TRANSCRIPTIONAL REGULATION DURING CARDIOVASCULAR DEVELOPMENT

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To my supportive family,
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TRANSCRIPTIONAL REGULATION DURING CARDIOVASCULAR DEVELOPMENT

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Heart development is a complex and tightly regulated process involving cell-fate specification, differentiation, and organogenesis. A network of transcription factors play pivotal roles in regulating each step during cardiogenesis. The zinc-finger proteins GATA4 and GATA6 are two closely related transcription factors expressed early during heart development. Although mice that are heterozygous for either a GATA4 or GATA6 null allele are normal, compound heterozygosity of GATA4 and GATA6 results in embryonic lethality by E13.5 accompanied by a spectrum of cardiovascular defects, abnormal smooth muscle development with reduced proliferation of cardiomyocytes and down

regulation of *MEF2C* and the embryonic contractile protein β-MHC. These results exemplify the strict regulation of heart development by revealing that the dose threshold of these two transcription factors is essential for proper cardiovascular development.

The basic helix-loop-helix transcriptional repressor Hairy-related transcription factor-2 (Hrt2), a direct target of Notch signaling, is expressed in ventricular but not atrial cardiomyocytes, as well as in endothelial and vascular smooth muscle cells. To elucidate the cell autonomous function of *Hrt2* in cardiac cell lineages, I generated a conditional *Hrt2* null allele using the loxP-Cre system. Cardiomyocyte-specific deletion of *Hrt2* in mice results in ectopic activation of atrial genes in ventricular myocardium with an associated impairment of cardiac contractility and a unique distortion in morphology of the right ventricular chamber. Furthermore, forced expression of *Hrt2* in atrial cardiomyocytes is sufficient to repress atrial cardiac genes. These results suggest that, consistent with its expression pattern, Hrt2 functions as a repressor in the ventricular cardiomyocyte to suppress atrial cell identity and the maintenance of post-natal cardiac function.

These studies reveal important functions of the transcription factors GATA4, GATA6 and Hrt2 in cardiovascular development.

TABLE OF CONTENTS

Chapter One

Chapter Two

Chapter Three

LIST OF PUBLICATIONS

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LIST OF FIGURES

LIST OF TABLES

ABBREIATIONS

□MHC	□-myosin heavy chain
A-P	anterior-posterior
ANF	atrial natriuretic factor
AV	atrioventricular
bHLH	basic helix-loop-helix
□MHC	□-myosin heavy chain
BNP	brain natriuretic peptide
BMP	bone morphogenetic protein
cx40	connexin 40
E	embryonic day
FS	fractional shortening
HDAC	histone deacetylase
Hrt	Hairy-related transcription factor
LV	left ventricle
LVID	left ventricular internal diameter
MEF2	myocyte enhancer factor2
Mlc1a	atrial myosin light chain 1
Mlc2a	atrial myosin light chain 2
Mlc2V	ventricular myosin light chain 2
MOI	multiplicity of infection

OFT	outflow tract
PTA	persistent truncus arteriosis
RV	right ventricle
SMC	smooth muscle cell
Sln	sarcolipin
VSD	ventricular septal defect

Chapter One

General Introduction

The heart is the first organ to form and function in the developing vertebrate embryo. The maturation of the heart depends on the interplay between various cell types, which dictate the contractile and electrophysiological properties necessary for normal pump function. Cardiogenesis is a highly orchestrated process, exquisitely sensitive to the expression level of genes mediating heart formation. Disruption of this process results in congenital heart disease, which is the most common human birth defect, occurring at an incidence of 1% of the population (Hoffman and Kaplan, 2002).

Mouse heart morphogenesis

Myocardial cells are derived from the mesoderm, emerging from the anterior region of the primitive streak during gastrulation (Garcia-Martinez and Schoenwolf, 1993; Tam et al., 1997). Bone morphogenetic protein (BMP) signals from the endoderm induce cardiomyocyte fate, whereas Wnt-mediated signals from the underlying neural tube and notochord suppress cardiomyocyte specification. In the mouse embryo at embryonic day 6.5 (E6.5), cardiac progenitor cells migrate in an anterior-lateral direction under the head folds to form two groups of cells on either side of the midline where cardiac markers are first detected. These cells further extend across the midline to form the cardiac crescent at E7.5 when differentiated myocardial cells become detectable. The cardiac crescent fuses at the midline, forming a beating heart tube which contains

cardiomyoctyes and the underlying endothelial cells. The heart tube is connected to the body through a posterior inflow, or the venous pole, and an anterior outflow, or arterial pole. The heart tube undergoes uneven growth and remodeling to form the primitive ventricles and atria followed by rightward looping which brings the inflow and outflow segments at the anterior pole of the heart and also positions the future cardiac chambers for proper development (Garry and Olson, 2006; Srivastava, 2006).

Recent studies have advanced our understanding of how the mammalian heart forms by describing multiple groups of cells that contribute to the cardiac lineage. The primary heart field derives from cells that form the cardiac crescent which gives rise to the atria and left ventricle. The secondary heart field derives from cells in the anterior heart field that supports the growth of the outflow tract (OFT, also referred to as conotruncus) and right ventricle. Additionally, non-mesodermal cardiac neural crest cells, which originate from the neural crest migrate into the heart via the pharyngeal arches and the OFT. These cardiac neural crest cells are involved in forming the septum of the heart and the cardiac valves (Buckingham et al., 2005).

Diverse cells types with specialized functions contribute to the muturation of the four chambered functional heart. For example, cardiac muscle cells contribute to ventricular and atrial myocytes, and smooth muscle cells contribute

to the coronary arteries and inflow and outflow vasculature. Endothelial cells form the endocardium and cardiac valves. The epicardium gives rise to the precursors of the coronary vasculature.

An important remodeling process after cardiac specification and differentiation of various cell lineages contributing to the formation of the heart is the septation and formation of the atria and ventricles. These two compartments of a vertebrate heart differ in the regulatory networks that control the expression of chamber specific structural, contractile and calcium binding proteins to facilitate the distinct morphology, electrophysiologic properties of the atria and ventricles.

Mlc2a and *Mlc2v* are two distinct isoforms of myosin light chains expressed in the atrial and ventricular chambers respectively, contributing to different contractile properties. Mis-expression of *Mlc2a* in ventricles to replace *Mlc2v* in *Mlc2v* null mice, results in a decrease in cardiac contractility (Chen et al., 1998). Chamber-specific expression of regulators of Ca^{2+} cycling is also responsible for different contractile properties between the atria and ventricles. *Sarcolipin* (*Sln*) encodes an atrial-specific inhibitor of the cardiac sarcoplasmic reticulum Ca^{2+} ATPase SERCA2a and suppresses Ca^{2+} uptake into the sarcoplasmic reticulum (MacLennan et al., 2003). Overexpression of *Sln* in the ventricle in transgenic mice leads to reduced cardiac contractility and heart failure (Asahi et al., 2004; Babu et al., 2006; Gramolini et al., 2006). The molecular

mechanism underlying the regulation of cardiac chamber specific gene expression still remains unclear.

Molecular control of vertebrate heart formation

Formation of the vertebrate heart is controlled by intrinsic and extrinsic signals, and cross talk between molecules in the primary and secondary heart fields. A network of evolutionarily conserved transcription factors has been revealed to play vital roles in keeping cardiogenesis under tight control in a timely and spatial manner. Cardiogenic signals activate several transcriptional regulators of cardiogenesis, such as the earliest molecular marker of the cardiac lineage, the homeobox gene *Nkx2-5* in vertebrates (Harvey, 1996), the orthologue of the *Drosophila Tinman* gene (Bodmer, 1993). *Tinman* has been shown to be necessary for specification of the cardiac lineage and directly activates transcription of the myocyte differentiation gene *MEF2* (Gajewski et al., 1998). Tinman and Nkx2.5 together with the zinc- finger transcription factors of the GATA family, synergistically activate expression of other cardiac genes.

Expression patterns of transcription factors are restricted to specific regions of the heart (Figure 1.1). There exist transcriptional hierarchies in cardiogenesis. In the primary heart field, Nkx2-5 and GATA4 are at the top of transcription hierarchy regulating down stream target genes such as *Hand1*, *Bmp10* and *Irx4*. Whereas, in the secondary heart field, *Isl1* and *Foxh1*, which are

at the top of the transcriptional hierarchy regulate the expressions of genes marking the secondary heart field, such as *Hand2*, the LIM-homeodomain transcription factor *Tbx1, Ptx2, Fgf8* and *Fgf10* (Kelly, 2001; cai 2003).

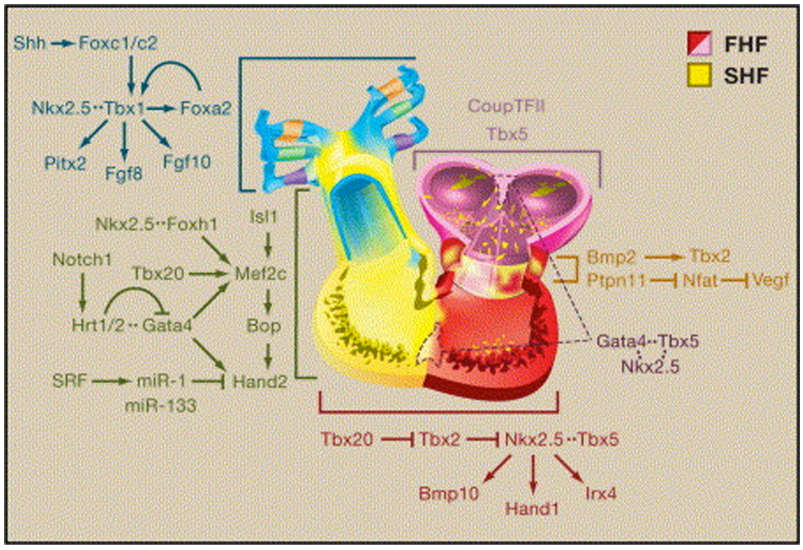

Figure 1.1 Pathways Regulating Region-specific Cardiac Morphogenesis

A partial list of transcription factors, signaling proteins, and miRNAs that can be placed in pathways that influence the formation of regions of the heart is shown. Positive influences are indicated by arrowheads, and negative effects by bars. Physical interactions are indicated by dashed lines between factors. (Adapted from Srivastava, 2006)

Nkx2-5

The homeobox gene *Nkx2-5*, the earliest gene expressed in the cardiac lineage, is a vertebrate orthologue of the *Drosophila Tinman* gene, serves as a target of inductive signals for initiation of cardiogenesis. It is expressed in the cardiogenic mesoderm upon specification of the lineage and its expression in the heart is maintained into adulthood (Komuro and Izumo, 1993; Lints et al., 1993).

Over-expression of *Nkx2-5* in zebrafish or frog embryos results in expansion of the heart field and upregulation of cardiac gene expression (Chen and Fishman, 1996; Cleaver et al., 1996). Mice lacking *Nkx2-5* are embryonically lethal due to abnormal morphogenesis of the heart tube and failure of left ventricular development (Lyons et al., 1995). Unlike *Tinman* in *Drosophila*, which has been shown to be necessary for specification of the cardiac lineage (Bodmer, 1993; Gajewski et al., 1998), *Nkx2-5* in mice is not required for the initial events of cardiogenesis. This suggests that other *Nkx* genes may have redundant functions during cardiac development. The importance of *NKX2-5* in humans has been evidenced by the identification of mutations of the *NKX2-5* gene that are associated with congenital heart disease (Schott et al., 1998).

MEF2

MEF2 genes encode the Myocyte Enhance Factor 2 (MEF2) transcription factor family which plays critical roles in muscle development (Gossett et al., 1989), chondrocyte maturation (Arnold et al., 2007) and brain development (Flavell et al., 2006; Mao et al., 1999). MEF2 proteins are members of the MADS box family of transcription factors. There are four members of the MEF2 protein family MEF2A, MEF2B, MEF2C and MEF2D. They share similar protein structures. The MADS box is followed by the MEF2 domain, which

mediates dimerization, and the transactivation domain, which mediates protein-protein interaction with activators or repressors.

MEF2C is the first detectable *MEF2* gene during cardiogenesis. It is detected in the early precardiac mesoderm of the primary and secondary heart fields at E7.5 (Dodou et al., 2004). *MEF2* enhancer studies have identified a cardiac-specific enhancer for the secondary heart field containing GATA and Isl1 consensus binding sites. Mutations of these sites disrupt *MEF2C* expression in the secondary heart field.

Functional studies of *MEF2A* and *MEF2C* genes have been carried out using targeted deletion of MEF2 in mouse model systems. *MEF2A* null mice die within the first two weeks of birth due to cardiac dilation and right ventricle failure, associated with a decrease in the number and function of mitochondria (Naya et al., 2002). *MEF2C* knockout mice die at E9.5 with severe cardiovascular defects (Lin et al., 1998; Lin et al., 1997), including failure of cardiac looping, pericardial effusion and disorganized vessel formation. Some cardiac markers for heart development such as cardiac □-actin, Mlc1A and HAND2 are significantly down-regulated in *MEF2C* null embryos.

