THE REGULATION OF GENE EXPRESSION DURING HEART DEVELOPMENT AND DISEASE

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ABSTRACT

Thomas E. Callis: The Regulation of Gene Expression During Heart Development and Disease

(Under the direction of Dr. Da-Zhi Wang)

Cardiovascular disease remains one of the most common fatal and disabling disorders in the United States. The development of the heart and pathological processes leading to heart disease are intimately linked to the regulation of gene expression. By understanding the complex genetic and molecular pathways controlling cardiac gene expression, new therapies might be developed for the prevention and treatment of heart disease. My research has focused upon the fundamental mechanisms of transcriptional and post-transcriptional regulation of gene expression. In particular, I have investigated how transcription factors and microRNAs (miRNAs) coordinate cardiac gene expression during development and in disease.

Myocardin is a cardiac and smooth muscle-specific transcriptional cofactor for serum response factor (SRF). Myocardin potently activates target gene expression by tethering with SRF bound to SRF-responsive elements. However the upstream signaling pathways controlling myocardin activity and specificity were unknown. Bone Morphogenetic Proteins (BMPs) play important roles in cardiovascular

development and I find that Smad1, an effector of the BMP signaling pathway, synergistically activates myocardin-dependent cardiac gene expression. This discovery that myocardin participates in a BMP signaling-dependent cardiac gene transcriptional program helps address how myocardin transactivation of cardiac versus smooth muscle genes is controlled.

Much of the current understanding of the genetic pathways controlling cardiac gene expression is based upon studies of transcription factors and regulatory enhancer sequences required for cardiac gene transcription. The discovery of miRNAs has further increased this complexity by adding another layer of regulation at the post-transcriptional level. I show that the miR-208 family, miR-208a and miR-208b, are differentially expressed during heart development, paralleling the expression of their respective host genes alpha- and beta-myosin heavy chain (αMHC and βMHC). Using genetically engineered mice that overproduce miR-208a specifically in the heart or lack miR-208a altogether, I show that miR-208a is an important regulator of cardiac hypertrophy and cardiac conduction. Collectively, my studies of the transcription factor myocardin and the miR-208 family extend the current understanding of how cardiac gene expression is regulated during heart development and disease.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

CHAPTER 1

INTRODUCTION

Mammalian Heart Development

The heart is the first organ to form and function during mammalian development (1). In the mouse, heart formation begins at embryonic day 7.5 (E7.5) when a population of cells in the anterior lateral plate mesoderm becomes committed to a cardiogenic fate, a process known as cardiac specification or cardiogenesis. Two distinct sources of cardiac precursor cells from the primary and secondary heart fields are needed for cardiogenesis (2, 3). Whereas the primary heart field is essential for the formation of the primary heart tube, additional cardiac precursor cells are recruited from the secondary heart field to contribute to the future right ventricle and outflow tract (4, 5). These cardiac cells, which are localized to a region known as the cardiac crescent, migrate ventromedially to form the linear heart tube at E8.0. Shortly after the formation of the linear heart tube, the initiation of rhythmic contraction begins. Subsequent events of looping morphogenesis, chamber specification, cardiac valve formation, and neural crest migration give rise to the multichambered heart by E10.5 (2, 6). Once formed, the four-chambered heart will continue to grow and mature, a process that includes ventricular trabeculation. Many features of vertebrate heart development, including morphological events and the complex genetic networks involved, are evolutionarily conserved from avians, fish, frog, and mice to humans, indicating that studying heart development and disease in those accessible animal model systems provides insight into human heart pathophysiology (7).

Transcriptional Control of Heart Development

The embryologic events associated with heart development have been studied for centuries, but the relatively recent identification and characterization of cardiac transcription factors has provided much needed insight into the molecular mechanisms underlying heart development (8). Many transcription factors regulate cardiac gene expression during development and include: Nkx2.5 (9-12); homeodomain only protein (Hop) (13, 14); Myocyte enhancer factor 2C (MEF2C) (15-18); GATA4 (19-22); Hand1 (23); serum response factor (SRF) (24, 25); myocardin (26-29); and the T-box (Tbx) transcription factors Tbx1, Tbx5 and Tbx20 (30-40). Genetic deletion studies in mouse models demonstrated critical roles for several of these transcription factors in different aspects and at different times during heart development. For example, genetic deletion of GATA4 results in embryonic lethality by E9.5 with disrupted heart looping and defective septation (41), while complete loss of Nkx2.5 in murine hearts resulted in arrested development by E10 after heart looping and disturbed the expression of several other cardiac transcription factors (42). Extremely complex genetic networks have been revealed by careful analyses of cardiac transcription factors, in which some of these proteins interact with one another, as well as additional cofactors, to promote or inhibit expression of specific genes during heart development. For example, SRF associates with Nkx2.5, GATA4, and myocardin as part of a multi-component transcriptional regulatory complex regulating the expression of cardiac-specific genes (26, 43-47). One research focus within this dissertation was to better

understand the interplay between SRF and myocardin and how they coordinate cardiac gene expression.

SRF, a MADS-box transcription factor, is an important regulator of both growth factor-inducible and muscle-specific genes. SRF regulates target genes by binding the DNA consensus sequence $CC(A/T)_6GG$, known as a CArG box or serum response element (SRE) (48-50). Although SRF is ubiquitously expressed, it is enriched in muscle tissue during development and in adulthood (51-53). SRF is essential for animal development because SRF knockout mice die during early embryonic development (54). Recently, the *in vivo* function of SRF during muscle development was clearly documented when SRF was conditionally knocked out in cardiac and skeletal muscle lineages (24, 25, 55-58). Interestingly, over-expression of a wild type or a dominant negative form of SRF specifically in the heart results in cardiomyopathy, suggesting there is an obligatory role for SRF in cardiogenesis (59, 60). It's thought that the spectrum of genes activated by SRF is dictated by its differential affinity for different CArG box sequences and by its association with a variety of cofactors, many of which are cell type-specific and signal-responsive (61, 62). Thus, the tissue-specific co-factors are critical for controlling SRF specificity, one of which is myocardin.

Myocardin is a SAP (SAF-A/B, Acinus, PIAS) domain transcription factor (26). During mouse embryogenesis, myocardin is initially expressed in the cardiac crescent, representing one of the earliest known markers of the cardiac lineage (26). Myocardin expression is maintained in the atrial and ventricular chambers of the heart throughout embryogenesis to adulthood. In addition, myocardin is expressed in

a subset of vascular and visceral smooth muscle cell types (26, 63-65). Unlike other cardiac transcription factors, such as Nkx2.5, MEF2C and GATA4, which bind to the conserved DNA sequences on the regulatory regions of their target genes, myocardin does not bind DNA alone, but forms a stable ternary complex with SRF bound to DNA (26). This interaction brings the powerful transcription activation domain (TAD) of myocardin to SRF-dependent target genes. Target genes that can be significantly transactivated by myocardin include cardiac-specific gene atrial natriuretic factor (ANF) and smooth muscle-specific gene SM22, both known targets for SRF. As an SRF co-factor, myocardin potently transactivates CArG box-containing reporter genes (26, 66). Interestingly, genetic deletion of myocardin in the mouse causes embryonic lethality by E10.5 with absence of smooth muscle cells but apparently normal cardiac development. However, the expression of myocardin in the heart and its ability to synergistically activate cardiac gene expression with SRF argues for a role in cardiac development. The lack of specific cardiac defects in myocardin null murine heart maybe explained, at least in part, by functional redundancy between myocardin and its related family members myocardin-related transcription factors (MRTFs) –A and –B, which share homology with the SAP, basic and glutamine-rich domains of myocardin (66). Whereas myocardin is expressed in a cardiac- and smooth muscle-specific manner, MRTF-A and MRTF-B are widely expressed in embryonic and adult tissues and are able to synergistically activate CArG box promoters (66). Given their overlapping expression patterns and their ability to cooperatively activate gene expression with SRF, it's formally possibly that the myocardin family of transcription factors play functionally redundant roles in the

heart. Several experimental evidences point to the importance of myocardin in heart development: Expression of a dominant-negative myocardin mutant or myocardin knockdown by morpholino in *Xenopus* blocks heart development (26, 28), while ectopic overexpression of myocardin in *Xenopus* embryos leads to activation of ectopic cardiac gene expression. Importantly, recent evidence demonstrates myocardin can induce hypertrophic growth of cardiomyocytes, suggesting that myocardin may have a function in the adult heart and disease process cardiac hypertrophy (67).

Transcriptional Mechanisms of Congenital Heart Disease

Over the past decade, clinical studies have identified a number of congenital heart diseases (CHDs) associated with mutations in cardiac transcription factors (Table 1.1). CHDs threaten nearly 1% of all newborns and pose a significant threat of infant death; however, the underlying genetic mechanisms of many CHDs remain elusive. Most likely, the majority of these defects have a basis in the complex process of cardiogenesis. Heart development involves a series of highly coordinated events including cell proliferation, differentiation, migration and morphogenesis, and a number of genes involved in these processes have been identified as potential causes of specific cardiac anomalies. Transcription factors are major regulators of developmental processes and play essential roles in cardiogenesis. Six CHDs associated with deletions or mutations of transcription factors and the current understanding of their molecular bases are described in this section (**Fig. 1.1**).

DiGeorge Syndrome: Tbx1

The presentation of DiGeorge syndrome is one consequence of the most common human genetic deletion, monoallelic microdeletion of chromosome 22q11.2. In most cases, the heterozygous deletion eliminates approximately 3 Mbp of the long arm of chromosome 22, resulting in the loss of an estimated 30 genes (68). Recent studies have described highly variable clinical indications of patients with chromosome 22q11.2 deletions, even within the same pedigree. However, CHDs are the most common feature of DiGeorge syndrome, or *del22q11*, and may include tetralogy of Fallot, interruption of the aortic arch type B, ventricular septal defects, pulmonary atresia, or persistent truncus ateriosus (**Fig. 1.1**) (68, 69).

The use of mouse genetics has recently clarified which of the numerous genes deleted in *del22q11* may be responsible for the DiGeorge syndrome phenotypes. Targeted mutations in the mouse genome have allowed the majority of the DiGeorge syndrome clinical manifestations to be attributed to haploinsufficiency of Tbx1, one of the genes deleted in *del22q11* patients (68, 70). Tbx1 is a member of the T-box family of transcription factors and is involved in the patterning of the pharyngeal endoderm and aortic arches, as well as cardiac outflow tract development in a gene dosage-dependent manner (69). Attempts to further connect Tbx1 to DiGeorge syndrome have led to searches for mutations in this gene in patients lacking the typical chromosomal deletion. To this end, five patients have been identified as carrying only a Tbx1 gene mutation (71). Though these individuals do not exhibit all characteristics of DiGeorge syndrome, this

demonstrates that mutations in human Tbx1, as in mouse, are capable of causing many of the defects associated with *del22q11*. In an effort to understand the molecular mechanisms of Tbx1 function, recent observations have resulted in a model in which Fgf8 in the pharyngeal endoderm is regulated by Tbx1 to control the proper patterning of the aortic arch through epithelial-mesenchymal interactions (69). Additionally, Tbx1 transcription has been shown to be regulated by the sonic hedgehog (shh) signaling pathway via the Foxc1 and Foxc2 transcription factors which are expressed in the head mesenchyme and the mesenchyme surrounding the aortic arch arteries (69). Together, studies such as these demonstrate signaling cascades by which Tbx1 is transcribed and may initiate proper patterning events; however, the complete mechanism of Tbx1 action remains unknown.

Familial Cardiac Septal Defects: Nkx2.5 and Gata4

Cardiac septal defects (CSDs) are a common form of CHD and are defined by a hole in the septal wall allowing blood transfer between the atria or ventricles. Atrial septal defects (ASDs) affect over one in 1000 live births, while ventricular septal defects (VSDs) are the most prevalent CHD, occurring in approximately one in 300 live births (**Fig. 1.1**). Over time, persistent left-to-right shunting of blood between the atria or ventricles leads to pulmonary hypertension, arrhythmias, and atrial and ventricular dysfunction. Fortunately, severe ASDs and VSDs can be treated by surgical- or catheter-based procedures that employ a prosthetic patch to close the defect. Despite the high incidence of CSDs, the precise molecular mechanisms directing septal morphogenesis remain unclear. However, genetic studies have

implicated mutations in the Nkx2.5 and GATA4 loci as genetic causes of familial CSDs.

Mutant alleles of the Nkx2.5 locus correlate with ASDs in rare families in which the defect is inherited (**Fig. 1.1**) (72). Genetic studies in a wide variety of organisms demonstrate that Nkx2.5 functions at many stages of cardiac development and in a variety of cardiac tissues (73, 74). Complete loss of Nkx2.5 in mice results in early embryonic lethality with severe cardiac defects (75), while mice heterozygous for the Nkx2.5 allele only occasionally suffer ASDs (76). This suggests that genetic modifiers are important for ASD penetrance. Nkx2.5 interacts with other transcription factors associated with CHDs such as GATA4 and Tbx5, and many cardiac-specific genes contain Nkx2.5 binding sites in their promoters, highlighting the importance of Nkx2.5 in the cardiac transcriptional program (**Table 1.1**) (77). Chien *et al.* (2004) reported that mice harboring a ventricular muscle-cell restricted knockout of Nkx2.5 mimic CHD and implicated persistent BMP-10 (Bone Morphogenetic Protein-10) expression as playing an important role in the onset and progression of observed cardiac defects (78). This study suggests that antagonizing BMP-10 signals could represent a new therapeutic approach to prevent progression of Nkx2.5-associated CHDs.

ASDs, as well as VSDs and atrioventricular septal defects, are also associated with GATA4 haploinsufficiency (**Fig. 1.1**) (79-81). A study of a large pedigree revealed a missense mutation in GATA4 linked to an autosomal dominant disorder where ASD was fully penetrant. GATA4 encodes a zinc-finger transcription factor essential for cardiogenesis, and directly interacts with the cardiac transcription

factors Nkx2.5 and Tbx5 to synergistically activate cardiac gene expression (77, 82). Inherited mutations in GATA4 result in reduced DNA binding and transactivation of target genes, as well as loss of Tbx5 interaction (79). In addition to Nkx2.5 and Tbx5, GATA4 associates with a variety of binding partners thought to create specific transcriptional complexes that confer tissue-specific gene expression during heart development (**Table 1.1**) (73, 77).

Holt-Oram Syndrome: Tbx5

Holt-Oram syndrome (HOS) is an autosomal dominant condition that occurs in approximately one of every 100,000 live births. HOS generally presents highly variable phenotypes including both upper limb and cardiac defects. Though rare, there is much to learn from its presentation of CHDs, which range from single or multiple ASDs and VSDs, to more complex malformations such as tetralogy of Fallot and hypoplastic left heart syndrome (**Fig. 1.1**) (83). Mild to severe cardiac arrhythmias are also common (84).

The genomic locus responsible for HOS phenotypes was previously mapped to chromosome 12q24.1. Since then, HOS has been linked to more than 30 mutations distributed throughout Tbx5, generally thought to result in Tbx5 haploinsufficiency (83, 85). Tbx5, like Tbx1, is a T-box containing transcription factor that is essential for proper vertebrate tissue patterning and differentiation (86). Though familial studies and studies in mouse have attempted to correlate the location of the many Tbx5 mutations along the locus with the wide variation in severity of limb and cardiac defects, there is currently insufficient evidence to

support such a hypothesis (83). Currently, it is thought that loss of transactivation, reduced interaction with other cardiac transcription factors such as Nkx2.5, GATA4, and Tbx20, or mis-sorting of mutant forms of Tbx5 are main causes for HOS pathogenesis (**Table 1.1**) (73, 82, 87).

Okihiro Syndrome: Sall4

Okihiro Syndrome is an autosomal dominant condition consisting of Duane anomaly, radial ray defects and deafness. The phenotype may include cardiac defects, anal stenosis, pigmentary disturbance, renal abnormalities, or facial asymmetries. The specific cardiac defects are most often ASDs, VSDs, or tetralogy of Fallot (**Fig. 1.1**) (88, 89).

Familial studies of individuals affected by Okihiro syndrome have identified mutations in the Sall4 (spalt-like 4) gene and suggest that haploinsufficiency of this gene is responsible for the clinical phenotype (90-92). Sall4 is a member of the Sal gene family, which encodes a group of four probable zinc-finger transcription factors (93). Thus far, a total of 11 different mutations over the entire Sall4 gene have been described in relation to Okihiro syndrome (88, 90, 91). In addition, Borozdin (2004) and colleagues demonstrated that Okihiro syndrome can also be caused by deletions of either the entire Sall4 gene or of single coding exons (92). Based on work with the closely related Sall1, it is likely that these mutations result in truncated proteins, possibly having the dominant effect of an upregulated repressor (94). However, at this point there are no known upstream effectors or downstream targets of Sall4.

Char Syndrome: TFAP2B

Char syndrome is an autosomal dominant trait characterized by facial dysmorphism, hand anomalies, and patent ductus arteriosus (**Fig. 1.1**). Char syndrome has been mapped to chromosome 6p12-p21 and further analyses point to inherited mutations within the TFAP2B (transcription factor AP-2 beta) locus as a genetic cause of Char syndrome (95-97). TFAP2B encodes a neural crest-related transcription factor belonging to the TFAP family, whose members play an important role in retinoic acid-induced differentiation (98). Char syndrome likely results from abnormal neural crest development, as neural crest cells are important for the development of several affected tissues (99). TFAP2B mutations associated with Char syndrome inhibit target gene activation through a dominant-negative mechanism or cause abnormal mRNA splicing resulting in TFAP2B haploinsufficiency (96, 97). However, the precise molecular mechanisms underlying the effects of aberrant TFAP2B activity resulting in Char syndrome remain to be elucidated.

Dilated Cardiomyopathy with Sensorineural Hearing Loss: Eya4

Cardiomyopathy is the leading cause of heart failure and is most commonly associated with a dilated cardiomyopathy (DCM) phenotype, defined by increased diastolic and systolic ventricular volumes and contractile dysfunction (100). Often, DCM is presented in conjunction with defects of the inner ear resulting in sensorineural hearing loss (SNHL). While not typically characterized as a classical

CHD, cardiomyopathy is discussed here as the origins of its pathophysiology may also result from mutations in cardiac transcription factors.

Several studies have demonstrated that approximately 25% to 30% of DCM cases may be familial (100). Until recently, the significant mortality and late onset of this disease hindered work to identify the genomic location of the responsible disease loci. Schönberger and colleagues have identified a human mutation that causes dilated cardiomyopathy and associated heart failure in addition to previously described sensorineural hearing loss (101, 102). The identified mutation is a 4846 bp deletion of the human gene Eya4, one of four vertebrate orthologs of the *Drosophila melanogaster* gene eyes absent (eya) (103). Eya4 is a transcriptional coactivator that interacts with members of the *sine oculis* family (Six1-Six6) and Dach transcription factors to lead to gene activation (**Table 1.1**) (103-105). The characterization of the human mutation is supported by work in zebrafish, as attenuated Eya4 levels produce the morphological and hemodynamic features of heart failure. In addition, Schönberger *et al.* (2002) demonstrate critical roles for Eya4-Six regulation of transcription in normal heart function (101).

