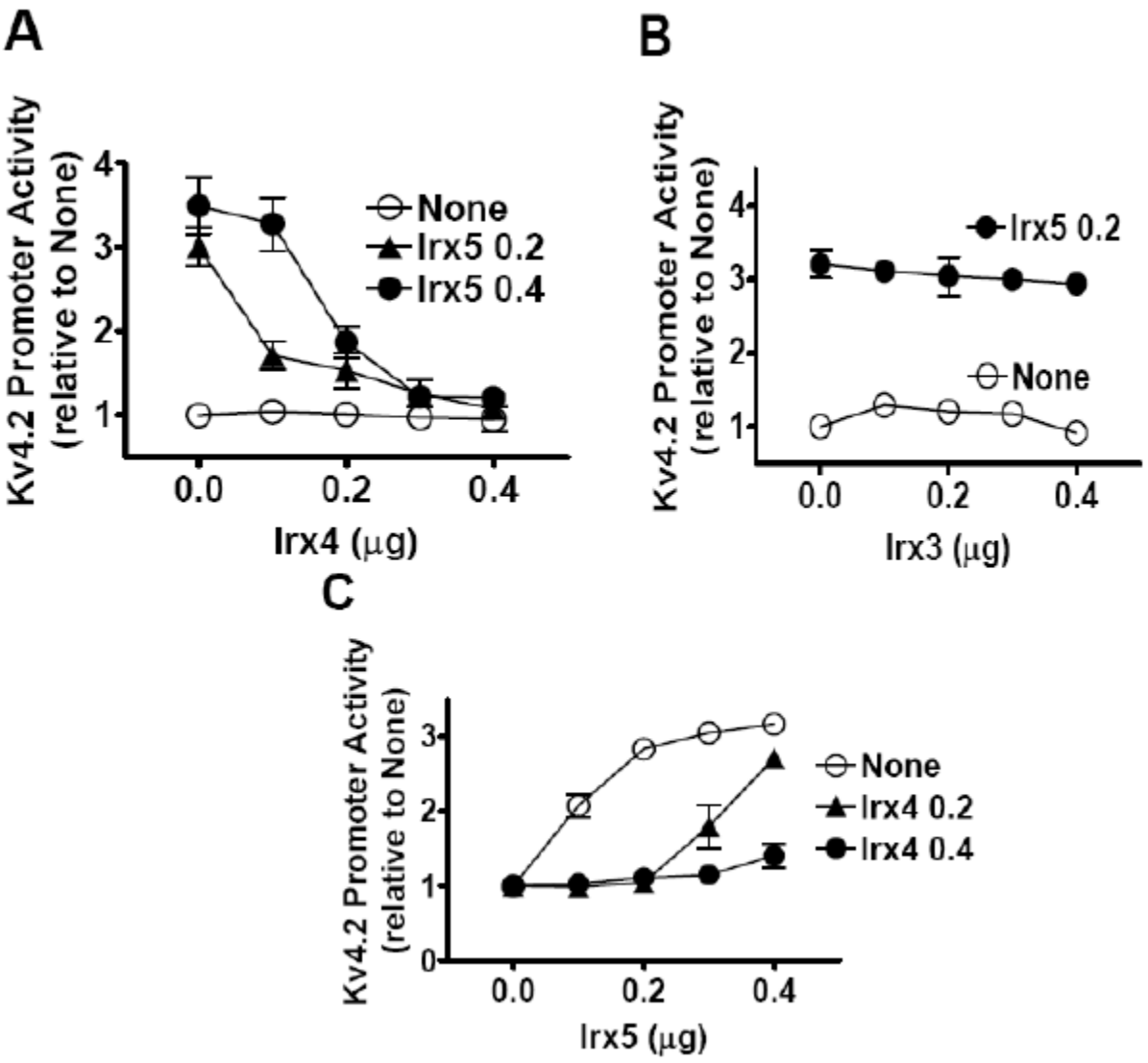

Figure 5. Irx4 suppresses the Irx5-induced increase in Kv4.2 promoter activity in 10T1/2 cells.

(A and B) Various amounts of Irx4 or Irx3 cDNA were transfected with a constant amount of Irx5 cDNA or empty vector (None). (C) Various amounts of Irx5 cDNA were used with a constant Irx4 cDNA or empty vector (None). Note that the ratio of Irx5/Irx4 is correlated with the degree of channel promoter activation.

2.4.5 Irx4 siRNA Suppresses the Inhibition of Irx5 on Kv4.2 Promoter Activity in Myocytes

Abundantly expressed Irx4 might constantly inhibit the activation action of Irx5 in cardiac myocytes. Thus, reducing Irx4 in neonatal myocytes might influence the outcome of Irx5-induced regulation in neonatal myocytes (Fig. 6). Two siRNAs for Irx4 with different efficacies were used to decrease Irx4 expression (Fig. 6A). Irx4 siRNA alone did not affect the basal promoter activity. This might be due to very low levels of Irx5 in neonatal myocytes (unpublished observation). A control siRNA produced no apparent effects on the Irx5-induced decrease in Kv4.2 promoter activity (Fig. 6B). In contrast, the two Irx4 siRNAs resulted in a reduction in the Irx5-induced inhibition of the channel promoter. At the higher dose of Irx4 siRNA, Irx5 even caused a small increase in channel promoter activity. Although it was unable to determine the level of Irx4 proteins in neonatal myocytes due to inability of the current commercially available anti-Irx4 antibody, the reduction and further increase in channel promoter activity were correlated with the efficacy of the two siRNAs to decrease Irx4 expression in a mammalian cell line (Fig. 6A). These results suggest that endogenous Irx4 prevents the ability of transfected Irx5 to increase Kv4.2 promoter activity.

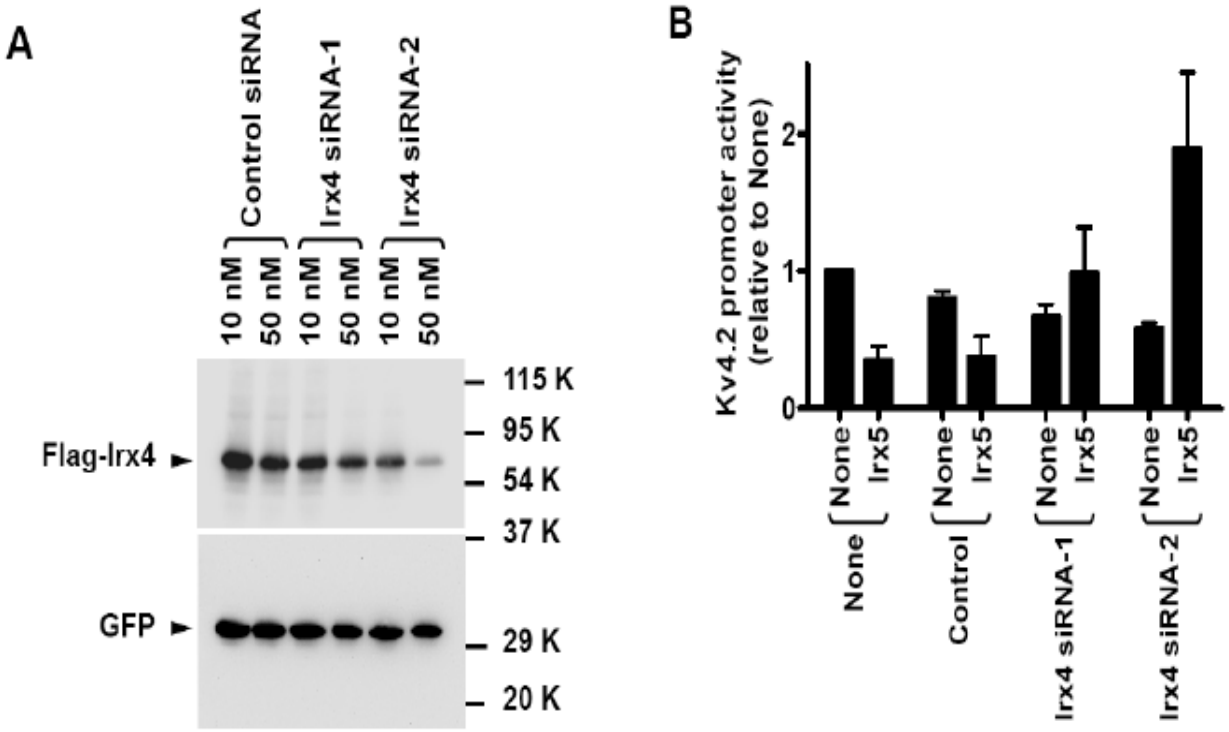

Figure 6. Irx4 siRNA reverses the Irx5-induced regulation from inhibition to activation in neonatal myocytes.

(A) HEK293 cells were transfected with Flag-Irx4, GFP-C1 and indicated siRNA. Immunoblot analysis was performed with anti-Flag and anti-GFP antibodies. (B) Neonatal myocytes were transfected with Kv4.2-luciferase, pRL-tk, Irx5 or empty vector (None) and indicated amounts of siRNA.

2.5 DISCUSSION

This chapter examined expression patterns of Irx members in various regions of the adult rat heart and their roles in controlling transcription of Kv4.2 channel gene. The two Irx members, Irx3 and Irx5, are distributed in an inverse relationship to Kv4.2 gene across the left ventricular wall, whereas Irx4 is equally abundant throughout the ventricular tissues. Only Irx5 appears to possess the ability to control Kv4.2 gene promoter by itself. Irx4 indirectly regulates the channel gene transcription by influencing the action of Irx5. Thus, the interaction of the two Irx transcription factors plays an essential role in maintaining the transmural gradient of Kv4.2 gene expression in rodent heart.

Homeobox transcription factors are generally characterized by their fundamental roles in organogenesis and pattern formations during development. Relatively less is known about their functions in adult organisms. Yet, the expression of Irx genes is not transient in the developing embryos, but remains significant into adulthood. For instance, Irx3 acts as a critical factor for patterning the intermediate region of the developing frog nephron, and remains segmentally in the mature nephron (51). Likewise, previous and present study all show that Irx4 is required for ventricle-specific gene expression and is abundant in these chambers of adult hearts (26). The data in this chapter further demonstrated that Irx4 and Irx5 possess the ability to control cardiac Kv4.2 gene transcription. These findings indicate that Iroquois homeobox genes are not just reminiscent of embryonic development, but subsequently impose their effects on gene expression as maintenance factors in adult organs.

Previous studies have suggested overlapping expression patterns of Iroquois proteins. The identical expression pattern of Ara and Caup is observed in *Drosophila* (52). The overlapping expression of Irx1 and Irx2 is also seen in a subset of ventricular cells on the left of

the interventricular groove in the developing mouse heart (26). Similarly, this study identified overlapping expression of Irx3, Irx4 and Irx5 in the rat left ventricle. Furthermore, the two Irx members, Irx3 and Irx5, are similarly distributed in a gradient across the left ventricular wall. The presence of highly conserved non-coding elements in the Irx clusters is likely to contribute to the expression pattern of Irx proteins (Fig. 7). In human and mouse genomes, the cluster A contains Irx1, Irx2 and Irx4, whereas the cluster B includes Irx3, Irx5 and Irx6. It has been proposed that these two clusters derive from a chromosomal duplication event. Shared enhancers present in the cluster B may contribute to the identical expression pattern of Irx3 and Irx5 in the left ventricle.

Despite the overlapping expression patterns of Irx proteins, it is noteworthy that Irx members play distinct roles in controlling transcription of a subset of genes. For example, a previous study reported distinct functions of Irx1 and Irx3 during *Xenopus* kidney development (53). In this study, Irx5, but not Irx3, is found to regulate transcription of Kv4.2 gene. The differentially-expressed Irx3 may regulate the expression of other, unidentified downstream genes. In addition, Irx5 and Irx4 possess distinct functions in controlling Kv4.2 gene transcription. These unique functional roles of individual Irx proteins are quite different from transcription factors in other families. For example, GATA transcription factors often exhibit redundant functions (54-56). Six mammalian Irx proteins contain substantially diverse N- and C-terminal regions (Fig. 7, alignment of rat Irx3, Irx4 and Irx5). Thus, this diversity may provide a unique transcriptional regulatory role for each Irx transcription factor.

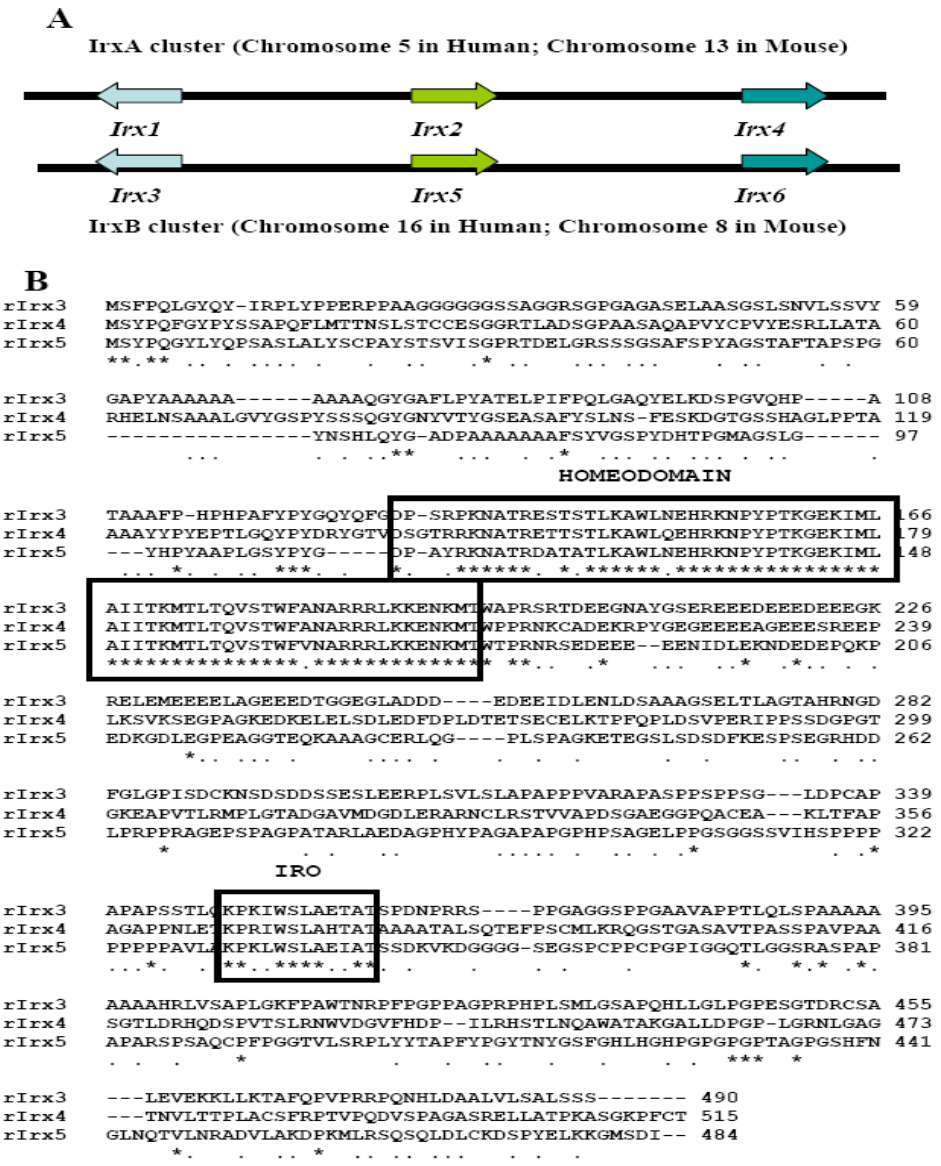

Figure 7. Genomic organization of *Iroquois* homeobox genes and alignment of the predicted amino acid sequence of rat Irx3, Irx4 and Irx5.

(A). Similar genomic organization of Irx genes in human and mouse. Two clusters of three Irx genes are located in the indicated chromosomes. Orientation of arrows represents direction of transcription. (B). Alignment of the predicted amino acid sequence of rat Irx3 (GenBank Accession No.XM_226322), rat Irx4 (GenBank Accession No. AF124732) and rat Irx5 (GenBank Accession No. DQ109812). Identical (*) and similar (·) residues are indicated underneath the sequence. The homeodomain and IRO box are boxed.

