THE ROLE OF HOMEODOMAIN TRANSCRIPTION FACTOR IRX5 IN CARDIAC CONTRACTILITY AND HYPERTROPHIC RESPONSE

By

KYOUNG HAN KIM

A THESIS SUBMITTED IN CONFORMITY WITH THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE DEPARTMENT OF PHYSIOLOGY
UNIVERSITY OF TORONTO

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ABSTRACT

Irx5 is a homeodomain transcription factor that negatively regulates cardiac fast transient outward K⁺ currents (I_{to,f}) via the Kv4.2 gene and is thereby a major determinant of the transmural repolarization gradient. While I_{to,f} is invariably reduced in heart disease and changes in I_{to,f} can modulate both cardiac contractility and hypertrophy, less is known about a functional role of Irx5, and its relationship with I_{to,f}, in the normal and diseased heart. Here I show that Irx5 plays crucial roles in the regulation of cardiac contractility and proper adaptive hypertrophy. Specifically, Irx5-deficient (Irx5⁻/⁻) hearts had reduced cardiac contractility and lacked the normal regional difference in excitation-contraction with decreased action potential duration, Ca²⁺ transients and myocyte shortening in sub-endocardial, but not sub-epicardial, myocytes. In addition, Irx5⁻/⁻ mice showed less cardiac hypertrophy, but increased interstitial fibrosis and greater contractility impairment following pressure overload. A defect in hypertrophic responses in Irx5⁻/⁻ myocardium was confirmed in cultured neonatal mouse ventricular myocytes, exposed to norepinephrine while being restored with Irx5 replacement. Interestingly, studies using mice
virtually lacking I_{to,f} (i.e. Kv4.2-deficient) showed that reduced contractility in \( Irx5^{+/−} \) mice was completely restored by loss of Kv4.2, whereas hypertrophic responses to pressure-overload in hearts remained impaired when both \( Irx5 \) and I_{to,f} were absent. These findings suggest that Irx5 regulates cardiac contractility in an I_{to,f}-dependent manner while affecting hypertrophy independent of I_{to,f}. On the other hand, \( Irx5 \)-ablation attenuated calcineurin (Cn)-induced hypertrophy in hearts and cultured cardiomyocytes, suggesting that the effect of Irx5 on hypertrophy involves the Cn-NFAT signalling cascade. Biochemical assessments further revealed that Irx5 can positively mediate Cn-NFAT activities as well as Nfatc3 and Gata4 expression, and interacts with Nfatc3 and Gata4, suggesting the formation of a transcription complex for hypertrophic gene regulation. Taken together, these studies have identified Irx5 as a vital cardiac transcription factor, important for contractile function of the heart by regulating I_{to,f}, and compensatory hypertrophic response to biomechanical stress in the heart by affecting the Cn-NFAT (and Gata4) signaling pathway.
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STATEMENT OF CONTRIBUTIONS

The Irx5-deficient mouse (Irx5<sup>−/−</sup>) was originally generated in the laboratory of Dr. Chi-chung Hui. The Kv4.2 knockout mice (Kv4.2<sup>−/−</sup>) and cardiac-specific over-expressed constitutively active calcineurin mice (CnA-TG) were kindly provided by Drs. Jeanne M. Nerbonne (Washington University School of Medicine) and Jeffery D. Molkentin (University of Cincinnati), respectively. Dr. Jie Liu, a post-doctoral fellow in the laboratory of Dr. Peter H. Backx, conducted patch-clamp electrophysiology experiments to measure voltage-activated outward K<sup>+</sup> currents and action potentials. Langendorff-perfused heart experiments were carried out by Dr. Kiwon Ban in the laboratory of Dr. Mansoor Husain (University of Toronto). Co-immunoprecipitation experiments and the subsequent Western blots were performed by Vijitha Puviindran in the laboratory of Dr. Chi-chung Hui (The Hospital for Sick Children). Vijitha Puviindran and Dongling Zhao generated Irx5 adenovirus, and virus amplifications were conducted by Dongling Zhao. Dr. M. Golam Kabir carried out some of transverse aortic banding and invasive hemodynamic studies.
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**LIST OF ABBREVIATIONS**

**A**  
Ad: Adenovirus  
ANF: Atrial Natriuretic Factor  
AngII: Angiotensin II  
AP: Action Potential  
APD: Action Potential Duration  
AR: Adrenergic Receptor  
AVC: Atrioventricular Canal

**B**  
BAND: Transverse Aortic Banding  
BNP: B-type Natriuretic Peptide  
B.P.M.: Beat Per Minute  
BW: Body Weight

**C**  
CARP: Cardiac Ankyrin Repeat Protein  
CBP: CREB-Binding Protein  
Cn: Calcineurin  
ΔCnA: Constitutively-active Calcineurin A  
CnA-TG: Cardiac Specifically ΔCnA Over-expressed Transgenic Mice  
Co-IP: Co-Immunoprecipitation  
CsA: Cyclosporine A  
CTRL: Control  
Cx: Connexin

**D**  
DBP: Diastolic Blood Pressure  
DNA: Deoxyribonucleic acid  
dp/dt: Derivatives of the Left Ventricular Pressure Change over time (sec)  
dSL/dt: Derivatives of the Sarcomere Length Change over time (sec)

**E**  
EA: Embryonic Atrium  
ECC: Excitation-Contraction Coupling  
ENDO: Sub-endocardial myocytes  
EPI: Sub-epicardial myocytes  
ER: Endoplasmic Reticulum  
ETC: Excitation-Transcription Coupling  
EV: Embryonic Ventricle

**F**  
FKBP: FK506 Binding Protein  
FLAG: A protein expression tag with DYKDDDDK residues
<table>
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<tr>
<td>FOG2</td>
<td>Friend of GATA–2</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead box subfamily O</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional Shortening</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GATA</td>
<td>Protein family of (A/T) GATA (A/G) binding zinc fingers</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GSK-3β</td>
<td>Glycogen Synthase Kinase-3β</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone Acetyltransferase</td>
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<td>HCM</td>
<td>Hypertrophic Cardiomyopathy</td>
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<tr>
<td>HD</td>
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<td>Histone Deacetylase</td>
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<tr>
<td>Irx5+/+</td>
<td><em>Irx5</em> Homozygous Wild-type</td>
</tr>
<tr>
<td>Irx5−/−</td>
<td><em>Irx5</em> Homozygous Knockout</td>
</tr>
<tr>
<td>ISO</td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>I_Ca,L</td>
<td>L-Type Ca²⁺ Current</td>
</tr>
<tr>
<td>I_K1</td>
<td>Inward-Rectifier K⁺ Current</td>
</tr>
<tr>
<td>I_Kr</td>
<td>Rapidly activating delayed rectifier K⁺ current</td>
</tr>
<tr>
<td>I_Ks</td>
<td>Slowly activating delayed rectifier K⁺ current</td>
</tr>
<tr>
<td>I_Kr,slow1</td>
<td>Rapidly Activating, Slowly Inactivating K⁺ Current</td>
</tr>
<tr>
<td>I_Kr,slow2</td>
<td>Slowly Activating, Slowly Inactivating K⁺ Current</td>
</tr>
<tr>
<td>I_Kv</td>
<td>Voltage-Activated Outward K⁺ currents</td>
</tr>
<tr>
<td>I_Na</td>
<td>Inward Na⁺ Current</td>
</tr>
<tr>
<td>I_Peak</td>
<td>Peak K⁺ currents</td>
</tr>
<tr>
<td>I_SS</td>
<td>Steady-State K⁺ Current</td>
</tr>
<tr>
<td>I_to</td>
<td>Transient Outward K⁺ Current</td>
</tr>
<tr>
<td>I_to,f</td>
<td>Fast, Transient Outward K⁺ Current</td>
</tr>
<tr>
<td>I_to,s</td>
<td>Slow, Transient Outward K⁺ Current</td>
</tr>
<tr>
<td>KChIP</td>
<td>K⁺ Channel Interacting Protein</td>
</tr>
</tbody>
</table>
KO: Knock Out
Kv4.2N: A dominant-negative N-terminal fragment of the Kv4.2 Channel
Kv4.2W362F: A point mutation at position 362 in the pore region of Kv4.2 Channel

L
LA: Left Atrium
LV: Left Ventricle
LVDP: Left Ventricular Develop Pressure
LVEDD: Left Ventricular End-Diastolic Dimension
LVEDP: Left Ventricular End-Diastolic Pressure
LVESD: Left Ventricular End-Systolic Dimension
LVESP: Left Ventricular End-Systolic Pressure

M
MEF2: Myocyte Enhance Factor–2
MHC: Myosin Heavy Chain
MLC: Myosin Light Chain
MLP
MiRP: MinK-Related channel subunit

N
NCX: Na⁺–Ca²⁺ Exchanger
NE: Norepinephrine
NFAT: Nuclear Factor of Activated T–cells
NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
Nkx: Nirenberg-Kim homeobox protein
NMVM: Neonatal Mouse Ventricular Myocytes
Non-TG: Non-Transgenic Mice

O
OFT: Outflow Track

P
PE: Phenylephrine
PICOT: PKC–interacting cousin of thioredoxin
PKB: Protein Kinase B, also known as Akt
PKC: Protein Kinase C
PMCA4: 
PWTD: LV Posterior Wall Thickness at Diastole

Q
qPCR: Quantitative Reverse-Transcriptase Polymerase Chain Reaction

R
RA: Right Atrium
RNA: Ribonucleic acid
RV: Right Ventricle
RyR: Ryanodine Receptor

S
SBP: Systolic Blood Pressure
SERCA2a: Sarcoplasmic Reticulum (SR) Ca^{2+} ATPase pump
SHAM: Sham-operated mice
SR: Sarcoplasmic Reticulum
SRF: Serum Response Factor

T
TALE: Three Amino acid Loop Extension
TBX: T-box Transcription Factor
TEA: Tetarethlammonium
TF: Transcription Factor
TL: Tibia Length

W
WB: Western Blot
WT: Wild Type

X
Xiro: Xenopus Irx

Z
Ziro: Zebrafish Irx

0 ~ 10
4-AP: 4-Aminopyridine
CHAPTER 1

INTRODUCTION
1.1 Synopsis

In 2006, cardiovascular disease accounted for 30% of all deaths in Canada, and it is estimated that there are 500,000 Canadians living with heart failure and 50,000 new patients diagnosed each year (Ross et al., 2006). Although the most effective therapy for end-stage heart failure is heart transplantation, this approach is limited by the availability of the donor organ (e.g. 162 heart transplants in Canada in 2008), and is not suitable for patients with milder forms of the disease. Despite advances in pharmacological treatments and improved mechanical devices, heart failure mortality remains very high.

Most types of heart failure are preceded by cardiac hypertrophy. Although cardiac hypertrophy is considered to be a compensatory response to various extrinsic and intrinsic biomechanical stresses by maintaining cardiac output with increased contractile function and normalized wall tension, prolonged hypertrophy is associated with unfavourable outcomes, such as arrhythmia, sudden death, dilated cardiomyopathy, and the development of heart failure (Frey et al., 2004; Frey and Olson, 2003). The hypertrophic growth program is accompanied by gene remodelling that is controlled by numerous cardiac transcription factors (Akazawa and Komuro, 2003; Frey and Olson, 2003). Notably, as induction of the fetal gene reprogramming is a signature of diseased hearts, these transcription factors play a crucial role in gene regulation in cardiogenesis (Bruneau, 2002). Thus, understanding the mechanism and function of cardiac transcription factors in heart disease is complementary to comprehension of cardiac development, potentially unlocking a key to successful transcriptional or stem cell therapies for acquired or congenital heart diseases (McKinsey and Olson, 2005; Bruneau, 2008).

We have previously shown that Iroquois homeobox transcription factor 5 (Irx5) is expressed in a gradient across the ventricular myocardium wherein it negatively regulates the transient outward K⁺ current (I_{to,f}). In addition, loss of Irx5 eliminates the I_{to,f} gradient, leading to increased susceptibility to ventricular arrhythmias (Costantini et al., 2005). Although I_{to,f} is evidently associated with cardiac contractility and hypertrophy, other roles of Irx5 in normal and diseased hearts are largely unknown. My studies have explored how Irx5 regulates cardiac contractility and hypertrophy, and have also examined whether these functions of Irx5 in the heart are dependent on I_{to,f}. In Chapter 1, the Iroquois homeodomain transcription factors, the fast transient outward K⁺ current (I_{to,f}), and cardiac transcription factors in hypertrophy are introduced and reviewed. Section 1.5 outlines the specific hypotheses tested in this study and the
experimental procedures conducted to support the conclusion. In Chapter 2 and Chapter 3, the methods and results of all experiments are described, respectively. Moreover, results and future directions are discussed in Chapter 4 and Chapter 5, respectively.

In addition, Appendix A demonstrates a method to reliably separate multiple components of K⁺ currents in adult mouse ventricular myocytes, which is also used in the Irx5 studies. Using this method, contrary to previous results (Guo et al., 2005), we observed that cardiomyocytes from mice lacking Kᵥ4.2 (Kᵥ4.2⁻/⁻) still expressed low levels of Iₒ,f. Thus we proceeded several additional experiments to confirm existence of functional Iₒ,f in Kᵥ4.2⁻/⁻ myocytes, which are described in Appendix B.
1.2 *Iroquois* Homeodomain Transcription Factors

1.2.1 Introduction of *Iroquois* Homeodomain Transcription Factors

*Iroquois Homeobox* (*Irx*) gene family members encode a conserved family of homeodomain-containing transcription factors (TF) that play fundamental “pre-patterning” roles in many developmental processes in both invertebrates (*Drosophila*) and vertebrates (*Xenopus*, zebrafish, chicken and mammals). While the homeodomain (HD) is a 60-polypeptide chain consisting of three alpha helices and is known to bind to DNA (Gehring, 1993), *Irx* family members contain an atypical homeodomain with three amino acids inserted between the first and second alpha helices, termed the three-amino acid-loop-extension (TALE), which places the *Irx* genes into the TALE family of transcription factors (Burglin, 1997) (*Figure 1.1A*). *Irx* family members also have a unique acidic activation domain with a conserved 13 amino acid residue motif (*Iro*-box) in the carboxyl-terminal region whose function has not been resolved (Jordan *et al*., 2000; Cavodeassi *et al*., 2001). It is believed that the homedomain (HD) and *Iro*-box, which are highly conserved amongst members of the *Irx* gene family and across species, confer DNA binding capability to the *Irx* proteins (Cavodeassi *et al*., 2001; Gomez-Skarmeta and Modolell, 2002; Matsumoto *et al*., 2004). However, its binding sequence has not completely been identified yet.

*Caenorhabditis elegans* have only one ancestral *Irx* gene (Peters *et al*., 2000), but in many species *Iroquois* genes are found in one or more genomic clusters and show evolutionarily conserved structural and functional similarities (Gomez-Skarmeta and Modolell, 2002). As shown in *Figure 1.1B*, *Drosophila melanogaster* expresses three closely related proteins, *araucan* (*ara*), *caupolican* (*caup*) and *mirror* (*mirr*) that were the first identified *Iroquois* genes (Gomez-Skarmeta *et al*., 1996; McNeill *et al*., 1997). These three *Drosophila Irx* genes are located in a gene cluster called the *Iroquois* Complex (*Iro-C*) that is involved in the development of the eye, head, wing vein as well as in patterning of sensory organ precursor cells on the dorsal mesothorax (notum) (Cavodeassi *et al*., 2001). Thus loss of the *Iro-C* leads to mis-regulation of developmental patterning genes on the notum of the fly (Gomez-Skarmeta and Modolell, 1996). In fact, the gene ‘*Iroquois*’ is named after a *Drosophila* mutant in which the lateral parts of the notum are completely naked with loss of bristle formation, while a median band of bristles is left unaffected, resembling the ‘Mohawk’ hair style common in native Iroquois Indians (Gomez-Skarmeta *et al*., 1996; Leyns *et al*., 1996). The *ara* and *caup* are closely related and have similar
expression pattern, suggesting functional redundancy of these proteins (Gomez-Skarmeta and Modolell, 1996). This notion was confirmed by the data that over-expression of one Irx gene is sufficient to rescue defects caused by the loss of one or even multiple Irx genes.

In mammals, six Irx genes have been identified and are clustered in two groups of three genes each; the IrxA cluster contains Irx1, Irx2 and Irx4 and is located on mouse chromosome 13 (human chromosome 5), and the IrxB consists of Irx3, Irx5, and Irx6 and is found on mouse chromosome 8 (human chromosome 16) (Bosse et al., 2000; Peters et al., 2000; Cavodeassi et al., 2001) (Figure 1.1B). Xenopus and chicken also have six Irx genes (Ogura et al., 2001; de la Calle-Mustienes et al., 2005; Kerner et al., 2009). As the Irx1, Irx2, and Irx4 proteins are paralogues of Irx3, Irx5, and Irx6, respectively, the similarity of the sequences suggested that the two Irx gene clusters in mammalian genomes were generated by segmental or chromosomal duplication of a single ancestral cluster during evolution (Peters et al., 2000). Interestingly, zebrafish and pufferfish express eleven Irx genes, organized into four genomic clusters; IrxAa (Ziro1a, 2a, 4a), IrxAb (Ziro1b, 4b), IrxBa (Ziro3a, 5a, 6a) and IrxBb (Ziro3b, 5b) as well as a divergent member, called Ziro7 (Lecaudey et al., 2001; Dildrop and Ruther, 2004; Feijoo et al., 2004; Lecaudey et al., 2005). This suggests that additional round of genomic duplication has likely occurred in the fish (Taylor et al., 2001).

1.2.2 The Role of Iroquois Homeobox Proteins in Development and Disease

Iroquois homeobox proteins have been implicated in a variety of early developmental processes to establish proper spatial and temporal patterns of target gene expression (Gomez-Skarmeta and Modolell, 2002). In Drosophila, Irx genes control specification of large territories by establishing planar polarity in the eye and wing disc and directing dorsal-ventral axis patterning in the ovary (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996; McNeill et al., 1997; Kehl et al., 1998; Jordan et al., 2000). In vertebrates, Irx proteins also participate in numerous developmental processes in different tissues. For instance, a number of studies of zebrafish have demonstrated the essential role of Irx genes in formation and patterning of the nervous system, including the brain (Lecaudey et al., 2004; Cheng et al., 2005; Lecaudey et al., 2005). Similarly, Irx2 is involved in cerebellum formation by facilitating the subdivision of the forebrain into anterior and posterior domains (Matsumoto et al., 2004), although mice lacking Irx2 exhibit no significant defect in brain development with a normal midbrain-hindbrain boundary.
**Figure 1.1**

*Iroquois Homeobox Genes*

(A) Structure of *Iroquois* proteins. HD, homeodomain; TALE, three-amino acid-loop-extension; IRO, *Iro*-box. (B) Genomic organization of *IrX* genes in mouse (Mm), human (Hs) and Drosophila (Dm).
(Lebel et al., 2003). The absence of a phenotype is likely due to functional compensation by other \textit{Irx} genes, since all \textit{Ir}x genes except \textit{Ir}x4 in mice participate in neurogenesis (Bosse et al., 1997; Cohen et al., 2000). In addition, \textit{Ir}x1 and \textit{Ir}x5 are implicated in the development of the retina (Cheng et al., 2005; Cheng et al., 2006). Kidney development is also contributed by \textit{Ir}x genes, as \textit{Ir}x3 expression is sufficient to specify nephron segment fate with intermediate tubule formation in \textit{Xenopus} (Schwab et al., 2006; Reggiani et al., 2007; Alarcon et al., 2008). Furthermore, \textit{Ir}x gene expression is detected in the developing lung, as well as in the female gonad during sex determination (Jorgensen and Gao, 2005; van Tuyl et al., 2006). A recent study has demonstrated that all \textit{Ir}x genes in mouse and chick are expressed in developing limbs (McDonald et al., 2010). \textit{Ir}x genes are also expressed in the heart which will be addressed below (see Section 1.3). Interestingly, the mouse Fused toes (Ft) mutation, which results in a deletion of 1.6 Mb of genomic sequences on mouse Chromosome 8 including the entire \textit{Ir}x\textit{B} genomic cluster and other three genes, causes severe limb and heart malformation, craniofacial, forebrain, and ventral neural tube development defects, and premature death (Peters et al., 2002). Consistent with these observations, \textit{Ir}x3, \textit{Ir}x5, and \textit{Ir}x6 in the \textit{Ir}x\textit{B} genomic cluster in mice are expressed with spatial-temporal pattern in the developing limbs, craniofacial areas, central nervous system, as well as heart of mice (Houweling et al., 2001).

The functions of \textit{Ir}x genes are not limited to normal developmental process, but are likely to contribute to diseases. For example, \textit{Ir}x1 and \textit{Ir}x2 serve an evolutionarily conserved role in the regulation of pancreatic alpha-cell-specific gene expression with neurogenin3 in mice, while a recent study suggests a direct link of pancreatic \textit{IRX3} function to both obesity and Type 2 diabetes (Ragvin et al., 2010). In addition, \textit{IRX1}, \textit{IRX2}, and \textit{IRX4} on chromosome 5 have been proposed as candidate genes of Kyphoscoliosis, a three-dimensional deformity of spinal growth (Miller et al., 2006). Furthermore, \textit{Ir}x genes are associated with several types of cancers, suggesting that \textit{Ir}x genes may be involved in the regulation of the cell cycle or apoptosis. Particularly, elevated expressions or hyper-methylation of \textit{Ir}x genes are frequently detected in various cancers in patients, carcinoma cell lines as well as mouse cancer models, such as head and neck squamous cell carcinoma (Bennett et al., 2008), oligodendrogliomas (Ordway et al., 2006), malignant breast cancer cell lines (Kadota et al., 2009), soft tissue sarcomas (Adamowicz et al., 2006), gastric cancer (Guo et al., 2010), prostate cancer (Morey et al., 2006), as well as Dupuytren's disease that is a benign fibroproliferative tumor with an unknown etiology and high recurrence postsurgery (Shih et al., 2009a; Shih et al., 2009b). Interestingly, it has been
suggested that Irx genes can be a promising new therapeutic target in cancer treatment as silencing of Irx5 induces apoptosis, leading to reduction in cell viability of human prostate cancer cells (Myrthue et al., 2008).

The mechanism of transcriptional regulation by Irx genes in these tissues still remains to be resolved as they intrinsically can act as either activators or repressors. In Drosophila, ara and caup in the lateral notum function as activators on the promoters of the achaete-scute genes to promote the development of sensory organs, such as the eyes, bristles and antennae (Gomez-Skarmeta et al., 1996). On the other hand, though Xenopus Irx (Xiro) genes play a role in neurogenesis, similar to their Drosophila counterparts, Xiro1 (and most likely Xiro2 and 3) can function as repressor(s) by binding to the promoter of Bmp4, thereby defining regions of the ectoderm that will acquire neural or epidermal fates (Gomez-Skarmeta et al., 2001) or by repressing the expression of the cell-cycle inhibitor XGadd45-γ to specify regions within the neural plate (de la Calle-Mustienes et al., 2002). A similar repressor function of Irx genes has been observed for zebrafish Ziro1, Ziro3, and Ziro7 in early spinal cord motor neuron development (Kudoh and Dawid, 2001) and at the midbrain-hindbrain boundary domain (Itoh et al., 2002). Furthermore, Irx proteins act either as activators or repressors of gene transcription in a context-dependent manner. For example, during cerebellum formation of chick, phosphorylation via Fgf8-MAPK signaling cascade can switch Irx2 from a transcriptional repressor to an activator (Matsumoto et al., 2004). During chick heart development, Irx4 regulates ventricular and atrial gene expression in a chamber-specific manner as an activator or a repressor, respectively (Bao et al., 1999). Therefore, loss of Irx4 in mouse hearts leads to reduction of ventricular chamber gene expression, but induction of an atrial chamber-specific transgene in the ventricle (Bruneau et al., 2001a). In addition, Irx5 acts as an activator of the Kv4.2 gene in non-cardiac cells, while functioning as a repressor in cardiomyocytes (Costantini et al., 2005; He et al., 2009). On the other hand, Irx3 is able to activate a gap junction channel gene by repressing a repressor (manuscript in preparation).

Irx homeobox transcription factors can form protein complexes that modulate transcriptional regulation. For example, in zebrafish, Ziro7 interacts with Meis factors to activate the expression of anterior hindbrain markers, such as hoxb1a, hoxa2 and krox20, ectopically in the anterior neural plate (Stedman et al., 2009). In chick, to prevent inappropriate expression of neuronal genes in surrounding non-neuronal tissue in spinal cord development, Irx3 and Nkx2.2 are thought to act as co-repressors by recruiting histone deacetylase (HDAC) proteins (Lee et al.,
which promote chromatin condensation and silencing of gene transcription (Gottlieb et al., 2002). Irx4 also forms an inhibitory protein complex by interacting with an RXRα/VDR heterodimer, thereby repressing slow MyHC3 expression in the ventricular chamber during chick heart development (Wang et al., 2001). In mouse ventricular cardiomyocytes, Irx5 interacts with the cardiac specific co-repressor mBOP (smyd1) to recruit HDAC (Gottlieb et al., 2002; Sims et al., 2002), thereby repressing Kv4.2 gene expression in the hearts (Costantini et al., 2005). Irx3 also interacts with Nkx2.5 and Tbx5, regulating gap junction gene expression (manuscript in preparation).

1.2.3 Cardiac Expression and Function of Iroquois Homeobox Genes

As illustrated in Figure 1.2, all six Iroquois homeobox genes are expressed in the developing mouse heart with unique and overlapping patterns (Christoffels et al., 2000b). Irx1 and Irx2 expression patterns are almost identical with expression in the interventricular septum and ventricular conduction system, whereas Irx3 expression is restricted to the ventricular conduction system (His-Purkinje), suggesting that they may play a potential role in establishing a ventricular conduction system. Irx4 is exclusively expressed in the ventricular myocardium, but absent in atria. It is the earliest marker that defines ventricular chamber, indicating that Irx4 is likely to be an important mediator of ventricular differentiation (Bruneau et al., 2000). Moreover, Irx5 and Irx6 show almost equal expression patterns in the ventricle with subendocardial-to-subepicardial transmural gradients (Mummenhöff et al., 2001).

Functions for Irx genes in the heart have been unveiled recently in chick and transgenic mice model, showing that they play significant roles in ensuring proper cardiac function (Bruneau et al., 2001a; Costantini et al., 2005). Irx4 has been shown to regulate chamber-specific gene expression by activating ventricular myosin heavy chain-1 (VMHC1) while suppressing atrial myosin heavy chain-1 (AMHC1) expression (Bao et al., 1999). Mis-expression of Irx4 in the atria leads to ectopic expression of VMHC1 in the atria while suppressing AMHC1 expression, thereby disrupting the chamber-specific MHC expression. Meanwhile, over-expressing the engrailed repressor form of Irx4 results in a decrease in VMHC1 expression, but an increase in AMHC1 in the ventricle, suggesting that Irx4 can play a dual role as an activator or a repressor of MHC gene regulation in a context-dependent manner. Consistent results have been found in mice lacking Irx4 (Irx4<sup>-/-</sup>), showing ventricular induction of an atrial chamber-specific transgene (Bruneau et al., 2001a). Consequently, Irx4-deficient mice develop cardiomyopathy manifested
Figure 1.2
Iroquois Homeobox Expression in the Heart
Irx1 and Irx2 are found in the interventricular septum while Irx3 is strongly expressed in the ventricular conduction system (His-Purkinje). Irx4 is expressed in whole ventricles. Irx5 and Irx6 are expressed in endocardial-to-epicardial myocardium with transmural gradients.
(Courtesy of John Wylie and Benoit G. Bruneau)
by impaired systolic function and hypertrophy. Loss of Irx5 in mice leads to disruption of transmural repolarization gradients in the heart by elevation in $I_{\text{to,f}}$ in endocardial myocytes (Costantini et al., 2005). This disturbance of $I_{\text{to,f}}$ heterogeneity results in increased susceptibility to inducible ventricular arrhythmia. The role of Irx5 will be elaborated further in the next section (see Section 1.2.4). Moreover, we have recently revealed the role of Irx3 in the heart (manuscript in preparation). Consistent with its expression in ventricular conduction system (Christoffels et al., 2000b), loss of Irx3 led to prolonged QRS duration, “R notch” combined with slowed His-Ventricle conduction times, and functional right bundle branch block. These phenotypes are associated with a decrease in connexin 40 (Cx40) throughout the conduction tissue and ectopic expression of connexin 43 (Cx43) in the proximal bundle branches. Furthermore, Irx3 directly represses $Gja1$ (encoding Cx43) transcription while indirectly activating $Gja5$ (encoding Cx40) by repressing a repressor. Together, our results establish that Irx3 regulates electrical conduction through the ventricular conduction system. On the other hand, no cardiac abnormalities were observed in mice deficient for Irx2 (Lebel et al., 2003) or Irx6 (unpublished data). As suggested (Lebel et al., 2003) and seen in Drosophila and zebrafish Irx genes (Cavodeassi et al., 2001; Itoh et al., 2002), loss of Irx2 or Irx6 was likely compensated by other Irx genes. Although mutation of the Irx1 gene ($Ziro1b$) causes bradycardia in zebrafish (Joseph, 2004), the role of Irx1 has not been studied in the mammalian heart.

1.2.4 The Role of Irx5 in the Heart

The Iroquois homeodomain protein 5 (Irx5) was first identified in mouse embryo during characterizing the genomic region of Irx3 (Bosse et al., 2000). Cardiac expression of Irx5 is detected as early as E9 at the ventral side of the looping tubular heart (Christoffels et al., 2000b). At E9.5, Irx5 expression is detected in endocardium lining the atrial and ventricular chamber myocardium, but absent from endocardium lining the atrioventricular canal, inner curvature, and outflow track. Irx5 expression in the embryonic heart is illustrated in Figure 1.3. The myocardium expressing Irx5 expresses ANF ($Nppa$), Chisel as well as various genes related to contractile proteins and sarcoplasmic reticulum components (Christoffels et al., 2000a; Moorman et al., 2000). Thus, the expression pattern of Irx5 suggests that it may be involved in the pathway for the formation of chamber myocardium. The function of Irx5 in the heart was further examined using the Irx5 homozygous null (Irx5$^{-/-}$) mice (Costantini et al., 2005). Despite the distinct expression of the Irx5 in developing heart, no noticeable aberration was found in Irx5-
Figure 1.3

*The Expression Pattern Of Irx5 during Cardiac Development*

Schematic diagrams represent *Irx5* mRNA expression assessed by *in situ* hybridization. AVC, atrioventricular canal; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; EA, embryonic atrium; EV, embryonic ventricle

(Modified from Christoffels et al., 2000)
Figure 1.4

Electrophysiological Phenotype Characterization of Irx5⁻/⁻ Heart

(A) Representative ECGs in the lead II configuration show that downward T-wave deflection (arrows) was not evident in Irx5⁻/⁻ mice compared to Irx5⁺/⁺. (B) Representative voltage-gated K⁺ currents in epicardial and endocardial myocytes of Irx5⁺/⁺ and Irx5⁻/⁻ mouse hearts. (C) Transmural difference in Iₒ is abolished in Irx5⁻/⁻ heart by elevated Iₒ in Irx5⁻/⁻ endocardial myocytes. (D) Programmed ventricular stimulation induced episodes of ventricular tachycardia (VT) in Irx5⁻/⁻ mice while no VT was found in Irx5⁺/⁺ mice.

(Adapted from Costantini et al., 2005).
Figure 1.5
Inverse Relationship Between Irx5 and Kv4.2 Expression
(A) Irx5 and Kv4.2 protein expression in adult myocardium shows inverse transmural gradient expression patterns. (B) Representative Western blots of Kv4.2 protein expression and (C) relative expression of Kv4.2 mRNA (Kcnd2) expression demonstrate up-regulation of Kv4.2 protein and mRNA expression in Irx5−/− sub-endocardial myocytes.
(Adapted from Costantini et al., 2005).
Figure 1.6
Transcriptional Regulation of Kv4.2 by Irx5
(A) Irx5 dose-dependently increases Kv4.2 (Kcnd2) promoter activity in non-cardiomyocyte cells. (B) In cardiomyocytes, on the other hand, Irx5 dose-dependently represses Kcnd2 promoter activities. (C) A cardiac co-repressor, mBop is necessary for Irx5-mediated repression of Kcnd2 expression. (D) A schematic model for the role of Irx5 in transcriptional regulation of Kv4.2 in the heart. (Adapted from Costantini et al., 2005).
deficient hearts, suggesting that other Irx genes might compensate for loss of Irx5. Interestingly, in vivo telemetric electrocardiography detected absence of the T-wave deflection in the heart of Irx5−/− mice, which reflects a defect in ventricular repolarization (Figure 1.4A). Since T-wave deflection in mice is contributed mainly by transmural difference of fast transient outward K+ current Ito,f (Gussak et al., 2000; Yan et al., 2003), we measured Ito,f in ventricular myocytes isolated from sub-endocardial (ENDO) and sub-epicardial (EPI) myocardium and found that Ito,f heterogeneity between ENDO and EPI was completely abolished in the Irx5−/− mouse heart by up-regulation of Ito,f in Irx5−/− ENDO cells (Figure 1.4, B and C). This loss of Ito,f heterogeneity led to increased susceptibility to inducible arrhythmia as the gradient of Ito,f is important to prevent arrhythmia (Figure 1.4D) (Antzelevitch and Sicouri, 1994; Guo et al., 2000; Kuo et al., 2001; Shah et al., 2005; Antzelevitch, 2007). The heterogeneity of Ito,f in rodent hearts is generated by a Kv4.2 expression gradient (see Section 1.3.2) (Dixon et al., 1996; Guo et al., 1999; Rosati et al., 2001; Costantini et al., 2005). As shown in Figure 1.5A, Kv4.2 expression mirrors that of Irx5, suggesting that Irx5 negatively correlates to Kv4.2. Consistent with the absence of Ito,f heterogeneity, loss of Irx5 in mouse hearts resulted in disruption of Kv4.2 mRNA and protein expression gradients by their up-regulation in Irx5−/− ENDO myocytes (Figure 1.5, A and B), suggesting that Irx5 negatively regulates Kv4.2 gene expression. To test this possibility, the effect of Irx5 on Kv4.2 gene (Kcnd2) promoter activity was examined in COS-7 cells and neonatal cardiomyocytes. Contrary to initial expectation, in COS-7 cells, Irx5 activated the Kcnd2 reporter construct (Figure 1.6A). In cardiomyocytes, on the other hand, Irx5 dose-dependently repressed Kcnd2 promoter activity (Figure 1.6B), suggesting that Irx5 can act as an activator or repressor in a context-dependent manner. This antithetical role of Irx5 in cardiomyocytes and non-cardiomyocytes as a repressor and activator, respectively, is achieved by recruitment of a cardiac co-repressor, mBop (smyd1), which binds to HDAC (Gottlieb et al., 2002). Consistent with this notion, silencing mBop by RNA interference eliminated Irx5-mediated repression on Kcnd2 promoter activity (Figure 1.6C). Taken together, this study has demonstrated that Irx5 is essential for generating Ito,f heterogeneity in the heart by negatively regulating Kv4.2 gene expression, which ensures proper repolarization of ventricular myocardium (Figure 1.6D).
1.3 The Cardiac Role of the Fast Transient Outward Potassium Current (I_{to,f})

1.3.1 Introduction to the Fast Transient Outward Potassium Current (I_{to,f})

Transient outward K\(^+\) current (I_{to}) is a key determinant of early repolarization, Phase 1 of the action potential (AP) in the heart. Due to its contribution to AP duration (APD), I_{to} can affect Ca\(^{2+}\) homeostasis via regulating the magnitude of the L-type Ca\(^{2+}\) current, thereby mediating cardiac contraction (Sah et al., 2002b; Sah et al., 2003). I_{to} is observed in atrial and ventricular myocytes (as well as Purkinje fibers) in mammals including human (Kaab et al., 1998; Rosati et al., 2001), dog (Dixon et al., 1996; Zicha et al., 2004), feline (Furukawa et al., 1990), ferret (Brahmajothi et al., 1999), rabbit (Bassani et al., 2004), rat (Clark et al., 1993; Casis et al., 1998; Wickenden et al., 1999a, 1999b; Brunet et al., 2004), and mouse (Xu et al., 1999b; Brunet et al., 2004). One exception is guinea pig, which completely lacks I_{to} (Zicha et al., 2003).

I_{to} can be classified into at least two types of currents, fast I_{to} (I_{to,f}) and slow I_{to} (I_{to,s}) based on the distinctive kinetics of recovery from steady-state inactivation (Oudit et al., 2001; Patel and Campbell, 2005). Both I_{to,f} and I_{to,s} have a very rapid activation rate (τ_{act} = 2 – 10 ms) and rapid, but slightly different inactivation rate (I_{to,f}, τ_{inact} = 25 – 80 ms; I_{to,s}, τ_{inact} = 80 – 200 ms). Although it is controversial whether I_{to,f} and I_{to,s} can be distinguished by different inactivation rates (Wickenden et al., 1999a; Xu et al., 1999b), they clearly have discernable recovery kinetics from inactivation since the rate of recovery from inactivation of I_{to,s} (τ_{slow} = 1 – 2 sec) is significantly slower than the recovery rate of I_{to,f} (τ_{fast} = 25 – 80 ms) (Clark et al., 1988; Giles and Imaizumi, 1988; Wettwer et al., 1994) (see Appendix A). Due to this slow recovery from inactivation compared to rapid heart rate of rodent, however, the function of I_{to,s} in rodent heart has been questioned (Roberds et al., 1993; London et al., 1998b).

1.3.2 Molecular Basis of I_{to,f}

Initially, a number of candidate Kv α-subunits, such as Kv1.4, Kv1.7, Kv3.4, Kv4.2 and Kv4.3, were extensively investigated to better understand the molecular basis of I_{to} (Oudit et al., 2001). Considerable evidence indicates that Kv4 α-subunits (Kv4.2 and Kv4.3) encode I_{to,f}. Heterologous expression of Kv4 α-subunits showed rapidly activating, inactivating and recovering I_{to,f}-like outward K\(^+\) currents (Wickenden et al., 1999a), which is sensitive to heteropodatoxins (HpTx) (Xu et al., 1999b), and phrixotoxin (PaTxs) (Diochot et al., 1999). 4-AP treatment in heterologously expressed Kv4.2 currents was able to reproduce ‘reverse use dependence’ block which property was thoroughly investigated for ferret I_{to,f} (Campbell et al.,
As assembly of four Kv α-subunits into a tetrameric structure forms a functional I_{to,f} channels (Jan and Jan, 1992), co-expression of Kv4.2 and Kv4.3 in HEK-293 or isolated cardiomyocytes mimics kinetic properties of I_{to,f}, suggesting that I_{to,f} in mouse ventricle is encoded by heterotetrameric KV4.2/4.3 channels (Guo et al., 2002a). Therefore, the biophysical, kinetic, and pharmacological characteristics of Kv4-mediated currents are very similar to native I_{to,f}. More importantly, several transgenic mouse studies have provided strong evidence for the molecular identity of I_{to,f}. Over-expression of truncated Kv4.2 (Kv4N) or Kv4.2 pore mutant (Kv4.2W362F) in mice, which functions in a dominant negative manner, leads to reduction or elimination of I_{to,f} in the heart (Barry et al., 1998; Wickenden et al., 1999b). In addition, antisense oligonucleotides (AdODNs) targeting Kv4.2 and Kv4.3 attenuate I_{to,f} in rat and mouse ventricular myocytes (Fiset et al., 1997; Guo et al., 2002a). Furthermore, Guo et al have shown that the targeted deletion of Kv4.2 in mice results in elimination of I_{to,f} with a significant reduction in the K_Chip2 (Guo et al., 2005), while loss of Kv4.3 gene in mice does not affect I_{to,f} currents (Niwa et al., 2008), suggesting obligatory co-assembly of Kv4.2 α-subunit to encode I_{to,f} (see Appendix B). Interestingly, loss of K_Chip2, a regulatory subunit of Kv4 α-subunit, leads to complete loss of I_{to,f} (Kuo et al., 2001). These data suggest that Kv4.2-K_Chip2 are critical players in constructing I_{to,f} in rodent, consistent with a previous study that most rat ventricular myocytes (> 87%) express both Kv4.2 and K_Chip2 (Schultz et al., 2005). Unlike small animals such as mouse, rat and ferret where I_{to,f} is contributed by both Kv4.2 and Kv4.3 (Dixon and McKinnon, 1994; Wickenden et al., 1999a), Kv4.3 is a predominant α-subunit of I_{to,f} in human and canine hearts (Dixon et al., 1996; Kong et al., 1998).

Species differences in the molecular profile of I_{to,f} are also shown in the regional heterogeneity that is one of the key electrophysiological characteristics of I_{to,f}, with epicardial-to-endocardial, as well as apical-to-basal expression gradients (Xu et al., 1999b; Brunet et al., 2004; Costantini et al., 2005). The heterogeneity of I_{to,f} in rodent hearts is due to Kv4.2 expression gradient, whereas K_Chip2 expression is almost uniform (Dixon et al., 1996; Guo et al., 1999; Rosati et al., 2001; Costantini et al., 2005). On the other hand, in human and canine, K_Chip2 contributes to the I_{to,f} gradient by modulating Kv4.3 protein gradient expression, although Kv4.3 mRNA is uniformly expressed (Rosati et al., 2001; Zicha et al., 2004).

Although Kv4.2/4.3 expression in heterologous systems encode K^+ currents similar to I_{to,f}, these currents are still different from native I_{o} currents, suggesting that additional factors or accessory subunits are necessary to modulate biophysical properties. For example, Kv4-encoded
I_{to,f}-like currents in HEK-293 cells show rapidly inactivating, but slower recovery kinetics compared to endogenous I_{to,f}, whereas Kv4 α-subunit expression in cardiomyocytes mimics rapid decay and recovery kinetics of I_{to,f}, suggesting the difference was likely to be made by a cardiac specific auxiliary subunit (Guo et al., 2002a). KChIPs (Wickenden et al., 1998; Bou-Abboud and Nerbonne, 1999; An et al., 2000; Guo et al., 2002a), neuronal calcium sensor subfamily of Ca\(^{2+}\) binding proteins (Neuronal Calcium Sensor-1, NCS-1) (Nakamura et al., 2001b; Guo et al., 2002b), MinK-related peptides (MiRPs) (Zhang et al., 2001; Delpon et al., 2008), dipeptidyl-aminopeptidase-like proteins (Dpps) (Nadal et al., 2003; Jerng et al., 2004), three homologous Kvβ accessory subunits (Kvβ1–3) (Accili et al., 1997; Yang et al., 2001; Deschenes and Tomaselli, 2002; Wang et al., 2003; Aimon et al., 2005), voltage-gated Na\(^+\) channel accessory subunit (Navβ) (Deschenes and Tomaselli, 2002; Deschenes et al., 2008) have been proposed to recapitulate native I_{to,f} in the heart.