Potential therapeutic strategies for transcription factor-associated CHDs

In general, transcription factors have historically been poor targets of drug therapy due to their nuclear localization, lack of enzymatic activity, and the difficulty associated with reprogramming transcriptional networks. Presently, the most effective therapy for cardiac diseases is heart transplantation. However, due to the shortage of organs, cost, and inaccessibility of treatment for most affected

individuals, this remains a limited therapeutic option. Alternative treatment is the administration of drugs that improve myocardial contractility, though this treatment is only effective as a short-term therapy, with the 5-year survival rate using current agents being less then 60% (106). More recently, new strategies have focused on two main approaches for treatment of transcription factor-associated heart disease, cardiac stem cell transplants and chemical modulators of transcriptional activity.

The ability to isolate and propagate cell populations that can differentiate into cardiomyocytes *in vivo* offers the opportunity to treat a wide range of cardiac diseases. The existence of cardiac precursor or stem cells in adults remains a contentious issue. However, recent reports suggest that cardiac precursor or stem cells are present, albeit, in a very low number. In addition to endogenous cardiac stem cells, other studies have shown that multipotential cells, most notably embryonic stem (ES) cells and bone marrow-derived stem cells have, under defined conditions, differentiated into cardiomyocytes. Although these studies offer the promise of growing cells for use in repairing damaged cardiac tissue, three major hurdles must be overcome before stem cells can be considered as a therapy for cardiac disease. First, the molecular, biochemical, and cellular properties of these different cell populations must be established. Second, studies must demonstrate that precursor cell populations can be maintained and expanded to suitable numbers to be used as a cardiac therapy while maintaining their multipotentiality. Finally, results must show multipotential cells, once transplanted to the heart, can give rise to functioning cardiomyocytes while not undergoing uncontrolled differentiation leading to cardiac teratomas or fibrosis (107).

An alternative therapeutic strategy for transcription factor-associated cardiac disease is to screen small chemical libraries to identify agents that either exacerbate or ameliorate transcription factor activities. These agents could either act in an intercellular signaling cascade that turns on, off, or modifies transcription factor activities, most notably agents that act in the calcium or phosphate signaling pathways, or act directly on transcription co-factors such as histone acetyltransferases (HATs) or histone deactylases (HDACs) (106). The major obstacle is the availability of an inexpensive, quick screen for these molecules. However, recent observations showing the sequence, expression, and function of many cardiac disease-associated transcription factors are evolutionarily conserved open the possibility of using fish or frog model systems as bioassays to test for agents that modulate these pathways.

Given the number of transcription factors demonstrated to play essential roles in vertebrate cardiogenesis, the current pool of CHD-associated transcription factors is likely underrepresented. It is very likely that more correlations between mutations in cardiac transcription factors and CHD will be made. To this end, disruptions in the function of at least six cardiac transcription factors have been associated with human CHD. Haploinsufficiency of the genes Tbx1, Tbx5, GATA4, and Sall4 have been correlated with CHDs such as DiGeorge syndrome, Holt-Oram syndrome, familial ASDs and VSDs and Okihiro syndrome while similar disruptions in Nkx2.5, TFAP2B, and Eya4 are associated with familial ASDs, Char syndrome and cardiomyopathies. While the identification of genes associated with CHD is an important first step towards the goal of curing cardiovascular disease, it has become

clear that understanding the genetic pathways and the molecular mechanisms of transcription factors will be key to our ability to identify therapeutic agents for CHD. Based on our current understanding of these mechanisms and of heart development in general, possible treatment options may eventually grow to include cardiac stem cell transplants and chemical agents. However, these possibilities lie in the future, and their development will rely upon studies using a variety of animal models and our growing knowledge of CHD.

Post-transcriptional Regulation of Cardiac Gene Expression by MicroRNAs

MicroRNAs (miRNAs) are an evolutionarily conserved class of small noncoding RNAs known to regulate translation of target messenger RNAs in animals (108). The first microRNA was discovered in 1993 in the worm *Caenorhabditis elegans* (109, 110). Many scientists dismissed this discovery as a singular instance of a genetic oddity found only in worm. The second microRNA was discovered in worm eight years later, but this microRNA was found to also exist in humans and other mammals, drawing the attention of many scientists to these molecules (111). In recent years, advances in sequencing technologies and bioinformatics have enabled scientists to catalog the existence of over 500 human microRNA genes (112). Strikingly, computer models predict that microRNAs may regulate the expression of more than one-third of human protein-coding genes, highlighting their potential importance in both human development and disease (113, 114). The discovery of this new and growing class of regulatory molecules has provided an additional layer

of spatial and temporal control of developmental and homeostatic events by altering levels of critical regulators within complex genetic pathways.

Some of the most exciting biological roles for microRNAs discovered so far have emerged from within the cardiovascular research community (115). Our laboratory and others have carefully analyzed the expression levels of hundreds of microRNAs in animal models of heart disease and changes were found in the levels of specific microRNAs (116-120). Expression profiling of microRNAs in severely diseased human hearts identified similar changes, indicating that microRNAs may play a role in heart disease. Furthermore, recent genetic studies identified roles for miRNAs in cardiogenesis, cardiac electrical conduction, and stress-dependent cardiac remodeling (121-123). The recent progress made at the intersection of the miRNA and cardiac muscle biology fields are more fully reviewed in Chapter 3.

Research Presented in this Dissertation

The direction of my dissertation research has been aimed at understanding the fundamental mechanisms of transcriptional and post-transcriptional regulation of gene expression. In particular, I studied how transcription factors and microRNAs coordinate cardiac gene expression during development and in disease.

A project started at the beginning of my graduate training increased our understanding of the regulation of myocardin, an important transcription factor expressed in cardiac and smooth muscle tissues. Despite the significant role of myocardin in controlling muscle gene expression, upstream signaling pathways that regulate myocardin activity remained unknown (26). Bone morphogenetic proteins

(BMPs) and downstream BMP signaling effectors are known to be essential for cardiovascular development (124-126); however, it was clear that BMP signaling alone was insufficient to activate the cardiac gene program because BMP signaling pathway components are expressed in a wide range of tissues and cell types outside of cardiac muscle. Indeed, the ability of BMP signaling to commit specific mesodermal cells to a cardiac fate requires that BMP signaling be interpreted in a cell type-specific mechanism. In light of the role of BMP signaling and myocardin in cardiovascular development, I investigated and discovered that BMP signaling could regulate myocardin-mediated cardiac gene expression. This work along with a collaborator's concurrent study addressed an important question facing the cardiovascular field (29, 127): how does myocardin discriminate between cardiac- and smooth muscle-specific genes? The answer appears to be, at least in part, that myocardin's target specificity is determined by which upstream signaling pathways and their downstream effectors interact with myocardin. This work is presented in Chapter 2.

After completing the BMP-myocardin study, my major research direction has centered on miRNA biology as it relates to cardiac muscle biology. Despite the prevalence of miRNAs found within mammalian genomes, miRNAs are a relatively new and understudied class of molecules, thus very few miRNAs have been assigned specific biological roles. Of the miRNAs reported as having muscle-specific expression patterns, miR-208 was reported as being solely expressed in cardiac muscle (128). I broadly hypothesized that the heart-specific expression pattern of miR-208 and its sequence conversation across species was indicative of an

important function in the heart. To identify a biological role for miR-208, I initiated studies of miR-208 using cardiomyocytes *in vitro*, genetic analyses in the mouse *in vivo*, and combined bioinformatics and experimental approaches to identify direct molecular targets of miR-208. I find that the miR-208 family, consisting of miR-208a and miR-208b, are differentially expressed during heart development, paralleling the expression of their respective host genes alpha- and beta-myosin heavy chain (αMHC and βMHC). Expression of miR-208a and miR-208b are subject to thyroid hormone regulation like their host genes, and miR-208b is induced during cardiac hypertrophy concurrent with βMHC expression, indicating that these intronic miRNAs are co-regulated with their respective host genes. In an effort to understand the function of miR-208a in the adult heart, I created genetic miR-208a gain- and loss-of-function mouse models. Through careful analysis of these mice, I found evidence that the miR-208 family is a key regulator of cardiac remodeling. Overproduction of miR-208a in transgenic mouse hearts induced hypertrophic growth and increased both βMHC and miR-208b expression levels. Conversely, deletion of miR-208a from the mouse genome resulted in lower levels of βMHC expression in the adult heart, providing complementary genetic evidence that miR-208a is involved in regulating βMHC expression. miR-208a and miR-208b share similar sequence identity and appear to repress the translation of the same regulatory targets, including Thyroid hormone associated protein 1 (Thrap1) and myostatin, two negative regulators of muscle growth and hypertrophy. From these studies, I proposed a model where the miR-208 family fine-tunes the expression of anti-hypertrophy genes and regulates hypertrophic growth during normal and pathological conditions. Interestingly, the

miR-208 also appears to be an important regulator of the cardiac conduction system. These novel findings contribute new knowledge to our understanding of the genetic networks important for cardiovascular pathophysiology. The work on the miR-208 family is presented in Chapters 4 and 5.

Figure 1.1

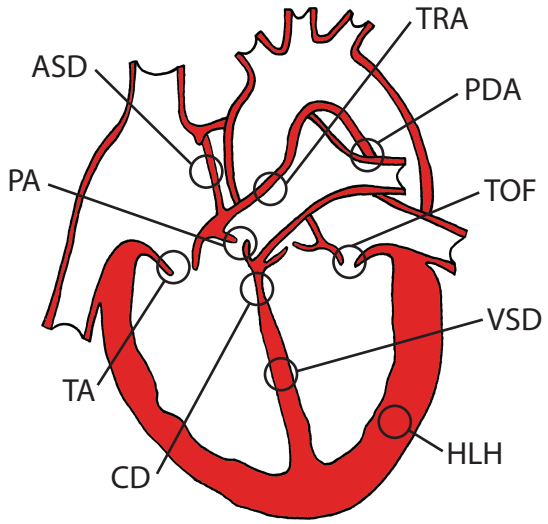

Figure 1.1. Congenital heart defects associated with mutations in cardiac transcription factors. Mutations in cardiac transcription factors such as Tbx1, Nkx2.5, Gata4, Tbx5, Sall4, and TFAP2B have been associated with multiple human congenital heart defects including atrial septal defects (ASD), ventricular septal defects (VSD), tetralogy of Fallot (TOF), conduction system defects (CD), hypoplastic left heart (HLH), pulmonary atresia (PA), patent ductus arteriosus (PDA), tricuspid atresia (TA), and truncus arteriosus (TRA). Schematic shows the relative position of each defect.

Table 1.1 Human congenital heart diseases, associated transcription factors and molecular interactions

Human Clinical Manifestation	Associated Transcription Factor	Co-factors/ Upstream Molecules	Potential Downstream Cardiac Genes	References
DiGeorge Syndrome	Tbx1	VEGF, FOXc1, FOXc2	Fgf8	(68-71, 73, 74, 77)
Familial ASD	Nkx2.5	GATA4, Tbx5	Nppa/ANF, Bnp, eHand, Mef2C Mlc2V, Msx2, N-Myc	(72-77, 82)
Familial ASD/VSD	GATA4	FOG2, GATA6, MEF2C, NFATc4, Nkx2.5, SRF, Tbx5	Nppa/ANF, α/β-MHC, Cardiac a-actin, Cardiac TnC,Cardiac TnI, Gata6, Nkx2.5	(73, 74, 77, 79-82)
Holt-Oram Syndrome	Tbx5	GATA4, Nkx2.5, Tbx20	Nppa/ANF, Cx40, Gata4, Hey2, Irx4, Mlc2v, Nkx2.5	(73, 74, 77, 82-87)
Okihiro Syndrome	Sall4	Unknown	Unknown	(73, 88-94)
Char Syndrome	TFAP2B	Unknown	Unknown	(82, 95-97)
Dilated Cardiomyopathy with Sensorineural Hearing Loss	Eya4	SIX, DACH	Unknown	(73, 101-105)

Abbreviations: VEGF-vascular endothelial growth factor; FOXc1/2-human forkhead-box subfamily c1/2; GATA-GATA binding protein; TBX-T-box; FOG2-friend of GATA; MEF2C-myocyte enhancer factor 2C isoform; NFATc4-nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent-4; NKX2.5-NK2-related homeobox; SRF-serum response factor; Fgf8-fibroblast growth factor 8; Nppa/ANF-natriuretic peptide precursor a/atrial natriuretic factor; Bnp-brain natriuretic peptide; eHAND-heart- and neural crest derivatives-expressed 1; mlc2v-myosin light chain-2 ventricular isoform; Msx2-muscle segment homeobox 2; N-Myc-neuroblastoma-myelocytomatosis viral-related oncogene; α/β-MHC-alpha-, beta- myosin heavy chain; TnC-troponin C; TnI-troponin I; Cx40-connexin 40; Hey2-hairy/enhancer of split-related with YRPW motif-2; Irx4-iroquois 4

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CHAPTER 2

BONE MORPHOGENETIC PROTEIN (BMP) SIGNALING MODULATES MYOCARDIN TRANSACTIVATION OF CARDIAC GENES

Abstract

Bone morphogenetic proteins (BMPs) play important roles in cardiovascular development. However, how BMP signaling pathways regulate cardiac gene expression is less clear. We have previously identified myocardin as a cardiac and smooth muscle-specific transcriptional cofactor for serum response factor (SRF). Myocardin potently activates target gene expression by tethering with SRF bound to SRF-responsive elements, the CArG box. Here, we show that Smad1, an effector of the BMP signaling pathway, synergistically activates myocardin-dependent cardiac gene expression. Interestingly, the CArG box is necessary and sufficient to mediate such synergy whereas no obvious Smad Binding Element (SBE) appears to be involved. Consistent with their functional interaction, we find that myocardin and Smad1 proteins interact directly. Furthermore, myocardin protein levels were dramatically increased by BMP-2 treatment in cardiomyocytes. These findings suggest myocardin participates in a BMP signaling-dependent cardiac gene transcriptional program.

Introduction

Myocardin is a serum response factor (SRF) cofactor expressed in cardiac and smooth muscle cell lineages (1). Although myocardin lacks intrinsic DNA binding ability, it forms a stable ternary complex with SRF to potently activate muscle-specific genes through the consensus sequence $CC(A/T)_6GG$, known as a CArG box (1-3). In addition to activating cardiac gene expression and the cardiogenesis program, myocardin is also a potent transactivator for smooth muscle cell (SMC) differentiation and smooth muscle (SM) related gene expression (1, 4-15). Despite the significant role of myocardin in controlling muscle gene expression, upstream signaling pathways that regulate myocardin activity remain unknown.

Bone morphogenetic proteins (BMPs) are growth and differentiation factors of the transforming growth factor β (TGF-β) superfamily (16). Signaling by this superfamily is mediated by Smad proteins. There are three classes of Smads: receptor-regulated Smads (R-Smads), co-Smad (Smad4), and inhibitory Smads. To date, three R-Smads (Smad1, 5, and 8) participate in BMP signaling (17). Once activated, R-Smads form a heteromeric complex with Smad4 that translocates into the nucleus to regulate expression of BMP-responsive genes (17). Smad proteins bind DNA relatively weakly, but are strongly recruited to specific target genes by interacting with other transcription factors (17).

BMPs and downstream BMP signaling effectors are essential for cardiovascular development (18-23). However, it is clear BMP signaling alone is insufficient to activate the cardiac gene program since BMP signaling pathway components are expressed in a wide range of tissues and cell types outside of cardiac muscle.

Indeed, the ability of BMP signaling to commit specific mesodermal cells to a cardiac fate requires the interpretation of BMP signaling in a cell-type specific mechanism. How BMP signaling interacts with cardiac transcriptional networks is largely unknown. In light of the role of BMP signaling and myocardin in cardiovascular development, we investigated whether BMP signaling might regulate myocardin-mediated cardiac gene expression.

In this report, we show that Smad1 synergistically activates myocardin-dependent cardiac gene expression. Interestingly, the CArG box is necessary and sufficient for such synergy, whereas no obvious Smad Binding Elements (SBEs) are involved. Consistent with their functional interaction, myocardin and Smad1 proteins physically interact. Myocardin transactivity was repressed by Smad7, an inhibitory Smad, and enhanced by constitutively activated ALK3, a type I BMP receptor. Furthermore, myocardin protein expression was dramatically induced by BMP-2 treatment in cardiomyocytes. These findings suggest a role for BMP signaling in regulating myocardin expression and activity to control cardiac gene expression.

Materials and Methods

Plasmid constructs

Myocardin and SRF expression plasmids are described (1, 24). Myocardin C-terminal deletion mutants (M1, M2 and M3) were cloned into a pcDNA expression vector with a N-terminal Myc tag. Myocardin and Smad1 cDNAs were cloned into pGEX-KG vector to generate GST-fusion proteins. Smad1, Smad4, Smad7, and constitutively active (Q233D) ALK3 (ALK3 QD) expression plasmids are described.(25-28) Smad1 cDNA was subcloned into pM1 vector to make GAL4-Smad1 fusion protein.

Cell culture and luciferase reporter assays

Alpha-cardiac actin (*α-CA*) (29), *myosin light chain 2V* (*MLC2V*) (30), *alpha-myosin heavy chain* (*α-MHC*) (1), and *atrial natriuretic factor* (*ANF*) (1) promoter luciferase reporters are described. The *Nkx2.5* promoter and Smad Binding Element (SBE) mutations are described and were cloned into pGL3 (31). Truncated *ANF* luciferase reporters were generated by cloning the –406, –226, –115 to +70 regions of the *ANF* promoter into pGL3. The –115 *ANF* reporter was further truncated by deleting the –5 to +70 region. The *ANF* SBE mutation (–5 to –2) was generated by site-directed mutagenesis. COS7 cells were cultured as described (1). Neonatal rat cardiomyocytes were prepared as described (32). Reporter assays were conducted in triplicate at least two times in 12 well plates. Transfections were performed with either Fugene6 (Roche) or Lipofectamine (Invitrogen) reagents. Unless otherwise indicated, 100 ng of reporter and 200 ng of activator plasmids were used. A CMV-

lacZ reporter was used as an internal control to normalize for transfection efficiencies, and total amount of DNA per well was kept constant by adding the corresponding amount of empty expression vector. Statistical analysis was performed using the Student *t* test; comparisons were considered significant where $P < 0.05$.

GST-protein Pulldown assays

GST alone, GST-Smad1, and GST-myocardin proteins were expressed and purified as described and used for *in vitro* binding assays (14). Smad1 and myocardin proteins were *in vitro* translated (Promega) and [^{35}S] labeled. Pulldowns were performed by incubating radiolabeled proteins with bead-bound GST-fusion proteins in buffer (20 mM Tris, pH 7.3, 150 mM NaCl, 0.5% NP-40, and protease inhibitors) for 2 hours at 4°C, followed by three washes in the same buffer. Samples were analyzed by SDS-PAGE and autoradiography.

Co-Immunoprecipitation (Co-IP) and Western blotting assays

COS7 cells were transfected with Myc-myocardin and Flag-Smad1 plasmids in 10 cm plates. After 48 hours post-transfection, whole cell extracts were prepared in 1 ml of PBS buffer containing 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitors. Extracts were cleared by 10,000 x *g* centrifugation for 10 min, incubated with anti-Flag M2 affinity gel resin (Sigma) for 2 hours at 4°C, then washed 3 times in the same buffer, and samples were subsequently analyzed by

SDS-PAGE and Western blot analysis using anti-Myc (A-14, Santa Cruz, 1:2500) or anti-Flag (M2, Sigma, 1:2500) antibodies.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed essentially as described using the *c-fos* CArG probe (33). Complementary oligonucleotides were annealed and labeled using Klenow polymerase and [α-^{32}P]dCTP. Labeled probe was incubated with *in vitro* translated SRF, Myc-tagged myocardin, and/or Smad1 proteins in gel shift buffer (10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) with poly(dI-dC). Antibody supershift experiments were performed with anti-SRF (G-20, Santa Cruz) or anti-Myc. DNA-protein complexes were separated by gel electrophoresis on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography.