One intriguing aspect of this study is the cell-type specific activity of Irx5 in regulating Kv4.2 gene promoter. In 10T1/2 fibroblasts, Irx5 acts as a transcription activator. A similar stimulation of the channel promoter was seen in Ad293, CHO and PC12 cells (unpublished observation). In contrast, Irx5 inhibits the channel promoter in neonatal ventricular myocytes. The simplest interpretation of this observation is the presence of cardiac myocytes-specific factor(s) that alter the outcome of Irx5 action. This study suggested that Irx4 is a plausible candidate for this role. Irx4 is abundantly and restrictedly expressed in the ventricle of heart and known for regulating the chamber-specific gene expression (26, 27, 29). Furthermore, Irx4 was found to inhibit the Irx5 effect on channel promoter in 10T1/2 cells. A similar repressor action of Irx4 has been seen in other systems. For example, retroviral vector-mediated expression of Irx4 specifically inhibits the expression of *Slit1* gene in certain cell populations in retina (57), though the precise mechanism of this Irx4 action remains obscure. Irx4 has also been shown to inhibit transcription of the gene for the atrial isoform of myosin heavy chain (29). In this latter regulation, Irx4 appears to exert its effect indirectly by binding to other transcription factors. The observed Irx4 action on Kv4.2 promoter in this study is similar to this effect, in which Irx4 itself produces no apparent effect on the channel promoter, but prevents the Irx5-induced increase. Moreover, siRNA-mediated reduction in Irx4 caused an increase in channel promoter activity in cardiac myocytes. These findings support the universal transcription activator action of Irx5 on Kv4.2 promoter in any cell types in the absence of Irx4. Endogenous Irx4 mRNA was detected at a relatively high level in the culture of neonatal myocytes. Therefore, the findings also suggest that abundant Irx4 may mask this activator role of Irx5 in myocytes.

However, the identified Irx4-mediated suppression of the Irx5-induced activation is insufficient to account for the inhibitory effect of Irx5 on Kv4.2 promoter in myocytes. First,

Irx5 at lower doses caused a marked reduction in channel promoter activity in myocytes. Second, coexpression of Irx4 and Irx5 at any ratio failed to lower channel promoter activity below the baseline in 10T1/2 cells. Therefore, Irx5 might produce the inhibition of channel promoter in myocytes independent of its activator action. It is conceivable that Irx5 possesses the ability to prevent the effect of unidentified myocyte-specific transcription activator. This may be analogous to the Irx4 action on the atrial myosin heavy chain gene in which Irx4 blocks the activation by retinoic receptors and PXPR. Further studies on identification of this putative myocyte-specific activator may be necessary for elucidating the region-selective expression of Kv4.2 gene in the heart.

3.0 THE REGULATORY MECHANISM BY WHICH IRX5 AND IRX4 CONTROL TRANSCRIPTION OF THE RAT KV4.2 GENE

3.1 ABSTRACT

The Iroquois (Irx) homeobox genes encode a conserved family of transcription factors that contain an atypical homeodomain with a three-amino acid extension. Irx proteins have been shown to play important roles in diverse developmental processes. Yet, little is known about their direct targets or regulatory mechanisms. The previous chapter established that Irx5 inhibits Kv4.2 gene promoter in neonatal ventricular myocytes, whereas the transcription factor stimulates the channel promoter in non-myocytes. Moreover, Irx4 appears to prevent this Irx5-induced regulation of channel promoter. This chapter is aimed at elucidating molecular mechanisms underlying these actions of the two Irx transcription factors on Kv4.2 promoter. Reporter gene assays using chimeras between Irx proteins show that the C-terminal region of Irx5 is necessary and sufficient for the regulation of channel promoter activity in both myocytes and non-myocytes. This region also confers the repression by Irx4 of the promoter activity in non-myocytes. Similarly, the N-terminal region of Irx4 is necessary and sufficient to suppress the regulation of promoter activity by Irx5. Dimer formation was supported by co-immunoprecipitation. However an *in vivo* interaction system (Checkmate Mammalian Two-hybrid System) failed to detect any interaction between Irx4 and Irx5. Addition of histone

deacetylase inhibitor (TSA) relieved the inhibitory effect of Irx4 suggesting the involvement of HDACs in the Irx4-mediated inhibition of promoter activity. These results indicate that diverse N- and C-terminal regions of Irx5 and Irx4 mediate their distinct actions on Kv4.2 gene promoter. These actions of Irx proteins may involve indirect influences on other transcription factors and recruitment of genome-modifying enzymes.

3.2 INTRODUCTION

Iroquois (Irx) proteins contain a highly conserved atypical homeodomain flanked by diverse N-terminal and C-terminal peptides, each with 100-150 amino acids and ~300 amino acids in length. Unlike classic homeodomain proteins, such as Hox transcription factors, which have a characteristic 60-amino acid homeodomain, the atypical homeodomain of Irx proteins includes three extra amino acids between the helix 1 and helix 2, which places the Irx proteins into a subfamily, called TALE (three amino acid loop extension) (37). There are four classes of TALE proteins in vertebrates: PBC (58), MEIS (59), TGIF (60) and IRO corresponding to Irx proteins (52). TALE proteins are also found in other organisms: two groups in fungi, the mating type genes (M-ATYP) and the CUP genes (37); two types in plant, maize Knotted-1 (61) and BEL (62). This classification is mainly based on either sequence identity within the atypical homeodomain or conserved protein motifs outside of the atypical homeodomain. Irx proteins have a conserved thirteen-amino acid domain, called the Iro box, in the C-terminal region (63), although no function has been assigned to this domain. In addition, the atypical homeodomain of Irx proteins possesses additional unique characteristics, such as the presence of an alanine in the position that is occupied by a polar residue in the typical homeodomain (37). These unique characteristics suggest that the DNA binding of Irx proteins is weaker than that of typical homeodomain proteins. It also implies that the DNA-binding specificity of Irx proteins may be partially compensated for by interaction with other proteins through their N-terminal and C-terminal regions. Recently, the DNA binding of the Irx's atypical homeodomain has been studied with the motif of the *Drosophila* Irx proteins, Mirror, using *in vitro* site selection. This biochemical study showed that the binding sequence of the atypical homeodomain is substantially different from the classic Hox consensus (50). Also the sequence outside the

identified core binding region affects the binding specificity. Thus, the target DNA-binding site of Irx proteins remains obscure and may be largely influenced by their interacting proteins.

One class of interacting proteins for the TALE-family proteins is the Hox proteins, the founding members of the homeodomain superfamily. Indeed, the role of TALE atypical homeodomain proteins as co-factors for typical homeodomain transcription factors has been documented for a long time. For example, the Pbx/exd atypical homeodomain proteins appear to influence the Hox developmental programs in arthropods and vertebrates (64). Although these Hox proteins possess a similar sequence preference for DNA binding as monomers *in vitro* (65, 66), they exhibit distinct target specificities when directing the developmental pathway *in vivo* (67). A number of studies have suggested that this diversity is dependent on the presence of various cofactors including atypical homeodomain-containing proteins (64, 68). The result of this protein-protein interaction is the enhanced DNA-binding affinity or specificity *in vitr*o (69-74). Similarly, a recent gain-of-function study of Irx3 showed that Irx3 acts as a transcriptional regulator of Nkx6.1 and Nkx2.2, two typical homeodomain proteins, in ventral neuronal patterning in *Drosophila* embryo (42).

The interaction between different TALE atypical homeodomain proteins has also been documented. This heteromeric association confers strong binding to DNA sites for which the single protein shows otherwise weak affinity. For example, Pbx proteins dimerize with Meis-hth and bind to a DNA element with a high affinity (75, 76). This interaction also promotes nuclear localization and stability of Pbx proteins (77-81). In addition, the formation of homodimers and heterodimers between Irx proteins has been suggested by an *in vitro* study (50).

In addition to typical and atypical homeodomain-containing proteins, TALE homeodomain proteins also interact with non-homeodomain proteins, such as nuclear hormone-

receptors (29). Irx4 interacts with retinoic X receptor and forms a trimeric complex with Vitamin D receptor to bind to the vitamin D response element. Another example is the repression of general transcriptional activators, such as Sp1 and E2F, by Irx3 in controlling neuronal gene expression (82). Irx5 has also been suggested to interact with the muscle-specific transcription factor, mBOP, in regulating Kv4.2 gene transcription in mouse cardiac myocytes (2). Taken together, TALE-family members including Irx proteins interact with a variety of factors to exert their effects on gene transcription.

It has been speculated that the N-terminal region of Irx proteins is essential for protein-protein interaction. Gel shift assays showed that the flag-tagged Mirr protein that lacks the N-terminal peptide fails to form a homodimer in the context of DNA-protein binding (50). Another study using reporter gene assays indicated the importance of the N-terminal peptide of Irx4 in mediating the inhibition of the atrial myosin heavy chain gene. This inhibition is believed to function through the interaction of Irx4 with the retinoic X receptor, suggesting the interaction of Irx4 N-terminal peptide with this hormone receptor (29). On the other hand, a study using coimmunoprecipitation suggested that the C-terminal region of Irx5 is required for its association with mBOP, a muscle-specific transcription factor, in mediating its transcriptional regulation (2). Therefore, Irx proteins may control gene transcription by their interaction with other proteins via its N- and/or C-terminal regions.

Finally, much progress has been made in the link between chromatin modifications and transcriptional regulation. Chromatin modifications provide a coarse adjustment to gene expression by making a region of DNA more or less available for transcription. Acetylation of the amino-termini of core histones has been shown to generate the formation of transcriptionally-competent chromatin. Histone deacetylation at the promoter region was generally shown to

correlate with impaired gene expression. DNA methylation is another well-known mechanism for silencing genes. For example, the methylation of CpG island near a promoter is known to silence certain genes in cancer (83). A previous study suggested the involvement of histone deacetylases (HDACs) in mediating the repressor activity of Irx5 in controlling Kv4.2 gene expression in cardiac myocytes (2). Thus, the regulation of channel gene transcription by Irx4 and Irx5 may involve chromatin modifications.

Hence, this chapter is to elucidate molecular mechanisms underlying Irx-mediated regulation of Kv4.2 gene promoter. Various regions of Irx5 and Irx4 polypeptides were examined for their roles in channel gene transcription and the association between Irx proteins was examined by different assays. This chapter also tested if the Irx-mediated regulation of gene transcription involves chromatin modifications.

3.3 MATERIALS AND METHODS

3.3.1 Cell Culture and Reagents

3.3.1.1 10T1/2 Cells

10T1/2 cells were grown at 37ºC under 5% CO_2 atmosphere in Basal Medium Eagle (BME) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% $_L$-glutamine (Invitrogen, Carlsbad, CA). Cells were passed just before confluence.

3.3.1.2 Reagents

Trichostatin A (TSA) and RG108 were purchased from EMD Chemicals, Inc. (Calibochem, Gibbstown, NJ). Stock solutions for these reagents were made in dimethyl sulfoxide (DMSO) at 1000-fold of the final working concentrations.

3.3.2 Constructions

3.3.2.1 Chimeras between Irx cDNAs

Rat Ix3, Irx4, Irx5-pcDNA3 constructs were generated as described in the previous chapter. Chimeras between two Irx cDNAs were generated by overlapped PCR or introduction of a unique restriction enzyme site near the border. N, H and C in these chimeras denote the N-terminal, homeobox and C-terminal peptides of Irx proteins, respectively: 3N, 3H and 3C indicate Irx3 polypeptides corresponding to the amino acid 1-134, 135-199 and 200-507; 4N, 4H and 4C represent Irx4 polypeptides to the amino acid 1-147, 148-212 and 213-515; 5N, 5H and

5C are Irx5 polypeptides to the amino acid 1-116, 117-181 and 182-484. All chimeras were made in pcDNA3. The obtained chimeric constructs were verified by DNA sequencing.

3.3.2.2 Vectors for rIrx4 and rIrx5 Interaction Assays

The rIrx4 and rIrx5 cDNAs were cloned into pACT and pBIND vectors for the interaction assay in mammalian cells. The full-length rIrx4 and rIrx5 fragments were obtained by PCR with primers each containing *Bgl*II and *Nhe*I restriction sites (Table 3), and cloned into the *Bam*HI-*Xba*I site of pACT and pBIND vectors (Promega, Madison, WI).

To increase the flexibility of the protein binding, the linker [$(GlyGlySer)_4$: GGTGGATCAGGCGGATCAGGTGGCTCAGGTGGGATC)] was added between rIrx4/5 cDNA and pACT/pBIND vector. This was done by inserting the double-stranded oligonucleotides corresponding to this linker (Table 3) at the *Bam*HI-*Mlu*I site of the vectors, followed by cloning the full-length Irx4 and Irx5 fragments into these linker-containing vectors.

Table 3. Oligonucleotides Used in rIrx4 and rIrx5 Interaction Assay

rIrx5	5'-CAGCTCAGATCTCCTACCCGCAGGGCTACTT-3'
	5'-CTGACGTCTAGATGTCGGACATACCTTTC-3'
rIrx4	5'-CGCAGCAGATCTCCTACCCGCAGTTTGGAT-3'
	5'-GCTGACGCTAGCTGCAGAAGGGTTTGCCACT-3'
linker	5'phospho-GATCGGTGGATCAGGCGGATCAGGTGGCTCAGGTGGGATC CA-3'
	5' phosphor-CGCGTGGATCCCACCTGAGCCACCTGATCCGCCTGATCCA CC-3'

3.3.3 Reporter Gene Assay

3.3.3.1 Transfection of Plasmid DNAs into 10T1/2 Cells

10T1/2 Cells were seeded on 6-well plates with 50% confluence. Kv4.2 promoter (-1094~ +592) -reporter gene, rIrx-pcDNA3, chimeras between two Irx cDNAs-pcDNA3 and

pRL-tk were transfected into 10T1/2 cells using Lipofectamine 2000 at 1 μg DNA: 2 μl transfection reagent ratio for 3 hours in the absence of serum. The transfected cells were cultured in the complete medium for one more day before harvesting for luciferase assay.

3.3.3.2 Luciferase Assay

The transfected cells were harvested with ice-cold PBS by scraping with a plastic lifter after washing with the same solution. The cell pellets were collected by centrifugation at 2000 rpm for 1 minute followed by one more wash with ice-cold PBS. The cells were lysed with 1 x passive lysis buffer provided by the dual-luciferase reporter assay system and the activities of the two different luciferases (firefly and renilla) were detected according to the manufacture's instruction.

3.3.4 Co-immunoprecipitation

3.3.4.1 Transfection

10T1/2 cells in a 100-mm plastic dish were transfected with 5 μg of Flag-Irx or empty vector and Myc-Irx5 cDNAs, as described above.

3.3.4.2 Immunoprecipitation

One day after transfection, cells were collected with ice-cold PBS. Cell lysate was prepared by suspending the collected cell pellet in 400 μl of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.2 M NaCl and 1 mM EDTA supplemented with protease inhibitor cocktails at ~5 mg protein/ml. After centrifugation at 10000 *g* for 10 minutes, proteins were incubated with protein-G agarose, monoclonal anti-Flag M2 or anti-Myc antibody (2 μg) at

4ºC for 2 hours for immunoprecipitation. The bound materials were collected by centrifugation and washed 4 times with the same ice-cold lysis solution. Bound materials were eluted with 2x SDS sample buffer and analyzed by immunoblot analysis.

3.3.4.3 Immunoblot Analysis

Samples were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 1x PBS supplemented with 0.1% Tween 20 (PBST) overnight at 4 ºC followed by probing with the same monoclonal anti-Flag or polyclonal anti-Myc antibodies at 4 ºC for 2 hours. The membrane was washed with PBST followed by incubation with a horseradish-peroxidase conjugated secondary antibody at room temperature for 1 hour. The membrane was washed 4 times with PBST and incubated with chemiluminescent reagents at room temperature for 1 minute. Chemiluminescence images were captured and analyzed using charged-coupled device camera-based system.

3.3.5 Checkmate Mammalian Two Hybrid System

3.3.5.1 Method Description and Strategy

Interaction between Irx proteins was analyzed using the Checkmate Mammalian Two-Hybrid system (Promega, Madison, MI). Two control transfections were routinely included in experiments: the VP16-fusion protein plus the non-fused GAL4 binding domain (provided by the empty pBIND plasmid); and GAL4-fusion protein plus the non-fused VP16 activation domain (provided by the empty pACT plasmid). These two controls were included to exclude the possibility that the firefly luciferase production is due to an interaction of either one of the fusion

proteins with the non-fused VP16 or GAL4 domains. One day after transfection, the cells were lysed, and dual luciferase activity was measured. The signal of Renilla luciferase was used for normalization.

3.3.5.2 Transfection and Reporter Gene Assay

10T1/2 cells seeded on 6-well plates were co-transfected with a pair of the GAL4 and VP16 fusion constructs, pRL-tk and pG5luc vector. Dual luciferase assay was performed 24 hours after transfection as described above.

3.3.6 Chromatin Modification Studies

10T1/2 cells were co-transfected with rKv4.2 promoter (-1094~ +592)-pGL3 basic, rIrx5-pcDNA3 and/or rIrx4-pcDNA3 in serum-free medium for 3 hours as described above. Immediately after transfection, cells were exposed to drug treatment in the complete serum-containing medium for 24 hours. Treatment conditions included DMSO (control), TSA (10 nM) or RG108 (0.2 μM). After treatment, cells were harvested and dual luciferase activity was performed.