1.3.3 The Cardiac Action Potential and I_{to,f}

The cardiac action potential (AP) is a momentary change in membrane potential driven by movement of ions across the membrane of a cell (Bers, 2002; Bassani, 2006). Typical mammalian AP waveforms have several phases (0 through 4), which include an initial, rapid depolarization, a slow repolarization phase (plateau), and a final rapid repolarization phase (Figure 1.7). In human ventricular myocytes or Purkinje fiber cells, the initial rapid depolarization (upstroke) to approximately +20 mV (Phase 0) is generated by Na\(^+\) influx into the cell (I_{Na}) as large numbers of voltage-gated sodium channels open. The peak of the AP occurs when I_{Na} is balanced by a net efflux of positive charge that results mainly from an outward flow of K\(^+\) ions. The brief repolarization (Phase 1), or “notch,” following the upstroke is driven by the Ca\(^{2+}\)-independent, voltage-dependent, fast transient outward K\(^+\) current (I_{to,f}) that rapidly activates and inactivates following membrane depolarization. The following plateau (Phase 2) is the result of a balance of K\(^+\) efflux through voltage-gated potassium channels and a Ca\(^{2+}\) influx through voltage-gated L-type calcium channels (I_{Ca,L}). During the repolarization phase (Phase 3), Ca\(^{2+}\) channels begin to inactivate and there is gradual increase in activation of delayed rectifier K\(^+\) channels (I_{K}). In human, I_{K} currents are classified into ultra-rapid, I_{kur}; rapid, I_{Kr}; and slow, I_{Ks}, based on their relative activation rate. In mice, on the other hand, two kinetically distinct components of I_{K} have been identified, a rapidly activating, slowly inactivating, micromolar 4-aminopyridine (4-AP)-sensitive component, called I_{K,slow1}, and a millimolar tetarethlammonium
Figure 1.7
Action Potential Profiles
Action potential (AP) profiles of human (left) and mouse (right) ventricular myocytes. The observed differences in AP waveforms reflect differences in ionic currents. Upward and downward arrowheads indicate outward and inward currents, respectively.

Figure 1.8
Effect of $I_{to,f}$ on Murine and Canine AP Profiles
(A) In mouse cardiomyocyte, reduction in $I_{to,f}$ densities results in prolongation of AP duration. (B) In canine myocyte, removal of $I_{to,f}$ markedly slows early repolarization, abolishes the AP notch, and shortens the overall AP duration. Note the difference in time scale between the canine and murine APs. (Adapted from Sah et al., 2003).
(TEA)-sensitive current, referred to as $I_{K,\text{slow}2}$ (Xu et al., 1999a; Kodirov et al., 2004) (see Appendix A). A non-inactivating, steady-state current ($I_{SS}$) also exists in mouse myocytes. When Ca$^{2+}$ influx becomes less than K$^+$ efflux, the membrane potential returns to more hyperpolarized potentials. As this occurs, voltage-gated potassium channels close, but the K$^+$ conductance remains high due to the opening of inwardly rectifying K$^+$ channels. The resting membrane potential (Phase 4) is maintained and stabilized by inward rectifier K$^+$ currents ($I_{K1}$) until the next AP stimulus (Lopatin and Nichols, 2001).

A number of investigations have provided evidence that $I_{to,f}$ play a considerable role in influencing AP waveform (i.e. duration and shape) (Beuckelmann et al., 1993; Kaab et al., 1996; Kaprielian et al., 1999; Greenstein et al., 2000; Sah et al., 2002b). In rodents that have spike-like morphologies of AP (Figure 1.7), $I_{to,f}$ density is relatively large and thus down-regulation of $I_{to,f}$ leads to significant prolongation of the action potential duration (APD) (Wickenden et al., 1998; Sah et al., 2002b) (Figure 1.8A). In higher mammals, on the other hand, the story is more complicated because $I_{to,f}$ densities are lower than in rodent. As it is classically known that $I_{to,f}$ is responsible for the ‘notch’ of the AP in the early repolarization (Liu et al., 1993), reduction in $I_{to,f}$ slows phase 1 repolarization, abolishes the AP notch, and elevates the AP plateau (Greenstein et al., 2000; Oudit et al., 2001; Sah et al., 2003) (Figure 1.8B).

1.3.4 The Role of $I_{to,f}$ in Excitation–Contraction Coupling

Cardiac excitation–contraction coupling (ECC) is the physiological process of converting an electrical stimulus (action potential) to a mechanical response (contraction) in the heart (Figure 1.9). When a cardiomyocyte is depolarized by action potential, the ubiquitous second messenger Ca$^{2+}$ ions enter the cell through voltage-gated L-type Ca$^{2+}$ channels ($I_{Ca,L}$) during Phase 2 of the action potential, which transiently raises cytosolic free Ca$^{2+}$ from 0.1 µM to 1 µM. Although this is significantly lower than the amount of Ca$^{2+}$ typically required for activation of the contractile proteins contraction (40 – 60 µM) (Bers, 2001; Bers, 2002; Sah et al., 2003), the bulk of Ca$^{2+}$ required for contraction is supplied by Ca$^{2+}$ released from the sarcoplasmic reticulum (SR), a process called ‘Ca$^{2+}$-induced Ca$^{2+}$-release’ (CICR). The SR Ca$^{2+}$ release is brought about through the organization of a cluster of 10 – 400 SR Ca$^{2+}$ release channels (called ryanodine receptor, RyR2) in close proximity to the one or several $I_{Ca,L}$ within the cleft of the myocyte. Thus, SR Ca$^{2+}$ release is affected by the amplitude and frequency of $I_{Ca,L}$, the SR Ca$^{2+}$ content, regulation of RyR2 by ions (e.g. Mg$^{2+}$), as well as phosphorylation and accessory
proteins (e.g. FKBP12.6, triadin, junctin, and calsequestrin) (Bassani et al., 1995; Lopez-Lopez et al., 1995; Marx et al., 2000; Xin et al., 2002). Consequently, the duration and amplitude of the Ca\(^{2+}\) transients during ECC control the force of contraction of cardiomyocytes. Relaxation of myocyte contraction requires the extrusion of Ca\(^{2+}\) from cytosol. The Ca\(^{2+}\)-ATPase in the SR (SERCA2a), which derives the energy from ATP to actively pump Ca\(^{2+}\) into the lumen of the SR, contributes the majority of Ca\(^{2+}\) removal (70 – 92% depending on the species), while Na\(^+\)–Ca\(^{2+}\) exchangers (NCX) extrudes remaining Ca\(^{2+}\) (5 – 30%) from the myocyte. The large contribution of SERCA2a to reuptake Ca\(^{2+}\) into the SR ensures that the myocyte is ready for the next round of ECC with fully replenished SR. In addition, the fraction of Ca\(^{2+}\) efflux by NCX is approximately equal to the amount of Ca\(^{2+}\) that enters the cell through the activity of I\(_{Ca,L}\).

The duration and shape of an AP, largely regulated by repolarization, determines the magnitude and time course of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels, which influences the SR Ca\(^{2+}\) release and loading, and ultimately determines contractility (Cannell and Soeller, 1999; Sah et al., 2001; Sah et al., 2002b; Kondo et al., 2006). The profile of the AP waveform in ventricular myocytes varies considerably between different species as well as within the different regions of the ventricle in the same species (Linz and Meyer, 2000; Oudit et al., 2001). In small animals such as mice and rats, the AP is characterized by a spike-like, triangular shape with an absence of a prominent plateau (Phase 2) (Kaprielian et al., 1999; Wickenden et al., 1999b). Particularly, the AP profile in rodent myocytes is largely affected by I\(_{to,f}\) (as compared to larger animals), which overpowers the depolarizing contribution of I\(_{Ca,L}\) (Barry et al., 1998; Guo et al., 2000). For example, an increase in I\(_{to,f}\) by over-expressing K\(_{V}\)4.2, K\(_{V}\)4.3 or KChIP2 leads to shortened APD and reduced Ca\(^{2+}\) transient and single cell contractility (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010). On the contrary, I\(_{to,f}\) reduction observed in diseased rodent hearts results in a decrease in early AP repolarization and prolonged APD, which enhances SR Ca\(^{2+}\) release and Ca\(^{2+}\) transient amplitude via increased I\(_{Ca,L}\), thereby compensating for contractility (Bouchard et al., 1995; Kaprielian et al., 1999; Kaprielian et al., 2002). In contrast to rodents, the role of I\(_{to,f}\) in contributing to ECC in larger animals is more complicated because I\(_{to,f}\) does not play a major repolarising role in setting the APD in larger species (Greenstein et al., 2000; Sun and Wang, 2005; Dong et al., 2006). In fact, it is well established that in larger animals such canine and humans, I\(_{to,f}\) is responsible for the early repolarization of the AP, leading to the separation of phase 1 and 2 by a notch and the characteristic spike and dome
Figure 1.9
Cardiac Excitation-Contraction Coupling
A schematic diagram of cardiac excitation-contraction coupling in ventricular myocytes. Inset illustrates the time course of an action potential, Ca$^{2+}$ transient and contraction measured in a rabbit ventricular myocyte.
(Adapted from Donald M. Bers, 2002)
profile of the AP (Lukas and Antzelevitch, 1993; Yu et al., 2000). Contrary to rodents, it has been shown that in larger animals, a slowing of early repolarization via a reduction in $I_{to,f}$, results in diminished peak amplitude and increased time to peak of $I_{Ca,L}$, leading to reduced $Ca^{2+}$ transient amplitude and impaired contractility (Linz and Meyer, 2000; Sah et al., 2002b). In addition, the slowing of $I_{Ca,L}$ kinetics causes desynchronized SR $Ca^{2+}$ release and reduces efficiency of ECC, thereby further decreasing the peak intracellular $Ca^{2+}$ transient (Sah et al., 2002b). These data suggest that in normal human and canine myocytes, $I_{to,f}$ and the associated AP notch may enhance ECC as required to transiently increase $I_{Ca,L}$ in order to synchronize and optimize SR $Ca^{2+}$ release. Moreover, in contrast to rodent myocytes, it is believed that $I_{to,f}$ reduction in failing canine and human myocytes contributes to further impairment of SR $Ca^{2+}$ release (Greenstein et al., 2000; Antzelevitch and Fish, 2001; Sah et al., 2002b; Sah et al., 2003). Thus, it has been hypothesized that there is a biphasic dependence of SR $Ca^{2+}$ release on repolarization rate between rodents versus larger animals (Sah et al., 2003). On the other hand, another study has shown in canine cardiomyocytes that the magnitude of initial $I_{Ca,L}$ influx is reduced when activated by an AP waveform with a prominent notch on Phase 1, suggesting that $I_{to,f}$ negatively controls ECC, similar to rodents (Zygmunt et al., 1997; Banyasz et al., 2003; Dong et al., 2010).

### 1.3.5 Molecular Regulation $I_{to,f}$ in the Heart

As changes in $I_{to,f}$ commonly occur in developing hearts as well as diseased hearts, many studies have been devoted to examining the transcriptional regulation of the molecular constituents of $I_{to,f}$, such as $K_V4.2/3$ and KChIP2, during heart development, as well as by hormonal and autocrine/paracrine factors. For example, in rodents, an elevation in $I_{to,f}$ during postnatal development, accompanied by a shortening of the APD, is the result of an increase in the levels of $K_V4.2$, $K_V4.3$, and KChIP2 transcripts in the ventricles (Grandy et al., 2007), and is largely affected by a rise in the plasma levels of tri-iodothyronine (T3) (Shimoni et al., 1997). The transactivating effect of T3 has been shown to be mediated by the thyroid receptor $TR\alpha 1$, through direct binding to the $K_V4.3$ promoter region (Gassanov et al., 2009). $I_{to,f}$ is also regulated in response to $\alpha$-adrenergic ($\alpha$-AR) and $\beta$-adrenergic ($\beta$-AR) receptor stimulation. Reductions in $I_{to,f}$ in response to $\alpha$-AR stimulation have been observed in rat ventricular myocytes, as well as in rabbit atrial and ventricular myocytes, which are accompanied by down-regulation of $K_V4.2$, $K_V4.3$ and KChIP2 mRNA levels (Apkon and Nerbonne, 1988; Ravens et al., 1989; Fedida et al., 2010).
On the other hand, the effect of β-AR stimulation on I_{to,f} is not very well known. A recent study has shown that isoproterenol (ISO) infusion to mice leads to I_{to,f} reduction with down-regulation of Kv4.2 mRNA in mouse ventricular myocytes, which is completely blocked in mice lacking β-AR (Rossow et al., 2009).

Several cardiac transcription factors have been revealed to be involved in transcriptional regulation of the molecular constituents of I_{to,f} such as Kv4.2/3 and KChIP2. In cardiomyocytes, the cardiac transcription factor, Gata4 is able to increase the promoter activity of the Kv4.2 gene (Kcnd2), while the co-repressor friend of Gata (Fog2) exerts repressive effects when binding to Gata4 (Jia and Takimoto, 2003). Our group has shown that the I_{to,f} transmural gradient across the ventricle of mice is determined by the transcription factor Irx5, which represses Kv4.2 transcription by recruiting the co-repressor mBop and HDAC (Costantini et al., 2005). On the other hand, another study has demonstrated that Irx5 represses the Kv4.2 promoter activity not with mBop, but Irx4 (He et al., 2009). In addition, Rossow et al. have reported that a regional heterogeneity of calcineurin-NFATc3 transcriptional repressive activities contributes to the transmural gradient of I_{to,f} in the heart; thus, Nfatc3-deficient mice exhibit disruption of the I_{to,f} transmural gradient (Rossow et al., 2006). They have also demonstrated that calcineurin-NFATc3 activity is involved in I_{to,f} down-regulation after myocardial infarction or β-adrenergic stimulation via transcriptional down-regulation of Kv4.2/4.3 (Rossow et al., 2004; Rossow et al., 2009). On the other hand, our group has reported that activation of calcineurin-NFATc3 in neonatal rat ventricular myocytes increases I_{to,f} via transcriptional up-regulation of Kv4.2 (Gong et al., 2006), suggesting that calcineurin-Nfatc3 may act as an activator or repressor in a context-dependent manner. Additionally, myocyte enhancer factor 2A and 2C (Mef2A and Mef2C), known to interact with Nfat and Gata4 (Morin et al., 2000; Passier et al., 2000), reduce I_{to,f} density with decreased Kv4.2 gene expression (Xu et al., 2006). Another study showed that a decrease in cyclic adenosine monophosphate response element binding protein (CREB) is associated with down-regulation of KChIP2 mRNA induced by left ventricular pacing of canine hearts (Ozgen et al., 2010). Furthermore, our group has found that NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), involved in cardiac hypertrophy (Zelarayan et al., 2009; Van der Heiden et al., 2010), plays an essential role in the transcriptional regulation of I_{to,f} molecular components in response to α-adrenergic stimulation (Unpublished data, Brian Panama).
1.3.6 \( I_{\text{to,f}} \) and Cardiac Hypertrophy

In many heart diseases including cardiac hypertrophy and myocardial infarction, \( I_{\text{to,f}} \) is consistently decreased, along with down-regulation of the molecular constituents of \( I_{\text{to,f}} \) such as \( K_{\text{V}4.2}, K_{\text{V}4.3} \) and KChIP2 (Beuckelmann et al., 1993; Kaab et al., 1998; Xu et al., 1999b; Yao et al., 1999; Radicke et al., 2006). Reduction in \( I_{\text{to,f}} \) results in APD prolongation, commonly observed in diseased myocardium, which strongly affects the \( \text{Ca}^{2+} \) transients (Beuckelmann et al., 1993; Kaab et al., 1996; Kaprielian et al., 1999; Greenstein et al., 2000; Sah et al., 2002b). While elevated \( \text{Ca}^{2+} \) is attributed to the positive inotropic actions of \( \alpha \)-AR stimulation occurring in heart disease (Fedida et al., 1990; Fedida and Bouchard, 1992; Nagashima et al., 1996), it is conceivable that an increase in \( \text{Ca}^{2+} \) transient may contribute to cellular hypertrophy via \( \text{Ca}^{2+} \)-mediated hypertrophic signaling pathways such as calcineurin, Protein Kinase C, and calmodulin-dependent protein kinase (CaMK) (Dolmetsch et al., 1997; Molkentin et al., 1998; Zhang and Brown, 2004). Indeed, transgenic mice with cardiac-specific over-expression of a dominant-negative N-terminal fragment of the \( K_{\text{V}4.2} \) \( \alpha \)-subunit (\( K_{\text{V}4.2\text{N}} \)), which show significant reductions in \( I_{\text{to,f}} \) with APD prolongation, develop severe cardiac hypertrophy with ventricular chamber dilatation and interstitial fibrosis around 10–12 weeks of age (Wickenden et al., 1999b). The connection between \( I_{\text{to,f}} \) reduction and hypertrophy has been also shown in vitro, as over-expressing dominant-negative \( K_{\text{V}4.2\text{N}} \) or mutant \( K_{\text{V}4.2\text{W362F}} \) subunits in cultured neonatal cardiomyocytes leads to reduction or elimination of \( I_{\text{to,f}} \) with APD prolongation, and induces cellular hypertrophy with elevations in calcineurin-NFAT activity (Kassiri et al., 2002). \( I_{\text{to,f}} \)-mediated hypertrophy in vivo and in vitro is inhibited by the calcineurin inhibitors, cyclosporin A (CsA) or Cabin1/Cain, as well as the Ca\(^{2+}\) channel blocker verapamil, suggesting that calcineurin activated by elevated \( \text{Ca}^{2+} \) through L-type \( \text{Ca}^{2+} \) channels underlies cardiac hypertrophy induced by reduction of \( I_{\text{to,f}} \) (Kassiri et al., 2002; Sah et al., 2002a). Furthermore, elevation of \( I_{\text{to,f}} \) by \( K_{\text{V}4.2}, K_{\text{V}4.3} \) or KChIP2 over-expressions can abrogate the in vitro and in vivo hypertrophic response to phenylephrine (PE, \( \alpha \)-AR), angiotensin II (AngII), and pressure-overload because an increase in \( I_{\text{to,f}} \) decreases \( \text{Ca}^{2+} \) transient amplitude with shortened APD, leading to a reduction in calcineurin-NFAT activity (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010). Taken together, these results evidently suggest that \( I_{\text{to,f}} \) can play a crucial role in modulating cardiac hypertrophy. However, other studies have yielded contrary results. For example, elimination of \( I_{\text{to,f}} \) in mice by expression of mutant \( K_{\text{V}4.2\text{W362F}} \) construct (Barry et al., 1998), targeted deletion of \( K_{\text{V}4.2} (K_{\text{V}4.2^{-}}) \) (Guo et al., 2005), as well as knock out of KChIP2...
(Kuo et al., 2001) does not induce spontaneous hypertrophy in adult mouse hearts despite APD prolongation. Therefore, future experiments will be necessary to understand why some rodent models exhibit a causal relationship between $I_{\text{to}, f}$ and hypertrophy while others do not.

1.4 Cardiac Hypertrophy
1.4.1 Introduction to Cardiac Hypertrophy

Hypertrophy [from the Greek hyper (over) and trophie (growth)] of the heart is clinically diagnosed and defined as the increase in myocardial mass, and is considered as an initial compensatory response to increased hemodynamic demands caused by extrinsic and intrinsic stimuli (Lips et al., 2003; Diwan and Dorn, 2007). Wall stress at ventricular end-systole is believed to be an important determinant of the degree and type of cardiac hypertrophy (Grossman et al., 1975; Grossman, 1980), and is normalized by the increase in myocardial mass (i.e. wall thickening), which can be demonstrated through LaPlace’s principle as follows:

$$\sigma = \frac{p r}{2h}$$

where $\sigma$ is wall stress, $p$ is intracavitary pressure, $r$ is the internal radius of the chamber, and $h$ is the wall thickness (Figure 1.10) (Lips et al., 2003; Diwan and Dorn, 2007). For example, when pressure ($p$) is increased due to hypertension, the elevation in wall stress ($\sigma$) can be alleviated by an increase in wall thickness ($h'$).

At the cellular level, hypertrophic myocardium is the consequence of an increase in cardiac myocyte size, not myocyte number. Since proliferation is impossible or at best limited in cardiomyocytes due to terminal differentiation shortly after birth by irreversibly withdrawing from the cell cycle (Brooks et al., 1998; Poolman and Brooks, 1998), their only means of the heart muscle augmentation is by hypertrophic cellular enlargement. Nevertheless, much evidence has demonstrated that cell cycle regulatory mechanisms are involved in cardiac hypertrophy (Sadoshima et al., 1997; Tamamori et al., 1998; Nozato et al., 2000; Ahuja et al., 2007). In response to hypertrophic stimuli, cardiomyocytes re-enter the cell cycle and synthesize DNA with the up-regulation of G1 cyclin/Cdns and reciprocal down-regulation of CdkIs, but do not undergo mitosis (Li and Brooks, 1997; Li et al., 1998; Poolman and Brooks, 1998). Karyokinesis in the absence of cytokinesis causes endoreduplication, resulting in tetraploid, octoploid or even more sets of chromosomes in cardiomyocytes after heart disease (Herget et al., 1997; Meckert et al., 2005). Although the functional role of this process still remains to be resolved, it has been
Figure 1.10
*Development and Progression of Cardiac Hypertrophy*

In response to hemodynamic stress and/or myocardial injury, the heart develops cardiac hypertrophy to normalize wall stress by increasing ventricular wall thickness \((h' > h)\). Hypertrophy is characterized by cardiomyocyte growth which is induced by neurohormonal activation or mechanical stretch. Prolonged hypertrophic stimulation can cause cardiomyocyte death or myocyte drop-out, which lead to transition to dilated cardiomyopathy manifested by chamber dilation \((r' > r)\) and wall thinning \((h'' < h')\). (Modified from Diwan and Dorn, 2007).
suggested that the polyploidisation probably enables the cardiomyocyte to develop hypertrophy without losing its normal cell volume/DNA content ratio. On the other hand, non-cardiomyocytes such as fibroblasts proliferate in response to hypertrophic stimuli, which is related to fibrosis, although it is not completely understood whether this contributes to reduction in wall stress.

Two different patterns of cardiac hypertrophy have been defined, which depend on the types of extrinsic hypertrophic stimuli, pressure-overload versus volume-overload (Lips et al., 2003; Diwan and Dorn, 2007). Pressure-overload, a condition with increased afterload due to systemic hypertension or aortic stenosis, results in concentric hypertrophy, characterized by thickening of ventricular wall with minimal or no increase in the ventricular chamber diameter. At the cellular level, the thickening of myocardium in pressure-overload induced hypertrophy is an outcome of sarcomeric assembly in parallel rather than in series, as shown by enlarged myocyte cross sectional area. Volume-overload, on the other hand, induces eccentric hypertrophy, defined as an increase in wall thickness in conjunction with LV cavity diameter enlargement. In the clinical setting, aortic and mitral valve regurgitation, intracardiac shunt, arteriovenous fistula, anemia, as well as other situations where cardiac output is increased at normotensive pressure, evoke volume-overload induced hypertrophy. Similar to the alteration in the morphology of the ventricle, myocytes with eccentric hypertrophy exhibit a proportional increase in myocyte cross-sectional area and length by sarcomeric assembly both in series and in parallel. Physiological hypertrophy induced by exercise or pregnancy resembles volume-overload induced hypertrophy, although there are significant differences in the underlying signaling pathways and hypertrophic gene programs (Dorn et al., 2003; Wakatsuki et al., 2004; Eghbali et al., 2005). Furthermore, cardiac hypertrophy can be caused by intrinsic stimuli. Specifically, mutations in genes related to sarcomeric contractile proteins, such as β-myosin heavy chain (MYH7), cardiac troponin T (TNNT2) and I (TNNI3), α-tropomyosin (TPM1), myosin-binding protein C (MYBPC3), myosin light chain 2 (MYL2) and 3 (MYL3), cardiac α-actin genes (ACTC), and titin (TTN), approximately account for 60% of hypertrophic cardiomyopathy (HCM) which is a common inherited cardiac disease, affecting 1 in 500 people (Keren et al., 2008).

Despite its possible function in the initial adaptive response to hemodynamic workload, sustained hypertrophy with persistent insult over a prolonged period of time is often accompanied by adverse outcomes, including sudden death, development of decompensation and
heart failure (Levy et al., 1990; Bikkina et al., 1993; Frey and Olson, 2003; Gosse, 2005). Long-term maladaptive remodelling of cardiac hypertrophy is often associated with progressive ventricular dilation. As shown in Figure 1.10, once ventricles start dilating, cardiac enlargement \((r')\) and wall thinning \((h')\), but constant pressure \((p)\) additionally increase wall stress \((\sigma)\), thereby further activating the hypertrophic response and eventually leading to the “chronic myocardial exhaustion” phase (Meerson, 1969). Although most of the signaling cascades involved in cardiac hypertrophy enhance myocyte survival via an anti-apoptotic pathways (e.g., the extracellular signal-regulated kinase 1/2 (ERK1/2), the protein kinase B (PKB, Akb), and calcineurin-NFAT), paradoxically over-stimulating the hypertrophic signaling pathways can lead to more deleterious results by tipping the balance from a cell growth response to cell death (van Empel and De Windt, 2004). These detrimental results have been also observed in animal models, such as mice over-expressing constitutively active calcineurin (CnA-TG) (Molkentin et al., 1998), as well as mice subjected to both pressure-overload and G\(_{aq}\) subunit over-expression (Sakata et al., 1998). During the last decades, numerous investigators have tried to identify mechanisms that underlie the transition from chronic hypertrophy to overt heart failure. Understanding mechanisms of hypertrophy and heart failure will enable us to dissect signals for the desirable effects of hypertrophy (i.e., elevated cardiac function with increased sarcomere organization), while preventing maladaptive outcomes (i.e., dilated cardiomyopathy, arrhythmia, cardiac dysfunction) (Frey et al., 2004b; Selvetella et al., 2004).

1.4.2 Transcriptional Factors in Cardiac Hypertrophy

During the development of cardiac hypertrophy, signaling cascades are initiated and activated by neurohormonal stimulation (e.g., angiotensin II, AngII; norepinephrine, NE, phenylephrine, PE; and endothelin-1, ET-1), and mechanical stretch via Gq-coupled mechanoreceptors or transient receptor potential channels (Lips et al., 2003; Sharif-Naeini et al., 2010). Activation of various intracellular signaling pathways target transcription factors that regulate a number of cardiac genes related to the cell cycle, growth, excitation-contraction coupling and metabolism. Hypertrophic phenotypes are also characterized by activation of immediate early genes (e.g. \(c\)-jun, \(c\)-fos, \(c\)-myc), as well as re-activation of the fetal gene program (e.g., atrial natriuretic factor, ANF; B-type natriuretic peptide, BNP; and \(\beta\)-myosin heavy chain, \(\beta\)-MHC). The transcriptional program is not limited to adaptive hypertrophy with cellular growth, but also is involved in pathological responses such as sarcomere structural
alterations, fibrotic pathogenesis, inflammation, or apoptosis, which leads to progression to heart failure. Understanding cardiac transcriptional regulation is, therefore, essential to improve heart failure therapy.

1.4.2.1 Calcineurin-NFAT

Calcineurin is a Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase (PP2B) that plays a crucial role in cardiac development, hypertrophy and heart failure (Frey and Olson, 2003). Calcineurin, evolutionarily conserved from yeast to humans in its overall structure and biochemistry, exists as a heterotrimer consisting of a catalytic A subunit (CnA) of approximately 57 – 61 kDa, and two small (16 – 19 kDa) EF-hand calcium binding proteins, the regulatory B subunit (CnB) and calmodulin (Klee et al., 1998). In vertebrates, three different genes encode three separate catalytic subunit isoforms (CnA\(\alpha\), CnA\(\beta\) and CnA\(\gamma\)), whereas two genes encode two isoforms of the regulatory subunit (CnB1 and CnB2) (Molkentin, 2000a; Rusnak and Mertz, 2000). While CnA\(\alpha\), CnA\(\beta\) as well as CnB1 are ubiquitously expressed in many tissues including the heart, CnA\(\gamma\) and CnB2 expression is restricted to the brain and testis. Its role and mechanism were initially investigated in T-cell activation. When the T cell receptor is activated, intracellular Ca\(^{2+}\) is elevated and binds to a Ca\(^{2+}\) sensor protein, calmodulin. Ca\(^{2+}\)-calmodulin complex then binds to and activates calcineurin (Crabtree and Olson, 2002; Gallo et al., 2006). Once activated, calcineurin directly dephosphorylates the nuclear factor of activated T cells (NFAT), a member of the family of Rel homology domain-containing transcription factors, which foster translocations from the cytosol to the nucleus, followed by NFAT-DNA binding and gene regulation. NFAT also can interact with other cardiac transcription factor, such as AP-1 (c-jun/c-fos), Gata4, and Mef2, thereby reinforcing NFAT-DNA interactions (Hogan et al., 2003). Conversely, re-phosphorylation of the serine-rich N-terminus of NFAT by glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)), c-Jun N-terminal kinase (JNK), casein kinase I (CK1), protein kinase A (PKA) and mitogen-activated protein kinase kinase kinase 1 (MEKK1) promotes nuclear export of NFATs, prevents calcineurin-mediated translocation to the nucleus, and inhibits the DNA-binding activity of NFAT (Neal and Clipstone, 2001; Vega et al., 2003a). Furthermore, the activity of calcineurin-NFAT signaling pathway is regulated by a number of proteins. Calcineurin phosphatase activity is inhibited by regulator of calcineurin 1 (Rcan1; Mcip1; Dscr1) (Vega et al., 2003a; Vega et al., 2003b), Cabin1/Cain (Lai et al., 1998; Sun et al., 1998), calcineurin homologous protein (CHP; calcineurin B homology protein) (Lin et al., 1999),
mitochondrial FK506-binding protein 38 (FKBP38) (Shirane and Nakayama, 2003), and A-Kinase anchor protein-79-KD (AKAP79) (Taigen et al., 2000). Calcineurin catalytic activity is also pharmacologically inhibited by the immunosuppressive drugs, cyclosporine A (CsA) and FK506 by forming complexes with cytoplasmic receptors cyclophilin and FKBP12, respectively (Klee et al., 1998; Crabtree, 1999). In addition, VIVIT, a synthetic peptide binding to the PxIxIT docking motif of NFAT to which calcineurin binds, distinctively blocks calcineurin-NFAT interactions while not affecting calcineurin phosphatase activity (Aramburu et al., 1999).

The calcineurin-NFAT signaling pathway is also operative in cardiomyocytes, and its important role in the development of cardiac hypertrophy has been well demonstrated in numerous studies (Figure 1.11). A number of studies have demonstrated that in myocardium, calcineurin-NFAT can be activated and/or affected by various intracellular Ca\(^{2+}\) regulators such as L-type Ca\(^{2+}\) channels (Kassiri et al., 2002; Sah et al., 2002a; Balijepalli et al., 2006), SR Ca\(^{2+}\) release via RyR2, T-type Ca\(^{2+}\) channels (Nakayama et al., 2009), transient receptor potential canonical (TRPC) channels (Kuwahara et al., 2006; Seth et al., 2009; Wu et al., 2010), InsP\(_3\)-dependent perinuclear Ca\(^{2+}\) release (Wu et al., 2006), and plasma membrane Ca\(^{2+}\)-ATPase isoform 4 (PCNA4) (Wu et al., 2009), which expressions are changed by hypertrophic stimulation. Calcineurin is also activated by mechanical stretch of the myocardium. Muscle LIM protein (MLP), which is anchored at the sarcomeric Z-disc and acts as a stress-sensing machinery, is physically associated with calcineurin, thereby playing a critical role in calcineurin-NFAT pathway in hypertrophy (Heineke et al., 2005). Thus, cardiac hypertrophy is attenuated when the MLP-calcineurin interaction is inhibited by competitive binding of PKC–interacting cousin of thioredoxin (PICOT) to MLP (Jeong et al., 2008).

The role of calcineurin in hypertrophy was first demonstrated by mice (CnA-TG) with cardiac specific over-expression of constitutively active calcineurin, which exhibited robust hypertrophy (Molkentin et al., 1998). Subsequently, a number of studies have reported elevations of calcineurin activity in hypertrophied hearts (Shimoyama et al., 1999; Eto et al., 2000; Lim et al., 2000). By contrast, calcineurin Aβ-null mice show impaired cardiac hypertrophy in response to pressure overload, ISO infusion, or AngII infusion (Bueno et al., 2002). In addition, pharmacological inhibition of calcineurin activity by CsA and FK506 (Sussman et al., 1998; Meguro et al., 1999), or over-expression of the calcineurin inhibitory proteins such as Rcan1 (Rothermel et al., 2001; Hill et al., 2002), Cabin1/Cain (De Windt et al., 2001), AKAP79 (Taigen et al., 2000), have been shown to blunt the hypertrophy induced by pressure-overload...
Figure 1.11
Calcineurin-NFAT Signalling Pathway in Cardiac Hypertrophy

In response to hypertrophic stimuli, calcineurin is activated by Ca\(^{2+}\) through L-type Ca\(^{2+}\) channels (LTCC), T-type Ca\(^{2+}\) channels (TTCC), ryanodine receptor (RyR) of sarcoplasmic reticulum, and transient receptor potential channel (TRPC) in cardiomyocytes. Activated calcineurin dephosphorylates Nfatc3, promoting translocation to the nucleus, while phosphorylation of Nfatc3 by GSK-3β, p38, and JNK leads to extrusion of Nfatc3 from the nucleus. Calcineurin activity is negatively regulated by cyclosporin A (CsA), FK506, regulator of calcineurin 1 (Rcan1), A-Kinase anchor protein-79-KD (AKAP79), and Cabin1/Cain.
and neurohormonal stimulation. Collectively, all evidence supports the conclusion that calcineurin is a key mediator of cardiac hypertrophy.

Five NFAT transcription factors have been identified so far. Four of them (NFATc1–c4) are regulated by calcineurin, whereas NFAT5, a novel member of the NFAT family of proteins, is regulated independent of calcineurin due to lack of the N-terminal NFAT homology region containing the calcineurin regulatory motif (Molkentin, 2004; Wilkins and Molkentin, 2004; Ito et al., 2007). As the mammalian heart expresses four different calcineurin-activated NFAT isoforms (NFATc1–c4) (van Rooij et al., 2002; Wilkins et al., 2002), their functional roles in cardiac hypertrophy have been investigated. In hypertrophied heart and cultured myocytes in response to constitutively-active calcineurin, PE, NE, and D-myoinositol 1,4,5-tris-phosphate (InsP3), NFAT mRNA and protein expressions are increased (Zhu et al., 2005; Bourajjaj et al., 2008; Kaludercic et al., 2010; Li et al., 2010). Over-expressing NFAT in cardiomyocytes and hearts can induce hypertrophy (Molkentin et al., 1998; van Rooij et al., 2002), while over-expression of VIVIT blunts the hypertrophic response to PE (Pu et al., 2003), suggesting that NFAT is required for calcineurin-mediated cardiac hypertrophy in response to various hypertrophic stimuli. Moreover, a few studies with transgenic mice provided direct evidence of the role of specific NFAT members in hypertrophy. For example, mice lacking NFATc2 (NFATc2−/−) showed attenuated hypertrophic response to constitutively active calcineurin, pressure-overload, as well as neurohormonal infusions (Bourajjaj et al., 2008). While NFATc4 over-expression is capable of inducing hypertrophy in vivo and in vitro (Molkentin et al., 1998; van Rooij et al., 2002), targeted disruption of NFATc4 (NFATc4−/−) failed to block or reduce calcineurin or pressure overload-induced hypertrophy in mice (Wilkins et al., 2002). Ablation of NFATc3 (NFATc3−/−), on the other hand, results in significant reductions in hypertrophy, suggesting that NFATc3, rather than NFATc4, is a critical modulator of ventricular hypertrophy. Nevertheless, incomplete elimination of hypertrophy in NFATc3−/− and NFATc2−/− mice suggests compensation by other factors, possibly due to functional redundancy of the NFAT transcription factor family (Wilkins et al., 2002; Bourajjaj et al., 2008).

In addition, for transcriptional gene regulation, NFATs interact and/or cooperate with other cardiac transcription factors, such as Gata4 (Molkentin et al., 1998), Mef2 (MADS Box Transcription Enhancer Factor-2) (Passier et al., 2000; Hannenhalli et al., 2006; Putt et al., 2009), E12 (Zhou et al., 2005; Zeini et al., 2009), CBP (CREB Binding Protein) (Garcia-Rodriguez and Rao, 1998; Meissner et al., 2007; Falvo et al., 2008), p300 (Kawamura et al., 2004), and Fox
(Hannenhalli et al., 2006). Although the exact mechanism by which NFAT regulates specific gene expression with other transcription factors remains to be resolved, a number of studies have shown that transcriptional complexes with NFAT regulate gene expression are required for hypertrophic (e.g. BNP, β-myosin heavy chain, tumor necrosis factor) and cyto-protective (e.g. B Cell Leukemia-2) responses mediated by calcineurin (Molkentin et al., 1998; Kawamura et al., 2004; Meissner et al., 2007; Falvo et al., 2008).

Similar to prolonged hypertrophy (see Section 1.4.1), over-expression of calcineurin or NFATs results in a detrimental outcome with severe dilated heart failure (Molkentin et al., 1998). However, prevention of hypertrophy by inhibition of calcineurin-NFAT activity appears to be not always beneficial. For example, attenuated hypertrophy by calcineurin inhibitor CsA conversely increases mortality with high susceptibility to decompensation and heart failure (Meguro et al., 1999). Similarly, NFATc3−/− mice that show attenuated hypertrophy, still undergo heart decompensation with a reduction in contractility (Wilkins et al., 2002). This maladaptive response of calcineurin-NFAT inhibition in cardiac hypertrophy may be associated with the cytoprotective or anti-apoptotic role of calcineurin-NFAT. In an in vitro setting, calcineurin inhibition with VIVIT or CsA that blunts PE-induced hypertrophy induces apoptosis in cardiomyocytes, while activation of Calcineurin-NFAT can prevent apoptosis of cardiac cells (De Windt et al., 2000; Kakita et al., 2001; Pu et al., 2003). These results suggest that during the development of cardiac hypertrophy, calcineurin-NFAT can act as a double-edge sword, by activating survival and cell-death pathways in cardiomyocytes.

1.4.2.2 The Zinc Finger-containing GATA Transcription Factors

Gata transcription factors (Gata1 – 6) are a zinc finger-containing transcription factor family (Pikkarainen et al., 2004) that bind to the specific consensus DNA sequence element 5’-(A/T)GATA(A/G)-3’ of target gene promoters (Evans and Felsenfeld, 1989). While Gata1 – 3 play an important regulator role in hematopoietic stem cells and their derivatives (Cantor and Orkin, 2002; Ohneda and Yamamoto, 2002), Gata4 – 6 are expressed in mesoderm and endoderm-derived tissues including the heart (Laverriere et al., 1994). Within the heart, Gata4 and Gata6 are expressed in the whole myocardium of embryonic and adult hearts, while Gata5 expression is found in endocardium (Arceci et al., 1993; Kelley et al., 1993; Heikinheimo et al., 1994; Morrisey et al., 1997). As Gata4 is one of the earliest transcription factors expressed in cardiac development as early as E7.0 – 7.5 in the precardiac mesoderm (Heikinheimo et al.,
1994), the role of Gata4 in cardiac development has been well investigated. Although Gata4 is not necessary for initiation of the cardiac differentiation pathway, Gata4 over-expression in P19 embryonic carcinoma cells accelerates differentiation into cardiomyocytes with an increase in the number of beating cardiomyocytes (Grepin et al., 1997). On the contrary, inhibition of Gata4 blocks cardiomyocyte differentiation with an increase in apoptotic cell death (Grepin et al., 1995; Grepin et al., 1997). The importance of Gata in cardiac development is further emphasized by transgenic mouse models. Gata4-deficient mice die around E8 – 9 due to the defects in ventral morphogenesis and heart tube formation (Kuo et al., 1997; Molkentin et al., 1997). Deletion of Gata6-loxP with Nkx2.5-cre also results in late embryonic lethality with heart defects (van Berlo et al., 2010). Interestingly, the cardiomyocyte differentiation is not impaired in the Gata4-deficient mice with normal expressions of ANF and α-myosin heavy chain (α-MHC), suggesting that loss of Gata4 is compensated by up-regulation of Gata6 (Molkentin et al., 1997; Xin et al., 2006).

Gata4 and Gata6 are thought to function as key cardiac transcriptional regulator of many cardiac genes, such as α-MHC, myosin light chain 1/3 (MLC1/3), cardiac troponin C and I, ANF, BNP, cardiac-restricted ankyrin repeat protein (CARP), NCX, cardiac m2 muscarinic acetylcholine receptor, A1 adenosine receptor, and carnitine palmitoyl transferase Iβ (Molkentin, 2000b; Akazawa and Komuro, 2003), which are greatly affected in hypertrophied hearts. Changes in Gata4 and Gata6 expressions are detected in cardiac hypertrophy. Specifically, Gata4 expression is elevated in calcineurin over-expressing (CnA-TG) mouse hearts (Ikeda et al., 2009), as well as hypertrophied cardiomyocytes induced by InsP3 (Zhu et al., 2005), electrical pacing (Xia et al., 2000), or prostaglandin F2α (PGF2α) (Jiang et al., 2007). In addition, mice subjected to pressure-overload show up-regulation of Gata4 and Gata6 mRNA and protein expression in the heart (Heineke et al., 2007; Tang et al., 2010; van Berlo et al., 2010). Gata4 expression is also significantly higher in hypertrophic cardiomyopathy patients (Kontaraki et al., 2007). Moreover, over-expression of Gata4 and Gata6 in neonatal cardiomyocyte or adult heart is capable of inducing cardiac hypertrophy (Liang et al., 2001; van Berlo et al., 2010). Transgenic mouse hearts with controlled Gata6 over-expression develop greater hypertrophy with pressure-overload stimulation (van Berlo et al., 2010). These observations suggest that Gata4 and Gata6 act as pro-hypertrophic transcriptional mediators. Consistent with this possibility, cardiac-specific deletion of Gata4 or Gata6 using α-MHC and β-MHC promoter driven Cre transgenes results in significantly attenuated cardiac hypertrophy in response to pressure-overload (Oka et
al., 2006; van Berlo et al., 2010). However, another mouse model with heterozygous Gata4 ablation (by deleting the second exon of Gata4) did not show a difference in pressure-overload induced hypertrophy (Bisping et al., 2006), suggesting a half dose of Gata4 gene is sufficient for hypertrophic response. More interestingly, mice lacking Gata4 or Gata6 in their cardiomyocytes as well as Gata4 heterozygous mice develop evident heart failure with increased apoptosis and fibrosis (Bisping et al., 2006; Oka et al., 2006; van Berlo et al., 2010). This increased susceptibility to heart failure by loss of Gata4 has been shown to be associated with reduced myocardial capillary density and prevention of pressure overload–augmented angiogenesis (Heineke et al., 2007). Although the role of Gata6 in adaptive angiogenesis has not been tested, in vitro tube formation experiments with Gata6 suggest that it may play a similar role in the heart disease. Taken together, these results strongly suggest that Gata4 and Gata6 are not only important for the development of cardiac growth, but also essential for comprehensive adaptive responses and maintenance of heart functions.

Transcriptional activity of Gata4 is affected through post-translational modifications, as hypertrophic stimuli can enhance Gata4 DNA-binding activity (Yamazaki et al., 1993; Yamazaki et al., 1995). Specifically, phosphorylation of Gata4 by ERKs and p38 MAPK activates its transcriptional activity (Morimoto et al., 2000; Charron et al., 2001; Liang et al., 2001), whereas phosphorylation at N-terminus of Gata4 by GSK-3β induces its nuclear extrusion (Morisco et al., 2001). Furthermore, Gata4 regulates transcriptional activity by interacting with several cardiac transcription factors such as serum response factor (SRF) (Belaguli et al., 2000), Nkx2-5 (Lee et al., 1998; Shiojima et al., 1999), Mef2 (Morin et al., 2000), NFAT (Molkentin et al., 1998), dHand / Hand2 (Dai et al., 2002), co-activator p300 (Dai and Markham, 2001; Dai et al., 2002), and the co-repressor FOG2 (Lu et al., 1999; Hirai et al., 2004).