BMP response assay

After serum starving overnight, cardiomyocytes were treated with (or without treatment in control) 20 ng/ml recombinant BMP-2 (R&D Systems) for 48 hours, then harvested in 200 μl lysis buffer composed of PBS containing 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, and protease inhibitors. Twenty μl of lysate was loaded onto SDS-PAGE for Western blot analysis. Antibodies used were anti-myocardin (sc-21559, Santa Cruz, 1:1000); anti-MEF2 (sc-313, Santa Cruz, 1:1000); anti-SRF (sc-335, Santa Cruz, 1:2500); anti-α-actinin (sc-17829, Santa Cruz, 1:2500); anti-β-tubulin (T-4026, Sigma, 1:5000).

Results

Synergistic activation of cardiac promoters by myocardin and Smad proteins

Smad1 is a downstream effector of BMP signaling that mediates target gene transcription; we therefore tested whether myocardin and Smad1 might activate cardiac gene expression in a cooperative manner. Whereas myocardin strongly activated the *ANF* promoter luciferase reporter ((1) and **Fig. 2.1a**), co-expression of myocardin and Smad1 synergistically activated this reporter in COS7 cells. In contrast, Smad1 by itself did not significantly activate the *ANF* reporter (**Fig 2.1a**). Myocardin and Smad1 also synergistically activated the *ANF* reporter in cardiomyocytes (**Fig. 2.1g**). Similarly, myocardin and Smad1 synergistically activated all other cardiac promoter reporters tested (**Fig. 2.1b-e**). Since Smad1 is known to heterodimerize with Smad4, we investigated the effects of Smad4 on myocardin/Smad1 synergy and found that Smad4 further increased myocardin/Smad1 activation (**Fig. 2.1e**).

To rule out the possibility that the myocardin/Smad1 synergy observed was an indirect effect mediated through SRF, we tested whether myocardin and GAL4-Smad1 fusion protein, could activate a GAL4-dependent luciferase reporter (UAS-luciferase). GAL4-Smad1 and myocardin activated the UAS-luciferase reporter in a dosage-dependent manner (**Fig. 2.1f**). Similarly, Smad1 synergized with GAL4-myocardin fusion protein to activate the UAS-luciferase reporter (data not shown). These results suggest myocardin/Smad1 synergy is directly mediated by myocardin and Smad1 interaction. Together, we conclude BMP signaling mediator Smad1 dramatically enhances myocardin transactivation of cardiac promoters.

CArG box is necessary and sufficient to mediate myocardin and Smad1 synergy

Deletion analysis of the *ANF* promoter was performed to determine the minimal region required for myocardin/Smad1 functional interaction. Co-expression of myocardin and Smad1 increased activation of the *ANF* reporter ~5-fold higher than myocardin alone (**Fig. 2.2a**). Truncating the *ANF* promoter to –406 to +70 did not significantly affect the activation by myocardin alone or the synergy between myocardin and Smad1 (**Fig. 2.2a**). The –226 to +70 *ANF* reporter, which excluded one of the two CArG boxes present within the *ANF* promoter (CArG-far, –397 to –77), dramatically decreased the activation of this reporter by myocardin alone (data not shown), however, the synergy between myocardin and Smad1 was unaffected (**Fig. 2.2a**). Further deletion of the *ANF* promoter (–115 to +70) only slightly reduced reporter activation by myocardin and Smad1 (**Fig. 2.2a**). Thus, the –115 to +70 region of the *ANF* promoter, containing a single CArG box, is sufficient to mediate myocardin/Smad1 synergy.

To test whether the CArG box is required for Smad1 and myocardin functional interaction, the two CArG boxes within the *ANF* promoter luciferase reporter were mutated (1). Whereas myocardin and Smad1 could synergistically activate the *ANF* luciferase reporter with a CArG-far mutation, such synergy was abolished by double CArG mutations (**Fig. 2.2a**). These data indicate Smad1 and myocardin functional interaction is CArG box-dependent.

To determine if the CArG box is sufficient to mediate myocardin/Smad1 synergy, we used a luciferase reporter controlled by four tandem copies of a consensus CArG box (1). Myocardin alone activated the 4XCArG reporter ~200 fold, whereas Smad1 and myocardin increased activation to ~625 fold (**Fig. 2.2b**). Thus, we conclude the CArG box is necessary and sufficient to mediate myocardin and Smad1 synergy.

Smad Binding Element (SBE) is not required for myocardin and Smad1 synergy

Most Smads weakly interact with DNA through a Smad Binding Element (SBE) sequence within the promoter of responsive genes (34). The *ANF* –115 to +70 promoter region does not contain a consensus SBE (AGAC GTCT), but does have three AGAC, a half-SBE previously shown to be sufficient for Smad MH1 domain binding (**Fig. 2.3a**) (35, 36). However, Smad1 or Smad4 did not bind to the three AGAC and flanking ~10 bp by electrophoretic mobility shift assay (data not shown). Mutating the AGAC closest to the TATAA box (–5 to –2), the only AGAC identified in the promoter region within the –115 to +70 of the *ANF* regulatory region (the other two are in the 5' UTR of this gene, **Fig. 2.3a**), did not affect activation by myocardin and Smad1 (**Fig. 2.3b**). Myocardin and Smad1 synergistically activated a truncated *ANF* reporter (–115 to –5) where all three AGAC sites were deleted (data not shown), suggesting the SBE site is not required for myocardin and Smad1 synergy. Furthermore, we found that Smad1 did not activate a luciferase reporter controlled by 6X SBE from *SM22*, nor could Smad1 synergize with myocardin on this reporter (data not shown). Finally, we tested whether Smad1 and myocardin could activate

the *Nkx2.5* promoter luciferase reporter containing a SBE mutation (31). As shown in Fig. 2.3c, SBE mutation did not affect the synergy of Smad1 and myocardin. Together, these data demonstrate that Smad1 can synergistically activate cardiac target gene expression with myocardin in a SBE-independent manner.

Myocardin and Smad1 interact directly

COS7 cells were co-transfected with expression plasmids encoding Flag-tagged Smad1 and Myc-tagged myocardin (or singly transfected with each of those constructs in controls). Anti-Flag antibodies were used to immunoprecipitate Smad1. Anti-Myc antibodies were then used to detect the presence of associated myocardin. The interaction of myocardin and Smad1 was detected in lysates prepared from cells expressing both proteins (**Fig. 2.4a**). Such interaction was further confirmed using a series of C-terminal deletion myocardin mutants (**Fig. 2.4b**).

To test whether myocardin and Smad1 interact directly *in vitro*, we performed GST-fusion protein pulldown assays. GST-Smad1 protein was bacterially expressed and immobilized to glutathione-agarose beads and incubated with *in vitro* translated radiolabeled myocardin. Myocardin specifically interacted with GST-Smad1 but not with GST alone (**Fig. 2.4c**).

To confirm the specificity of such interaction as well as to determine the region of myocardin that mediates Smad1 interaction, we generated a deletion series of GST-myocardin fusion proteins and tested their interaction with Smad1 by pulldown assay. Radiolabeled Smad1 specifically interacted with amino acids (aa) 1-560 and 129-689 of myocardin, but not with GST alone or with aa 382-670 or aa 669-935 of

myocardin (**Fig. 2.5a**). This result suggests Smad1 directly interacts with myocardin at a region between aa 129 to aa 560 (**Fig. 2.5b**). We have previously identified several conserved domains within this region of myocardin protein, including the basic domain (aa 243-260), Q domain (aa 287-320), and SAP domain (380-414) (1, 24). We therefore tested whether the functional interaction of myocardin and Smad1 is affected by mutations in those domains by luciferase reporter assay. Basic domain mutation completely abolished myocardin transactivation as well as its synergy with Smad1 (data not shown). Deletion of the SAP domain or Q domain dramatically decreased the synergy of myocardin and Smad1 (**Fig. 2.5c**). The mutations did not alter the expression of those proteins (**Fig. 2.5c**). Together, these data demonstrate a direct interaction between myocardin and Smad1 and suggest such physical interaction is important for their synergistic activation of cardiac promoters.

Smad proteins did not directly affect formation of the myocardin/SRF/CArG complex

Myocardin does not bind to DNA directly, instead it is recruited to target genes by forming a stable complex with its cofactor SRF bound to DNA element, the CArG box (1). The interaction of myocardin and Smad1 led us to examine whether Smad1 directly associates with myocardin/SRF protein complexes bound to CArG box. Electrophoretic mobility shift assays (EMSAs) were performed using a radiolabled oligonucleotide containing a consensus CArG box. Addition of *in vitro* translated SRF to the labeled probe resulted in a specific band (**Fig. 2.6**, lane 2). Addition of both SRF and Myc-tagged myocardin resulted in an additional specific band

corresponding to the myocardin/SRF/CArG complex (**Fig. 2.6**, lane 7). This myocardin/SRF/CArG complex was diminished when anti-Myc antibodies were added (**Fig. 2.6**, lane 13), demonstrating it contained myocardin. Similarly, both SRF and myocardin/SRF complexes were supershifted by anti-SRF antibodies, demonstrating these complexes contained SRF (**Fig. 2.6**, lane 14). However, when *in vitro* translated Smad1 was added to the incubation mixtures, the SRF/CArG or myocardin/SRF/CArG complexes were neither supershifted nor diminished (**Fig. 2.6**, lanes 8). A similar result was obtained despite a several-fold increase in the amount of Smad1 relative to the fixed amounts of SRF and myocardin (**Fig. 2.6**, lanes 11 and 12). We also found Smad4 alone or Smad1 plus Smad4 could not affect the myocardin/SRF/CArG complex (**Fig. 2.6**, lanes 9 and 10). These results indicate, under the experimental conditions employed, Smad1 is not a stable component of the myocardin/SRF/CArG complex.

Myocardin activity is modulated by BMP signaling

Interestingly, BMP signaling can be antagonized by inhibitory Smad6 and Smad7 (16, 17). Smad7 repressed myocardin transactivation *cardiac α-actin* and *ANF* promoters in a dosage-dependent fashion (**Fig. 2.7a** and **2.7b**), presumably by interfering with activation of endogenous BMP signaling components. Constitutively activated ALKs activate BMP signaling in the absence of BMP ligands (25). To test whether upstream BMP signaling components stimulate myocardin transactivity, we employed a constitutively activated BMP receptor, ALK3 QD, in luciferase reporter assays. ALK3 QD stimulated myocardin transactivation of the *cardiac α-actin* and

Nkx2.5 reporters (**Fig. 2.7c** and **2.7d**). While stimulation of myocardin transactivity by ALK3 QD is comparable to Smads 1 and 4, co-transfection of both ALK3 QD and Smads1/4 with myocardin further increased the activation of the *Nkx2.5* reporter (**Fig. 2.7d**). Those results suggest myocardin transactivity is stimulated by BMP signaling originating from the cell surface.

BMP signaling increases myocardin protein expression

BMPs induce the expression of cardiac transcription factor Nkx2.5 and other cardiac markers in treated chick embryos (20), as well as the P19CL6 cell line (21, 22). We asked whether BMPs have the same effect upon myocardin expression. Neonatal rat cardiomyocytes treated with BMP-2 dramatically increased myocardin and MEF2 protein expression, but not of SRF or α-actinin (**Fig. 2.8**). BMP-2 did not induce global protein synthesis, as β-tubulin protein expression was unchanged in BMP-2 treated cardiomyocytes (**Fig. 2.8**). This result demonstrates BMP signaling induces myocardin expression in cardiomyocytes and suggests a positive feedback mechanism for BMP signaling and myocardin to activate cardiac genes.

Discussion

In this report, we identified the molecular interaction of myocardin and the BMP signaling pathway to synergistically activate cardiac gene expression. Along with the accompanying study demonstrating that myocardin and TGF-β signaling pathway synergistically activate smooth muscle (SM) gene expression, our results clearly establish that myocardin is involved in TGF-β superfamily signaling pathways that regulate cardiac and SM specific gene expression.

Transactivation of cardiac gene expression by myocardin and BMP signaling

We have recently uncovered several mechanisms by which myocardin regulates cardiac and SM gene expression. GATA4 represses or activates myocardin-mediated transactivation depending on the specificity of target genes (37). Myocardin transactivity is also positively and negatively regulated by p300 and HDAC5, suggesting an additional layer of regulation at the chromatin level (14). Interestingly, myocardin is also involved in a molecular switch controlling SRF-dependent cell differentiation versus proliferation processes, where myocardin directly competes with Elk1 for SRF association and target gene activation (15). Together, those studies indicate the transcriptional activity of myocardin is tightly controlled.

In this study, we demonstrated myocardin transactivation of cardiac gene expression is modulated by BMP signaling through a protein-protein interaction between myocardin and BMP downstream effector Smad1, providing another novel mechanism in which myocardin is integrated into an important signaling pathway to

regulate gene expression. Most importantly, we found BMP signaling was also able to induce expression of myocardin, suggesting a potential positive feedback mechanism. This mechanism could be used where both myocardin and BMP initiate early cardiac gene expression, whereas myocardin is later employed for the maintenance of the cardiac program. Interestingly, such mechanisms exist in skeletal muscle and other biological systems (38, 39).

SBE-dependency or SBE-independency?

The synergy between Smad1 and myocardin in activating the *ANF* promoter appears to be SBE-independent. Those results were distinct from the response of Smad3 and myocardin, which synergistically activate a SBE controlled reporter (40). In contrast, myocardin and Smad1 synergistically activated a luciferase reporter driven by four CArG box copies, where absolutely no SBE is involved. These data suggest Smad1 can activate cardiac gene expression independently of inherent DNA binding. Similar SBE-independent mechanisms have been recently reported for a variety of target genes regulated by BMP/Smads (41-43).

Then how does Smad1 activate target gene expression independent of DNA binding? Several mechanisms may apply: Smad1 could be recruited by myocardin and SRF to the CArG boxes in the *ANF* promoter. However, whereas we detected protein-protein interaction between myocardin and Smad1 *in vitro* and *in vivo*, we were unable to obtain evidence for the formation of a potential ternary complex among those proteins bound to DNA under our experimental conditions. Interestingly, some SRF cofactors are suggested to enhance the affinity of SRF/DNA

association, despite not forming a stable ternary complex with SRF bound to DNA (3, 44). Our data suggest that Smad1 enhances the activity of the myocardin/SRF transcriptional complex through alternative mechanisms. Those include the recruitment of coactivators, such as p300, or by repelling transcriptional repressors from this transcriptional complex. It will be important to investigate how the physical and functional interaction of endogenous Smad1 and myocardin proteins is influenced by BMP signaling as we cannot rule out the possibility that Smad1 might affect myocardin/SRF/CArG complex formation in a BMP-dependent manner.

Cardiac or Smooth Muscle?

Myocardin is a cardiac and smooth muscle-specific transcriptional cofactor for SRF and activates target gene expression in a CArG-dependent manner (1, 3). It is currently unclear how myocardin discriminates between cardiac and smooth muscle target genes, though the SAP domain has been suggested to be involved (1). In this study, we showed myocardin and Smad1 interact directly to synergistically activate cardiac reporter gene expression. Such activation requires the CArG-box and appears to be SBE-independent. Additionally, the functional interaction between myocardin and Smad1 was completely abolished in SRF null embryonic stem cells (data not shown), further supporting the notion of CArG box/SRF-dependency. We suggest the target specificity (cardiac versus smooth muscle) for myocardin is determined, at least in part, by which upstream signals, TGF-β or BMP, and their downstream effectors, Smad3 or Smad1, are used. This hypothesis is consistent with the notion that BMPs are key regulators for cardiac gene expression, whereas

TGF-β appears to play a significant role in controlling smooth muscle cell gene expression (18, 45, 46). Interestingly, the SAP domain of myocardin appears involved in mediating the functional interaction between myocardin and Smad1 since SAP domain mutation dramatically decreased the synergy of myocardin and Smad1 on a CArG-dependent reporter gene (**Fig. 2.5c**). Together, our data suggest the SAP domain of myocardin may serve as a nodal point to integrate BMP signaling pathway in activating myocardin-mediated cardiac gene expression. Future investigation, in particular *in vivo* studies, will be needed to further clarify this issue. Nevertheless, our studies establish a direct molecular and functional interaction between myocardin and BMP signaling and suggest a molecular mechanism for the transcriptional regulation of the cardiac gene program. Given the importance of myocardin and BMPs, it is intriguing to speculate mutations in either molecule or disruption of their functional interaction may contribute to human cardiovascular diseases.

Figure 2.1

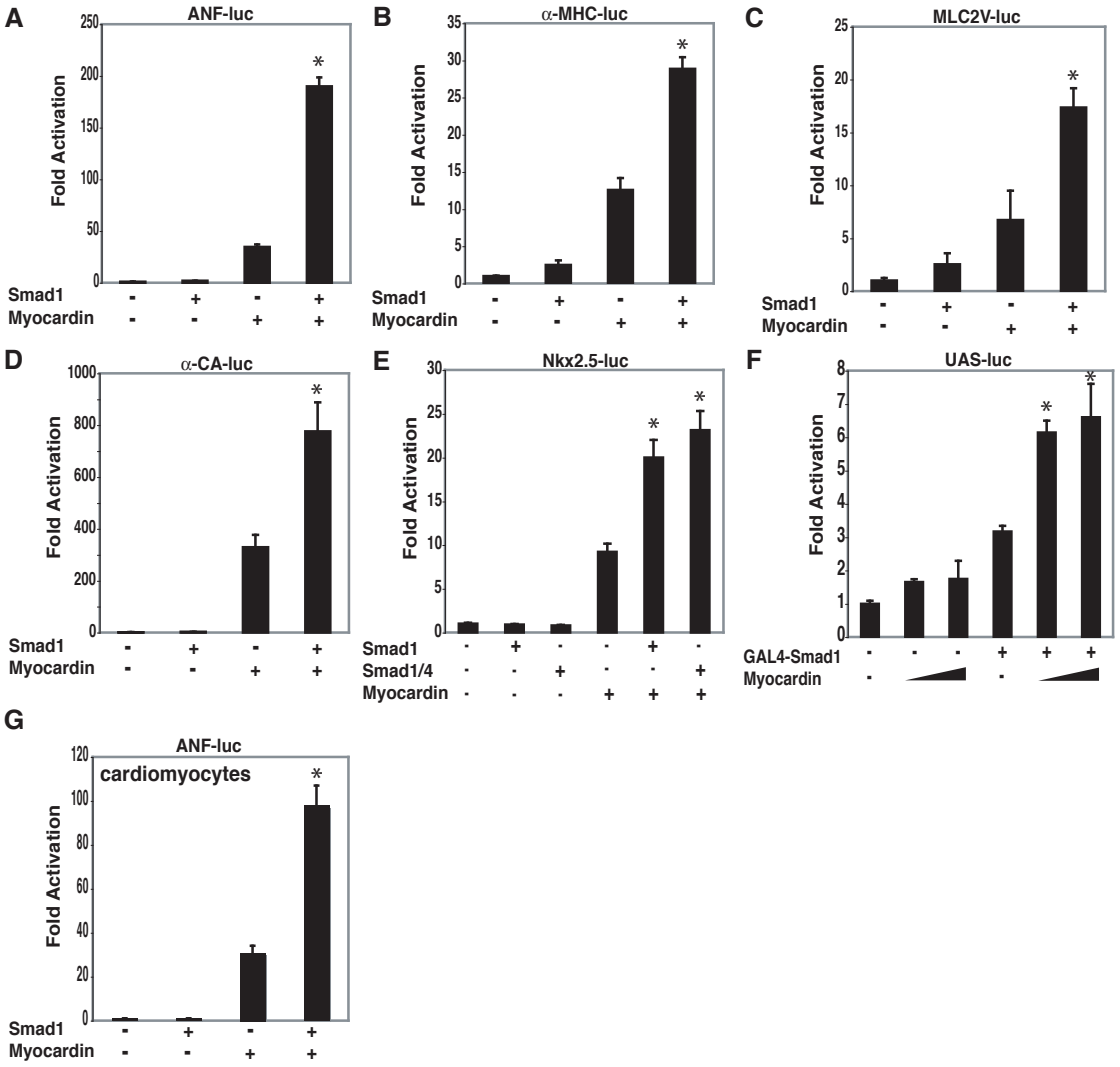

Figure 2.1. Synergistic activation of cardiac promoters by myocardin and Smad1. Luciferase reporters controlled by (A) *ANF*, (B) α-*MHC*, (C) *MLC2V*, (D) α-*CA*, and (E) *Nkx2.5* promoters were transfected into COS7 cells with Smad1 and/or myocardin expression plasmids. (F) COS7 cells were transfected with UAS-luciferase reporter and/or GAL4-Smad1 and myocardin (400 and 800 ng as indicated) expression plasmids. (G) Cardiomyocytes were transfected with *ANF* luciferase reporter with Smad1 and/or myocardin expression plasmids. Values are the fold-increase in luciferase activity relative to activation of the reporter alone. Error bars represent standard deviation of at least two experiments. Student *t* test, $P < 0.05$: *myocardin alone vs myocardin plus Smad1.