3.3.7 Statistical Analysis

Each data value was obtained from a duplicate assay. Data values from at least three independent experiments were used for statistical analysis. Statistical comparison was performed by one-way ANOVA, followed by Bonferroni's posthoc test with a significance level

of 0.05 or greater using Prism 5.0 (GraphPad Software, San Diego, CA). Results are expressed as mean ± SEM.

3.4 RESULTS

3.4.1 The C-terminal Region of Irx5 Mediates the Regulation of Kv4.2 Gene Promoter

Irx proteins contain a highly conserved atypical homeodomain flanked by diverse N- and C-terminal peptides, each with 100-150 amino acids and ~300 amino acids in length. To identify the region of Irx5 that mediates the regulation of Kv4.2 promoter, various chimeras between Irx3 and Irx5 were generated (Fig. 8A). In 10T1/2 cells, substituting the N-terminal region of Irx5 with the corresponding portion of Irx3 (3N-5HC and 3NH-5C) had no effect on the ability to increase channel promoter activity (Fig. 8B). In contrast, replacing the C-terminal portion of Irx5 with that of Irx3 (5N-3HC and 5NH-3C) significantly and substantially reduced this induction. Moreover, adding the C-terminal region of Irx5 to Irx3 (3N-5HC and 3NH-5C) transferred the ability to induce promoter activity. In addition to the ability to induce channel promoter activity, the C-terminal portion of Irx5 conferred the suppression by Irx4: Irx4 reduced the upregulation caused by 3N-5HC and 3NH-5C, as well as the original Irx5. Therefore, the C-terminal region of Irx5 is necessary and sufficient for the induction of channel promoter and its suppression by Irx4 in these fibroblastic cells.

The generated chimeras were also used to test if the C-terminal region of Irx5 might also be responsible for the reduction in channel promoter activity in neonatal ventricular myocytes (Fig. 9). Chimeras containing the C-terminal region of Irx5 (3N-5HC and 3NH-5C) were capable of inhibiting channel promoter activity. Substituting this region with the corresponding Irx3 peptide (5N-3HC and 5NH-3C) eliminated the ability to produce this inhibition, although the latter chimera tended to cause a small reduction in promoter activity. Thus, the C-terminal region of Irx5 is mainly responsible for the inhibition of Kv4.2 promoter in neonatal myocytes.

Taken together, the C-terminal Irx5 peptide mediates the regulation of channel promoter activity in both myocytes and non-myocytes.

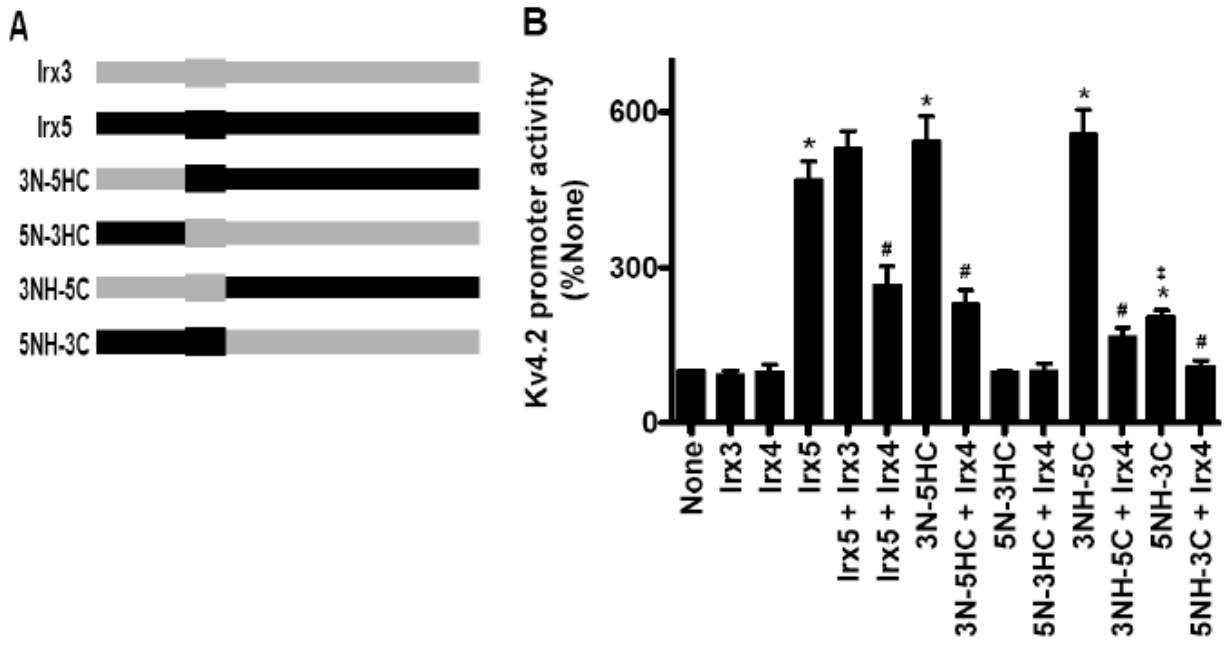

Figure 8. The C-terminal region of Irx5 mediates activation of Kv4.2 promoter in 10T1/2 cells.

(A) Chimeras between Irx3 and Irx5 used for the study are shown with grey and black bars indicating regions originated from the former and latter, respectively. N, H, and C represent N-terminal, homeobox and C-terminal portions, respectively. (B) 10T1/2 cells were transfected with indicated individual or combination of wild-type or chimeric Irx proteins. Total amount of expression vector was kept constant by supplementing empty vector. *3N-5HC, 3NH-5C and 5NH-3C, as well as Irx5, significantly increased Kv4.2 promoter activity compared to None or Irx3 ($p<0.05$, $n>4$ for each condition). ‡However, the increase produced by 5N-3HC was significantly smaller than that by 3N-5HC, 3NH-5C or Irx5 ($p<0.05$). #Coexpression of Irx4 significantly reduced the increase produced by 3N-5HC, 3NH-5C, 5NH-3C or Irx5 ($p<0.05$).

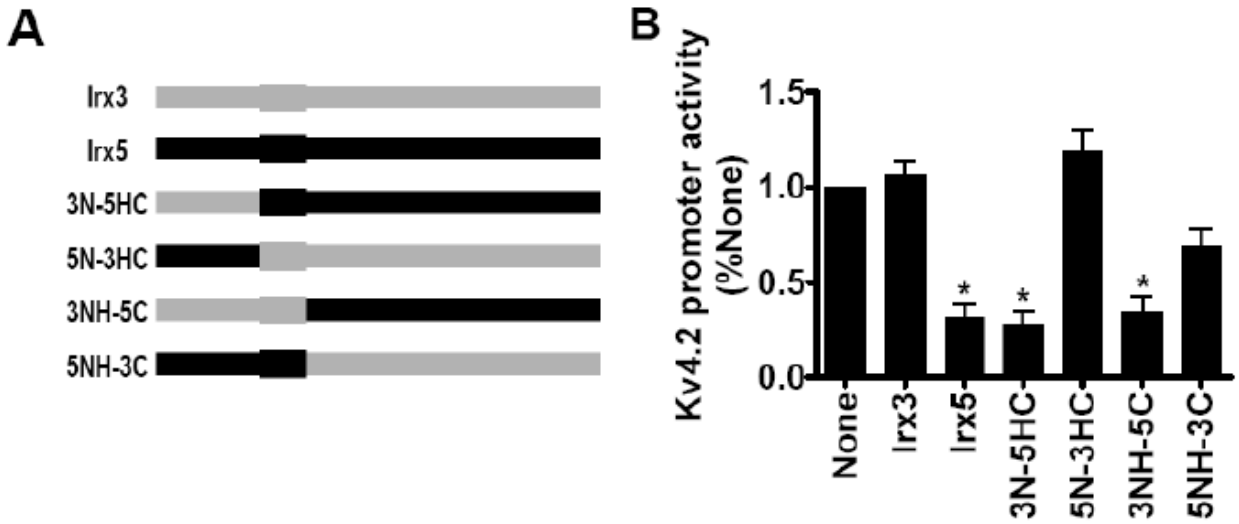

Figure 9. The C-terminal region of Irx5 confers the reduction in Kv4.2 promoter activity in neonatal ventricular myocytes.

(A) Chimeras between Irx3 and Irx5 used for the study are shown with grey and black bars indicating regions originated from the former and latter, respectively. N, H, and C represent N-terminal, homeobox and C-terminal portions, respectively. (B) Neonatal myocytes were transfected with indicated wild-type or chimeric Irx proteins. *3N-5HC, 3NH-5C and Irx5 significantly decreased Kv4.2 promoter activity compared to None or Irx3 ($p<0.05$, n=4 for each condition).

3.4.2 The N-terminal Region of Irx4 is Responsible for the Suppression of the Irx5-induced Regulation

The region of Irx4 responsible for the suppression of the Irx5-induced regulation was next examined using various chimeras between Irx3 and Irx4 (Fig. 10A). These chimeras, as well as the original Irx3 and Irx4, produced only minor changes in Kv4.2 promoter activity by themselves (Fig. 10B). Substitution of the N-terminal portion of Irx4 with the corresponding region of Irx3 (3N-4HC and 3NH-4C) disrupted the inhibition of the Irx5-induced increase in promoter activity. Furthermore, addition of the N-terminal region of Irx4 to the corresponding portion of Irx3 (4N-3HC and 4NH-3C) exhibited the suppression of Irx5-induced activation. Hence, the N-terminal region of Irx4 is necessary and sufficient for the suppression of the Irx5-induced increase in Kv4.2 promoter activity.

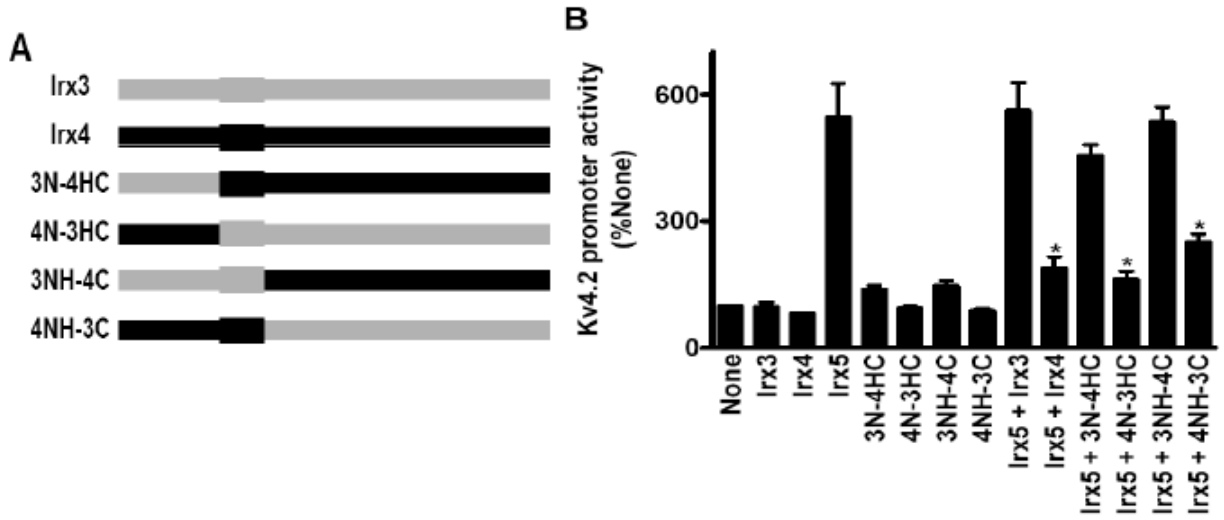

Figure 10. The N-terminal region of Irx4 mediates the suppression of the Irx5-induced activation of Kv4.2 promoter.

(A) Chimeras between Irx3 and Irx4 used for the study are shown with grey and black bars indicating regions originated from the former and latter, respectively. N, H, and C represent N-terminal, homeobox and C-terminal domains, respectively. (B) 10T1/2 cells were transfected with indicated individual or combination of wild-type or chimeric Irx proteins. *4N-3HC, 4NH-3C, as well as Irx4, significantly decreased the Irx5-induced activation of Kv4.2 promoter compared to None or Irx3: Irx5 + 4N-3HC, Irx5 + 4NH-3C or Irx5 + Irx4 < Irx5 or Irx5 + Irx3 ($p<0.05$, $n=4$ for each condition).

3.4.3 Iroquois Proteins Less Efficiently Form Homomeric and Heteromeric Complexes *In Vitro*

To test if the observed suppression by Irx4 might be due to its selective association with Irx5, immunoprecipitation with N-terminally-tagged Irx proteins was performed (Fig. 11). Irx5 proteins interacted indiscriminately with Irx3 and Irx4, as well as its own kind. However, the efficiency of complex formation seemed very low compared to the input Irx proteins. Changing detergent concentrations or composition (0.2-1% Triton X-100 and 1% Triton X-100 + 0.1% SDS) or salt concentrations (0 to 0.5 M NaCl) did not affect the observed non-selective association or the efficiency. These findings suggest that Irx members can form homomeric and heteromeric complexes albeit less efficiently.

To further verify the association of Irx4-Irx5 identified by co-immunoprecipitation of ectopically expressed Irx cDNAs, a mammalian two-hybrid system (Checkmate Mammalian Two-hybrid System, Promega) was employed to test this putative interaction. The entire Irx5 and Irx4 proteins were cloned into the two-hybrid plasmids pACT and pBIND and luciferase reporter gene (pG5) assay was performed 24 hours after transfection. However, no significant association was observed between Irx5 and Irx4 fusion proteins (Fig. 12A and B). To enhance the sensitivity of the two-hybrid system, a flexible linker with twelve amino acids $(GlyGlySer)_4$ between reporter domains and Irx polypeptides was introduced. Again, no detectable interaction between the two Iroquois proteins was detected (Fig. 12C and D).

The homodimerization between the same Irx proteins was also examined using the two-hybrid system. When Irx5-pBIND construct was co-transfected with their homologous VP16-tagged construct, a significant (~3 fold) transcriptional activation resulting from the formation of homomeric complexes of Irx5 proteins was observed (Fig. 13A). However, no interaction

between Irx4-GAL4 or Irx4-VP16 fusion proteins was detected (Fig. 13B). Therefore, Irx5, but not Irx4, proteins can form homomeric complexes under this assay condition.

Taken together, we failed to observe consistent complex formation between Irx proteins. The discrepancy between immunoprecipitation and two-hybrid assays might be due to the lack of nucleus localization of putative complexes (i.e. Irx4-Irx4 and Irx4-Irx5) or the introduction of a large reporter polypeptide at the N-terminus of Irx proteins. Importantly, the selective suppression of the Irx5-induced promoter activation by Irx4 is not due to its specific physical interaction.

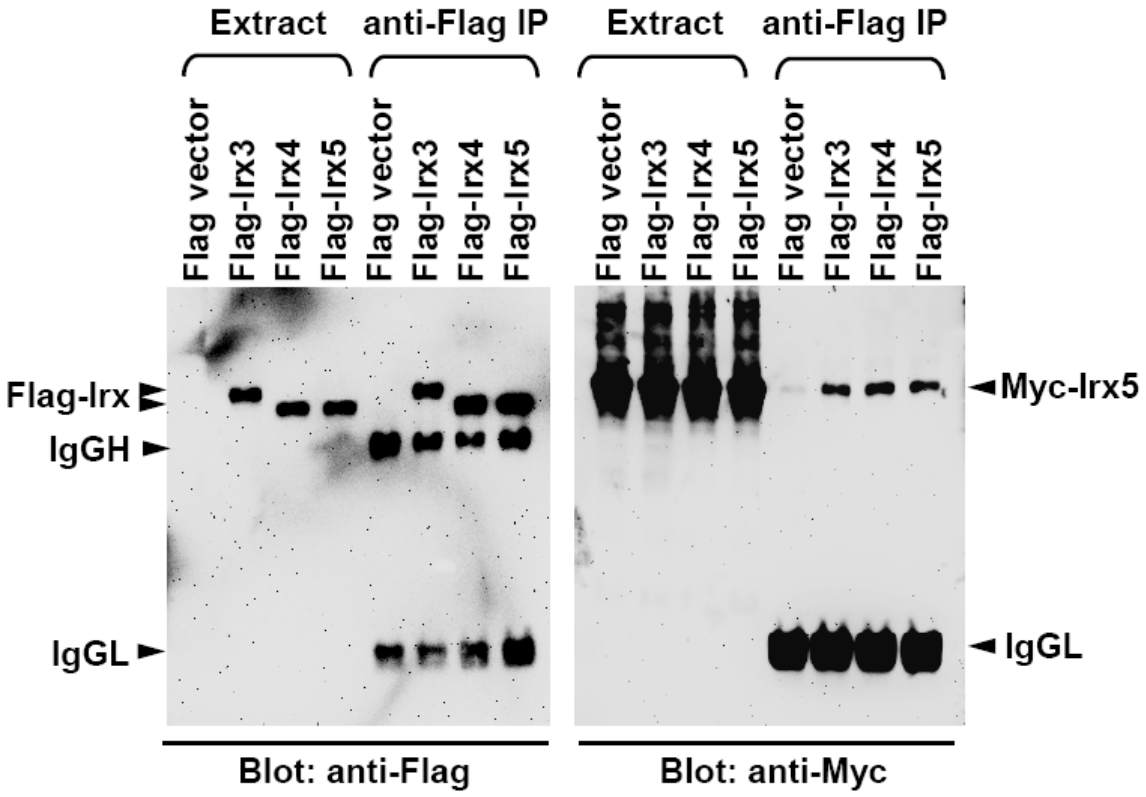

Figure 11. Iroquois proteins form homodimers and heterodimers *in vitro*.