1.4.2.3 NK-2 Class Homeobox Transcription Factor Csx/Nkx2-5

Csx/Nkx2-5 is a member of the NK homeobox gene family that is conserved in evolution and acts as transcriptional activator by binding to the specific consensus DNA sequence 5’- T(C/T)AAGTG-3’ (Kim and Nirenberg, 1989; Chen and Schwartz, 1996; Harvey, 1996). It was originally identified as the vertebrate homologue of Drosophila tinman (Komuro and Izumo, 1993; Lints et al., 1993) that is vital for the primitive heart formation of Drosophila (Azpiazu and Frasch, 1993; Bodmer, 1993). Similarly, Nkx2-5 is highly expressed in the heart primordia in both primary and secondary heart fields during development, and continues to be expressed at
a high level in the adult heart (Komuro and Izumo, 1993; Chen and Schwartz, 1995; Kasahara et al., 1998; Stanley et al., 2002). Thus, the role of Nkx2-5 in cardiogenesis has been extensively examined. Three different mice lacking Nkx2-5 have been generated and revealed fundamentally identical phenotypes, exhibiting embryonic lethality around E9 – 10 due to cardiac structural defects and growth retardation (Lyons et al., 1995; Biben et al., 2000). In fact, a variety of congenital heart diseases and atrioventricular conduction delay in humans are associated with mutations in Nkx2-5 (Schott et al., 1998; Benson et al., 1999). Loss of Nkx2-5 (Nkx2-5−/−) in the embryonic mouse heart results in reduction in numerous cardiac genes, such as myosin light chain 2v (MLC2v), ANF, BNP, CARP, (Lyons et al., 1995; Biben and Harvey, 1997; Zou et al., 1997; Tanaka et al., 1999; Bruneau et al., 2000; Shin et al., 2002), which are up-regulated in mouse heart over-expressing Nkx2-5 (Takimoto et al., 2000). In addition, expression of cardiac transcription factors including Mef2C, eHand/Hand1, N-myc, Irx4, homeodomain-only protein (Hopx), Gata4, and a zinc finger–type transcription factor (Zac1) are decreased in the embryonic heart of Nkx2-5−/− mice (Lyons et al., 1995; Biben and Harvey, 1997; Tanaka et al., 1999; Bruneau et al., 2000; Shin et al., 2002; Riazi et al., 2009; Yuasa et al., 2010). Taken together, these data clearly demonstrate that Nkx2-5 is essential for cardiac developmental pathways by regulating transcription of cardiac-specific genes, and that Nkx2.5 is one of the most upstream transcriptional mediators in the hierarchical cascade of cardiac transcription factors.

Although Nkx2-5 is expressed in the adult heart (Komuro and Izumo, 1993; Lints et al., 1993), little is known about the physiological and pathological functions of Nkx2-5 in the adult heart due to embryonic lethality of Nkx2-5−/− mice (Lyons et al., 1995; Tanaka et al., 1999). Interestingly, previous studies have shown that Nkx2-5 is up-regulated in animal and cellular models of cardiac hypertrophy induced by pressure-overload and adrenergic stimulations (e.g. PE and ISO) (Thompson et al., 1998; Saadane et al., 1999) as well as in hypertrophic cardiomyopathy (HCM) patients (Kontaraki et al., 2007), indicating that Nkx2-5 may be implicated in the transcriptional regulation during the generation of cardiac hypertrophy. On the other hand, transgenic mice over-expressing human Nkx2-5 exhibit no hypertrophy with normal sized hearts while up-regulating several cardiac genes including ANF, BNP, CARP, and MLC2v (Takimoto et al., 2000). These findings suggest that, although Nkx2-5 is not sufficient for generating cardiac hypertrophy, it regulates cardiac gene expression in both embryonic and adult hearts. It is very possible that Nkx2-5 contributes to transcriptional regulation in cardiac
hypertrophy by forming transcription complexes with other cardiac transcription factors, since it is very well known to interact with numerous cardiac transcription factors, such as Gata4 (Lee et al., 1998; Shiojima et al., 1999), Mef2c (Vincentz et al., 2008), SRF (Chen and Schwartz, 1996), T-box-containing transcription factor Tbx5 (Bruneau et al., 2001b), Tbx2 (Habets et al., 2002), and Tbx20 (Stennard et al., 2003), as well as eHand/Hand1 (Thattaliyath et al., 2002a). Furthermore, the cardio-protective role of Nkx2-5 may play an additional role in cardiac hypertrophy by promoting transcription of the genes in the anti-apoptotic pathway (Toko et al., 2002).

1.4.2.4 Other Transcription Regulators in Cardiac Hypertrophy

In addition to calcineurin-NFAT, Gata4 and Nkx2-5, several other cardiac transcription factors, co-factors and transcription modulators are also implicated or expected to be involved in cardiac hypertrophy. The identification and elucidation of networks of cardiac transcription factors will be necessary to further understand mechanism of cardiac hypertrophy.

Myocyte Enhancer Factor 2 (Mef2)

Myocyte enhancer factor 2 (Mef2) transcription factors, which are able to physically interact with Nfat, Gata4, and Nkx2-5, have been extensively investigated in the context of cardiac development and hypertrophy (Czubryt and Olson, 2004). Members of the Mef2 family contain a MADS (MCM1, agamous, deficiens and SRF) domain and an adjacent Mef2-specific domain in the N-terminus (Akazawa and Komuro, 2003; Potthoff and Olson, 2007). In vertebrates, four Mef2 factors (Mef2A, Mef2B, Mef2C and Mef2D) have been identified, displaying overlapping expression patterns in embryonic and adult tissue. Mef2 factors play a crucial role in cardiac development, as mice lacking Mef2a and Mef2c develop lethal arrhythmia and embryonic death, respectively (Lin et al., 1997; Naya et al., 2002). In response to hypertrophic stimuli, Mef2 transcriptional activity is increased by promoting the nuclear export of its suppressor, class II histone deacetylases (HDACs) (see below). Mef2 down-regulation by over-expressing a dominant-negative Mef2 and loss of Mef2D is able to reduce hypertrophy induced by constitutively active calcineurin, while more provocatively, preventing chamber dilation and improving cardiac contractility (van Oort et al., 2006; Kim et al., 2008b). In contrast, over-expression of Mef2A, Mef2C, or Mef2D results in severe ventricular dilation and
cardiomyopathy with impaired contractile function and abnormal gene expression (Xu et al., 2006; Kim et al., 2008b). Collectively, these observations suggest that Mef2 family members act as a key transcription factors for pathological remodelling in cardiac hypertrophy, proposing that they may be strong candidates for therapeutic targets for heart failure.

**Histone Acetyltransferase (HAT) and Histone Deacetylase (HDAC)**

As histones control gene transcription by modulating the structure of chromatin and the accessibility of DNA promoter sequences to transcription factors, numerous studies have investigated the roles of histone modifications by histone acetyltransferases (HAT) and histone deacetylases (HDAC) in cardiac gene programming during cardiogenesis and hypertrophy (Backs and Olson, 2006). Notably, both HAT and HDAC associate, directly or indirectly, with numerous cardiac transcription factors engaged in hypertrophy, including Gata4, SRF, Mef2, and NFAT, and act as adaptors to modulate transcriptional activities in cardiac hypertrophy.

Acetylation of histone (conserved lysine residue) by HATs results in transcriptional activation by destabilizing the histone-DNA complex, which makes DNA more accessible to transcription factors. Among the many HATs, p300 and CREB-binding protein (CBP) are the most extensively examined and have been found to play vital roles in cardiac growth in physiological and pathological contexts. The transcriptional activities of p300 and CBP are increased in response to hypertrophic stimuli (Gusterson et al., 2002; Gusterson et al., 2003), and p300 over-expression in the heart leads to hypertrophy and heart failure (Yanazume et al., 2003). Conversely, inhibition of p300 activity by dominant-negative mutant p300 or curcumin treatment, as well as CBP down-regulation, inhibits PE-induced myocyte hypertrophy (Gusterson et al., 2003; Morimoto et al., 2008).

In contrast to HATs, HDACs basically suppress transcriptional activity by promoting chromatin condensation. The 18 known HDACs are categorized into three classes. However, their effects on cardiac growth can be opposite depending on the class of HDAC. Specifically, class II HDACs (HDAC4, -5, -6, -7, -9 and -10) have been reported to repress cardiac hypertrophy, while several studies have shown that class I HDACs (HDAC1, -2, -3, and -8) promote cardiac growth. Thus, mice lacking class II HDAC, such as HDAC5 (Chang et al., 2004) or HDAC9 (Zhang et al., 2002), develop spontaneous hypertrophy or greater hypertrophy in response to pressure-overload and activated calcineurin. On the other hand, inhibition of class I HDAC such as HDAC1 prevents myocyte hypertrophy, whereas promoting class I HDAC by
over-expression of homeodomain only protein (Hop; Hopx) induces severe cardiac hypertrophy (Kook et al., 2003). Furthermore, a recent study reported that sirtuin 3 (Sirt3), which belongs to class III HDAC that act as NAD-dependent deacetylases, represses cardiac hypertrophy (Sundaresan et al., 2009). Therefore, developing class-specific HDAC inhibitors would be an interesting therapeutic approach for heart failure treatment.

**Nuclear Factor kappa-B (NF-κB)**

The NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) family is comprised of p65 (RelA), RelB (RelB), c-Rel (Rel), p105/p50 (NFκB1) and p100/p52 (NFκB2). They share an N-terminal RHD (Rel-homology domain) required for regulation of transcription, including association with inhibitory proteins, such as IκB (inhibitory κB) (Van der Heiden et al., 2010). Several studies have demonstrated that NF-κB plays a necessary role in cardiac hypertrophy, because hypertrophy in vitro (i.e. myotrophin, PE, ET-1, and AngII) and in vivo (i.e. pressure-overload and angiotensin Type I receptor over-expression) is attenuated by inhibition or ablation of NF-κB (Purcell et al., 2001; Gupta et al., 2002; Hirotani et al., 2002). Interestingly, NF-κB inhibition in the heart following aortic constriction accelerates LV remodelling with impaired contractility and increased fibrosis, suggesting that NF-κB is necessary for the adaptive hypertrophic response. This early decompensation may be associated with cytoprotective role of NF-κB against many apoptotic stimuli (van Empel and De Windt, 2004; Hall et al., 2006).

**The Basic Helix-Loop-Helix Factor Hand**

eHand/Hand1 and dHand /Hand2 are basic helix-loop-helix transcription factors, and play unique roles in cardiogenesis (Srivastava, 1999). Their expression patterns are very distinctive with predominant eHand/Hand1 expression in the LV in contrast to dHand /Hand2 expression restricted to the RV (Firulli, 2003). While detectable in the adult heart, their expression is down-regulated in hypertrophied hearts in animals and human (Natarajan et al., 2001; Thattaliyath et al., 2002b). In addition, the transcriptional activities of eHand/Hand1 and dHand /Hand2 on ANF, BNP and α-MHC are enhanced by interaction with Nkx2-5, Gata4, and p300, strongly suggesting that Hand transcription factors may be implicated in gene remodelling during cardiac hypertrophy (Dai et al., 2002; Thattaliyath et al., 2002a). Elucidations of their roles and mechanisms in the cardiac disease will be of interest.
The Forkhead box subfamily O (FoxO)

FoxO transcription factors are members of the Forkhead/winged helix family, characterized by a conserved DNA binding domain known as the Forkhead box (Fox) (Ronnebaum and Patterson, 2010), and play a essential role in cardiac development (Papanicolaou et al., 2008). It has been shown that three members of the FoxO subfamily, such as FoxO1, FoxO3, and FoxO4, are crucial for maintaining cardiac function and mediating cardiac stresses in the adult heart, and hypertrophic stimuli can inactivate FoxO proteins via the PI3K-Akt pathway (Ronnebaum and Patterson, 2010). In addition, over-expression of FoxO1 or FoxO3 decreases myocyte hypertrophy (Skurk et al., 2005; Ni et al., 2006), whereas FoxO3-deficient mice show mildly bigger hearts. Interestingly, the effect of FoxO appears to involve the calcineurin-NFAT pathway because FoxO over-expression can decrease calcineurin phosphatase activity, while calcineurin activity is reduced in FoxO3-deficient mice.
1.5 Study Rationale and Experimental Design

The studies presented in this thesis were designed to investigate the role of Irx5 in normal and diseased hearts. Previously, we have demonstrated that a member of the Iroquois (Irx) family of pre-patterning, homeodomain transcription factors, Irx5, is expressed in a gradient across the mouse ventricular wall (endocardial > epicardial) and creates an inverse transient outward K\(^+\) current (\(I_{\text{to,f}}\)) gradient by negatively regulating Kv4.2 gene expression across the ventricular wall (Costantini et al., 2005). Previous studies have also demonstrated that Irx5 may act as a major contributor to the electrical heterogeneity of repolarization in rodents, as well as potentially in large mammals and humans (Costantini et al., 2005; Rosati et al., 2006; He et al., 2009; Gaborit et al., 2010). The fast transient outward K\(^+\) currents (\(I_{\text{to,f}}\)) are major determinants of excitation-contraction coupling efficiency, suggesting that \(I_{\text{to,f}}\) gradients are critical factors in co-ordinating and orchestrating the sequence of contraction of the heart (Sah et al., 2003).

Importantly, \(I_{\text{to,f}}\) levels rise during development, particularly at birth, and are invariably reduced in heart disease and cardiac hypertrophy, conditions associated with altered contractility, electrical heterogeneity and lethal arrhythmias (Wickenden et al., 1997; Oudit et al., 2001; Kaprielian et al., 2002; Sah et al., 2003). In addition, changes in \(I_{\text{to,f}}\) can modulate hypertrophic responses in myocyte cultures, as well as in adult hearts (Kassiri et al., 2002; Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010). In light of these observations, we sought to explore the role of Irx5 in modulating the physiological and pathological properties of the heart by regulating \(I_{\text{to,f}}\). Since \(I_{\text{to,f}}\) is elevated in the mouse heart lacking Irx5 (Costantini et al., 2005), I specifically hypothesized that loss of Irx5 will lead to a reduction in contractility and a decreased hypertrophic response to pressure-overload via elevated \(I_{\text{to,f}}\) (Figure 1.12).

Patch-clamp experiments, Ca\(^{2+}\) transient and single contractility measurements, the Langendorff-perfusion system, and in vivo hemodynamic measurements were used to examine the role of Irx5 on excitation-contraction coupling and cardiac contractility. These studies revealed that loss of Irx5 in mice resulted in reduced cardiac contractility, consistent with abbreviated APD in the endocardium resulting from increased \(I_{\text{to,f}}\). These effects of Irx5 were abolished when \(I_{\text{to,f}}\) was absent as a consequence of Kv4.2 ablation, establishing that the effects of Irx5 on cardiac contractility are \(I_{\text{to,f}}\)-dependent. On the other hand, the hypertrophic response was severely attenuated in hearts subjected to pressure-overload and cultured isolated cardiomyocytes lacking Irx5, and restored by Irx5 over-expression. However, the Irx5-mediated
hypertrophic response was independent of $I_{\alpha,f}$. Interestingly, previous studies have shown that mice lacking the cardiac hypertrophic transcriptional factor $Nfatc3$ exhibit very similar phenotype to $Irx5^{-/-}$ mice with the disruption of $I_{\alpha,f}$ heterogeneity and attenuated hypertrophy (Wilkins et al., 2002; Costantini et al., 2005; Rossow et al., 2006). Therefore, it was hypothesized again that $Irx5$ modulates calcineurin-Nfat signaling, possibly via regulation of $Nfatc3$ expression or its association with $Nfatc3$ in a transcriptional complex (Figure 3.22). This possibility was tested using quantitative PCR, Western blots and co-immunoprecipitation, revealing that $Irx5$ indeed positively regulates $Nfatc3$ (and Gata4) while physically interacting with $Nfatc3$ (and Gata4). Therefore, these studies demonstrated that $Irx5$ coordinates cardiac contractility dependent of $I_{\alpha}$, while regulating hypertrophy in a Calcineurin-NFAT-dependent, but $I_{\alpha}$-independent manner.
Figure 1.12

Hypothesis
It was originally hypothesized that Irx5 would modulate cardiac contractility and hypertrophy via $I_{to,f}$ regulation. Thus, loss of Irx5 would lead to reduction in contractility and decreased hypertrophic response to pressure-overload via elevated $I_{to,f}$. 

Irx5 $\rightarrow$ $I_{to,f}$

Contractility
Hypertrophy
CHAPTER 2

METHODS
2.1 Mice

2.1.1 Transgenic Mice

All experimental protocols conformed to the standards of the Canadian Council on Animal Care. Irx5 knock-out mice (Irx5<sup>−/−</sup>), K<sub>v</sub>4.2 knock-out mice (K<sub>v</sub>4.2<sup>−/−</sup>), and cardiac-specific constitutively active calcineurin over-expression mice (CnA-TG) were described previously (Molkentin <i>et al.</i>, 1998; Cheng <i>et al.</i>, 2005; Guo <i>et al.</i>, 2005). K<sub>v</sub>4.2<sup>−/−</sup> and CnA-TG mice were gifts from Dr. Jeanne Nerbonne (Washington University School of Medicine) and Dr. Jeffrey D. Molkentin (University of Cincinnati), respectively. To generate Irx5-K<sub>v</sub>4.2 double knock-out (Irx5<sup>−/−;K<sub>v</sub>4.2<sup>−/−</sup></sup>) mice, Irx5<sup>−/−</sup> mice were crossed with K<sub>v</sub>4.2<sup>−/−</sup> mice. The progeny of this cross were interbred for 5 to 7 generations to establish mice homozygous for both transgenes. For Irx5-CnA double transgenic mice, CnA-TG mice were backcrossed twice with CD1 mice, and then interbred with Irx5 mice. To ensure proper comparison with identical genetic background, littermate controls were analyzed when crossed with CnA-TG mice.

2.1.2 Genotyping of Transgenic Mice

Genotype analysis was carried out to verify the presence of the transgenes in several mouse lines. Genomic DNA was isolated from mouse tails by digestion with 300 µL of 0.2N NaOH for 1 – 1.5 hours at 95 – 100°C, followed by neutralization with 100 µL of 0.5M Tris-HCl (pH 8.0). PCR was conducted with specific primers, illustrated in the Table 2.1. PCR products were separated on a 1% agarose gel in TAE (Tris/Acetate/EDTA) buffer.

2.2 Preparation of Isolated Myocytes

2.2.1 Adult Mouse Ventricular Myocytes

Left ventricular (LV) myocytes were obtained from 10 – 12 week old adult male Irx5 mice using an isolation procedure described previously (Costantini <i>et al.</i>, 2005). Mice were anesthetised with 2.5% isoflurane, and hearts were rapidly removed and retrogradely perfused with Ca<sup>2+</sup>-free Tyrode’s solution [(mmol/L) 137 NaCl, 5.4 KCl, 1.0 MgCl<sub>2</sub>, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, 10 D-glucose, 10 HEPES, pH 7.4] at 37°C through the aorta for 3 – 4 min. After perfusing the heart with collagenase (1.0 mg/mL, Worthington) for 10 – 12 min, endo- and epicardial layers were dissected from the LV free wall followed by gentle trituration to dissociate cardiomyocytes.
<table>
<thead>
<tr>
<th>Mice</th>
<th>Target</th>
<th>Primer Sequence (5’-3’)</th>
<th>Band Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irx5 mice</td>
<td>WT:Irx5a+Irx5b KO:Irx5a+Irx5c</td>
<td>Irx5a: GCCACCCAAAAGACTGAAACC Irx5b: TAAACCTATCTTTGGAATCC Irx5c: ACCGCTCCTCGTCTTTAC</td>
<td>WT: 600 KO: 800</td>
</tr>
<tr>
<td>Kᵥ4.2 mice</td>
<td>KV42EX1FOR:KV42EX1REV:</td>
<td>KV42EX1FOR: GTGGATGCTTGTGCTTC KV42EX1REV: CCCACAAGGCAGTTTTATA</td>
<td>WT: 613</td>
</tr>
<tr>
<td></td>
<td>Neo-specific</td>
<td>NEO Fwd: AAGATCTCCTGGCTACCTCGCCTTGCTTGNEO Rev: AAGACTCGTCAAGAGGCAGTTTTATA</td>
<td>KO: 500</td>
</tr>
<tr>
<td>Calcineurin</td>
<td>Human growth hormone</td>
<td>CNA-Fwd: GTCTGACTAGGTGCTCTTCT CNA-Rev: CGTCTCCTGCTGTTATAG</td>
<td>TG: 300</td>
</tr>
<tr>
<td>mice</td>
<td>sequence</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Cells were stored in Krebs-Hensleit-bicarbonate (KB) solution [(mmol/L) 120 potassium glutamate, 20 KCl, 20 HEPES, 1.0 MgCl\textsubscript{2}, 10 D-glucose, 0.5 K-EGTA, and 0.1% bovine serum albumin, pH 7.4] until use.

2.2.2 Neonatal Mouse Ventricular Myocytes

Neonatal mouse ventricular cardiomyocytes (NMVM) were isolated by a modification of the previous methods used to culture neonatal rat cardiomyocytes (Deng et al., 2000; Kassiri et al., 2002) and neonatal mouse cardiomyocytes (Kim et al., 2008a). Hearts were removed from 1 day old neonatal Irx5 mice. Using aseptic technique, the atria and blood vessels were removed, and ventricles were cut into 2 - 4 pieces. Ventricular tissue was washed repeatedly in ice-cold calcium- and bicarbonate-free Hanks with HEPES buffer (CBFHH) [(mmol/L) 137 NaCl, 5.36 KCl, 0.81 MgSO\textsubscript{4} · 7H\textsubscript{2}O, 0.44 KH\textsubscript{2}PO\textsubscript{4} · 7H\textsubscript{2}O, 0.34 NaH\textsubscript{2}PO\textsubscript{4} · 4H\textsubscript{2}O, 20 HEPES, and 5.6 dextrose, pH 7.4] and predigested overnight in 0.5mg/mL trypsin in CBFHH at 4°C with constant rocking. Twelve to sixteen hours later, cardiomyocytes were dissociated at 37°C by gentle stirring (about 40 - 60 rpm) in 4 ml of digestion media containing 50 U/mL of collagenase type II (Worthington Biochemicals, Lakewood, NJ), 0.2 mg/ml trypsin (Invitrogen), and 0.1% Gentamycin (50 µg/mL, Sigma) in bicarbonate-free Hanks with HEPES buffer (BFHH). Dispersed cells were collected every 6 – 12 minutes into conical polycarbonate tubes containing ice cold 10% fetal bovine serum (FBS) to stop the digestion. Dissociated cell suspensions were centrifuged at 1000 rpm at 4°C for 5 minutes, and resuspended in serum media (SM) containing DMEM:HAM F-12 (1:1, v/v), 10% FBS and 100 units/mL penicillin/streptomycin (P/S). Non-myocyte contamination with fibroblast and endothelial cells were prohibited by differential plating for 1 hour onto 100 mm Primaria\textsuperscript{®} culture dishes (25382-1701, BD Biosciences). Purity of NMVM has been confirmed previously by immunofluorescence microscopy using anti-cardiac α-actinin antibody (A7811, SIGMA), which showed that 93% of isolated cells are cardiomyocytes (Kim et al., 2008a). Myocytes were counted using trypan blue exclusion (0.4%, T8154, Sigma) on a hematocytometer (Z35, 962-9, Sigma), plated at a density of 2.5 x 10\textsuperscript{5} cells/mL and cultured at 37 °C in a humidified atmosphere of 5% CO\textsubscript{2}. 0.1 mM bromodeoxyuridine (BrdU, B9285, Sigma) and 20 µM arabinosylcytosine (Ara-C, #C1768, Sigma) were added to the culture medium to inhibit non-myocyte proliferation (Deng et al., 2000). After culturing in 10% SM for 24 hours, cells were infected with adenovirus (see below), then medium was replaced by serum-free medium (SFM) minimally supplemented with 1%
insulin-transferrin-selenium Supplements-X (ITS-X, Invitrogen), which contains Insulin 1.0 g/L, Transferrin 0.55 g/L, and Sodium Selenite 0.67mg/L. After culturing in SFM for 24 hours, all experiments were conducted at day 2 as follows:

- To investigate the cellular hypertrophy response of Irx5 NMVM, cells were stimulated by 20 µM of norepinephrine (NE) for 48 hours as shown previously (Tsoporis et al., 1998; Deng et al., 2000; Kaludercic et al., 2010). Cell images (10 – 20 per dish) were randomly taken for hypertrophy assessments using a phase-contrast light microscope equipped with a CoolSNAP digital camera (Roper Scientific), followed by harvesting cells for quantitative PCR or protein experiments.

- For co-immunoprecipitations, and mRNA & protein expression measurements, NMVM infected with Ad-GFP or Ad-Irx5 were cultured in 10% SM for 48 hours, and harvested for experiments.

- To assess cell viability of Irx5 NMVM under biological stress, NMVM were exposed to various concentrations of hydrogen peroxide (H₂O₂) for 7 hours at day 2. Cell survival rate was examined with MTT assay (see below).

2.3 Recombinant Adenoviral Construct Generation and Infection

2.3.1 Adenoviral Construct Generation

The adenoviral Irx5 construct (Ad-Irx5) was generated using the Adeno-X™ ViraTrak™ Expression System 2 (Clontech). A mouse cDNA encoding the entire Irx5 coding sequence was sub-cloned into the Creator Donor vector pDNR-CMV. The Irx5 sequence between two loxP sites of the pDNR-CMV vector was transferred into the single loxP site in the adenoviral genome of the Adeno-X ViraTrak Acceptor vector by Cre-mediated recombination. After selecting recombinants with chloramphenicol, the recombinant adenoviral genome was released by enzyme digestion with PacI to produce infectious recombinant adenovirus, and transfected into low passage HEK-293 cells. Two days later, transfection efficiency was visually confirmed under a fluorescent microscope. Seven to ten days later, recombinant adenovirus was harvested.
and stored at -80°C after the titer was determined. Adenovirus expressing constitutively active calcineurin (Ad-CnA) was described previously (Gong et al., 2006). Adenoviral construct generation was performed in collaborations with Dongling Zhao and Vijitha Puviindran.

2.3.2 Adenovirus Infection

For adenovirus-mediated gene delivery, NMVM were infected with Ad-Irx5 (5), Ad-GFP (5), and/or Ad-CnA (40) at an optimized multiplicity of infection (MOI). After infection for 3 – 4 hours in SFM without antibiotics, cells were washed three times and maintained in SFM until day 2. Infection efficiency, confirmed under a fluorescent microscope, was typically more than 98% with minimal cell death with these levels of MOI.

For cellular hypertrophy assessments, adenovirus-infected NMVM were fixed and permeabilized in 4% para-formaldehyde with 0.2% Triton X-100 in phosphate-buffered saline (PBS) at room temperature, and then stained with rhodamine phalloidin for 1 hour. Fluorescent images were captured from 12 – 14 random fields at different wave lengths (GFP: 480/520 nm; rhodamine phalloidin : 543/585 nm; excitation/emission) and superimposed. Myocyte surface area was measured using the Image-Pro® Plus (Media Cybernetics).

2.4 Cellular and Electrophysiological Assessments

2.4.1 Patch-Clamp Experiments

Voltage-activated K⁺ currents from isolated adult mouse ventricular myocytes were recorded using the whole-cell patch clamp technique in voltage-clamp mode, using an Axopatch 200B amplifier and pClamp 9 software (Axon Instrument, CA, USA). Cardiomyocytes which were Ca²⁺ tolerant and rod-shaped were selected and superfused with 1mM Ca²⁺ Tyrode solution for 15 minutes before electrophysiological recording at room temperature (21 – 24°C). The bath solution contained (mmol/l): 140 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 D-glucose, and 0.3 CdCl₂ (pH 7.4). The pipette resistance ranged between 1.2 – 2.0 MΩ when filled with a pipette solution containing (mmol/l): 120 potassium aspartate, 20 KCl, 5 NaCl, 1 MgCl₂, 5 MgATP, 10 HEPES, and 10 EGTA (pH 7.2). After membrane rupture, the cell capacitance was measured by integrating the capacitance transient in response to 10 mV steps from a holding potential of -50 mV and used to normalize current magnitudes. Cell capacitance and series
resistance were then electronically compensated by 85%. Voltage-gated K\(^+\) currents were measured by 25 second depolarizations from a holding potential of -80 mV to +60 mV, the reversal potential of Na\(^+\). The electrophysiological recording data were analyzed using pClamp software (Clampfit 10.0, Axon, CA, USA) and Prism 5 (GraphPad Software, San Diego, CA, USA), as fully described in Appendix A. In brief, the decay phase of outward K\(^+\) currents was fitted to a sum of tri-exponentials, and separated into fast (I\(_{o}\)), intermediate (I\(_{K,slow1}\)) and slow (I\(_{K,slow2}\)) components. Action potentials are recorded with the whole-cell patch clamp technique under current-clamp mode. APs were evoked by a brief (4 ms) injection of depolarizing current (1 – 4 nA). Patch-clamp experiments were performed in collaboration with Dr. Jie Liu.

2.4.2 \([\text{Ca}^{2+}]\)\(_i\) Transient Measurement

Cardiomyocytes in KB solution were loaded with the fluorescent Ca\(^{2+}\) indicator, Indo-1 by incubating with Indo-1/AM (Invitrogen) with Pluronic F-127 for 3-5 minutes at room temperature. Cells were plated in the chamber, and then perfused with 1 mM Ca\(^{2+}\) Tyrode solution for at least 20 minutes. The Ca\(^{2+}\) Tyrode solution contained (mmol/l): 130 NaCl, 5.4 KCl, 0.4 NaH\(_2\)PO\(_4\), 0.5 MgCl\(_2\)·6H\(_2\)O, 1.0 CaCl\(_2\), 25 HEPES, and 22 D-glucose (pH 7.4). Cardiomyocytes were stimulated by square-pulse (1 Hz, 4 ms duration, and amplitude of 1.5\times threshold). Following excitation at 360 nm, fluorescence emissions at 410 and 485 nm from a single beating myocyte were acquired using Felix software (Photon Technology International, ON, Canada). The Indo-1/AM emission ratio of 405/485 nm was calculated after subtracting the background autofluorescence. \([\text{Ca}^{2+}]\) transients in this study are expressed as the fluorescence ratio rather than as absolute Ca\(^{2+}\) concentration.

2.4.3 Single Cell Contractility Measurement

Cardiomyocytes were plated and superfused with 1 mM Ca\(^{2+}\) Tyrode solution for 20 minutes before recording at room temperature (21 – 24\(^\circ\)C). Cardiomyocytes, which were Ca\(^{2+}\) tolerant and rod-shaped with diastolic length > 1.70 \(\mu\)m, were selected and stimulated with a square-pulse (1 Hz, 4 ms duration, and amplitude of 1.5\times threshold). Single cell contractility was measured using the high-speed video sarcomere length system (HVSL; Aurora Scientific Inc., ON, Canada). Cell shortening (\%) was calculated by the equation: (diastolic sarcomere length – systolic sarcomere length) / diastolic sarcomere length.
2.5 *In vivo* Biomechanical Stress.

To investigate the role of Irx5 and Ito in the progression of heart disease, 8 week old male mice were subjected to pressure-overload by transverse aortic banding (BAND). Mice were anesthetised with 2% isoflurane and placed in the supine position, and then endotracheal intubation was performed using a plastic needle. Then the tube was connected to a volume-cycled rodent ventilator (Harvard Apparatus) with a tidal volume of 0.2 – 0.3 ml and respiratory rate of 135 breathes/min. The chest cavity was entered via a left medial thoracotomy incision, and the transverse aorta between the right (proximal) and left (distal) carotid arteries was isolated. A 7.0 silk suture was placed around the transverse aorta and tied tight around both the aorta and a 26-gauge needle. The needle was removed promptly to yield consistent aortic constriction. Sham-operated animals (SHAM) underwent the identical procedure with the exception of aortic constriction. After the chest was closed, mice were allowed to recover from anaesthesia on the ventilator and heating pads. Transverse aortic banding was partly performed in collaboration with Dr. M. Golam Kabir.

2.6 Cardiovascular Functional Assessments

2.6.1 Langendorff-Perfused Heart

Irx5 male mice at 10 – 12 weeks of age were used to compare cardiac contractility function as previously described (Ban et al., 2010). Following injection with heparin (1000 IU/kg) to prevent the blood from clotting, the mice were anesthetized with sodium pentobarbital (200 mg/kg). The heart was rapidly excised and placed in warm Krebs-Hensleit solution [(mmol/L) 118 NaCl, 4.7 KCl, 11 glucose, 1.2 MgSO₄, 25 NaHCO₃, and 1.2 KH₂PO₄, pH 7.4] for a few seconds to empty the remaining blood from the heart, and then was transferred to a dissecting dish containing ice-cold Krebs-Hensleit solution which immediately stopped heart contractions and reduced energy expenditure. The aorta was cannulated with a grooved 20-gauge stainless steel blunt needle and perfused immediately at a constant perfusion pressure (CPP) of 80 mmHg. The heart was surrounded by a water jacket maintaining the ambient air surrounding the heart at 37°C. The temperature of Krebs-Hensleit solution was maintained at 37°C by a thermo-circulating system (Harvard apparatus). After allowing 3 min perfusion, excess tissue was removed and a small plastic balloon connected to the pressure transducer was inserted into
left ventricle (LV) via the left atrium through a left atrial incision. The balloon, hand-made by
securing some plastic Saran Wrap™ around a piece of plastic tubing, was tested before insertion
to ensure no water leakage or bubbles inside the tubing. LV end diastolic pressure (LVEDP) was
measured while inflating the balloon in LV, and maintained between 4 – 8 mmHg throughout the
equilibrium phase of the experimental protocol. Cardiac performance parameters such as heart
rate (HR), LV end systolic pressure (LVESP), and LVEDP were acquired using Acqknowledge
software (Biopac System, Goleta, CA). LV developed pressure (LVDP), an index of cardiac
pump function, was calculated by subtracting LVEDP from LVESP. Only data derived from
hearts demonstrating intrinsic heart rates greater than 350 beats/min were used in the study. The
Krebs-Hensleit solution was freshly prepared from a 10× stock solution and filtered through a
0.45 μm membrane filtration system. After adding 2.5 mM of CaCl₂, the solution was
oxygenated with 95% O₂ and 5% CO₂. The solution was bubbled throughout the experiments to
maintain a specific level of oxygen and pH 7.4. Langendorff-perfused heart experiments were
performed in collaboration with Dr. Kiwon Ban.

2.6.2 Echocardiography

Two-dimensional guided echocardiography was used to non-invasively assess left
ventricular (LV) function of animal models using the Vevo 770 system equipped with a 30 MHz
micro visualization scanhead (VisualSonics, ON, Canada), as well as an Acuson Sequoia C256
system equipped with a 15 MHz linear transducer (15L8) (Version 4.0, Acuson Corp). Mice
were anesthetized with 1 – 1.5% isoflurane while body temperature, measured by a rectal
temperature probe, was monitored and maintained at 36.8 ± 0.3°C using a heating pad and a heat
lamp throughout the entire procedure. Motion (M)-mode were recorded in both short- and
parasternal long-axis views while the cursor was positioned perpendicular to the interventricular
septum and posterior LV wall at the level of the papillary muscles. LV end-systolic dimension
(LVESD) and end-diastolic dimension (LVEDD), and diastolic posterior LV wall thickness
(PWT D) were measured. Left ventricular fractional shortening (FS, %) in the long axis view was
calculated as FS = (LVEDD - LVESD) / LVEDD × 100.

2.6.3 Invasive Hemodynamic Measurements

Invasive hemodynamic assessments were performed using 1- or 1.4-French scale pressure
transducer catheters from Millar Instruments and Scisense. The right carotid artery was isolated
and cannulated with the catheter which was then advanced into the left ventricle through the aortic valve. During recording, mice were anesthetized with 1 – 1.2% isofluorane and body temperature was maintained at 36.8 ± 0.3°C. Arterial and left ventricular pressures as well as rates of LV pressure change (±dP/dt) were acquired and analyzed using Acqknowledge data acquisition and analysis software. Invasive hemodynamic measurements were partly performed in collaboration with Dr. M. Golam Kabir.

2.6.4 Other Physiological Assessments

After functional assessments, mice were sacrificed. The hearts were first harvested, rinsed in PBS, blotted dry, weighed, and snap-frozen with liquid nitrogen for molecular experiments or fixed in 10% phosphate-buffered formalin for histological experiments. Other organs, such as lung, liver, and kidney, also were collected and weighted. Finally, tibia length was measured for heart weight normalization as tibia length remains constant after maturity, compared to the fluctuating body weight in heart disease models (Yin et al., 1982).

After surgery, animals were followed, and survival curves were analyzed by the Kaplan-Meier method, considering only heart disease mortality. Mortality within 2 days of the surgery was not counted, as it was considered to be a surgery-related complication.

2.6.5 Tail-cuff Plethysmography

Blood pressure (BP) was measured non-invasively using tail-cuff plethysmography (Kent Scientific). During BP measurements, mice were under light anaesthesia with 1 – 1.2% isofluorane while body temperature was maintained at 36.8 ± 0.3°C using a heating pad. The pressure gradient after transverse aortic banding was calculated as arterial systolic pressure in the right carotid artery – systemic blood pressure.

2.7 Histological Assessments

Histology and staining were conducted at the Center for Modeling Human Disease (CMHD) in the Toronto Centre for Phenogenomics and analyzed for hypertrophy and fibrosis. In brief, hearts were harvested, rinsed in PBS, blotted dry, weighed, fixed in 10% phosphate buffered formalin and embedded in paraffin. Tissues were sectioned into 5 µm thick slices,
followed by staining with hematoxylin and eosin (H&E) for gross morphology, and Masson’s trichrome for fibrosis. Images were taken using the ScanScope XT (Aperio Technologies) slide scanner. To measure myocyte cross-sectional area, images were taken where nuclei were in the center of the myocytes. 7 images containing a minimum of 500 myocytes from 3 different animals were quantified for each experimental group using Image-Pro® Plus (Media Cybernetics).

To examine interstitial fibrosis (fibrosis in interstitial spaces), Masson’s trichrome staining was quantified using the Photoshop software (Adobe), as described previously (Baba et al., 1999; Dahab et al., 2004). Green pixels were expressed as a percentage of the entire pixels of an image, giving the interstitial collagen fraction. A total of 5 – 6 images covering the entire heart section was analyzed in each of at least 3 mice per experimental group. Perivascular fibrosis (fibrosis around intramyocardial arteries) on cross sections of vessels was calculated as the ratio of fibrosis area to vessel lumen area, as described previously (Baba et al., 1999). The fibrosis area around the vessel and the cross-sectional area of the corresponding vessel were measured using Image-Pro® Plus.

2.8 Gene Expression Assays

2.8.1 RNA Isolation and cDNA Synthesis

Total RNA was isolated with Trizol (Invitrogen) following a protocol provided by the manufacturer. In brief, dissected heart tissues or NMVM were homogenized in 1 mL of Trizol, followed by incubation at room temperature for 5 minutes to completely dissociate nucleoprotein complexes. After adding 200 µL of chloroform, tubes were vigorously shaken and centrifuged at 12,000g for 15 minutes at 4°C. The colorless upper aqueous phase was collected into new tube, and then 0.5mL of isopropyl alcohol was added to precipitate RNA. Following incubation at 15 to 30°C for 10 minutes, a gel-like RNA pellet was formed by centrifugation at 12,000g at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol, and then centrifuged at 7,500g for 5 minutes at 4°C. After air-drying the pellet for 10 minutes, the RNA was dissolved in RNase-free water with incubation for 10 minutes at 55°C. RNA was quantified using a spectrophotometer at A260. RNA with an A260/280 ratio > 1.8 was used for cDNA synthesis. cDNA was synthesized from 1.5 µg of RNA using Superscript II reverse transcriptase (Invitrogen) as described in the manufacturer’s protocol. cDNA was diluted 20× for quantitative PCR reaction.
Table 2.2
List of Oligonucleotide Primers for Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Protein (Gene)</th>
<th>Gene Bank Accession #</th>
<th>Primer Sequence (5'-3')</th>
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<td>Irx5</td>
<td>NM_018826</td>
<td>TaqMan® Probe Assay ID: Mm00502107ml</td>
</tr>
<tr>
<td>ANF (Nppa)</td>
<td>Fwd: GGAGGAGAAGATGCCGGTAGA (700nM) Rev: GCTTCCTCAGTCTGCTCACTCA (700nM)</td>
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<tr>
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<tr>
<td>α-Sk-Actin (Acta1)</td>
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<tr>
<td>Kv4.2 (Kcnd2)</td>
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<tr>
<td>Rcan1, ex-4/5 (Rcan1.4)</td>
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<tr>
<td>Nfatc3</td>
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<tr>
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<td>TaqMan® Rodent GAPDH Control Reagents</td>
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<tr>
<td>18s rRNA</td>
<td>NM_00804</td>
<td>TaqMan® Ribosomal RNA Control Reagents</td>
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</tbody>
</table>
2.8.2 Quantitative Real-Time RT-PCR

Gene expression assay was conducted on 10 ng of template cDNA by Quantitative PCR (qPCR) using Taqman and SYBR green PCR methods equipped with ABI 7900HT (Applied Biosystems). Primers were designed using Primer Express® software and Primer-BLAST on the National Center for Biotechnology Information (NCBI) website, followed by confirmation with nucleotide BLAST. Other Primer sequences were obtained from previously published articles. Primers for SYBR green based PCR, optimized at different primer concentrations and dissociation curves, and validated by the PCR efficiencies near 100%, were used for experiments. Primer sequences and information are listed in Table 2.2. PCR results described as threshold cycle value (C\text{T}) were compared using relative quantitation of gene expression with Comparative C\text{T} Method (ΔΔC\text{T} Method). The amount of target, normalized to an endogenous reference (GAPDH) and relative to a control group, is given by: \(2^{-\Delta\Delta C_{T}}\).

2.9 Co-Immunoprecipitation and Immunoblots

2.9.1 Co-Immunoprecipitation

Co-immunoprecipitation and immunoblotting were conducted as described previously (Cheung et al., 2009). NVMV infected with Ad-Irx5 or Ad-GFP were used to examine Irx5-Nfatc3 interaction. To examine Irx5-Gata4 interaction, COS-7 cells at 90% confluence in 10 cm plates were transfected with FLAG-Irx5 and Gata4 constructs using Lipofectamine 2000 (Invitrogen). NMVM and COS-7 cells were harvested in lysis buffer (200 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, and pH 7.5) containing protease inhibitors for 30 minutes on ice. Lysates were classified by spinning for 10 minutes at 4°C, and supernatants were incubated with the indicated primary antibodies (0.5 – 2 ug/ml) overnight at 4°C. Lysates were spun for 10 minutes at 4°C, and the supernatants were incubated with Protein G–Sepharose beads to collect immunoprecipitates. Immune complexes were washed 3-5 times with 1 ml lysis buffer, and then immunoprecipitated proteins were subjected to immunoblotting analysis (described below). Co-immunoprecipitation experiments were performed in collaboration with Vijitha Puvindran in the laboratory of Dr. Chi-Chung Hui.
2.9.2 Immunoblots

Immunoblotting was performed using standard protocols by overnight incubation at 4°C with the following primary antibodies: Nfatc3 (Santa Cruz Biotechnology), Gata4 (Santa Cruz Biotechnology), and β-actin (Oncogene). Cultured NMVM were harvested in ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 20 mM EDTA, 100 mM NaCl, 1% Triton X-100, a protease inhibitor cocktail (Roche). Cell lysates or immunoprecipitated proteins were resuspended in 40 µL of 2X SDS loading buffer (250 mM Tris pH 6.8, 20% glycerol, 2% SDS, 200 mM β-mercaptoethanol, bromophenol blue to color), boiled for 5 minutes, and resolved electrophoretically by 6-10% SDS-PAGE. Proteins on gel were transferred to Polyvinylidene difluoride (PVDF) membranes (Roche), and were blocked with 5% BSA or 5% skim milk at room temperature for 1 hour. After incubating with the primary antibodies at 4°C overnight, the membranes were blotted with horseradish peroxide (HRP)-conjugated secondary antibodies (Amersham Biochem) for 1 hour at room temperature. Immunoreactive signals were visualized by enhanced chemiluminescence (ECL, Amersham Biochem). β-actin or a nonspecific cross-reacting band was used as loading control as previously described (Helfer and Gladfelter, 2006).