Figure 2.2

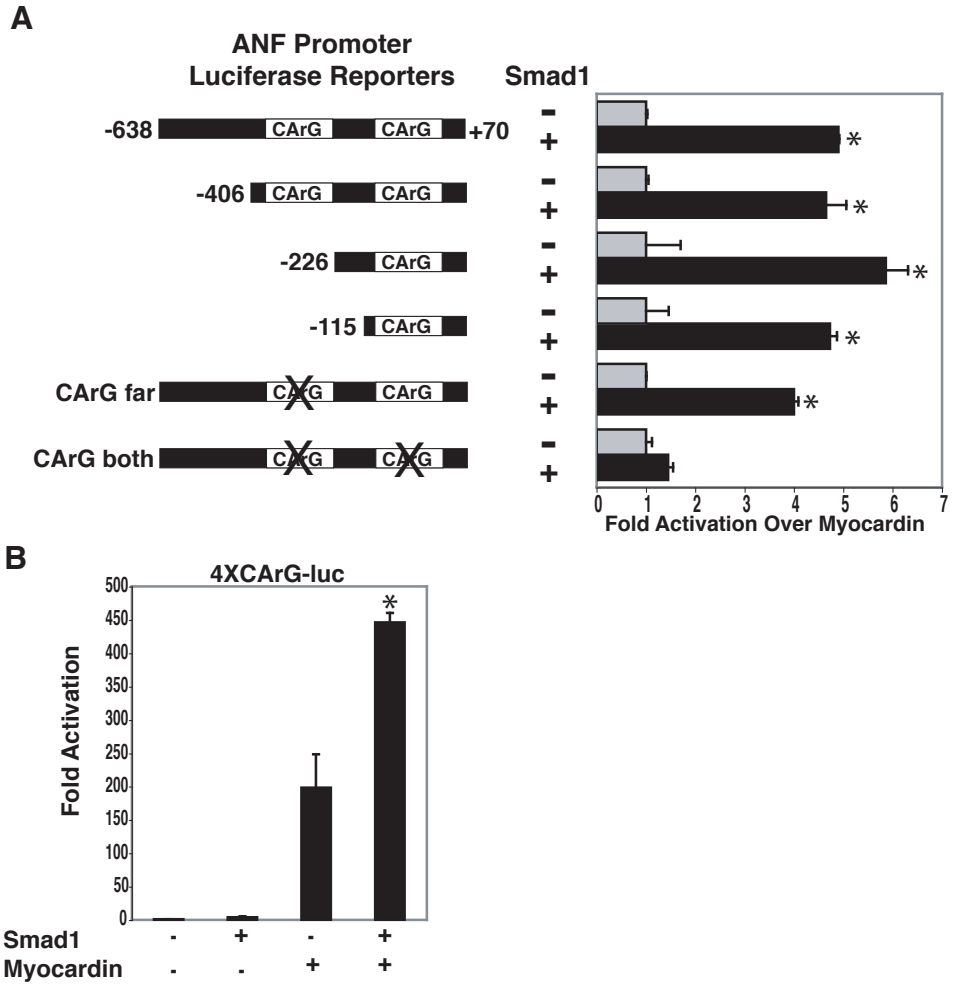

Figure 2.2 CArG box is necessary and sufficient to mediate myocardin and Smad1 synergy. (A) COS7 cells were transfected with the indicated *ANF* promoter luciferase reporters and/or myocardin and Smad1 expression plasmids. Values are the fold-increase of luciferase activity by myocardin and Smad1 (black bars) relative to luciferase activity by myocardin alone (gray bars), which is assigned the value of 1. (B) A luciferase reporter controlled by four copies of a consensus CArG box were transfected into COS7 cells with myocardin and/or Smad1 expression plasmids. Values are the fold-increase in luciferase activity relative to activation of the reporter alone. Error bars represent standard deviation of at least two experiments. Student *t* test, $P < 0.05$: *myocardin alone vs myocardin plus Smad1.

Figure 2.3

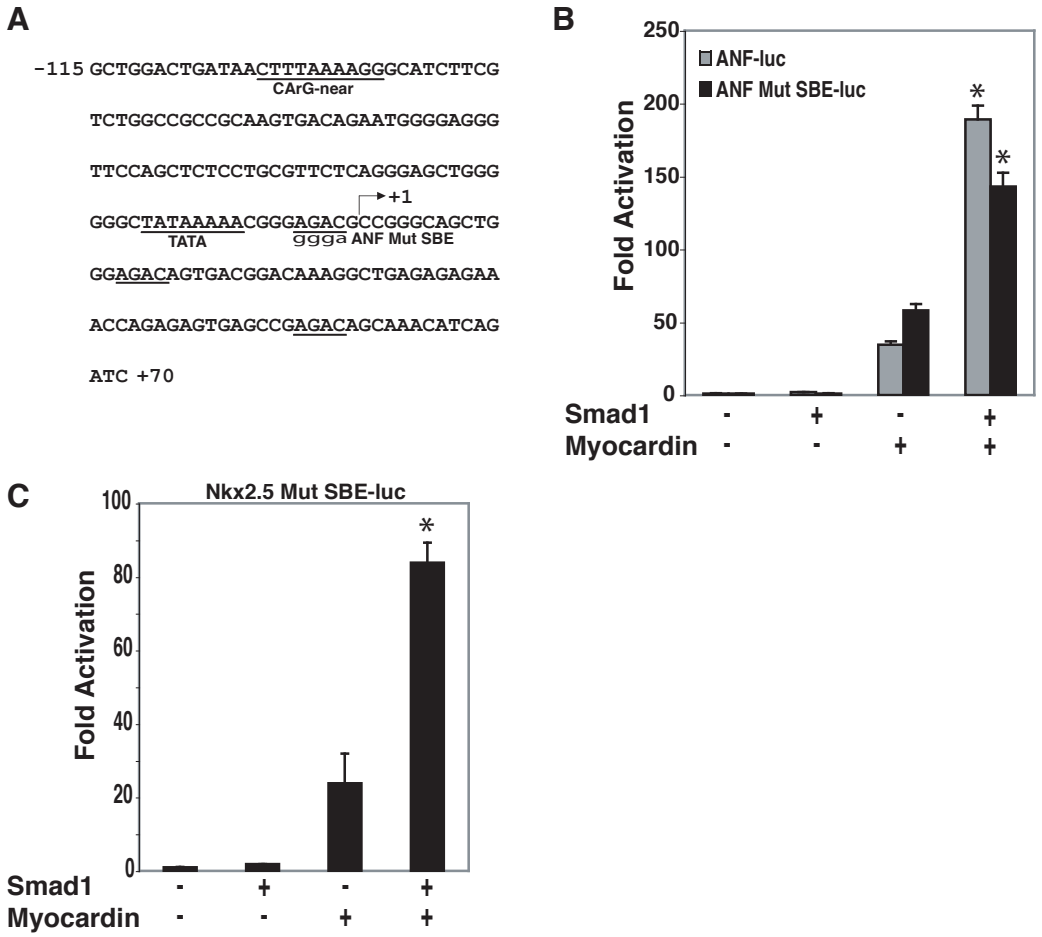

Figure 2.3. Smad Binding Element (SBE) is not required for myocardin and Smad1 synergy. (A) The DNA sequence of the –115 to +70 region of the *ANF* promoter. Underlined are CArG-near, TATA box, the mutated half-SBE site, and two other half-SBE sites (AGAC) within the 5' untranslated region (UTR). (B) Luciferase reporters controlled by the *ANF* promoter (gray bars) or the ANF promoter with mutated half-SBE (black bars) were transfected into COS7 cells with myocardin and/or Smad1 expression plasmids. (C) A luciferase reporter controlled by the *Nkx2.5* promoter containing SBE mutations was transfected into COS7 cells with myocardin and/or Smad1 expression plasmids. Values are the fold-increase in luciferase activity relative to activation of the reporter alone. Error bars represent standard deviation of at least two experiments. Student *t* test, $P < 0.05$: *myocardin alone vs myocardin plus Smad1.

Figure 2.4

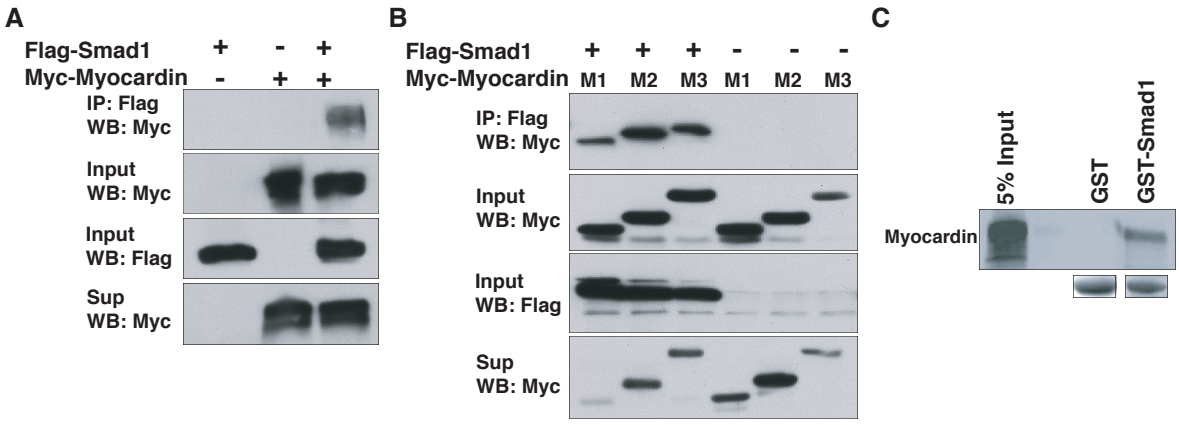

Figure 2.4. Myocardin and Smad1 interact directly. (A) Myocardin immunoprecipitates with Smad1. COS7 cells were transfected with plasmids encoding Flag-tagged Smad1 and/or Myc-tagged myocardin as indicated. Smad1 was immunoprecipitated by anti-Flag antibodies, and anti-Myc antibodies were used to detect the presence of myocardin in the immunoprecipitates by Western blot (WB) analysis (top panel). One-fifteenth of cell extracts were directly immunoblotted to detect the presence of myocardin and Smad1 proteins (middle panels). One-fifteenth of superants (after immunoprecipitation) were immunoblotted to detect myocardin proteins (bottom panel). (B) Myc-tagged myocardin aa 1-274 (M1), aa 1-351 (M2), and aa 1-421 (M3) were detected in Flag-Smad1 immunoprecipitates (top panel). Ten percent of cell extracts were directly immunoblotted to detect the presence of truncated myocardin proteins or Smad1 (middle panels). One-fifteenth of supernatants (after immunoprecipitation) were immunoblotted to detect truncated myocardin proteins (bottom panel). (C) Myocardin specifically interacted with GST-Smad1, but not with GST alone. Coomassie stained proteins corresponding to the amount of GST and GST-Smad1 protein used in the pulldown assay are shown below the autoradiograph and 5% of the input protein is shown left.

Figure 2.5

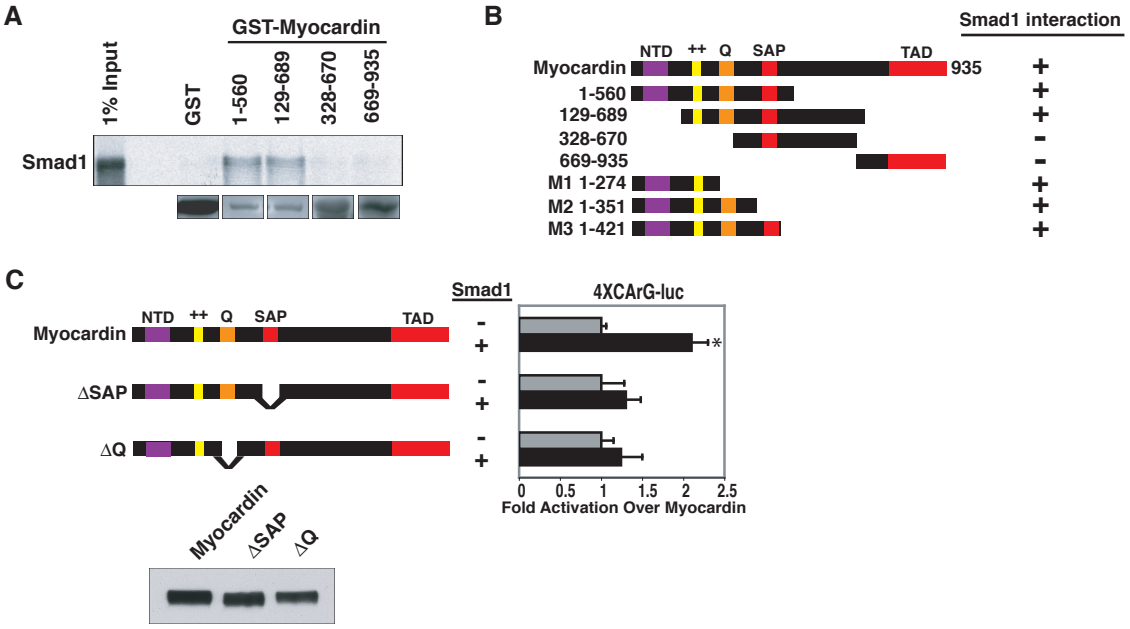

Figure 2.5. Myocardin and Smad1 interaction is required for transactivity. (A) Smad1 interacts with GST fused to myocardin aa 1-560 and aa 129-689, but not with aa 328-670 nor aa 669-935, nor with GST alone. Coomassie stained proteins corresponding to the amounts of GST and GST-myocardin protein used in the pulldown assay are shown directly below the autoradiograph and 1% of the input protein is shown left. (B) Myocardin and Smad1 interaction summary. Myocardin domains abbreviated as follows: NTD, amino-terminal domain; ++, basic domain; Q, a stretch of glutamine residues; SAP, SAF A/B, Acinus, PIAS domain; TAD, transactivation domain. (C) A luciferase reporter controlled by four copies of a consensus CArG box and expression plasmids for myocardin, myocardin ΔSAP domain, or myocardin ΔQ domain, and/or Smad1 were transfected into COS7 cells. Values are the luciferase activity by myocardin or myocardin mutants, and Smad1 (black bars) relative to the activation of reporter by myocardin alone or myocardin mutants (gray bars). Error bars represent standard deviation of at least two experiments. Student *t* test, $P < 0.05$: *myocardin alone vs myocardin plus Smad1. Myc-tagged myocardin, myocardinΔSAP, and myocardinΔQ protein expression are shown by Western blot.

Figure 2.6

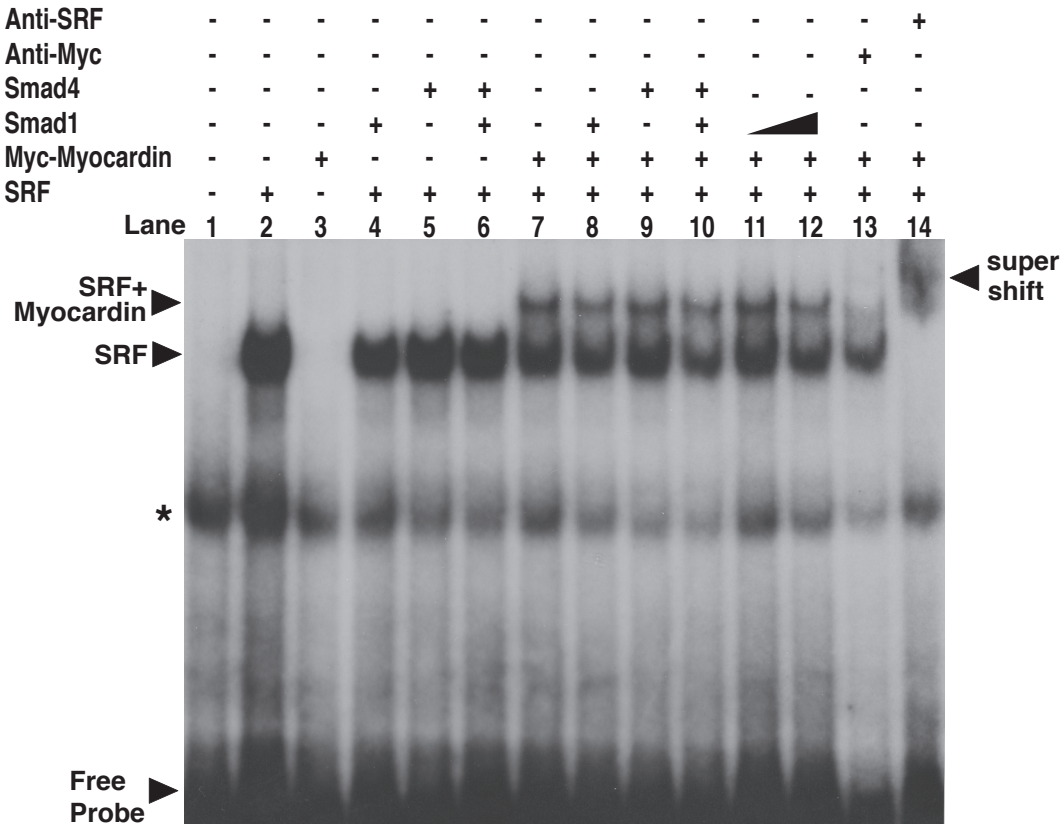

Figure 2.6. Smad proteins did not affect the formation of the myocardin/SRF/CArG complex. SRF, Myc-tagged myocardin, and Flag-tagged Smad1 and Smad4 proteins were *in vitro* translated and incubated with radiolabeled CArG probe, as described in Materials and Methods. Protein-DNA complexes were separated by non-denaturing PAGE and analyzed by autoradiography. Anti-SRF and anti-Myc antibodies were indicated. Asterisk denotes non-specific band.