10T1/2 cells were transfected with Myc-Irx5 and indicated Flag-Irx or empty vector. Extract was prepared with 1% Triton X-100 and immunoprecipitation was performed anti-Flag antibody. Extract and immunoprecipitate (anti-Flag IP) were subjected to immunoblot analysis. Arrow heads indicate the position of indicated proteins. IgGH and IgGL represent IgG heavy and light chains, respectively.

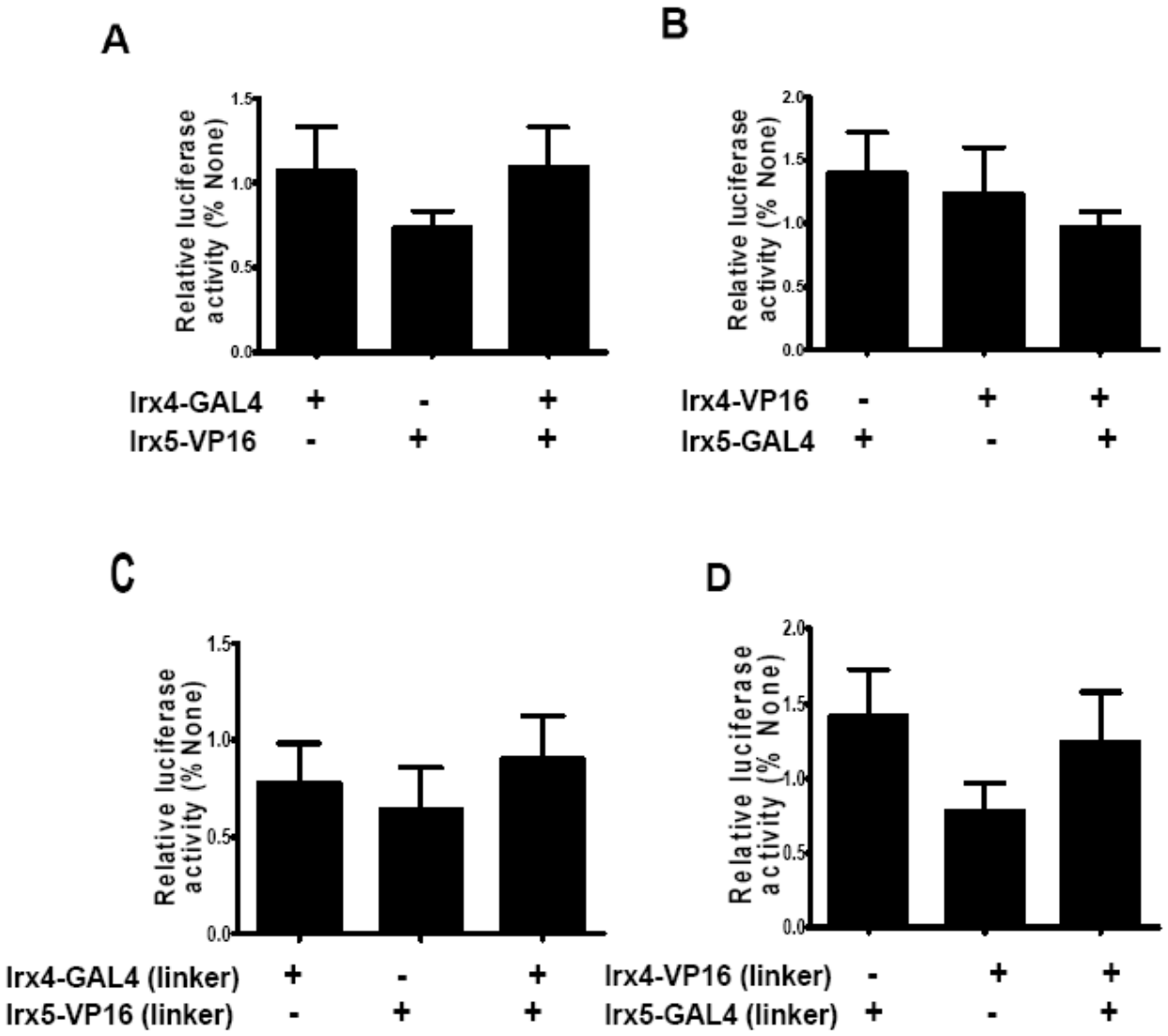

Figure 12. No interaction was detected between Irx5-Irx4 in a mammalian two-hybrid system.

Rat Irx cDNA were fused to VP16 activation domain or GAL4 DNA-binding domain as described in Materials and Methods. 10T1/2 cells were co-transfected with a pair of the indicated constructs, pG5 (None) and pRL-tk (Normalization). Hyphens indicate the expression of an unfused VP16 or GAL4 protein. Columns indicate the mean of luciferase activity relative to None (pG5 only) with error bars representing S.E.M. (n=3 for Fig. A, C; n=4 for Fig. B, D).

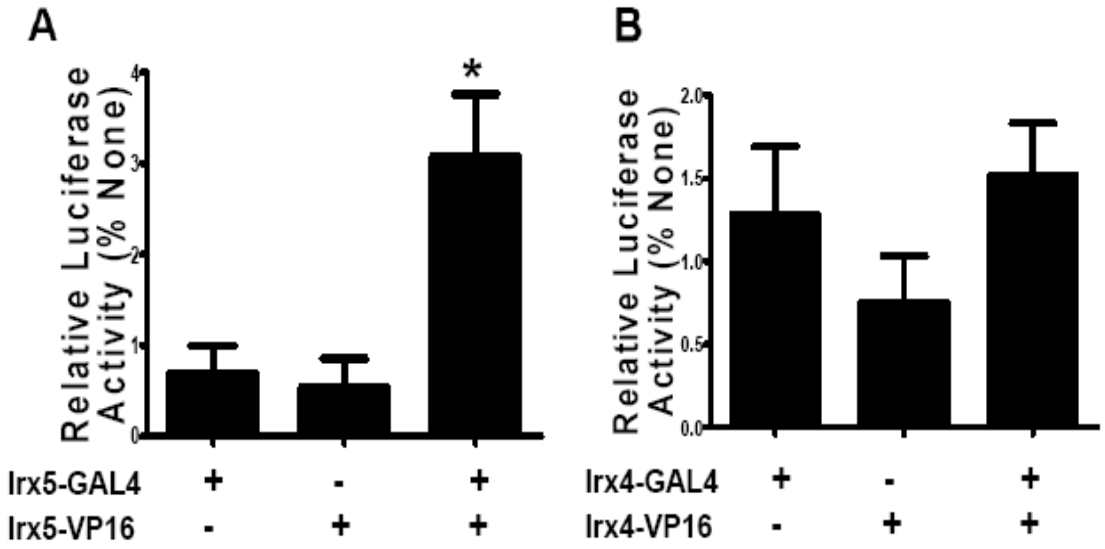

Figure 13. Irx5, but not Irx4 forms homomeric complexes in a mammalian two-hybrid system.

Rat Irx cDNA were fused to VP16 activation domain or GAL4 DNA-binding domain as described in Materials and Methods. 10T1/2 cells were co-transfected with a pair of the indicated constructs, pG5 (None) and pRL-tk (Normalization). Hyphens indicate the expression of an unfused VP16 or GAL4 protein. Columns indicate the mean of luciferase activity relative to None (pG5 only) with error bars representing S.E.M. (n=3 for Fig. A; n=5 for Fig. B).

3.4.4 Histone Deacetylase Inhibitor Relieves the Inhibition of Irx5 Effect by Irx4

It is known that the degrees of histone acetylation and/or DNA methylation in the promoter region of gene influence the transcription initiation. It has also been proposed by Costantini DL *et al*., that the recruitment of histone deacetylases (HDACs) is involved in the action of Irx5 on Kv4.2 promoter activity (2). To investigate whether the observed Irx5 and Irx4 effects on the promoter activity of Kv4.2 gene occur via histone acetylation or DNA methylation, transfected cells were treated with 10 nM HDAC inhibitor Trichostatin A (TSA) (Fig. 14B), 0.2 μM DNA methyltransferase inhibitor (RG108) (Fig. 14A), or vehicle control for 24 hours before luciferase assay. Addition of TSA or RG108 did not significantly change the Irx5-induced increase in promoter activity. In contrast, TSA, but not RG108, relieved the inhibition by Irx4. These suggest that Irx4 recruits HDACs to suppress the Irx5-mediated activation of channel promoter.

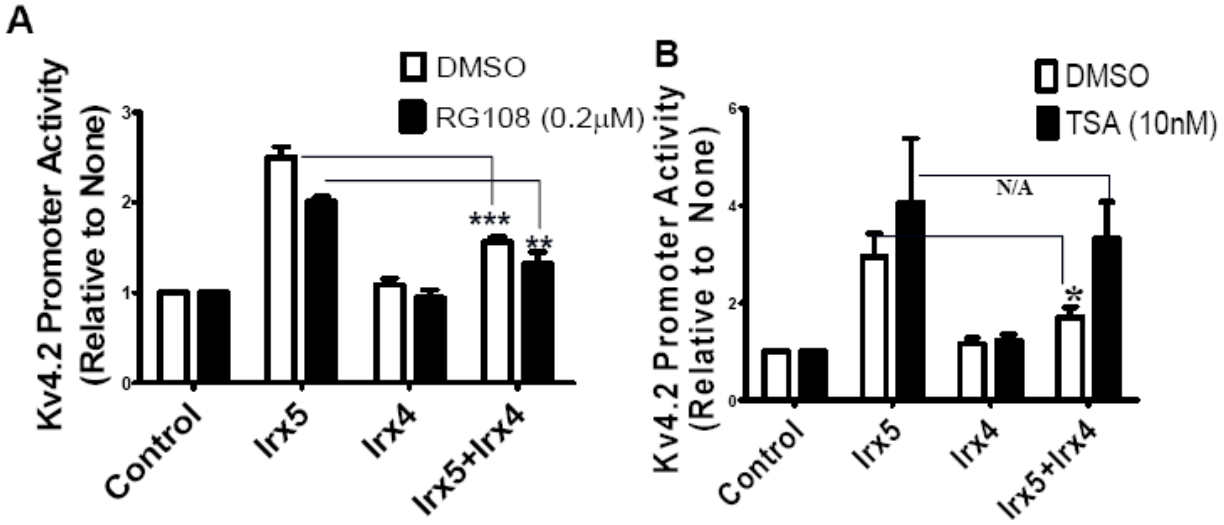

Figure 14. Histone deacetylase inhibitor relieves the inhibition of Irx5-induced activation by Irx4.

10T1/2 cells were co-transfected with a luciferase reporter gene vector (pGL3-basic) containing rKv4.2 promoter (-1094~ +592), rIrx5 and/or Irx4, and pRL-tk in a serum-free medium for 3 hours as described in Methods and Materials. The empty vector pCDNA3 was used to keep the total amount of DNA constructs constant. The transfected cells were then changed to complete medium containing DMSO (control), TSA (10 nM) (B) or RG108 (0.2 μM) (A) for 24 hours before harvest for luciferase assay. Irx4 significantly inhibited the promoter activity induced by Irx5 in DMSO and RG108 (* $p<0.05$, ** $p<0.01$, *** $p<0.005$, n=3). Note there is no significant inhibition of the Kv4.2 promoter by Irx4 in TSA (n=8).

3.5 DISCUSSION

Homeodomains are generally considered to act as a DNA-binding motif. However, a number of studies have suggested Iroquois (Irx) proteins possess a weaker DNA-binding affinity or produce its transcriptional regulation indirectly by interacting with other transcription factors. The present study using various chimeras between Irx proteins revealed that the unique N- and C-terminal peptides of Irx proteins are necessary and sufficient for the regulation of Kv4.2 channel promoter. The C-terminal peptide of Irx5 mediates the upregulation of channel promoter, whereas this enhancement is suppressed by the N-terminal portion of Irx4. Taken together with the extremely high conservation of atypical homeodomains in Irx members, these findings support the possibility that the Irx proteins control Kv4.2 promoter by influencing other transcription factors.

A previous study suggests that Irx proteins may form homomeric or heteromeric complexes *in vitro* (50). In this study, the physical interactions between Irx proteins were examined with co-immunoprecipitation and two-hybrid system. While the former detected non-selective interactions between any two Irx members, the latter detected homomeric association of Irx5, but not complexes between Irx4 and Irx5. This discrepancy might arise from a number of reasons. First, the two-hybrid system uses addition of a relatively large peptide at the N-terminus of the bait. Potentially, it prevents the formation of Irx-Irx complexes. However, the positive association of Irx5 homomeric complexes argues against this possibility. Moreover, the introduction of a flexible linker also failed to detect Irx4-Irx5 heteromeric complexes. Another possibility is that the two-hybrid system is based on transcriptional activity and thus requires nuclear localization of the newly formed complexes. Therefore, if Irx4-Irx5 complexes are only formed in cytosol and fail to import to the nucleus, then the assay would yield a negative result.

This possibility is supported by the fact that Irx4-VP16 appears to give lower firefly signal in this system compared with Irx5-VP16. Thus, Irx4 may be less efficiently imported to or more efficiently exported from the nucleus. Future studies on subcellular localization of Irx complexes may be required to solve this issue.

It has been observed that TGIF2, a TALE atypical homeodomain protein, represses gene transcription at least in part by recruiting histone deacetylase 1 (HDAC1) (84). To test the involvement of chromatin modification in the Irx4-mediated transcriptional repression, the histone deacetylase inhibitor TSA and the DNA methyltransferase inhibitor RG108 were used in this study. Unlike sodium butyrate that elicits pleiotropic effects, TSA is thought to specifically inhibit histone deacetylase activity (85). TSA significantly counteracted the repression elicited by Irx4, whereas RG108 did not affect the repression activity of Irx4. These suggest the participation of HDAC in the Irx4-mediated inhibitory effect. The same observation was made by Costantini DL *et al*. in dissecting the regulatory mechanism of Irx5 on the Kv4.2 expression, although the proposed co-repressor was different (2). They postulated that HDACs was recruited by mBOP to repress the transcription of Kv4.2 channel gene. To address the question whether HDACs are associated on the promoter region (-1094~ +592) of Kv4.2 gene, other approaches for a direct detection such as chromatin immunoprecipitation may be required.

The N-terminal region of Iroquois proteins is proposed to act as a protein interaction domain. *In vitro* translation of flag-Mirr, a fly Irx protein, lacking the N-terminal peptide failed to form homodimer in a pull-down assay (50). However, since deletion might result in misfolding of truncated proteins, one can argue that the failure of homodimerization with N-terminal truncation may arise from impaired conformation of the protein. Another study using CAT reporter gene assay showed the importance of the N-terminal region of Irx4 in mediating

the inhibitory effects on transcription of the atrium-specific myosin heavy chain gene (29). Concordantly, the N-terminal region was identified to interact with retinoic X receptor α subunit (29). Analysis with chimeric Irx proteins in this study also revealed the importance of the N-terminal region of Irx4 in the suppression of Irx5-induced activation of Kv4.2 promoter. Taken together, these results support a model where Irx4 interacts with an as yet identified molecule via its N-terminal region to inhibit channel gene transcription. Since the suppression by Irx4 was prevented by inhibition of histone deacetylase, the putative Irx4-containing complexes may involve these enzymes.

While this work, as well as several previous studies (29, 50), indicates that the N-terminal region of Irx proteins interact with cofactors, little is known about the roles of their C-terminal peptides. The C-terminal region of Irx proteins contains a 13-amino-acid homology domain, named the Iro box. Outside the Iro box, the similarity in amino acid sequence is very low. The study by Costantini *et al*. suggests that the region proximal to the atypical homeodomain of Irx5 is required for its regulation of Kv4.2 promoter (2). The chimera analysis in this chapter also demonstrated that the entire C-terminal region of Irx5 is necessary and sufficient for the regulation of channel promoter. Moreover, the same region confers the inhibitory effect by Irx4. Thus, the C-terminal peptide of Irx5 may also recruit cofactors to activate transcription of the Kv4.2 gene. Irx4 may prevent the Irx5's complex formation with this putative cofactor or its activity.