2.10 MTT Assay (Cell Survival Measurements)

Cell viability of Irx5-/- NMVM in response to oxidative stress using hydrogen peroxide (H₂O₂) was assessed by the MTT assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazodium Bromide) is cleaved by active mitochondria in living cells into formazan, the amount of which is directly proportional to the number of living cells. Cells were washed two times with PBS followed by a 4 hour incubation at 37°C with 1 mg/ml of MTT in a reaction volume of 100 µL. After removing the MTT solution, DMSO was added to dissolve the formazan crystals, and the plate was shaken at 55°C for 5 min in order to completely dissolve the formazan crystals. Dye absorbance in viable cells was measured at 595 nm with 630 nm as the reference wavelength. All experiments were repeated at least 3 times, and each condition tested was performed in quadruplicate for each experiment.
2.11 Plasma Catecholamine Assay

Plasma concentrations of epinephrine, norepinephrine, and dopamine were determined by Dr. Jeff Tong of the Toronto Medical Laboratories (TML, University Health Network, Toronto, ON, Canada) using high-performance liquid chromatography (HPLC) with electrochemical detection, as previously described (Smith and Betteridge, 1984; Weir et al., 1986). Mouse blood was collected from the right carotid artery into a syringe containing sodium citrate and kept on ice. Nine parts of blood were anti-coagulated with one part of 3.8% trisodium citrate (Vacutainer blue-top, Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were immediately centrifuged at 12,000g for 20 min at 4°C, and the plasma was separated and stored at -80°C until measurements. Plasma catecholamine assay was performed in collaboration with Peter C. Papageorgiou in Drs. John Floras and Peter H. Backx Labs.

2.12 Statistical Analysis

All results are expressed as mean ± S.E.M. (the standard error of the mean). Significance and difference of multiple groups was determined by Student’s t-test, and one-, two-, or three-way ANOVA with post-hoc analysis using Student-Newman-Keuls test. Differences at $P < 0.05$ were considered statistically significant. Calculations and statistical test were performed using the Sigma Stat 3.0 program.
CHAPTER 3

RESULTS
3.1 Loss of *Irx5* Results in Disruption of the Regional Heterogeneity in Excitation-Contraction Coupling

For the heart to pump synchronously, precise timing of electrical activation of the myocardium is crucial, which is achieved by gradients of the action potential (AP) profile within the working ventricular myocardium (Antzelevitch *et al*., 1991). It has been well established that this heterogeneity of AP profile in rodent is strongly influenced by the transient outward K\(^+\) currents (I\(_{to,f}\)), which are major repolarizing currents in the early phase of AP (Sah *et al*., 2003). We previously found that Irx5 is responsible for the regional difference in I\(_{to}\) in mice by negatively regulating Kv4.2 gene expression, and the loss of *Irx5* leads to disruption of transmural gradients of I\(_{to,f}\) (Costantini *et al*., 2005). To examine whether loss of *Irx5* might affect the heterogeneity of excitation-contraction coupling via changes of I\(_{to,f}\), K\(^+\) currents were examined in cardiomyocytes isolated from sub-endocardium (ENDO) and sub-epicardium (EPI) of the left ventricular (LV) free wall. As shown in Table 3.1, the peak of total K\(^+\) current densities (I\(_{peak}\)) was higher (P < 0.05) in *Irx5\(^{+/+}\*) EPI myocytes compared with *Irx5\(^{+/+}\*) ENDO cells, consistent with previous results (Brunet *et al*., 2004; Costantini *et al*., 2005). Further dissection of K\(^+\) currents using 3-exponential fits (see Appendix A) revealed that transmural gradients of current density from EPI to ENDO (P < 0.05) were observed in all K\(^+\) current components, I\(_{o}\), I\(_{K,slow1}\) and I\(_{K,slow2}\). *Irx5\(^{-/-}\*) hearts also showed a regional difference (P < 0.05) in I\(_{peak}\) between ENDO and EPI, whereas I\(_{peak}\) in *Irx5\(^{-/-}\*) ENDO myocytes was higher (P < 0.05) compared with that in *Irx5\(^{+/+}\*) ENDO cells. Consistent with previous results (Costantini *et al*., 2005), the regional difference in I\(_{to,f}\) was eliminated by specific increases in *Irx5\(^{-/-}\*) ENDO myocytes compared to *Irx5\(^{+/+}\*) ENDO cells while I\(_{to,f}\) in *Irx5\(^{-/-}\*) ENDO myocytes was not different from *Irx5\(^{+/+}\*) EPI cells (Table 3.1). On the other hands, other K\(^+\) currents such as I\(_{K,slow1}\) and I\(_{K,slow2}\) were not affected by loss of *Irx5* with similar current densities (P > 0.3), suggesting that elevated I\(_{to,f}\) in *Irx5\(^{-/-}\*) ENDO myocytes accounted for an increase in I\(_{peak}\) in *Irx5\(^{-/-}\*) ENDO myocytes compared to *Irx5\(^{+/+}\*) ENDO cells.

Accordingly, the regional difference in action potential duration (APD) between ENDO and EPI, which was evident (P < 0.05) in *Irx5\(^{+/+}\*) mouse hearts, was completely disrupted (P > 0.7) in the *Irx5\(^{-/-}\*) mouse heart, consistent with loss of the I\(_{to,f}\) transmural gradient (Figure 3.1). Specifically, analysis of APDs revealed that APD at 20%, 50%, and 75% repolarization (APD20, APD50, and APD75) in *Irx5\(^{-/-}\*) ENDO were shortened (P < 0.05) compared to that in *Irx5\(^{+/+}\*)
ENDO. Conversely, APDs were indistinguishable ($P > 0.6$) between $I_{rx5}^{+/+}$ and $I_{rx5}^{-/-}$ EPI, which was consistent with the finding that changes of $I_{lo,f}$ were found only in $I_{rx5}^{-/-}$ ENDO myocytes.

Next it was determined whether the electrical gradients (e.g. AP and $I_{lo,f}$) within the working ventricular myocardium were associated with mechanical gradients via cardiac excitation-contraction coupling (ECC). As expected (Dilly et al., 2006; Kondo et al., 2006; Sengupta et al., 2006b), $I_{rx5}^{+/+}$ ENDO myocytes showed a higher ($P < 0.01$) amplitude of Ca$^{2+}$ transients (Figure 3.2) and better ($P < 0.01$) cellular contractile function (Figure 3.3), as measured by the percentage of cell shortening and the velocity of sarcomere length shortening ($dS/L/dt$), compared with $I_{rx5}^{+/+}$ EPI cells. Time to peak in Ca$^{2+}$ transients was also longer in $I_{rx5}^{+/+}$ ENDO cells compared to $I_{rx5}^{+/+}$ EPI cells (Figure 3.2C), consistent with previous studies (Fowler et al., 2005; Fu et al., 2006). On the other hand, no regional differences in Ca$^{2+}$ transient amplitude or cell shortening were detected in $I_{rx5}^{-/-}$ cardiomyocytes. Furthermore, direct comparisons between $I_{rx5}^{+/+}$ and $I_{rx5}^{-/-}$ myocytes revealed no differences in Ca$^{2+}$ transients and cell shortening for EPI cardiomyocytes, whereas ENDO myocytes from $I_{rx5}^{-/-}$ hearts had reduced ($P < 0.01$) contractility with decreased ($P < 0.01$) Ca$^{2+}$ transients, compared with $I_{rx5}^{+/+}$ ENDO cells. Selective contractility reductions in ENDO cardiomyocytes from $I_{rx5}^{-/-}$ hearts correlated with selective abbreviation of APD in this subset of myocytes, as a result of $I_{lo,f}$ elevations in this region. These results are consistent with previous studies establishing that APD is a powerful determinant of contractility in mice and other species (Bouchard et al., 1995; Volk et al., 1999; McIntosh et al., 2000; Kaprielian et al., 2002; Dilly et al., 2006; Kondo et al., 2006). In addition, these results suggest that Irx5 establishes transmural gradients of ECC by negatively regulating $I_{lo,f}$ in the heart.
Table 3.1
Summary of Voltage-gated Outward $K^+$ Current Densities (pA/pF) of Left Ventricular Sub-endocardial (ENDO) and Sub-epicardial (EPI) Cardiomyocytes in *Irx5*+/+ and *Irx5*−/− Mice

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>$I_{\text{peak}}$</th>
<th>$I_{\text{to}}$</th>
<th>$I_{\text{K,slow1}}$</th>
<th>$I_{\text{K,slow2}}$</th>
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<tr>
<td><em>Irx5</em>+/+ ENDO</td>
<td>8</td>
<td>28.5 ± 3.0*</td>
<td>7.9 ± 1.3*</td>
<td>8.9 ± 0.8*</td>
<td>11.6 ± 1.4*</td>
</tr>
<tr>
<td><em>Irx5</em>+/+ EPI</td>
<td>8</td>
<td>57.4 ± 8.4</td>
<td>22.5 ± 2.7</td>
<td>19.0 ± 5.0</td>
<td>16.0 ± 1.6</td>
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<tr>
<td><em>Irx5</em>−/− ENDO</td>
<td>24</td>
<td>42.0 ± 4.2**</td>
<td>20.2 ± 2.4</td>
<td>10.2 ± 1.8**</td>
<td>11.4 ± 1.0</td>
</tr>
<tr>
<td><em>Irx5</em>−/− EPI</td>
<td>20</td>
<td>63.0 ± 4.1</td>
<td>22.5 ± 2.5</td>
<td>21.1 ± 2.1</td>
<td>19.2 ± 1.6</td>
</tr>
</tbody>
</table>

ENDO, sub-endocardial myocytes; EPI, sub-epicardial myocytes. *, $P < 0.05$ vs. *Irx5*+/+ EPI; **, $P < 0.05$ vs. *Irx5*−/− EPI; #, $P < 0.05$ vs. *Irx5*−/− ENDO; N.S., not significant vs. *Irx5*−/− EPI.
Figure 3.1

Loss of Irx5 Abolishes Transmural Difference in Action Potential Duration in the Heart. (A) Representative action potential traces from Irx5+/+ and Irx5−/− cardiomyocytes isolated from sub-endocardium (ENDO) and sub-epicardium (EPI) of left ventricle. (B) Mean action potential duration (APD), measured at 25, 50, and 75% repolarization, demonstrated that Irx5+/+ ENDO has longer APD than Irx5+/+ EPI. (C) Irx5−/− ENDO has shortened action potential compared to Irx5+/+ ENDO. Consequently, the regional difference in APD shown in Irx5+/+ hearts was abolished in Irx5−/− hearts. n = 7 – 8 per group; *, P < 0.05 vs. Irx5+/+ ENDO.
Figure 3.2

Loss of Irx5 Leads to Disruption of Ca\textsuperscript{2+} Transient Heterogeneity in the Heart.

(A) Representative [Ca\textsuperscript{2+}]\textsubscript{i} transients, measured by the ratio of emitted fluorescence at 405 and 485 nm, in \textit{Irx5}\textsuperscript{+/+} and \textit{Irx5}\textsuperscript{-/-} cardiomyocytes isolated from sub-endocardium (ENDO) and sub-epicardium (EPI) of left ventricle. (B) \textit{Irx5}\textsuperscript{+/+} hearts exhibited a regional difference in the peak amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} transients (A405/485) with higher amplitude in \textit{Irx5}\textsuperscript{+/+} ENDO than \textit{Irx5}\textsuperscript{-/-} EPI. On the contrary, \textit{Irx5}\textsuperscript{-/-} hearts showed a loss of heterogeneity in Ca\textsuperscript{2+} transients since the amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} transients in \textit{Irx5}\textsuperscript{-/-} ENDO was lower, compared to \textit{Irx5}\textsuperscript{+/+} ENDO, while there was no difference in the amplitude in EPI between \textit{Irx5}\textsuperscript{+/+} and \textit{Irx5}\textsuperscript{-/-}. (C) \textit{Irx5}\textsuperscript{+/+} hearts also showed the regional difference in the time to peak of [Ca\textsuperscript{2+}]\textsubscript{i} transients between ENDO and EPI, which was abolished in \textit{Irx5}\textsuperscript{-/-} hearts. n = 12 – 14 per group; *, P < 0.01 vs. \textit{Irx5}\textsuperscript{+/+} ENDO; **, P < 0.01 vs. \textit{Irx5}\textsuperscript{+/+} ENDO.
Figure 3.3
Loss of Irx5 Results in Reduction in Cardiomyocyte Contractility.
(A) Representative single cell shortening measured in Irx5+/+ and Irx5−/− cardiomyocytes isolated from sub-endocardium (ENDO) and sub-epicardium (EPI) of left ventricle. (B) Irx5+/+ hearts exhibited a regional difference in the cell contractility with greater cell shortening in Irx5+/+ ENDO than Irx5+/+ EPI. By contrast, Irx5−/− hearts showed loss of heterogeneity in cell shortening by reduced cell contractility in Irx5−/− ENDO compared to Irx5+/+ ENDO. (C) These results were consistently found in ±dSL/dt, the velocity of sarcomere length (SL) shortening. n = 30 – 35 per group; *, P < 0.01 vs. Irx5+/+ ENDO; **, P < 0.01 vs. Irx5+/+ ENDO.
3.2 The Absence of Irx5 in the Heart Leads to Decreased Cardiac Contractility, But Not Heart Disease

Given that the gradients of electrical and mechanical properties in the heart are associated with cardiac contraction and relaxation (Sengupta et al., 2006b), it was determined whether selectively reduced contractility in Irx5−/− ENDO myocytes with loss of electrical and mechanical heterogeneity had consequences on global contractile function in Irx5−/− hearts. Although invasive hemodynamic measurements revealed no difference ($P = 0.886$) in left ventricular end-systolic pressure (LVESP) between 12 week old Irx5+/+ and Irx5−/− mice (Figure 3.4B), the rates of LV pressure change (±dp/dt) of the heart were lower ($P < 0.02$) in Irx5−/− mice than in Irx5+/+ mice (Figure 3.4C), suggesting that Irx5−/− heart had lower contractile function compared with Irx5+/+ hearts. To further examine overall pressure generation ability of the heart, independent of neural and humoral influences (Skrzypiec-Spring et al., 2007), Irx5 mouse hearts were studied using the Langendorff-perfused system. As shown in Figure 3.5, left ventricular developed pressure (LVDP) and ±dp/dt were lower ($P < 0.001$) in Irx5−/− hearts than in Irx5+/+ hearts, confirming that contractile function of Irx5−/− hearts was significantly attenuated. These results suggest that it is the I$_{to,f}$-mediated abbreviation of APD in the ENDO myocytes that is responsible for the reduced global cardiac contractility in Irx5−/− mice, and support the conclusion that Irx5 is necessary for maintaining cardiac contractility via regulating regional variations in I$_{to,f}$, APD and ECC. This conclusion is further supported later by restoration of contractility with loss of I$_{to,f}$ (see Section 3.6).

Compensatory hypertrophy is commonly observed in response to impaired cardiac function as typified by the hypertrophic cardiomyopathy seen with mutations of β-myosin heavy chain (β-MHC) (Arad et al., 2002) and titin (Peng et al., 2007) as well as other functional proteins involved in cardiac contraction such as Serca2a (Ver Heyen et al., 2001) and phospholamban (Zhao et al., 2006). Despite reduced cardiac function in Irx5−/− mice, however, no morphological evidence of heart disease was noted in 12 week old Irx5−/− hearts, compared to Irx5+/+ hearts (Figure 3.6A), consistent with a previous study (Costantini et al., 2005). In addition, heart weight normalized to body weight (HW/BW) was indistinguishable ($P = 0.641$) between Irx5+/+ and Irx5−/− mice (Figure 3.6B), indicating no compensatory hypertrophic response. Consistent with the absence of morphological changes, echocardiographic assessments revealed that LV end-diastolic dimension (LVEDD; $P = 0.592$) and LV posterior wall thickness
at diastole (PWTD; \( P = 0.274 \)) were indistinguishable between \( I_{rx5^{+/+}} \) and \( I_{rx5^{-/-}} \) hearts, except that fractional shortening (FS, \( \% \)) was lower \( (P < 0.01) \) with increased LV end-systolic dimensions (LVESD) in \( I_{rx5^{-/-}} \) mouse hearts than in \( I_{rx5^{+/+}} \) hearts (Table 3.2). These results indicate that Irx5 plays a regulating role in compensatory response of the heart and possibly might be associated with the development of heart disease. In addition, this lack of compensatory response in \( I_{rx5^{-/-}} \) hearts might be associated with elevated \( I_{to,f} \) since several previous studies have demonstrated that an increase in \( I_{to,f} \) can prevent hypertrophy induced by biomechanical stress (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010).
Figure 3.4

Absence of Irx5 in the Heart Leads to Decreased Cardiac Contractility.

(A) Representative left ventricular pressure traces and derivatives of Irx5<sup>+/+</sup> and Irx5<sup>-/-</sup> mice, measured by an invasive pressure catheter. (B & C) While left ventricular end-systolic pressure (LVESP) was indistinguishable between Irx5<sup>+/+</sup> and Irx5<sup>-/-</sup> mice, the rate of LV pressure change (LV dp/dt) was significantly reduced in Irx5<sup>-/-</sup> mice, compared with Irx5<sup>+/+</sup> mice. n = 5 – 6 per group; *, P < 0.02 vs. Irx5<sup>+/+</sup> mice.
Figure 3.5
Absence of Irx5 Leads to Reduction in Contractile Function of the Heart.

(A) Representative left ventricular pressure traces and derivatives of $Irx5^{+/+}$ and $Irx5^{-/-}$ mice measured by the Langendorff-perfused heart system. (B & C) $Irx5^{-/-}$ hearts showed reduced pressure generation ability, as indicated by lower left ventricular developed pressure (LVDP = LV systolic pressure – LV diastolic pressure) as well as LV dp/dt, compared with $Irx5^{+/+}$ hearts. n = 7 – 10 per group; *, P < 0.001 vs. $Irx5^{+/+}$ mice.
Figure 3.6
No Heart Disease in Irx5⁻/⁻ Mice Despite Lower Cardiac Contractile Function.

(A) Representative histological sections of the hearts from Irx5⁺/+ and Irx5⁻/⁻ mice revealed no evidence of heart disease. The scale bar indicates 800 µm. (B) Heart weight normalized to body weight (HW/BW) was indistinguishable between Irx5⁺/+ and Irx5⁻/⁻ hearts. n = 4 per group; NS, not significant.
Table 3.2
Echocardiographic Assessments of 12 week-old Irx5 Mice

<table>
<thead>
<tr>
<th></th>
<th>Irx5&lt;sup&gt;+/-&lt;/sup&gt; (n = 4)</th>
<th>Irx5&lt;sup&gt;-/-&lt;/sup&gt; (n = 4)</th>
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<tbody>
<tr>
<td>LVEDD (mm)</td>
<td>4.12 ± 0.02</td>
<td>4.18 ± 0.10</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.44 ± 0.03</td>
<td>2.91 ± 0.11*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>40.7 ± 1.1</td>
<td>30.3 ± 1.1*</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>0.61 ± 0.03</td>
<td>0.57 ± 0.01</td>
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</table>

LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; PWTD, LV posterior wall thickness at diastole. Values are presented as means ± S.E.M. *, P < 0.05 vs. Irx5<sup>+/-</sup>.
3.3 *Irx5*−/− Mice Develop Early Onset of Dilated Cardiomyopathy with Reduced Cardiac Contractile Function in Response to Biomechanical Stress

To further explore whether loss of *Irx5* could affect the compensatory response of the heart to biomechanical stress, *Irx5* mice were subjected to additional hemodynamic stress induced by transverse aortic banding (BAND) for 12 weeks, and their cardiac function was examined using echocardiography at 2, 8 and 12 weeks post-banding. As shown in Figure 3.7A, it was clear that BAND-*Irx5*−/− hearts developed early onset dilated cardiomyopathy. Specifically, BAND-*Irx5*−/− displayed significant left ventricular (LV) dilation with increases (P < 0.05) in LVEDD and LVESD with prolonged pressure-overload (over 8 weeks), while the progression of LV dilation was less severe in BAND-*Irx5*+/− hearts (Figure 3.7, B and C). Accordingly, while cardiac contractile function in BAND-*Irx5*−/−, as indicated by FS (%), was maintained (P = 0.370) until 2 week post-banding, it began severely impaired (P < 0.05) after 8 week post-banding (Figure 3.7D). On the contrary, BAND-*Irx5*+/− showed mild reduction (P < 0.05), but relatively well maintained pump function over time compared to BAND-*Irx5*−/−. *In vivo* hemodynamic measurements at 12 week post-banding also shown that +dp/dt was well maintained (P = 0.361) in BAND-*Irx5*+/− compared to sham-operated (SHAM) animals, whereas it was significantly reduced (P < 0.05) in BAND-*Irx5*−/− mice (Table 3.3). Interestingly, the degree of diastolic posterior LV wall (PWTD) thickening of *Irx5*−/− mice (~58%, P < 0.01) in response to pressure-overload was much attenuated (P < 0.01), compared to *Irx5*+/− mice (~27%, P < 0.01), implicating attenuated hypertrophy in BAND-*Irx5*−/− mice compared to BAND-*Irx5*+/− mice (Figure 3.7E). These results suggest that *Irx5* is necessary for compensatory contractile and possibly hypertrophic responses to pressure-overload, and its absence leads to accelerated decompensation with the early onset of ventricular dilation under biomechanical stress.
**Figure 3.7**

Absence of Irx5 Leads to Heart Dilation with Reduced Hypertrophic Response to Pressure-overload.

Left ventricular remodeling after transverse aortic banding (BAND) was assessed by echocardiography. (A) Representative M-mode figures of Irx5+/+ and Irx5−/− hearts measured at 2, 8 and 12 week post-banding. (B & C) LV end-diastolic and systolic dimensions (LVEDD and LVESD). (D) Fractional shortening (%). (E) Mean values of anterior and posterior wall thickness in diastole showed reduced thickening in response to pressure-overload. n = 4 – 12 per group; *, P < 0.05 vs. Irx5+/+ SHAM; **, P < 0.05 vs. Irx5−/− SHAM; #, P < 0.05 vs. Irx5+/+ BAND.
Table 3.3
Invasive Hemodynamic Measurements of Irx5 Mice at 12 Week Post-Banding

<table>
<thead>
<tr>
<th></th>
<th>Irx5+/+ SHAM (n = 4)</th>
<th>BAND (n = 6)</th>
<th>Irx5−/− SHAM (n = 6)</th>
<th>BAND (n = 5)</th>
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<tr>
<td>SBP (mmHg)</td>
<td>108.5 ± 2.7</td>
<td>163.6 ± 9.7*</td>
<td>91.8 ± 4.8</td>
<td>123.4 ± 5.3**,#</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.4 ± 2.8</td>
<td>70.3 ± 4.3</td>
<td>66.1 ± 3.5*</td>
<td>71.6 ± 6.5</td>
</tr>
<tr>
<td>HR (B.P.M.)</td>
<td>474 ± 13</td>
<td>526 ± 25</td>
<td>469 ± 20</td>
<td>486 ± 26</td>
</tr>
<tr>
<td>LVEESP (mmHg)</td>
<td>111.8 ± 2.1</td>
<td>162.5 ± 10.3*</td>
<td>95.8 ± 2.2</td>
<td>120.4 ± 9.3**,#</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>7.9 ± 0.8</td>
<td>12.4 ± 5.0</td>
<td>14.3 ± 4.0</td>
<td>15.5 ± 3.2</td>
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<td>+dP/dt (mmHg/s)</td>
<td>10024 ± 314</td>
<td>9290 ± 971</td>
<td>7572 ± 286*</td>
<td>5692 ± 161**,#</td>
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<td>-dP/dt (mmHg/s)</td>
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<td>-6474 ± 3300</td>
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SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; B.P.M., beats per minute; LVEESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt and -dP/dt, derivatives of the left ventricular pressure change over time (sec). Values are presented as means ± standard error. *, P < 0.01 vs. Irx5+/+ SHAM; **, P < 0.05 vs. Irx5−/− SHAM, #, P < 0.01 vs. Irx5+/+ BAND.
3.4 Irx5 Is Necessary For Compensatory Hypertrophic Response to Pressure-Overload

To test whether Irx5 plays a role in the development of hypertrophy, mice were subjected to pressure-overload for 2 weeks, which time point is known to show concentric hypertrophy (Maillet et al., 2009). Cardiac hypertrophy was evaluated by heart weight normalized to body weight (HW/BW; Figure 3.8B) as well asibia length (HW/TL; Figure 3.8C). No difference ($P > 0.300$) in HW/BW and HW/TL ratios was observed in between SHAM- $Irx5^{+/+}$ mice (4.37 ± 0.21 mg/g; 8.12 ± 0.13 mm/mm) and $-Irx5^{-/-}$ mice (4.58 ± 0.10 mg/g; 7.42 ± 0.25 mm/mm), respectively. As expected (Ling et al., 2009; Nakayama et al., 2009), BAND-$Irx5^{+/+}$ mice exhibited 54% and 49% increases ($P < 0.01$) in HW/BW (6.75 ± 0.14 mg/g) and HW/TL (12.11 ± 0.34 mm/mm) ratios, respectively, compared to SHAM- $Irx5^{+/+}$ mice, indicating pressure-overload induced cardiac hypertrophy. Although BAND-$Irx5^{-/-}$ mice also developed hypertrophy with 30% and 21% increases in HW/BW (5.95 ± 0.16 mg/g) as well as HW/TL (8.99 ± 0.28 mg/mm) ratios, respectively, the degree of hypertrophy response to pressure-overload was significantly attenuated ($P < 0.02$) in BAND-$Irx5^{-/-}$ mice compared with BAND-$Irx5^{+/+}$ mice, which was consistent with the echocardiography results. The reduced hypertrophy in $Irx5^{-/-}$ mice following BAND was further observed in the histological analysis of LV tissue section (Figure 3.8A). Image analysis to quantify histological sections revealed that enlargement of myocyte cross-section area, indicating cellular hypertrophy, was significantly less ($P < 0.01$) in BAND-$Irx5^{-/-}$ mice than in BAND-$Irx5^{+/+}$ mice (Figure 3.8D), suggesting that the reductions in HW/BW and HW/TL were not due to alteration in cell number. On the other hand, the lack of a difference ($P = 0.187$) in myocyte cross-section area between sham-operated $Irx5^{+/+}$ and $Irx5^{-/-}$ mice confirmed the absence of baseline cellular hypertrophy in $Irx5^{-/-}$ mice despite of reduced cardiac function.

Although cardiac hypertrophy is considered to be a compensatory response by normalizing the elevated wall stress, persistent hypertrophic stimuli and stresses is associated with unfavourable consequences such as sudden cardiac death and heart failure with functional and structural deterioration. In addition, several previous studies have demonstrated that prevention or reverse of hypertrophy in the setting of pressure-overload may be beneficial (Frey and Olson, 2003). However, as cardiac dilation was observed in $Irx5^{-/-}$ heart in response to prolonged long-term stress (Figure 3.7), reduced hypertrophy in BAND-$Irx5^{-/-}$ heart was not a
favourable response, and did not indicate attenuation of cardiac remodelling. In addition, lung weight normalized to body weight (Lung W/BW), a pulmonary congestion indicator, was greatly elevated ($P < 0.02$) in BAND-Irx5$^{-/-}$ mice compared to BAND-Irx5$^{+/+}$ mice (Figure 3.9C). Since pressure overload-induced hypertrophy is known to induce fibrosis (Frey and Olson, 2003), histological analysis with Masson’s trichrome staining was conducted, and the fibrotic area was quantified. As shown in Figure 3.9, A and D, the interstitial fibrosis was clearly observed in both Irx5$^{+/+}$ and Irx5$^{-/-}$ hearts subject to BAND, yet the degree of its increase was much greater ($P < 0.05$) in BAND-Irx5$^{-/-}$ hearts than BAND-Irx5$^{+/+}$ hearts. On the other hand, the increase in perivascular fibrosis following BAND was indistinguishable between Irx5$^{+/+}$ and Irx5$^{-/-}$ hearts (Figure 3.9, B and E). Furthermore, invasive hemodynamic measurements at 2 week post-bandling revealed that BAND-Irx5$^{-/-}$ mice showed lower ($P < 0.05$) left ventricular end-systolic pressure (LVESP) than BAND-Irx5$^{+/+}$ mice (Table 3.4). Although it could have been questioned a difference in the degree of surgical constriction, other unfavourable responses in BAND-Irx5$^{-/-}$ mice, such as the increased LV end-diastolic pressure (LVEDP) at 2 week-post banding, and further reductions in LVESP and $+dP/dt$ with severe ventricular dilation at 12 week post-bandling, suggested that decreased LVESP in BAND-Irx5$^{-/-}$ mice was due to impaired cardiac force generation as contractile dysfunction in congestive heart failure often results in a reduction in LV ejection pressure (Xu et al., 2006). Indeed, the conclusion was further supported later by restored LVESP in BAND-Irx5$^{-/-}$; Kv4.2$^{-/-}$ mice at 2 week-post banding (Table 3.5) (see Results 3.6).

Cardiac hypertrophy accompanies gene remodelling, and especially re-activation of fetal genes such as Nppa (ANF), Myh7 ($\beta$-MHC), and Acta1 ($\alpha$-sk-actin) is a hallmark of heart disease (Razeghi et al., 2001; Frey and Olson, 2003). Gene expression assays in the heart by quantitative RT-PCR (qPCR) showed results corresponding to an altered hypertrophic response in Irx5$^{-/-}$ mice subjected to BAND. For examples, the hypertrophy hallmark gene, ANF (Nppa) expression was elevated ($P < 0.01$) far less in BAND-Irx5$^{-/-}$ hearts compared to BAND-Irx5$^{+/+}$ hearts (Figure 3.10A). On the other hand, the opposite pattern of changes ($P < 0.05$) was seen for a cardiac remodelling marker, $\beta$-MHC (Myh7) mRNA expression, consistent with the tight spatial relationship between Myh7 increases and regions of fibrosis (Pandya et al., 2006). Elevations ($P < 0.05$) in another cardiac remodelling marker, $\alpha$-sk-actin (Acta1), were indistinguishable ($P = 0.213$) between Irx5$^{+/+}$ and Irx5$^{-/-}$ mouse hearts subjected to BAND. Taken together, these results suggest that Irx5 is necessary for compensatory hypertrophic response to biomechanical stress in the heart, and is required for regulating hypertrophic gene expression.
Furthermore, Kv4.2 (Kcnd2) gene expression was examined since the reduction in I_{to,f}, as a result of decreased expression of Kv4.2, is commonly found in the heart diseases (Oudit et al., 2001). Consistent with our previous results (Costantini et al., 2005), Kcnd2 mRNA level was higher (P < 0.02) in SHAM-Irx5^{−/−} hearts than SHAM-Irx5^{+/+} hearts (Figure 3.10B). While pressure-overload down-regulated (P < 0.05) Kcnd2 expression in both Irx5^{+/+} and Irx5^{−/−} hearts by a similar proportion, the amount of Kcnd2 mRNA expression remained higher (P < 0.03) in BAND-Irx5^{−/−} hearts than in BAND-Irx5^{+/+} hearts. These results implied potential contributions of higher I_{to,f} to the decreased hypertrophic responses seen with Irx5 ablation (see below).
Figure 3.8
Assessment of Pressure-overload Induced Cardiac Hypertrophy in \(Ir\times 5^{+/+}\) and \(Ir\times 5^{-/-}\) Mice at 2 Week Post-banding. (A) Representative figures of H&E staining showing myocytes cross-sectional area. Scale bars indicate 50 µm. (B & C) Heart weight / body weight ratio (HW/BW) as well as heart weight / tibia length ratio (HW/TL) at 2 week post-banding revealed that \(Ir\times 5^{-/-}\) mice developed attenuated hypertrophy, compared with \(Ir\times 5^{+/+}\) mice. \(n = 11 \text{–} 16\) per group; *, \(P < 0.01\) vs. \(Ir\times 5^{+/+}\) SHAM; **, \(P < 0.01\) vs. \(Ir\times 5^{-/-}\) SHAM; #, \(P < 0.01\) vs. \(Ir\times 5^{+/+}\) BAND. (D) This result is consistently observed in the histological measurements of myocyte cross-sectional area at 2 week post-banding. \(n = 3\) per group; *, \(P < 0.01\) vs. \(Ir\times 5^{+/+}\) SHAM; **, \(P < 0.01\) vs. \(Ir\times 5^{+/+}\) SHAM; #, \(P < 0.01\) vs. \(Ir\times 5^{-/-}\) BAND.
Figure 3.9

**Cardiac Remodeling in Irx5^{+/+} and Irx5^{-/-} Mice at 2 week Post-banding**

Representative figures of Masson's trichrome staining for interstitial fibrosis (A) and perivascular fibrosis (B). Scale bars indicate 50 µm. (C) Lung weight / body weight ratio (LungW/BW) was significantly elevated in Irx5^{-/-} hearts at 2 weeks after banding, compared with Irx5^{+/+} hearts. n = 11 -16 per group; **, P < 0.05 vs. Irx5^{-/-} SHAM; #, P < 0.05 vs. Irx5^{+/+} BAND. (D) Quantification of fibrosis revealed that Irx5^{-/-} hearts developed severe interstitial fibrosis in response to pressure-overload compared to Irx5^{+/+} hearts while perivascular fibrosis was indistinguishable between Irx5^{+/+} and Irx5^{-/-} hearts. n = 3 per group; *, P < 0.05 vs. Irx5^{+/+} SHAM; **, P < 0.05 vs. Irx5^{-/-} SHAM; #, P < 0.05 vs. Irx5^{+/+} BAND.
Table 3.4

*Invasive Hemodynamic Measurements of Irx5 Mice at 2 Week Post-Banding*

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<tr>
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SBP, systolic blood pressure; DBP, diastolic blood pressure, HR, heart rate; B.P.M., beats per minute; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt and -dP/dt, derivatives of the left ventricular pressure change over time (sec). Values are presented as means ± standard error.

<sup>*</sup>, <i>P</i> < 0.01 vs Irx5<sup>+/+</sup> SHAM; <sup>**</sup>, <i>P</i> < 0.01 vs Irx5<sup>−/−</sup> SHAM; <sup>##</sup>, <i>P</i> < 0.05 vs Irx5<sup>+/+</sup> BAND.
Gene Expression Analysis of Irx5+/+ and Irx5-/- Mouse Hearts at 2 Week Post-banding. (A) Quantitative PCR results revealed that gene expressions of a hypertrophic marker, ANF (*nppa*) was less elevated in *Irx5-/-* mouse hearts at 2 week post-banding, compared to *Irx5+/+* mouse hearts. On the other hands, heart disease markers, α-skeletal actin (*acta1*), and β-MHC (*Myh7*), were both increased in *Irx5+/+* and *Irx5-/-* hearts after banding. (B). Kᵥ4.2 (*Kcnd2*) mRNA expression was higher in banded *Irx5-/-* hearts, compared with banded *Irx5+/+* hearts. n = 3 – 5 per group; *, P < 0.05 vs. *Irx5+/+* SHAM; **, P < 0.05 vs. *Irx5+/+* BAND.
3.5 Loss of \textit{Irx5} Impairs Cardiomyocyte Hypertrophy Induced by Adrenergic Stimulation

To further investigate the role of Irx5 in the hypertrophic response at cellular level, we examined the effects of adrenergic stimulation with 20 μM of norepinephrine (NE) for 48 hours on cultured neonatal mouse ventricular myocytes (NMVM) isolated from 1 day-old mice. In the absence of NE, myocyte surface area was indistinguishable between \textit{Irx5}\textsuperscript{+/-} and \textit{Irx5}\textsuperscript{-/-} NMVM with a normal distribution of cell size (Figure 3.11). NE stimulation induces a rightward shift in the size distribution of NMVM with increased mean ($P < 0.01$) in both \textit{Irx5}\textsuperscript{+/-} and \textit{Irx5}\textsuperscript{-/-} NMVM, indicating hypertrophic growth. However, the relative rightward shift in the cellular distribution curve caused by NE was much blunted ($P < 0.01$) in \textit{Irx5}\textsuperscript{-/-} NMVM, compared to \textit{Irx5}\textsuperscript{+/-} NMVM.

Clearly, the effects of Irx5 on NE-induced hypertrophy in NMVMs mirror closely the results in intact hearts following TAC. This similarity is bolstered by the gene expression patterns. For example, \textit{Nppa} mRNA levels increased ($P < 0.05$) far more in \textit{Irx5}\textsuperscript{+/-} NMVM compared to \textit{Irx5}\textsuperscript{-/-} NMVM following NE treatment while \textit{Acta1} mRNA expressions changes were indistinguishable between the groups (Figure 3.12A). On the other hand, unlike the pressure-overload results, \textit{Myh7} levels were ($P < 0.05$) reduced, consistent with previous results in NMVM (Deng \textit{et al}., 2000), but did not differ ($P = 0.100$) between the groups. This may be related to results that \textit{Myh7} is primarily elevated in fibrotic regions of diseased hearts (Pandya \textit{et al}., 2006). Changes in \textit{Kcnd2} mRNA level caused by adrenergic stimulation in Irx5 NMVM were also consistent with results in BAND-Irx5 mouse hearts. Specifically, in control treatment, \textit{Irx5}\textsuperscript{-/-} NMVM showed higher ($P < 0.05$) \textit{Kcnd2} mRNA level than \textit{Irx5}\textsuperscript{+/-} NMVM, as expected (Costantini \textit{et al}., 2005) (Figure 3.12B). Similar to a previous study (Bru-Mercier \textit{et al}., 2002), NE administration reduced ($P < 0.05$) \textit{Kcnd2} mRNA expression in both \textit{Irx5}\textsuperscript{+/-} and \textit{Irx5}\textsuperscript{-/-} NMVM, whereas \textit{Kcnd2} mRNA level remained higher ($P < 0.05$) in \textit{Irx5}\textsuperscript{-/-} NMVM compared with \textit{Irx5}\textsuperscript{+/-} NMVM.

To determine whether exogenous Irx5 expression was sufficient to induce hypertrophy, and was able to rescue the reduced hypertrophic response of \textit{Irx5}\textsuperscript{-/-} NMVM, cells were infected with adenovirus containing \textit{Irx5} (Ad-Irx5). Infections with GFP (Ad-GFP) were used as a control. Ad-GFP infected myocytes showed identical hypertrophic response to NE as shown in non-infected cells (Figure 3.13, A and B; compare with Figure 3.11). Interestingly, Ad-Irx5 infection in the absence of NE stimulation mildly, but significantly ($P < 0.05$) increased
myocytes surface area in both Irx5+/+ and Irx5−/− NMVM, suggesting that Irx5 may act as a pro-hypertrophic transcription mediator. Indeed, over-expression of Irx5 in wild-type NMVM for 48 hours, cultured in 10% serum, resulted in greater (P < 0.01) hypertrophy response compared to Ad-GFP-infected NMVM (Figure 3.14). Most importantly, blunted hypertrophy in Irx5−/− NMVM was restored by Irx5 replacement. Specifically, after Ad-Irx5 infection, the hypertrophic response of Irx5−/− NMVM to NE was not different (P = 0.163) from Irx5+/+ NMVM infected with either Ad-Irx5 or control Ad-GFP, but significantly higher (P < 0.05) compared to Ad-GFP-infected Irx5−/− NMVM. Taken together, these data further support the conclusion that Irx5 modulates hypertrophic responses in the myocardium.

Several studies have suggested that apoptosis is a critical factor during disease progression from compensatory hypertrophy to heart failure, and is also associated with cardiac fibrosis and dilated cardiomyopathy (van Empel and De Windt, 2004; Anselmi et al., 2008; Dorn, 2009). Interestingly, a previous study has shown that silencing Irx5 gene expression induces apoptosis in human prostate cancer cells (Myrthue et al., 2008), implying that Irx5−/− cardiomyocyte might be more susceptible to biomechanical stimuli, leading to apoptotic cell death. To test hypothesis, NMMV were exposed to hydrogen peroxide (H2O2), known to cause oxidative stress and commonly found in pressure overload-induced hypertrophy (Date et al., 2002; Kaludercic et al., 2010), and then were examined for cell survival. As shown in Figure 3.15, H2O2 dose-dependently caused cell death in Irx5+/+ and Irx5−/− NMVM, while the susceptibility to oxidative stress-induced cell death was indistinguishable between two genotypes. This result suggests that early development of dilated cardiomyopathy may not be due to greater susceptibility to apoptotic stress in Irx5−/− myocardium while it may be related to secondary effect such as decreased oxygen delivery with a defect in capillary angiogenesis.
Figure 3.11
Assessment of Cellular Hypertrophy in Irx5<sup>+/+</sup> and Irx5<sup>-/-</sup> Neonatal Mouse Ventricular Myocytes (NMVM) in Response to Adrenergic Stimulation

(A) Representative phase-contrast microscope images of NE-induced hypertrophy in Irx5<sup>+/+</sup> and Irx5<sup>-/-</sup> NMVM. Scale bars indicate 50 µm. (B) Histogram of quantified cell surface area of Irx5<sup>+/+</sup> and Irx5<sup>-/-</sup> NMVM after NE treatment. n = 6 – 14 per group; *, P < 0.01 vs. Irx5<sup>+/+</sup> Control; **, P < 0.01 vs. Irx5<sup>-/-</sup> Control; #, P < 0.01 vs. Irx5<sup>+/+</sup> NE..
Figure 3.12
Gene Expression Analysis of Irx5+/+ and Irx5−/− Neonatal Cardiomyocytes in Response to Adrenergic Stimulation. (A) Quantitative PCR results revealed that gene expression of a hypertrophic marker, ANF (nppa) was less elevated in Irx5−/− NMVM in the presence of NE (20 µM), compared to Irx5+/+ NMVM. On the other hand, a heart remodeling marker, α-skeletal actin (acta1) mRNA level was increased in Irx5+/+ and Irx5−/− NMVM in response to NE stimulation. β-MHC (Myh7) mRNA expression, down-regulated by NE, was indistinguishable between Irx5+/+ and Irx5−/− NMVM. (B). Kv4.2 (Kcnd2) mRNA expression was higher in NE-treated Irx5+/− NMVM, compared with NE-treated Irx5+/+ NMVM. n = 4 – 7 per group; *, P < 0.05 vs. Irx5+/+ NMVM - Control; **, P < 0.05 vs. Irx5+/− NMVM - Control; #, P < 0.05 vs. Irx5+/+ NMVM - NE.
Figure 3.13
Assessments of Cellular Hypertrophy in Adenovirus-infected *Irx5*+/+ and *Irx5*−/− Neonatal Cardiomyocytes in Response to Adrenergic Stimulation

(A) Representative fluorescent microscope images of NE-induced cellular hypertrophy in *Irx5*+/+ and *Irx5*−/− NMVM. Scale bars indicate 50 µm. (B) Quantification of myocyte surface area revealed that *Irx5* over-expression restored the attenuated hypertrophic response of *Irx5*−/− NMVM. n = 4 – 7 per group; *, P < 0.01 vs. *Irx5*+/+ Ad-GFP-Control; **, P < 0.01 vs. *Irx5*−/− Ad-GFP-Control; †, P < 0.01 vs. *Irx5*+/+ Ad-GFP-NE; †, P < 0.05 vs. *Irx5*−/− Ad-GFP-NE.
Figure 3.14  
Assessment of Cellular Hypertrophy in Adenovirus-infected Neonatal Cardiomyocytes Cultured in 10% Serum Media.  
(A) Representative phase-contrast microscope images of 10% serum-cultured NMVM infected with GFP or Irx5 adenovirus for 48 hours. Scale bars indicate 50 µm.  
(B) Quantification of myocyte surface area revealed that Irx5 over-expression in NMVM accelerated hypertrophic response to serum, compared to Ad-GFP infected NMVM. * , P < 0.01 vs. Ad-GFP.
Figure 3.15  
_Cell Survival Assessments of Neonatal Mouse Ventricular Myocytes (NMVM) in the Presence Of H$_2$O$_2$. _Different concentrations of hydrogen peroxide (H$_2$O$_2$) treatments in NMVM for 7 hours resulted in dose-dependent cell death, whereas no difference in cytotoxic susceptibility was observed in between $lrx5^{+/+}$ and $lrx5^{-/-}$ NMVM._
3.6 Loss of I÷f Restores the Diminished Cardiac Contractility, But Not the Hypertrophic Defect in Irx5−/− Mice

The finding that Irx5−/− hearts and NMVM expressed higher levels of Kv4.2 transcript compared with Irx5+/+ hearts and NMVM after hypertrophic stimuli enabled us to postulate that the attenuated hypertrophic response by loss of Irx5 might be mediated by elevated I÷f, especially because I÷f elevation, by over-expressing Kv4.2, Kv4.3 or KChIP2, can prevent hypertrophy in vitro and in vivo (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010). To test whether effects of Irx5 ablation on contractility and hypertrophy are mediated by I÷f, Irx5−/− mice were crossed with Kv4.2−/− mice that virtually lack I÷f (Guo et al., 2005) (see Discussion 4.1 and Appendix B).