Figure 2.7

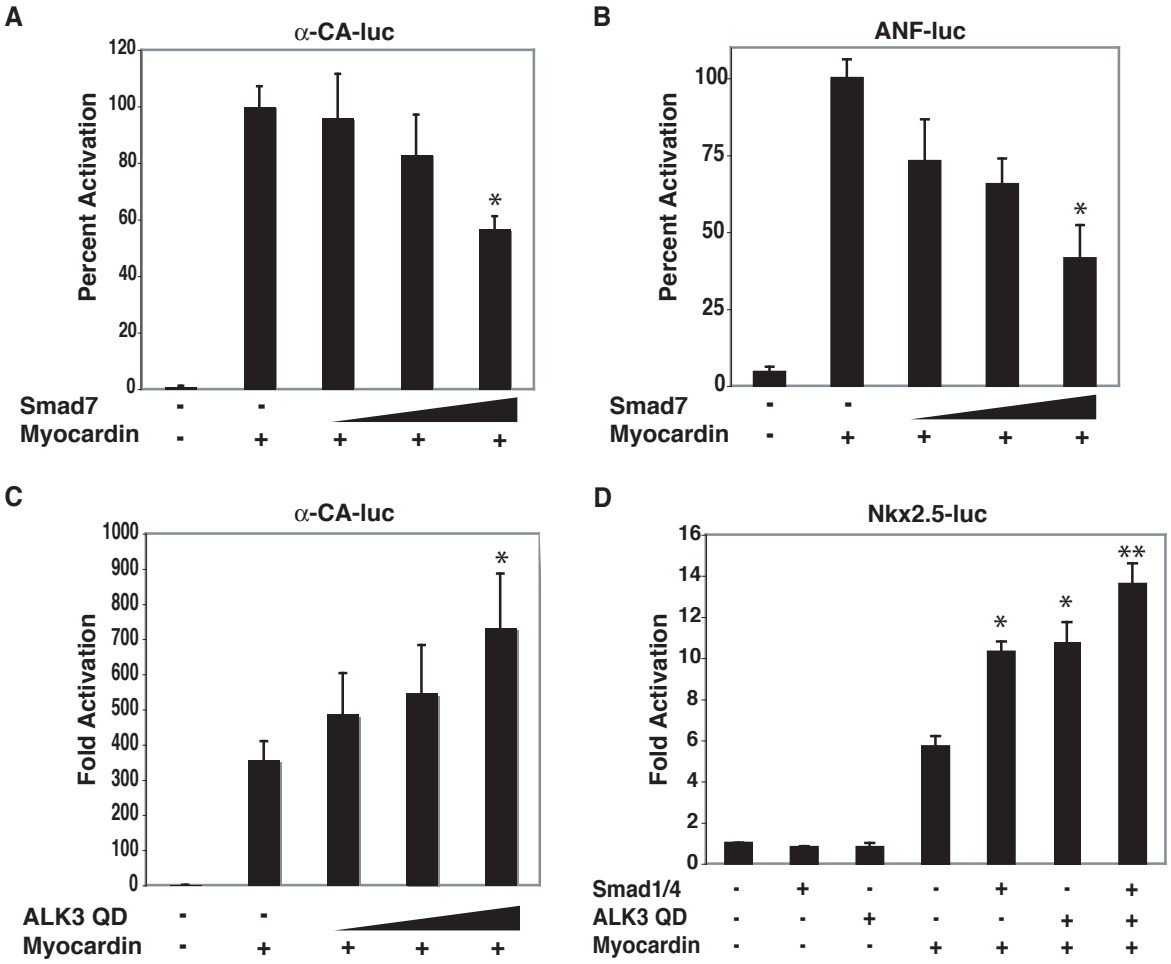

Figure 2.7. Myocardin activity is modulated by BMP signaling. Myocardin and/or Smad7 (100, 150, and 200 ng) expression plasmids and (A) α-*CA* or (B) *ANF* luciferase reporter were transfected into COS7 cells. Values are the percent luciferase activity relative to activation of the reporter by myocardin alone. (C) The α-*CA* or *Nkx2.5* luciferase reporter, Smads 1 and 4, ALK3 QD, and/or myocardin expression plasmids were transfected into COS7 cells. Values are the fold-increase in luciferase activity relative to activation of the reporter alone. Error bars represent standard deviation of at least two experiments. Student *t* test, $P < 0.05$: *myocardin alone vs myocardin plus Smad7, Smad1/4, or ALK3 QD; **myocardin alone vs myocardin plus Smad1/4 and ALK3 QD.

Figure 2.8

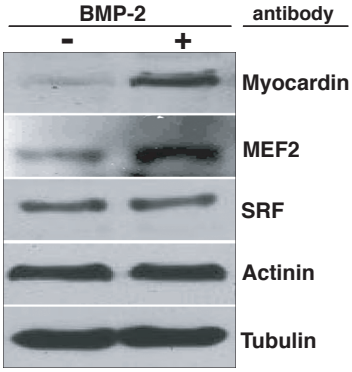

Figure 2.8. Myocardin protein level is increased in BMP-2 treated cardiomyocytes. Rat neonatal cardiomyocytes were treated with 20 ng/ml BMP-2 (or without in negative control) for 48 hours prior to harvesting and lysate production for SDS-PAGE and Western Blot analysis with indicated antibodies, as described in Materials and Methods.

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CHAPTER 3

MICRORNAS: A NEW PARADIGM FOR THE REGULATION OF CARDIAC GENE EXPRESSION

Introduction

MicroRNAs (miRNAs) are an evolutionarily conserved class of small regulatory RNAs that have recently gained status as important regulators in cardiac developmental and pathology processes. They are genomically encoded and are initially transcribed as part of much longer molecules that become processed into a mature ~22 nucleotide-long form. MiRNAs are generally regarded as negative regulators of gene expression that inhibit translation and/or promoting mRNA degradation by base pairing to complementary sequences within protein-coding messenger RNA (mRNA) transcripts (1-3). Although not yet a well-established phenomenon, some evidence suggest miRNAs may function to enhance translation under particular circumstances (4). Hundreds of human miRNA genes have been identified and bioinformatic analyses indicate that miRNAs may the regulate expression of more than one-third of human protein-coding genes (5), highlighting the potential magnitude of their influence upon gene expression.

Heart development and pathology are intimately linked to the regulation of complex genetic pathways, and much effort has been expended in attempts to understand the molecular mechanisms underlying these pathways with the ultimate goal of improving the prognosis of heart patients (6). Much of our current understanding of how cardiac gene expression is controlled is at the level of transcriptional regulation, in which transcription factors associate with their regulatory DNA elements (enhancer/promoter sequences) to activate gene expression (7). The regulation of cardiac gene expression is complex, with individual cardiac genes being controlled by multiple independent enhancers that direct very

restricted expression patterns in the heart. MiRNAs have reshaped our view of how cardiac gene expression is regulated by increasing this complexity even further by adding another layer of regulation at the post-transcriptional level. Here, we review recent progress in understanding the role of miRNAs in heart development and disease.

The global role of miRNA function in the heart has been addressed by conditionally inhibiting miRNA maturation in the murine heart, and has revealed that miRNAs play an essential role during development (8, 9). miRNA expression profiling studies demonstrate that the expression of specific miRNAs changes in diseased human hearts, pointing to their involvement in cardiomyopathies (10, 11). Furthermore, studies on specific miRNAs in animal models have identified distinct roles for miRNAs both during heart development and under pathological conditions, including the regulation of key factors important for cardiogenesis, the hypertrophic growth response, and cardiac conductance (9, 12-16). Cumulatively, these findings clearly indicate that miRNAs are important regulators of gene expression in heart development, function and pathology. These understudied and previously unknown relationships between miRNAs and heart biology also suggest the potential for miRNAs as diagnostic markers and therapeutic targets in human cardiovascular disease.

Biology of microRNAs

A little over ten years ago, the *lin-4* gene, which controls the timing of *C. elegans* larval development, was discovered to unexpectedly produce a 21-nucleotide long

noncoding RNA that suppressed *lin-14* protein expression without noticeably affecting *lin-14* mRNA levels. This small RNA was found to base pair to complementary sites in the 3' untranslated region (UTR) of *lin-14* mRNA and negatively affect its translation (17, 18). Although this phenomenon was initially treated as a genetic oddity and virtually ignored for nearly a decade, we now recognize that thousands of these small RNAs, now called miRNAs, similar to lin-4 exist in the genomes of divergent species and post-transcriptionally regulate gene expression.

miRNAs are part of the RNA interference (RNAi) pathway, the general term for RNA-guided regulation of gene expression that is conserved in most eukaryotes (19). Another class of non-coding RNAs, known as small interfering RNAs (siRNAs) also shares common downstream components of the RNAi pathway with miRNAs. Although mature miRNAs and siRNAs are structurally similar and both negatively regulate gene expression, their origins and upstream processing pathways differ significantly: miRNAs are genomically encoded whereas siRNAs arise from foreign dsRNA, and miRNAs undergo more extensive post-transcriptional processing than siRNAs (20, 21). The RNAi pathway is thought to have first evolved using siRNAs as a form of innate immunity against viruses and later endogenously encoded miRNAs were selected as beneficial post-transcriptional regulators of gene expression. The discovery of RNAi and miRNAs offers a new paradigm for understanding the control of gene expression during development and disease. Indeed, miRNAs are now recognized to regulate gene expression in a variety of fundamental biological

processes including cell proliferation, differentiation, apoptosis, tumorigenesis, and recently have been linked to cardiac hypertrophy and disease (22-24).

miRNA biogenesis and mechanism of miRNA function

miRNAs arise endogenously from independent transcriptional units or from within the introns of messenger RNA (mRNA) transcripts (**Fig. 3.1**) (25). miRNAs are initially part of immature primary transcripts that undergo extensive post-transcriptional processing to yield mature miRNAs, whose lengths are approximately 18 to 24 nucleotides. The lengths of the primary transcripts range from several hundred to several thousand nucleotides and may harbor a single miRNA or sometimes several (26). Mature miRNAs become part of the RNA-induced silencing complex (RISC) that facilitates miRNA-mediated regulation of gene expression through complementary base-pairing between a miRNA and sequence(s) within the 3' untranslated region (UTR) of targeted mRNAs (27, 28). The majority of animal miRNAs base pair imperfectly to their targeted mRNAs, which generally results in suppression of translation (25). The mechanism underlying this suppression is thought to occur at the initiation step of translation, where the RISC component Ago2 precludes binding of eIF4E, an essential translation factor, to the 7-methylguanosine cap of a targeted mRNA (1). Interestingly, miRNAs have also been shown to affect stability of targeted mRNAs and mediate their degradation (2). Furthering our understanding of gene expression regulation by miRNAs has been their connection to discrete cytoplasmic foci called processing bodies (P-bodies), which are sites of programmed mRNA degradation (29-33). Components of RISC,

miRNAs and their targeted mRNAs have been shown to co-localize to P-bodies, suggesting that miRNAs may mediate both translation suppression and mRNA degradation by directing targeted mRNAs to P-bodies. Moreover, release of mRNAs targeted by miRNAs from P-bodies and subsequent re-expression of those mRNAs indicate that P-bodies may also function as mRNA storage centers (34). Major advances have been made towards understanding the mechanisms underlying the RNAi phenomenon; nevertheless many aspects of miRNA biogenesis, trafficking of RNAi machinery, RISC assembly, and the mechanisms underlying RISC function await clarification.

Identification and expression of miRNAs

A variety of experimental approaches have been used to identify miRNAs and study their expression patterns. The cloning and sequencing of small RNAs from size fractioned RNA samples has uncovered many miRNAs that are tissue-specifically expressed (35, 36). Complementing small RNA cloning approaches, bioinformatics screens that searched genomic databases for the characteristic stem-loop structures of precursor miRNAs have predicted the existence of hundreds of mammalian miRNAs (37, 38). Other techniques, such as northern blotting, real-time RT-PCR, *in situ* hybridization, and repressible *in vivo* reporter transgenes have been adapted to verify such predictions and study the expression patterns of specific miRNAs (39-41). Recently, a comprehensive sequencing of over 250 small RNA libraries revealed additional new miRNAs and documented the expression patterns of most miRNAs (42).

To facilitate the analysis of global miRNA expression, microarray technology has been implemented with great success to quickly analyze the expression of hundreds of miRNA genes simultaneously (11, 43-46). These types of studies have shown that miRNA expression, like that of protein-coding genes, is highly regulated according to the cell's developmental lineage and stage: whereas some miRNAs are ubiquitously expressed, others are expressed in a cell- and tissue-specific manner (47), implying that miRNAs may participate in a variety of biological processes.

Prediction and validation of miRNA regulatory targets

Identifying the targets of specific miRNAs will be the key to our understanding the precise roles of miRNAs. Most animal miRNAs are imperfectly complementary to their target site, which thwarts using simple homology searches to identify animal miRNA target sites. To overcome this obstacle, several computational methods have been developed that incorporate sequence conservation and characteristics of known miRNA targets as criteria to predict new animal miRNA targets (12, 48-53). A major determinant for miRNA targeting is the perfect or near perfect complementary base pairing between the second and eighth nucleotides of the miRNA with its mRNA target site, known as the "seed" region. Other factors also deemed important for miRNA targeting include additional base pairing in the 3' portion of the miRNA and the degree of local AU nucleotide content flanking the target site (12, 53). The positive influence of increased AU content is attributed to a weaker secondary structure in the vicinity of the target site thus offering increased accessibility to RISC (12, 53). Computational approaches taking these determinants into account, as well

as sequence conservation of the target sites, have successfully predicted mammalian miRNA target sites, albeit the set of predictions produced for any particular miRNA almost certainly contains many false positives. Any prediction must be verified experimentally and, most importantly, placed into a relevant biological context before being considered a valid target. Given the vast number of known miRNAs and their potentially thousands of regulatory targets, it is hoped that a direct and facile method to identify miRNA target genes, possibly employing a proteomics-based strategy or from functional screening of cDNA libraries composed of 3' UTRs of regulatory target genes, will become available.

Regulation of miRNA expression in skeletal and cardiac muscle tissues

Several microRNA genes are specifically expressed or highly enriched in skeletal and/or cardiac muscle. The expression of muscle-specific miRNAs miR-1, miR-133, miR-206, and miR-208a, appears largely regulated by well-established and evolutionarily conserved muscle transcriptional networks involving SRF, MyoD, Twist, MEF2, and myocardin (12, 46, 54, 55). For example, miR-1 was highly conserved during evolution and, in addition to mouse and human, it is found in the genomes of organisms as diverse as worm, fly, zebrafish, and chicken. The pathways controlling miR-1 expression also appear highly conserved: *Drosophila* miR-1 expression in the presumptive and early mesoderm occurs downstream of Twist and MEF2, two transcription factors that are major regulators of mammalian muscle development (12, 55). In vertebrates, there are two polycistronic genes that encode miR-1 along with miR-133 (46). Accordingly, the expression of miR-1 and

miR-133 mirror one another in skeletal and cardiac muscle, where they are solely expressed. Their muscle-specific expression pattern is explained by promoter analyses demonstrating that both miR-1/miR-133 loci have upstream enhancers with SRF binding sites, and that myocardin activity increases the expression of those promoters in the heart, whereas as their skeletal muscle expression is controlled by MyoD (12, 46). Similarly, MyoD, a transcription factor sufficient to activate the program of skeletal muscle differentiation, stimulates the skeletal muscle-specific expression of miR-206 (56).

In contrast to miR-1, miR-133, and miR-206, which are expressed as independent transcriptional units, miR-208a is encoded by an intron of its host gene alpha myosin heavy chain (αMHC) (16). More than 127 human miRNAs have been identified within the introns of protein-coding genes, and findings support the idea that these intronic miRNAs are generally co-expressed with their host genes (16, 26, 43, 57). In agreement, both miR-208a and αMHC are heart-specific and concurrently expressed during development, suggesting that their expression is controlled by a common regulatory element. The promoter region of the αMHC gene contains several binding elements important for muscle-specific gene expression, such GATA4 and MEF2 site, and thyroid hormone signaling is also known to play an important role in controlling αMHC expression. Collectively, these studies indicate that muscle miRNA expression is under tight spatial and temporal regulation by transcriptional networks important for muscle gene expression.

MicroRNAs are required for normal heart development and function

Dicer is an endonuclease in the miRNA biogenesis pathway that is required to fully process miRNAs to their mature, active form (**Fig. 3.1**). One approach taken to understand the importance of miRNAs during development has been to disrupt Dicer function in mice and zebrafish, thus effectively removing all mature functional miRNAs (58-60). Dicer deletion in mice caused arrested development during gastrulation before the body plan was fully configured, suggesting that miRNA function is critical for early development (58). Similarly, creation of Dicer zebrafish mutants resulted in abnormal morphogenesis during gastrulation with somitogenesis and heart development both proving abnormal (59, 60). These genetic studies have provided convincing evidence that miRNAs are required for animal development.

To better understand the role of miRNAs in specific tissues, studies that conditionally deleted Dicer from the mouse genome using the Cre-LoxP system have further supported a crucial role for miRNAs in development (8, 9, 61-64). Cardiac-specific deletion of Dicer, using Cre-recombinase controlled by the αMHC promoter, did not affect specification or patterning of the mouse heart (8). However, the hearts of those mice exhibited aberrant cardiac contractile protein expression and profound sarcomere disarray coupled with significantly reduced cardiac function, and rapidly progressed to dilated cardiomyopathy, heart failure, and post-natal lethality (8). The cardiac phenotype associated with the Dicer mutant mice resembles the human clinical features of dilated cardiomyopathy and heart failure. Intriguingly, low levels of Dicer protein have been reported in human failing hearts,

suggesting the involvement of miRNAs in dilated cardiomyopathies and heart failure in human patients (8).

In contrast, the use of Cre-recombinase controlled by the Nkx2.5 promoter, which is expressed in the developing mouse heart, to delete Dicer instead led to embryonic lethality with defects in heart morphogenesis (9). The embryonic versus postnatal lethality observed in those studies likely reflects differences in the spatial-temporal expression patterns of the Nkx2.5-Cre and αMHC-Cre transgenes within the mouse heart. Aberrant tissue morphogenesis has also been observed in Dicer-deficient skin (61), skeletal muscle (62), limb (63), and lung (64). On a cautionary note, the interpretation that a global loss of miRNAs is solely responsible for the observed Dicer deletion phenotypes hinges upon whether or not Dicer serves any critical roles outside of miRNA biogenesis.

Genetic studies of specific microRNAs reveal distinct roles in the developmental heart

The conditional deletion of Dicer from the heart presumably down-regulated all cardiac-expressed miRNAs. In order to understand the contribution of specific miRNAs in cardiac development several groups have undertaken gain- and loss-of-function studies on individual miRNAs (9, 12, 16, 46). The outcomes of those studies clearly indicate that single miRNAs are capable of playing crucial and specific roles in both cardiac development and function (**Fig. 3.2**).

miR-1 and miR-133 are highly conserved and found expressed in the musculature of flies, mice, and humans. miR-1 and miR-133 are produced from the

same polycistronic transcripts, which are encoded by two separate genes in the genomes of both mouse and human; e.g. miR-1-1 and miR-133a-2 are clustered on mouse chromosome 2, while miR-1-2 and miR-133a-1 are clustered on mouse chromosome 18 (46). Well-known muscle transcriptional networks consisting of SRF/myocardin for cardiac muscle expression and MyoD/MEF2 for skeletal muscle expression have been demonstrated to regulate the expression of these muscle-specific miRNA genes (12, 46, 54).