Tbx

T-box transcription factors bind to the consensus sequence of GGTGT via their highly conserved DNA binding domain, the T-box, and direct transcription

of their target genes in different tissues. Out of the 18 T-box proteins in mice, Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20 are expressed in the developing heart. It has been implied that T-box proteins are essential for early cardiac lineage determination, chamber formation, and diversification of the specialized conduction system. The secondary heart field marker *Fgf10* is regulated by Tbx5 and Tbx1. *TBX1*, *TBX3* and *TBX5* are associated with human congenital heart diseases. Mutations in *TBX5* genes cause Holt-Oram syndrome in humans promoting limb and cardiac malformation (Basson et al., 1997; Benson et al., 1998; Huang et al., 2002). Mice lacking the *Tbx5* gene die at E9.5 with a deformed linear heart tube acompanied by a hypoplastic posterior heart segment. (Bruneau et al., 2001b). *ANF* and *Connexin 40* (*Cx40*) are down regulated in *Tbx5* mutant mice. Moreover, Tbx5 interacts with other important cardiac transcription factors, such as Nkx2-5 and GATA factors to synergistically activate transcription (Bruneau et al., 2001b; Garg et al., 2003).

Hand

The basic helix-loop-helix (bHLH) family of transcription factors has been shown to be important for specification, differentiation, and morphogenesis of multiple tissues and organs during embryogenesis (Massari and Murre, 2000). Hand1 and Hand2 represent the earliest cardiac chamber-specific transcription factors (Cserjesi et al., 1995; Srivastava et al., 1995; Srivastava et al., 1997).

Hand1 is expressed exclusively in the outer curvatures of the left ventricle and outflow tract (Biben and Harvey, 1997; Srivastava et al., 1997). In contrast, the highest level of Hand2 expression in the heart was detected in the right ventricle. Functions of Hand proteins were revealed from studies of targeted mutagenesis of *Hand1* and *Hand2* genes in mouse models. *Hand1* null mouse embryos die at E 8.0 due to severe extraembryonic defects. In contrast, consistent with its expression pattern, targeted deletion of *Hand2* results in embryonic lethality at E10.5 with cardiac and vascular defects (Srivastava et al., 1997; Yamagishi et al., 2000). This suggests that the *Hand2* gene is essential for morphogenesis of the right ventricle, and formation of the neural crest-derived aortic arches.

GATA

GATA proteins are zinc finger transcription factors that bind to HGATAR DNA motif and contain two class IV zinc-finger domains. They can be divided into two subgroups: *GATA1*, *GATA2* and *GATA3*, which are expressed predominantly in hematopoietic cells, and regulate differentiation-specific gene expression in T-lymphocytes, erythroid cells, and megakaryocytes (Orkin et al., 1998). Subgroup 2 consists of *GATA4*, *GATA5* and *GATA6*, which are expressed in several endodermal and mesodermal linages, such as the developing heart, liver, lung, gonad, and gut where they play critical roles in regulating tissue-specific gene expression (Molkentin, 2000).

A) GATA genes and cardiovascular development

Null mutations in the *Drosophila GATA4* orthologue, *pannier*, zebrafish *GATA5* (Reiter et al., 1999) or mouse *GATA4* (Kuo et al., 1997; Molkentin et al., 1997) result in early defects in cardiogenesis. Mouse embryos lacking *GATA4* die at E8.5 due to cardiac bifida and failure of ventral foregut closure. To circumvent early lethality caused by conventional deletion of *GATA4* in the mouse, conditional deletion of *GATA4* alleles have been generated. Early cardiac-specific deletion of *GATA4* also results in myocardial thinning, abnormal endocardial cushion development and right ventricular hypoplasia (Zeisberg et al., 2005), while cardiac-specific deletion at later time points results in reduced cardiac function and an inability to undergo hypertrophy following pressure overload or exercise (Oka et al., 2006).

Recent studies have suggested that there is a dose sensitivity of *GATA4* in regulating cardiac function. Reduction in *GATA4* dosage from a hypomorphic *GATA4* allele causes cardiac septal and other congenital heart defects (Pu et al., 2004). Heterozygous mutations in *GATA4* are also associated with congenital heart defects in humans (Garg et al., 2003). These mutations not only reduced GATA4 DNA binding affinity and transcriptional activity of downstream target genes, but also disrupt the interaction between GATA4 and Tbx5, suggesting interactions between GATA and other transcription factors play important roles in heart development.

GATA6 null mice die post implantation due to defects in visceral endoderm function and extraembryonic development (Morrisey et al., 1998). Tetraploid rescue experiments implicate a role for GATA6 in liver differentiation and growth, and suggest that GATA4 provides functional redundancy in liver specification (Zhao et al., 2005). *GATA6* conditional knockout mice have also been generated. Tissue-specific deletion of *GATA6* in smooth muscle or neural crest suggests a role for this factor in patterning the cardiac outflow tract and the aortic arch (Lepore et al., 2006). To date, defects in myocardial development have not been observed in *GATA6* mutant mice.

Moreover, *in vitro* studies suggest GATA4 and GATA6 interact and function synergistically in activating atrial natriuretic factor (*ANF*) and *BNP* gene expression in cardiomyocytes (Charron et al., 1999). Furthermore, GATA4 and 6 have been shown to be sufficient to drive extraembryonic endoderm differentiation in embryonic stem cells, suggesting a critical role for these factors in early development.

Given the similarities in protein structure, their overlapping expression patterns, and their ability to homodimerize and synergistically drive the expression of cardiac genes, we postulated that GATA4 and 6 might play redundant roles during embryonic development. To further examine the function of these closely related genes in mouse development, we generated double heterozygous mice for *GATA4* and *GATA6* mutant alleles, the results of which are

presented in Chapter 2. Briefly, whereas mice heterozygous for either a *GATA4* or *GATA6* null allele are normal, the combination of heterozygous mutations in the *GATA4* and *GATA6* genes results in embryonic lethality at E13.5, accompanied by a spectrum of defects in liver development, hematopoiesis and the cardiovascular system.

B) Transcriptional regulation by GATA proteins

Regulation of GATA downstream targets in cardiovascular development shows that GATA4 is an important cardiac lineage marker. Specifically, GATA4 not only regulates the expression of genes critical for cardiac contractility, such as □□*MHC* and □□*MHC* (Charron et al., 1999), but also the expression of important cardiac transcription factors, such as *Nkx2.5*, *MEF2* and *Hand2* (Dodou et al., 2004; Jiang et al., 1999; Lien et al., 1999; McFadden et al., 2000; Molkentin et al., 1994; Nemer and Nemer, 2001; Nemer and Nemer, 2003; Pikkarainen et al., 2004; Reecy et al., 1999). In addition, GATA4 and 6 have been implicated as regulators of liver-specific gene expression through consensus GATA sites (A/T)GATA(A/G) within the promoters of liver-specific genes. The regulation of GATA downstream targets is achieved by association of GATA with tissue specific co-activators such as nuclear factor of activated T cells 3 (NFAT3) (Molkentin et al., 1998), Nkx2-5 ((Lee et al., 1998; Sepulveda et al., 1998), Tbx5 (Garg et al., 2003), serum response factor (SRF) (Belaguli et al., 2000), MEF2C

(Morin et al., 2000), Hand 2 (Dai et al., 2002) and myocardin (Oh et al., 2004) to activate transcription synergistically. On the other hand, transcriptional activities of GATA proteins have also been shown to repress gene activity by association with transcriptional co-repressors, such as FOG2 (friend of GATA2) (Hirai et al., 2004) and Hrt2 (Fischer et al., 2005; Kathiriya et al., 2004).

Hrt2

Hrt (Hairy-related transcription factor) proteins belong to a small family of basic helix-loop-helix (bHLH) transcription factors that are related to the Drosophila *hairy* and *Enhancer-of-split* genes. Other names such as *Hey*, *Hesr*, *CHF*, *Herp* have also been given to Hrt genes by different research groups (Chin et al., 2000; Iso et al., 2001a; Kokubo et al., 1999; Leimeister et al., 1999). Hrt proteins are one of the direct targets of the Notch signaling pathway. There are three structurally related members of the *Hrt* gene family identified in human and mouse: *Hrt1*, *Hrt2* and *Hrt3* (Nakagawa et al., 1999). They share high homology in the basic DNA binding domain, the Helix-loop-Helix domain for protein dimerization and the orange domain, an additional interface domain for potential protein-protein interactions. In addition, Hrt1 and Hrt2 proteins share a conserved domain in the C-terminus with a consensus sequence of YRPW. Although no function has been attributed to the YRPW domain in Hrt proteins, it has been

speculated that the YRPW domain is important due to strong evolutionary conservation in many hairy-related factors.

A) *Hrt* genes and cardiovascular development

The *Hrt2* gene is expressed in ventricular cardiomyocytes, in the cardiac outflow tract and aortic arch arteries, as well as in precursors of the pharyngeal arches and subsequently in the pharyngeal clefts. The *Hrt2* gene is expressed in other sites of epithelial-mesenchymal interactions, including the developing kidneys, brain, limb buds, and vasculature (Leimeister et al., 1999; Nakagawa et al., 1999). The *Hrt1* gene is only expressed in atrial cardiomyocytes.

Functional studies of Hrt2 protein have been performed in several laboratories including ours by using targeted mutagenesis in embryonic stem cells. Mice lacking *Hrt1* are normal, whereas mice lacking *Hrt2* expression die shortly after birth and display a spectrum of congenital heart defects including ventricular septal defects, tetralogy of Fallot, and tricuspid atresia, indicating that Hrt2 plays important roles in regulating cardiac morphogenesis and mediating Notch signaling in the developing heart (Donovan et al., 2002; Gessler et al., 2002; Kokubo et al., 2004; Sakata et al., 2002; Sakata et al., 2006). However, the molecular pathway leading to ventricular septal defect (VSD) and associated defects in *Hrt2* null mice is not currently known.

A recent gene expression profiling study revealed that *Hrt2* was selectively expressed in arterial endothelial cells and induced the expression of several arterial–specific genes (Chi et al., 2003). This is consistent with the results obtained from the study of the *Hrt2* orthologue in Zebrafish, *gridlock (grl)*. A mutation of *grl* leads to a localized defect of the aorta that resembles human coarctation of the aorta (Weinstein et al., 1995; Zhong et al., 2001; Zhong et al., 2000).

B) Transcriptional regulation by Hrt2 protein

Hrt2 protein belongs to the bHLH family, which bind DNA consensus E-box sequences (CANNTG) as homo-dimers or hetero-dimers through the bHLH domain. Although it has been shown that Hrt2 binds to a putative E-box *in vitro* (Nakagawa, 1999), its real *in vivo* target still remains unknown. Hrt2 has been shown to be a transcriptional repressor of GATA4 by directly associating with GATA4 and repressing GATA4-dependent transcriptional activation of its target genes, such as *ANF* (Fischer et al., 2005; Kathiriya et al., 2004). A recent study demonstrated that Hrt2 could affect transcriptional regulation of the smooth muscle myosin heavy chain promoter *in vitro*, by physically associating with GATA6 leading to repression of GATA6 dependent transcriptional activity (Shirvani et al., 2006). In both cases, Hrt2 functions as a repressor in a similar manner through direct protein-protein interaction with a tissue specific

transcription factor. So far, no Hrt2 direct target has been identified. Therefore, it has been postulated that Hrt2 could specifically repress target genes by association with other transcription factors instead of directly binding to DNA sequences.

Objectives

In this study, I took a genetic approach using the mouse as a model system, to determine the critical functions of GATA4, GATA6 and Hrt2 in cardiac development.

I. To characterize the phenotype of *GATA4/GATA6* heterozygous mutant mice.

Given the similarities in protein structure, their overlapping expression patterns, and their ability to homodimerize and synergistically drive the expression of cardiac genes, we postulated that GATA4 and 6 might play redundant roles in embryonic development. To further examine the function of these closely related genes in mouse development, we generated mice doubly heterozygous for *GATA4* and *GATA6* null alleles. *GATA4/GATA6* compound heterozygosity causes embryonic lethality by E13.5 with complete penetrance. My goal was to determine the cause of lethality in the *GATA4/GATA6* compound mutant embryos by using a variety of approaches, such as histology, immunohistochemistry and candidate gene analysis.

II. To use the LoxP/Cre system to generate a conditional Hrt2 knockout mouse line to study the cell autonomous function of Hrt2 in cardiomyocytes.

The neonatal lethality of Hrt2 null mice makes it difficult to study the role of the *Hrt2* gene in adult hearts. Also, using a conventional knockout strategy precludes the ability to study the function of the Hrt2 protein in a tissue-specific manner.

To achieve my goal of elucidating the requirement of *Hrt2* in cardiomyocytes, I generated a floxed *Hrt2* allele in which exons 2 and 3, containing most of coding sequence of the DNA binding and protein dimerization bHLH domain were flanked by loxP sites. Cardiomyocyte-specific Cre recombinase-mediated site-specific recombination of chromosomally integrated loxP sites results in deletion of exons 2 and 3 leading to a frame shift of the rest of the Hrt2 protein, yielding a null allele in the heart. Characterization of the morphology, gene expression profiles and function of hearts of mice with cardiac deletion of Hrt2 was carried out.

Chapter Two

A threshold of *GATA4* and *GATA6* expression is required for cardiovascular development

Abstract

The zinc-finger transcription factors GATA4 and GATA6 play critical roles in embryonic development. Mouse embryos lacking *GATA4* die at embryonic day (E) 8.5 due to failure of ventral foregut closure and cardiac bifida, whereas GATA6 is essential for development of the visceral endoderm. While mice heterozygous for either a *GATA4* or *GATA6* null allele are normal, we show that compound heterozygosity of *GATA4* and *GATA6* results in embryonic lethality by E13.5 accompanied by a spectrum of cardiovascular defects including thin-walled myocardium, ventricular and aortopulmonary septal defects, and abnormal smooth muscle development. Myocardial hypoplasia in *GATA4/GATA6* double heterozygous mutant embryos is associated with reduced proliferation of cardiomyocytes, diminished expression of the myogenic transcription factor MEF2C, and down-regulation of beta-myosin heavy chain expression, a key determinant of cardiac contractility. These findings reveal a threshold of GATA4 and GATA6 activity required for gene expression in the developing cardiovascular system and underscore the potential of recessive mutations to perturb the delicate regulation of cardiovascular development.