The structure-function studies in this chapter suggest that Irx proteins interact with various cofactors via diverse N- and C-terminal peptides to control gene transcription. The presence and absence of these cofactors likely underlie the observed cell type-specific and region-selective expression of Kv4.2 gene. Given the high homology of the DNA-binding

atypical homeodomain in Irx proteins, it is plausible that they require cofactors to achieve their diverse biological functions. Incorporation of these cofactors may enhance DNA-binding specificity or stability, or even alter the outcome of the DNA binding, as has been seen with typical homeodomain transcription factors. Hence, the observations made in this study and previous reports by others support the proposal that heteromeric complex formation of TALE atypical homeodomain proteins including Irx proteins with homeodomain-containing and/or other factors may be a generalized feature of their transcriptional functions.

4.0 THE CIS-ELEMENT REQUIRED FOR THE IRX5-INDUCED RAT KV4.2 GENE TRANSCRIPTION

4.1 ABSTRACT

Iroquois (Irx) proteins are transcription factors with an atypical homeodomain. However, their target genes and binding sequences remain largely unknown. A previous animal study (2) and previous chapters in this thesis have demonstrated the importance of Irx5 in regulating Kv4.2 gene transcription. This chapter uses deletion and mutation analyses of the channel promoter, and electromobility shift assays to identify Irx5-regulatory elements. Deletion analysis of the Kv4.2 5'flanking region indicated that multiple regions between -1002 and -414 are required for the full activation of channel promoter by Irx5 in 10T1/2 cells. The most dramatic reduction of Irx5-mediated activation was seen when a 36-bp portion (-1002~ -966) was eliminated. The 5'flanking region also activated a minimum heterologous promoter (SV40) in response to Irx5 in a direction-dependent way. Similarly, the 36-bp fragment is sufficient to mediate the Irx5 response in a direction-dependent manner. Point mutations further identified a GT-rich element (GGTGGGTGG) in this 36-bp region that is required for the activation by Irx5. Electrophoretic mobility shift assay (EMSA) failed to detect any specific binding of Irx5 in this region, but suggested a binding of an unidentified endogenous protein in fibroblast cells. In conclusion, these results demonstrate the presence of a previously unidentified Irx5-response

element in the Kv4.2 distant promoter region. They also suggest that Irx5 indirectly regulates Kv4.2 gene transcription by influencing other factors that control channel promoter activity.

4.2 INTRODUCTION

Iroquois (Irx) proteins possess a 63-amino acid atypical homeodomain with three extra amino acids (proline-tyrosine-proline) between the helix 1 and helix 2. This insertion is found in various transcription factors, which constitute the TALE atypical homeodomain superclass (37). The DNA-binding specificity of homeodomain is primarily determined by its third helix, called the recognition helix, which inserts itself into the major groove of the recognition site (86, 87). It has been proposed that the 9th position of the recognition helix (the 50th position of the homeodomain) plays a critical role in differential DNA recognition (88, 89). In most cases, homeodomain proteins have a polar residue at the 50th position, such as glutamine, lysine, cystein, histidine or serine. In contrast, proteins in the TALE atypical homeodomain superclass invariably contain a non-polar residue, isoleucine, with the exception of alanine in Irx proteins and glycine in Pbx proteins (37).

Much of knowledge about the DNA recognition sites for homeodomain proteins come from studies of classic homeodomains. These typical homeodomains bind to the sequence consisting of the common "TAAT" core with additional two residues at the 3' end which differ depending on individual homeodomain proteins (90). Very little is known about the binding sites of the atypical homeodomains. An early study found that the *Drosophila* Irx protein, Arauca, protected a region containing the classic consensus sequence, TAAT, in footprinting assays (52). A later study examined the same region carefully and suggested a different binding site (ACAnnTGT) for the *Drosophila* Irx protein, Mirr, using *in vitro* site selection and electrophoretic mobility shift assays (EMSA) (50). Another biochemical study suggested that TGIF (5'TG3' Interacting Factor), a member of TALE atypical homeodomain proteins, binds to a TGTCA core sequence and represses retinoid X receptor α-mediated transcription activation as

competing overlapping DNA binding sites (60). Thus, the binding sequence of Irx proteins remains obscure.

The Irx5-mediated regulation of Kv4.2 gene promoter provides an excellent opportunity to identify cis element(s) that is required for transcriptional regulation by atypical homeodomain transcription factors. A previous study in this laboratory using 5' RACE and RNase protection assays have determined the transcription start site of Kv4.2 gene (36). The transcription of this gene was found to initiate at 552 bp upstream from the translation site in the brain and heart. Approximately 200-bp fragment encompassing this transcription start site drives significant transcription in neonatal cardiac myocytes. Nothing is known about the distant promoter region of Kv4.2 gene. This study and reports by others (2) indicate that the region between -1073 and -432 is required for the full activation of channel promoter by Irx5, suggesting the presence of Irx-responsive element(s) in this region. Therefore, this chapter is aimed at identifying cis element(s) that mediate Irx5-induced regulation of Kv4.2 gene.

4.3 MATERIALS AND METHODS

4.3.1 Cell Culture and Reagents

4.3.1.1 Cell Culture

10T1/2 cells were cultured as described in the previous chapters. Ad293 human embryonic kidney epithelial cells were obtained from Stratagene (La Jolla, CA). This cell line was derived directly from the HEK293 cells and possesses improved adherence to plastic dishes. These cells were cultured in DMEM supplemented with 10% FBS at 37ºC under 5% CO_2 atmosphere.

4.3.1.2 Reagents

Poly-(deoxyinosinic-deoxycytidylic) acid sodium salt was from Sigma-Aldrich (St. Louis, MO). Ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Bio-Rad (Hercules, CA).

4.3.2 Constructions

4.3.2.1 Kv4.2 Promoter-pGL3 Basic Constructs

Various 5' deletion fragments of Kv4.2 5'flanking region were generated by PCR-based methods using primers with convenient restriction enzyme sites (Table 4 with restriction enzyme sites underlined). These fragments were then subcloned into pCR-Blunt II TOPO vector (Invitrogen, Carlsbad, CA) and digested at the introduced enzymes sites to release the fragments,

followed by cloning the released fragments into the identical or compatible restriction enzyme sites in the polylinker of pGL3-basic vector.

Table 4. Primers Used in Deletion Analysis

Constructs	Forward	Reverse
-1002/ +488	5'- TTGGATCCATTTGGCGTC GAGGCTGG-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-966/ +488	5'-GCTAGCGCGAGAGGAGCC CGA-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-934/ +488	5'-GCTAGCAAGGTCAAGGCG AG-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-906/ +488	5'-CAGGATCCGCCTTTGCCTA ACCTGC-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-858/ +488	5'-CTGGATCCCTCCCTGCTTA TTTATCG-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-547/ +488	5'-CGGGATCCCCGCACCTTTT TGAACTTG-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-491/ +488	5'-CTGGATCCTGGGAAGGTG ACAAGGAG-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'
-434/ +488	5'-GAGGATCCGCCAGAGCTT TATTTATGC-3'	5'-AAGCTTCCCCACTTCTTC ACCTC-3'

4.3.2.2 Kv4.2-SV40 Promoter Constructs

The effects of Kv4.2 5' flanking region on a heterologous promoter were tested using pGL3-promoter vector which contains the multiple cloning sites in front of the SV40 minimum promoter. Various lengths of the Kv4.2 5'flanking region were generated by PCR using primers with unique or convenient restriction enzyme sites (Table 5 with restriction enzyme sites underlined). These fragments were subcloned into pCR-Blunt II TOPO vector and digested with appropriate restriction enzymes to release the inserts. The released fragments were then cloned into the identical or compatible enzyme sites in the polylinker of pGL3-promoter vector.

Table 5. Primers Used for Heterologous Promoter Assay

Constructs	Forward	Reverse
-1073bp~-98bp **-1073bp~-98bp (R)**	5'- AAGCTTCCCTTTG TTCATCC-3'	5'-CGTCGGTCTAGAGTTCT CTG-3'
-1073bp~-572bp	5'-AAGCTTCCCTTTG TTCATCC-3'	5'-GCTCCTGCCAACTTACTG CG-3'
-867bp~-329bp	5'-CTGGATCCCTCCC TGCTTATTTATCG-3'	5'-GCATAGAGACTTTGCTGT CG-3'
-1013bp~-903bp	5'-TCTCCCTTGGTGA CATTTGG-3'	5'-TCTCCTGCCTTTCCCTCG CC-3'

4.3.2.3 Kv4.2 (-1002~ -966) x1, x2 and x4-pGL3-promoter Vectors

Heterologous promoter constructs containing the 36-bp (-1002~ -966) Kv4.2 5'flanking region were made in pGL3-promoter vector. The 5'-end phosphorylated double-strand oligonucleotides corresponding to one or two copies of the 36-bp fragment were chemically synthesized (Table 6 with restriction enzyme sites underlined). Because the fragment with two copies was very long, one (sense) strand with the full sequence and the other that corresponded to the 3'-end portion of the long oligonucleotide were used. The two partially-compatible oligonucleotides were annealed and excessive end was filled by PCR. This fill-in PCR protocol was: 10 PCR cycles consisting of 65ºC for 20 seconds and 72ºC for 20 seconds; followed by a final extension at 72ºC for 2 minutes. The obtained blunt-ended fragment was cloned into the topoisomerase-based vector, pCR-Blunt TOPO II.

The construct containing one 36-bp fragment [Kv4.2 (-1002~ -966) x1] was generated by a simple linker ligation, whereas the one with two fragments [Kv4.2 (-1002~ -966) x2] was made from the pCR-Blunt TOPO II containing two 36-bp fragments in tandem. For the former, the two oligonucleotides contained sticky ends compatible to the *Bgl*II site at the 5'end and the *Nhe*I site at the 3'end. Followed by annealing of the complementary oligonucleotides, the annealed oligonucleotides were inserted to the *Bgl*II-*Nhe*I site of pGL3-promoter. For the latter, the insert

was liberated with *Bgl*II and *Nhe*I from the TOPO construct and subcloned into the corresponding site of the vector.

The construct with four copies of the 36-bp fragment was then generated by inserting an additional two 36-bp-containing fragments into Kv4.2 (-1002~ -966) x2. To provide the fragment with two 36-bp region suitable for subcloning, the TOPO clone with correct direction: *Kpn*I-the insert-*Xba*I, was selected. The insert was liberated from the TOPO clone with these two enzymes and subcloned in front of the two elements of Kv4.2 (-1002~ -966) x2 at the *Kpn*I-*Xba*I site. The generated four 36-bp fragment-containing constructs have extra sequences between the front two and the back two 36-bp fragments.

Table 6. Oligomers Used for Construction of 36-bpx1, x2, x4-pGL3-promoter Vectors.

Constructs	Forward	Reverse
36-bp x1	5'phospho-CTAGCATTTGGCGTCGAGGCTGGTGGGTGGCTAGGGTGGA-3'	5'phospho-GATCTCCACCCTAGCCACCCACCAGCCTCGACGCCAAATG-3'
36-bp x2 36-bp x4	5'phospho-CATTTGGCGTCGAGGCTGGTGGGTGGCTAGGGTGGAGATCCATTTGGCGTCGAGGCTGGTGGGTGGCTAGGGTGGAAGATCTGATC-3'	5'phospho-GATCAGATCTTCCTCCCTAG-3'

4.3.2.4 Kv4.2-(-1002~ -966)-mutation-pGL3-promoter Vectors

Kv4.2-(-1002~ -966)-mutation constructs contain four copies of the identified 36-bp region from -1002 to -966 with nucleotide substitutions eliminating the consensus for either an E-box motif (5'-**ACTTGT**GCGTCGAGGCTGGTGGGTGGCTAGGGTGGA -3')
or a GT-rich motif (5'- CATTTGGCGTCGAGGCT**GCAGGCAGG**CTAGGGTGGA-3'). The four 36-bp elements with mutations were cloned upstream of the SV40 minimum promoter in pGL3-promoter vector. These constructs were generated using simultaneous ligation of two

double-stranded oligonucleotides into a standard cloning vector, followed by transferring a two-36-bp-containing fragment from the cloning vector to the pGL3-promoter vector with a two-mutant 36-bp fragment. Three sets of compatible oligonucleotides containing altered E-box or GT-rich motif with sticky ends (Table 7 with restriction enzyme sites underlined) were designed. The first set contained the 5' *Nhe*I-compatible end and the 3' *Hind*III-compatible end; the second the 5' *Hind*III-compatible end and the 3' *Xho*I-compatible end; and the third the 5' *Hind*III-compatible end and the 3' *Bgl*II-compatible end. The first and second sets of annealed oligonucleotides were simultaneously cloned into the *Spe*I-*Xho*I site of pCR-BluntTOPOII vector. These clones were used to supply the fragment for a two-mutant-containing-36-bp region. The first and third sets were similarly cloned into the *Nhe*I-*Bgl*II site of pGL3-promoter vector to generate intermediate constructs with two 36-bp fragments in a heterologous promoter vector. The two 36-bp-containing fragments from the cloning vector were released by digestion with *Kpn*I and *Xba*I in the polylinker region. The released fragments were then cloned into the *Kpn*I-*Nhe*I site of the intermediate constructs. The inserts of the obtained constructs were verified by DNA sequencing.

Table 7. Oligomers Used for Mutation Analysis

Constructs	Forward	Reverse
E-box mutation	5'phospho-CTAGCACTTGTGCGTCGAGGCTGGTGGGTGGCTAGGGTGGA-3'	5'phospho-AAGCTTCCACCCTAGCCACCCACCAGCCTCGACGCACAAGTG-3'
	5'phospho-AGCTTACTTGTGCGTCGAGGCTGGTGGGTGGCTAGGGTGGA-3'	5'phospho-TCGATCCACCCTAGCCACCCACCAGCCTCGACGCACAAGT-3'
	5'phospho-AGCTTACTTGTGCGTCGAGGCTGGTGGGTGGCTAGGGTGGA-3'	5'phospho-GATCTCCACCCTAGCCACCCACCAGCCTCGACGCACAAGT-3'
GT-rich mutation	5'phospho-CTAGCCATTTGGCGTCGAGGCTGCAGGCAGGCTAGGGTGGA -3'	5'phospho-AAGCTTCCACCCTAGCCTGCCTGCAGCCTCGACGCCAAATGG-3'
	5'phospho-AGCTTCATTTGGCGTCGAGGCTGCAGGCAGGCTAGGGTGGA-3'	5'phospho-TCGATCCACCCTAGCCTGCCTGCAGCCTCGACGCCAAATG-3'
	5'phospho-AGCTTCATTTGGCGTCGAGGCTGCAGGCAGGCTAGGGTGGA-3'	5'phospho-GATCTCCACCCTAGCCTGCCTGCAGCCTCGACGCCAAATG-3'

4.3.3 Reporter Gene Assay and Luciferase Assay

10T1/2 cells were seeded on 6-well plates with 50% confluence. Kv4.2 promoter-reporter gene, pRL-tk, and rIrx5-pcDNA3 were co-transfected into 10T1/2 cells using Lipofectamine 2000 at 1 μg DNA: 2 μl transfection reagent ratio for 3 hours in the absence of serum. The transfected cells were cultured in the complete medium for one more day before harvesting for luciferase assay as described above.

4.3.4 Electrophoretic Mobility Shift Assay (EMSA)

4.3.4.1 Nuclear Extraction

Ad293 cells in a 100-mm plastic dish were transfected with 5 μg of rIrx5-pcDNA3 or empty vector as described above. One day after transfection, Ad293 cells were scraped off from plastic surface using a plastic lifter with ice-cold PBS. The cell pellets were collected by centrifugation at 2000 rpm for 1 minute. Nuclear proteins were prepared by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Perbio Science, Erembodegem, Belgium) according to the manufacturer's protocol.

4.3.4.2 Binding Reaction

The oligonucleotides encompassing the putative Irx5 binding site in the Kv4.2 promoter (-1013~ -958) were generated by PCR using primers with or without a biotin group at the 5'-end (Table 8). The amplified fragment was then purified using QIAquick gel extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. The biotin-labeled fragment was used as a probe, while the unlabeled products were served for competition with the probe in the binding.

The binding reactions were performed with the biotin-labeled probe using LightShift chemiluminescent EMSA kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instruction. Briefly, nuclear proteins were first added to the reaction mixture containing 1x binding buffer, 50 ng/μl Poly (dI·dC) and incubated for 5 minutes at room temperature. Then the biotin-labeled oligonucleotide (20 fmol) was added in a final volume of 20 μl and the incubation was further continued for 20 minutes. In competition assays, 200-fold molar excess of unlabeled oligonucleotides were added at the start of the reaction.