In the developing mouse heart, overexpression of miR-1 caused defective ventricular myocyte proliferation (12), while introduction of miR-1 into developing *Xenopus* embryos also interfered with heart development (46). The reported phenotypes of other transgenic mice that overexpress miRNAs specifically in the heart range from benign to castastrophic: miR-214 caused no apparent cardiac defects, miR-195 induced hypertrophic growth in the adult heart, while miR-24 overexpression resulted in embryonic lethality (11).

Complementing the overexpression studies of miR-1, Zhao and colleagues targeted the mouse miR-1-2 gene for deletion, one of the two miR-1 genes expressed in skeletal and cardiac muscle (9). Although separate genes encode miR-1-1 and miR-1-2, they are identical in sequence and thus appear to target the same mRNAs. However some questions seem to remain regarding the temporal and spatial expression patterns, which appear largely overlapping (9, 12, 40, 46). The authors report that approximately half of miR-1-2 null animals die by weaning age and some suffer from incomplete ventricular septation, indicative of abnormal cardiac morphogenesis. Analysis of miR-1-2 null animals *in utero* found pericardial

edema, consistent with embryonic myocardial dysfunction. The miR-1-2 null animal phenotype suggests that miR-1-2 plays non-redundant roles with miR-1-1 in the heart despite their apparent overlapping expression patterns. Previously, miR-1 was found to promote skeletal muscle myogenesis (46), however loss of miR-1-2 did not appear to affect skeletal muscle development by gross morphological analysis. Potentially, the different requirement for miR-1 in cardiac versus skeletal muscle development might reflect a difference in the tissue-specific genes that are targeted. It would certainly be exciting to know whether deletion of miR-1-1 invokes a similar phenotype as miR-1-2 deletion, and whether deletion of both miR-1 genes causes a more severe cardiac phenotype and/or affects skeletal muscle development.

Identifying the targets of specific miRNAs is a prerequisite for understanding the precise molecular mechanisms underlying their function. Most animal miRNAs are partially complementary to their target sites, which thwart simple homology searches to identify target sequences. In response, several bioinformatic prediction algorithms that weigh various criteria, including sequence conservation and thermal stability, were developed and are proving an indispensable guide for advancing miRNA research (5, 49, 65). However, these *in silico* predictions require experimental testing and to date only a handful of miRNA targets with roles in the heart have been validated in biological systems (**Table 3.1**). One such validated target of miR-1 in the heart is Hand2, an important cardiac transcription factor whose genetic ablation in the mouse produced a similar failure in ventricular myocyte as miR-1 overexpression in the developing mouse heart (66). Accordingly, miR-1 overexpression reduced Hand2 protein levels, while Hand2 was conversely up-

regulated in the miR-1-2 null animals (9, 12). Although the targeting of Hand2 partially explains the phenotypes observed in the gain- and loss-of-function miR-1 animal studies, like most miRNAs, miR-1 has been predicted to target hundreds of genes. Future studies aimed at determining physiologically relevant targets directly regulated by miRNAs are clearly needed.

MicroRNA expression during cardiac remodeling

The heart is very sensitive to many stimuli and stresses, and even slight perturbations during cardiogenesis or in the adult heart can result in catastrophic consequences. The major response of the heart to biomechanical stress and pathological stimuli is to undergo extensive cardiac remodeling known as hypertrophic growth (67). Cardiac hypertrophy is defined by an increase in myocyte size and/or myofibrillar volume without a change in myocyte number and helps to sustain cardiac output in the face of such stress. Cardiac hypertrophy is also accompanied by re-activation of fetal cardiac genes normally expressed in the heart before birth. The reactivation of cardiac fetal genes in post-natal cardiomyocytes suggests the molecular events that control cardiac gene expression during development are redeployed to regulate hypertrophic growth or heart regeneration (68). Although hypertrophy induced by pathological stimuli is an adaptive mechanism that is beneficial in the short term, prolonged hypertrophy has adverse consequences associated with heart failure and sudden death (69).

Several groups have implemented microarray technology to analyze the expression of hundreds of miRNA genes simultaneously (10, 11, 45, 70). Studies

profiling miRNA expression using mice with thoracic aortic-banded hearts or with constitutively activated calcineurin signaling, two models of pathological cardiac hypertrophy, demonstrate that the expression of miRNAs are both up and down regulated during cardiac hypertrophy (11, 45, 70). Profiling studies in human samples reveal that changes in miRNA expression also occurs in human failing hearts, including the up-regulation of miRNAs normally expressed in the developing heart (10, 11). Furthermore, functional analyses using both gain- and loss-of-function approaches in mice have begun to establish a correlation between miRNAs and cardiac hypertrophy by demonstrating that stress-regulated miRNAs can both positively and negatively influence the cardiac hypertrophic growth response (11, 15, 16).

MicroRNAs modulate cardiac hypertrophy

miR-195 is up-regulated during cardiac hypertrophy in both human and mouse hypertrophic hearts and was found sufficient to induce hypertrophic growth in cultured rat cardiomyocytes (11). Furthermore, overexpression of miR-195 in mouse hearts induced hypertrophy within several weeks after birth. Continued miR-195 overexpression led to dilated cardiomyopathy and heart failure in young mice (11). The mechanisms underlying miR-195 is not yet clear as no target genes have yet been verified. miR-214 is also up-regulated during hypertrophy, however transgenic mice overexpressing miR-214 caused no abnormal phenotype in the heart (11). These studies indicate that some miRNAs, but not others, are sufficient to induce cardiac hypertrophy. Clearly, future loss-of-function studies to determine if these

miRNAs are necessary for the hypertrophic response, as well identification of their target genes, is worthy of pursuit.

Unlike miR-195 and miR-214, miR-1 and miR-133 are down-regulated during hypertrophy (11, 15, 71). Their matching expression patterns are not surprising since miR-1 and miR-133 are both transcribed together from the same polycistronic genes. Overexpression of miR-1 or miR-133 inhibited hypertrophic growth in an *in vitro* model of cardiac hypertrophy using primary cardiomyocytes (15, 71). Conversely, prolonged inhibition of miR-133 *in vivo* using chemically-modified oligonucleotides antisense to miR-133, delivered by an osmotic minipump implanted into the mouse heart, was sufficient to cause a marked hypertrophic response (15). While miR-1 expression is down-regulated during cardiac hypertrophy (11, 15, 71), additional genetic studies are needed to clearly demonstrate a direct role for miR-1 in the regulation of cardiac hypertrophy. Both miR-1 and miR-133 are proposed to regulate expression of growth-related genes (12, 15, 71), suggesting that these miRNAs may act as growth suppressors that are relieved during cardiac hypertrophy. Intriguingly, a recent report suggested that miR-1 and miR-133 may also play a distinct role in the regulation of cardiomyocyte apoptosis: while miR-1 seems to be pro-apoptotic, miR-133 appears anti-apoptotic (72). Clearly, understanding how miRNAs and their regulatory targets integrate into relevant genetic pathways is the crux for future studies.

miR-208a is expressed specifically in the heart and was recently deleted from the mouse genome by van Rooij and colleagues (16). miR-208a null animals were viable and appeared normal without any apparent gross developmental defects.

However, the miR-208a null animals exhibited a slight reduction in contractility at two months of age, and a continued reduction in cardiac function in later life. Although miR-208a does not appear to be necessary for cardiogenesis, a requirement for miR-208a in the cardiac hypertrophic growth response was identified. The loss of miR-208a protects mice against cardiac hypertrophy and up-regulation of βMHC induced by hypothyroidism, activated calcineurin signaling and cardiac pressure-overload induced stress (16). Those results suggest that the genetic pathways coordinating cardiac hypertrophy share a common component regulated by miR-208a. One such candidate proposed is thyroid hormone receptor associated protein 1 (Thrap1), a co-factor of the thyroid hormone nuclear receptor, which can positively and negatively influence transcription. The 3' untranslated region (3'UTR) of Thrap1 is directly targeted by miR-208a and Thrap1 protein levels were found elevated in miR-208a null hearts, suggesting that miR-208a may function, at least in part, by regulating the expression of a thyroid hormone signaling pathway component (16). Those observations linked miRNA function to classical hormone-regulated muscle physiology and are likely to bring about a renaissance in this important research field.

miR-21, a miRNA implicated in tumor-related cell growth and apoptosis (73-75), is consistently reported up-regulated in response to agonist-induced cardiac hypertrophy in cell culture experiments and in pressure-overload induced hypertrophy *in vivo* (11, 45, 70, 71). However, the exact nature of miR-21 function remains unclear. Inhibition of miR-21 using antisense oligonucletides was reported to suppress agonist-induced hypertrophic growth in primary cardiomyocytes (70).

Whereas inhibition of miR-21 using locked nucleic acid-modified miR-21 antisense oligonucleotides stimulated hypertrophic growth *in vitro* (45). While the basis for these differences is unclear, it is interesting to note that other studies on miR-21 function also appear contradictory: miR-21 was reported to stimulate cell growth (74), while also reported to activate apoptosis and inhibit cell proliferation (73, 75). Clearly, further genetic studies and delineation of the molecular pathways modulated by miR-21 in different biological systems are needed to better understand the biological function of this miRNA.

Collectively, emerging evidence has established miRNAs, in particular miR-1, miR-21, miR-133, miR-195, and miR-208a, as newly identified players in animal models of cardiac hypertrophy. The establishment of the hypertrophic miRNA signature has yielded many hitherto unrecognized candidate genes involved in cardiac hypertrophy that await further scrutiny. Given the complexity of the cardiac remodeling occurring during hypertrophy, the identification of specific targets for miRNAs involved in the hypertrophic response will provide insight into the molecular mechanisms underlying this disease process.

MicroRNAs regulate cardiac conduction system components

The electrical conduction system, which is required to maintain proper heart rhythmicity, is composed of specialized muscle cells and distinct sets of ion channels. Functional defects in the conduction system can result in arrhythmias, which may occur from congenital disorders and often accompany heart disease. The consequences of arrhythmias vary from silent defects to sudden and unexpected

death. Interestingly, recent studies have pointed to two miRNAs, miR-1 and miR-133, which have been implicated in cardiac development, muscle proliferation and differentiation, as regulating components of the cardiac conduction system and having the potential to induce arrhythmias (13, 14).

Interestingly, miR-1 levels are elevated in human hearts with coronary artery disease and infarcted rat hearts (14). Further investigation revealed that overexpression of miR-1 in both normal and infarcted rat hearts slowed cardiac conduction and lead to arrhythmias. Those effects appear to be mediated, at least in part, through post-transcriptional repression of potassium channel subunit KCNJ2 and gap junction protein connexin 43 (14). Conversely, blocking miR-1 function by releasing chemically-modified oligonucleotides antisense to miR-1 in infarcted rat hearts inhibited arrhythmogenesis (14). The homeodomain transcription factor Irx5, which regulates cardiac repolarization by repressing potassium channel KCND2, has also been identified as a direct miR-1 target (9), further supporting a role for miR-1 in cardiac conduction.

Similar to miR-1, miR-133 is down-regulated in failing human hearts as well as in several animal models of cardiac hypertrophy (11, 15, 71), however miR-133 was found elevated in a rabbit model of diabetes (13). The elevated miR-133 levels occurred concurrently with lowered protein levels, but without reduction in mRNA levels, of ether-a-go-go (ERG), a cardiac potassium ion channel important for myocyte repolarization and associated with congenital arrhythmias. A target site partially complementary to miR-133 was identified within the 3'UTR of ERG, indicating that miR-133 may directly regulate ERG expression. In support,

introduction of miR-133 into isolated cardiomyocytes reduced ERG expression post-transcriptionally and accordingly delayed myocyte repolarization. Collectively, an emerging portrait has come to the central stage where muscle miRNAs are playing a much larger and broader role in the regulation of the cardiovascular system, including cellular proliferation and differentiation, apoptosis, cardiomyocyte hypertrophy and cardiac conduction.

MicroRNAs as novel heart disease genes

Congenital heart disease affects nearly 1% of all newborns and is responsible for more deaths in the first year of life than any other birth defect (76). Over the past decade, clinical studies have identified a number of congenital heart diseases associated with mutations in specific genes, with the majority those reported mutations affecting cardiac transcription factors and structural proteins (77, 78). Given the increasingly important roles being identified for miRNAs in heart development and function, we speculate that mutations in miRNA genes or their targeted sequences will be correlated to congenital heart disease in humans. A proof-of-principle lies in the identification of a single nucleotide polymorphism that affected the 3'UTR of the myostatin transcript in Texel sheep, which are known for their exceptional muscularity (79). Myostatin is a well-known repressor of skeletal muscle growth and mutant alleles of myostatin are associated with abnormally large skeletal muscles in animals and humans (80, 81). This particular single nucleotide polymorphism in Texel sheep myostatin created an aberrant miR-1 target site, so that the highly expressed and muscle-specific miR-1 repressed the myostatin

expression at the translational level (79). While not directly related to heart disease, this evidence provides convincing evidence that single nucleotide polymorphisms affecting miRNA function could act as causative factors for human heart disease. The movement towards next-generation high-throughput sequencing technologies that will enable scientists to rapidly sequence entire genomes may identify allelic mutations in miRNA genes and/or their target sites associated with human disease (82).

microRNAs as novel therapeutic targets

Given their profound role in the cardiovascular system, the question is whether miRNAs are good targets for therapeutic applications. In fact, several properties of miRNAs could make them clinically relevant: Firstly, miRNA expression changes have been documented in diseased hearts, making miRNAs likely biomarkers or diagnosis indicators for cardiovascular disease. Secondly, miRNAs are small molecules, making their *in vivo* delivery feasible (83, 84). Thirdly, single miRNAs are predicted to have multiple mRNA targets (many into the hundreds) and most importantly, some of those miRNA regulatory targets seem to work in a concert to control a common pathway and/or biological function. This will make miRNAs much more efficient tools to target a disease pathway/process. Yet this feature of miRNAs could be a two-edged sword that brings about “off-target” side effects. For example, miR-133 is thought to repress cardiac hypertrophy, raising the possibility for a therapeutic application where synthetic miR-133 molecules are introduced into patients to control pathological hypertrophy (15). However, the overexpression of

miR-133 has been shown to induce arrhythmias (13). Clearly, caution and future studies directed at understanding the pathways regulated by cardiac miRNAs are needed before clinical treatments be seriously considered.

Concluding remarks

The biology of miRNAs is a young research area and as an emerging field, there are many more questions than answers. miRNAs are now conceived as ‘tiny players with big roles’ in diverse biological processes. Within the cardiovascular research field, studies in animal models demonstrate that miRNAs are required for proper heart development and function. The involvement of miRNAs in human heart disease is evidenced by dysregulated expression of miRNAs and Dicer, a miRNA pathway component, in human failing hearts. The expression signatures of miRNAs in disease may eventually provide an additional diagnostic tool to assess heart disease. Future studies aimed at understanding how miRNAs are integrated into the complex genetic networks important for heart disease is prerequisite for their development as potential therapeutic targets. In the course of taking miRNAs to heart, we face big challenges but with the big promise that miRNAs may provide us powerful tools to battle cardiovascular disease.

Figure 3.1

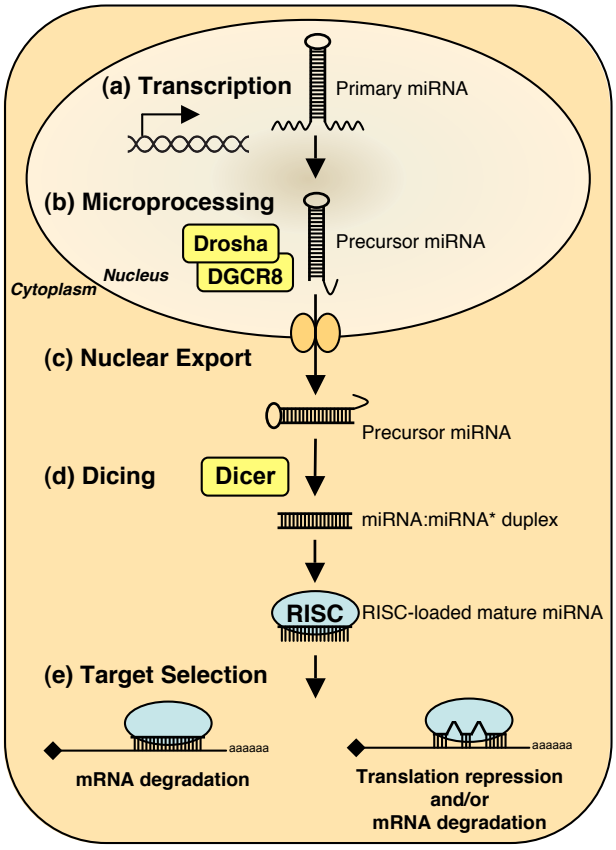

Figure 3.1. MicroRNA biogenesis and mechanism. **(a)** MiRNA biogenesis begins with the transcription of primary-miRNAs by RNA polymerase II from independent transcriptional units with lengths ranging from several hundred to several thousand nucleotides that may encode a single miRNA or sometimes two or more miRNAs. In addition to independent transcriptional units, some miRNAs originate from within the introns of mRNA transcripts. **(b)** Primary-miRNAs enter the miRNA-processing pathway and undergo nuclear cleavage by the Microprocessor complex in which RNase III endonucleases Drosha and DGCR8 produce an approximately 70-nucleotide long intermediate precursor-miRNA whose hallmarks are a stem-loop-like structure and a staggered cut at the stem-loop base. **(c)** Exportin-5 recognizes the staggered cut and exports the precursor-miRNA to the cytoplasm. **(d)** Once cytoplasmic, Dicer, another RNAase III endonuclease, cleaves both stem arms of the precursor-miRNA and generates a miRNA duplex. A single stem arm of the resulting ~22-nt duplex is selectively incorporated into the RNA-induced silencing complex (RISC), while the other stem arm is presumably degraded. **(e)** Regulation of target gene expression by a miRNA-loaded RISC is facilitated by miRNA complementary base pairing to target sequence(s) within the 3' UTR of target mRNAs. Generally in animals perfect or near-perfect complementary base pairing between RISC-bound miRNA and targeted mRNA results in immediate mRNA cleavage. However the vast majority of animal miRNAs are imperfectly complementary to their targeted mRNAs, which has been shown to suppress translation as well as affect stability of targeted mRNAs and mediate their degradation.

Figure 3.2

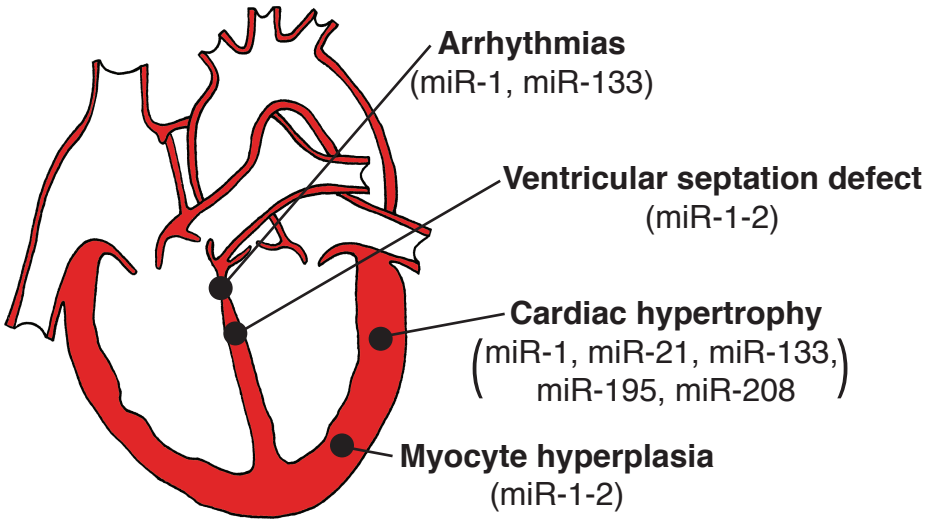

Figure 3.2. Known roles of miRNAs in heart development and function. Recent studies have demonstrated an association of several miRNAs with various cardiac defects. miR-1 contributes to numerous cardiac abnormalities, including arrhythmias, defective ventricular septation, cardiac hypertrophy and myocyte hyperplasia, while miR-133 was shown to have arrhythmogenic potential and play a role in cardiac hypertrophy. Additionally miR-21, miR-195, and miR-208 are indicated in the control cardiac hypertrophy. It is expected that more miRNAs will be added to this growing list.