Introduction

The GATA family of transcription factors plays important roles in differentiation, growth and survival of diverse cell types (Molkentin, 2000; Weiss and Orkin, 1995). Six GATA family members have been identified in vertebrates, all of which contain two zinc finger domains that bind the consensus site (A/T)GATA(A/G) and mediate co-factor interactions. GATA1, 2, and 3 are primarily expressed in haematopoietic lineages (Weiss and Orkin, 1995) and GATA4, 5, 6 are expressed in mesoderm and endoderm derived tissues such as the heart, liver, lung, and gut (Molkentin, 2000).

GATA4 regulates the expression of genes critical for cardiac contraction, as well as the expression of cardiac transcription factors such as Nkx2.5, Hand2, and MEF2C (Dodou et al., 2004; Jiang et al., 1999; Lien et al., 1999; McFadden et al., 2000; Molkentin et al., 1994; Nemer and Nemer, 2001; Nemer and Nemer, 2003; Pikkarainen et al., 2004; Reecy et al., 1999). *GATA4* null mice display defects in heart morphogenesis and ventral foregut closure (Molkentin et al., 1997), resulting in embryonic lethality by E8.5. Tetraploid rescue experiments with *GATA4* null embryonic stem cells give rise to embryos with abnormal looping of the heart tube and thin-walled myocardium (Watt et al., 2004). Early

cardiac-specific deletion of *GATA4* also results in myocardial thinning, abnormal endocardial cushion development and right ventricular hypoplasia (Zeisberg et al., 2005), while cardiac-specific deletion at later time points results in reduced cardiac function and an inability to undergo hypertrophy following pressure overload or exercise (Oka et al., 2006). Mice homozygous for a hypomorphic *GATA4* mutation display a variety of heart defects including double outlet right ventricle and hypoplasia of the compact myocardium (Pu et al., 2004). Heterozygous mutations in *GATA4* are also associated with congenital heart defects in humans (Garg et al., 2003).

GATA6 null mice die post implantation due to defects in visceral endoderm function and extraembryonic development (Morrisey et al., 1998). Tetraploid rescue experiments have implicated GATA6 in liver differentiation and growth and suggest that GATA4 provides functional redundancy in liver specification (Zhao et al., 2005). Tissue-specific deletion of *GATA6* in smooth muscle or neural crest suggests a role for this factor in patterning the cardiac outflow tract and the aortic arch (Lepore et al., 2006). To date, defects in myocardial development have not been observed in *GATA6* mutant mice.

Mice heterozygous for *GATA4* or *GATA6* null mutations are viable and without obvious cardiovascular phenotypes. However, given the similarities in protein structure and expression pattern of GATA4 and 6, and their ability to physically interact and synergistically enhance gene transcription (Charron et al.,

1999), we postulated that GATA4 and 6 might act cooperatively to regulate cardiovascular development. Here we show that *GATA4/6* compound heterozygous mice die by E13.5 with 100% penetrance. These mutant mice display a spectrum of cardiovascular defects that include ventricular-septal defects (VSDs), a persistent truncus arteriosis (pta), myocardial hypoplasia, reduced myocardial proliferation, and impaired differentiation of vascular smooth muscle cells (SMCs). Our findings reveal an exquisite sensitivity of the developing cardiovascular system to the levels of GATA4 and GATA6 and suggest that these GATA factors act cooperatively to regulate downstream target genes in cardiac and SMCs *in vivo*.

Materials and Methods

Mice

Heterozygous *GATA6*$^{+/-}$ mice were maintained in both a pure SV129 and a mixed background of SV129 and C57BL6. SM22-lacZ transgenic mice have been described previously (Li et al., 1996). Heterozygous *GATA4*$^{+/-}$ (Molkentin et al., 1997) and *GATA6*$^{+/-}$ mice (Xin et al., 2006) of mixed background were intercrossed and the appearance of the vaginal plug was taken as E0.5. Genomic DNA was isolated from embryos, and genotypes of fetuses were determined by

PCR. For GATA4 223 bp and 600 bp fragments were obtained from wild-type and *GATA6* homozygous mutant animals, respectively. For the *GATA6* genotyping, 150 and 430 bp PCR fragments were obtained from wild-type and *GATA6* homozygous mutant animals, respectively. , Primer sequences for PCR genotypings are:

G4mutrp (5'GGAGGTGGGGCATAGACACAGC),

neo3'fp (5'TTGGCTACCCGTGATATTGCTGAAGAGC)

G4WTrp (5'GGGACTATCCTGGCCTAGCCC)

G6mutrp(5'TGTAAGCTGTGGAGCACCGGCG),

neo3'fp (5'TTGGCTACCCGTGATATTGCTGAAGAGC)

GATA6WTrp(5'CACCAGTACAGCTCGCTGTCCG)

Histology and immunostaining

Embryos were collected at various time points of gestation from E10.5 to E14.5 and fixed in 4% paraformaldehyde in PBS at 4°C overnight followed by dehydration and embedding in paraffin as previously described (Moller and Moller, 1994). Sections of 5 micron thickness were mounted on slides followed by hematoxylin/eosin staining according to standard procedures. For immunostaining, sections were deparaffinzed in xylene, rehydrated through graded ethanol to phosphate buffered saline (PBS), and permeabilized in 0.3% Triton X-100 in PBS. Sections were then blocked by 1.5% normal horse serum in

PBS followed by incubation with anti-smooth muscle alpha actin clone 1A4 (Sigma-Aldrich, St. Louis, MO) or rabbit anti-phosphohistone H3 (Upstate Cell Signaling Solutions, Charlottesville, VA) at a 1:200 dilution in 0.1% BSA in PBS overnight at 4°C. Sections were washed in PBS, and Cy3 or fluorescein-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) were applied at a 1:200 dilution in 1% normal horse serum for 1 hour. For the phosphohistone H3 studies, the measurements represent the average of six sections of control and mutant embryonic hearts with standard deviations.

Whole mount staining for β-galactosidase

Embryos were collected at E12.0 and stained for β-galactosidase as previously described (Naya et al., 1999). Following overnight staining, embryos were fixed in 4% paraformaldehyde/0.2% glutaraldehyde, dehydrated through graded methanol and cleared in benzyl benzoate: benzyl alcohol (2:1).

Whole Mount Immunostaining

E10.5 embryos were fixed in 4% paraformaldehyde/PBS overnight at 4°C and bleached with 5% H_2O_2 for 4 hours to block endogenous peroxidase. Embryos were blocked with PBSMT (3% milk, 0.1%Triton X-100 in PBS) for 2 hours at room temperature followed by overnight incubation with 10 μg/ml anti-PECAM

(BD Biosciences, San Jose, CA) at 4°C. Embryos were subsequently washed in PBSMT and incubated with 1:100 dilution of anti-rat HRP-coupled secondary antibody at 4°C overnight. Staining was visualized using 0.3mg/ml 3.3'diaminobenzidine (Sigma-Aldrich, St. Louis, MO), 0.5% NiCl2, 0.03% H_2O_2.

India Ink Injections

E12.5 embryos were harvested and subjected to intracardial injection of India ink using custom made glass pipettes. After injection, embryos were fixed in 4% paraformaldehyde for 12 hours, dehydrated through graded methanol and cleared in benzyl benzoate: benzyl alcohol (2:1) as previously described (Oh et al., 2005).

RNA isolation, Real-time PCR and Microarray analyses

Total RNA was isolated from embryonic hearts collected at E11.75 using the TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. For RT-PCR, 1 microgram of total RNA was used as a template for reverse transcription with random hexamer primers. 25 ng of cDNA was amplified in each real-time PCR reaction using the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA). Mean relative gene expression was calculated using standard curves from serial dilutions of cDNA from wild type hearts and normalized to GAPDH, n=3 per group.

Results

Compound heterozygosity of *GATA4/6* results in embryonic lethality by E13.5.

To investigate whether a threshold of GATA4 and GATA6 activity might be required for normal embryonic development, we intercrossed $GATA4^{+/-}$ and $GATA6^{+/-}$ mice. No *GATA4/6* compound heterozygous mice were observed at birth (Table 2.1), suggesting that the combined heterozygous mutations resulted in embryonic lethality. Analysis of embryos from timed matings revealed that compound heterozygous offspring were viable up to E13.5. However, no viable *GATA4/6* compound heterozygous animals were observed at E14.5 or later, indicating that this genotype results in embryonic lethality with complete penetrance by E13.5. Embryonic lethality of *GATA4/6* compound heterozygous embryos was observed in C57 BL6/129 mixed genetic backgrounds, suggesting its independence of possible strain variability.

Table 2.1. Genotypes of Offspring from *GATA4*$^{+/-}$, *GATA6*$^{+/-}$ Intercrosses

	Offspring of Each Genotype			
Age	+/+	*GATA4*$^{+/-}$	*GATA6*$^{+/-}$	*GATA4*$^{+/-}$/*6*$^{+/-}$
P1	29(36%)	27(34%)	24(30%)	0(0%)
E14.5	8(30%)	9(33%)	10(37%)	0(0%)
E10.5-E13.5	26(26%)	28(28%)	26(26%)	19(19%)

GATA4$^{+/-}$ mice were mated with *GATA6*$^{+/-}$ mice. Numbers of viable offspring of each genotype and percent of total at each developmental stage are shown.

Compound heterozygosity of *GATA4/6* results in abnormal vascular development.

GATA4/6 compound heterozygous embryos displayed widespread hemorrhages by E11.5 and edema at E13.5, as well as reduced liver size as compared to control littermates (Fig. 2.1 and data not shown). We did not observe a difference in hepatic gene expression in the *GATA4/6* mutants although we did observe a reduction in the number of mature erythrocytes in the peripheral blood (data not shown). These results are consistent with *GATA4/6* loss of function studies in zebrafish which demonstrate their redundant roles in growth of the specified liver bud (Holtzinger and Evans, 2005).

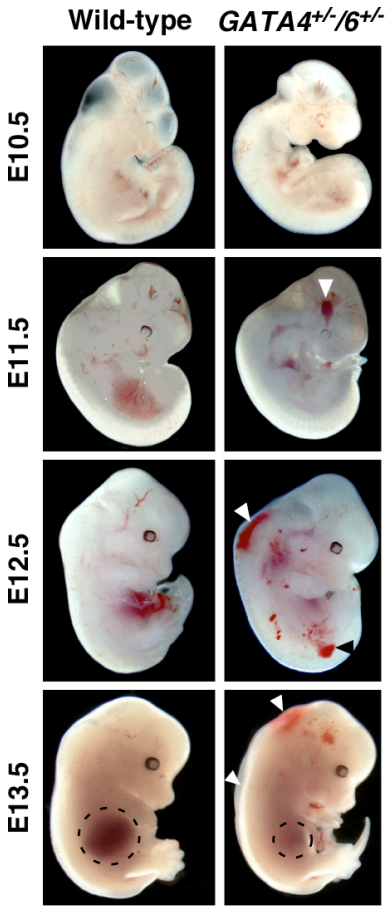

Figure 2.1 Edema and hemorrhage in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos at E13.5.

Wild type and *GATA4*$^{+/-}$/*6*$^{+/-}$ mutant embryos at E10.5, E11.5, E12.5, and E13.5 are shown. Arrowheads denote hemorrhage and edema. Dotted lines outline the liver in both control and *GATA4/6* mutants.

The heartbeat of *GATA4/6* mutants at E11.75 was sluggish and irregular as compared to that of control littermate embryos (data not shown), suggesting possible abnormalities in cardiac function. To visualize potential vascular abnormalities in *GATA4/6* mutant embryos, we stained the vasculature of E10.5

embryos for platelet/endothelial cell adhesion molecule (PECAM). As shown in Figure 2.2, the cranial and intersomitic vasculature in the *GATA4/6* double heterozygous embryos was enlarged and disorganized compared to wild type embryos, indicative of possible defects in blood circulation and/or vessel development and remodeling.

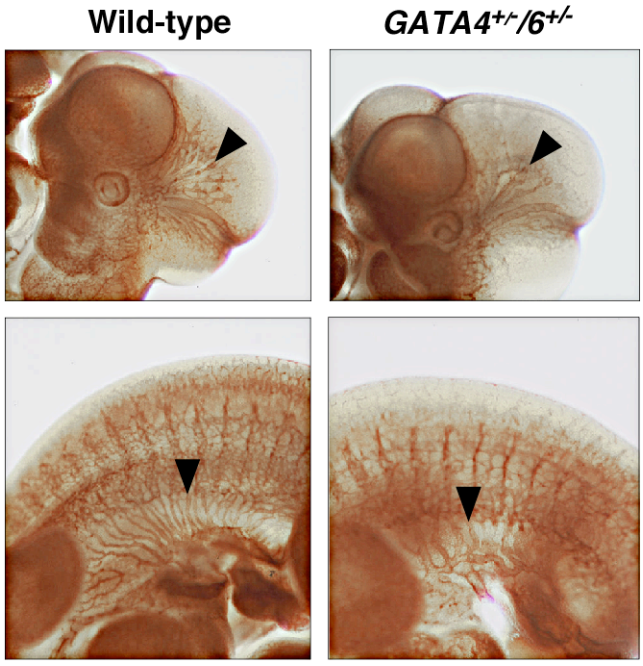

Figure 2.2 Vascular defects in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. Whole mount staining of wild type and mutant embryos at E10.5 using anti-platelet/endothelial cell adhesion molecule (PECAM) antibodies. Arrowheads denote dilated and less developed vessels within the cranial and intersomitic vasculature in the *GATA4*$^{+/-}$ */6*$^{+/-}$ embryos.