Table 8. Oligonucleotides Used in EMSA

DNA Fragment	Forward	Reverse
-1013~ -958	5'-TCTCCCTTGGTGACATTTGG-3'	5'-TCTCGCTCCACCCTAGCCAC-3'
-1002~ -966	5'phospho-CATTTGGCGTCGAGGCTGGTGGGTGGCTAGGGTGGA-3'	5'phospho-TCCACCCTAGCCACCCACCAGCCTCGACGCCAAATG-3'

4.3.4.3 Electrophoresis

The bound complexes were separated on a 6% polyacrylamide gel at 4 ºC in 0.5% TBE and transferred to Hybound-N^+ membrane (GE Healthercare, Piscataway, NJ). The biotin-labeled DNA was detected using the LightShift chemiluminescent EMSA kit according to the manufacturer's instruction. Chemiluminescence images were captured and analyzed using charged-coupled device camera-based system.

4.3.5 Statistical Analysis

Each data value was obtained from duplicate assay. Data values from at least three independent experiments were used for statistical analysis. Statistical comparison was performed by one-way ANOVA followed by Bonferroni's posthoc test with a significance level of 0.05 or greater using Prism 5.0 (GraphPad Software, San Diego, CA). Results are expressed as mean ± SEM.

4.4 RESULTS

4.4.1 Multiple Regions Are Required for the Full Activation of Kv4.2 Promoter by Irx5

This laboratory previously isolated and characterized the promoter for the rat Kv4.2 gene (36). Transcription of this gene initiates at 552 bp upstream from the translation site in the brain and heart. In addition, the region between -3162 to -1094 exhibits inhibitory effects in cardiac myocytes and neuron-like PC12 cells. Hence, to identify the sequence that might be responsible for the Irx5 effects, a series of deletion from the 5'end of the 5'flanking region (-1094~ +488) of rat Kv4.2 promoter were generated. The generated fragments were tested for the promoter activity in the presence and absence of Irx5 in 10T1/2 cells (Fig 15A). The basal activity in the absence of the transcription factor was similar in all deletion constructs. The degree of Irx5-induced activation significantly differed between deletion constructs (Fig 15B). The longest promoter construct (-1073 ~+519) drove a ~8 fold increase (Fig 15B). Deletion of the region from -1002 to -966 halved the induction by Irx5 (fold change by Irx5 in a deletion construct compared to that of the longest promoter construct, $p<0.05$, $n>5$ for each construct, Bonferroni's test). These findings indicate that multiple portions in the Kv4.2 5'flanking region mediate Irx5-induced activation of the channel promoter. They also suggest that the 36-bp portion (-1002~ -966) contains an element that is required for the full activation of channel promoter by Irx5.

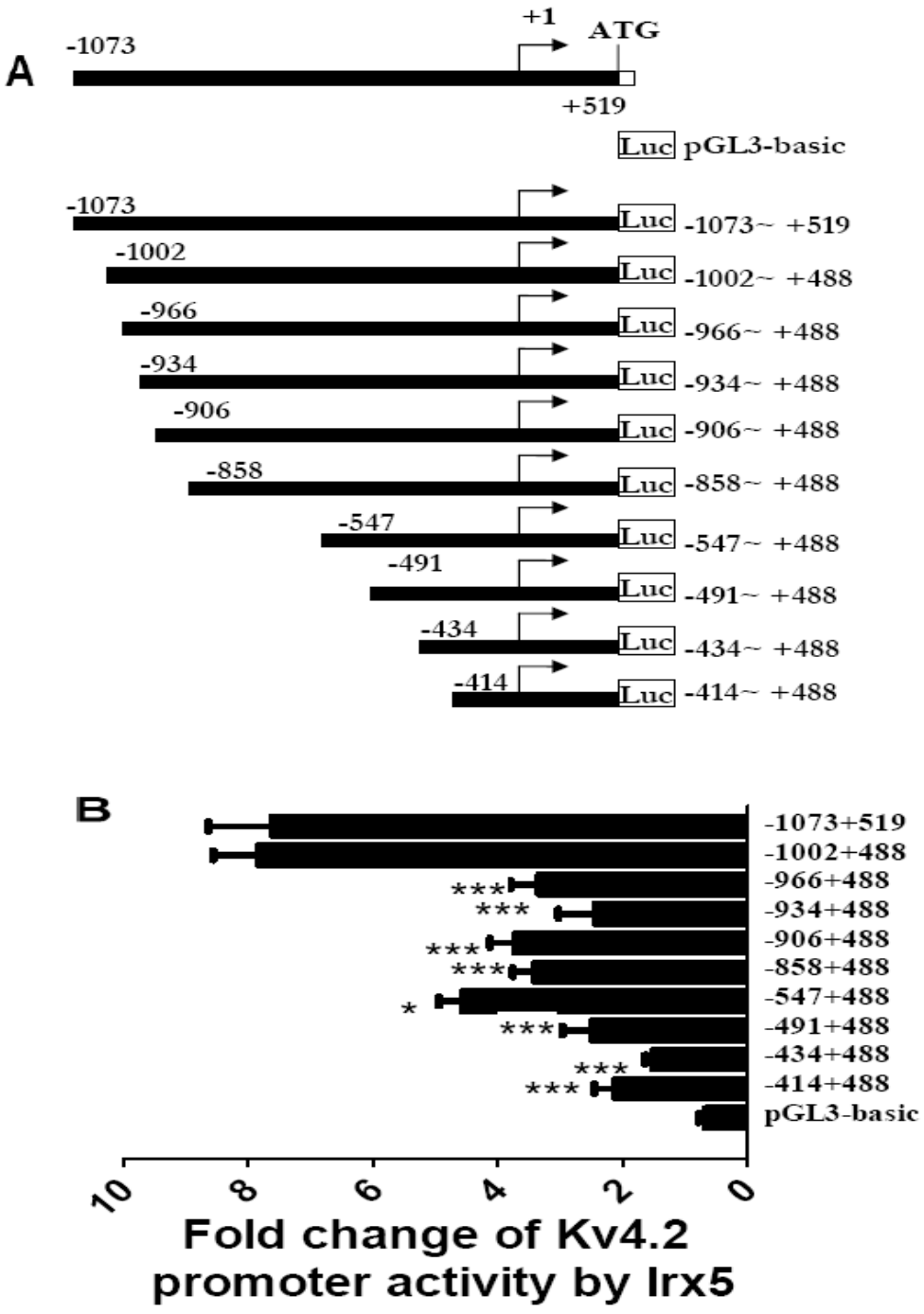

Figure 15. Multiple regions are required for the full activation of Kv4.2 promoter by Irx5.

A. Various portions of the Kv4.2 5'flanking region (filled bars) were fused to the upstream of the luciferase gene (open bars) of pGL3-basic vector. Numbers represent the positions in bp with respect to the transcription initiation site. B. 10T1/2 cells were co-transfected with one of these 5'-deletion constructs containing different promoter regions, expression vector for rIrx5 or empty vector and pRL-tk. Luciferase activity was normalized with the activity of co-transfected pRL-tk, and is presented as fold increases by Irx5 (Irx5 expression vector/empty vector). Columns and error bars represent the means and S.E.M., respectively. N≥5 for each construct. *p<0.05 and *** p<0.005 compared to the longest promoter construct (-1073~ +519).

4.4.2 The ~1-kb Kv4.2 5'-flanking Region Enhances a Heterologous Promoter in Reponses to Irx5

A classical enhancer acts independently of the promoter, direction or location. To test if the distal promoter region of Kv4.2 may possess these characteristics, the ~1-kb region (-1073~ -98) was cloned into the vector containing a heterologous SV40 core promoter. When the ~1-kb region (-1073~ -98) was placed in front of the SV40 minimum promoter, Irx5 significantly increased promoter activity by ~four-fold (Fig 16A). The ~1-kb fragment in the reverse orientation did not produce any change in promoter activity in response to Irx5. Thus, this region contains a direction-dependent Irx5-responsive element. To narrow down the region responsible for the Irx5-induced activation of the heterologous promoter, the ~1-kb region was divided into three portions (Fig 16B). The 5' and 3' part of the region supported significant (~two-fold) increases in promoter activity by Irx5, but none of them restores the activity mediated by the full length (~1-kb) of the region, suggesting a synergetic effect of the two parts. The middle region also tended to increase promoter activity upon Irx5 expression. These data further support the presence of multiple Irx5-responsive elements in the distal 5'flanking region of Kv4.2 promoter.

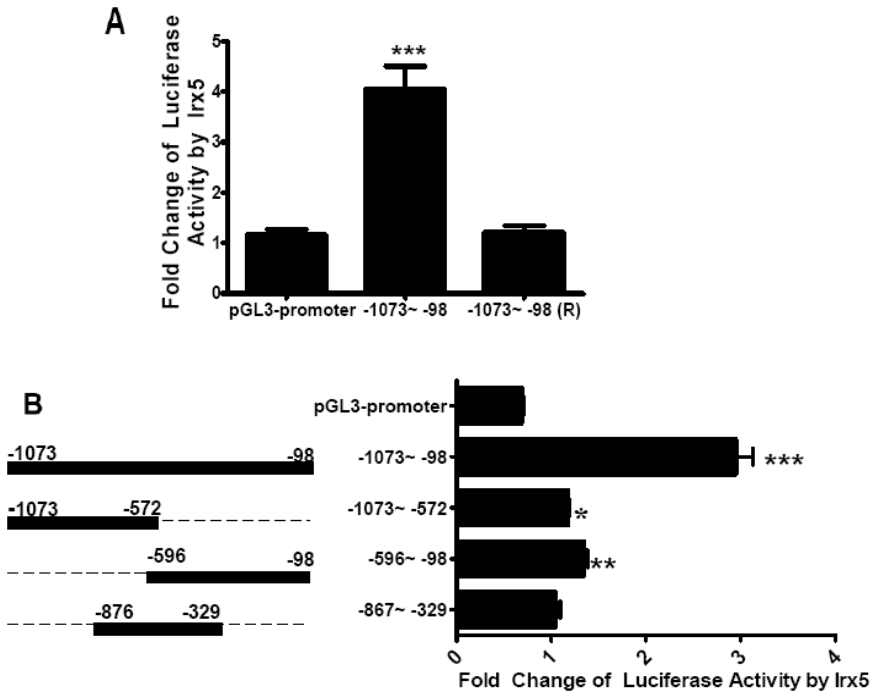

Figure 16. The distal 5'-flanking region of Kv4.2 promoter activates the SV40 minimum promoter in a direction-dependent manner.

A. The ~1-kb fragment of the 5'-flanking region of Kv4.2 promoter (-1073~ -98) was fused to pGL3-promoter vector which contains the SV40 promoter upstream of the luciferase gene. R denotes the ~1-kb fragment (-1073~ -98) in the reverse direction. Fold changes by Irx5 determined as described in the legend for Fig. 14B. Columns and error bars represent the mean and SEM. *p<0.05 compared to pGL3-promoter vector (n=4 for each construct). B. The ~1-kb fragment of the 5'-flanking region of Kv4.2 promoter (-1073~ -98) was divided into three parts with each ~500 bp in length: -1073~ -572, -596~ -98, and -876~ -329. These different regions were fused to the heterologous promoter (SV40). Columns and error bars represent the mean and SEM. *p<0.05 compared to pGL3-promoter vector (n=3 for each construct).

4.4.3 A GT-rich Element in the 36-bp Region is Required for the Irx5-induced Enhancement of Promoter Activity.

Deletion analysis indicated that the 36-bp region (-1002~ -966) is required for the maximum activation of Kv4.2 promoter by Irx5. Thus, the identified 36-bp region (-1002~ -966) might be sufficient to stimulate a heterologous promoter in response to Irx5. One, two or four copies of the 36-bp region were placed in front of the SV40 core promoter in a luciferase vector. Reporter gene assays showed that this 36-bp fragment mediates stimulation of the heterologous promoter by Irx5 (Fig. 17). The increase tended to be larger when more copies were introduced. These results indicate that the identified 36-bp region (-1002~ -966) contains an Irx5-responsive element. They also suggest that this region mediates, in part, the upregulation of Kv4.2 promoter by Irx5.

The sequence of this 36-bp region contains several potential elements that can mediate the Irx5 response. Notably, a non-canonical E-box (CANNTG) is located at the 5'end, whereas a GT-rich sequence (GGTGGGTGG) is seen in the middle (Fig. 18A). Previous studies have shown the importance of E-box and GT-rich elements in the regulation of various cardiac and other genes (91-95). To determine if these elements are required for the Irx5-induced activity in 10T1/2 cells, four copies of the 36-bp fragment that contains mutations in either the E-box or GT-rich motif were inserted into pGL3-promoter vector (Fig 18B). Transient transfection assays indicated that Irx5 caused 2~3-fold enhancement of the promoter activity with wild-type element *(-1002~ -966) x4* (Fig. 18B) ($p<0.01$). Mutations of the E-box motif did not affect the Irx5 response ($p<0.01$). In contrast, destroying the GT-rich sequence eliminated the activation of the promoter activity by Irx5. These results indicate that the GT-rich element in the identified 36-bp region acts as an Irx5-responsive element.

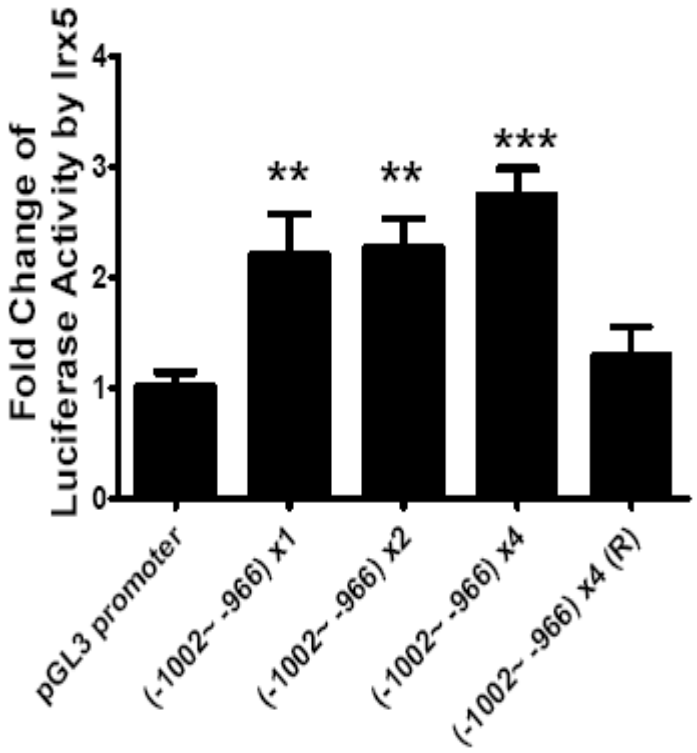

Figure 17. The 36-bp fragment (-1002~ -966) shows enhancement of a heterologous promoter (SV40) in response to Irx5.

One, two, or four copies of the 36-bp fragment (-1002~ -966) in the Kv4.2 distal promoter region were placed in tandem in the pGL3-promoter vector: construct (-1002~ -966) x1, (-1002~ -966) x2 and (-1002~ -966) x4. Fold changes by Irx5 are shown with columns and error bars indicating the mean and SEM. *p<0.05, **p<0.01 and *** p<0.005 compared to pGL3-promoter vector (n=5 for each construct).

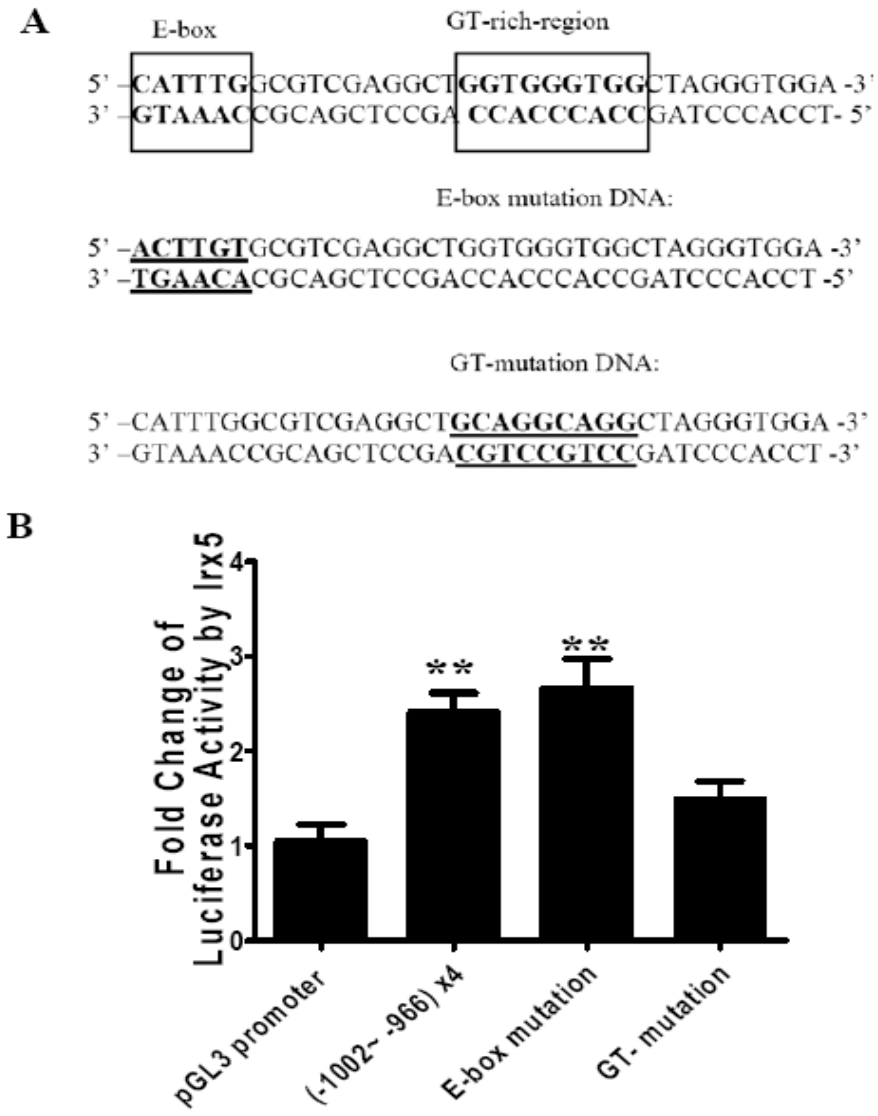

Figure 18. A GT-rich element (GGTGGGTGG) in the 36-bp fragment is responsible for the activity by Irx5.