In vivo cardiac contractile function was first examined by invasive hemodynamic measurements. Although LVESP was not significantly affected by loss of Kv4.2 (Figure 3.16A), consistent with previous studies establishing links between I÷f and contraction (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010), in vivo LV +dP/dt was elevated (P < 0.05) in 12 – 16 week old Kv4.2-deficient mice compared to control littermates (Irx5+/+;Kv4.2+/+) (Figure 3.16B). More importantly, LV +dP/dt in mice lacking both Irx5 and Kv4.2 (Irx5−/−;Kv4.2+/+) did not differ from mice lacking Kv4.2, but was higher (P < 0.05) than in WT littermates, and much higher (P < 0.001) than either Irx5−/−;Kv4.2+/+ littermates or littermates lacking Irx5 and one allele of Kv4.2 (Irx5−/−;Kv4.2+/+). As might be expected, littermate Irx5−/−;Kv4.2+/+ and Irx5−/−;Kv4.2+/+ mice had reduced (P < 0.02) contractile function compared to WT littermates. These observations establish that Irx5 regulates cardiac contractility via alterations in I÷f.

The role of I÷f in hypertrophic response was next examined using Irx5-Kv4.2 mice. Contrary to previous studies suggesting that I÷f plays a role in hypertrophy (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010), hypertrophy after 2 weeks post-banding, as assessed by PWTD and HW/BW, did not differ (P = 0.593) between Kv4.2+/− and WT littermate mice (Figure 3.17). On the other hand, unlike our initial expectation that absence of Kv4.2 would restore the reduced hypertrophic response in Irx5−/− hearts, Irx5−/−;Kv4.2+/− mice subjected to pressure-overload for 2 weeks had an attenuated hypertrophic response with lower (P < 0.02) PWTD and HW/BW compared with banded control or Kv4.2+/− mice, while being similar (P = 0.561) to the hypertrophy seen in BAND-Irx5−/− mice. This result suggests that the effects of Irx5 on the hypertrophic response are independent of I÷f. Furthermore, invasive hemodynamic
measurements showed that LVESP and +dp/dt of Irx5−/−;Kv4.2−/− mice was maintained after banding with normal LVEDP compared with Irx5−/− mice, suggesting that increased contractile function may protect the heart from decompensation (Table 3.5).
Figure 3.16
Absence Of $K_v4.2$ in the Heart Restores Decreased Cardiac Contractility of $Irx5^{+/+}$ Hearts. (A) Loss of $K_v4.2$ did not significantly affect LVESP in the background of $Irx5^{+/+}$ or $Irx5^{-/-}$ mice. (B) $Irx5^{+/+};K_v4.2^{-/-}$ mice showed an increase in cardiac contractile function, indicated by elevated LV $+dP/dt$, compared to wild-type mice ($Irx5^{+/+};K_v4.2^{+/+}$). Furthermore, loss of $K_v4.2$ completely restored attenuated contractile function caused by the absence of $Irx5$. $n = 3–8$ per group; *, $P < 0.02$ vs. $Irx5^{+/+};K_v4.2^{+/+}$ mice; #, $P < 0.001$ vs. $Irx5^{-/-};K_v4.2^{+/+}$ mice.
**Figure 3.17**

*Irx5 Regulates Hypertrophic Response Independent of *I*o*

*Irx5*-Kv4.2 double transgenic mice were subjected to pressure-overload for 2 weeks. While no difference in hypertrophic response was observed in *Irx5*+/+;Kv4.2−/− mice, compared to control (*Irx5*+/+;Kv4.2+/+) mice, *Irx5*−/−;Kv4.2−/− showed attenuated hypertrophic response with less thickening of diastolic LV posterior wall (PWTD) (A) and lower HW/BW ratio (B). n = 5 - 10 per group; *, P < 0.001 vs. SHAM of each genotype; #, P < 0.02 vs. *Irx5*+/+;Kv4.2+/−-BAND.
Table 3.5  
*Invasive Hemodynamic Measurements of Irx5-Kv4.2 Mice at 2 Week Post-Banding*

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SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; B.P.M., beats per minute; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt and -dP/dt, derivatives of the left ventricular pressure change over time (sec). Values are presented as means ± standard error.  
*, P < 0.01 vs. Irx5<sup>+/−</sup>;Kv4.2<sup>/−</sup> SHAM; **, P < 0.01 vs. Irx5<sup>−/−</sup>;Kv4.2<sup>/−</sup> SHAM.
3.7 Modulation of Hypertrophy by Irx5 Depends on the Calcineurin-NFAT Signaling Pathway

A number of cardiac transcription factors (TFs) such as Nfat, Gata, Mef2, Csx/Nkx2-5, Hand1/2, and NF-kB are implicated in hypertrophy and the genetic remodelling associated with heart disease (Akazawa and Komuro, 2003; Frey and Olson, 2003). Among these cardiac TFs, the calcineurin-Nfat pathway was of particular interest, because calcineurin (Cn) is an important modulator of cardiac hypertrophy (Molkentin et al., 1998), and Nfatc3-deficient mice have diminished hypertrophy following BAND (Wilkins et al., 2002) and lose the normal Ito,f transmural gradient without affecting Irx5 expression (Rossow et al., 2006). Consistent with a possible contribution of Nfat to the altered hypertrophic responses in Irx5-deficient mice, mRNA levels of Rcan1 (exon 4; Rcan1.4), a marker of Cn-Nfat activity (Rothermel et al., 2003; Kuwahara et al., 2006; Jin et al., 2010), were up-regulated far less (P < 0.03) in Irx5−/− hearts (subjected to BAND) and myocytes (treated with NE), compared to Irx5+/+ (Figure 3.18, A and B). On the other hand, Irx5 over-expression in NMVM cultured in 10% serum media showed elevated Rcan1 mRNA expression, compared with Ad-GFP infected control cells (Figure 3.18.C). The above results suggest that Irx5 modulates cardiac hypertrophic growth by altering Cn-Nfat activity. To test this possibility, we generated mice lacking Irx5 and cardiac specifically over-expressing constitutively active Cn (ΔCnA) (i.e., Irx5-CnA mice) (Molkentin et al., 1998). Consistent with previous studies (Molkentin et al., 1998; Wilkins et al., 2002), 6 week-old Irx5+/+;CnA-TG mice showed severe hypertrophy with increases (P < 0.001) in HW/BW and HW/TL ratios as well as PWTD by 270%, 261% and 79%, respectively, compared with non-transgenic WT littermates (Irx5+/+;Non-TG) (Figure 3.19 and Table 3.6). More importantly, the extent of hypertrophy, as reflected in HW/BW (230%), HW/TL (215%) ratios and PWTD (27%), was attenuated (P < 0.02) in Irx5−/−;CnA-TG mice compared with littermate Irx5+/+;CnA-TG mice. Interestingly, cardiac contractile function was markedly compromised (P < 0.05) in Irx5−/−;CnA-TG hearts compared to Irx5+/+;CnA-TG hearts without cardiac ventricular dilation (Table 3.6). Attenuation of Cn-mediated hypertrophy by the loss of Irx5 was confirmed in NMVMs, showing that increases in myocyte surface area induced by ΔCnA, using Ad-CnA (40 MOI), were attenuated (P < 0.05) in Irx5−/− NMVM, compared to Irx5+/+ NMVM (Figure 3.20). Moreover, the reduced Cn-induced hypertrophic response in Irx5−/− NMVM was restored (P < 0.05) by Irx5 over-expression. Taken together, our results support the conclusion that Irx5
modulates hypertrophy mediated by Cn-Nfat, which is activated by pressure-overload and adrenergic-receptor activation (Wilkins et al., 2004; Wu et al., 2009; Heineke et al., 2010a).
Figure 3.18
Irx5 is Associated with Calcineurin-NFAT at Signaling Pathway
Rcan1 mRNA expression, an indicator of calcineurin-Nfat activity, was up-regulated by hypertrophic stimuli such pressure-overload (A) in heart and an adrenergic agonist (B) in cultured cardiomyocytes. This hypertrophic stimuli–induced elevations in Rcan1 mRNA level were attenuated by loss of Irx5. (C) Irx5 over-expression in NMVM cultured in 10% serum media elevated Rcan1 mRNA level. n = 3 – 8 per group; *, P < 0.03 vs. Irx5+/+ SHAM or Irx5+/+ NMVM-Control; #, P < 0.03 vs. Irx5+/+ BAND or Irx5+/+ NMVM-NE.
**Figure 3.19**

*Irx5 is Necessary for Calcineurin-mediated Cardiac Hypertrophy*

Cardiac-specific constitutively active calcineurin over-expression in 6 week old mice (CnA-TG) resulted in severe hypertrophy compared with non-transgenic mice (Non-TG), as indicated by increases in HW/BW (A) as well as HW/TL (B) ratios. Compared with *Irx5<sup>+/+</sup>;CnA-TG, *Irx5<sup>−/−</sup>;CnA-TG mice developed attenuated hypertrophy, suggesting that calcineurin-mediated hypertrophic response requires *Irx5*. n = 6 – 11 per group; *, P < 0.001 vs. *Irx5<sup>+/+</sup>;Non-TG; **, P < 0.001 vs. *Irx5<sup>−/−</sup>;Non-TG; #, P < 0.001 vs. *Irx5<sup>+/+</sup>;CnA-TG.
Figure 3.20
Calcineurin-induced Cellular Hypertrophy in Irx5+/+ and Irx5−/− Neonatal Cardiomyocytes

Myocyte surface area measurements revealed that Irx5−/− NMVM developed attenuated CnA-induced cellular hypertrophy, compared with Irx5+/+ NMVM. NMVMs were infected with 40 MOI of Ad-CnA or Ad-GFP. Additionally, Irx5 overexpression restored reduced CnA-mediated hypertrophy response of Irx5−/− NMVM. n = 4 – 8 per group; *, P < 0.01 vs. Irx5+/+ Ad-GFP; **, P < 0.01 vs. Irx5−/− Ad-GFP; #, P < 0.01 vs. Irx5+/+ Ad-CnA; †, P < 0.05 vs. Irx5−/− Ad-CnA; NS, not significant.
Table 3.6
Echocardiography Assessments of 6 week old Irx5-CnA Mice

<table>
<thead>
<tr>
<th></th>
<th>Irx5+/+</th>
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<th>Irx5−/−</th>
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<tr>
<td></td>
<td>Non-TG</td>
<td>CnA-TG</td>
<td>Non-TG</td>
<td>CnA-TG</td>
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<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 9)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.99 ± 0.09</td>
<td>4.48 ± 0.15*</td>
<td>3.85 ± 0.08</td>
<td>4.45 ± 0.12**</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.52 ± 0.07</td>
<td>2.66 ± 0.08</td>
<td>2.72 ± 0.07</td>
<td>3.50 ± 0.10**#</td>
</tr>
<tr>
<td>FS (%)</td>
<td>37.1 ± 0.5</td>
<td>40.5 ± 0.9*</td>
<td>29.3 ± 0.7*</td>
<td>21.4 ± 1.3**#</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>0.59 ± 0.01</td>
<td>1.05 ± 0.02*</td>
<td>0.58 ± 0.02</td>
<td>0.74 ± 0.02**#</td>
</tr>
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</table>

LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening; PWTD, LV posterior wall thickness at diastole. Values are presented as means ± standard error. *, P < 0.05 vs. Irx5+/+; Non-TG; **, P < 0.05 vs. Irx5−/−; Non-TG; #, P < 0.05 vs. Irx5+/−; CnA-TG.
3.8 Irx5 Interacts with and Regulates the Cardiac Transcription Factors, Nfatc3 and Gata4

Whereas a number of studies have shown the implications of the Cn-Nfat pathway in cardiac hypertrophy and heart diseases in various aspects (Molkentin et al., 1998; Kuwahara et al., 2006; Nakayama et al., 2009; Wu et al., 2009), less is known about Irx5 in heart diseases in relationship with other cardiac transcription factors. First it was tested whether changes in Irx5 expression were involved in cardiac hypertrophy. qPCR and Western blot analyses in cardiomyocytes stimulated with NE (20 µM) revealed that Irx5 transcripts and protein expression were up-regulated ($P < 0.02$) in NE-treated hypertrophied NMVM compared with control NMVM (Figure 3.21). These observations may be associated with microarray and RNA Sequencing results showing that Irx5 expression is elevated in the heart failure patients with dilated cardiomyopathy (Beisvag et al., 2006) as well as in mice over-expressing Gαq in the heart (Matkovich et al., 2010). In addition to modulation of Irx5 expression in hypertrophy, we found that, as expected (Heineke et al., 2007; Kaludercic et al., 2010; Tang et al., 2010), NE administration mildly increased ($P < 0.05$) Nfatc3 and Gata4 mRNA and protein expression.

Based on our results suggesting that Cn-Nfat activity is affected by Irx5, and other data showing loss of $I_o$ heterogeneity in Nfatc3-deficient mice without affecting Irx5 (Wilkins et al., 2002; Rossow et al., 2006), we tested whether Irx5 directly regulates Nfatc3 or if Irx5 works with Nfatc3 by forming a transcriptional complex, thereby modulating Cn-Nfat activity; the proposed mechanisms are illustrated in Figure 3.22, A and B, respectively. Gata4, well known to bind and work with Nfat in hypertrophy (Molkentin et al., 1998; van Rooij et al., 2002; Oka et al., 2005), was also examined. Consistent with the mild increases in the myocyte surface area shown in Figure 3.13B, Irx5 over-expression with Ad-Irx5 increased ($P < 0.05$) mRNA and protein expression of both Nfatc3 and Gata4 (Figure 3.23), compared with Ad-GFP infected myocytes. In addition, NE treatment of myocytes infected with Ad-Irx5 caused further elevations ($P < 0.01$) in the mRNA and protein for these genes. These results demonstrate that the increase in expression of Nfatc3 and Gata4 induced by hypertrophic stimuli are intensified in the presence of Irx5, thereby promoting hypertrophy by enhancing Cn-Nfat and Gata4-mediated signaling pathways. Unexpectedly, Nfatc3 and Gata4 expression levels were also similarly augmented in myocytes over-expressing Irx3 (Figure 3.23B), another member of Iroquois transcription factor family, suggesting that Irx3 and Irx5 might be implicated in the similar transcriptional pathway.
(see Discussion 4.7). Interestingly, elevation in Nfatc3 and Gata4 protein expression caused by Irx5 appeared to be greater than their mRNA levels, suggesting that there might be post-transcriptional or translational modification, leading to protein stabilization (Wang et al., 2004). Moreover, Irx5-mediated regulation of Nfatc3 and Gata4 expression in hypertrophy was further confirmed since elevations of Nfatc3 and Gata4 mRNA expressions in response to hypertrophic stimuli (pressure-overload and adrenergic stimulation) were blunted in Irx5−/− hearts and myocytes, compared to Irx5+/+ (Figure 3.24). Interestingly, in the absence of hypertrophic stimuli, Nfatc3 and Gata4 expressions in Irx5−/− heart and cells were higher than in WT, which might be compensatory response due to lack of Irx5. It also suggests that Nfatc3 and Gata4 might be indirectly regulated by Irx5.

Iroquois transcription factor family members can form a transcription complex with a cardiac TF, Nkx2.5 (Figure 3.25), just like the well-known interaction between Nkx2.5 and Tbx5 (Moskowitz et al., 2007), suggesting a possible interaction of Irx5 with other cardiac TFs. Thus, it was determined whether Irx5 could physically associates with Nfatc3. Co-immunoprecipitation (Co-IP) experiments performed in cultured NMVM revealed that Nfatc3-specific antibodies enriched for Irx5 as well as Gata4 (Figure 3.26), suggesting that Irx5 physically associate with Nfatc3 and Gata4, thereby forming a transcription complex. In addition, Co-IP in mammalian cells further exhibited that Irx5 is directly associated with Gata4. Deletion of N-terminal HD (the homeobox domain), but not C-terminal IRO (Iro-box domain) of Irx5 protein resulted in a complete loss of interaction with Gata4 (Figure 3.27). These results strongly suggest that Irx5, Nfatc3 and Gata4 can form a transcriptional complex, possibly regulating hypertrophic gene expression.
Figure 3.21

*Ir*5 and Other Cardiac Transcription Factors Are Up-regulated by Hypertrophic Stimulation

Hypertrophic stimulation with 20 µM norepinephrine (NE) in cultured neonatal cardiomyocytes led to mild up-regulation in mRNA (A) and protein (B) expression of I*r*5, Nfatc3 and Gata4. n = 4 – 5 per group; *, P < 0.02 vs. Control.
Two Proposed Mechanisms of Irx5 Associated with Calcineurin-NFAT Pathway

(A) Irx5 would positively regulate Nfatc3 expression. (B) Irx5 would interact with Nfatc3, forming a transcription complex.
Figure 3.23

*Irx5 Positively Regulates Nfatc3 and Gata4 Expressions in Cardiomyocytes*

(A) *Irx5* over-expression mildly elevated *Nfatc3* and *Gata4* mRNA levels, while they were further increased when *Irx5* was over-expressed in the presence of NE. n = 3 – 5 per group; *, P < 0.05 vs. Ad-GFP; #, P < 0.01 vs. Ad-Irx5.

(B) *Irx5* over-expression in cardiomyocytes cultured in 10% serum conditions led to up-regulation of *Nfatc3* and *Gata4* protein expression. Consistent with mRNA expression results, hypertrophic stimulation with NE resulted in increases in the *Nfatc3* and *Gata4* protein levels. Arrows indicate bands corresponding to target proteins.
Figure 3.24

Up-regulation of Nfatc3 and Gata4 Expressions in Response to Hypertrophic Stimuli are Affected by Irx5

Nfatc3 and Gata4 mRNA expression was mildly increased by hypertrophic stimuli such as pressure-overload (A) in heart and the adrenergic agonist (B) in cultured cardiomyocytes, whereas these elevations were blunted by loss of Irx5. Interestingly, hearts and myocytes lacking Irx5 showed higher expressions of Nfatc3 and Gata4 in the absence of hypertrophic stimuli, compared to WT. For heart samples, n = 3 – 5 per group; *, P < 0.05 vs. Irx5+/+ SHAM; For neonatal myocyte sample, n = 4 – 5 per group; *, P < 0.05 vs. Irx5+/+ Control.
Figure 3.25

**Irx5 Interacts with a Cardiac Transcription Factor, Nkx2.5**

Co-immunoprecipitation experiments reveal that the cardiac transcription factor, Nkx2.5 can physically interact with Iroquois transcription factors including Irx1, Irx2, Irx3, and Irx5, just like the well-known interaction between Nkx2.5 and Tbx5, whereas it is not associated with Sufu (suppressor of fused), a component in the Hedgehog signaling. These results suggest that Irx5 can form a transcriptional complex with other cardiac transcription factors.
Irx5 Physically Interacts with Nfatc3 and Gata4 Transcription Factors in Cardiomyocytes

Co-immunoprecipitation (Co-IP) experiments performed in cardiomyocytes infected with Ad-GFP or Ad-Irx5 by pulling down endogenous Nfatc3, showing that Irx5 physically interacted with Nfatc3. In addition, Gata4 was associated with Nfatc3. Arrowheads indicates bands corresponding to Gata4 while the asterisk indicates the position of the immunoglobulin G (IgG) heavy chain.
Figure 3.27

Irx5 Directly Interacts with Gata4 Transcriptional Factor

(A) A schematic representation of Irx3 and two modified constructs. HD and IRO stands for the homeobox domain and the Iro-box domain, respectively. (B) Co-IP, performed in mammalian cells expressing FLAG-Irx5 and Gata4, revealed that Irx5 directly interacts with Gata4. Deletion of the IRO domain of Irx5, FLAG-Irx5(C), resulted in a loss of interaction with Gata4 while deletion of the HD, FLAG-Irx5(N) did not affect the interaction with Gata4.
CHAPTER 4

DISCUSSION
4.1 Summary of Findings

This dissertation has presented the first experimental evidence on the roles of Irx5 in maintenance of cardiac contractility and the adaptive response of the heart to biomechanical stress. Although we have shown previously that the Irx5 homeodomain transcription factor is responsible for the transmural gradient of the transient outward $K^+$ current ($I_{to,f}$) in heart by negatively regulating $K_V4.2$ genes (Costantini et al., 2005), and many previous studies have revealed that changes in $I_{to,f}$ can modulate both cardiac contractility via effects on action potential profile and cardiac hypertrophy (Kassiri et al., 2002; Zobel et al., 2002; Lebeche et al., 2004; Cheung et al., 2009; Jin et al., 2010), other roles of Irx5, possibly associated with $I_{to}$, in the heart had not been explored. This study demonstrates that Irx5 coordinates heterogeneity of excitation-contraction coupling (ECC), and maintains cardiac contractility in an $I_{to,f}$-dependent manner. In addition, Irx5 is involved in the development of cardiac hypertrophy, induced by hemodynamic overload, adrenergic stimulation or constitutive activation of calcineurin ($\Delta CnA$), which is found to be regulated in an $I_{to,f}$-independent manner. Furthermore, characterization of novel interactions between Irx5 and other key cardiac transcription factors such as Nfatc3 and Gata4, and positive modulation of calcineurin-Nfat activity (assessed by $Rcan1$) as well as Nfatc3 and Gata4 expressions by Irx5 suggest that Irx5 is a critical regulator of cardiac gene expression that acts by forming a transcription factor complex, thereby regulating cardiac contractility and hypertrophy (Figure 4.1).
An increase in Irx5 in response to hypertrophic stimuli leads to up-regulation of the cardiac transcription factors, Nfatc3 and Gata4 expression while it forms a transcription complex with Nfatc3 and/or Gata4, thereby regulating gene expression.
4.2 Irx5 Establishes Heterogeneity of Excitation-Contraction Coupling, and Affects Cardiac Contractility via \( I_{\text{to,f}} \) Regulation

It has been well established that \( I_{\text{to,f}} \) is a major contributor to the electrical heterogeneity of repolarization in rodents, large mammals and humans, thereby generating regional differences in action potential (AP) profile (particularly in the early repolarization phase) (Liu et al., 1993; Antzelevitch and Fish, 2001; Kuo et al., 2001; Sah et al., 2003; Costantini et al., 2005). In addition, the regional differences in AP waveform determines heterogeneity of cardiac excitation-contraction coupling (ECC) by altering the amplitude and time course of the L-type \( \text{Ca}^{2+} \) current, \( \text{Ca}^{2+} \) release via RyR2 channels in the sarcoplasmic reticulum and \( \text{Ca}^{2+} \) transient amplitudes (Bouchard et al., 1995; Volk et al., 1999; McIntosh et al., 2000; Kaprielian et al., 2002; Sah et al., 2003; Kondo et al., 2006). This electrophysiological relationship between \( I_{\text{to,f}} \), AP and cardiomyocyte shortening was clearly observed in wild-type mouse hearts, which showed smaller \( I_{\text{to,f}} \), slower repolarization with prolonged APD, larger \( \text{Ca}^{2+} \) transients and greater sarcomere shortening in LV endocardial (ENDO) myocytes than in LV epicardial (EPI) myocytes. On the contrary, consistent with our previous study (Costantini et al., 2005), Irx5\(-/-\) mouse hearts lack transmural gradients of \( I_{\text{to,f}} \), which leads to complete abrogation of APD and ECC heterogeneity. Specifically, as summarized in Figure 4.2, elevated \( I_{\text{to,f}} \) in Irx5\(-/+\) ENDO myocytes abbreviated APs and suppressed \( \text{Ca}^{2+} \) transients as well as cell shortening, compared to \( \text{Irx5}^{+/-} \) ENDO cells. This \( I_{\text{to,f}} \)-mediated ECC is consistent with previous results showing that increased \( I_{\text{to,f}} \) by \( K_{\text{V}}4.2 \), \( K_{\text{V}}4.3 \) or KChIP2 over-expression leads to shortened APD, reduced \( \text{Ca}^{2+} \) transients and single cell contractility (Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010), whereas reduction in \( I_{\text{to,f}} \), as routinely occurs early in response to cardiac stress, elevates \( \text{Ca}^{2+} \) transients and cardiac contractility, thereby promoting sustained increases in contractile strength, independent of \( \beta \)-adrenergic stimulation (Kaprielian et al., 1999; Wickenden et al., 1999b; Kaprielian et al., 2002; McCrossan et al., 2004). Consequently, loss of \( \text{Irx5} \) leads to reduced pump function of the heart, although it was notable that suppressed cell shortening exclusively in \( \text{Irx5}^{-/-} \) ENDO myocytes translated into the global reduction in cardiac contractile function. This is consistent with the greater force generating capacity of the sub-endocardium compared to the sub-epicardium under physiological conditions (Gallagher et al., 1985; Schulz and Heusch, 1998). In addition, decreased cardiac contractile functions may be related to asynchronous pumping action of the heart since the regional heterogeneity in electrophysiological properties of
In *Irx5*<sup>-/-</sup> endocardial myocytes, $I_{to,f}$ elevation results in shortened action potential duration (APD), reduced intracellular calcium ([Ca<sup>2+</sup>]$_i$), leading to a decrease in cell contractility.

**Figure 4.2**
*A Schematic Diagram of Reduced Contractility by Loss of Irx5*
In LV endocardial myocytes, loss of *Irx5* results in $I_{to,f}$ elevation, shorten action potential duration (APD), and Ca$^{2+}$ transient reduction, leading to a decrease in cell contractility.
the hearts is required to coordinate and synchronize activation and relaxation of heart muscle, thus ensuring efficient pump function (Kondo et al., 2006; Sengupta et al., 2006a; Sengupta et al., 2006b).

Collectively, our results support the conclusion that \( I_{\text{to,f}} \) elevations underlie decreased contractility in \( Irx5^{-/-} \) mice. However, it is conceivable, particularly since \( Irx5 \) is a transcriptional factor, that the effects of \( Irx5 \)-ablation are \( I_{\text{to,f}} \)-independent. Nevertheless, we found that when mice lacked \( K_{V4.2} \) gene (\( Kcnd2 \)) (Guo et al., 2005), cardiac contractility was unaffected by the loss of \( Irx5 \), establishing the essential role of \( I_{\text{to,f}} \) in the reduced contractility of \( Irx5^{-/-} \) myocardium. The dominant role of \( I_{\text{to,f}} \) in mouse contractility is further supported by the observation that \( K_{V4.2}^{-/-} \) mice showed marked elevations in contractility compared to wild-type. Interestingly, although a previous study had presented that \( I_{\text{to,f}} \) was completely eliminated by loss of \( K_{V4.2} \) (Guo et al., 2005), we, in fact, found that \( I_{\text{to,f}} \) still remains in \( K_{V4.2}^{-/-} \) cardiomyocytes with an approximately 80% reduction, compared to littermate controls (see Appendix B). This result becomes more intriguing because ~80% reductions in \( I_{\text{to,f}} \) are sufficient to eliminate \( I_{\text{to,f}} \)-dependent effects, emphasizing the potent role of \( I_{\text{to,f}} \) in contractility. The elevated contractility in \( Irx5^{-/-};K_{V4.2}^{-/-} \) mouse hearts should be related to eliminated, or very low density of \( I_{\text{to,f}} \), thereby leading to prolonged APD, increased \( Ca^{2+} \) transients and enhanced cardiomyocyte contractility. Our preliminary results showed that APDs (\( APD_{20}, APD_{50}, \) and \( APD_{75} \)) were significantly prolonged in \( K_{V4.2}^{-/-} \) ENDO myocytes, compared with \( K_{V4.2}^{+/+} \) ENDO cells (data not shown; Jie Liu, unpublished data). However, a previous study has claimed that APD is indistinguishable between \( K_{V4.2}^{+/+} \) and \( K_{V4.2}^{-/-} \) LV apex myocytes (Guo et al., 2005). While this discrepancy might come from regional difference (i.e. ENDO vs. Apex), further investigation on ECC in \( K_{V4.2}^{-/-} \) and \( Irx5^{-/-};K_{V4.2}^{-/-} \) mouse hearts will be required to resolve this difference.

Taken together, these results strongly suggest that \( Irx5 \) establishes electrophysiological heterogeneities, a key property of synchronous contraction and relaxation, in the ventricle of the mouse heart by regulating \( K_{V4.2} \) potassium-channel gene expression, thereby ensuring cardiac contractile function.

Although our studies were conducted using mice, these results are likely to have relevance to humans and other species for a number of reasons. First, \( Irx5 \) is also expressed in an endocardial-to-epicardial gradient in rats, dogs and humans (Costantini et al., 2005; Rosati et al., 2006; Gaborit et al., 2010). Moreover, \( Irx5 \) mutations found in arrhythmia patients is associated with abnormal KChIP2 expression in the heart (personal communication with Charles
Antzelevitch), and Irx5 can negatively regulate KChIP2 gene expression (John Wylie and Benoit G. Bruneau, unpublished data). Since $I_{to,f}$ heterogeneity in canine and humans is generated by KChIP2, not by $K_V 4\alpha$-subunits (Kaab et al., 1998; Gaborit et al., 2007), these findings suggest that Irx5 also can underlie the heterogeneity of $I_{to,f}$ in human hearts by KChIP2 regulation. Since human and canine myocytes have much longer APD of an order of 500 ms, unlike rodent APD typically around 50 ms, $I_{to,f}$ contributions to APD in human and canine myocytes are relatively small (Greenstein et al., 2000; Sah et al., 2003; Sun and Wang, 2005; Dong et al., 2006), but it rather affects AP notch on early phase 1 which synchronizes and optimizes SR Ca$^{2+}$ release (Lukas and Antzelevitch, 1993; Yu et al., 2000; Antzelevitch and Fish, 2001; Sah et al., 2003). Interestingly, although it has been suggested that reductions in $I_{to,f}$, which leads to a loss of the AP notch, may contribute to impaired contractility seen in patients with heart disease and heart failure (Greenstein et al., 2000; Antzelevitch and Fish, 2001; Sah et al., 2002b; Sah et al., 2003), a recent study has demonstrated that in canine ventricular myocytes, $I_{to,f}$ elevation, inducing prominent AP notch on Phase 1, results in reduction in initial Ca$^{2+}$ influx ($I_{Ca,L}$), Ca$^{2+}$ transients, leading to suppression of contractility (Dong et al., 2010). These workers have also shown that canine hearts have electrical and mechanical heterogeneity across the LV free wall, similar to rodents, strongly suggesting that Irx5 may act as an essential coordinator of the electrical and contractile properties of the human heart. Furthermore, regional differences in $I_{to,f}$, between the epicardium and endocardium also appear to be critical for transmurral re-entry events occurring in the lethal arrhythmias like those seen in the Brugada syndrome (Di Diego et al., 2002; Delpon et al., 2008). Additional studies are clearly necessary to assess the role of Irx5 in physiological and pathological properties of the human heart.

4.3 $I_{to,f}$ regulated by Irx5 Does Not Affect Cardiac Hypertrophy: The Role of $I_{to,f}$ in Hypertrophy

Since $I_{to,f}$ reduction is a hallmark of early electrical remodelling in heart disease (Yao et al., 1999), the modulatory role of $I_{to,f}$ in cardiac hypertrophy has been investigated in many studies (Kassiri et al., 2002; Zobel et al., 2002; Lebeche et al., 2004; Perrier et al., 2004; Jin et al., 2010). For example, $I_{to,f}$ reduction or elimination by over-expressing dominant-negative $K_V 4.2N$ or mutant $K_V 4.2W362F$ subunits in cultured neonatal cardiomyocytes results in
elevations in calcineurin (Cn)-Nfat activity following increased intracellular Ca$^{2+}$ concentration, leading to hypertrophy (Kassiri et al., 2002). This I$_{\text{to,f}}$-mediated hypertrophy is prevented by administration of either a Cn inhibitor, cyclosporin A or a Ca$^{2+}$ channel blocker, verapamil, suggesting that elevations in intracellular Ca$^{2+}$ via L-type Ca$^{2+}$ channels and Cn activity play a central role in hypertrophy after I$_{\text{to,f}}$ reduction (Sah et al., 2002a). In addition, I$_{\text{to,f}}$ elevations by over-expressing Kv4.2, Kv4.3 or KChIP2 can prevent hypertrophy in vitro and in vivo, induced by phenylephrine (PE), angiotensin II (AngII), or pressure-overload, via decreasing Ca$^{2+}$ transient amplitude, which is associated with reduced Cn-Nfat activity (Zobel et al., 2002; Lebeche et al., 2004; Lebeche et al., 2006; Jin et al., 2010). These data suggest that the role of I$_{\text{to,f}}$ in excitation-transcription coupling (ETC) in cardiomyocytes is to affect intracellular [Ca$^{2+}$], as shown in other types of cells such as neuronal cells (Dolmetsch, 2003), smooth muscle cells (Santana and Navedo, 2009), and skeletal myocytes (Schiaffino and Serrano, 2002). Consistent with these studies, Irx5$^{-/-}$ hearts and cultured myocytes, showing reduced hypertrophy, expressed higher levels of Kv4.2 transcript, suggesting that higher I$_{\text{to,f}}$ might diminish Ca$^{2+}$-mediated Cn activity in Irx5$^{-/-}$ heart and cells. However, unlike our initial hypothesis that attenuated hypertrophy by loss of Irx5 would be restored by loss of I$_{\text{to,f}}$, eliminating (or reduction) in I$_{\text{to,f}}$, by Kv4.2-ablation (Guo et al., 2005), had no effect on the reduced hypertrophy in Irx5$^{-/-}$ mice. This suggested that Irx5 modulates hypertrophy via other mechanisms, independent of I$_{\text{to,f}}$. This was initially surprising to me because changes in I$_{\text{to,f}}$ can modulate activation of Cn, possibly as a result of altered Ca$^{2+}$ entry into myocytes (Kassiri et al., 2002; Sah et al., 2002a). Meanwhile, mice lacking Kv4.2 did not develop spontaneous hypertrophy, consistent with a previous report in this mouse model (Guo et al., 2005). More interestingly, no difference in the degree of pressure-overload induced hypertrophy was observed between Kv4.2$^{+/+}$ and Kv4.2$^{-/-}$ mice although it was expected that mice lacking I$_{\text{to,f}}$ would develop greater hypertrophy than WT since I$_{\text{to,f}}$ can modulate hypertrophy (Kassiri et al., 2002; Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010).

These results raise a few important questions with respect to the role of I$_{\text{to,f}}$ in hypertrophy. First, why does reduction or elimination of I$_{\text{to,f}}$ not induce spontaneous hypertrophy in adult heart while causing it in neonatal cardiomyocytes? Specifically, expression of Kv4.2W362F mutant constructs that virtually eliminate I$_{\text{to,f}}$ in dominant-negative manner (Barry et al., 1998), can induce spontaneous hypertrophy in neonatal cardiomyocytes (Kassiri et al., 2002), whereas transgenic mice expressing the identical construct do not exhibit spontaneous
hypertrrophy (Barry et al., 1998). No spontaneous hypertrophy was observed in other \(I_{o,f}\)-deficient mice by loss of \(K_v4.2\) (Guo et al., 2005) or KChIP2 (Kuo et al., 2001). Although there is compensatory up-regulation of \(K_v1.4\), which encodes \(I_{o,s}\) in \(K_v4.2^{−}\) or \(K_v4.2W362F\) transgenic mouse hearts (Barry et al., 1998; Guo et al., 2005), this does not appear to be responsible for the lack of hypertrophy or failure because double transgenic mice lacking both \(K_v4.2\) and \(K_v1.4\) (\(K_v1.4^{−}/K_v4.2W362F\)) have structurally normal hearts, despite significant QT prolongation and frequent ventricular tachycardia (Guo et al., 2000). Therefore, these results suggest a difference of \(I_{o,f}\)-mediated ETC in between neonatal and adult cardiomyocytes. In fact, a similar question has been raised and discussed with respect to the effects of contractile Ca\(^{2+}\) on the Cn-Nfat signaling pathway (Houser and Molkentin, 2008). Houser and Molkentin have suggested that neonatal cardiomyocytes lack specialized cellular microdomains to separate (or insulate) different Ca\(^{2+}\)-dependent signaling pathways, such as protein kinase C (PKC), Ca\(^{2+}/\)calmodulin-dependent protein kinase (CaMK), as well as Cn, from the contractile Ca\(^{2+}\). This assumption is supported by absence of T-tubules or an organized junctional microdomain for SR coupling in neonatal cardiomyocytes (Cohen and Lederer, 1988), along with a diffuse pattern of calcineurin-Nfat (Santana et al., 2002). It is indeed a very plausible model because beating cardiomyocytes involve huge tidal wave of contractile Ca\(^{2+}\), which should naturally be designed to minimize unnecessary contamination of Ca\(^{2+}\)-dependent signaling. Additionally, a few recent studies have provided additional evidence of Ca\(^{2+}\) signaling compartmentalization between ECC and ETC in cardiomyocytes (Balijepalli et al., 2006; Wu et al., 2006; Wu et al., 2009). Therefore, I would propose that lack of insulation of contractile Ca\(^{2+}\) in neonatal cardiomyocytes may account for the different effect of \(I_{o,f}\) on hypertrophy between neonatal and adult cardiomyocytes. However, if there is a compartmentalization of Ca\(^{2+}\) signaling for ETC and ECC, how can increases in \(I_{o,f}\) by over-expressing \(K_v4\) \(\alpha\)-subunit or KChIP2 prevent hypertrophy in the adult mouse heart? (Lebeche et al., 2004; Jin et al., 2010) Although these studies have suggested that \(I_{o,f}\) elevations result in attenuated hypertrophy with shortened APD, reduced Ca\(^{2+}\) transients and decreased contractility, the molecular mechanism(s) affected by \(I_{o,f}\) elevations in hypertrophy has been only tested in an \textit{in vitro} system using neonatal myocytes. In fact, over-expression (gain-of-function) effect could be very different from an endogenous or loss-of-function system. Over-expression of \(K_v4\) channels or KChIP2 might override endogenous Ca\(^{2+}\) regulation, which affects microdomain Ca\(^{2+}\)-dependent signalling. It might be also associated with mis-localization of over-expressed proteins since subcellular localization
and trafficking to membrane of ion channels is tightly regulated by kinesin (An et al., 2000; Chu et al., 2006; Zadeh et al., 2009); several Rab GTPases (Strutz-Seebohm et al., 2006; McEwen et al., 2007; Zadeh et al., 2008) and dynamin (Nesti et al., 2004; Choi et al., 2005). Further studies to examine the difference in \( I_{\text{to,f}} \)-mediated ETC and hypertrophy between neonatal and adult cardiomyocytes will be of interest (See Future Directions 5.2).

Secondly, if reduction in \( I_{\text{to,f}} \), one of early markers in electric remodelling in the diseased heart, does not cause hypertrophy, then what could be the role of \( I_{\text{to,f}} \) reduction in hypertrophy? It may be related to increasing or maintaining contractility under biomechanical stress, as a previous study showed that pressure-overload decreased \( I_{\text{to,f}} \), proceeded hypertrophy (Perrier et al., 2004), which led to APD prolongation. Our preliminary results indicate that cardiomyocyte contractility is significantly augmented at 2 weeks after BAND with prolonged APD and increased \( \text{Ca}^{2+} \) transient amplitude (Jie Liu, unpublished data). Similar results have been reported in various hypertrophy animal models (Nakamura et al., 2001a; McCrossan et al., 2004). Thus, our results and others suggest that \( I_{\text{to,f}} \) reduction followed by APD prolongation with increased contractility is likely to be an adaptive event of cardiac hypertrophy. Indeed, our results support this notion because the contractile function of \( \text{Irx5}^{-/-};K\text{v}4.2^{-/-} \) mice was well maintained after banding without significant hypertrophy while BAND-\( \text{Irx5}^{-/-} \) mice expressing higher \( K\text{v}4.2 \) transcripts showed lower contractile function. Thus, it would be of interest if \( \text{Irx5}^{-/-};K\text{v}4.2^{-/-} \) mice subject to pressure-overload are protected from cardiac dilation which was shown earlier in \( \text{Irx5}^{-/-} \) mice in response to prolonged pressure-overload.

### 4.4 Transcriptional Regulation of \( I_{\text{to,f}} \) by Irx5, Calcineurin-NFAT and Gata4

A previous study has shown that \( \text{Nfatc3} \)-ablation eliminates the \( I_{\text{to,f}} \) transmural gradient by increasing \( K\text{v}4.2 \) and \( I_{\text{to,f}} \) in endocardium (Rossow et al., 2006), which is remarkably similar phenotype to mice lacking \( \text{Irx5} \) (Costantini et al., 2005). Interestingly, the \( \text{Irx5} \) expression gradient is intact in \( \text{Nfatc3}^{-/-} \) mouse hearts (Rossow et al., 2006), suggesting that \( \text{Irx5} \) alone in the absence of \( \text{Nfatc3} \) is not sufficient to evoke heterogeneous \( K\text{v}4.2 \) expression and \( I_{\text{to,f}} \) function across the left ventricular wall. Characterizations of the novel interaction between \( \text{Irx5} \) and \( \text{Nfatc3} \), and modulated \( \text{Cn-Nfat} \) activity by \( \text{Irx5} \) presented in this study may account for these phenotypic resemblances, suggesting that \( \text{Irx5} \) regulates \( K\text{v}4.2 \) gene expression by forming a
transcriptional complex with Nfatc3. Nevertheless, it remains unclear how Irx5 and Cn-Nfat coordinate I_{o,t} regional heterogeneity in the heart. For example, while this study demonstrates that Irx5 positively regulates Nfatc3 expression, Rossow et al. showed that Nfatc3 protein expression is indistinguishable between ENDO and EPI despite intact Irx5 gradients (Rossow et al., 2006). This discrepancy might be associated with differences in physiological versus pathological conditions because an increase in Nfatc3 expression by Irx5 over-expression was more significant in hypertrophic condition. In addition, although Rossow et al. reported that increased calcineurin-Nfat activity in ENDO is constructed by higher intracellular [Ca^{2+}] in ENDO compared to EPI, the mechanism underlying this regional difference in intracellular [Ca^{2+}] remains unclear. Therefore, it would be interesting to investigate whether loss of Irx5 affects regional differences in Cn-Nfat activity and intracellular [Ca^{2+}]. Furthermore, although Nfatc3^{-/-} mouse hearts show elevated I_{o,t} in ENDO myocytes, contractile function of Nfatc3^{-/-} heart has not been mechanistically investigated using in vivo hemodynamic measurements or the Langendorff-perfusion system. A previous study merely presented fractional shortening (FS), but showed no difference between 10-week old Nfatc3^{+/-} and Nfatc3^{-/-} mice (Wilkins et al., 2002). It would be of interest to examine whether loss of Nfatc3 results in reduced cardiac contractility via I_{o,t} up-regulation, similar to Irx5^{-/-} hearts.