To better visualize the defects in vascular patterning in mutant mice, we intercrossed *GATA4* and *GATA6* heterozygous mice with mice transgenic for *SM22-LacZ*, a cardiac and arterial smooth muscle-specific reporter (Li et al., 1996). LacZ staining at E12 revealed patterning defects of the outflow tract in *GATA4/6* mutants, as evidenced by the presence of a pta, a defect resulting from incomplete septation of the conotruncus into the aorta and pulmonary artery (see arrowhead in Fig. 2.3 and Fig 2.6). Although the process of aortopulmonary septation occurs between E10.5 and E13.5 (Sharma et al., 2004), the pta observed in the *GATA4/6* mutants was completely penetrant (n>10) when compared with control littermates, suggesting either a delay in the mutants or an inability to completely septate. As the *GATA4/6* compound heterozygotes die by E13.5, we cannot rule out the possibility that this component of the phenotype is a result of general delay in cardiovascular development beginning at E11.5. LacZ staining in the descending aorta (da) and vertebral artery (va) of compound mutant embryos was also diminished compared to littermate controls (Fig 2.3 arrows). As the *SM22* promoter is not regulated directly by GATA factors (Li et al., 1997), its down-regulation suggests a general defect in smooth muscle differentiation. Notably, the *SM22-LacZ* reporter, which is normally expressed specifically in

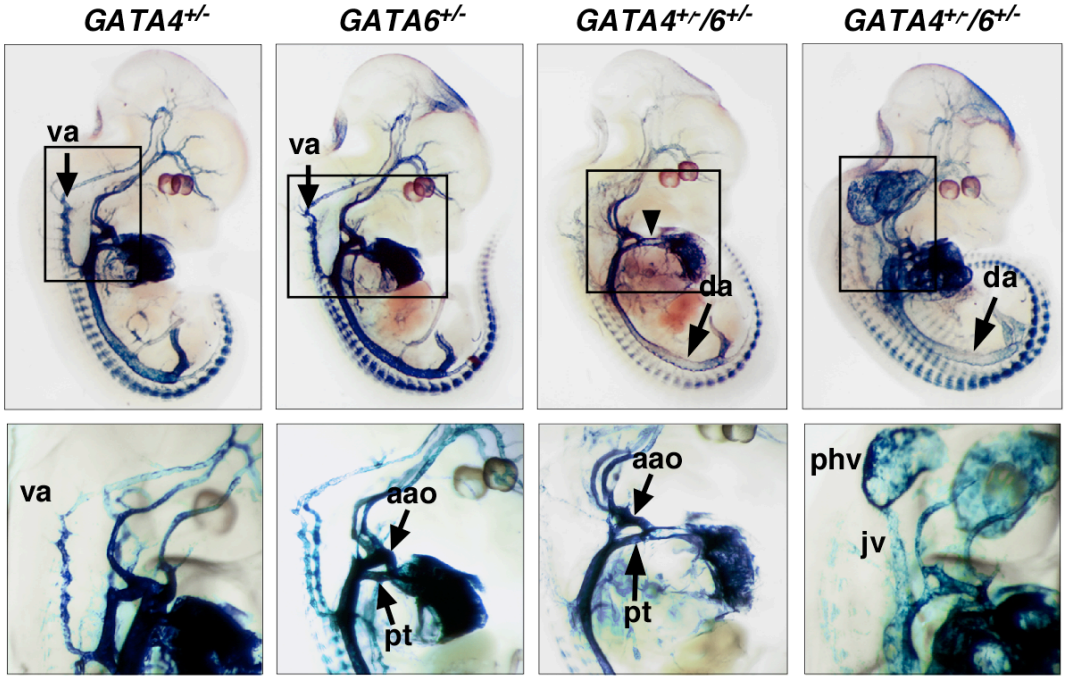

Figure 2.3 Vascular defects in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. □□glactosidase staining of *GATA4*$^{+/-}$, *GATA6*$^{+/-}$, or *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos at E12 reveals reduced expression of the *SM22-LacZ* transgene (arrows) and the presence of a pta (see arrowheads). jv and phv denote expression of the *SM22-LacZ* transgene in venous SM of *GATA4*$^{+/-}$/*6*$^{+/-}$ mutants. Areas within boxes in upper panels have been enlarged in lower panels. ba, branchial arch; va, vertebral artery; jv, jugular vein; phv, primary head vein; da, dorsal aorta; aao, ascending aorta; pt, pulmonary trunk.

arterial SMCs, was activated in venous SMCs of the *GATA4/6* mutants (see Fig. 2.3; jugular vein (jv), and primary head vein (phv)). Transverse sections of stained embryos showed that the primary head vein (see arrow) and jugular veins (arrowhead) of mutant embryos were positive for LacZ expression, suggesting an abnormality in SMC identity within the developing vasculature (Fig. 2.4). A large arterial-venous malformation can also be seen in the embryo shown in Figure 2.3.

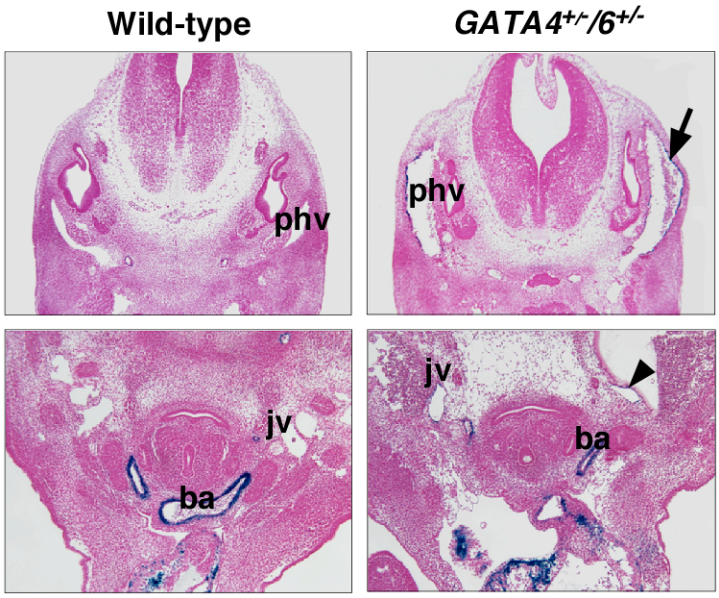

Figure 2.4 Vascular defects in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. Transverse sections of b-galactosidase stained embryos in B demonstrate LacZ staining within the primary head (arrow) and jugular veins (arrowhead). ba, branchial arch; jv, jugular vein; phv, primary head vein.

Similar defects were seen in multiple embryos, although their severity varied. Whether GATA4 and 6 participate directly in the establishment of vascular identity, or whether the *SM22* promoter becomes inappropriately activated in venous SMCs in response to pathological signals, such as hypoxia, requires further study.

Histological sections also revealed thin dilated vessels in *GATA4/6* mutants compared to littermate controls. Immunostaining for smooth muscle-alpha actin at E12.5 showed that there was less smooth muscle in the medial layer of the aorta (Fig. 2.5) and supported our observation of a reduction in arterial

smooth muscle. These data suggest that GATA4 and 6 are required for proper development of vascular SMCs and suggest a role for these factors in maintaining overall vessel integrity.

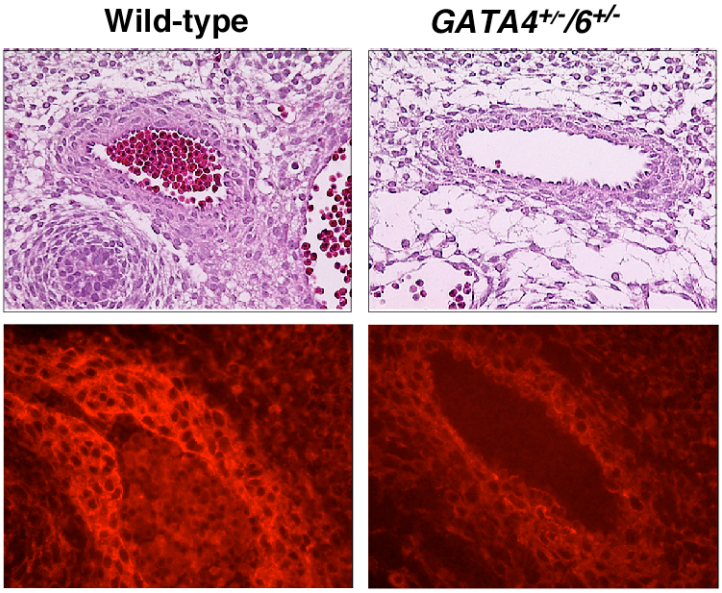

Figure 2.5 Vascular smooth muscle defects in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. H&E staining (top) of E12.5 embryos shows a hypoplastic and dilated aorta in the *GATA4/6* mutants. Smooth muscle-actin staining (bottom) shows reduced smooth muscle differentiation in the *GATA4*$^{+/-}$/*6*$^{+/-}$ mutants.

***GATA4/6* compound heterozygotes display outflow tract defects.**

To further examine the pta and abnormalities in the great vessels and cardiac outflow tract in the *GATA4/6* mutants, we performed ventricular India ink injections into the beating heart. As shown in Figure 2.6, wild type animals display complete septation (or division) of the cardiac outflow tract by the aorticopulmonary septum by E12.5. In the mutants, only a single outlet was

visible, indicating a failure of septation between the aorta and pulmonary trunk, and confirming the abnormalities seen in transverse sections of E12.5 embryos (Fig. 2.6B and D). We also observed a hypoplastic transcending aortic arch in the *GATA4/6* double heterozygous embryos, further suggesting patterning defects of the great vessels.

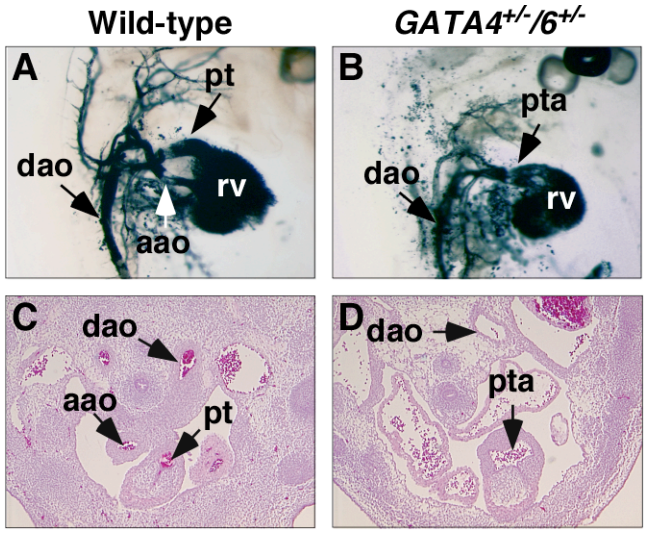

Figure 2.6 Defects in the cardiac outflow tract of *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. India ink was injected into the left ventricle of beating hearts of wild-type (A) and *GATA4*$^{+/-}$/*6*$^{+/-}$ (B) embryos at E12.5. The atria were removed and the right lateral views of the heart and great vessels are shown. (C) and (D). Transverse sections of wild type and *GATA4*$^{+/-}$/*6*$^{+/-}$ mutants at E12.5 displaying incomplete septation between the outflow tract and the pulmonary trunk. aao, ascending aorta, dao, descending aorta; pt, pulmonary trunk; pta, persistent truncus arteriosis; rv, right ventricle, lv; left ventricle.

GATA4/6 mutants display myocardial thinning and VSDs due to a reduction in myocyte proliferation

Histological sections of E10.5-E13.5 embryos revealed a narrow temporal window for the onset of cardiac defects in *GATA4/6* mutants (Fig. 2.7). There was a modest delay in the formation of the ventral septum beginning at E11.5. Myocardial thinning first became apparent at E12.5. Hearts of mutant embryos at E13.5 contained only two myocardial cell layers within the compact zone compared to a five-cell layer in wild type embryos. In addition, *GATA4/6* mutant embryos displayed VSDs that persisted until the time of death at E13.5, a finding consistent with the phenotype resulting from hypomorphic *GATA4* alleles (Pu et al., 2004). As defects in cardiovascular development have been shown to be secondary to placental defects (Sapin et al., 1997), we examined *GATA4/6* mutant embryos for perturbations in placental structure and found no obvious morphological abnormalities, suggesting that the cardiovascular defects observed are not secondary to defects in early embryonic development.