A. The 36-bp region contains the consensus sequence for E-box and GT-rich elements (highlighted with boxes), which are known to control transcription of various cardiac and other genes (top). Point mutations were introduced to eliminate the consensus for these elements (middle for E-box and bottom for GT-rich region). B. Four copies of the original and mutated 36-bp elements were inserted in pGL3-promoter vector containing the SV40 promoter upstream of the luciferase gene. Each wild-type or mutated construct was cotransfeted with pRL-tk with Irx5 expression construct or empty vector into 10T1/2 cells. Normalized luciferase activity (GL/RL) was used for comparison. Columns indicate the ratio of Irx5 expression vector/empty vector with error bars representing SEM. ** $p<0.01$ compared to pGL3-promoter vector ($n\geq3$ for each construct).

4.4.4 The 36-bp Region of Kv4.2 Promoter (-1002~ -966) Binds to an Endogenous Nuclear Protein.

Irx5 might activate promoter activity by directly binding to the 36-bp region (-1002~ -966). To test this hypothesis, nuclear extracts were prepared from rIrx5-pcDNA3- or empty vector-transfected Ad293 cells. The obtained nuclear proteins were tested for the binding to this identified region using electrophoretic mobility shift assay (EMSA) with a biotin-labeled 55-bp fragment (-1013~ -958) encompassing the 36-bp region (-1002~ -966). A specific band was observed in EMSA assays in both control and ectopic Irx5-expressing extracts (Fig 19). This band was eliminated when excess competitor was included in the binding reaction. No apparent differences in the size or intensity of this band were detected between control and Irx5-containing extracts. A similar binding of endogenous proteins was also seen with nuclear extracts from 10T1/2 cells, although the intensity of the band was much weaker (data not shown). These results indicate that Irx5 does not bind to the 36-bp region directly. The observed enhancement of promoter activity by Irx5 may involve a more complex indirect action of the transcription factor.

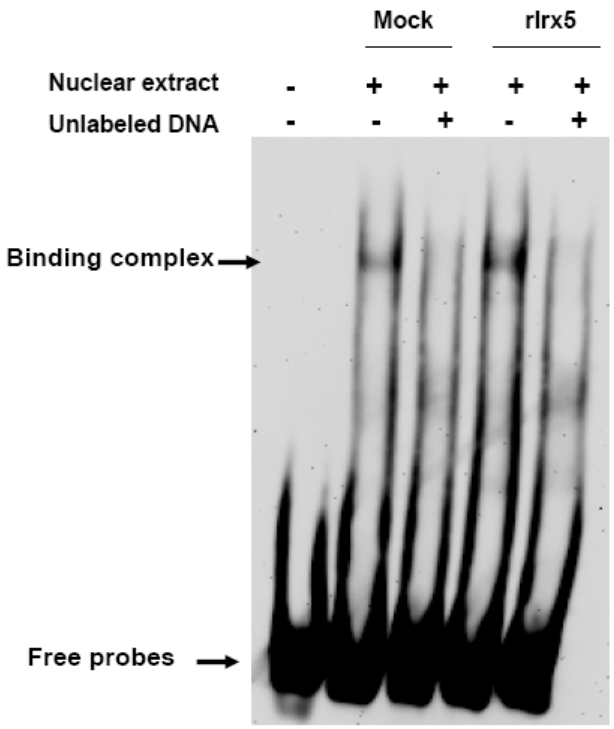

Figure 19. The 36-bp region (-1002~ -966) of Kv4.2 promoter binds the endogenous proteins in 10T1/2 cells.

EMSA experiments were performed with nuclear extracts from rIrx5-pcDNA3 or empty vector transfected ad293 cells using biotin-labeled probes containing the identified 36-bp region (-1002~ -966). Unlabeled DNA (200-fold excess) was used to eliminate specific binding to the probe. An arrow indicates the position of binding complex which was eliminated with excess unlabeled probes. Note that no apparent difference in the size or intensity between Irx5 and Mock.

4.5 DISCUSSION

During the last 20 years, many studies have shown the importance of Iroquois (Irx) atypical homeodomain-containing proteins for various developmental processes. Using loss- and gain-of-function approaches, these studies have resulted in a wealth of information about their roles in neural, cardiac and lung development (26, 27, 42, 96, 97). Yet, far less is known about their target genes and regulatory DNA-binding sequences. In a recent study by Costantini *et al*., Kv4.2 has been identified for the first time as a target gene of Irx5 using Irx5-defient mice (2). However, no cis-element that regulates the transcription of Kv4.2 gene has been defined in any system. This study carried out a thorough analysis of the ~1-kb 5'flanking region of rat Kv4.2 gene. Deletion analysis indicated that multiple portions are required for the full activation by Irx5. In particular, the 36-bp fragment (-1002~ -966) appeared to respond to Irx5 to increase the activity of its own and heterologous promoter (SV40). This Irx5-induced enhancement with the 36-bp, as well as the entire 5'flanking region, depended on the orientation of the fragment. EMSA assay failed to detect a specific binding of this region with Irx5. These observations suggest that Irx5 exerts its transcriptional regulation by influencing other as yet unidentified factors via diverse *cis*-regulatory elements.

The data in this chapter identified a 36-bp region (-1002~ -966) that is critical for Irx5-dependent regulation of Kv4.2 gene transcription in 10T1/2 cells. This fragment was also able to confer the Irx5-responsiveness to a heterologous promoter. Mutation analysis showed a GT-rich element (GGTGGGTGG) is responsible for the activity of this region. This GT-rich element was originally reported as a retinoblastoma control element (RCE) (93). This element appears to be essential for the retinoblastoma (RB) protein-mediated transcription of many genes, including c-Fos, c-Myc and TGF beta 1. It has been shown that Sp1 proteins are also capable of binding to

this motif (98) and interacts with RB to regulate gene transcription. This Sp1-like element is thus proposed to cooperate in the Sp1-mediated transcription system, which is further modulated by other transcription factors, including Rb and p53, to regulate the expression of some housekeeping genes (94, 99). More importantly, this GT-rich element has also been implicated in the regulation of gene transcription by other Iroquois proteins. The expression of *Hb9*, an embryonic motor neuron gene, is repressed by Irx3 and Nkx2.2 in chicken embryo (82). It is proposed that Irx3 together with Nkx2.2 inhibits the transcriptional activation of Sp1 on the *Hb9* promoter. In addition, the 36-bp fragment containing this element was found to bind to some endogenous proteins in this study, although the specific DNA binding sequence within this fragment is presently unclear. Taken together, these observations suggest that Irx5 influences Kv4.2 channel promoter by, at least in part, altering the activity of transcription factor complexes that bind to this element.

10T1/2 cells were used for analysis of Kv4.2 promoter in this chapter. This raises the issue of whether Irx5 regulates channel promoter via the same DNA elements in cardiac myocytes. The deletion analysis in this and previous studies from other laboratory (2) made similar observations that Irx5 has opposite regulatory effects on the Kv4.2 gene promoter activity in myocytes and non-myocytes. In particular, the most dramatic decline in the Irx5-induced regulation is seen when the region between -1073 and -414 is eliminated in both cell types. Moreover, the previous chapter established the pivotal role of Irx5 in controlling channel gene transcription in both cell types. These observations justify analysis in this study focusing on this region. However, it is certainly possible that distinct elements located within this region differentially mediate the activation in 10T1/2 cells and the inhibition in cardiac myocytes. Thus, further studies focused on the roles of the identified 36-bp region and GT-rich element in

regulating Kv4.2 gene expression in cardiac myocytes are needed to provide insights into detailed molecular mechanisms underlying the regulation of Kv4.2 gene transcription in cardiac myocytes.

Although the identification of the partners of Iroquois proteins await further investigation, it is reasonable to conclude that this 36-bp fragment (-1002~ -966) is essential for the transcriptional regulation of Kv4.2 gene by Irx5 in fibroblast cells. The observations made in this chapter favor a model in which multiple regions are involved in the regulation of Kv4.2 gene transcription. Interestingly, a recent study revealed that Irx7 activates transcription of a neuron-specific gene, *Krox20*, via a cis-regulatory element in GFP reporter assay (100). While Irx7 failed to bind to this cis element, the *in vivo* ChIP assays showed that Irx7 binds to the core promoter region, suggesting the existence of a chromatin loop formed between distal and proximal promoter. Therefore, the Irx5 regulation of Kv4.2 promoter may also involve a similar mechanism of the interaction between the distal and proximal promoters at the chromatin level. In addition, this process may also require complex interactions of Irx5 with other factors. Thus, it is of great interest to determine the roles of not only distal but also proximal elements, as well as their interactions in future studies.

5.0 SUMMARY AND CONCLUDING REMARKS

5.1 SUMMARY

It has been several decades since the importance of electrical heterogeneity for the proper cardiac function has been recognized. Yet, only the last 10 years have seen molecular understanding of this physiologically-important phenomenon. The cloning of a wide variety of voltage-gated ion channels revealed that various shapes and durations of cardiac action potential are generated by the assortment of ion channels present in each cell. While the upward movement of membrane potential is primarily due to molecularly-identical voltage-gated sodium channel in atrial and ventricular myocytes, the downward repolarization is set by diverse potassium (K^+) channels. Specifically, the voltage-gated transient outward K^+ (I_{to}) channels consisting of Kv4.2 and/or Kv4.3 pore-forming and auxiliary KChIP2 subunits are the major determinant of cardiac action potential. The expression level of Kv4.2 largely determines the shape and duration of action potential in rodent hearts. Thus, the present study is aimed at elucidating transcriptional regulation of the rat Kv4.2 gene.

This study has demonstrated that the atypical homeodomain Iroquois (Irx) family proteins, Irx4 and Irx5, cooperatively regulate the transcription of Kv4.2 gene. Irx5 acts as a primary regulator of Kv4.2 gene transcription, while Irx4 is a suppressor of the Irx5 action. In non-myocytes where no endogenous Irx4 or Irx5 is present, Irx5 activates Kv4.2 gene promoter

(Fig. 20). Irx4 prevents this stimulation of channel promoter by Irx5. A reduction in Irx4 with siRNA restores the stimulatory effect of Irx5 in neonatal ventricular myocytes. These results indicate that Irx5 acts as a transcription activator in both cardiac myocytes and non-myocytes. They also suggest that abundantly-expressed Irx4 continuously prevents the activation of Kv4.2 gene transcription by Irx5 in cardiac myocytes. However, this interplay between Irx4 and Irx5 cannot account for the inhibition of Kv4.2 promoter by Irx5 in cardiac myocytes. Instead, this inhibition by Irx5 could only be explained by assuming the presence of an additional myocyte-specific molecule that interacts with Irx5 to decrease the activity of channel promoter (Fig. 20).

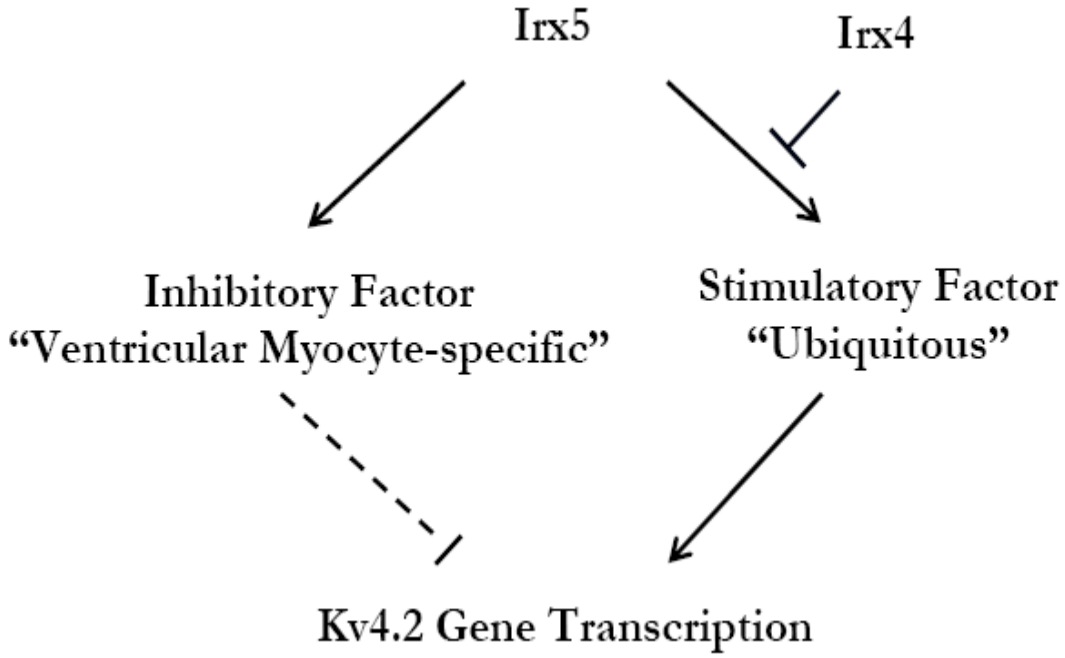

Figure 20. Proposed model for the regulation of Kv4.2 gene transcription by Iroquois family proteins.

The present study also provides new insights into the molecular actions of TALE atypical homeodomain proteins. TALE atypical homeodomain proteins may produce their transcriptional regulation by directly binding to a specific DNA sequence; alternatively, these proteins indirectly control gene transcription by affecting other transcription factors. The present study supports the

latter possibility for the activator action of Irx5. Deletion and mutation analyses of the Kv4.2 5'flanking region revealed that multiple regions are required for the full activation by Irx5. One small region, a 36-bp portion, appears to stimulate own and heterologous promoters in response to Irx5. This enhancement requires a GT-rich element (GGTGGGTGG), a potential binding site for SP1 and other factors. Furthermore, the C-terminal peptide of Irx5 is necessary and sufficient for its activation of channel promoter. These findings indicate that Irx5 activates Kv4.2 gene promoter indirectly by interacting with endogenous transcription factors via its C-terminal peptide. The results in this study also indicate that the regulatory action of Irx4 involves other proteins. The N-terminal peptide of Irx4 appears to mediate this effect. Moreover, addition of histone deacetylase inhibitor (TSA) relieves the inhibitory effect of Irx4. Therefore, it is possible that Irx4 produces its suppression of the Irx5 action by modifying the structure of chromatin and recruitment of histone deacetylase.

Taken together, the present study has shown that the interplay between the two atypical homeodomain proteins, Irx4 and Irx5, controls the transcription of rat Kv4.2 gene. These actions of Irx proteins involve some housekeeping and/or cell-type specific transcription factors. Hence, the levels of these factors, as well as the ratio of the two Irx proteins, determine the outcome of channel transcription and electrical phenotype in each cell.

5.2 HETEROGENEOUS EXPRESSION OF KV CHANNEL GENES AND IROQUOIS FAMILY MEMBERS IN THE HEART

Studies conducted over the past twenty years or so have demonstrated that mammalian ventricular myocardium is not homogeneous with respect to repolarization characteristics. Ventricular epicardium and midmyocardium display action potentials with a prominent transient outward potassium current (I_{to})-mediated early phase, giving rise to a notched appearance of the action potential. The absence of this prominent notch in the endocardium is a consequence of a much smaller I_{to}. Similar regional differences in I_{to} are found in canine, feline, rabbit, rat and human ventricular myocytes. Consistent with these previous findings, the data in chapter 1 showed that there is a transmural gradient of Kv4.2 gene expression in rat heart with higher expression in epicardium. Also there is a uniform expression of Kv4.3 gene across the left ventricle wall of rat heart.