Cn-Nfat activity is also associated with I_{o,t} remodelling in the diseased hearts. Rossow et al. have demonstrated that Nfatc3 is necessary for I_{o,t} down-regulation in response to myocardial infarction (Rossow et al., 2004) and β-adrenergic stimulation (Rossow et al., 2009). However, unlike Nfatc3^{+/-} mice, which show complete abrogation of I_{o,t} reduction in MI or hypertrophy, Irx5 appears to be partially necessary, but not essential for I_{o,t} reductions in cardiac hypertrophy, because Kv4.2 mRNA expression obviously declined in Irx5^{-/-} heart and NMVM in response to pressure-overload and adrenergic stimulation. This might be related to compensation of other Iroquois proteins such as Irx3 (see Section 4.6). Meanwhile, higher Kv4.2 mRNA levels in hypertrophied Irx5^{-/-} hearts and NMVM still suggest that Irx5 is an upstream regulator of I_{o,t}.

On the other hand, the molecular mechanism by which Cn-Nfat contributes to I_{o,t} regulation is still a conundrum. For example, expressing constitutively active calcineurin (ΔCnA) in neonatal rat ventricular cardiomyocytes and young CnA mouse hearts (3–4 week old), which induces evident hypertrophy, leads to up-regulation of I_{o,t} (Petrashevskaya et al., 2002; Gong et al., 2006), whereas adult CnA hearts (3 month old) show down-regulation of I_{o,t} (Dong et al., 2003; Gong et al., 2006). Moreover, ΔNfatc3, constitutively active form of Nfatc3, induces two
different results showing $I_{to,f}$ up-regulation and down-regulation in neonatal and adult cardiomyocytes, respectively (Rossow et al., 2004; Gong et al., 2006). This discrepancy may be due to a structural or developmental difference such as $Ca^{2+}$ compartmentalization between neonatal and adult cardiomyocytes, as discussed above (see Section 4.3). It might also be a context-dependent phenomenon between physiological versus pathological conditions or possibly related to other unknown binding partners for Nfat. Nevertheless, it is notable that this inconsistency becomes an opportunity to dissociate the roles of calcineurin-Nfat in hypertrophy as well as in $I_{to,f}$ regulation. Understanding these mechanisms would also help us to comprehend the role of $I_{to,f}$ in hypertrophy. On the other hand, Irx5 does not show this type of inconsistency as Irx5 over-expression results in both hypertrophy and reduction in $I_{to,f}$ in neonatal cardiomyocytes. Thus it would be interesting to examine the role of Cn-Nfat in $I_{to,f}$ regulation in the absence of Irx5.

### 4.5 Regulation of Cardiac Hypertrophy by Irx5 with Calcineurin-Nfat and Gata4

This study provides the first evidence that Irx5 modulates calcineurin-Nfat (and Gata4) activities. The extent of hypertrophy in the Irx5$^{+/}$ mice correlated closely with the extent of Cn-Nfat activation as assessed by Rcan1 levels (Rothermel et al., 2003; Kuwahara et al., 2006; Jin et al., 2010), in both BAND-hearts and NE-treated cultured myocytes. In addition, in Irx5$^{+/}$ mice and cardiomyocytes following hypertrophic stimuli, we observed markedly lower expression in the hypertrophic marker ANF ($Nppa$), which correlates especially strongly with Cn-Nfat activation levels (Taigen et al., 2000; De Windt et al., 2001; Frey et al., 2004a; Frank et al., 2007; Alarcon et al., 2008; Bourajjaj et al., 2008; Prasad and Inesi, 2009). Collectively, these results suggest that Irx5 is a modulator of Cn-Nfat signaling in cardiac hypertrophy. Consistent with this possibility, Irx5 over-expression increases Rcan1 expression, indicating higher calcineurin-Nfat activity. Moreover, over-expression of Irx5 positively regulates Nfatc3 (and Gata4) expression levels. These results might account for greater hypertrophy in myocytes over-expressing Irx5, because Nfatc3 or Gata4 over-expressions can induce hypertrophy in vivo and in vitro (Molkentin et al., 1998; Molkentin, 2000a; Liang et al., 2001; van Rooij et al., 2002; Akazawa and Komuro, 2003). More importantly, the Nfatc3 (and Gata4) elevations induced by
hypertrophic stimuli were also found to require Irx5, and Irx5 and Nfatc3 (along with Gata4) co-assemble in a transcriptional complex. These findings support the model of Cn-Nfat (and Gata4) regulation by Irx5 shown in Figure 4.1. This model is further underscored by a number of observations such as the attenuation of constitutively active Cn-induced hypertrophy in Irx5−/− mouse hearts and cultured myocytes, which was restored in myocytes by Irx5 over-expression. The functional relevance of this relationship between Irx5, Cn-Nfat and Gata4 is also emphasized by elevations in cardiac Irx5 levels in response to hypertrophic stimuli. These observations are consistent with microarray and RNA sequencing results showing that Irx5 expression is elevated in the heart failure patients with dilated cardiomyopathy (Beisvag et al., 2006) as well as in hypertrophied hearts induced by Goq over-expression (Matkovich et al., 2010). Taken together, hypertrophic stimuli result in up-regulation of Irx5, Cn-Nfat, and Gata4 expression and activity, thereby forming a critical signalling complex for regulating the hypertrophic response of the heart. Counter-intuitively, however, in the absence of hypertrophic stimuli, hearts and cardiomyocytes lacking Irx5 expressed higher levels of Nfatc3 and Gata4 compared to WT. This may be compensatory feedback since the lower contractility of Irx5−/− hearts would activate various adaptive responses. In fact, we found that Irx5−/− mice have higher plasma concentration of norepinephrine (NE) than WT (Figure 4.3), which is often observed in heart failure patients with impaired contractile function (Lamba and Abraham, 2000; Kaye and Esler, 2005). It also suggests that basal levels of Nfatc3 and Gata4 may be controlled by other cardiac transcription factors. Moreover, it is worth emphasizing that, despite elevated expressions of Nfatc3 and Gata4 as well as higher plasma NE level, Irx5−/− hearts, as well as myocytes, did not show spontaneous hypertrophy, further underlining the conclusion that Irx5 is necessary, but not sufficient for cardiac hypertrophy. Nevertheless, it should be noted that expression levels of Nfatc3 and Gata4 do not always correlate with their transcription activities since nuclear translocation is required for their transcriptional regulations (Hogan et al., 2003; Wang et al., 2004; Arminan et al., 2009; Oliveira et al., 2009). In addition, it is not known whether Irx5 translocates to the nucleus in response to hypertrophic stimulation. Although reduced Rcan1 mRNA expression indirectly reports lower transcriptional activity of Nfat in Irx5−/− hearts and myocytes under hypertrophic stimulations, future experiments will be necessary to further examine the molecular mechanism of how Irx5 can affect nuclear translocations of Nfatc3 and Gata4 as well as transcriptional activity and DNA binding activity (see Future Directions 5.1).
Figure 4.3
Plasma Concentrations of Catecholamine in Irx5 Mice
Plasma catecholamine assay was conducted on blood collected from 10 - 12 week old Irx5 mice. Irx5<sup>−/−</sup> mice exhibited significantly higher plasma concentration level of norepinephrine, compared to Irx5<sup>+/+</sup> mice. n = 5 per group; *, P < 0.04 vs. Irx5<sup>+/+</sup> mice
When receiving biomechanical stress (i.e. pressure-overload), hearts develop greater hypertrophy in sub-endocardium (ENDO) than in sub-epicardium (EPI) (Omens et al., 1996). This pattern of hypertrophic responses is also shown by preferential re-expression of ANF or BNP (Prestle et al., 1999; Wong et al., 2000; Dosch et al., 2001) and the earlier modification of myosin heavy chain (MHC) expression from α-MHC to β-MHC (Toffolo and Ianuzzo, 1994) in ENDO. Moreover, given that Irx5 levels correlate with Cn-Nfat activities, which are higher in the ENDO (compared to EPI) (Rossow et al., 2006), we expected to find regional gradients of fibrosis and hypertrophy within WT hearts subjected to pressure-overload. However, no differences in interstitial fibrosis could be resolved, suggesting that the Irx5 levels in the epicardium may not be sufficiently low to impair Cn-Nfat activities to levels seen in the Irx5^{-/-} hearts. Differences in myocyte cross-sectional area also did not seem to differ between regions of the WT BAND heart, similar to a previous study (Hill et al., 2000), although this comparison was hampered by systematic changes in fiber orientation across the ventricular free wall (Ashikaga et al., 2007).

4.6 Loss of Irx5 Leads to Early Decompensation under Biomechanical Stress

Although cardiac hypertrophy is considered as a compensatory response, sustained hypertrophy causes adverse responses such as sudden death or progression to heart failure (Frey and Olson, 2003). Accordingly, a number of previous studies have focused on inhibition of hypertrophy to reduce unfavourable outcomes (Frey et al., 2004b). However, despite reduced hypertrophy, Irx5^{-/-} hearts subjected to pressure-overload developed early onset of dilated cardiomyopathy accompanied by deteriorated contractile function, severe interstitial fibrosis as well as elevated cardiac remodelling markers such as β-MHC and Sk-α-actinin. Though the cause and mechanism of this earlier decompensation in the Irx5^{-/-} mice are unclear, Cn-Nfat activation is not only critical for hypertrophy, but generally provides potent cardio-protection in conditions of both acute and chronic cardiac stress (De Windt et al., 2000; Heineke et al., 2010b), despite being detrimental when persistent and excessive activation (Molkentin et al., 1998). Specifically, mice lacking Nfatc3 or mice treated with a Cn inhibitor CsA, have reduced hypertrophy, but undergo cardiac decompensation (contractile dysfunction and dilation) (Meguro et al., 1999; Wilkins et al., 2002), suggesting that Cn-Nfat activation in Irx5^{-/-} mice may also be
important for appropriate adaptive responses. While activation of Cn-Nfat can prevent apoptosis (De Windt et al., 2000), no difference in \( \text{H}_2\text{O}_2 \)-induced cell death between \( \text{Ir}x5^{+/−} \) and WT was observed, unlike a previous study showing that loss of \( \text{Ir}x5 \) results in apoptosis in prostate cancer cells (Myrthue et al., 2008). Thus, this suggests that dilated cardiomyopathy in \( \text{Ir}x5^{+/−} \) mice subjected to pressure-overload may be independent of apoptosis in cardiomyocyte, similar to a previous study (De Windt et al., 2000). Although it was not tested in this study, it is possible that apoptosis in non-cardiomyocytes can be a cause of failing myocardium (Takemura et al., 1998; De Windt et al., 2000; Hayakawa et al., 2003; Khoynezhad et al., 2007). On the other hand, severe interstitial fibrosis in BAND-\( \text{Ir}x5^{+/−} \) hearts may be associated with impaired up-regulation of ANF (\( \text{Nppa} \)) in response to hypertrophic stimuli, as ANF acts as anti-fibrotic factor (Nishikimi et al., 2006). For example, ANF treatments inhibit DNA synthesis as well as collagen synthesis stimulated by angiotensin II or endothelin-1 in cardiac fibroblasts (Fujisaki et al., 1995; Maki et al., 2000), whereas disruption of guanylyl cyclase (GC) A, a natriuretic peptide receptor, results in cardiac hypertrophy and severe interstitial fibrosis in mice (Lopez et al., 1995; Oliver et al., 1997).

Early onset of cardiac decompensation in BAND-\( \text{Ir}x5^{+/−} \) mice may be associated with Gata4, as \( \text{Ir}x5 \) interacts with Gata4. Under pressure-overload, cardiac-specific Gata4-deficient mice or mice heterozygous for deletion of the second exon of Gata4 (\( \text{G4D} \)) develop cardiac decompensation and dilation with increased fibrosis and apoptosis (Bisping et al., 2006; Oka et al., 2006). This rapid decompensation in Gata4 mutant mice has been shown to be related to angiogenesis because Gata4 in cardiomyocytes functions as a regulator of angiogenesis in mouse heart. Loss of Gata4 attenuates pressure overload-induced increase in angiogenesis, leading to rapid decompensation (Heineke et al., 2007), suggesting that loss of \( \text{Ir}x5 \) might abrogate the Gata4-mediated adaptive response to biomechanical stress. It will be of interest to examine whether \( \text{Ir}x5 \) ablation can affect angiogenesis required for compensatory hypertrophy (Izumiya et al., 2006). Taken together, these results suggest that \( \text{Ir}x5 \) provides cardio-protection against biomechanical stress by facilitating the activation of the Cn-Nfat- and Gata4-mediated signalling pathways.

Furthermore, lower contractile function may contribute to the early failure of \( \text{Ir}x5^{+/−} \) hearts because cardiac hypertrophy compensates for demands of the heart by decreasing wall tension as well as increasing contractility (Nakamura et al., 2001a; Frey and Olson, 2003; McCrossan et al., 2004). Indeed, a causal role of depressed contractility in the initiation of heart failure has been
shown in various animal studies (Bailey and Houser, 1992; Bailey and Houser, 1993; Lorell, 1997; Ito et al., 2000; DiPaola et al., 2001; Houser and Margulies, 2003). On the contrary, increased contractility by elevated thyroid hormone (Trivieri et al., 2006), a high Ca$^{2+}$ affinity mutant of SERCA2a (Nakayama et al., 2003), or loss of PKC-α (Braz et al., 2004) can protect against heart failure induced by pressure overload. These results are consistent with Irx5$^{-/-}$;Kv4.2$^{-/-}$ mice following BAND for 2 weeks; despite attenuated hypertrophy, they maintained cardiac contractility (i.e. +dp/dt and LVESP) and even relaxation (LVEDP), compared to Irx5$^{-/-}$ mice. This suggests that increased contractility by loss of Kv4.2 may attenuate decompensation by Irx5 ablation. Thus, it would be of interest whether Irx5$^{-/-}$;Kv4.2$^{-/-}$ mice subjected to pressure-overload are protected from cardiac decompensation, which was shown earlier in Irx5$^{-/-}$ mice in response to prolonged pressure-overload.

4.7 Loss of Irx5 Does Not Completely Abolish the Hypertrophic Response: Compensatory Mechanism for loss of Irx5

It is notable that previous studies have shown that, remarkably similar to Irx5$^{-/-}$, Nfatc3-ablation eliminates the I$_{o,f}$ transmural gradient by increasing Kv4.2 and I$_{o,f}$ in endocardium (Rossow et al., 2006) while impairing the hypertrophic response and reductions in I$_{o,f}$ to a number of stimuli (i.e. TAC, myocardial infarction, β-adrenergic stimulation and ΔCnA over-expression) (Wilkins et al., 2002; Rossow et al., 2004; Rossow et al., 2009). However, unlike Nfatc3$^{-/-}$ mice, which show complete abrogation of Kv4.2 mRNA down-regulation and I$_{o,f}$ reduction in response to myocardial infarction (Rossow et al., 2004) and β-adrenergic stimulation (Rossow et al., 2009), Kv4.2 mRNA expression levels were mildly but significantly reduced in both BAND-hearts and NE-treated cardiomyocytes from Irx5$^{-/-}$ mice, albeit to a much lesser extent than in WT littermates. Furthermore, similar to Nfatc3$^{-/-}$ mice (Wilkins et al., 2002), loss of Irx5 in mice could not completely block hypertrophy induced by pressure-overload, adrenergic stimulation and calcineurin activation. These results might argue that Irx5 is not essential for I$_{o,f}$ reductions and hypertrophic responses, but this may be due to compensation or functional redundancy of other Iroquois proteins as all Irx members are expressed in the heart (Christoffels et al., 2000b). For example, the loss of Irx4 in the heart results in compensatory up-regulation of Irx2 gene expression (Bruneau et al., 2001a). Although no significant changes in
other Irx members are observed in Irx5+/− hearts (Thesis of Danny L. Costantini), it is possible that Irx3, which has a similar expression pattern to Irx5 (Rosati et al., 2006; He et al., 2009), may compensate for the loss of Irx5. In fact, we found that over-expression of Irx3 was able to increase Nfatc3 and Gata4 protein and mRNA expressions (Figures 3.23 and 4.4A). Irx3 was also able to bind to Nfatc3 and Gata4 (Figure 4.4B; Unpublished data, Vijitha Puvvidran and Chi-chung Hui). Mouse hearts lacking Gata4 show decreased Irx3 expression, suggesting a possible relationship of these TFs (Oka et al., 2006). Moreover, we observed that Irx3 over-expression in serum-cultured NMVM was able to repress Kv4.2 mRNA (Kcnd2) expression to a similar extent to Irx5 over-expression, while mice lacking Irx3 showed increases in Kv4.2 gene in LV freewall without affecting LV transmural heterogeneity of Kv4.2 (Figure 4.5).

Furthermore, cardiac specific over-expression of a repressor form of Irx3 can induce severe hypertrophy (Unpublished data, Jieun Kim and Dr. Chi-chung Hui), although the mechanism of action has not been completely understood yet. These results strongly suggest that Irx3 may play a similar role to Irx5 in regulating the electrical properties as well as the hypertrophic response of the heart. Furthermore, since Irx5 can bind to other Irx members (He et al., 2009) (unpublished data, Vijitha Puvvidran and Chi-chung Hui), several Irx genes might perform overlapping essential functions and perhaps act in a combinatorial manner. A further characterization of the role of Irx3 in the heart will be the subject of a future study.
Figure 4.4

*Irx3 is Associated with Cardiac Transcription Factors, Nfatc3 and Gata4*

(A) *Irx3* over-expression in cardiomyocytes cultured in 10% serum-media results in elevations in *Nfatc3* and *Gata4* mRNA expression. n = 3 – 4 per group; *, P < 0.05 vs. *Ad-GFP.* (B) Anti-*Irx3* antibody enriches *Nfatc3* while anti-HA antibody shows no precipitation. Arrowhead indicates *Nfatc3* band. This data suggests a physically interaction between *Irx3* and *Nfatc3.*
**Figure 4.5**

*Kv4.2 Gene Expression (Kcnd2) is Negatively Regulated by Irx3 in Cardiomyocytes*

(A) *Irx3* over-expression in cardiomyocytes cultured in 10% serum conditions led to down-regulation of *Kcnd2* mRNA expression while neonatal myocytes lacking *Irx3* resulted in its up-regulation. n = 4 – 6 per group; *, P < 0.05 vs. Ad-GFP; #, P < 0.05 vs. Ad-*Irx3*. (B) *Irx3−/−* mice lacking *Irx3* (*Irx3−/−*) showed elevated *Kcnd2* mRNA level in both endocardial (ENDO) and epicardial (EPI) myocytes compared to littermate controls while transmural gradients was intact. n = 5 per group; *, P < 0.05 vs. *Irx3*+/+; #, P < 0.05 vs. ENDO.
4.8 Other Possible Mechanisms of Irx5 in Cardiac Hypertrophy

The hypertrophic growth program is accompanied by gene remodelling that is controlled by numerous cardiac transcription factors, which commonly form transcription complexes (Akazawa and Komuro, 2003; Frey and Olson, 2003). For instances, Nfat and Gata4 are known to physically interact with Mef2 (Molkentin et al., 1998; Morin et al., 2000; van Oort et al., 2006), which is one of the key transcriptional mediators in cardiac hypertrophy (van Oort et al., 2006; Xu et al., 2006; Kim et al., 2008b). In addition, the evolutionally conserved homeobox transcription factor Csx/Nkx2-5, which is indispensible for cardiac development and disease (Prall et al., 2002; Akazawa and Komuro, 2003; Akazawa and Komuro, 2005), interacts with other transcription factors such as Gata4 (Durocher et al., 1997; Lee et al., 1998; Shiojima et al., 1999) and SRF (Chen and Schwartz, 1996), thereby modulating its transcriptional activity. Therefore, identification of an interaction between Irx5 and Nkx2-5 suggests a possible transcription complex regulating hypertrophic genes. Irx5-Nkx2-5 may contribute to protecting the heart and prevent cardiac decompensation in response to hypertrophic stimuli (Toko et al., 2002). In addition, like Nfatc3 and Gata4, Irx5 might positively regulate Nkx2-5 expression since up-regulation of Nkx2.5 is observed in hypertrophied hearts induced by pressure-overload and adrenergic stimulation (Thompson et al., 1998; Saadane et al., 1999; Kontaraki et al., 2007). Future investigations about possible transcription complex with Irx5 and identification of target genes will be necessary to further understand the molecular mechanism of Irx5 in normal and diseased hearts (see Future Directions 5.1).

Regulation of hypertrophy by Irx5 may be associated with microRNA (Cordes and Srivastava, 2009; Mishra et al., 2009; Small et al., 2010). Specifically, a recent study has demonstrated that miR-1-2, one of the two miR-1 family members, negatively regulates Irx5, so that loss of miR-1-2 in mouse heart leads to an increase in Irx5 expression (Zhao et al., 2007). Several studies have also shown that miR-1 is an important regulator of cardiomyocyte hypertrophy because miR-1 attenuates hypertrophy via down-regulation of Cn-Nfat activity as well as Gata4 and Mef2a expressions, whereas loss of miR-1 induces cellular hypertrophy in neonatal cardiomyocytes (Ikeda et al., 2009). Notably, miR-1 is down-regulated in pressure-overload induced hypertrophy (Sayed et al., 2007; Luo et al., 2008) and ΔCnA-induced heart failure (Ikeda et al., 2009), which is expected to increase Irx5 (Zhao et al., 2007), thereby possibly reinforcing the actions of miR-1 to regulating hypertrophy.
Lastly, Irx5 may affect cardiac hypertrophy by regulating cardiomyocyte cell cycle signals. Although adult myocytes are withdrawn from cell cycle and not capable of proliferation, cell cycle regulators are important for hypertrophy development (Sadoshima et al., 1997; Tamamori et al., 1998; Nozato et al., 2000; Ahuja et al., 2007). Several studies have demonstrated that the cyclin-dependent kinase inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ are crucial for hypertrophic growth of cardiomyocytes (Li et al., 1998; Hauck et al., 2007; Hauck et al., 2008), and their expression is down-regulated in pressure overload-induced hypertrophy (Li and Brooks, 1997). These observations are very interesting because $p21$ expression is elevated by silencing $Irx5$, while down-regulated by $Irx5$ over-expression, in human prostate cancer cells (Myrthue et al., 2008). In addition, as cell cycle regulators (e.g. c-Myc) that control hyperplastic growth can mediate hypertrophic growth in adult post-mitotic myocytes (Ahuja et al., 2007), Irx5 appears to be associated with cardiomyocyte proliferation in developing heart. Although mice lacking $Irx5$ did not show any signs of morphological defects in the heart, ablation of both $Irx5$ and $Irx3$ led to embryonic death and often showed narrow ventricular chamber indicating hypoplasia (Unpublished data, Jieun Kim and Dr. Chi-chung Hui). On the other hand, loss of miR-1-2, which up-regulates Irx5 expression, results in hyperplastic heart with increased myocyte proliferation, although underlying exact mechanism is still unknown (Zhao et al., 2007).

Therefore, as elevated $Irx$ genes are frequently found in various cancers, Irx5 might mediate cell cycle signaling that is essential for hypertrophy. In addition, it would not be too surprising to find even if Irx5 is involved in signaling pathways for both hypertrophy and cancerogenesis, as previously suggested that “There are numerous parallels between signaling pathways that drive tumorigenesis and signaling pathways that regulate hypertrophic responses and survival in cardiomyocytes” (Hoshijima and Chien, 2002; Cheng and Force, 2010). Truly, Cn-NFAT signaling pathways, which is modulated by Irx5, have been recently recognized as central players in the development of a number of very different human maligancies, indicating their role in cell cycle (Buchholz and Ellenrieder, 2007). Future studies to investigate if Irx5 modulates cell cycle with calcineurin-NFAT pathways in both cardiac hypertrophy and cancer will be of interest.
CHAPTER 5

FUTURE DIRECTIONS
5.1 Molecular Mechanisms of Irx5

5.1.1 Transcription Factor Interactions of Irx5 in the Heart

The molecular interaction of Irx5 presented in this thesis clearly identified Irx5 as a novel partner of various cardiac transcription factors (TF) in transcription complexes. Specifically, since the calcineurin (Cn)-Nfat activity is reduced by loss of Irx5, it was intriguing to identify a novel interaction between Irx5 and Nfatc3 in cardiomyocytes. Although Co-IP experiments in a heterologous system showed Irx5 directly binds to Gata4, potentially via a homeodomain (HD) in N-terminus of Irx5, it is not known whether Irx5 directly binds to Nfatc3 or indirectly interacts with Nfatc3 via other cardiac TFs such as Gata4. This can be tested by performing Co-IP experiments in heterologous system expressing both Irx5 and Nfatc3 or by a GST pull-down assay. Irx5 deletion constructs, used for Irx5-Gata4 Co-IP experiments, should also be useful to examine the structural importance of the HD and/or Iro-box (IRO) of Irx5 in protein-protein interaction. In addition, it will be necessary to confirm Irx5-Nfatc3 interaction in endogenous cardiomyocyte in the absence or presence of hypertrophic stimuli.

Since the development of cardiovascular system is extremely interrelated and requires precise gene regulation, mapping and understanding the interactions among cardiac TFs are very important (Bruneau, 2002; Bruneau, 2008). In addition to Nfatc3, Gata4, and Nkx2-5, we have found that Irx proteins can interact with other cardiac TFs, strongly suggesting that Irx is profoundly involved in the cardiac transcription network during development and disease. To explore the interaction network, our group (Drs. Backx and Chi-chung Hui) are currently performing a mass spectrometer-based screen to identify proteins that are associated with Irx3. *Irx3<sup>3myc-6his</sup>* knock-in mouse line that expresses Irx3 fused with three myc-tags (EQKLISEEDL) and six histidines will be utilized. Since several evidence has claimed that Irx3 plays a similar role to Irx5, identified TFs from high-throughput results can be reconfirmed individually with Irx5. These investigations will provide insight into the transcriptional network of Iroquois TF.

5.1.2 Transcriptional Regulation of Irx5

We have previous shown that Irx5 represses Kv4.2 gene (*Kcnd2*) promoter activity (Costantini et al., 2005). In addition, results presented in this thesis also suggested that some cardiac genes such as ANF (*Nppa*), β-MHC (*Myh7*), and Rcan1 (*Rcan1*) appeared to be affected by Irx5 in the presence of hypertrophic stimulation, suggesting that a number of cardiac genes...
may be regulated by Irx5. Nevertheless, far less is known about Irx5’s target genes, regulatory DNA-binding sequences as well as a mechanism of transcriptional regulation. As summarized in Table 5.1, each cardiac TF can impact differently on each gene. For example, *Nppa* expression can be elevated by Irx5, NFAT, and Gata4, whereas *Kcnd2* expression is repressed by Irx5 and/or NFAT (Rossow et al., 2004; Costantini et al., 2005; Rossow et al., 2006; Rossow et al., 2009), but activated by Gata4 (Jia and Takimoto, 2003). To understand the transcription mechanisms of Irx5 with NFAT and Gata4, it will be necessary to perform promoter activity assays using *Nppa, Kcnd2, Myh7*, and *Rcan1* reporter constructs with different compositions of these TF expressions. In addition, transient transfection assays in cultured native (i.e. neonatal cardiomyocytes) or heterologous cell-lines should demonstrate whether these regulations are cardiac-specific (Costantini et al., 2005).

### Table 5.1

*Gene Regulations by Cardiac Transcription Factors*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Irx5</th>
<th>NFAT</th>
<th>Gata4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF (<em>Nppa</em>)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-MHC (<em>Myh7</em>)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rcan1 (<em>Rcan1</em>)</td>
<td>+</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>Kv4.2 (<em>Kcnd2</em>)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

‘+’ indicates for transcription activation while ‘–’ presents suppression; N.D., not determined.
Iroquois TFs can act as activators or repressors depending on context (Bao et al., 1999; Bruneau et al., 2000; Matsumoto et al., 2004). Since Irx5−/− hearts under hypertrophic stimuli showed reductions in Nppa and Rcan1, but an elevation in Myh7, it is possible that Irx5 may act as an activator on Nppa and Rcan1 while serving as a repressor on Myh7. However, this possibility can be very complicated because I have observed that a repressor form of Irx3 is able to activate gene expression by repressing a potential repressor. Therefore, to further gain mechanistic insight of Irx5 transcriptional mechanisms, dominant Irx5 activator (VP16-Irx5) and dominant Irx5 repressor (EnR-Irx5) can be generated by fusing Irx5 to VP16 activation domain and the Engrailed suppressor domain, respectively. NMVM infected with adenoviral forms of these constructs will reveal how these target genes are regulated by Irx5 either as an activator or repressor. Furthermore, microarray experiments on these cells would provide high-throughput outcomes of candidate genes that are specifically regulated by Irx5 in the heart. Recently, our group successfully conducted these types of experiments for Irx3.

There are many unanswered questions with regard to the actions of Irx5. Although several putative Iroquois consensus sequences have been suggested (Bilioni et al., 2005), it is not known whether Irx5 requires a direct interaction with DNA for transcriptional regulation. Despite evident transcriptional regulation of Irx5 on Kcnd2, no cis-element has been identified. As it has been suggested that the HD and IRO are required for DNA binding and protein-protein interaction (Cavodeassi et al., 2001; Matsumoto et al., 2004), our results and others suggest that the C-terminal of Irx5, possibly IRO, is required for Kcnd2 transcriptional regulation, while the N-terminal of Irx5, HD, is necessary for interaction with Gata4 (Jia and Takimoto, 2003). However, the roles of these domains are not clear yet. Further studies on Irx5 proteins and other cardiac transcription factors, as well as detailed analysis of target gene promoters, will be necessary to reveal mechanisms of Irx5.

5.2 The Role of I_{to,f} in Excitation-Contraction and Excitation-Transcription Couplings

I_{to,f} as a modulator of cardiac hypertrophy has been demonstrated in a number of studies (Wickenden et al., 1999b; Kassiri et al., 2002; Sah et al., 2002a; Zobel et al., 2002; Lebeche et al., 2004; Jin et al., 2010). However, the present study showed that reduced (or eliminated) I_{to,f}
by loss of Kv4.2 in adult mouse hearts, despite enhancing ECC (i.e., higher contractility and intracellular Ca\(^{2+}\)), did not result in spontaneous hypertrophy or difference in hypertrophic response to pressure-overload compared to control mice. Moreover, with respect to the impact of \(I_{\text{to,f}}\) down-regulation on hypertrophy (Table 5.2), it was noted from results in previous studies and the present study that lack of spontaneous hypertrophy in Kv4.2\(^{-/-}\) mice might be due to a difference between neonatal and adult myocytes, suggesting that hypertrophic signaling in adult cardiomyocytes could be much less affected by \(I_{\text{to,f}}\) reduction (see Section 4.3). Thus it will be of interest to investigate the role of \(I_{\text{to,f}}\) in ECC and ETC (i.e. hypertrophy) and to examine whether there is a difference of ECC and ETC in between neonatal and adult cardiomyocytes.

<table>
<thead>
<tr>
<th>Source of (I_{\text{to,f}}) Down-regulation</th>
<th>Spontaneous Hypertrophy</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMVM (In vitro)</td>
<td>Adult Heart (In Vivo)</td>
</tr>
<tr>
<td>K(_{\text{V4.2N}})</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K(_{\text{V4.2W362F}})</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>K(_{\text{V4.2}})</td>
<td>N.D</td>
<td>–</td>
</tr>
<tr>
<td>KChIP2(^{-/-})</td>
<td>N.D</td>
<td>–</td>
</tr>
</tbody>
</table>

K\(_{\text{V4.2N}}\), a dominant-negative N-terminal fragment of the Kv4.2 Channel; K\(_{\text{V4.2W362F}}\), a point mutation at position 362 in the pore region of Kv4.2 Channel; K\(_{\text{V4.2}}\)\(^{-/-}\), targeted deletion of Kv4.2 gene; KChIP2\(^{-/-}\), genetic deletion of KChIP2 gene. ‘+’ indicates for spontaneous hypertrophy while ‘–’ presents no hypertrophy; NMVM, neonatal mouse ventricular myocytes; N.D., not determined.
To examine whether neonatal mouse ventricular myocytes (NMVM) are more susceptible to hypertrophy induced by \( I_{\text{to,f}} \) reduction, \( K_v 4.2^{-/-} \) NMVM can be used. I expect that, contrary to \( K_v 4.2^{-/-} \) adult heart, cultured \( K_v 4.2^{-/-} \) NMVM will develop spontaneous hypertrophy or higher propensity to cellular hypertrophy in response to adrenergic stimulation, compared to WT cells. Patch-clamp experiments can be performed to examine \( I_{\text{to,f}} \) and ECC on these cells. Since it is believed that hypertrophy induced by reduced \( I_{\text{to,f}} \) is mediated by Cn-Nfat (Kassiri et al., 2002; Sah et al., 2002a; Zobel et al., 2002; Jin et al., 2010), measuring Nfat activity is most direct way to quantify activated ETC. Specifically, generation of double transgenic mice by breeding \( K_v 4.2^{-/-} \) mice with NFAT-luciferase reporter mice should be an exceedingly useful tool to determine \( I_{\text{to,f}} \)-mediated ETC in cultured NMVM, the postnatal heart as well as the adult heart (Wilkins et al., 2004).

In contrast to \( I_{\text{to,f}} \) reduction, it might be possible to decrease Ca\(^{2+}\) for ETC using over-expression of plasma membrane Ca\(^{2+}\)-ATPase isoform 4 (PMCA4) that pumps Ca\(^{2+}\) out of the cell. It has been shown that PMCA4 over-expression does not affect ECC in the adult heart while it reduces ETC activity (i.e. Cn-NFAT) and hypertrophy (Wu et al., 2009). However, it has not been tested if PMCA4 over-expression affects ECC in neonatal myocytes. Thus, if neonatal myocytes lack Ca\(^{2+}\) compartmentalization for ECC and ETC, PMCA4 over-expression in myocytes would negatively affect both ECC and ETC.

I expect these experiments will be able to determine whether there are differences in the role of \( I_{\text{to,f}} \) in ECC and ETC between neonatal and adult myocytes. If so, further studies may be needed to identify the mechanism behind the discrepancy. Ultimately, uncovering differences in neonatal and adult cardiomyocytes will promote valuable information that is required for stem cell-based regenerative strategies for heart failure.

### 5.3 Transmural Gradients of Ca\(^{2+}\)/Calcineurin-NFAT activity

Rossow et al. have reported that regional differences in Cn-Nfate3 activities establish \( I_{\text{to,f}} \) transmural gradients in the heart. They also have shown that this heterogeneity of Cn-Nfate3 activity is not generated by Nfate3 protein expression, which are uniform across the wall, but by higher intracellular Ca\(^{2+}\) in ENDO compared to EPI. However, the mechanism to build the regional difference in intracellular Ca\(^{2+}\) remains unclear. While the \( I_{\text{to,f}} \) gradient definitely
contributes to Ca\textsuperscript{2+} influx by modulating APD (Sah et al., 2002a), previous studies have demonstrated that regional differences in AP profiles partially account for differences in intracellular Ca\textsuperscript{2+}, and the rest of which are contributed to by ECC components proteins such as SERCA2a (ENDO < EPI), NCX (ENDO < EPI), and RyR2 (ENDO > EPI) (Laurita et al., 2003; Xiong et al., 2005; Dilly et al., 2006). As Irx5 is one of a few transcription factors known to express with a transmural gradient, it would be of interest to investigate whether Irx5 may regulate transcripts of those Ca\textsuperscript{2+} regulatory proteins, thereby, combined Ito,f regulation, globally contributing to electrophysiological heterogeneity in the heart.

5.4 The Role of Irx5 in the Physiological Hypertrophy

Cardiac hypertrophy is induced by not only pathological stimuli, but also physiological stimuli such as exercise and pregnancy (Selvetella et al., 2004; Heineke and Molkentin, 2006; Catalucci et al., 2008). Previous studies have demonstrated that exercise-induced physiological hypertrophy does not require Cn-Nfat activation (Wilkins et al., 2004) while loss of Gata4 results in reduced hypertrophy following exercise stimulation (Oka et al., 2006). Since Irx5 modulates Cn-Nfat (and Gata4) signaling in pathological hypertrophy, it is natural to ask if Irx5 plays a similar role in development of the physiological hypertrophy. Interestingly, a recent study has shown that Irx5 expression is not altered by voluntary exercise-induced hypertrophy while Ito,f is mildly reduced (Stones et al., 2009), suggesting that Irx5 may not be involved in physiological hypertrophy. Nonetheless, in the future it will be interesting to investigate the role of Irx5 in physiological hypertrophy induced by exercise or pregnancy.
APPENDIX A

DISSECTION OF THE
VOLTAGE-ACTIVATED K⁺ OUTWARD CURRENTS
IN ADULT MOUSE VENTRICULAR MYOCYTES

: $I_{to,f}$, $I_{to,s}$, $I_{K,slow1}$, $I_{K,slow2}$, & $I_{ss}$
A.1 Abstract

Voltage-activated outward K\(^+\) currents (I\(_{Kv}\)) are essential for cardiac repolarization and are major factors in the electrophysiological remodeling and arrhythmias seen in heart disease. Mouse models have been useful for understanding cardiac electrophysiology. However, previous methods for separating and quantifying the components of I\(_{Kv}\) in mouse myocardium have yielded inconsistencies. In this study, we developed a statistically rigorous method to uniquely quantify various I\(_{Kv}\) in adult mouse ventricular myocytes, and concluded that tri-exponential functions as well as depolarizing pulses with durations greater than 20 seconds are essential to adequately separate the different I\(_{Kv}\) components. This method enabled us to reliably dissect the kinetic components of the decay phase of I\(_{Kv}\) into fast (I\(_{to}\)), intermediate (K\(_V\)1.5-encoded I\(_{K,slow1}\)) and slow (K\(_V\)2-encoded I\(_{K,slow2}\)) components. The most rapid kinetic phase, I\(_{to}\), can be further dissected into fast (K\(_V\)4-encoded I\(_{to,f}\)) and slow (K\(_V\)1.4-encoded I\(_{to,s}\)) components by measuring recovery from inactivation, voltage-dependence of activation and sensitivity to HpTx-2 and 4-AP. The applicability of our dissection method was validated using transgenic mice over-expressing dominant-negative K\(_V\)1.1 transgene which largely abolished the 4-AP-sensitive portion of I\(_{to}\) (i.e. I\(_{to,s}\)) and the I\(_{K,slow1}\) component. This method should prove useful in future electrophysiological studies using mouse.

A.2 Introduction

Voltage-activated K\(^+\) currents (I\(_{Kv}\)) are major determinants of the cardiac action potential profile. Because altered repolarization occurs in heart disease and is linked to arrhythmias, I\(_{Kv}\) have been extensively studied in humans and animal models, particularly in mice which can be genetically manipulated. Previous mouse studies attempted to separate and quantify the components of I\(_{Kv}\) by fitting the decay phase current traces lasting 4 – 5 seconds to either bi-exponential (Xu et al., 1999a; Xu et al., 1999b; Wang et al., 2007; Niwa et al., 2008; Haim et al.) or tri-exponential functions (Zhou et al., 2003; Costantini et al., 2005). While these previous studies have been very helpful in identifying the underlying molecular basis of I\(_{Kv}\) in mouse, the number of kinetic components in mouse ventricular cardiomyocytes remains uncertain leading to inconsistencies in the molecular identification of various kinetic components of I\(_{Kv}\) (Xu et al., 1999b; Zhou et al., 2003; Costantini et al., 2005; Guo et al., 2005).
We find that step depolarizations lasting 4 – 5 sec are too short to accurately estimate the kinetics and magnitude of the components making up $I_{Kv}$. By using established curve-fitting algorithms along with statistically rigorous criteria for separating and quantifying multiple overlapping first-order kinetic processes (i.e. exponential time-courses), we find that $I_{Kv}$ currents in response to depolarizing pulses lasting greater than 20 seconds allow reliable separation of $I_{Kv}$ into fast, intermediate and slow components, corresponding to a Ca$^{2+}$-independent transient outward K$^+$ current ($I_{to}$), a rapidly activating, slowly inactivating K$^+$ current ($I_{K,slow1}$), and a slowly inactivating outward K$^+$ current ($I_{K,slow2}$), respectively. The most rapid kinetic fraction of $I_{Kv}$ can be further dissected into fast ($I_{to,f}$) and slow ($I_{to,s}$) components by assessing recovery from inactivation, voltage-dependence of activation and sensitivity to both HpTx-2 and 4-AP. Our approach is validated using a transgenic mice over-expressing dominant-negative Kv1.1 transgene (Kv1DN) which selectively eliminates the intermediate component and $I_{to,s}$.

A.3 Methods

A.3.1 Isolation of Adult Mouse Ventricular Myocytes

Left ventricular (LV) myocytes were obtained from 8 – 12 week old adult male C57B6 mice (35 animals, Charles River) as well as adult male Kv1DN (13 animals) in a CD1 background (bred in our animal facility) using an isolation procedure described previously (Costantini et al., 2005). All experimental protocols conformed to the standards of the Canadian Council on Animal Care. Mice were anesthetised with 2.5% isoflurane, and hearts were rapidly removed and retrogradely perfused with Ca$^{2+}$-free Tyrode’s solution [(mmol/L) 137 NaCl, 5.4 KCl, 1.0 MgCl$_2$, 0.33 NaH$_2$PO$_4$, 10 D-glucose, 10 HEPES, pH 7.4] at 37°C through aorta for 3 – 4 min. After perfusing the heart with collagenase (1.0 mg/mL, Worthington) for 10 – 12 min, endo- and epicardial layers were dissected from the LV free wall followed by gentle trituration to dissociate cardiomyocytes. Cells were stored in Krebs-bicarbonate solution [(mmol/L) 120 potassium glutamate, 20 KCl, 20 HEPES, 1.0 MgCl$_2$, 10 D-glucose, 0.5 K-EGTA, and 0.1% bovine serum albumin, pH 7.4] at 4°C until use.
A.3.2 Patch Clamp Electrophysiology

Voltage-activated K⁺ currents from isolated adult mouse ventricular myocytes were recorded with the whole-cell patch clamp technique under voltage-clamp mode, using Axopatch 200B amplifier and pClamp 9 software (Axon Instrument, CA, USA). Cardiomyocytes which were Ca²⁺ tolerant and rod-shaped were selected and superfused with bath solution for 15 minutes before electrophysiological recording at room temperature (21 – 24°C). The bath solution contained (mmol/l): 140 NaCl, 4 KCl, 1 MgCl₂, 1.2 CaCl₂, 10 HEPES, 10 D-glucose, and 0.3 CdCl₂ (pH 7.4). The pipette resistance ranged between 1.2 – 2.0 MΩ when filled with a pipette solution containing (mmol/l): 120 potassium aspartate, 20 KCl, 1 NaCl, 1 MgCl₂, 5 MgATP, 10 HEPES, and 10 EGTA (pH 7.2). Appropriate concentrations of recombinant heteropodatoxin-2 (HpTx-2) (Zarayskiy et al., 2005) and 4-aminopyridine (4-AP; Sigma, St. Louis, MO) were used in the bath solution. To examine the effect of tetraethylammonium (TEA; Sigma) in I_Kv, NaCl in the bath solution was replaced by equimolar TEA chloride. The pH was verified after pharmacological inhibitors were added. After membrane rupture, cell capacitance was measured by integrating capacitance transient in response to 10 mV steps from a holding potential of -50 mV and used to normalize current magnitudes. Cell capacitance and series resistance were then electronically compensated by 85%. Voltage-gated K⁺ currents were measured by 5 – 25 second depolarizations from a holding potential of -80 mV to +60 mV, the reversal potential of Na⁺. To examine recovery from steady-state inactivation, a double-pulse protocol was used. Cells were first depolarized to +60 mV for 20 sec (pre-pulse), subsequently hyperpolarized to the holding potential of -80 mV for various times ranging from 10 ms to 6 sec, and then stepped to +60 mV for another 5 – 20 sec (test-pulse) to activate the currents and assess the extent of recovery in the same cells (Wickenden et al., 1999a). To ensure complete recovery, minimum 30 sec interval was given between sweeps. In addition, to ensure reversal potential through the measurements, we measured I_K1 as a reference in between pulses as it is very sensitive to K⁺ concentration and subsarcolemmal accumulation (Piao et al., 2007; Jost et al., 2009).