To determine the molecular mechanism underlying the thin myocardium observed in the *GATA4/6* mutants, we evaluated myocardial apoptosis and proliferation. We observed no differences in TUNEL staining between wild type and mutant hearts at embryonic days 10.5, 11.5, 12.5 and 13.5 (data not shown), suggesting that the *GATA* mutants might have defects in myocyte proliferation. Indeed, *GATA4/6* compound heterozygous hearts displayed reduced cellular proliferation at E10.5 as assayed by phosphohistone H3 staining (Fig. 2.8),

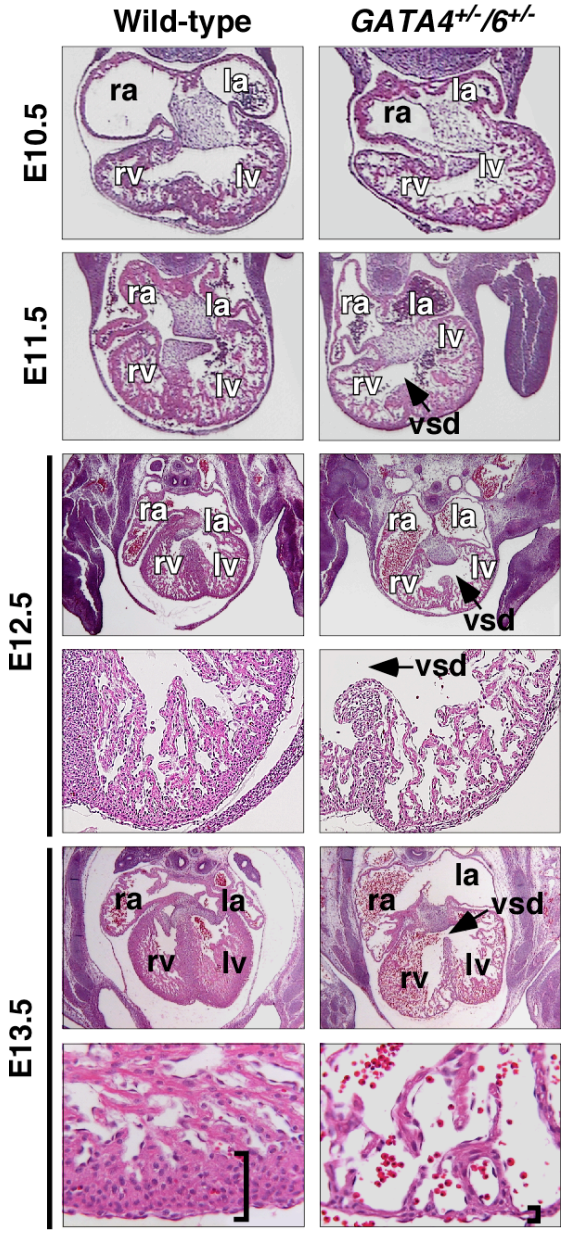

Figure 2.7 **Cardiac defects in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos.** H&E staining of transverse sections of wild type and *GATA4*$^{+/-}$/*6*$^{+/-}$ mutants. Upper panels of E12.5 and 13.5 embryos show the entire heart and lower panels show 10x magnifications of the left ventricle of each heart. Arrows point to VSDs. Higher magnification (40x) of E13.5 hearts reveals thinning due to a decrease in myocardial cell layers in mutant embryos. Brackets show thickness of myocardial layer of the left ventricle (lv). ra, right atrium, la, left atrium, rv, right ventricle; vsd, ventricular-septal defect.

myocyte proliferation and not apoptosis. These data are supported by our observation of a reduced number of oxygen carrying red blood cells, which may impede the proliferative potential of mutant cardiomyocytes.

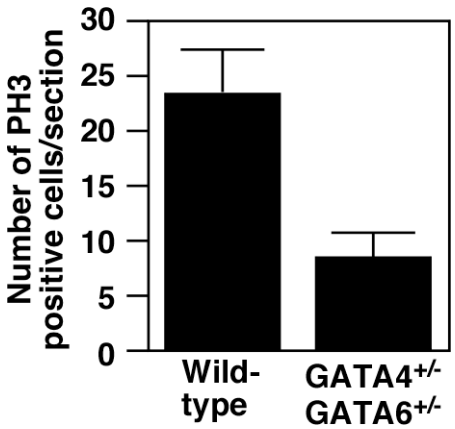

Figure 2.8 Reduced cardiomyocyte proliferation in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. Phosphohistone H3 staining of sections of hearts from wild-type and *GATA4*$^{+/-}$ */6*$^{+/-}$ embryos at E10.5 revealed reduced cardiomyocyte proliferation in the double mutant embryos.

Altered cardiac gene expression in *GATA4/6* mutants.

Quantitative RT-PCR on RNA isolated from hearts of wild-type and compound heterozygous mutant mice at E11.75 showed that *GATA4* and *GATA6* expression was reduced to 60% in mutants compared to wild type littermates (Figure 2.9). In addition, expression of the *beta-myosin heavy chain* (*β-MHC*) gene, a known GATA target, which encodes the major protein involved in embryonic cardiac contractility (Hasegawa et al., 1997), was also down-regulated in the *GATA4/6* mutants, suggesting possible defects in cardiac function.

Intriguingly, *MEF2C* transcripts were also markedly reduced in hearts from *GATA4/6* mutant embryos at E11.75. The reduction of *MEF2C* expression provides a potential explanation for the paucity of cardiomyocytes, and the patterning defects seen in *GATA4/6* compound heterozygotes.

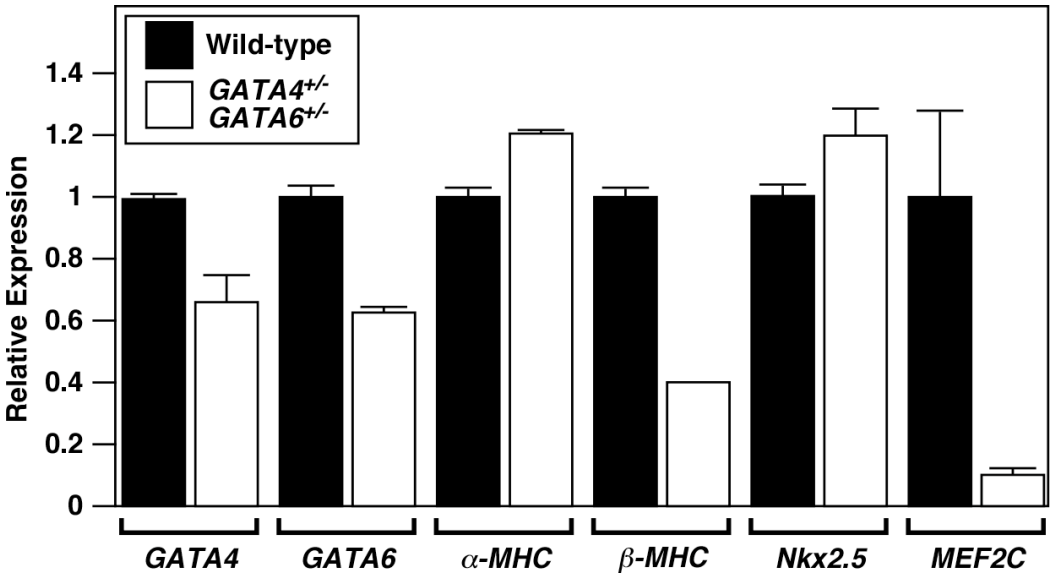

Figure 2.9 Modulation of myocardial gene expression in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos. Real time PCR was performed using RNA isolated from hearts of E11.75 embryos. Relative expression normalized to GAPDH in wild type (black) and *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos (white) is shown. Note the down regulation of *GATA4*, *GATA6*, *b-MHC* and *MEF2C* in *GATA4*$^{+/-}$/*6*$^{+/-}$ embryos.

Discussion

Mice heterozygous for a null mutation in either *GATA4* or *GATA6* are viable, whereas we show in the present study that compound heterozygosity of *GATA4* and *6* null alleles results in a spectrum of lethal cardiovascular phenotypes, including VSDs, myocardial hypoplasia, a pta, and abnormalities in vascular smooth muscle development. The defects observed in *GATA4/6* heterozygous embryos are distinct from those in embryos homozygous for either a *GATA4* or *GATA6* null allele, suggesting that these GATA factors play a cooperative role in cardiovascular development. Dosage sensitivity in development has not been previously observed with other combinations of heterozygous GATA mutations.

Cardiovascular phenotypes of *GATA4/6* mutant mice

The VSDs and thin ventricular myocardium accompanied by reduced myocyte proliferation in *GATA4/6* mutant embryos resemble the phenotype resulting from conditional deletion of *GATA4* using an Nkx2-5-Cre transgene (Pu et al., 2004; Zeisberg et al., 2005). The fact that vascular defects were not observed upon cardiac specific deletion of *GATA4* (Pu et al., 2004; Zeisberg et al., 2005) suggests that such abnormalities in *GATA4/6* compound heterozygous embryos reflect a cell autonomous function of *GATA4* and *6* in the vascular system, rather than a secondary response to cardiac demise. It is also interesting

to note that down-regulation of □-*MHC* and *MEF2C* expression was not observed following cardiac-specific deletion of *GATA4*, suggesting a combinatorial role for both GATA4 and GATA6 in maintaining adequate levels of these transcripts for proper cardiogenesis.

GATA6 has been implicated in maturation of cardiac mesoderm in Xenopus and zebrafish embryos (Peterkin et al., 2003), but to our knowledge, there has been no prior evidence for a specific role of GATA6 in heart development based on phenotypes of *GATA6* mutant mice. In fact, conditional deletion of *GATA6* using either *Wnt1*-Cre or *SM22*-Cre transgenes, resulted in perinatal lethality from defects in septation of the cardiac outflow tract and patterning of the aortic arch arteries, without apparent myocardial abnormalities (Lepore et al., 2006). Moreover, rescue of *GATA6* null embryos by tetraploid embryo complementation revealed no specific requirement for *GATA6* in cardiac development (Zhao et al., 2005). Such results are intriguing and suggest that GATA4 and GATA6 may still function independently of one another in select tissues or at certain times in development, despite the functional redundancy suggested by the compound heterozygous phenotype described here.

While we presume that GATA4 and 6 act synergistically in the heart and vasculature such that deletion of one copy of each gene diminishes GATA activity below a threshold required for activation of essential genes in these muscle cell types, we cannot be certain from the present studies whether GATA4 and 6 are

required cell autonomously in both of these tissues or, alternatively, whether developmental abnormalities in the heart or vasculature cause abnormalities in the other tissue secondarily. It is also conceivable, though we think unlikely, that GATA4 and 6 are required in different cell types such that deletion of one copy of both genes compromises development.

GATA5 has also been shown to be required for production of normal numbers of myocardial precursors and for expression of multiple cardiac genes in zebrafish (Reiter et al., 2001). In contrast, mice lacking *GATA5* do not show cardiac abnormalities (Molkentin et al., 2000). Moreover, *GATA4/5* or *GATA5/6* compound heterozygous mutant mice are normal (J.D.M. and E.N.O., unpublished), demonstrating the specificity in cardiac functions of *GATA4* and *6*.

Functions of GATA factors

GATA 4 and 6 heterodimerize and synergistically activate the *ANF* and *BNP* genes in cardiomyocytes (Charron et al., 1999). However, neither of these genes was down-regulated in *GATA4/6* mutants, indicating that the phenotype of these mutants does not result from a general diminution in expression of GATA target genes in the cardiovascular system. Instead, it seems more likely that specific GATA target genes, such as *b-MHC* and *MEF2C* or others yet to be identified, are highly sensitive to the combined level of GATA4 and 6, perhaps reflecting preferential responsiveness to GATA4/6 heterodimers compared to GATA homodimers. As a subset of transcriptional coactivators have been shown

to interact with GATA4 and not GATA6 (Durocher et al., 1997), it is tempting to speculate that the heterodimerization of these factors is required to mediate full transcriptional competency.

In light of the down-regulation of *MEF2C* in GATA4/6 mutant mice and the synergistic interactions between MEF2 and GATA4 (Morin et al., 2000), we analyzed the expression several MEF2 target genes, including *Srpk23* (Nakagawa et al., 2005) and *Bop* (Phan et al., 2005), and found no change in their expression patterns. Thus, we cannot attribute the *GATA4/6* mutant phenotype to a general diminution in expression of MEF2 targets. It is possible, however, that genes regulated cooperatively by GATA4/6 and MEF2C might be especially sensitive to the reduced expression of these factors in *GATA4/6* mutant embryos.

The transcriptional activities of GATA4 and GATA6 are influenced by co-repressors and non DNA-bound transcriptional activators (Dai and Markham, 2001; Wada et al., 2000; Yanazume et al., 2003). In concert with Tbx20, GATA4 synergistically activates the expression of *MEF2C* in the anterior heart field (Takeuchi et al., 2005). In addition, GATA4 has also been shown to interact with NFAT3 (Molkentin et al., 1998), MEF2C (Morin et al., 2000), Nkx2.5 (Lee et al., 1998; Sepulveda et al., 1998), SRF (Belaguli et al., 2000), Hand2 (Dai et al., 2002) and myocardin (Oh et al., 2004) to regulate cardiac gene expression. Full transcriptional activation by both GATA4 and GATA6 also requires interaction with the histone acetyltransferase p300 (Dai and Markham, 2001; Wada et al.,

2000); and this interaction is lost in the presence of the competing co-repressors FOG-2 (Hirai et al., 2004) and Hey-2 (Fischer et al., 2005; Kathiriya et al., 2004). It will be of interest to determine whether mutations in the genes encoding any of these GATA cofactors, when combined with *GATA4* or *6* mutations, evokes unique phenotypes not seen with the single heterozygous mutations.

Implications for human congenital heart disease

A variety of GATA4 mutations have been linked to cardiac septal defects in humans (Garg et al., 2003; Okubo et al., 2004). The results of the present study point to the potential involvement of *GATA6* mutations in human congenital heart disease. It is unclear why heterozygous mutations in *GATA4* or *GATA6* alone do not result in cardiac defects in mice, although it is well established that human heart development is more sensitive to subtle genetic abnormalities (Srivastava and Olson, 2000).