Table 3.1. Experimentally validated targets of cardiac -expressed microRNAs.

microRNA	Expression Pattern	Validated Targets	References
miR-1	Heart, Skeletal Muscle	Cdk9, Delta, Fibronectin, GJA1, Hand2, Irx5, KCNJ2, HDAC4, HSP60, HSP70, KCNE1, nPTB, RasGAP, Rheb	(12, 24, 25, 36, 49-51)
miR-21	Heart, Spleen, Small Intestine, Colon	PTEN, TPM1	(52, 53)
miR-133	Heart, Skeletal Muscle	Caspase-9, Cdc42, ERG, KCNQ1, nPTB, RhoA, SRF, WHSC2	(13, 15, 24, 36, 51)
miR-208	Heart	Thrap1	(16)

Abbreviations: Cdc42, Cell division cycle 42; Cdk9, Cyclin-dependent kinase 9; ERG, Ether-a-go-go potassium channel; GJA1, Gap junction protein alpha 1; Hand2, Heart and neural crest derivatives expressed 2; HSP60, heat-shock protein 60; HSP70, heat-shock protein 70; HDAC4, Histone deacetylase 4; Irx5, iroquois homeobox protein; KCNE1, Potassium voltage-gated channel, Isk-related family, member 1; KCNJ2, Potassium inwardly-rectifying channel, subfamily J, member 2; KCNQ1, Potassium voltage-gated channel, KQT-like subfamily, member 1; nPTB, polypyrimidine tract-binding protein 2; PTEN, phosphatase and tensin homolog; RasGAP, Ras GTPase-activating protein; Rheb, Ras homolog enriched in brain; RhoA, Ras homolog A; SRF, Serum response factor; Su(fu), suppressor of fused; Thrap1, thyroid hormone receptor associated protein 1; TPM1, tropomyosin 1; WHSC2, Wolf-Hirschhorn syndrome candidate 2.

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CHAPTER 4

ELEVATED MICRORNA EXPRESSION PROVIDES A FEED-FORWARD MECHANISM FOR CARDIAC REMODELING

Abstract

Chronic heart disease is often associated with cardiac remodeling accompanied by maladaptive hypertrophic growth. Despite intensive investigation, the molecular mechanisms underlying hypertrophy are not well understood. Here we show that the miR-208 family, miR-208a and miR-208b, are differentially expressed in the heart, paralleling the expression of their respective host genes alpha- and beta-myosin heavy chain (αMHC and βMHC). Cardiac overexpression of miR-208a is sufficient to induce hypertrophy and βMHC/miR-208b expression, which results in pronounced repression of miR-208 regulatory targets Thyroid hormone associated protein 1 and myostatin, two negative regulators of muscle growth and hypertrophy. We further found miR-208a-induced βMHC expression is restricted to a subset of cardiomyocytes associated with fibrosis, providing an explanation why reactivation of fetal gene expression is not beneficial. Together, our studies uncover a novel miRNA-dependent feed-forward mechanism where miRNAs repress anti-hypertrophy genes and modulate hypertrophic growth during normal and pathological conditions.

Introduction

MicroRNAs (miRNAs or miRs) are a conserved class of ~22-nucleotide long noncoding RNA molecules that regulate gene expression through complementary base pairing with the 3' untranslated region (UTR) of targeted mRNAs (1). miRNAs are thought to provide a post-transcriptional layer of spatial and temporal control of developmental and homeostatic events by altering levels of critical regulators within complex genetic pathways (2-4). We and others have shown that miRNAs play important roles in cardiogenesis, electrical conduction, and stress-dependent cardiac remodeling (5-7). Recent studies describe dysregulated miRNA expression in animal models of cardiac hypertrophy and in failing human hearts (8-12), indicating that miRNAs were previously unrecognized factors in the regulation of the hypertrophic growth response.

Cardiac hypertrophy, defined broadly as heart enlargement resulting from increased cardiomyocyte size, is initially an adaptive response of the heart to increased demand for cardiac output during chronic physiological or pathological stress (13). Although initially beneficial, pathological cardiac remodeling caused by prolonged hypertrophic growth is associated with heart failure and sudden death (14). Most treatments of heart failure aim to attenuate or even reverse maladaptive cardiac remodeling to improve heart patient prognosis. A paradigm in cardiac hypertrophy is activation of a gene program including cardiac hormone atrial natriuretic factor (ANF) and re-expression of fetal cardiac beta-myosin heavy chain (βMHC) (13, 14). However, the molecular events that induce hypertrophy and

modulate hypertrophic gene expression are not well defined, nor is the significance of the changes fully understood.

Here we show that cardiac-specific miR-208a and miR-208b are developmentally and pathologically regulated. We find that adult isoform miR-208a is sufficient to induce cardiac remodeling and regulate the expression of hypertrophy pathway components, including specific up-regulation of βMHC. Conversely, genetic deletion of miR-208a resulted in decreased βMHC expression. We find that miR-208a and miR-208b share similar sequence identity and repress the same target genes involved in repressing hypertrophy. Taken together, our data suggests a feed-forward mechanism where miR-208a and miR-208b repress the expression of anti-hypertrophy genes and regulate hypertrophic growth during normal and pathological conditions.

Materials and Methods

Northern blot analysis and RT-PCR

RNA analyses by miRNA Northern blot, semi-quantitative RT-PCR, and quantitative RT-PCR were essentially performed as described (15-17).

Generation of miR-208a transgenic mice

All procedures were approved by and performed in accordance with the University of North Carolina Institutional Animal Care and Use Committee. A genomic fragment encoding the miR-208a precursor and flanking region was amplified by PCR using mouse genomic DNA as a template. This fragment was cloned into the pUHG10-3 tetracycline responsive vector plasmid at the XbaI site. A 2 kb fragment containing tetracycline-responsive element (TRE), miR-208a precursor, and SV40 poly signal was excised by XhoI/AseI digestion and purified. The TRE-miR-208a gene was injected into the pronuclei of C57/Bl6 X C3H hybrid embryos and implanted into pseudo-pregnant recipient females by the University of North Carolina Animal Models Core. Five TRE-miR-208 founders were established and crossed with C57BL/6 and expanded. Separate strains derived from two founders were maintained by mating animals heterozygous for TRE-miR-208a or αMHC-tTA that expresses heart-specific tTA controlled by the heart-specific alpha myosin heavy chain (αMHC) promoter (kindly provided by Dr. Glenn I. Fishman, New York University) (18). Single transgenic animals genotyped αMHC-tTA (referred to as 'control' throughout this study) were compared to double transgenic littermates genotyped αMHC-tTA/TRE-miR-208 (referred to as 'miR-208a Tg'). Oligonucleotide

sequences available upon request.

Generation of miR-208a null mice

The miR-208a targeting vector was generated by digesting a 4.5 kb fragment (5' arm) with NheI/XhoI and ligated upstream of a positive selection neomycin cassette flanked by loxP sites. A 1.8 kb fragment (3' arm) was digested with PmeI/NotI and ligated downstream of the neomycin cassette and upstream of a negative selection thymidine kinase cassette. Targeted ES-cells were identified by PCR and Southern blot analyses and used for blastocyst injection by the University of North Carolina Animal Models Core. The resulting chimeric mice were bred to C57BL/6 mice to obtain germline transmission of the floxed allele. Subsequently, the neomycin cassette was excised by breeding to mice that ubiquitously express Cre recombinase (19).

Pressure-overload induced hypertrophy model

Male C57BL6 mice (6–8 wk old) were subjected to pressure overload by thoracic aortic banding (TAB) (8). Mice were sacrificed after 3 weeks banding and hearts harvested for RNA extraction.

Histological analysis of miR-208a transgenic mice

Histological analyses of heart tissues were performed according to standard procedures. Samples were stained with H&E for routine examination and agglutinin-wheat germ-TRITC conjugate to identify sarcolemmal membranes so that myofiber

diameter could be quantified. Antibodies against desmin (AB17156, Abcam) were used to visualize sarcomeric structure. Images were collected on an epifluorescent microscope (Eclipse E800, Nikon). Quantification of cardiomyocyte surface area was performed using ImageJ software (NIH) on fluorescent micrographs from 4 hearts per genotype using ~225 cells per heart across multiple sections.

Transthoracic echocardiography

Unanaesthetized mice were restrained on a temperature-controlled mouse board (Indus Instruments) and echocardiography was performed on 208a Tg and control mice using a Vevo 660 ultrasound system (Visual Sonics) equipped with a 30-MHz transducer. An echocardiographer blind to animal genotype captured two-dimensional parasternal long axis views of the left ventricle. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and the posterior wall of the left ventricle at the level of the papillary muscles. The following measurements were obtained for systole and diastole using 4 cardiac cycles averaged: interventricular septal thickness, left ventricular posterior wall thickness, left ventricular internal diameter, heart rate and fractional shortening.

Pressure-volume loops in anesthetized mice

Mice were placed in a supine position, secured on the operating table, anesthetized with inhaled isoflurane (3% for induction and 1-2% for maintenance) and connected to a rodent ventilator after endotracheal intubation. Cardiac catheterization was performed using a 1.4 French (0.46 mm) conductance catheter (Millar Instruments)

inserted retrograde through the right carotid artery into the left ventricle. The catheter shaft was gently rotated to achieve optimal placement of the tips along the axis of the left ventricle. After catheter placement, steady-state pressure and volume measurements were recorded at baseline. Data were recorded digitally at 1,000 Hz and analyzed with PVAN software (Millar Instruments).

Immunoblotting and immunostaining

Immunoblotting and immunostaining was essentially performed as described (15) using antibodies to βMHC (M8421, Sigma), MHC (MF20, University of Iowa DSHB), myostatin (AB3239, Chemicon), Thrap1 (gift from Dr. Robert G. Roeder, Rockefeller University), β–tubulin (C4585, Sigma) and GAPDH (AB374, Chemicon).

Cultured cardiomyocyte experiments

Preparation of neonatal rat cardiomyocytes was as described (16). Cardiomyoyctes were treated with triiodothyronine (T3, Sigma) essentially as described (20). Antisense 2'O-methyl-modified oligonucleotides were transfected using Lipofectamine (Invitrogen). Cardiomyocytes were transduced with miR-208a expressing adenovirus at MOI 10. Immunostaining performed using antibodies against a-actinin (A5044, Sigma), βMHC (M8421, Sigma), and ANF (sc-20158, Santa Cruz Biotechnology). For fluorescence intensity analysis, individual cardiomyocytes (at least 100 cell bodies per condition) were measured on a 0-255 gray-value scale using ImageJ software (NIH). The intensity of immunostaining was reported as the fold change in mean gray value ± SEM.

Confocal analysis of hearts from miR-208a Tg; YFP-βMHC mice

The miR-208a Tg line was bred with the YFP-βMHC reporter line. The YFP-βMHC mice harbor a YFP-βMHC fusion allele that allows precise and accurate assessment of βMHC expression in adult hearts (17). Hearts from heterozygous triple transgenic mice expressing YFP-βMHC, tTA and miR-208a (YFP-βMHC; αMHC-tTA; TRE-miR-208a) were compared to heterozygous double transgenic littermates expressing YFP-βMHC and tTA (YFP-βMHC; αMHC-tTA). Paraformaldehyde-fixed hearts were sectioned with a vibrotome at 150-mm thicknesses. Individual sections were treated with sodium borohydride (1 mg/ml in PBS) for 30 minutes to reduce fixative-induced fluorescence. Sections were stained with Alexa Fluor 633-agglutinin wheat germ and analyzed with a FV500 confocal microscope (Olympus). Morphometric analyses were conducted on coronal sections using individual cell areas from the left ventricular free wall that were traced with ImageJ software.

Cloning and expression of miR-208a, miR-208b, and miR-124.

Genomic fragments of miR-208a, miR-208b, and miR-124 precursors were amplified by PCR using mouse genomic DNA as a template. Primer sequences are available upon request. PCR products were cloned into pcDNA(+)3.1 vector (Invitrogen) and miRNA expression was confirmed by Northern blot analysis of transfected 293T cells (Fugene6, Roche).

Luciferase assays

A modified pGL3-control vector (pGL3cm) for 3' UTR-luciferase reporter assays was described previously (15). A 575 bp fragment of the Thrap1 3' UTR was amplified from a mouse cDNA library and cloned downstream of the luciferase gene to create the luc-Thrap1 construct. Duplication of the two Thrap1 target sites was accomplished by PCR subcloning a portion of the Thrap1 3' UTR directly into the initial Thrap1 construct to create luc-Thrap1 4x. Seed region mutations were generated by site-directed mutagenesis (Fig. 5.9). The luc-myostatin 4x reporter construct was generated by annealing oligionucleotides encoding two mouse miR-208 target sites separated by 10 bp and cloning them in tandem downstream of the luciferase gene. To confirm miRNA expression in the reporter assays, we generated miRNA sensor constructs consisting of perfectly complementary sequences to miR-208a or miR-124 directly downstream of the luciferase gene. Reporter assays were conducted using human embryonic kidney 293T cells in triplicate at least three times in 24 well plates. Transfections were performed with 50 ng of reporter and 50, 100, 150 ng of miRNA plasmids (Fugene6, Roche). A CMV-lacZ reporter was used as an internal control to normalize for transfection efficiencies, and total amount of DNA per well was kept constant by adding the corresponding amount of empty expression vector.

Statistics

Values are reported as means ± SEM, unless indicated otherwise. The two-tailed Mann-Whitney test was used for comparing two means (Prism; GraphPad). Values of $P < 0.05$ were considered statistically significant.

Results

Cardiac Myosin Heavy Chain Genes Encode Intronic miRNAs

The cardiac αMHC and βMHC isoforms are the major contractile proteins in cardiomyocytes. They differ primarily in their ability to convert ATP to mechanical work at different rates, and their relative protein expression ratio affects contractility of the cardiac sacromeres (21-24). During development, the αMHC and βMHC isoforms are expressed in a developmental stage-specific manner (25). In mouse hearts, the slower isoform βMHC is fetal-specific while the faster isoform αMHC becomes the predominant isoform in the adult heart. In humans and other large mammals, βMHC expression continues into adulthood. However, the expression of βMHC increases during pathological remodeling of both mouse and human hearts, which is thought to negatively impact cardiac function (14). Interestingly, an intron from each of the αMHC and βMHC genes host a conserved miRNA, respectively miR-208a and miR-208b (**Fig. 4.1a**) miR-208a expression was detected specifically in the adult mouse heart (**Fig. 4.1b**), and could be detected at very low levels in the heart as early as embryonic day 13.5 (E13.5) (**Fig. 4.1c**). The switch from fetal isoform βMHC to the adult isoform αMHC in mouse occurs shortly after birth (**Fig. 4.1d**). We found that a similar switch from miR-208b to miR-208a expression also occurs, suggesting they are co-transcribed with their MHC host genes (**Fig. 4.1d**). miR-208a and miR-208b are of similar sequence with identical seed regions (**Fig. 4.1a**), which suggests they might be functionally redundant (26). However, miR-208b was not detectable in the adult heart, indicating that if miR-208a and miR-208b do target the same mRNAs, they do so at different developmental stages.

Thyroid hormone signaling is a well-known regulator of αMHC and βMHC transcription (27). A surge of circulating thyroid hormone that occurs shortly after birth represses βMHC expression and activates αMHC expression though negative and positive cis-acting elements within their respective promoters. We treated isolated rat cardiomyocytes with thyroid hormone and observed reduced βMHC/miR-208b expression, while dramatically inducing αMHC/miR-208a expression (**Fig. 4.1e**). Together, those data suggest that the intronic miR-208 family and their MHC host genes are co-expressed and regulated by common transcriptional events and signaling pathways.

Cardiac Overexpression of miR-208 Is Sufficient to Drive Cardiac Hypertrophy

In an effort to understand the function of miR-208a in the adult heart, we overexpressed miR-208a specifically in the heart under the control of the α-myosin heavy chain (*αMHC*) promoter using a bigenic system. An advantage of this strategy is that miR-208a is overexpressed specifically at the same time and place it would normally be expressed. The overexpression strategy consisted of a transgene encoding miR-208a downstream of a tetracycline-responsive promoter (*TRE-miR-208a*) and a second transgene encoding the tetracycline-controlled transactivator protein driven by the αMHC promoter (*αMHC-tTA*) (18). Using this system, we found that cardiac-specific overexpression of miR-208a does not cause embryonic lethality, thus administration of tetracycline to delay transgene expression was unnecessary. Multiple founder *TRE-miR-208a* transgenic lines were established. Primary analyses indicated that miR-208a is overexpressed at similar levels,

therefore we combined results from the studies of different transgenic lines. Throughout our studies, we compared heterozygous mice carrying the *αMHC-tTA* and *TRE-miR-208a* transgenes (simply referred to hereafter as 'miR-208a Tg') to mice heterozygous for αMHC-tTA (referred to hereafter as 'control').

Northern blot analysis showed miR-208a levels were ~4-fold higher in miR-208a Tg hearts compared to control hearts (**Fig. 4.2a**). The gross heart morphology of 4 month-old miR-208a Tg hearts was dramatically larger relative to control littermates (**Fig. 4.2b**). Accordingly, the heart to body weight ratios of miR-208a Tg animals were significantly higher compared to control animals (**Fig. 4.2c**). Histological sectioning and H&E staining revealed the appearance of enlarged chambers and thickened ventricular walls in the miR-208a Tg hearts, suggestive of hypertrophic growth (**Fig. 4.2d)**. Analysis of desmin, an intermediate filament found near the sarcomeric Z line, revealed no changes in the integrity of the sarcomeric structure of miR-208a Tg cardiomyocytes (**Fig. 4.2e**). Quantitative measurement of miR-208a Tg cardiomyocytes revealed a 52% increase in cell size relative to controls (**Fig. 4.2f**). Together, these results indicate that miR-208a overexpression in the mouse heart induced hypertrophic growth.

Analysis of cardiac function by echocardiography on 3 month-old animals revealed that miR-208a Tg hearts displayed thickening of the ventricular walls (anterior wall in diastole and systole, posterior wall in diastole), an increase in left ventricular diameter (left ventricular diameter in diastole and systole) and deterioration in cardiac function, as indicated by decreased fractional shortening (**Fig. 4.2g, Table 4.1**). Cardiac output, a measure of contractile performance, was

assessed by invasive hemodynamic monitoring and found consistently decreased in miR-208a Tg mice, though the difference was not statistically significant (**Fig. 4.2h**). We also measured cardiac function in 7 month-old animals and obtained similar results (**Table 4.2**).