A.3.3 Data Analysis of Outward K⁺ Currents and Statistics

The electrophysiological recording data were analyzed using pClamp software (Clampfit 10.0, Axon, CA, USA) and Prism 5 (GraphPad Software, San Diego, CA, USA). The decay
phase of outward K⁺ currents was fitted to a sum of exponentials with various terms (n) from 2 to 4 using the following equation:

$$F(t) = \sum_{i=1}^{n} A_i \times \exp\left(-\frac{t}{\tau_i}\right) + A_s$$

in which $A_i$ is the amplitude of the kinetic component decaying with a time constant of $\tau_i$, and $A_s$ is the amplitude of the steady-state, non-inactivating component. $\sum_{i=1}^{n}$ represents the sum over the “n” components.

Recovery from steady-state inactivation was estimated for each of the kinetic components by fitting the decay phase of $I_{Kv}$ traces in a pre-pulse and a subsequent test-pulse applied after a variable period following repolarization to -80 mV. The magnitude of each kinetic component observed in the test-pulse was normalized by (i.e. divided by) the magnitude of the corresponding kinetic component in the pre-pulse ($I_o$) and plotted as a function of the repolarization time to yield the fractional recovery. These results were fit with the biphasic equations:

$$\frac{I(t)}{I_o} = A_1 \cdot [1 - \exp(-t/\tau_1)] + A_2 \cdot [1 - \exp(-t/\tau_2)]$$

where $A_1$ and $A_2$ are the proportions of the recovery that occur with time constants of $\tau_1$ and $\tau_2$, respectively, and $A_1 + A_2 = 1$. When $\tau_1$ and $\tau_2$ were statistically identical, the recovery data was fit according to a mono-exponential equation (Wickenden et al., 1999a; Volk et al., 2001).

Conductance ($G$) was estimated for the various kinetic components using the equation:

$$G(V_m) = \frac{I(V_m)}{(V_m - E_{rev})}$$

where $I(V_m)$ is the current measured at the applied membrane potential, $V_m$, and $E_{rev}$ is the calculated Nernst potential (i.e. -90.7 mV) for K⁺ ions under our experimental conditions. The conductance as a function of the applied step potential, $V_m$, was fit with a multi-component Boltzmann equation:

$$G(V_m) = G_{max} \times \sum_{i=1}^{n} A_i \times (1 + \exp((-V_{1/2,i} - V_m) / k_i))$$

where $G_{max}$ is the maximum conductance, and $A_i$, $V_{1/2,i}$, $k_i$ represent the amplitude, membrane voltage for 50% activation, and slope factor for “i-th” component. $\sum_{i=1}^{n}$ represents the sum over the number of components, “n”. The amplitudes were constrained by the equation: $A_1 + A_2 + \ldots + A_n = 1$. 
Parameter estimation for all fits to data was based on the goodness-of-fit method using the reduced Chi-square ($\chi^2_{\text{red}}$) statistic (David M. Glover, 2005) defined as:

$$\chi^2_{\text{red}} = \frac{\chi^2}{v} = \sum_{i=1}^{n} \frac{(O_i - E)^2}{(E \times v)}$$

where $\sum_{i=1}^{n}$ is the sum over the “i” observations, $O_i$, $E$ is the estimated mean of $O$ and $v$ is the number of degrees of freedom. When $\chi^2_{\text{red}} \leq 1$, we concluded that an adequate fit was achieved.

To assess the number of components providing the statistically best fit to the data, we utilized the F-distribution test ($P < 0.05$) using the $T$-statistic which is defined as:

$$T = \frac{\text{SSE}_i - \text{SSE}_{i+1}}{\text{SSE}_{i+1}} \cdot \frac{n - k_{i+1}}{k_i}$$

where $\text{SSE}_i$ is $\sum_{i=1}^{n} = (O_i - E)^2$, $k_i$ and $n$ are the number of parameters and observations, respectively. The value of $T$ calculated from fits with different model equations was compared using the standard $F$-distribution (Rao, 1973).

When comparing the estimates of measurements between groups, we used the Student’s $t$ test or one-way analysis of variance combined with the Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered statistically significant. Results are expressed as mean ± S.E.M.

A.4 Results

A.4.1 K⁺ current Curve Fitting and Model Selection

Because the expression of $I_{Kv}$ is known to vary between regions of the myocardium, we first present data from myocytes obtained from the endocardium of the LV free wall. Results for LV epicardial myocytes are presented later for comparison. In our analysis, we assumed that the components of mouse $I_{Kv}$ inactivate exponentially (Xu et al., 1999b; Rossow et al., 2004; Costantini et al., 2005) and that membrane depolarization durations needed to be ~5-fold longer than the time constant of decay of the slowest kinetic component. Based on preliminary recordings, we initially conjectured (justified below) that a depolarizing pulse lasting 25 seconds would be sufficiently long to allow accurate quantification of the slowest kinetic component(s) present in the mouse endocardial $I_{Kv}$. We then analyzed these 25 sec traces in order to identify the $I_{Kv}$ components by fitting the traces with functions comprised of 2 – 4 exponential
components. **Figure A.1** shows typical results of these fits on a slow (A) and fast (B) time scale (original current trace in black, best fits in red, the individual components in green). The difference between the current trace and the fit (i.e. the residual), shown in blue, demonstrates that fits with a 2-exponential function are poor relative to either 3- or 4-exponential fits. Indeed, as summarized in **Figure A.2A**, only 24 out of 106 traces (one trace per cell) showed $\chi^2_{\text{red}} \leq 1$ when 2-exponential functions was used, while $\chi^2_{\text{red}}$ was $\leq 1$ for all traces when 3- and 4-exponential fits were used. The inadequacy of the 2-exponential fit compared to 3- and 4-exponential fits is further demonstrated by comparing the correlation coefficients (R) for the different fits (**Figure A.2B**).

The results above support the conclusion that adult mouse ventricular myocytes generally have at least three resolvable kinetic components. Since fitting experimental data with functions containing more parameters will invariably improve the "goodness-of-fit" (See Methods), we next sought to determine how many components are required to achieve the statistically best fit to the data. Applying the $F$-distribution test to the least squares fitting statistic (See Methods) revealed that 56 out of 106 traces were better fit with 4-exponential than a 3-exponential function. However, further analysis of the distribution of time constants (predicted from our fits) shown in **Figure A.2C**, suggests that $I_{Kv}$ has 3 kinetic components. Specifically, there is far more spread in time constant distributions with 4-exponential fits compared to 3-exponential fits with five clusters of time constants being observed with 4-exponential fits: 3 clusters matching the time constants seen with the 3-exponential fits (albeit with greater spread), one very broad cluster with time constants below 10 ms and one cluster with very long time constants. Thus, the concordance of kinetic estimates between myocytes is actually reduced when 4-exponential fits are used. Since the molecular constituents and biophysical properties underlying the different kinetic components of $I_{Kv}$ are expected to be the same between myocytes, these observations support the conclusion that $I_{Kv}$ in mouse endocardial myocytes are comprised of 3 separable and distinct kinetic components. It is worth noting that the time constants derived from the 2-exponential fits also show a larger spread compared to the 3-exponential fits and are found midway between the time constants estimated from the 3-exponential fits.

The findings above support the conclusion that $I_{Kv}$ in adult mouse endocardial myocytes is comprised of 3-exponential with the slowest time constants invariably being ~5 seconds in duration. Thus, depolarizations steps lasting 4 – 5 seconds, as used previously (Zhou et al., 1998;
Xu et al., 1999b), are not expected to be long enough to allow accurate estimation of the various kinetic components of $I_{Kv}$. Indeed, changing the pulse durations (5 – 25 seconds) revealed systematic inaccuracies in the magnitude (Figure A.3B) and kinetics (Figure A.3C) of the $I_{Kv}$ components, with clear convergence when the duration was above 20 seconds. In addition, the sustained (non-inactivating) $K^+$ current magnitude, $I_{SS}$, was overestimated with 5 second pulses (i.e. $5.21 \pm 0.70$ pA/pF; $P < 0.05$), compared to 25 sec pulse ($3.12 \pm 0.30$ pA/pF), as might be expected since the current has only declined by ~75% after 5 seconds. These results demonstrate that pulse durations greater than 20 seconds are required to correctly quantify the components of the $I_{Kv}$ in mouse ventricular myocytes.
Figure A.1

**Curve Fitting Comparison with 2 – 4 Exponential Models.** (A) Representative figures of $I_{KV}$ traces fitted with the sum of 2, 3, and 4 exponentials are demonstrated with a current trace (black), a fitted trace (red), fitted components (green) and a residual (blue), suggesting that $I_{KV}$ are well described with 3- and 4-exponential fittings while 2 exponential fitting led to incomplete dissection of outward $K^+$ currents, which is visually illustrated in the enlarged peak current traces (B).
Figure A.2

$I_{K_v}$ is Well Described with a Sum of 3-Exponentials

The reduced Chi-squared values (A) and the correlation coefficient $R$ (B) of 2 – 4 exponential functions statistically evaluates the goodness of fit, indicating that 3- and 4-exponential function ($X^2 \leq 1$ and $R \geq 0.98$) adequately fit $I_{K_v}$ traces well while 2-exponential function leads to improper fitting. (C) the distribution of time constants estimated from 2 – 4 exponential fits shows that 3-exponential fits lead to a tight clustering of the time constants into 3 distinct groups.
A

B

C

Inactivation Time Constant (ms)

Depolarization Duration (sec)

Density (pA/pF)

**Inactivation Time Constant (ms)**

**Depolarization Duration (sec)**

**Density (pA/pF)**

**A**

**B**

**C**

* * *
Figure A.3
Comparisons of Depolarization Durations in Curve Fitting

(A) Tri-exponential fittings on different time points at 5, 15 and 25 sec are displayed with a current trace (black), a fitted trace (red), fitted components (green) and a residual (blue). (B) Depolarization duration affects the amplitudes of $I_{\text{p0}}$, $I_{\text{K,slow1}}$, and $I_{\text{K,slow2}}$ estimated from the 3-exponential fit that becomes converged with longer pulse duration. These data clearly demonstrates that $I_{\text{K,slow2}}$ and $I_{\text{SS}}$ are overestimated while $I_{\text{K,slow1}}$ is underestimated in the shorter pulse duration. (C) Inactivation time constants of each component are systematically underestimated by short depolarizations. *, $P < 0.05$ vs. 25 sec.
A.4.2 Kinetic Dissection of $I_{to}$ into $I_{to,f}$ and $I_{to,s}$

The most rapidly inactivating component ($I_{to}$) in mouse endocardial myocytes had a time constant ($\tau$) of $68 \pm 2$ ms ($n = 106$) and a current density of $15.9 \pm 0.8$ pA/pF. Based on previous reports (Oudit et al., 2001; Patel and Campbell, 2005), we speculated that the fastest component represents a transient outward $K^+$ current which, on the basis of recovery from inactivation kinetics, is reported to be comprised of a fast ($K_V4$-encoded $I_{to,f}$) and a slow ($K_V1.4$-encoded $I_{to,s}$) currents in human (Wettwer et al., 1994) and ferret (Brahmajothi et al., 1999), rat (Wickenden et al., 1999a; Volk et al., 2001). Although previous studies have attempted to dissect mouse $I_{to,f}$ and $I_{to,s}$ using fits to $I_{Kv}$ inactivation (Xu et al., 1999b; Brunet et al., 2004), the inactivation kinetics of these currents in other species are very similar (Wickenden et al., 1999a; Oudit et al., 2001). Therefore, we assessed the contribution of $I_{to,f}$ and $I_{to,s}$ to the rapid kinetic component of $I_{Kv}$ by examining the recovery from inactivation properties of $I_{to}$. As shown in Figure A.4A, increasing the duration of repolarization to -80 mV, following a pre-pulse to +60 mV causes progressive increases in the current density measured in response to a second depolarizing test-pulse to +60 mV. Fitting the currents in the pre- and test-pulse with 3-exponential functions allows the recovery properties of $I_{Kv}$ to be quantified by plotting the relative amplitude of each kinetic component in the test-pulse as a function of the repolarization (recovery). These plots are shown in Figure A.4 for $I_{to}$, $I_{K,slow1}$ and $I_{K,slow2}$. Figure A.4B clearly establishes that $I_{to}$ in endocardial myocytes is comprised of fast ($33 \pm 3$ ms, $n = 8$) and slow ($1869 \pm 457$ ms) recovering components with the rapid recovering component ($I_{to,f}$) accounting for ~71% of $I_{to}$. Consistent with the presence of fast ($I_{to,f}$) and slow ($I_{to,s}$) components, the conductance-voltage relationships of $I_{to}$ revealed the existence of two components with half-maximum values for activation (i.e. $V_{1/2}$) of $-6.2 \pm 1.2$ and $28.6 \pm 3.1$ mV (Figure A.5E, see below for more details), which match closely those reported previously for $K_V1.4$-encoded $I_{to,s}$ and $K_V4.2$-encoded $I_{to,f}$, respectively, in the presence of Cd$^{2+}$ (see Discussion) (Agus et al., 1991; Wickenden et al., 1999c). In addition, the fraction of the current activating with a $V_{1/2}$ of ~28 mV (i.e. $I_{to,f}$-like) is 0.78 which matches closely the rapidly recovering fraction of $I_{to}$. These findings support the conclusion that $I_{to}$ is comprised of $I_{to,f}$ and $I_{to,s}$.

To further characterize and quantify $I_{to}$, we examined the effects of HpTx-2, a specific inhibitor of $K_V4$-based $I_{to,f}$ (Sanguinetti et al., 1997) and low concentrations of 4-AP, which preferentially inhibits $I_{to,s}$ (Clement-Chomienne et al., 1999; Faivre et al., 1999). As summarized in Figure A.5F, HpTx-2 at 5 µM, which should completely block $I_{to,f}$ (Sanguinetti et al., 1997;
Zarayskiy et al., 2005), reduced ($P < 0.01$) the density of the rapid $K_V$ current, $I_{o}$ by $78.7 \pm 3.9\%$ ($n = 9$) without affecting ($P > 0.22$) the intermediate ($I_{K,slow1}$) and slow ($I_{K,slow2}$) $I_{Kv}$ components. Consistent with preferential block of $I_{to,f}$, the fraction of $I_{o}$ blocked by $HpTx-2$ was comparable to the amplitude (70.7%) of the rapidly recovering portion of $I_{o}$ (i.e. $I_{to,f}$) Moreover, after application of 5 µM $HpTx-2$, the relationship of conductance-voltage relationship could be best fit ($P < 0.05$) using a single component Boltzmann function with a $V_{1/2}$ value of -7.0 ± 2.1 mV, thereby eliminating the current activating at ~28 mV seen in non-treated myocytes (Figure A.5E).

Because 4-AP has been shown to preferentially block $K_V1$ channels with an $IC_{50} \approx 30 – 100$ µM, compared to $K_V4$ channels with an $IC_{50} \approx 1.5$ mM (Clement-Chomienne et al., 1999; Faivre et al., 1999), we examined the effects of 4-AP on $I_{o}$. As expected (see below), treatment with 250 µM 4-AP blocked 47.7 ± 8.6% of $I_{o}$ (Figure A.6F). Moreover, the conductance-voltage relationship of $I_{o}$ was now well fit ($P < 0.05$) using a single Boltzmann function with a $V_{1/2}$ of 23.6 ± 0.7 mV (Figure A.6, D and E). These results are consistent with the elimination of $I_{to,s}$ by 250 µM 4-AP and the partial blockade of $I_{to,f}$. Higher doses of 4-AP caused progressive reductions in $I_{o}$ density (Figure A.6F), as expected from 4-AP block of $K_V4$-encoded $I_{to,f}$ at higher concentrations. We also examined the affects of 4-AP on the recovery from inactivation. These studies were predicted to eliminate the slow recovering component of $I_{o}$. While the results are consistent with the preferential loss of $I_{to,s}$, these results are complicated by the appearance of an overshoot in the recovery (Figure A.7), which reflect state-dependent binding properties of 4-AP to $I_{to,f}$, as shown previously (Campbell et al., 1993; Tseng et al., 1996). It should be noted that this level of 4-AP also potently blocked most of the intermediate kinetic component ($I_{K,slow1}$) of $I_{Kv}$, as discussed further in the next section. Taken together, our results are consistent with the conclusion that the $I_{to}$ component of $I_{Kv}$ in mouse endocardial myocytes is comprised of ~70% $K_V4$-based $I_{to,f}$ with the remainder being $K_V1.4$-based $I_{to,s}$.

### A.4.3 Properties of $I_{K,slow1}$ and $I_{K,slow2}$

We found that the intermediate kinetic component of $I_{Kv}$ in mouse endocardial ventricular myocytes (i.e. $I_{K,slow1}$) decayed with a time constant of 803 ± 21 ms ($n = 106$) and had a current density of 21.3 ± 1.2 pA/pF. In addition, Figure A.4C shows that the recovery from inactivation of $I_{K,slow1}$ was best fit ($P < 0.05$) with bi-exponentials with recovery time constants, 349 ± 91 ms (54.2%) and 5412 ± 1941 ms (46.8%). While this could suggest that $I_{K,slow1}$ is comprised of
currents generated by two different channels, currents generated by Kv1.5 channels (Li et al., 2004), the likely molecular candidate for IK,slow1 (London et al., 2001; Li et al., 2004) as discussed below, shows two components for recovery from inactivation (Kurata et al., 2005). Consistent with this, Figure A.6F shows that IK,slow1 was potently inhibited (P < 0.05) by 250 mM 4-AP, a dose that potently inhibits Kv1.5 channels (Bouchard and Fedida, 1995). Collectively, these results establish that the intermediate component of the IK in mouse endocardial myocytes are highly sensitive to 4-AP, consistent with this current originating from Kv1.5 channels (See Discussion).

The slowest kinetic component of IK (IK,slow2) had a decay time constant of 5334 ± 128 ms (n = 106) and a density of 11.7 ± 0.5 pA/pF. This component recovers from inactivation with single time constant of 422 ± 25 ms (Figure A.4D). A previous report using bi-exponential fits showed that a portion of IK,slow2 is TEA-sensitive and is generated by Kv2.X channels (Xu et al., 1999a). Consistent with this, Figure A.8 reveals that 50 mM TEA blocks 49 ± 7% of IK,slow2, while having more modest effects on Ito and IK,slow1. By contrast, IK,slow2 is completely insensitive to HpTx-2 (Figure A.5F) and relatively insensitive to 4-AP (Figure A.6F). Indeed, IK,slow2 is the only current that remains after co-treatment with HpTx-2 and 4-AP (Figure A.9).

A.4.4 K+ Current Dissection of Kv1DN Mouse Ventricular Myocytes

To interrogate the applicability of our dissection method, we analyzed the IK in endocardial myocytes from transgenic mice (Kv1DN) over-expressing a truncated Kv1.1 polypeptide in the myocardium (London et al., 1998a). Because the truncated Kv1.1 peptide assembles into heteromultimeric complexes with other α-subunits of Kv1.X-subfamily including Kv1.5 and Kv1.4 (Babila et al., 1994; Folco et al., 1997), Ito,s and IK,slow1 are expected to be reduced or eliminated by a dominant-negative mechanism (London et al., 1998a; Brunner et al., 2001; Zhou et al., 2003). IK in Kv1DN myocytes could be adequately fit with either 2-exponential functions in 5 of 21 myocytes (Figure A.10A) or 3-exponential functions in the remaining myocytes (Figure A.10B). In the myocytes fit with 2-exponential functions, IK,slow1 was absent while 3-exponential fits yielded estimates of IK,slow1 amplitudes that were reduced (P < 0.05) markedly (to 5.5 ± 1.5 pA/pF) compared to littermate control myocytes (30.4 ± 4.4 pA/pF). When present in Kv1DN myocytes, IK,slow1 had a decay time constant of 925 ± 115 ms which did not differ (P = 0.97) from WT myocytes (919 ± 109 ms) and was eliminated by low levels of 4-AP (Figure A.11B). Although IK,slow2 was not altered in Kv1DN myocytes (Figure
A.10C), the $I_{o}$ density was paradoxically slightly increased ($P < 0.05$) to $38.5 \pm 3.7$ pA/pF compared to littermate controls, $27.9 \pm 2.9$ pA/pF. These increases in $I_{o}$ appeared to result from elevated $I_{o,f}$ because $5 \, \mu$M of HpTx-2 virtually eliminated $I_{o}$ in $K_{v1DN}$ myocytes (Figure A.11C). This conclusion was further supported by the observation that $I_{o}$ recovery from inactivation $K_{v1DN}$ myocytes showed a mono-exponential time course with a time constant of $21 \pm 1$ ms ($n = 5$) which did not differ ($P = 0.35$) from the fast time constant in WT myocytes ($24 \pm 2$ ms) (Figure A.10E). In addition, the conductance-voltage of $I_{o}$ was best fit ($P < 0.05$) by a single Boltzmann function with $V_{1/2} = 22.8 \pm 0.5$ mV ($n = 5$), corresponding to that of $I_{o,f}$ (Figure A.10F). Furthermore, the time constant of $I_{o}$ decay ($\tau_1 = 42 \pm 4$ ms) in $K_{v1DN}$ myocytes was reduced ($P < 0.05$) compared to WT myocytes ($64 \pm 6$ ms) (Figure A.10D), suggesting slightly different inactivation properties of $I_{o,f}$ and $I_{o,s}$ (see Discussion). These findings are consistent with the conclusion that $I_{o}$ in $K_{v1DN}$ ventricular myocytes is almost exclusively comprised of $I_{o,f}$.

A.4.5 $I_{K_{v}}$ in Mouse Epicardial Ventricular Myocytes

Since regional differences of $I_{K_{v}}$ is invariably observed across species (Antzelevitch and Fish, 2001), we also examined $I_{K_{v}}$ in epicardial myocytes from the LV free wall. As in the endocardial myocytes, all $I_{K_{v}}$ traces in epicardial myocytes were also best fit using 3-exponential function (data not shown). Importantly, we found that all $I_{K_{v}}$ components ($I_{o}$, $I_{K_{s,slow1}}$, $I_{K_{s,slow2}}$ and $I_{ss}$) of epicardial myocytes showed higher densities ($P < 0.01$) than in endocardial myocytes (Table A.1), similar to previous studies (Xu et al., 1999b; Brunet et al., 2004). On the other hand, relative amplitude of each component was not different between regions, suggesting that proportions of the molecular components are similar in cardiomyocytes from LV free wall. One notable difference in epicardial, compared to endocardial, myocytes is faster ($P < 0.05$) inactivation rate and the monophasic recovery from inactivation of $I_{o}$ (i.e. $\tau = 33 \pm 7$ ms, $n = 11$) (data not shown), suggesting that $I_{o,f}$ is a dominant form of $I_{o}$ in epicardial myocytes, consistent with previous studies (Xu et al., 1999b; Brunet et al., 2004; Costantini et al., 2005) and even other species such as rat (Wickenden et al., 1999a; Volk et al., 2001) and ferret (Brahmajothi et al., 1999). However, this conclusion is complicated by the presence of ‘overshoot’ in the recovery from inactivation, which has been reported in human epicardial myocytes (Wettwer et al., 1994).
**Figure A.4**  

**I_{Kv} Recovery from Steady-State Inactivation.** (A) Representative superimposed $I_{Kv}$ traces for recovery from inactivation. The initial phase of recovery of the currents is shown on an expanded time scale (inset). (B) $I_{to}$ recovery from inactivation was described by bi-exponential fitting with two distinct fast ($A_1$) and slow ($A_2$) components, corresponding to $I_{to,f}$ and $I_{to,s}$. $I_{K,slow1}$ (C) and $I_{K,slow2}$ (D) were fitted with bi- and mono-exponential fittings which recovery time constants were also comparable to $K_v1.5$- or $K_v2.1$-encoded currents, respectively.
Figure A.5

The Effects of HpTx-2 on $I_{Kv}$

(A) Representative $K^+$ current traces before and after HpTx-2 (5 μM) treatment are demonstrated. (B) HpTx-2 sensitive current was obtained using arithmetic subtraction of insensitive trace from control, showing that a rapid, $I_{to,f}$-like, current is blocked by HpTx-2. Peak current is shown with expanded time scale on the right panel. (C) Representative $K^+$ current traces in the absence (left) or presence (right) of HpTx-2 elicited by a series of pulses from −40 to +60 mV with 10 – 15 mV increments. (D) Conductance-voltage ($G_{max} – V$) relationship of $I_{to}$ with or without HpTx-2 (5 μM). (E) Normalized conductance -voltage ($G / G_{max} – V$) relationship of $I_{to}$ was well described by bi-Boltzman equation using different half-maximum values for activation ($V_{1/2}$) of $I_{to,f}$ and $I_{to,s}$. Administration of HpTx-2 eliminated $V_{1/2}$ of $I_{to,f}$, leading to monophasic $G / G_{max} – V$ relationship. (F) HpTx-2 selectively decreased $I_{to}$ without affecting $I_{K,slow1}$ and $I_{K,slow2}$.

Figure A.6

The Effects of 4-AP on $I_{Kv}$

(A) Representative $I_{Kv}$ traces before and after 250 μM of 4-AP application are displayed. (B) 4-AP sensitive current is obtained using arithmetic subtraction of insensitive trace from control. Peak current is shown with expanded time scale on the right panel. (C) Representative $I_{Kv}$ traces in the absence (left) or presence (right) of 4-AP elicited by a series of pulses from −40 to +60 mV with 10 – 15 mV increments. (D) Conductance-voltage ($G_{max} – V$) relationship of $I_{to}$ revealed that approximately 50% of $I_{to}$ is blocked by 4-AP. (E) 4-AP eliminates $V_{1/2}$ corresponding to $I_{to,s}$, leading to monophasic $G / G_{max} – V$ relationship of $I_{to}$. (F) 4-AP dose-dependently reduced current density of all $I_{Kv}$ components. $I_{K,slow1}$ is the most sensitive to 4-AP.
Figure A.7
4-AP induced Overshoot in the Recovery From Inactivation of $I_{to}$
(A) Representative superimposed $I_{Kv}$ traces for recovery from inactivation in the presence of 4-AP (250 µM). (B) $I_{to}$ recovery from inactivation shows overshoot of $I_{to}$ recovery in the test-pulse which phenomenon was previously described as “reverse use-dependent” behaviour.
Figure A.8

*The Effect of TEA on $I_{Kv}$*

(A) Representative $I_{Kv}$ traces before and after 50 mM of TEA administration are displayed. (B) Very slow inactivating, $I_{K, slow2}$-like, component was found to be TEA sensitive current. (C) Indeed, TEA (50 mM) selectively reduced $I_{K, slow2}$, compared to $I_{to}$ and $I_{K, slow1}$.
Figure A.9
The Effect of 4-AP and HpTx-2 Co-Treatments on $I_{KV}$

(A) Representative $I_{KV}$ traces before and after both HpTx-2 (5 µM) and 4-AP (250 µM) administrations are displayed. (B) Rapid inactivating currents were found to be HpTx-2 and 4-AP sensitive current. (C) $I_{K,slow2}$ was relatively insensitive to concurrent administrations of 4-AP (250 µM) and HpTx-2 (5 µM) while these selectively reduced $I_{to}$ and $I_{K,slow1}$. 
**A**

- Time Constant (ms)
  - $\tau_1 = 35 \text{ ms (81\%)}$
  - $\tau_2 = 7456 \text{ ms (19\%)}$

- Voltage range:
  - +60 mV
  - -80 mV

**B**

- Time Constant (ms)
  - $\tau_1 = 64 \text{ ms (81\%)}$
  - $\tau_2 = 1011 \text{ ms (4\%)}$
  - $\tau_3 = 6084 \text{ ms (15\%)}$

- Current Density: 10 pA/pF, 4 sec
- Conductance: 10 pA/pF, 400 ms

**C**

- Current Density (pA/pF) vs. Current (I/I0)
  - Control
  - $K_v1DN$

**D**

- Time Constant (ms)
  - I\_to
  - I\_K,s\_slow1
  - I\_K,s\_slow2

**E**

- Normalized Current (I/I0) vs. Recovery Time (ms)
- $\tau (\text{ms}) = 21 \pm 1$

**F**

- Normalized I\_to Conductance vs. Voltage (mV)
- $V_{1/2} = 22.8 \pm 0.5 \text{ mV}$

163
Figure A.10

\( I_{Kv} \) in \( K_v1DN \) Cardiomyocytes

(A) Representative \( I_{Kv} \) trace of \( K_v1DN \) cardiomyocytes fitted with 2-exponential function (5 out of 21) are displayed with 25 (left) and 2 (right) sec time scale, suggesting the intermediate component (\( I_{K,slow1} \)) is eliminated. (B) On the other hand, the rest of \( I_{Kv} \) traces in \( K_v1DN \) cardiomyocytes are fitted with 3-exponentials. (C) \( I_{Kv} \) density in \( K_v1DN \) cardiomyocytes reveal that \( I_{K,slow1} \) density is significantly reduced while \( I_{to} \) density is increased. No effect is observed in \( I_{K,slow2} \). *, \( P < 0.05 \) vs. Control. (D) Inactivation rate of \( I_{to} \) is faster in \( K_v1DN \) cardiomyocytes, compared to littermate control while decay time constants of \( I_{K,slow1} \) and \( I_{K,slow2} \) are indistinguishable. (E) \( I_{to} \) recovery from inactivation is well described by mono-exponential fitting with fast time constant of 21 ± 1 ms, suggesting that \( I_{to,s} \) is eliminated, leaving only \( I_{to,f} \) in \( K_v1DN \) myocytes. (F) This conclusion is supported by monophasic \( G/G_{max} – V \) relationship with \( V_{1/2} \) at 22.8 ± 0.5 mV, which is similar to that shown in 4-AP treated cells.
Figure A.11

**Pharmacological Inhibitors on $I_{Kv}$ in $Kv1DN$ Cardiomyocytes**

(A) In $Kv1DN$ cardiomyocytes, $I_{Kv}$ fitted 2-exponential function is virtually lacking 4-AP sensitive current, whereas small 4-AP sensitive current is observed in the 3-exponential fitted $I_{Kv}$ (B). (C) Representative $I_{Kv}$ of $Kv1DN$ myocytes in the absence or presence of HpTx-2 (5 µM) is illustrated that it blocked most of the fast component, leaving a very slow component.
The Regional Differences in $I_{Kv}$ Between LV Endo- and Epicardial Myocytes

Current densities were determined at +60 mV (Holding potential = -80 mV). Current densities of $I_{to}$, $I_{K,slow1}$, $I_{K,slow2}$ and $I_{SS}$ in epicardial myocytes are greater than in endocardial myocytes while amplitude of each components are not different between regions. Inactivation rate of $I_{to}$ in epicardial myocytes is faster than in endocardial myocytes while there is no regional difference in inactivation time constants of $I_{K,slow1}$, and $I_{K,slow2}$. ENDO and EPI indicate LV endocardial and epicardial myocytes, respectively. $\tau_{\text{inact}}$, inactivation time constant. Values are means ± S.E.M. ($n = 41$ for ENDO; $n = 13$ for EPI; $N = 12$). *, $P < 0.05$ vs. ENDO. $N = 13$.

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<th>$I_{\text{Peak}}$</th>
<th>$I_{to}$</th>
<th>$I_{K,\text{slow1}}$</th>
<th>$I_{K,\text{slow2}}$</th>
<th>$I_{SS}$</th>
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<td>743 ± 61</td>
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Table A.1
A.5 Discussion

We found that \( I_{Kv} \) in adult mouse ventricular cardiomyocytes have 5 components that can be separated based on their kinetic properties: \( I_{to,f}, I_{to,s}, I_{K,slow1}, I_{K,slow2} \) and \( I_{ss} \). While our findings generally agree with previous reports (Xu et al., 1999b; Zhou et al., 2003), we demonstrate that accurate estimation of the kinetic components of \( I_{Kv} \) in mouse cardiomyocytes using mathematical fitting of their decay phase requires a minimum of 20 seconds to incorporate the slow component (with a time constant of \( \sim 5 \) seconds). Indeed, when pulses durations were shorter than 20 seconds, systematic deviations occurred in the estimates of both time constants and current density for all kinetic components (see example below). When 25-sec pulses are used, our results rigorously establish, using the reduced Chi-squared goodness-of-fit test and \( F \)-distribution test of the \( T \)-statistic, that all \( I_{Kv} \) traces are adequately fit with a 3-exponential functions but not with 2-exponential functions. The preference for the 3-exponential fits was further supported by the observation that the time constants estimated using the 2-exponential fits (100 – 700 and 2000 – 6000 ms) were far more scattered than for the 3-exponential fits and were, as might be expected, intermediate between the 3 time constants predicted from the 3-exponential fits. Choosing between the 3- and 4-exponential fits was more challenging. Applying the \( F \)-distribution test to the \( T \)-statistic revealed that about 50% of the traces were better fit by a 3-exponential function. However, 3-exponential fits provided a much tighter clustering of the estimated time constants (40 – 100, 500 – 1000, and 3500 – 8000 ms) with each kinetic component having highly consistent amplitudes compared to 4-exponential fits. Moreover, 4-exponential fits yielded either very short or extremely long time constants with magnitudes that were either very small or negative. Thus, we conclude that 3-exponential fits to \( I_{Kv} \) in adult mouse ventricular myocytes are superior to either 2- or 4-exponential fits. Meanwhile, previous studies have also concluded that, when \( I_{to,f} \) is present in septal cardiomyocytes, 3-exponential fits are required with the components being \( I_{to,f} \) (\( \tau \sim 55 \) ms), \( I_{to,s} \) (\( \tau = 150 – 400 \) ms) and \( I_{K,slow} \) (\( \tau \sim 2200 \) ms) (Xu et al., 1999b; Brunet et al., 2004). Although remarkably similar kinetic components were identified when 5 second depolarizing pulses are used (Figure A.3C), our results establish that the fast component is \( I_{to} \) (consisting of \( I_{to,f} \) and \( I_{to,s} \)) with the other components being \( I_{K,slow1} \) and \( I_{K,slow2} \), as further discussed below.

Our results show that the most rapidly decaying kinetic component of \( I_{Kv} \) in mouse ventricular myocyte is \( I_{to} \), comprised of two currents with properties resembling \( I_{to,f} \) and \( I_{to,s} \) with
about 70% of $I_{to}$ originating from $I_{to,f}$ in endocardial myocytes. This conclusion is based on a number of complementary observations. For instance, $I_{to}$ activation properties (as quantified using the conductance-voltage relationship) showed biphasic properties (i.e. were comprised of two components) since external Cd$^{2+}$ (300 µM), added to block Ca$^{2+}$ currents, profoundly induces positive shift in a half-maximal activation voltage ($V_{1/2}$) in $K_V4.2$-encoded currents while modestly affecting $V_{1/2}$ of $K_V1.4$-encoded currents, without affecting $I_{to}$ inactivation properties (Agus et al., 1991; Wickenden et al., 1999c). Remarkably, the components of $I_{to}$ that activated at more positive potentials comprised about 70% of the current and showed a $V_{1/2}$ similar to heterologously expressed $K_V4.2$ currents, while the smaller component activating at more negative potentials had properties similar to $K_V1.4$ currents (Wickenden et al., 1999c). Moreover, the addition of HpTx, a selective blocker of $I_{to,f}$ with an IC$_{50}$ of about 140 nM (Sanguinetti et al., 1997), eliminated the component activating at more positive potentials while low doses of 4-AP eliminated the component activating at more negative potentials, as expected from the high sensitivity of $I_{to,s}$ to 4-AP (Zhang et al., 1998) (and see below). These differential activation properties of $I_{to,f}$ and $I_{to,s}$ have been exploited previously to separate these currents in mouse ventricular myocytes using different holding potentials (Brouillette et al., 2004). In addition, the biphasic recovery kinetics of $I_{to}$ supported the presence of $I_{to,f}$ and $I_{to,s}$, as previously described (Oudit et al., 2001; Patel and Campbell, 2005). For example, about 70% of $I_{to}$ recovered rapidly with kinetics similar to $K_V4.2/4.3$ currents ($\tau = 40 – 50$ ms) (Guo et al., 2002a) and with the remainder recovering slowly with kinetics comparable to $K_V1.4$ currents ($\tau = 4.7 – 9.4$ s) (Hashimoto et al., 2000; Kurata et al., 2004). These kinetics of the two $I_{to}$ components and the relative amplitudes ($I_{to,f}$:$I_{to,s}$ = 70:30) in mouse also match closely those found in rat ventricular myocytes ($I_{to,f}$: 30 – 50 ms, $I_{to,s}$: 2.0 – 3.5 s) (Wickenden et al., 1999a; Volk et al., 2001). As expected, low doses of 4-AP selectively eliminated the slow recovering components. The conclusion that $I_{to}$ is comprised of $K_V4$-based $I_{to,f}$ and $K_V1.4$-based $I_{to,s}$ is further supported by observation that the component with properties linked to $I_{to,s}$ is essentially absent in $K_V1DN$ cardiomyocytes. Specifically, $I_{to}$ showed rapid monophasic recovery from inactivation properties (< 40 ms) as well as a monophasic conductance versus voltage curve.

Again, our results support the conclusion that most rapidly inactivating component of $I_{K_V}$ in mouse ventricle (i.e. $I_{to}$) is comprised of a fast $K_V4$-based $I_{to,f}$ and a slower $K_V1.4$-based $I_{to,s}$. Previous studies have similarly concluded that $I_{to,f}$ and $I_{to,s}$ coexist in mouse ventricular myocytes while these studies identified $I_{to,f}$ with a 50 – 60 ms inactivating component (rapid) and $I_{to,s}$ with
a 150 – 400 ms inactivating component (intermediate) based on 3-exponential fits (Guo et al., 1999; Xu et al., 1999b; Brunet et al., 2004). However, we showed that the intermediate component is likely to be $k_{V1.5}$-encoded $I_{K,slow1}$ (see below). Furthermore, in our studies, $I_{to,f}$ and $I_{to,s}$ could not be kinetically separated when long $k_{V}$ traces (i.e. > 20 seconds) or even short $k_{V}$ traces (i.e. ~ 5 sec) were fit with 3-exponential functions, presumably because of kinetic similarities in their inactivation properties of $k_{V4}$-encoded ($\tau = 40 – 50$ ms) and $k_{V1.4}$-encoded ($\tau = 35 – 65$ ms) currents (Wickenden et al., 1999a; Hashimoto et al., 2000; Guo et al., 2002a; Jow et al., 2004). Although 4-exponential fits should, in principle, allow separation of $I_{to,f}$ and $I_{to,s}$, rigorous statistical tests using $F$-distribution test of $T$-statistic establish that $I_{to,f}$ and $I_{to,s}$ cannot be reliably separated using exponential fitting methods alone. This conclusion is consistent with results in rat (Wickenden et al., 1999a; Volk et al., 2001) and human (Wettwer et al., 1994) ventricular myocytes, showing that $I_{to,f}$ and $I_{to,s}$ cannot be separated on the basis of fitting alone but contrast with previous mouse studies using 3-exponential fits to 5 second traces (Xu et al., 1999b; Brunet et al., 2004). However, $I_{to}$ inactivation rates were affected by pharmacological interventions, genetic manipulation, and regional differences. Specifically, $HpTx-2$ treatment slightly increases ($P < 0.01$) the inactivation time constant of $I_{to}$ from 68 ± 2 ms to 92 ± 13 ms while it was mildly accelerated ($P < 0.01$) in both 4-AP treated WT myocytes (39 ± 5 ms) and $k_{V1DN}$ myocytes (42 ± 4 ms). In addition, epicardial myocytes, where $I_{to,s}$ is poorly expressed (Xu et al., 1999b; Brunet et al., 2004), revealed faster ($P < 0.02$) inactivation rate of $I_{to}$ (54 ± 4 ms) than endocardial myocytes (68 ± 2 ms) while there was no difference in decay rates of $I_{K,slow1}$ and $I_{K,slow2}$. Taken together, these results support a notion that inactivation rate of $I_{to,s}$ may be slightly slower compared to $I_{to,f}$ (Xu et al., 1999b; Brunet et al., 2004).

In the present study, we observed that the intermediate component of $I_{Kv}$ with an inactivation time constant ~800 ms was preferentially blocked by 250 µM 4-AP. This current is likely to be $k_{V1.5}$-encoded $I_{K,slow1}$ for several reasons. For example, $k_{V1.5}$-encoded currents in HEK-293 cells show inactivation time constants of 500 – 700 ms (Rampe et al., 1993; Rampe et al., 1995; Lacerda et al., 1997; Kwan et al., 2006), and is highly sensitive to low concentration of 4-AP with IC$_{50}$ of ~50 µM (Bouchard and Fedida, 1995). Moreover, the intermediate component of $I_{Kv}$ in mouse myocytes had bi-exponential recovery kinetics ($\tau_{fast} \sim 350$ ms and $\tau_{slow} \sim 5400$ ms) which is remarkably similar to the time course seen in $k_{V1.5}$ channels expressed in HEK-293 cells ($\tau_{fast}$, 170 ms; $\tau_{slow}$, 3190 ms) (Kurata et al., 2005). These two components in the recovery might reflect the two inactivation mechanisms of $k_{V1.5}$ (P/C-type and U-type) with distinct
kinetic properties (Klemic et al., 2001; Kurata et al., 2005). Regardless, $I_{K_{\text{slow}1}}$ is almost eliminated in $K_{\text{V}1\text{DN}}$ myocytes as over-expression of the truncated $K_{\text{V}1.1}$ peptide is expected to form non-functional heteromultimeric complexes with native $K_{\text{V}1.5}$ channels (Folco et al., 1997). Together, these results support the conclusion that the intermediate component of $I_{K}$ is $I_{K_{\text{slow}1}}$, encoded by $K_{\text{V}1.5}$.