It is not uncommon for congenital heart disease to appear in human pedigrees with incomplete penetrance and variable expressivity, which has suggested the existence of modifier genes that influence cardiac phenotypes (Srivastava and Olson, 2000). The finding that heterozygous mutations in *GATA4* and *GATA6* cause no observable phenotype alone in mice, whereas together they result in complete embryonic lethality illustrates the power of recessive genetic interactions to influence heart development. Moreover, the realization that

cardiovascular development is exquisitely sensitive to the threshold of GATA4/6 activity suggests that therapeutic strategies to augment the activity of these transcription factors, even subtly, might overcome certain congenital cardiac abnormalities. In light of the repressive influence of co-repressors and histone deacetylases (Dai and Markham, 2001; Wada et al., 2000) on GATA factors, we are currently investigating whether partial inhibition of such negative regulators or augmentation of positive effectors of GATA activity *in vivo* might restore cardiac function in *GATA4/6* mutant mice.

Chapter Three

Essential roles of the bHLH transcription factor Hrt2 in repression of atrial gene expression and maintenance of postnatal cardiac

Abstract

The basic helix-loop-helix transcriptional repressor Hairy-related transcription factor-2 (Hrt2) is expressed in ventricular but not atrial cardiomyocytes, as well as in endothelial and vascular smooth muscle cells. Mice homozygous for a null mutation of *Hrt2* die perinatally from a spectrum of cardiac abnormalities, raising questions about the specific functions of this transcriptional regulator in individual cardiac cell lineages. Using a conditional *Hrt2* null allele, we show that cardiomyocyte-specific deletion of *Hrt2* in mice results in ectopic activation of atrial genes in ventricular myocardium with an associated impairment of cardiac contractility and a unique distortion in morphology of the right ventricular chamber. Consistent with the atrialization of ventricular gene expression in *Hrt2* mutant mice, forced expression of *Hrt2* in atrial cardiomyocytes is sufficient to repress atrial cardiac genes. These findings reveal a ventricular myocardial cell-autonomous function for Hrt2 in the suppression of atrial cell identity and the maintenance of post-natal cardiac function.

Introduction

Formation of the four-chambered vertebrate heart requires complex morphogenetic events and interactions among diverse cell types with specialized functions. Atrial and ventricular cardiomyocytes, for example, display distinct gene expression patterns, contractile properties, and hormonal responses required for coordinated cardiac contractility. Endothelial cells give rise to the endocardium and cardiac valves, and smooth muscle cells contribute to the coronary arteries and inflow and outflow vasculature. Abnormalities in the developmental events associated with the differentiation, growth, migration or cell-cell interactions of these different cardiac cell types result in congenital heart disease, the most common human birth defect (Fishman and Chien, 1997; Harvey, 2002; Olson, 2006; Srivastava and Olson, 2000).

The Hairy-related transcription factor (Hrt) family of basic helix-loop-helix (bHLH) proteins, also referred to as Hey, Hesr, CHF and HERP (Chin et al., 2000; Iso et al., 2001a; Kokubo et al., 1999; Leimeister et al., 1999), consists of three members, Hrt-1, -2, and -3 (Nakagawa et al., 1999). These proteins share homology in their bHLH regions, which mediate DNA binding and dimerization, as well as in an Orange domain of unknown function and a unique C-terminal YXXW domain. Similar functional domains are contained in the Hairy/Enhancer of Split (HES) proteins (Kageyama et al., 2005). During embryogenesis, *Hrt2* is expressed in the ventricular myocardium, but not in the atrial myocardium, as

well as in the cardiac outflow tract and aortic arch arteries (Leimeister et al., 1999; Nakagawa et al., 1999). Hrt proteins function as transcriptional repressors downstream of Notch signaling, which regulates binary cell fate decisions during development. Upon activation by ligands such as Delta or Jagged on the surfaces of adjacent cells, the intracellular domain of the Notch receptor is cleaved and translocated to the nucleus where it cooperates with CSL/RBP-J to stimulate transcription of *Hrt* genes (Maier and Gessler, 2000; Nakagawa et al., 2000). In addition to binding to an E-box DNA sequence motif, Hrt proteins physically interact with GATA4/-5/-6 and repress the transcriptional activity of GATA-dependent genes (Kathiriya et al., 2004). Hrt proteins also dampen Notch-dependent activation of their own genes independent of DNA binding (King et al., 2006; Nakagawa et al., 2000). The repressive influence of the Hrt2 protein may result in part from an interaction with the mSin3, N-CoR and HDAC1 co-repressor complex (Iso et al., 2001b).

Mice lacking *Hrt1* are normal, whereas homozygous deletion of *Hrt2* in mice results in a remarkably variable spectrum of cardiovascular defects, including ventricular septal defects (VSDs), valvular defects, postnatal cardiac hypertrophy, cardiomyopathy, and vascular abnormalities (Donovan et al., 2002; Gessler et al., 2002; Kokubo et al., 2004; Sakata et al., 2002; Sakata et al., 2006) Misexpression studies have also suggested a role of Hrt1 and Hrt2 in boundary formation within the atrioventricular canal (Kokubo et al., 2007). Because *Hrt2* is expressed in

cardiomyocytes, vascular smooth muscle cells, and endothelial cells, it is unclear which of these abnormalities reflect cell-autonomous functions of Hrt2 in one particular cell type. They may also be due to secondary consequences of the loss of Hrt2 function in one cell type that indirectly affects another. The issue of cell autonomy is especially complex in the heart in which a defect in one cell type can have profound effects on growth and morphogenesis of other cardiac structures.

Here we show that cardiomyocyte-specific deletion of *Hrt2* results in ectopic activation of atrial genes in ventricular myocardium accompanied by contractile dysfunction and a unique distortion of right ventricular morphology. Our results indicate that Hrt2 acts in the ventricular myocardium to repress atrial gene expression, thereby functioning as a key regulator of cardiac cell identity and function.

Materials and Methods

Gene targeting

To generated *Hrt2loxP* targeting vector, a 4.5 kb fragment containing exon 1 was digested with BglII and XhoI. The fragment was ligated into the pGKnewF2L2dta targeting backbone upstream of the loxP sites and FRT-flanked neomycin resistance cassette. A 1.8 kb XhoI/BglI fragment containing exon 2 and 3 was ligated into the vector between the 5' loxP site and the neomycin resistance cassette. Finally, a 3.5 kb fragment containing exon 4 was digested

with BglI and NheI and ligated into the vector between the neomycin resistance and dta negative selection cassettes. Integrity of the targeting vector was confirmed by restriction mapping and DNA sequencing. PCR primer sequences are available upon request.

The *Hrt2neo-loxP* targeting vector was linearized with PvuI and electroporated into SM-1 ES cells. Following selection by G418, resistant colonies were screened by Southern analysis of BamHI digested genomic DNA using a probe (Figure 1A) from the 5' flanking region. Recombination of the 3' was confirmed by Southern analysis of PstI digested genomic DNA using a probe (Figure 1A) from the 3' flanking region. Four correctly targeted ES clones were expanded and injected into E3.5 c57BL/6 mouse blastocysts to produce chimeric mice which were mated with C57BL/6 females to obtain F1 mice carrying the targeted allele. Chimeric males from one clone transmitted the targeted allele through the germline. Mice from clone 1F2 were used for all subsequent analysis.

Genotyping

Tail and yolk sac DNA was isolated as previously described (McFadden et al., 2000). PCR reactions were used to detect Cre transgenes, and *Hrt2* knockout loci. Briefly, 1 µl of tail or yolk sac DNA was used a template in 25 µl PCR reactions using Promega *Taq* polymerase and 4 mM MgCl2. Thermal cycle reactions were as follows: 2 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30

seconds at 55°C, 45 seconds at 72°C and a final 5 minute extension at 72°C. Reactions were visualized on 1% agarose gels in TAE. Primer sequences are available upon request.

Conditional gene deletion with Cre transgenic mice

Heterozygous *Hrt2*$^{neo\text{-}loxP}$ mice were intercrossed with *hACTB::FLPe* transgenic mice to remove the neomycin resistance cassette in the germline (Rodriguez et al., 2000). The following Cre transgenic mice were used to delete the conditional *Hrt2* null allele: *Nkx2.5-Cre* (McFadden et al., 2005), *SM22-Cre* (Holtwick et al., 2002), and *Tie2-Cre* (Kisanuki et al., 2001).

Histology

Hearts from adult mice and embryos and were harvested from timed matings and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Following fixation, embryos were rinsed in PBS then dehydrated through graded ethanols and embedded in paraffin wax as previously described (McFadden et al., 2000). Histological sections were cut and stained with Hematoxylin and Eosin.

In situ hybridization

Section in situ hybridization was performed as described (Nakagawa et al., 1999). Plasmids for in situ probes have been previously described and were linearized and transcribed as follows: *ANF, Xho*I and T7 (Miller-Hance et al., 1993); *Tbx5, Spe*I and T7 (Bruneau et al., 1999); Mlc1a, EcoRI and T3 (Lyons et al., 1990); Mlc2a, BamHI and T7 (Kubalak et al., 1994); and *SLN*, *Bam*HI and T7 (Minamisawa et al., 2003).

Echocardiography

Cardiac function and heart dimensions were evaluated by two-dimensional echocardiography in conscious mice using a Vingmed System (GE Vingmed Ultrasound, Horten, Norway) and an 11.5-MHz linear array transducer. The data were analyzed by a single observer blinded to mouse genotype.

Cardiomyocyte cell culture and adenovirus infections

Primary rat cardiomyocytes were prepared as described (Molkentin et al., 1998), except that the atrial cardiomyocytes were isolated along with ventricle cardiomyocytes. Forty-eight hours after plating, cells were infected with adenovirus for 3 hours in 10% FBS containing media at 50 MOI. After an additional forty-eight hours, the cells were harvested and RNA was isolated for

RT-PCR. *GFP* and *Myc-Hrt2* expressing adenoviruses have been previously described (Kathiriya et al., 2004; Rybkin et al., 2003).

RNA purification, microarray analysis and real-time PCR

Total RNA was purified from mouse ventricular tissues and cultured cardiomyocytes using Trizol (Invitrogen) as described (Kathiriya et al., 2004). Microarray analysis was performed using Affymetrix Mouse Genome 430 2.0 array. Real-time PCR was performed using 7000 Sequence Detection System from Applied Biosystems.

Results

Targeting the mouse *Hrt2* gene

To create a conditional *Hrt2* null allele, we introduced *LoxP* sites into introns 1 and 3 of the mouse *Hrt2* gene by homologous recombination in ES cells (Fig. 3.1A). Deletion of the region of the gene between the two *LoxP* sites eliminates amino acids 29 to 82, which includes all of the basic and most of the HLH region, and splicing of exon 1 to exon 4 alters the reading frame of the remainder of the transcript. The deleted gene therefore does not encode a functional protein. A neomycin resistance cassette flanked by sites for Flp recombinase was incorporated into intron 3. Chimeric mice obtained by blastocyst injection of ES

cells heterozygous for the targeted *Hrt2* allele transmitted the mutant allele through the germline, yielding mice heterozygous for this $Hrt2^{neo\text{-}loxP}$ allele. Breeding of these mice to mice expressing the FLPe recombinase in the male germline allowed for the removal of the neomycin resistance cassette, creating the $Hrt2^{loxP}$ allele (Fig. 3.1B). A PCR strategy was also designed to genotype the various Hrt2 alleles, (Fig. 3.1C). Homozygous $Hrt2^{loxP}$ mice were phenotypically normal, demonstrating that the $Hrt2^{loxP}$ allele did not function as a hypomorphic allele.

We also created a null *Hrt2* allele by replacing exons 1-4 with a lacZ-neo cassette, referred to as $Hrt2^{KO}$. Mice homozygous for this mutant allele, in either an isogenic 129 or a mixed 129/C57Bl6 background, died in the neonatal period and all showed VSDs, which is consistent with studies by other groups (Donovan et al., 2002; Gessler et al., 2002; Kokubo et al., 2004; Sakata et al., 2002).

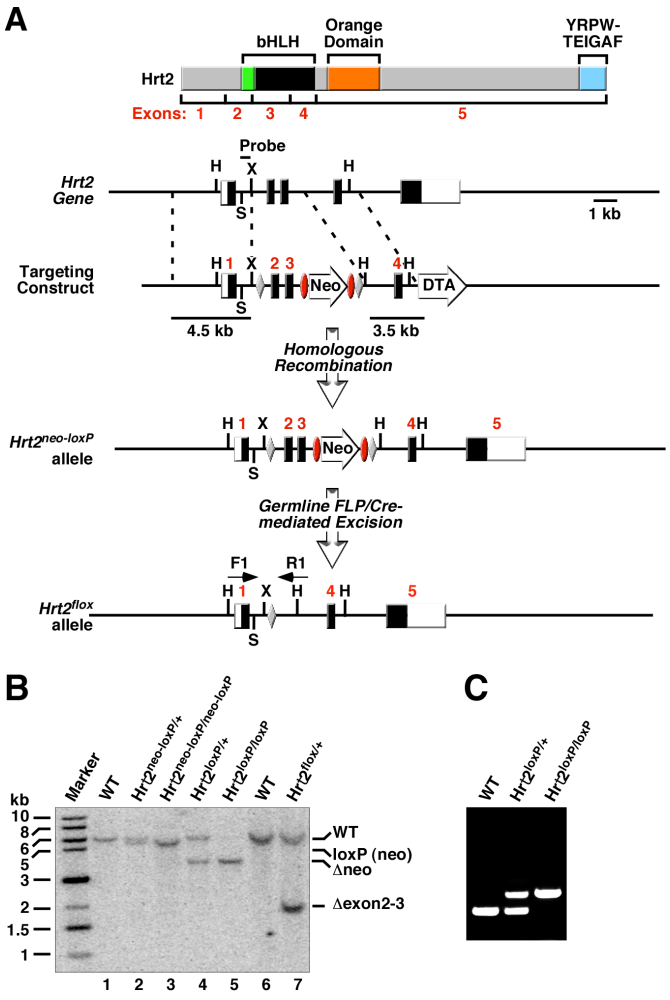

Figure 3.1 Targeting the *Hrt2* locus.
A) The structure of the mouse Hrt2 protein, denoting position of the exons, is shown at the top, along with the genomic locus, targeting vector and targeted allele. Lox P sites were inserted into introns 1 and 3 of the *Hrt2* gene, along with a neomycin resistance cassette flanked by FRT sites in intron 3. The neomycin resistance cassette was removed in the mouse germline by breeding heterozygous mice to *hACTB::FLPe* transgenic mice. Conditional deletion of exons 2 and 3 was achieved by breeding $Hrt2^{loxP/loxP}$ to $Hrt2^{loxP/+}$ mice harboring transgenes that expressed Cre recombinase tissue-specifically. Positions of the probes used for Southern analysis are shown.
B) Southern blot analysis of various *Hrt2* alleles. Genomic DNA isolated from mice of the indicated genotypes was digested with HindIII and analyzed by Southern blot with the SalI/XhoI probe.
C). PCR strategy for genotyping various Hrt2 alleles. Primers flanking the 5' loxP site are labeled in panel A. Genotypes are shown on the top.