Several studies have shown that mRNA stability plays an important role in controlling Kv4.3 expression in cardiac myocytes (45, 101, 102). However, previous studies have found no change in Kv4.2 mRNA stability upon treatment with various hormones or chemicals in neonatal myocytes, or consensus sequences for known elements in the 3' untranslated region of Kv4.2 mRNA that potentially influence mRNA stability [(45), unpublished observation in our laboratory]. Therefore, it is likely that this regional variation in channel gene expression reflects a pattern of differential transcriptional regulation within the ventricle. On the other hand, a recent microarray analysis identified differential expression of the two Iroquois transcription factors, Irx3 and Irx5, across the left ventricular wall (1). Since Irx proteins control various developmental patterning, they may control the region-selective transcription of downstream functional genes in the heart. The full examination of the expression patterns of all Irx family

members and some related factors in this study demonstrated that Irx3 and Irx5 are expressed in a steep gradient across the left ventricular wall in an inverse pattern to the expression of Kv4.2 gene. Irx4 is equally abundant throughout the ventricular tissue and absent from atrium. Other Irx members were either undetectable or very low without any region selectivity (data not shown). These findings strongly suggested that differentially-expressed Irx3 and Irx5, as well as abundant Irx4, act as upstream regulators to control the region-selective transcription of downstream functional genes including Kv4.2 in rodent hearts. Although less is known about regional differences in expression of functional genes except I_{to}-forming channel subunits, it is possible that Irx3 and Irx5 are involved in transcriptional control of other functional genes that play pivotal roles in cardiac functioning. It is also important to note that differential expression of Irx3 and Irx5 is also seen in the heart of large animals, such as dogs (1). In the left ventricular wall of large animals, the auxiliary subunit KChIP2, but not the pore-forming Kv4.2, is differentially expressed (7). Therefore, Irx3 and/or Irx5 may also control differential transcription of KChIP2 gene in the ventricle of large animals.

While the identified roles of Irx proteins are consistent with the expression pattern of Kv4.2 gene across the left ventricular wall, the relationship in expression levels between Irx proteins and Kv4.2 gene does not hold true for some other parts of the heart. For example, the expression of Irx5 mRNA was very low in atria and Irx4 is nearly undetectable in these tissues. If the ratio of Irx4/Irx5 is the sole indicator of Kv4.2 gene transcription, then Kv4.2 gene expression should be very low in atria. Surprisingly, the level of Kv4.2 mRNA was comparable to that in the right ventricle. A simple explanation for this discrepancy is the presence of factors that confer the inhibitory action of Irx5 in ventricles, but not in atria. If this is the case, then Irx5 could act as an activator of Kv4.2 gene transcription in atrial myocytes, similar to the action of

Irx5 in 10T1/2 cells in this study. Thus, a putative factor that confers the inhibitory action of Irx5 may be specifically expressed in ventricular myocytes, but not in atrial myocytes.

5.3 ROLE OF IRX5 ON KV4.2 GENE EXPRESSION

Despite the significant roles of Iroquois (Irx) proteins in regulating pattern formation during development and adulthood, very little is known about the mechanism by which these proteins exhibit their transcriptional and functional specificities. This is in part due to very few direct targets of Irx proteins that have been identified. Irx5-induced regulation of Kv4.2 gene provides a prime candidate to address this issue. The promoter analysis of Kv4.2 gene in fibroblast cells in the chapter 3 suggests the indispensability of a 36-bp region (-1002~ -966) of Kv4.2 gene promoter in mediating the action of Irx5, although this region alone cannot fully reproduce the stimulation by Irx5 observed with the longer region (-1073~ -98). Indeed, there were significant effects with other parts of the promoter region in response to Irx5 in heterologous promoter assays. These results suggest a synergetic effect of the different promoter regions. The data presented here favors a model where the transcriptional activity of Irx5 on Kv4.2 gene in non-myocytes requires the involvement of unidentified proteins at multi-loci over the distance, while the chromatin might form a loop between the distal and proximal promoters to facilitate the interaction of these transcription factors including Irx5.

During the course of this study, the role of Irx5 on the regulation of Kv4.2 gene (Kcnd2) expression within ventricular myocytes has also been reported by a couple of groups. The one group has generated Irx5-deficient mice. These mice showed increased expression of Kv4.2 gene in endocardium, resulting in ventricular repolarization abnormalities with predisposition to develop arrhythmias (2). The other work has located Irx5 expression in the downstream of microRNA. Upregulation of Irx5 in microRNA-deficient mouse correlates well with decreased transcription of Kv4.2 gene in the heart (103). Consistent with these observations, Irx5 appears to decrease the channel promoter activity in neonatal ventricular myocytes in this study. This

supports the general role of Irx proteins as transcriptional repressors, as has been observed with other Irx members. For example, Irx3 has been shown to repress the transcription of the *Hb9* gene in chicken embryo (82). Likewise, Xiro 1, a member of Irx proteins in *Drosophila*, is reported to function as a transcriptional repressor to down-regulate the expression of Wnt by direct binding to the promoter to allow neural plate formation (40). Since Irx5 produces the opposite outcomes in myocytes and non-myocytes, it is likely that Irx5 inhibits the transcriptional activation of unidentified myocyte-specific factors, but not directly binds to the channel promoter region. Hence, further studies on the identification of this putative myocyte-specific activator may be necessary for understanding the role of Irx5 in the region-selective expression of Kv4.2 gene in the heart.

In contrast to the inhibitory action in myocytes, Irx5 activates Kv4.2 gene promoter in non-myocytes. This activator action of Irx5 was seen in various non-myocytes, such as 10T1/2, Ad293 (HEK293) and PC12 cells. In addition, reducing Irx4 expression unmasked the activator action of Irx5 in neonatal ventricular myocytes. Moreover, this study revealed that Irx5 is moderately expressed in atria, whereas Irx4 is nearly undetectable in these tissues. Therefore, significant expression of Kv4.2 in these tissues is likely due to the activator action of Irx5. These findings support a universal role of Irx5 as a transcription activator of Kv4.2 gene in any cell types in the absence of Irx4. These observations also reflect different levels of coregulators for Irx actions present in ventricular myocytes and non-myocytes. First, the lack of the certain myocytes-specific activators may give rise to the low basal level of Kv4.2 gene in non-myocytes. Second, the C-terminal region of Irx5 is required for promoter activity suggests an indirect effect of Irx5 by association with some as yet unidentified ventricular myocyte-specific proteins in regulating the channel promoter activity. For instance, Irx5 may direct Kv4.2 gene transcription

via its C-terminal peptide to either activate or inhibit channel promoter by contact with local factors depending on the context.

The current study provides several potential candidates for the unidentified co-factors of Irx5 in transcriptional activation of Kv4.2 gene in non-myocytes. The data in the chapter 3 demonstrated an important GT-rich element (GGTGGGTGG) in mediating the promoter activation by Irx5. This DNA sequence has been documented as the binding site of Sp1, Sp2, and Sp3 which share extensive homology at the zinc-finger domain (92). Similarly, Irx3, another Irx family member, has been proposed to repress the transcription of the *Hb9* gene in chick embryo by preventing the transcriptional activity of Sp1 on the promoter (82). This process requires the involvement of Nkx2.2, a classic homeodomain protein. Identification of the proteins that binds to this region will clarify the regulatory roles of these putative and Irx proteins in the regulation of Kv4.2 gene.

5.4 ROLE OF IRX4 ON KV4.2 GENE EXPRESSION

Irx4 has been shown to inhibit the transcription of the cardiac *slow MyHC3* gene expression in myocytes and to down-regulate the expression of *Slit 1* within the retina. However, Irx4 does not exclusively function as a repressor in the regulation of gene transcription. For example, a loss-of-function study in Zebrafish has suggested that Irx4 positively regulates the expression of *Sox2* during development of the hindbrain placodes (104). Likewise, the disruption of Irx4 in mice increased expression of ventricular *eHand* among other genes whose expression were de-repressed (26). However, most of these studies employed gain-of-function or loss-of-function approaches in the entire animals. Given the complex situation *in vivo*, it is difficult to predict the direct actions of Irx4. Also, the dominant-negative form of Irx4 created by fusion of the Irx4 homeodomain with the repressor domain of Engrailed used in many studies is predicted to interfere with the actions of multiple Irx family members. Thus, the precise role of Irx4 in regulating gene transcription remains obscure. This study used a well-defined culture system to demonstrate that Irx4 possesses the ability to regulate Kv4.2 promoter. It further established a repressor action of this transcription factor in channel gene transcription.

It is important to note that Irx4 alone does not affect Kv4.2 channel promoter activity in either myocytes or non-myocytes. Rather, it exclusively prevents the promoter activity induced by Irx5. This effect is mediated by the N-terminal peptide of Irx4. This is very similar to a previous report that Irx4 lacks direct action on the *slow MyHC3* promoter, but represses the gene transcription by association with co-factors via its N-terminal region (29). Therefore, it may be a general scheme that Irx4, like other Irx members, controls gene transcription indirectly by interacting with other transcription factors, instead of directly binding to a DNA element.

5.5 THE INTERPLAY OF IRX4 AND IRX5 IN REGULATING KV4.2 GENE EXPRESSION

Iroquois proteins are known to be redundant and functionally interchangeable in flies. A deletion of at least two of the three *Drosophila* Iroquois genes *araucan*, *caupolican*, and *mirror* is required to cause a morphological defect, while deletion of all three results in more-profound abnormalities (52, 105, 106). However, there is always suspicion that this is not the case in vertebrates. For example, in the mouse developing heart, Irx genes exhibit distinct expression patterns (26, 27, 44, 107). Moreover, the data in the chapter 1 demonstrated the functional specificity of Irx members in regulating Kv4.2 promoter. Irx5, not Irx3 or Irx4, directly controls the activity of channel promoter via its unique C-terminal peptide, whereas Irx4, but not Irx3, prevents the Irx5-induced regulation of channel promoter by its N-terminal region. These findings establish the unique functional roles of individual Irx members. Hence, Irx proteins are not only expressed in distinct patterns, but also possess the unique structural features that are necessary for their individual roles.

This study established that Irx4 represses transcriptional activation of Kv4.2 promoter by Irx5. This action of Irx4 can be achieved by several possible ways. At molecular interaction level, all Irx proteins contain a highly conserved atypical homeodomain. Therefore, it is conceivable that Irx4 might prevent the Irx5 action by competing for a DNA element with Irx5. However, Irx3 appears to lack the ability to inhibit the Irx5-induced activation of channel promoter. Thus, a simple competition of a *cis* element is less likely. Similarly, Irx4 might interfere with the binding of Irx5 to a putative regulatory protein. Again, the lack of inhibition by Irx3 is against this possibility. Another possible molecular interaction on which Irx4 may influence is homomeric association of Irx5. In this case, the lack of inhibitory action by Irx3 can

arise from the neutrality of this factor, in which Irx5-Irx3 heteromers are still capable of inducing transcriptional activation. The data in the chapter 2 suggest non-selective association between Irx family members, although complex formation is very inefficient *in vivo*. This is consistent with a previous biochemistry study that *Drosophila* Irx proteins can form homo- or hetero-dimers (50). Therefore, Irx4 might form heteromeric complexes with Irx5 to reduce the formation of Irx5 homomeric dimers that is required for the activation of channel promoter. However, no heteromeric complexes between Irx4 and Irx5 were detected using a mammalian two-hybrid system. Although this discrepancy might be due to methodological reasons, such as inefficient nuclear localization of the heteromeric complexes, the results suggest less effective association between Irx proteins. Furthermore, N-terminal and most C-terminal regions of Irx proteins outside the highly-conserved atypical homeodomain are highly diverse. Thus, potential self-association may be mediated by very limited homologous regions within Irx polypeptides. Therefore, complex formation between Irx proteins is considered to be less stable or weak, if any, under physiological conditions. Finally, Irx4 might produce its inhibitory action independently of the molecular interaction of Irx5. For example, Irx4 may act at a downstream of the Irx5-induced signaling pathway leading to the activation of Kv4.2 promoter. However, the similarity between Irx member proteins and the observed dose dependency argue against this possibility. Further studies on the structure and roles of individual domains of Irx proteins may be needed to identify the precise molecular interactions involved in the inhibitory action of Irx4.

It is also important to identify cellular mechanisms by which Irx4 produces its inhibitory action on the Irx5-induced activation of Kv4.2 promoter. One possible mechanism is enhanced exportation of Irx5 transcriptional complexes from the nucleus. This is consistent with the observations that Irx4-fusion proteins in a mammalian two-hybrid system generated low

luciferase signals. There is evidence that TALE atypical homeodomain proteins regulate gene transcription by affecting the nuclear localization of other transcription factors (77). Another possibility is that the participation of Irx4 prevents transcriptional initiation from the channel promoter. The N-terminal peptide of Irx4 appears to be sufficient to produce the inhibition of the Irx5-induced activation of Kv4.2 promoter activity. A previous mechanistic study on the transcriptional regulation of the *slow MyHC3* gene also suggested the requirement of the N-terminal region of Irx4 in interacting with other co-factors in regulating gene transcription (29). Therefore, this peptide region is considered to interact with other proteins to exert its inhibitory action on the Irx5-induced regulation. Taken together, Irx4 may form unstable complexes with Irx5 to incorporate other proteins via its N-terminal peptide. These incorporated proteins may in turn prevent Irx5 to exhibit the activator action. The drug experiments in this study also suggested that Irx4 might incorporate histone deacetylase into complexes. The same observation was made by Costantini DL *et al*. in dissecting the regulatory mechanism of Irx5 on Kv4.2 expression, although they proposed a different co-repressor (2). The importance of histone acetylation has been gaining much attention and found in many instances in various regulatory processes. In general, acetylated histones marked active chromosomes. Thus, incorporation of histone deacetylase reduces gene transcription. It remains unknown whether the Irx4 N-terminal peptide directly binds to any histone deacetylase or indirectly incorporates these enzymes into complexes.

5.6 FUTURE DIRECTION

The regulation of Kv4.2 gene transcription by Iroquois (Irx) proteins represents not only a physiologically important phenomenon, but also a prime situation to elucidate molecular actions of atypical homeodomain transcription factors. There are many unanswered questions with regard to the actions of Irx4 and Irx5. First, in non-myocytes, a 36-bp region was found to exert the activation of channel promoter by Irx5. This 36-bp fragment appears to bind to endogenous proteins. Therefore, one future experiment should direct at the identification of this endogenous protein. Second, in addition to the 36-bp region, other regions are also required for the full activation of channel promoter by Irx5. A better overall picture may be obtained by more detailed sequence analysis and mutagenesis with the entire ~1-kb fragment. Third, the results in this study support the possibility that the N-terminal peptide of Irx4 interacts with endogenous proteins present in any cell types. Therefore, N-terminal peptide of Irx4 may be a good probe to identify putative interacting proteins. These investigations will provide insight into the activator action of Irx5 and its inhibition by Irx4.

In contrast to the activator action of Irx5 in non myocytes, the inhibitory action of Irx5 in ventricular myocytes largely remains unexplored. Although deletion constructs containing various lengths of Kv4.2 gene promoter show similar responses to Irx5 in myocytes and non-myocytes, detailed deletion and mutation analyses have not been performed in the former cell type. Therefore, transient transfection assays with various constructs will be used to determine whether the identified 36-bp fragment and GT-rich element responds similarly in myocytes. In addition to the identification of *cis* elements, it would be important to identify transcription factors that confer the inhibitory action of Irx5 in these cells. Further biochemical studies may be used to identify the partners of Irx5 in the heart. Possible approaches for this purpose include

immunoisolation of Irx5-containing protein complexes from ventricular tissues, followed by proteomic identification of proteins in the isolated complexes. The identified proteins would then be examined for their roles in regulating Kv4.2 gene transcription using reporter gene assays, siRNA and possibly gene targeting or virus-mediated siRNA expression in ventricular myocytes and animal models.

Transmural gradient of I_{to} density across the left ventricular wall is seen in various animal species. However, molecular mechanisms underlying this size difference differ between rodents and large animals. Since I_{to} channels are multimeric complexes consisting of pore-forming Kv4.2 and/or Kv4.3 and auxiliary KChIP2 subunits, the availability of either subunit can determine the level of functional channels. Whereas the expression of Kv4.2 gene correlates with I_{to} density in rodent hearts, the auxiliary subunit KChIP2 is differentially expressed across the left ventricular wall and may be responsible for the production of I_{to} gradient in large animals. This species difference may arise from a number of possibilities. Since Irx5 is similarly and differentially expressed in both rodents and dogs, one possibility is the presence or absence of Irx5-responsive element(s) in the promoter of rodent and canine Kv4.2 and KChIP2 genes. However, preliminary results from this laboratory suggest that human Kv4.2 promoter behaves similarly to the rat counterpart in response to Irx5, as well as Irx4 in the presence of Irx5 (data not shown). Another possibility is species differences in transcription factors that control Kv4.2 and KChIP2 genes. Further studies on Irx proteins and other transcription factors, as well as detailed analysis of channel subunit promoters, may reveal mechanisms underlying species differences in the region-selective expression of these genes.

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