The slow component of $I_{K}$ in mouse ventricular myocytes ($I_{K_{\text{slow}2}}$) inactivated with a time constant of $\sim 5$ seconds. It is the presence of this slow component that dictates the use of depolarization pulses lasting more than 20 seconds, particularly when multiple kinetic processes are present. Indeed, as already mentioned, we found that short depolarization steps led to a severe over-estimation of $I_{K_{\text{slow}2}}$ and $I_{SS}$ largely at the expenses of $I_{K_{\text{slow}1}}$. This component recovered from inactivation with a single time constant of $\sim 450$ ms. The inactivation property of $I_{K_{\text{slow}2}}$ is similar to $K_{\text{V}2.1}$-encoded currents ($\tau = 5 – 7$ sec) in the HEK-293 cells and *Xenopus* oocytes, whereas the recovery from inactivation rate is considerably faster than that of $K_{\text{V}2.1}$-encoded currents ($\tau = 1.3 – 1.6$ sec) (Kramer et al., 1998; Leung et al., 2003). The faster recovery of $I_{K_{\text{slow}2}}$ in cardiomyocytes might reflect the interactions of the $K_{\text{V}2.1}$ channels with other regulatory proteins such as $K_{\text{V}9.3}$, $K_{\text{V}5.1}$, or $K_{\text{V}6.1}$ (Kramer et al., 1998; Kerschensteiner and Stocker, 1999). This possible heteromeric composition of $I_{K_{\text{slow}2}}$ might also account for relatively low sensitivity to TEA ($\sim 50\%$ block by 50 mM TEA) since $K_{\text{V}2.1}$ is blocked by TEA with an IC$_{50}$ of 5 mM in the presence of physiological $[K^+]$ (Ikeda and Korn, 1995). Nevertheless, we found that $I_{K_{\text{slow}2}}$ was selectively blocked by high doses by TEA (50 mM), while showing a relatively low sensitivity to 4-AP and HpTx-2. Consistent with this pharmacological profile, no change in the current density and the inactivation time constant of $I_{K_{\text{slow}2}}$ in the $K_{\text{V}1\text{DN}}$ myocytes further support the conclusion that $K_{K_{\text{slow}2}}$ arises from $K_{\text{V}2}$ channels, and is not associated with $K_{\text{V}1.X}$ channels. Interestingly, a previous study suggested that the current density of $I_{K_{\text{slow}2}}$ was increased in the $K_{\text{V}1\text{DN}}$ mice while its time constant was unaffected (Zhou et al., 2003). This discrepancy may have resulted from regional differences; the up-regulation of $I_{K_{\text{slow}2}}$ was selectively found in apical myocytes while all experiments in the present study were conducted in LV endocardial cells that is close to basal myocytes.

Although pharmacological inhibitors can assist in dissecting various $I_{K}$ components, we found their use to be limited by non-specificity as well as complications associated with state-dependence of blockade. As has been reported previously (Xu et al., 1999b), we found that 4-AP reduces all the kinetic components in a dose-dependent manner, albeit with distinct potencies.
Specifically, 250 µM of 4-AP blocks more than 90% of the intermediate component (I_{K,slow1}) while essentially eliminating the component of conductance associated with I_{to,s} as evidenced by systematic changes in the conductance properties of I_{to} as well as the loss of the slow component in the recovery from inactivation of I_{to} (data not shown and see discussion below). These observations are consistent with previous studies showing that the I_{to,s} and I_{K,slow1} are formed by Kv1.4 and Kv1.5 channels, respectively, which are both highly sensitive to 4-AP (i.e. IC_{50} for Kv1.4 ≃ 125 µM; IC_{50} for Kv1.5 ≃ 50 µM) (Bouchard and Fedida, 1995; Zhang et al., 1998).

Although examination of the dose-dependence of 4-AP on I_{to,s} and I_{K,slow1} could in principle provide further evidence for this conclusion, we found that doses of 4-AP below ~100 µM induced systematic and complex changes in the kinetics of I_{K,slow1}, which can be attributed to closed- and/or open-state binding of 4-AP to Kv1.5 channels (Bouchard and Fedida, 1995). On the other hand, we found, as reported previously (Patel et al., 1997; Faivre et al., 1999; Tseng, 1999; Xu et al., 1999b), that 4-AP concentration higher than 250 µM also reduced I_{to,f} and I_{K,slow2}, consistent with the conclusion that these currents are produced by Kv4.x (IC_{50} ≃ 1 – 1.5 mM) and Kv2.1 (IC_{50} ≃ 5 mM) (Patel et al., 1997; Clement-Chomienne et al., 1999; Faivre et al., 1999; Wickenden et al., 1999b; Xu et al., 1999a; Lu et al., 2002). In addition, after 250 µM of 4-AP administration, I_{to} recovery was monophasic with a fast recovery time constant (i.e. 41 ± 0 ms) (Figure A.7). It should be mentioned that the recovery in the presence of 4-AP showed a prominent overshoot in the test-pulse, a phenomenon described previously as “reverse use-dependence” behaviour resulting from a closed-state blocking mechanism of Kv4-encoded I_{to,f} by 4-AP, which was not shown in Kv1.4-encoded I_{to,s} via activated-state block (Campbell et al., 1993; Tseng et al., 1996). This suggests that the overshooting in the I_{to} recovery results from dissociation of 4-AP from Kv4 channels during channel inactivation. Interestingly, I_{to} recovery from inactivation in epicardial myocytes also revealed ‘overshoots’ effect in the test-pulse (unpublished data). Similar phenomenon has been reported in human LV epicardial myocytes (Wettwer et al., 1994), which is linked to effects of minK-related peptide 1 (MiRP1) (Zhang et al., 2001). A further characterization of the overshoot properties will be the subject of a future publication. On the other hands, we found that TEA was relatively selective for I_{K,slow2} although both I_{to} and I_{K,slow1} were mildly (~10%) reduced with 50 mM TEA. These mild reductions in I_{to} and I_{K,slow1} may be due to direct inhibition by TEA on Kv4 (IC50 ≃ 320 mM) and Kv1.5 channels (IC50 ≃ 330) (Grissmer et al., 1994; Colinas et al., 2008). Meanwhile, decrease in I_{to} may be
associated with reduced Na$^+$ concentration replaced by TEA, which can have impact on $I_{to}$ (Dukes and Morad, 1991).

In conclusion, we have combined rigorous statistical analyses method with selected pharmacological interventions for dissecting outward K$^+$ currents. Our approach allows accurate quantification of $I_{to,t}$, $I_{to,s}$, $I_{K,slow1}$, $I_{K,slow2}$, and $I_{ss}$ in adult mouse ventricular myocytes. Although our studies quantify currents at room temperature, our rigorous approach is expected to be also useful at more physiological temperature (37°C). Moreover, our method should be valuable for dissection of outward K$^+$ currents in other tissues as well as in other species.
APPENDIX B

PRESENCE OF $I_{\text{to,fast}}$ IN $K_v4.2$ KNOCK-OUT MICE DUE TO FUNCTIONAL $K_v4.3$ EXPRESSION IN ADULT MOUSE VENTRICULAR MYOCYTES
B.1 Abstract

The fast component of transient outward potassium currents, $I_{\text{to,f}}$, is encoded by pore-forming $\alpha$-subunits (i.e. $K_{\text{V}}4.2$ and $K_{\text{V}}4.3$ in rodents and $K_{\text{V}}4.3$ in human) and auxiliary KChIP2 subunit in the heart. A recent study has demonstrated that $I_{\text{to,f}}$ is eliminated in mice lacking $K_{\text{V}}4.2$ ($K_{\text{V}}4.2^{-/-}$), suggesting obligatory co-assembly of $K_{\text{V}}4.2$ $\alpha$-subunit to make $I_{\text{to,f}}$-encoding functional channels with $K_{\text{V}}4.3$ $\alpha$-subunit. Unexpectedly, outward $K^+$ currents during its recovery from inactivation with 50 ms interval in the $K_{\text{V}}4.2^{-/-}$ cardiomyocytes were well described by a sum of tri-exponentials with a fast time constant less than 110 ms, strongly suggesting the existence of $I_{\text{to,f}}$. More importantly, this fast inactivating current was HpTx-2 sensitive, and biphasic in the recovery from inactivation, demonstrating that $I_{\text{to,f}}$ was evident without $K_{\text{V}}4.2$ $\alpha$-subunit. To examine whether $I_{\text{to,f}}$ in $K_{\text{V}}4.2^{-/-}$ myocytes was encoded by $K_{\text{V}}4.3$ $\alpha$-subunit, immunofluorescent staining with 3D reconstruction was conducted, and illustrated strong $K_{\text{V}}4.3$ expression on the membrane of $K_{\text{V}}4.2^{-/-}$ cardiomyocytes. Taken together, these findings demonstrate that $K_{\text{V}}4.3$ can assemble function channels encoding $I_{\text{to,f}}$ in the $K_{\text{V}}4.2^{-/-}$ hearts.

B.2 Introduction

The fast transient outward $K^+$ current, $I_{\text{to,f}}$, is an important determinant of early repolarization period of the action potentials, thereby regulating cardiac excitation-contraction coupling through its influence on $Ca^{2+}$ currents (Sah et al., 2003). $I_{\text{to,f}}$ down-regulation is commonly found as a hallmark in the heart diseases (Tomaselli and Marban, 1999; Oudit et al., 2001), while changes in $I_{\text{to,f}}$ can modulate hypertrophic responses in myocytes cultures as well as intact hearts (Bassani et al., 1995; Wickenden et al., 1999b; Kassiri et al., 2002; Sah et al., 2002a; Zobel et al., 2002; Lebeche et al., 2004; Lebeche et al., 2006; Jin et al., 2010). Furthermore, $I_{\text{to,f}}$ is related to arrhythmia. A recent study has presented that the mutation in KCNE3 (MiRP2) increases $I_o$ density by regulating $K_{\text{V}}4.3$ in human, leading to Brugada syndrome (Delpon et al., 2008). Regional differences of $I_{\text{to,f}}$ density are also thought be a major player of arrhythmia because an increment or loss of the regional heterogeneity in action potentials underlies arrhythmogenesis (Antzelevitch and Fish, 2001; Kuo et al., 2001; Costantini et al., 2005; Antzelevitch, 2007). In rodent, $I_{\text{to,f}}$ heterogeneity is parallel to $K_{\text{V}}4.2$ gene and protein expression, which is regulated by Irx5 or Nfatc3 in mice (Costantini et al., 2005; Rossow et al., 2006). In
large animals such as humans and canine, on the other hand, the $I_{to,f}$ gradient correlates with KChIP2 expression, not pore-forming $\alpha$-subunit of $K_{V4}$ channels (Oudit et al., 2001).

Furthermore, $K_{V4.2}$ is not expressed in large mammals, indicating that $I_{to,f}$ can be encoded by homotetrameric $K_{V4.3}$ channels, whereas in rodent both $K_{V4.2}$ and $K_{V4.3}$ express in the heart (Wickenden et al., 1999a; Brunet et al., 2004). $I_{to,f}$ is also affected by an auxiliary subunit KChIP2, which binds to $K_{V4}$ $\alpha$-subunit and augments $K_{V4}$-encoded $I_{to,f}$ (Bou-Abboud and Nerbonne, 1999; Wickenden et al., 1999c; An et al., 2000; Guo et al., 2002a). In addition, loss of KChIP2 results in complete loss of $I_{to,f}$ in mouse hearts although $K_{V4.2}$ and $K_{V4.3}$ mRNA and protein expression are intact, suggesting that KChIP2 are critical players in constructing $I_{to,f}$ gradients by facilitating the trafficking of $K_{V4}$ $\alpha$-subunit in rodent (An et al., 2000; Kuo et al., 2001). Interestingly, a recent study has concluded that in mice cardiac $I_{to,f}$ is formed by obligatory co-assembly of $K_{V4.2}$ because $I_{to,f}$ is eliminated in cardiomyocytes isolated from mice lacking $K_{V4.2}$ ($K_{V4.2}^{-/-}$) in spite of intact mRNA and protein expression of $K_{V4.3}$ (Guo et al., 2005). Meanwhile, mice lacking $K_{V4.3}$ ($K_{V4.3}^{-/-}$) express $I_{to,f}$ that is unaffected compared with wild-type mice (Niwa et al., 2008). These findings are somewhat surprising, because in the heterologous system, homotetrameric $K_{V4.3}$ as well as $K_{V4.2}$ can assemble function channels encoding $I_{to,f}$ (Guo et al., 2002a). Moreover, $I_{to,f}$ in large animals is encoded by homomeric $K_{V4.3}$ channels which sequence is highly conserved between all mammalian species (van der Heyden et al., 2006).

We have recently demonstrated a rigorous method to adequately dissect voltage-gated $K^+$ currents ($I_{K^+}$) in mouse ventricular myocytes (see Appendix A), and concluded that tri-exponential functions as well as depolarizing pulses with durations greater than 20 seconds are essential to adequately separate the different $I_{K^+}$ components, whereas bi-exponential fitting can lead to improper interpretation. In particular, while verifying our dissection method with various transgenic mice showing phenotypes related to $I_{K^+}$, such as mice over-expressing a truncated $K_{V1.1}$ ($K_{V1DN}$; loss of $I_{to,s}$ and $I_{K_s,slow}$) (London et al., 1998a; Zhou et al., 2003), Irx5 deficient mice ($I_{to,f}$ gradient) (Costantini et al., 2005), and $K_{V4.2}^{-/-}$ (loss of $I_{to,f}$) (Guo et al., 2005), we found that $I_{K^+}$ in $K_{V4.2}^{-/-}$ cardiomyocytes were fitted well with tri-exponentials, and $I_{to,f}$-like current was still observed in the most of LV cardiomyocytes, which was contrast to a previous study (Guo et al., 2005). Thus, in this study, we further examined whether $I_{to,f}$ was functionally available in $K_{V4.2}^{-/-}$ myocytes, and concluded that $K_{V4.3}$ can assemble homotetrameric functional channels encoding $I_{to,f}$ without $K_{V4.2}$.
B.3 Methods

B.3.1 Transgenic animals and genotyping

K\textsubscript{V}4.2\textsuperscript{+} mice generated in 129/SvEv background (Guo et al., 2005) were out-bred into the C57B6 mouse background for 8 generations. Genotypes of littermates were examined using following primers: K\textsubscript{V}4.2-specific primers (613 bps; forward, GTG GAT GCC TGT TGC TTC; reverse, CCC ACA AGG CAG TTC TTT TA) and neo-specific primers (500 bps; forward, AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG; reverse, AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG) (Chen et al., 2006). All the experiments in this study were conducted using 12 – 16 week old mice, conforming to the standards of the Canadian Council on Animal Care.

B.3.2 Isolation of Adult Mouse Ventricular Myocytes

Hearts were rapidly removed from anesthetised mice and retrogradely perfused with Ca\textsuperscript{2+}-free Tyrode’s solution [(mmol/L) 137 NaCl, 5.4 KCl, 1.0 MgCl\textsubscript{2}, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 10 D-glucose, 10 HEPES, pH 7.4] at 37° C through the aorta for 3 – 4 min. Then it was perfused with collagenase (1.0 mg/mL, Worthington) for 10 – 12 min, followed by gentle triturating to dissociate cardiomyocytes and storing cells in Krebs-bicarbonate solution [(mmol/L) 120 potassium glutamate, 20 KCl, 20 HEPES, 1.0 MgCl\textsubscript{2}, 10 D-glucose, 0.5 K-EGTA, and 0.1% bovine serum albumin].

B.3.3 Patch Clamp Electrophysiology & Data Analysis

Voltage-activated K\textsuperscript{+} currents (I\textsubscript{Kv}) from isolated adult mouse ventricular myocytes were recorded with the whole-cell patch clamp technique in voltage-clamp mode, using an Axopatch 200B amplifier and pClamp 6 software (Axon Instrument, CA, USA). Ca\textsuperscript{2+} tolerant rod-shape cardiomyocytes were selected and perfused with bath solution for 15 minutes before electrophysiological recording at room temperature (21 – 24° C). The bath solution contained (mmol/l): 140 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 1.2 CaCl\textsubscript{2}, 10 HEPES, 10 D-glucose, and 0.3 CdCl\textsubscript{2} (pH 7.4). To block K\textsubscript{V}4-encoded I\textsubscript{to,f}, 5 µM of the recombinant heteropodatoxin-2 (HpTx-2; gift from Dr. Michael Morales) (Zarayskiy et al., 2005) was used in the bath solution. The pipette resistance ranged between 1.2 – 2.0 MΩ when filled with a pipette solution containing (mmol/l): 120 potassium aspartate, 20 KCl, 5 NaCl, 1 MgCl\textsubscript{2}, 5 MgATP, 10 HEPES, and 10 EGTA (pH
7.2. After membrane rupture, the cell capacitance was measured by integrating the capacitance transient in response to 10 mV steps from a holding potential of -50 mV and used to normalize current magnitudes. Cell capacitance and series resistance were then electronically compensated by 85%. Voltage-gated K⁺ currents were measured by 20 second depolarization from a holding potential of -80 mV to +60 mV, where was the reversal potential of Na⁺ current. To examine the recovery from steady-state inactivation, a double-pulse protocol was used. Cells were first depolarized to +60 mV for 20 sec (pre-pulse), subsequently hyperpolarized to the holding potential of -80 mV for various times ranging from 10 ms to 6 sec, and then stepped to +60 mV for another 5 – 20 sec (test-pulse) to activate the currents and assess the extent of recovery in the same cells. To allow the complete reloading of the slowly recovering currents, the repetition interval of more than 45 sec was given.

**B.3.4 Data Analysis**

Electrophysiological data were analyzed using pClamp software (Clampfit 10.0, Axon, CA, USA) and Prism 4.03 (GraphPad Software, San Diego, CA, USA). Inactivation of the outward K⁺ currents was fitted to a sum of exponentials with various terms from 2 to 4 using the following equation:

\[ F(t) = A_1 \cdot \exp\left(-\frac{t}{\tau_1}\right) + A_2 \cdot \exp\left(-\frac{t}{\tau_2}\right) + \ldots + A_n \cdot \exp\left(-\frac{t}{\tau_n}\right) + A_s, \]

in which \( A_s \) is the amplitude of the steady-state, non-inactivating component. As shown in Appendix A, the goodness of fitting was evaluated by visual inspection and statistical examination using Chi-square (\( \chi^2 \)) analysis as well as weighted F-test.

To quantify the recovery from steady-state inactivation, K⁺ currents traces, stimulated by pre-pulse and test-pulse, were first fitted using the equation above. Then, the magnitude of each current, induced by the test-pulse, was expressed as a percentage of the corresponding current, induced by the pre-pulse on the same cell. The fractional recovery of \( I_{10} \) from steady-state inactivation was plotted as a function of time on a logarithmic scale and fit according to a biphasic equation:

\[ \frac{I}{I_{10}} = A_1 \cdot \left(1 - \exp\left(-\frac{t}{\tau_1}\right)\right) + A_2 \cdot \left(1 - \exp\left(-\frac{t}{\tau_2}\right)\right) \]

where \( A_1 \) and \( A_2 \) are the amplitudes of two different components \( (A_1 + A_2 = 1) \), \( t \) is the time spent at the recovery potential, and \( \tau_1 \) and \( \tau_2 \) are the recovery time constants. When \( \tau_1 \) and \( \tau_2 \) were statistically identical such as for \( I_{K_{slow2}} \), the recovery data was fit according to a mono-exponential equation (Wickenden *et al*., 1999a; Volk *et al*., 2001):
\[ I/I_o = A \cdot [1 - \exp(-t/\tau)] \]

B.3.5 Immunofluorescence Staining

Immunofluorescence staining was performed as described elsewhere (Kim et al., 2008a). In brief, myocytes isolated from \( \textbf{K}_\text{V}4.2^{+/+} \) and \( \textbf{K}_\text{V}4.2^{-/-} \) mouse ventricles were plated in laminin-coated (0.5 mg/mL, Roche) glass coverslips, followed by fixation with 4% paraformaldehyde in PBS for 1 hour at room temperature and permeation with 0.2% (v/v) Triton X-100 for 10 min. After blocking with 1% bovine serum albumin in PBS, cells were then exposed to the primary antibody against \( K_\text{V}4.3 \) (1:100) in blocking solution overnight at 4°C. The anti-\( K_\text{V}4.3 \) mouse monoclonal antibodies (NeuroMab) were raised against a fusion protein corresponding to amino acids 415 – 636 in the cytoplasmic C-terminus of a rat \( K_\text{V}4.3 \) protein (Menegola and Trimmer, 2006). A secondary Alexa Fluor 488 goat anti-mouse IgG antibody (Amersham Bioscience) was subsequently used for visualization. For cell imaging, an argon laser beam equipped confocal microscope was used (excitation 488 nm, emission 519 nm; Olympus). To control for background and cell autofluorescence, parameters were normalized based on fluorescence intensities measured in control samples treated with no antibody, primary antibody only or secondary antibody alone. The expression pattern of \( K_\text{V}4.3 \) protein was further determined by the optical Z sections (0.5 µm thickness) at 60X magnification in conjunction with a Kalman filter (n = 3), and 3D construction was performed using ImagePro Plus (Media Cybernetics).

B.4 Results

B.4.1 Voltage-activated \( K^+ \) currents in \( K_\text{V}4.2^{-/-} \) Cardiomyocytes Are Well Described by the Sum of Tri-exponentials

Voltage-activated \( K^+ \) currents (\( I_{K_v} \)) in adult mouse ventricular myocytes are well described by the sum of three exponentials, corresponding to \( I_{to} \), \( I_{K_{slow1}} \), and \( I_{K_{slow2}} \) (see Appendix A). In addition, \( I_{to} \) is further dissected into \( I_{to,f} \) and \( I_{to,s} \) using the recovery from inactivation and the conductance-voltage relationships. Guo et al. have presented that loss of \( K_\text{V}4.2 \) in mice completely eliminates \( I_{to,f} \) while \( K_\text{V}1.4 \)-encoded \( I_{to,s} \) is up-regulated (Guo et al., 2005). To verify our dissection method, \( K_\text{V}4.2^{+/+} \) cardiomyocytes were first depolarized by a double-pulse protocol with 50 ms interval. It was initially expected that \( K_v \) in the pre-pulse would be fitted by tri-exponentials, corresponding to \( I_{to} \) (potentially only containing \( I_{to,s} \), \( I_{K_{slow1}} \).
and $I_{K,\text{slow}2}$, whereas only two components would be observed in the test-pulse as $I_{t0,s}$ recovers very slowly from inactivation (1–2 sec) (Oudit et al., 2001; Patel and Campbell, 2005). Indeed, $I_{Kv}$ of $K_{V4.2^{-}}$ cardiomyocytes in the pre-pulse was well described by tri-exponential fittings, whereas bi-exponential function led to improper fitting with the residual (Figure B.1A). As summarized in Figure B.2A, inactivation time constant of $I_{t0}$ on $K_{V4.2^{-}}$ cardiomyocytes ($\tau_1 = 101 \pm 4$ ms) was mildly increased ($P < 0.05$), compared with littermate controls ($\tau_1 = 67 \pm 6$). Unexpectedly, in the test-pulse, $I_{Kv}$ of $K_{V4.2^{-}}$ cells were still fitted well with tri-exponentials rather than bi-exponentials (Figure B.1B). More importantly, the decaying rate of $I_{t0}$ ($\tau_1 = 91 \pm 7$) was fairly rapid, but was slower ($P < 0.01$) than that of $K_{V4.2^{+/+}}$ cardiomyocytes ($\tau_1 = 53 \pm 2$) (Figure B.2B). Therefore, these findings enabled us to postulate that $I_{t0,f}$ was expressed in $K_{V4.2^{-}}$ cardiomyocytes, which might be encoded by $K_{V4.3}$.

### B.4.2 Heteropodatoxin-Sensitive Currents are Evident in $K_{V4.2^{-}}$ Cardiomyocytes

To test whether this $I_{t0,f}$–like currents in $K_{V4.2^{-}}$ cardiomyocytes are encoded by homotetrameric $K_{V4.3}$ channels, we used HpTx-2, a highly selective blocker for $K_{V4}$-encoded currents (Sanguinetti et al., 1997; Zarayskiy et al., 2005). In $K_{V4.2^{+/+}}$ cardiomyocytes, 5 µM of HpTx-2 selectively blocked $I_{t0}$ about 80.4% ($P < 0.05$), while not affecting $I_{K,\text{slow}1}$ and $I_{K,\text{slow}2}$ (Figures B.3 and B.4A). Notably, the equal concentration of HpTx-2 selectively reduced ($P < 0.05$) $I_{t0}$, approximately 33.2% (Figure B.4B). These results strongly suggested that $K_{V4.2^{-}}$ cardiomyocytes expressed the functional $I_{t0,f}$ that was encoded by $K_{V4}$ channels, although the amplitude of HpTx-2 sensitive current in $K_{V4.2^{-}}$ cardiomyocytes was much smaller than that in $K_{V4.2^{+/+}}$ cells.

Furthermore, densities of HpTx-2 insensitive currents were indistinguishable between $K_{V4.2^{+/+}}$ and $K_{V4.2^{-}}$ cardiomyocytes, suggesting that $I_{t0,s}$ might not be affected by the loss of $K_{V4.2}$.

### B.4.3 Dissection of $I_{t0}$ into $I_{t0,\text{fast}}$ and $I_{t0,\text{slow}}$ in $K_{V4.2^{-}}$ Cardiomyocytes

To further determine whether the HpTx-2 sensitive current in $K_{V4.2^{-}}$ cardiomyocytes has an electrophysiological property of $I_{t0,f}$, a protocol to measure the recovery from inactivation was used to separate $I_{t0}$ into $K_{V4}$–encoding $I_{t0,f}$ and $K_{V1.4}$–encoding $I_{t0,s}$ (Oudit et al., 2001). $K_{V4.2^{-}}$ cells revealed a biphasic $I_{t0}$ recovery with two different recovery time constants of 74 ± 5 and 2261 ± 276 ms (Figure B.5, B and C). The fast $I_{t0}$ recovery time constant of ($I_{t0,f}$) in $K_{V4.2^{-}}$ cells was significantly slower ($P < 0.05$) than that in $K_{V4.2^{+/+}}$ (34 ± 4 ms), whereas the slow recovery time constants corresponding to $I_{t0,s}$ were indistinguishable between $K_{V4.2^{-}}$ and
$K_V4.2^{+/+}$ cardiomyocytes (2284 ± 359 ms) (Figure B.5C). Based on the amplitudes of $I_{to,f}$ and $I_{to,s}$ in the recovery from inactivation (Figure B.5B), current densities of $I_{to,f}$ and $I_{to,s}$ were calculated. As shown in Figure B.5D, $I_{to,f}$ density was lower ($P < 0.05$) in $K_V4.2^{-/-}$ cells (4.1 ± 0.6 pA/pF) than in $K_V4.2^{+/+}$ cells (11.6 ± 4.1 pA/pF). $I_{to,s}$ densities, on the other hand, were indistinguishable ($K_V4.2^{+/+}$: 3.6 ± 1.4; $K_V4.2^{-/-}$: 3.4 ± 0.6 pA/pF), similar to the $HpTx-2$ insensitive current density (see Figure B.4). Taken together, these findings confirmed that $I_{to,f}$ was evidently expressed in $K_V4.2^{-/-}$ cardiomyocytes. Furthermore, an increase in the $I_{to}$ inactivation time constants as well as its recovery time constants also suggested that electrophysiological properties of $I_{to,f}$ in $K_V4.2^{-/-}$ cardiomyocytes was different from those in $K_V4.2^{+/+}$ cells, but similar to the homometric $K_V4.3$-encoded $I_{to,f}$ (Guo et al., 2002a) (See Section B.5).

**B.4.4 $K_V4.3$ Expression on the Membrane of $K_V4.2^{-/-}$ cardiomyocytes**

The existence of $I_{to,f}$ in $K_V4.2^{-/-}$ cardiomyocytes could be only explained by $I_{to,f}$ encoded by homotetrameric $K_V4.3$ channels. However, this is directly in conflict with the previous conclusion that, despite unaffected $K_V4.3$ mRNA and protein expressions in $K_V4.2^{-/-}$ myocytes, homotetrameric $K_V4.3$ α-subunits are not capable of forming functional channels in mice without $K_V4.2$ due to the inability of coassembly and/or trafficking (Guo et al., 2005). We thus examined whether $K_V4.3$ proteins were expressed on the membrane of $K_V4.2^{-/-}$ cardiomyocytes using immunofluorescence labeling and confocal microscopy. As shown in Figure B.6A, both $K_V4.2^{+/+}$ and $K_V4.2^{-/-}$ myocytes showed a positive fluorescent staining compared to negative controls (a primary or secondary antibody alone). The localization of $K_V4.3$ channels was further examined for using 3D reconstruction (Figure B.6B). The longitudinal and transverse cross-section of cardiomyocytes illustrated the binding of the anti-$K_V4.3$ antibody to the outer region of the myocytes in both $K_V4.2^{+/+}$ and $K_V4.2^{-/-}$, indicating the membrane localization of $K_V4.3$ expression. Taken together, these results demonstrated that $K_V4.3$ subunits can form the homotetrameric functional channels on the membrane, encoding $I_{to,f}$ in the absence of $K_V4.2$ α-subunit in the adult mouse ventricular myocytes.
Figure B.1

Curve Fitting Comparison of $I_{Kv}$ in $K_{v}4.2^{-/-}$ Myocytes

Representative figures of $K^+$ current traces in $K_{v}4.2^{-/-}$ cardiomyocytes fitted with the sum of bi- and tri-exponentials. $K^+$ currents in $K_{v}4.2^{-/-}$ cardiomyocytes were obtained using double pulse protocol with 50 ms interval as illustrated above. $K^+$ currents in both pre-pulse (A) and test-pulse (B) are well described with tri-exponential fitting.
Figure B.2
_Inactivation Time Constant of I_{to} in the Double-pulse Protocols_

Current decay of I_{to} in Kv4.2^{−/−} cells in both pre-pulse (A) and test-pulse (B) were rapid with time constants less than 110 ms while these inactivation were significantly slower compared to littermate control.
**Figure B.3**

*The Effects of HpTx-2 on $K^+$ currents in $K_v4.2^{-/-}$ Myocytes*

Representative $K^+$ current trace of HpTx-2 (5 μM) insensitive (red) and sensitive $K^+$ currents. HpTx-2 sensitive current that was obtained using arithmetic subtraction of insensitive trace from baseline clearly existed in $K_v4.2^{-/-}$ cardiomyocytes. Peak current is shown with expanded time scale on the right panel.
The Effects of HpTx-2 on current densities of $I_{to}$, $I_{K,slow1}$, and $I_{K,slow2}$ in $Kv4.2^{-/-}$ Myocytes

$Kv4.2^{-/-}$ myocytes showed significantly lower $I_{to}$ density compared to control, whereas $IK_{slow1}$ and $IK_{slow2}$ densities were indistinguishable between genotypes. Moreover, HpTx-2 (5 μM) selectively decreased $I_{to}$ in both $Kv4.2^{-/-}$ and littermate control cells while no effect was noticed in $I_{K,slow1}$ and $I_{K,slow2}$.
Figure B.5
Recovery from Steady-State Inactivation of $I_{to}$ in $K\alpha 4.2^{-/-}$ Myocytes

(A) Representative superimposed K$^+$ current traces in $K\alpha 4.2^{-/-}$ cells for recovery from inactivation. The initial phase of recovery of the currents is shown on an expanded time scale (inset). (B) $I_{to}$ recoveries from inactivation in both $K\alpha 4.2^{+/-}$ and $K\alpha 4.2^{-/-}$ cardiomyocytes were described by bi-exponential fitting with two distinct components, corresponding to $I_{to,f}$ and $I_{to,s}$. (C) Recovery time constant of $I_{to,f}$ was significantly increased in $K\alpha 4.2^{-/-}$ cells while there was no difference in $I_{to,s}$ recovery kinetics, compared to $K\alpha 4.2^{+/-}$. (D) Densities of $I_{to,f}$ and $I_{to,s}$ were calculated based on amplitude ratio, showing that $I_{to,f}$ density was decreased in $K\alpha 4.2^{-/-}$ while $I_{to,s}$ density was indistinguishable.
Figure B.6
Membrane Expression of Kv4.3 in Ventricular Cardiac Myocytes of Kv4.2+/+ and Kv4.2−/− Mice

(A) Adult, Kv4.2+/+ (top panel) and Kv4.2−/− (bottom panel), mouse cardiac myocytes labelled with anti-Kv4.3 antibody show positive membrane staining at 10X magnification. (B) In 3D reconstruction of images taken by confocal microscope, cross sectional (left side of panel), transverse (bottom of panel) and longitudinal (right of panel) slices show membrane specific localization of the Kv4.3 α-subunit in both Kv4.2+/+ (left panel) and Kv4.2−/− (right panel) isolated cardiomyocytes. Blue and red lines indicate the position of the transverse and longitudinal section through the cells, respectively.
B.5 Discussion

B.5.1 Homotetrameric K\textsubscript{V}4.3 Channels Encode I\textsubscript{to,f} Without K\textsubscript{V}4.2 Subunit

In this study, we conclude that K\textsubscript{V}4.2\textsuperscript{-/-} cardiomyocytes express the fast transient outward K\textsuperscript{+} current I\textsubscript{to,f}, encoded by K\textsubscript{V}4.3 channels, which is supported by clear evidences such as HpTx-2 sensitive currents as well as biphasic I\textsubscript{to} recovery from inactivation in K\textsubscript{V}4.2\textsuperscript{-/-} cells. Moreover, immunofluorescence labelling has revealed that this I\textsubscript{to,f} in K\textsubscript{V}4.2\textsuperscript{-/-} cells is most likely to be encoded by homotetrameric K\textsubscript{V}4.3 channels. Interestingly, the inactivation time constant of I\textsubscript{to} (\(\tau_1 = 101 \pm 6\) ms), as well as the recovery time constant of I\textsubscript{to,f} (\(\tau_{\text{recov,fast}} = 65 \pm 8\) ms), in K\textsubscript{V}4.2\textsuperscript{-/-} myocytes are comparable to electrophysiological properties of the homotetrameric K\textsubscript{V}4.3 channels in mouse cardiomyocytes and the heterologous system, compared to those in K\textsubscript{V}4.2\textsuperscript{+/-} cells (\(\tau_1 = 64 \pm 7\) ms; \(\tau_{\text{recov,fast}} = 34 \pm 4\) ms). For example, a previous study has demonstrated that I\textsubscript{to,f} decay of the homotetrameric K\textsubscript{V}4.3 channels (\(\tau_{\text{decay}} = 108 \pm 10\) ms) is significantly slower than that of either homotetrameric K\textsubscript{V}4.2 (56 \pm 7 ms) or heterotetrameric K\textsubscript{V}4.2/4.3 channels (51 \pm 6 ms) in myocytes (Guo et al., 2002a). In addition, the recovery time constant of K\textsubscript{V}4.3 (83 \pm 2 ms) is longer than that of heterotetrameric K\textsubscript{V}4.2/4.3 (40 \pm 3 ms). These similarities in electrophysiological properties of I\textsubscript{to,f} support our conclusion that K\textsubscript{V}4.3 can form homotetrameric functional I\textsubscript{to,f} encoding channels in the absence of K\textsubscript{V}4.2 in mouse cardiomyocytes. It is well established that in humans and canine, I\textsubscript{to,f} is mainly encoded by homotetrameric K\textsubscript{V}4.3 since K\textsubscript{V}4.2 expression are not detected in the heart (Oudit et al., 2001; Akar et al., 2004). In spite of the highly conserved K\textsubscript{V}4.3 sequence among all mammalian species, however, electrophysiological properties of I\textsubscript{to,f} in humans or canine are slightly different from those in K\textsubscript{V}4.2\textsuperscript{-/-} myocytes. Since a previous study has demonstrated that the different predominant form of K\textsubscript{V}4.3 in humans (75 kD) or in canine (70 kD) can results in a phenotypic difference of I\textsubscript{to} (Akar et al., 2004), investigation of K\textsubscript{V}4.3 splice variants in mice will help us understand how K\textsubscript{V}4.3 subunits assemble to encode I\textsubscript{to,f} in the mouse heart, and furthermore how I\textsubscript{to,f} is regulated in diseased hearts.

Our data demonstrate that I\textsubscript{to,f} density is significantly reduced in K\textsubscript{V}4.2\textsuperscript{-/-} cells, whereas Niwa et al. have shown that I\textsubscript{to} in K\textsubscript{V}4.3\textsuperscript{-/-} cells is not distinguishable compared with control cells (Niwa et al., 2008). It is unclear what make a difference in these two animal models? This may be because K\textsubscript{V}4.2 is a major K\textsubscript{V}4 pore-forming \(\alpha\)-subunits in adult mouse ventricular cardiomyocytes, although a previous study has suggested that native I\textsubscript{to,f} in mouse hearts is likely
to be encoded by the heterotetrameric $\mathrm{K}_4\text{V}_2/4.3$ channels (Guo et al., 2002a). In addition, quantitative RT-PCR data from the left ventricles of 12 week old CD1 mice showed that $\Delta \text{CT}$ (normalized with 18srRNA) of $\mathrm{K}_4\text{V}_2$ (11.1 ± 0.4) was significantly lower than that of $\mathrm{K}_4\text{V}_3$ (13.7 ± 0.1), indicating approximately 6 fold higher mRNA expression of $\mathrm{K}_4\text{V}_2$ than $\mathrm{K}_4\text{V}_3$ (Unpublished data). A similar result has been also observed in rat hearts, showing that $\mathrm{K}_4\text{V}_2$ mRNA expression is 8 - 15 fold higher than $\mathrm{K}_4\text{V}_3$ expression (normalized with $\beta$-actin) (Goltz et al., 2007). Although our observation and others only are limited to the amount of mRNA, not protein, these may account for the indistinguishable $I_{\text{to,f}}$ density in $\mathrm{K}_4\text{V}_3^{-/-}$ mice compared with wild-type mice (Niwa et al., 2008). Furthermore, it is possible that lower $I_{\text{to,f}}$ density in $\mathrm{K}_4\text{V}_2^{-/-}$ may be due to reduced KChIP2 protein expression (Guo et al., 2005). For instances, epicardial-to-endocardial gradients of KChIP2 expression in human or canine hearts regulate a transmural heterogeneity of $\mathrm{K}_4\text{V}_3$-encoded $I_{\text{to,f}}$ density (Rosati et al., 2001; Zicha et al., 2004), and loss of KChIP2 in mice leads complete elimination of $I_{\text{to,f}}$ (Kuo et al., 2001). Collectively, these observations suggest that a reduction in KChIP2 expression may contributed to a decrease in $I_{\text{to,f}}$ density of $\mathrm{K}_4\text{V}_2^{-/-}$ cells. Further studies will be necessary to measure actual densities and propositions of $\mathrm{K}_4\text{V}_4$ channel mRNA and protein expression in mouse and other species, as well as to understand the molecular mechanism of KChIP2 modulating cell surface expression of $\mathrm{K}_4\text{V}_4$ channels.

B.5.2 Relationship with Previous Studies

Contrary to the previous report that $I_{\text{to,f}}$ is eliminated in $\mathrm{K}_4\text{V}_2^{-/-}$ myocytes (Guo et al., 2005), our results clearly demonstrated that functional $I_{\text{to,f}}$ is encoded by $\mathrm{K}_4\text{V}_3$ $\alpha$-subunits in the absence of $\mathrm{K}_4\text{V}_2$. It should be noticed that Guo et al. have shown that $\mathrm{K}_4\text{V}_3$ mRNA and protein expression is unaffected by loss of $\mathrm{K}_4\text{V}_2$, and indistinguishable compared with those in $\mathrm{K}_4\text{V}_2^{+/+}$ cells. Thus, one major question would be how ours and the other study reach to different conclusions on the identical mouse model. We believe that the main discrepancy results from differences of the dissection methods for outward $\mathrm{K}^+$ currents. As demonstrated in Appendix A, $I_{K_v}$ in mouse cardiomyocytes are well described by the sum of tri-exponentials, whereas bi-exponential fitting lead to improper dissection of $I_{K_v}$ components. In addition, the duration of a current trace is important for a proper mathematical separation, because a trace longer than 20 second is required to avoid over- or under-estimation. For example, fitting the first 4.5 sec of $\mathrm{K}^+$ current traces in $\mathrm{K}_4\text{V}_2^{+/+}$ (n = 6) with bi-exponentials shows decay time constants of 248 ± 46 (39
± 3%) and 1207 ± 115 ms (61 ± 3%), while tri-exponential fittings on the same traces shows 86 ± 17 (10 ± 2%), 466 ± 77 (54 ± 5%), and 2087 ± 445 ms (36 ± 4%). Specifically, these values obtained using the bi-exponential function are very similar to those of 212 ± 19 (37%) and 1140 ± 85 ms (63%) presented in the previous study (Guo et al., 2005). It clearly demonstrated that two exponential fitting leads to improper separation of K⁺ currents.

Finally, our results are supported by a number of neuroscience studies conducted in the identical Kᵥ4.2⁻/⁻ mice. For example, immunofluorescent stainings in Kᵥ4.2⁻/⁻ brain sections have shown that there are no apparent changes in the cellular or sub-cellular expression of Kᵥ4.3, while Kᵥ4.2 or KChIP2 expression is eliminated or down-regulated, respectively (Menegola and Trimmer, 2006). A similar result has been also shown that there is a strong Kᵥ4.3 expression in the superficial layers of the spinal cord dorsal in Kᵥ4.2⁻/⁻ mice (Hu et al., 2006). Furthermore, their results have illustrated that the recovery from inactivation of A-type currents (encoded by Kᵥ4 family subunits (Kᵥ4.1, Kᵥ4.2 & Kᵥ4.3), Kᵥ1.4, or Kᵥ3.4) in Kᵥ4.2⁻/⁻ is biphasic with very fast recovery and slow recovery components, which are quite similar to our Iᵪₒ recovery results in terms of the amplitude ratio between the fast and slow components, whereas the density of A-type currents is decreased. Therefore, our results, combined with other supportive evidences in Kᵥ4.2⁻/⁻ mice, demonstrate that Kᵥ4.3 can assemble function channel encoding Iᵪₒ,f in Kᵥ4.2⁻/⁻ hearts.
REFERENCES


maladaptive hypertrophic pathways: points of convergence and divergence." *Cardiovasc 

Sengupta, P. P., Khandheria, B. K., Korinek, J., Wang, J., Jahangir, A., Seward, J. B. and 

Sengupta, P. P., Korinek, J., Belohlavek, M., Narula, J., Vannan, M. A., Jahangir, A. and 
Khandheria, B. K. (2006b). "Left ventricular structure and function: basic science for 

Seth, M., Zhang, Z. S., Mao, L., Graham, V., Burch, J., Stiber, J., Stiokas, L., Winn, M., 

**112**(16): 2517-29.

Sharif-Naeini, R., Folgering, J. H., Bichet, D., Duprat, F., Delmas, P., Patel, A. and Honore, E.  

expression analysis of subcutaneous fat, fascia, and skin overlying a Dupuytren's disease 
nodule in comparison to control tissue." *Hand (N Y)* **4**(3): 294-301.

"Identification of biomarkers in Dupuytren's disease by comparative analysis of 
fibroblasts versus tissue biopsies in disease-specific phenotypes." *J Hand Surg Am* **34**(1): 
124-36.


"Thyroid hormone regulates postnatal expression of transient K+ channel isoforms in rat 


van Oort, R. J., van Rooij, E., Bourajjaj, M., Schimmel, J., Jansen, M. A., van der Nagel, R.,
"MEF2 activates a genetic program promoting chamber dilation and contractile

van Rooij, E., Doevendans, P. A., de Theije, C. C., Babiker, F. A., Molkentin, J. D. and de Windt,

van Tuyl, M., Liu, J., Groenman, F., Ridsdale, R., Han, R. N., Venkatesh, V., Tibboel, D. and

Vega, R. B., Bassel-Duby, R. and Olson, E. N. (2003a). "Control of cardiac growth and function

Vega, R. B., Rothermel, B. A., Weinheimer, C. J., Kovacs, A., Naseem, R. H., Bassel-Duby, R.,
Williams, R. S. and Olson, E. N. (2003b). "Dual roles of modulatory calcineurin-

Ver Heyen, M., Heymans, S., Antoons, G., Reed, T., Periasamy, M., Awede, B., Lebacq, J.,
Vangheluwe, P., Dewerchin, M., Collen, D., Sipido, K., Carmeliet, P. and Wuytack, F.
(2001). "Replacement of the muscle-specific sarcoplasmic reticulum Ca(2+)-ATPase
isoform SERCA2a by the nonmuscle SERCA2b homologue causes mild concentric

"Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart

outward K+ current and Ca2+ influx in rat cardiac myocytes of endo- and epicardial

alterations of repolarizing K+ currents among the left ventricular free wall of rats with