Cardiac defects resulting from myocardial deletion of *Hrt2*

To analyze the cardiomyocyte-specific functions of Hrt2 and their involvement in the phenotypes in *Hrt2* null mice, we bred $Hrt2^{loxP/loxP}$ female mice to $Hrt2^{KO/+}$ mice harboring an *Nkx2-5-Cre recombinase* (*Cre*) transgene, which is highly specific for cardiomyocytes (McFadden et al., 2005). In contrast to $Hrt2^{KO/KO}$ mice, $Hrt2^{loxP/KO}$*; Nkx2-5-Cre* mice with a cardiomyocyte-specific *Hrt2* deletion are viable to adulthood. They did not have significant valve defects or myocardial hypertrophy (Fig. 3.2), and the heart weight/body weight ratios were normal (data not shown). $Hrt2^{KO/KO}$ mice displayed a complete penetrance of VSDs, while we observed no VSDs in six adult $Hrt2^{loxP/KO}$*; Nkx2-5-Cre* mice. However, the hearts of adult $Hrt2^{loxP/KO}$*; Nkx2-5-Cre* mice were grossly enlarged with an aberrant architecture of the ventricular chambers in which the base of the dilated right ventricle (RV) merged with the interventricular septum above the apex of the heart (Fig. 3.2). Histological analysis of $Hrt2^{loxP/KO}$*; Nkx2-5-Cre* embryos at various stages revealed that this morphological defect was apparent by E13.5 and persisted in adult animals. A small fraction (2/7) of $Hrt2^{loxP/KO}$*; Nkx2-5-Cre* embryos showed VSDs or the delay of ventricular septal formation at E13.5 to 17.5 (Fig. 3.2).

The rarity of VSDs observed in $Hrt2^{loxP/KO}$*; Nkx2-5-Cre* mice suggested that cell populations other than cardiomyocytes contributed to the formation of VSDs observed in all $Hrt2^{KO/KO}$ mice. Consistent with this hypothesis, we recently

observed that deletion of *Hrt2* with an *SM22-Cre* transgene, which is expressed in the heart until E10.5 and in a subset of arterial smooth muscle cells thereafter (Holtwick et al., 2002), resulted in VSDs and partial perinatal lethality, while deletion of *Hrt2* in the endothelium with *Tie2-Cre* did not evoke detectable defects of cardiac structure (Table 3.1 and data not shown). Thus, it seems likely that the VSDs of $Hrt2^{KO/KO}$ mice arise as a result of the deletion of *Hrt2* in smooth muscle cells or, possibly, from the combined deletion in smooth and cardiac muscle cells. Detailed comparison of the phenotypes in various conditional mutant mice will be described elsewhere.

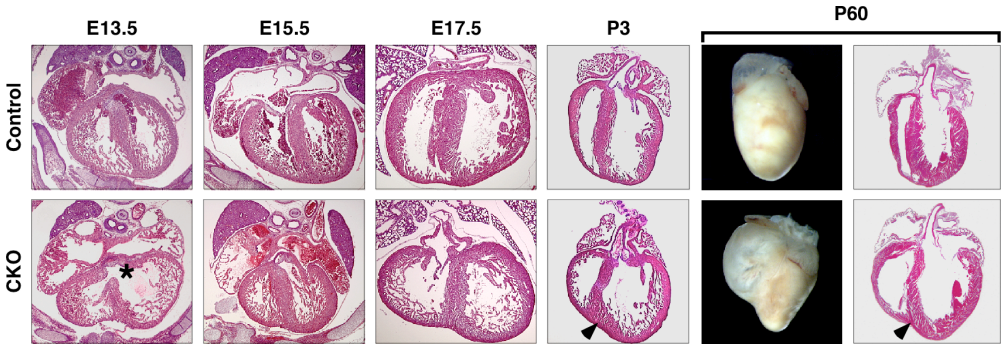

Figure 3.2 Cardiac defects resulting from myocardial deletion of *Hrt2*.
Whole-mount and H&E sections of hearts of wild type and $Hrt2^{CKO}$ mice at various embryonic and post-natal (P) time-points. Note the distention of the RV in the whole mount and the abnormal right ventricular chamber morphology in the H&E section of the mutant. Also note a VSD observed in one $Hrt2^{CKO}$ embryo at E13.5 denoted by an asterisk. Arrowhead denotes the base of the interventricular septum, which is shifted upward and towards the right ventricle in the hearts of $Hrt2^{CKO}$ mice.

Table 3.1

Consequences of conditional *Hrt2* deletion

Cre transgene	Cell Type of Deletion ___ Viability	Cardiac Phenotype	Viability
Nkx2-5-Cre	Cardiomyocytes	RV dilation contractile dysfunction	Viable
SM22-Cre	Smooth and Perinatal Cardiac muscle	RV dilation contractile dysfunction VSDs	Lethal
Tie2-Cre	Endothelial cells	Normal	Viable

Contractile dysfunction resulting from cardiac deletion of *Hrt2*

Hrt2$^{KO/KO}$ mice showed perinatal lethality in either an isogenic 129 or a mixed background, preventing the evaluation of cardiac function at adult stages. To study the effects of myocardial-specific *Hrt2* deletion on contractile function, we performed echocardiography on *Hrt2*$^{loxP/KO}$*; Nkx2-5-Cre* mice (hereafter referred to as *Hrt2*^{CKO} mice) at 6 weeks of age (Fig. 3.3A). Myocardial deletion of *Hrt2* caused an increase in the systolic left ventricular internal diameter (LVIDs) and a corresponding deterioration in cardiac contractility, as indicated by decreased

fractional shortening (FS) (Fig. 3.3B). The diastolic left ventricular internal diameter (LVIDd) did not change appreciably in mutant mice. Since the decrease in FS primarily reflects an increase in LVIDs, rather than an increase in both LVIDs and LVIDd as is associated with general dilative remodeling, the cardiac dysfunction in *Hrt2*^{CKO} mice is likely to arise from contractile dysfunction .

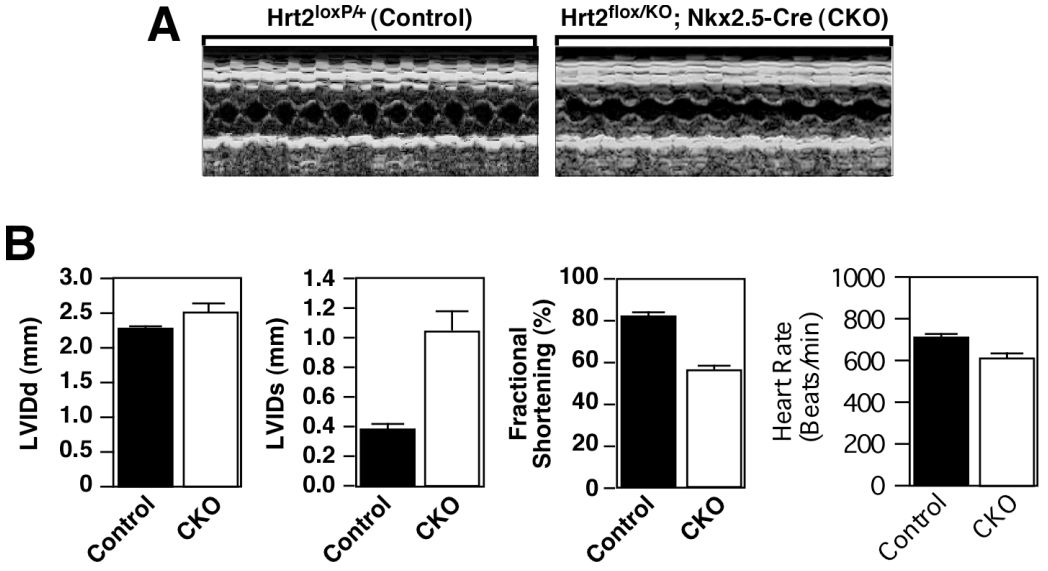

Figure 3.3 Functional defects resulting from myocardial deletion of *Hrt2*.
A) Representative M-mode images of control mice or *Hrt2*^{CKO} mice at 6 weeks of age demonstrate an increase in systolic left ventricular internal diameter (LVIDs), which results in a decrease in cardiac function.
B) Bar graph representation of LVIDd, LVIDs, FS and heart rate indicate that cardiac removal of Hrt2 reduces systolic function and attenuates cardiac contractility.

Ectopic activation of atrial genes in ventricular myocardium of *Hrt2*^{CKO} mice

In an effort to determine the molecular basis of cardiac dysfunction in *Hrt2*^{CKO} mice, we compared the pattern of gene expression in wild type and mutant hearts at 6 weeks of age by microarray analysis. Notably, atrial-enriched regulatory and structural genes, such as those encoding sarcolipin (*Sln*), myosin light chain 1a (*Myl4*) and -2a (*Myl7*) were up-regulated in the ventricles of *Hrt2*^{CKO} mice (Fig. 3.4A). Quantitative real time PCR demonstrated that expression of all three genes increased more than 10-fold compared to wild type littermates (Fig. 4B). In contrast, mRNA levels of *GATA4* and alpha myosin heavy chain (*MHC*α) (Fig. 3.4B), myosin light chain 1v, myosin light chain 2v, *Cx40* and phospholamban (data not shown), did not significantly change in the ventricles of *Hrt2*^{CKO} mice. In addition, the hypertrophic markers BNP, alpha-skeletal actin (*Acta1*) and beta-myosin heavy chain (*MHC*β) were unchanged in *Hrt2*^{CKO} mice (data not shown).

A

Gene	Fold-Change (MicroArray)
Sarcolipin	8.7
Mlc2a	5.6
Mlc1a	4.9
Tbx5	2.2
ANF	1.6

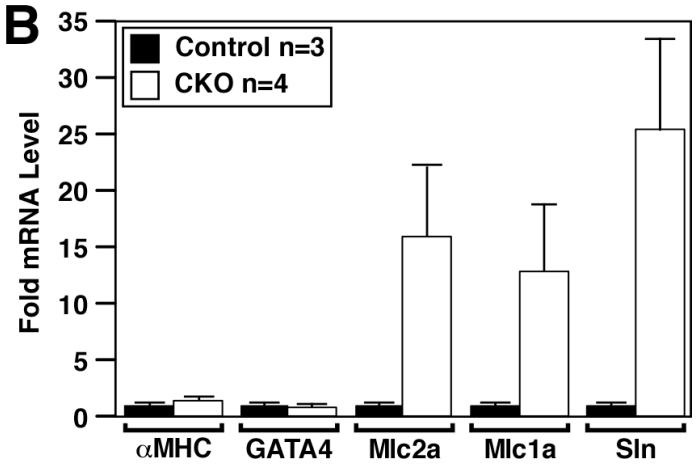

Figure 3.4 . Ectopic activation of atrial genes in ventricles of *Hrt2* mutant mice.
A) Microarray analysis showing the fold upregulation of atrial genes in the ventricles of $Hrt2^{CKO}$ mice at 6 weeks of age.
B) Quantitative RT-PCR analysis showing upregulation of the atrial specific genes *Mlc1a*, *Mlc2a* and *Sln* in ventricles of 6 week-old $Hrt2^{CKO}$ mice. The expression of *GATA4* and *αMHC* are not altered. The error bars represent standard deviation.

We next examined the tissue distribution of the dysregulated genes by *in situ* hybridization. The expression of *Mlc1a* and *Mlc2a* was expanded in the ventricles of E17.5 *Hrt2*^{CKO} mouse embryos, particularly in the interventricular septum and compact myocardium of both the left and right ventricles (Fig. 3.5). Wild type mice expressed these genes predominantly in the developing atria, while low levels of expression were observed in the ventricular trabeculation. Expanded expression of *Mlc1a* and *Mlc2a* was also observed in the ventricles of E15.5 *Hrt2*^{CKO} embryos (data not shown). The expression of *Sln* was observed ectopically in the compact myocardium of the ventricles of E17.5 *Hrt2*^{CKO} mouse embryos (Fig. 3.5), while this transcript is virtually absent from ventricles of wild type mice.

We also found the expression of atrial natriuretic factor (*ANF*) and *Tbx5* to be significantly increased in the left ventricles of *Hrt2*^{CKO} mice (Fig. 3.5). *ANF* and *Tbx5* normally display expression in the atria and trabecular cells of the left ventricle in wild type embryos. Since *Cx40* is also expressed predominantly in the atria and trabeculation of the left ventricle, we examined the expression of this gene by *in situ* hybridization at E17.5 and quantitative RT-PCR in adult ventricular tissue. *Cx40* did not show an increase in expression in the ventricles (data not shown), suggesting that Hrt2 regulates a subset of atrial-enriched target genes.

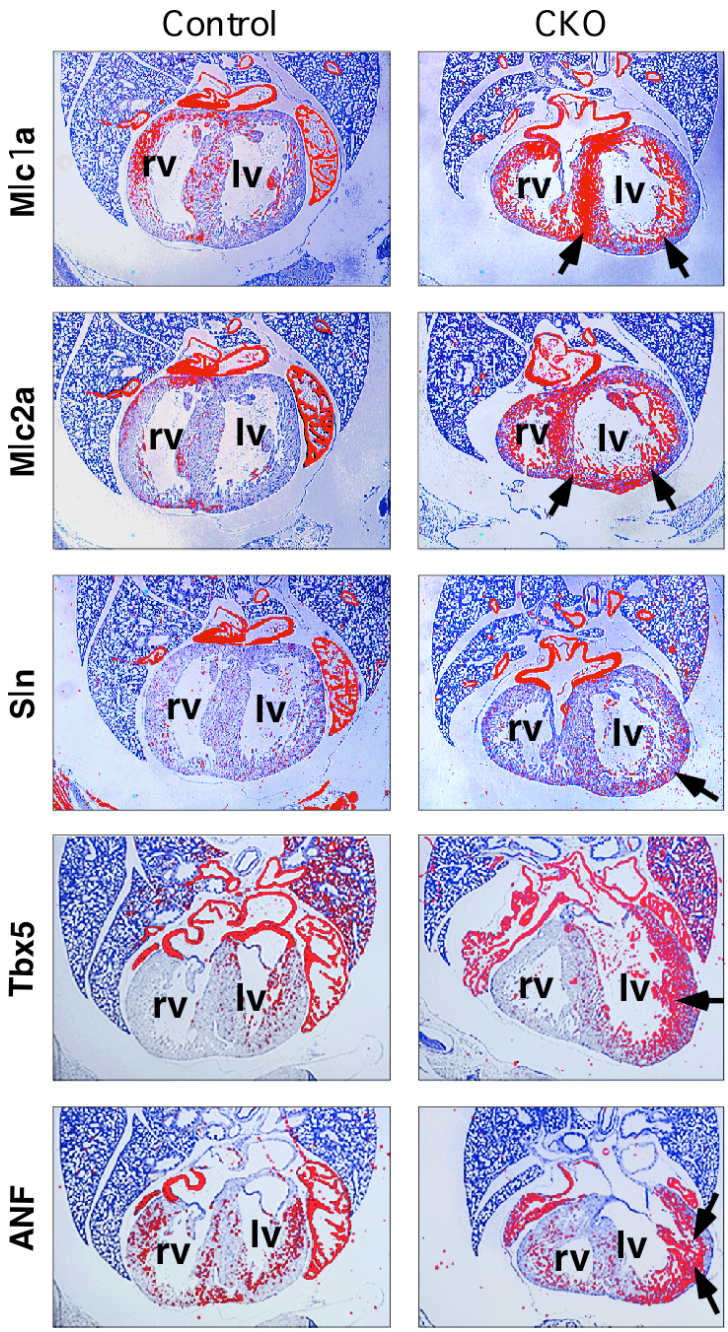

Figure 3.5 Expression of atrial genes in *Hrt2* mutant mice by *in situ* hybridization. *In situ* hybridization of E17.5 hearts showing the ectopic expression of *Mlc1a*, *Mlc2a*, *Sln*, *ANF* and *Tbx5* in ventricles of $Hrt2^{CKO}$ mice, compared to control mice. Arrows depict ectopic expression in the ventricle. lv, left ventricle; rv, right ventricle.

Repression of atrial genes by Hrt2

To determine whether Hrt2 was sufficient to repress endogenous atrial gene expression, we infected atrial and ventricular cardiomyocytes with adenovirus expressing Myc-Hrt2 or GFP as a control (Fig. 3.6). Because *Sln*, *Mlc1a* and *Mlc2a* were expressed at low levels in ventricular myocytes, it was difficult to measure further reduction of their expression by Hrt2. However, in atrial cardiomyocytes, the expression levels of *Sln*, *Mlc1a* and *Mlc2a* were all repressed by adenovirus-mediated Hrt2 expression. Furthermore, a reduction in expression of *ANF* and *Tbx5* was apparent upon Hrt2 overexpression in both atrial and ventricle cardiomyocytes (data not shown). Importantly, *Cx40* did not display an alteration of expression levels in cardiomyocytes infected with Ad-Hrt2, further demonstrating the specificity of Hrt2 target genes (Fig. 3.6). These observations are consistent with the increase in atrial gene expression observed in the $Hrt2^{CKO}$ mice and suggest that Hrt2 is both necessary and sufficient to limit the expression of atrial genes.

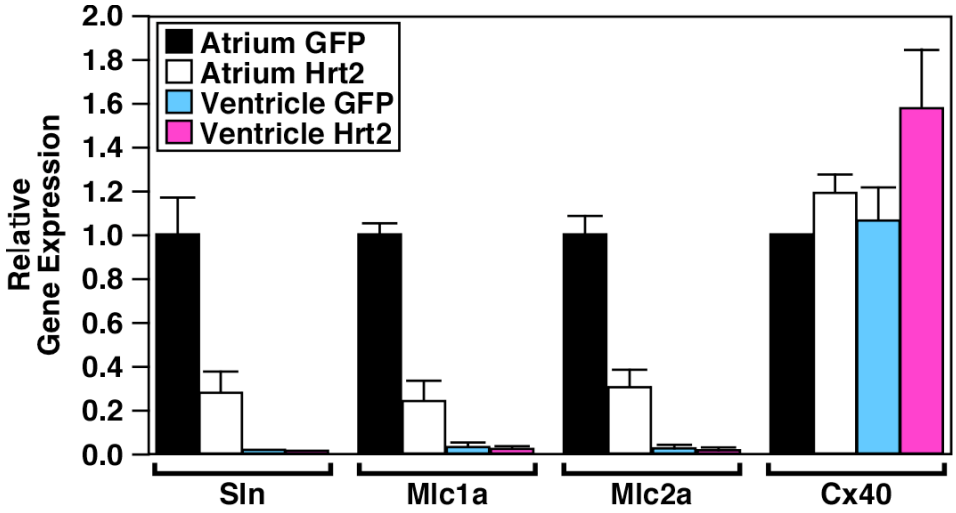

Figure 3.6 Hrt2 activity is sufficient to repress atrial genes in cardiomyocytes. Quantitative RT-PCR analysis showing repression of atrial specific genes in rat neonatal atrial and ventricle cardiomyoctyes infected with adenovirus expressing *Myc-Hrt2*. The error bars represent standard deviation.

Discussion

Global deletion of *Hrt2* in mice results in myriad cardiac defects, which have been difficult to interpret because of the expression of *Hrt2* in numerous cell types (Chin et al., 2000; Leimeister et al., 1999; Nakagawa et al., 1999), including cardiomyocytes, smooth muscle cells, and endothelial cells, all of which are required for cardiac development and function. Through the conditional deletion of *Hrt2* in each of these lineages, our results uncover a myocardial cell-autonomous function of Hrt2 in repression of atrial gene expression in the ventricular myocardium and maintenance of normal function of the adult heart. Misregulation of atrial genes in the ventricle likely contributes to the abnormalities in cardiac contractility resulting from cardiac-specific deletion of Hrt2.

Repression of atrial gene expression by Hrt2

Based on the up-regulation of multiple atrial genes including *Mlc1a*, *Mlc2a*, *Sln, ANF*, and *Tbx5* in ventricular cardiomyocytes of mice lacking cardiac expression of *Hrt2*, we conclude that Hrt2 is required to maintain ventricular identity and that activation of atrial genes represents a default gene program resulting from the absence of Hrt2-dependent repression. The atrialization of ventricular gene expression is likely to alter contractile properties that are required for ventricular function. For example, sarcomeric incorporation of atrial-

specific myosin light chains in the ventricles of *RXRα* or *Mlc2v* null mice leads to a reduced left ventricle ejection fraction (Chen et al., 1998; Dyson et al., 1995). *Sln* encodes an atrial-specific inhibitor of the cardiac sarcoplasmic reticulum Ca^{2+} ATPase SERCA2a and suppresses Ca^{2+} uptake into the sarcoplasmic reticulum (MacLennan et al., 2003). Notably, overexpression of *Sln* in the ventricle in transgenic mice leads to reduced cardiac contractility and heart failure (Asahi et al., 2004; Babu et al., 2006; Gramolini et al., 2006). Misregulation of these genes has also been associated with human cardiac pathologies. For example, SLN levels have been shown to be reduced in patients with atrial fibrillation (Uemura et al., 2004) and MLC1a and MLC2a upregulation has been observed in human cardiomyopathies (Lim et al., 2001; Trahair et al., 1993). Thus, the up-regulation of these atrial genes, and *Sln* in particular, in ventricular myocardium could contribute to the functional defects observed in the $Hrt2^{CKO}$ mice. Atrialization of gene expression in the ventricle may also play a role in the distended RV phenotype observed in the hearts of $Hrt2^{CKO}$ mice. It is interesting to note that retrovirus mediated misexpression of *Tbx5* in the presumptive RV results in a shift of the interventricular septum to the right and a distended and hypoplastic RV (Takeuchi et al., 2003). The abnormal RV phenotype accompanying the ectopic expression of atrial-enriched genes, especially *Tbx5* in the LV of $Hrt2^{CKO}$ mice, may suggest a role for Hrt2 in the specification or maturation of the right and left ventricles. It is curious that Hrt1, which shares a high structural similarity

to Hrt2, is expressed in atrial myocytes (Nakagawa et al., 1999), but appears not to repress endogenous atrial gene expression. Mice homozygous for *Hrt1* deletion are viable and have not been reported to display an increase in atrial gene expression, as might be expected (Fischer et al., 2004; Kokubo et al., 2005). One possibility is that ventricle-restricted co-repressors, such as the homeodomain protein Irx4, which has been implicated in suppression of atrial gene expression in ventricular myocardium, mediate the negative influence of Hrt2 on atrial-specific genes (Bao et al., 1999; Bruneau et al., 2001a; Garriock et al., 2001; Houweling et al., 2001; Wang et al., 2001). Alternatively, amino acid differences between Hrt1 and Hrt2 might confer unique functions to the proteins, possibly allowing the association of Hrt2 with specific co-repressors or other transcriptional regulators.

The configuration of transcription factor binding sites in atrial specific genes may also confer Hrt2-specific responsiveness. The *ANF* promoter, for example, contains a 17-bp element that seems to impart intrinsic repressive activity specifically in postnatal ventricular cardiomyocytes (Durocher et al., 1996). Mutation of this element in the *ANF* promoter abolishes atrial specificity and results in robust expression in the ventricles of transgenic *Xenopus* embryos (Small and Krieg, 2003). It is interesting to note that sequences upstream of the *Sln*, *Mlc1a* and *Mlc2a* genes contain a similar combination of *cis*-elements, including conserved binding sites for GATA factors, which serve as sensitive targets for repression by Hrt2 (Kathiriya et al., 2004).

Regulation of cell fate decisions by Hrt proteins and Notch signaling

Hrt proteins function as targets of Notch signaling, which regulates binary cell fate decisions during embryogenesis (Lai, 2004). The role of Hrt2 in repression of atrial gene expression in ventricular cardiomyocytes is reminiscent of its proposed role in determining arterial versus venous identity, as shown in zebrafish lacking expression of *gridlock*, the orthologue of *Hrt2*, which display the inappropriate development of venous instead of arterial smooth muscle cells from vascular precursors (Weinstein et al., 1995; Zhong et al., 2001; Zhong et al., 2000). However, mutations in genes encoding Notch family members or their ligands have not, to our knowledge, been reported to induce atrial gene expression in the ventricular myocardium. Thus, it remains to be determined whether repression of the atrial gene program by Hrt2 reflects a role of Notch signaling in this process or occurs through a Notch-independent mechanism. In this regard, the recent demonstration that members of the *Hrt/Hey* family of genes act downstream of the BMP-Smad pathway (Korchynskyi et al., 2003) raises the possibility that other signaling pathways may also employ these factors to regulate downstream target genes.

Conclusions and implications

While we have focused here on the consequences of cardiac deletion of *Hrt2*, the conditional allele of the *Hrt2* gene will make it possible to determine the role of *Hrt2* in a temporal and tissue-specific manner and to uncover possible functional redundancies with other members of the *Hrt* family. In this regard, mice lacking *Hrt1* are viable and do not display obvious phenotypic abnormalities, whereas *Hrt1/Hrt2* double mutant mice die during embryogenesis from global vascular deficiencies (Fischer et al., 2004; Kokubo et al., 2005). Combining the conditional *Hrt2* allele with an *Hrt1* null allele should permit the identification of additional cellular processes that rely on the expression of these genes in specific cell types. In the future, it will also be interesting to determine whether mutations in *Hrt2* contribute to congenital heart defects and whether Hrt2 modulates cardiac contractility in humans as predicted from the phenotype of *Hrt2* mutant mice.

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VITAE

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