A molecular hallmark of cardiac hypertrophy is the up-regulation of βMHC and cardiac hormone ANF in the adult heart (13, 14). Consistent with hypertrophic growth, we observed increased expression of βMHC transcripts and proteins, by real-time PCR and western blot analyses respectively, in miR-208a Tg hearts (**Fig. 4.3a,b**). Unexpectedly, no significant changes in ANF transcript levels were detected (**Fig. 4.3a**).

Changes in the expression levels of specific miRNAs have been reported in diseased human hearts and in animal models of heart disease, pointing to their potential roles in cardiomyopathies (8-12). Since βMHC expression is a hallmark of cardiac hypertrophy and because βMHC and miR-208b appear to be co-regulated, we surmised that miR-208b expression would also increase during cardiac hypertrophy. Using a mouse model of cardiac hypertrophy, in which the aorta was surgically constricted to produce chronic pressure overload, we indeed found miR-208b expression induced during hypertrophic growth (**Fig. 4.3c**). As another molecular indicator of hypertrophic growth, we analyzed the expression of miRNAs whose expression levels were reported altered in cardiac hypertrophy. Consistent with previous studies reporting decreased miR-1, miR-133 and miR-29a expression levels in cardiac hypertrophy (8-12), the expression levels of those miRNAs were also found decreased in miR-208a Tg hearts relative to control hearts (**Fig. 4.3d**).

However, while miR-125b levels were consistently reported as elevated in cardiac hypertrophy (9-11), its expression appeared unaltered in miR-208a Tg hearts. Together, those data demonstrate that miR-208a induced hypertrophic growth without affecting all aspects of the hypertrophic growth response pathway.

We also determined whether the effects of miR-208a overexpression on hypertrophy could be recapitulated *in vitro* using isolated rat neonatal cardiomyocytes. Cardiomyocytes were transduced with miR-208a expressing or control adenoviruses, then immunostained for α-actinin or βMHC (**Fig. 4.3e**). Consistent with the role of miR-208a in the induction of cardiac hypertrophy *in vivo*, overexpression of miR-208a in isolated cardiomyocytes increased cell size and βMHC expression, but did not affect ANF levels (**Fig. 4.3e-h**). Conversely, knockdown of miR-208a by introducing chemically modified oligonucleotides (2'O-methyl modified) into isolated cardiomyocytes resulted in decreased bMHC expression, but the size of cardiomyocytes and ANF expression were not affected (**Fig. 4.3e-h**). Taken together, these results using *in vivo* and *in vitro* strategies suggest that miR-208a influences subset of genes important in cell growth rather than activating a broader hypertrophic pathway.

Spatial Distribution of βMHC in miR-208a Tg Hearts Is Focal

Increased βMHC expression during cardiac hypertrophy is a well-established phenomenon and is thought to contribute to the overall poor functioning of the hypertrophic heart (22, 23, 28). To better assess the effects of miR-208a on the expression of βMHC, we employed a mouse strain harboring a βMHC indicator

allele, in which the yellow fluorescent protein (YFP) sequence is fused to the βMHC gene (17). We bred this allele into the miR-208a transgenic line. The YFP-βMHC was highly expressed in neonatal cardiomyocytes and essentially absent in adult hearts, which mimics wild type βMHC allele expression ((17), **Fig. 4.4a** and data not shown). We observed dramatically increased YFP-βMHC protein levels in the miR-208a Tg hearts (**Fig. 4.4a** and **4.5**). However, YFP-βMHC expression did not increase in all cardiomyocytes. Rather it was intensely up-regulated only in areas associated with interstitial fibrosis (**Fig. 4.4b,c**), which is consistent with previous report where the distribution of βMHC was analyzed and found correlated with fibrosis in an animal model of cardiac hypertrophy (17). Thus, even though miR-208a is presumably overexpressed in all cardiomyocytes, βMHC re-expression occurs only in subset of cardiomyocytes associated with fibrosis.

We next tested whether βMHC re-expression correlated with the miR-208a-induced hypertrophic growth of individual cardiomyocytes. To do this, we compared the cell areas of miR-208a Tg/YFP-βMHC cardiomyocytes to control YFP-βMHC cardiomyocytes lacking the miR-208a transgene. No association between the state of YFP-βMHC expression and cell area increase was observed. Thus, cardiomyocytes from miR-208a Tg hearts were significantly larger than the cardiomyocytes from control hearts (**Fig. 4.4d**) independently of whether they were positive or negative for YFP-βMHC expression. Taken together, these observations demonstrate that βMHC expression is not an obligate component of miR-208a-induced hypertrophic growth, and that overexpression of miR-208a alone is sufficient

to induce hypertrophic growth in cardiomyocytes even when they show no changes in βMHC expression.

Targeted Deletion of miR-208a Alters Cardiac Gene Expression

Having demonstrated that miR-208a is sufficient for hypertrophy and βMHC expression, we sought to examine the miR-208a loss-of-function mouse phenotype. We replaced the genomic sequence encoding miR-208a by homologous recombination with a neomycin selection cassette flanked by loxP sites (**Fig. 4.6a,b**). The selection cassette was subsequently excised by Cre-mediated recombination, leaving only a small footprint of exogenous DNA in place of miR-208a (**Fig. 4.6c**). Since miR-208a is located within an intron of the αMHC gene, we confirmed the splicing pattern of the αMHC transcript was unaffected by the mutant miR-208a allele (**Fig. 4.8**).

Progeny resulting from mating miR-208a$^{+/-}$ mice were viable and born in an expected Mendelian ratio (**Fig. 4.7a**). miR-208a expression was halved in miR-208$^{+/-}$ hearts compared to the wild type hearts, and undetectable in miR-208$^{-/-}$ hearts (**Fig. 4.7b**). Hearts of 12 to 16 week-old miR-208$^{-/-}$ mice did not display any gross morphological abnormalities and appeared normal compared to wild type littermates (data not shown). Furthermore, no differences in heart weight to body weight ratios were observed when comparing miR-208$^{-/-}$ and wild type mice (**Fig. 4.7c**). Those results are consistent with a recent report in which miR-208a was shown not required for normal heart development and function (5).

Consistent with the role of miR-208a in the regulation of cardiac hypertrophic growth and βMHC expression, we found that βMHC transcript and protein levels were significantly reduced, while αMHC and ANF transcript levels were unchanged in $208^{-/-}$ hearts (**Fig. 4.7d,e**). This result is complementary to the elevation of βMHC transcript and protein levels observed in miR-208a Tg hearts (**Fig. 4.3a,b**). Together, those genetic data provide convincing evidence that miR-208a is important for regulation of βMHC expression. We also examined the expression of miRNAs that were up-regulated in miR-208a Tg hearts (**Fig. 4.3d**). Surprisingly, we found the expression of those miRNAs unchanged in miR-$208a^{-/-}$ hearts, indicating that their expression is not dependent upon miR-208a (**Fig. 4.7f**).

miR-208a and miR-208b repress the expression of Thrap1 and myostatin

Utilizing the web-based 'Targetscan' database, we selected several predicted miR-208a target genes for experimental scrutiny (29, 30). A target site located in the 3' UTR of Thyroid hormone associated protein 1 (Thrap1) is among the most heavily weighted targets for miR-208a and was chosen for study since thyroid hormone signaling is a known repressor of βMHC transcription (31, 32). Upon closer inspection of the Thrap1 3' UTR, we identified a second conserved miR-208a target site located ~60 bp downstream of the first target site (**Fig. 4.9a and 4.11a**).

In addition, myostatin, a member of the transforming growth factor-β family, is a predicted miR-208a regulatory target and harbors a single miR-208a target site in its 3' UTR (**Fig. 4.9a** and **4.11c**). Myostatin is abundantly expressed by skeletal muscle and acts as an important repressor of hypertrophic growth (33, 34).

Myostatin is secreted into the plasma as a latent form and acts systemically (35). Myostatin is also expressed in heart muscle, although to a much lesser degree than found in skeletal muscle, and genetic inactivation of myostatin in mice has been recently linked to cardiac hypertrophy (36-40).

As a first step towards determining which genes are targeted by miR-208a and miR-208b, we cloned genomic fragments encoding miR-208a, miR-208b, and miR-124 into plasmids for overexpression in cultured cells (**Fig. 4.9b**). We hypothesized that similar sequence and identical seed region of miR-208a and miR-208b would enable them to repress similar sets of genes, while miR-124 is a brain-specific miRNA and served as a control miRNA for specificity.

Thrap1 is part of the thyroid hormone nuclear receptor complex and can positively and negatively influence transcription (41, 42); thus we reasoned that repression of Thrap1 by miR-208a might account for the increased βMHC expression in miR-208a Tg hearts (**Fig. 4.3a,b**). In agreement this notion, co-transfection of a luciferase gene with the Thrap1 3'UTR cloned immediately downstream (luc-Thrap1) and the miR-208a expression plasmid in cultured cells resulted in repressed luciferase activity (**Fig. 4.9c**). Expression of miR-124 with luc-Thrap1 had no such effect upon luciferase activity, indicating that miR-208a repression of luc-Thrap1 was specific. To further confirm such specificity, we mutated the candidate miR-208a target sites (luc-Thrap1 mutant), which resulted in the complete loss of miR-208a mediated repression (**Fig. 4.9c** and **4.11b**). As we predicted from the sequence similarity shared by miR-208a and miR-208b, we found that miR-208b also repressed the luc-Thrap1 luciferase activity (**Fig. 4.9c**). As

another demonstration of miR-208a and miR-208b targeting of Thrap1 and to test whether increasing the number of target sites would also increase the degree of repression, we duplicated the Thrap1 target sites downstream of the luciferase gene (luc-Thrap1 4x). Indeed, increasing target site number resulted in a pronounced decrease in luciferase activity, indicating that target site number is an important factor for miRNA-mediated repression (**Fig. 4.10a**).

In order to directly test whether miR-208a could repress the expression of myostatin, we constructed four repeats of the myostatin target sequence downstream of a luciferase gene (luc-myostatin 4x) and co-transfected with miRNA expression plasmids. Co-transfection of either miR-208a or miR-208b and luc-myostatin 4x plasmids repressed luciferase activity (**Fig. 4.10b**). Co-transfection of miR-124 and luc-myostatin 4x plasmids caused no decrease in luciferase activity and confirmed that miR-208a and miR-208b specifically target the myostatin 3' UTR (**Fig. 4.10b**).

To determine whether Thrap1 and myostatin were regulated *in vivo* by miR-208a, we tested whether their expression was altered in our miR-208a gain- and loss-of-function mouse models. The transcript levels of Thrap1 and myostatin appeared unchanged in the miR-208a Tg and $208a^{-/-}$ hearts (**Fig. 4.11d**). However, the protein levels of Thrap1 and myostatin were repressed in miR-208a Tg hearts compared to control hearts (**Fig. 4.10c**). Conversely, the protein levels of Thrap1 and myostatin were elevated in miR-$208^{-/-}$ hearts compared to hearts from wild type littermates (**Fig. 4.10c**). Taken together, these observations demonstrated that Thrap1 and myostatin are *bona fide* miR-208a targets.

Discussion

Increased expression of βMHC is a common feature of cardiac hypertrophy and heart failure (13, 14). The αMHC to βMHC isoform shift that occurs during cardiac disease reduces contractile performance and is thought be a maladaptive response (22, 23, 28). The shift towards βMHC is reversible under particular conditions that are associated with improved cardiac performance, including the regression of hypertrophy and human patients that respond favorably to beta-blocker therapy (43-47). Furthermore, mutations in the βMHC gene are commonly associated with hypertrophic cardiomyopathies (48). It is recognized that therapies that inhibit the maladaptive features of hypertrophy might be useful in improving the function of the diseased heart (14). However, the development of such therapies has been limited by an incomplete understanding of the molecular mechanisms underlying the maladaptive features of hypertrophy.

In this report, we show that miR-208a is an essential regulator of cardiac remodeling. Our experiments demonstrate that miR-208a is sufficient to induce cardiac hypertrophy. The hypertrophic growth induced by miR-208a is accompanied by increased βMHC expression. In contrast, deletion of miR-208a resulted in decreased βMHC expression in the adult heart, providing genetic evidence that miR-208a modulates βMHC expression. An important part of our study is the finding that βMHC is induced in a patchy pattern in miR-208a Tg hearts. Strikingly, up-regulated βMHC expression in miR-208a Tg hearts is tightly associated with regional fibrosis, similar to what we have previously found in renin-induced hypertrophy (17). The

strong correlation of re-expression of βMHC and fibrosis development may explain why such reactivation of 'fetal' genes is associated with a maladaptive phenotype. Further studies to understand how miR-208a regulates βMHC expression, as well as fibrosis, will likely shed light on the biology of cardiac hypertrophy.

Our findings demonstrate that miR-208a post-transcriptionally represses expression of Thrap1, a component of the thyroid hormone nuclear receptor complex. Thyroid hormone signaling has long been an established regulator of cardiac myosin heavy chain isoform expression: a surge of circulating thyroid hormone occurring after birth transcriptionally represses βMHC expression while activating αMHC expression (31, 32). Studies have also shown that excessive administration of thyroid hormone leads to the development of cardiac hypertrophy, but the molecular mechanism was elusive (49-52). Our findings, in which increasing the level of miR-208a in transgenic hearts reduced Thrap1 levels and induced hypertrophic growth, provide a link between the action of miR-208a and thyroid hormone in cardiac hypertrophy. We also found that miR-208a post-transcriptionally represses the expression of myostatin, a well-known repressor of hypertrophic growth. However, it's unlikely that myostatin repression is solely responsible for the hypertrophy observed in miR-208a Tg hearts since genetic deletion of myostatin results in a comparatively mild cardiac hypertrophy phenotype (36). Myostatin repression is instead likely one of several additive factors contributing to miR-208a-induced hypertrophy.

Intriguingly, miR-208a induced hypertrophy has also increased the expression level of miR-208b in hypertrophic hearts. Since both miR-208a and miR-208b likely

target similar mRNAs, we speculate the outcome of increasing miR-208 family levels beyond the normal miR-208 threshold increasingly represses miR-208 regulatory target genes. The induction of miR-208b expression during hypertrophy suggests a feed-forward mechanism in which miR-208b and miR-208a cooperatively promote hypertrophy by repressing Thrap1 and myostatin (**Fig. 4.10d**). Our results, which provide genetic evidence that miR-208a modulates βMHC expression and is sufficient to induce hypertrophy, are consistent with a recent study showing that miR-208a is necessary for the hypertrophic growth response (5). Together, our studies support a role for miR-208a in repressing anti-hypertrophy genes as part of the genetic program needed for hypertrophic growth. We anticipate that inhibition of miR-208a may be a viable therapeutic strategy to repress βMHC expression and could remove some of the maladaptive features of hypertrophy.

Figure 4.1

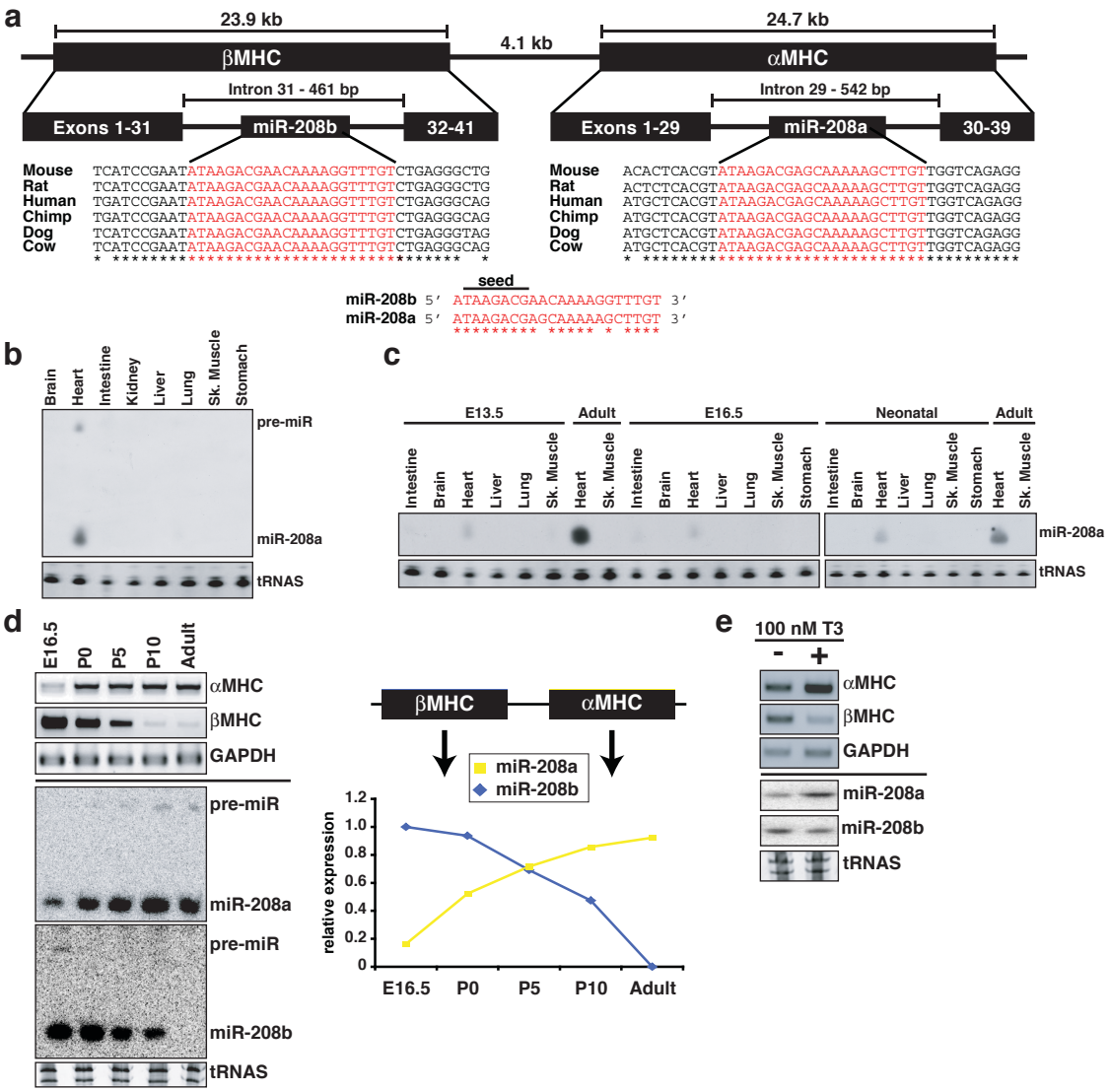


Figure 4.1 Expression of miR-208a and miR-208b parallels the expression of their respective host genes αMHC and βMHC. (**a**) miR-208a is encoded by intron 29 of the αMHC gene, while miR-208b is encoded by intron 31 of the βMHC gene. miR-208a and miR-208b are highly conserved and share similar sequence identity (asterisks). (**b**) Detection of mature and precursor miR-208a in adult mouse tissues. (**c**) Detection of mature and precursor miR-208a in embryonic day 13.5 (E13.5), E16.5 and neonatal tissues by Northern analysis. (**d**) Upper left, αMHC and βMHC transcripts were detected in E16.5, postnatal day 0 (P0), P5, P10 and adult mouse hearts by RT-PCR. Lower left, miR-208a and miR-208b expression was detected in the samples by Northern analysis. Right, relative levels of miR-208a and miR-208b during heart development (**e**) Upper, αMHC and βMHC transcripts were detected in isolated rat neonatal cardiomyocytes following treatment with thyroid hormone (T3) by RT-PCR. Lower, miR-208a and miR-208b were detected by Northern analysis.

Figure 4.2

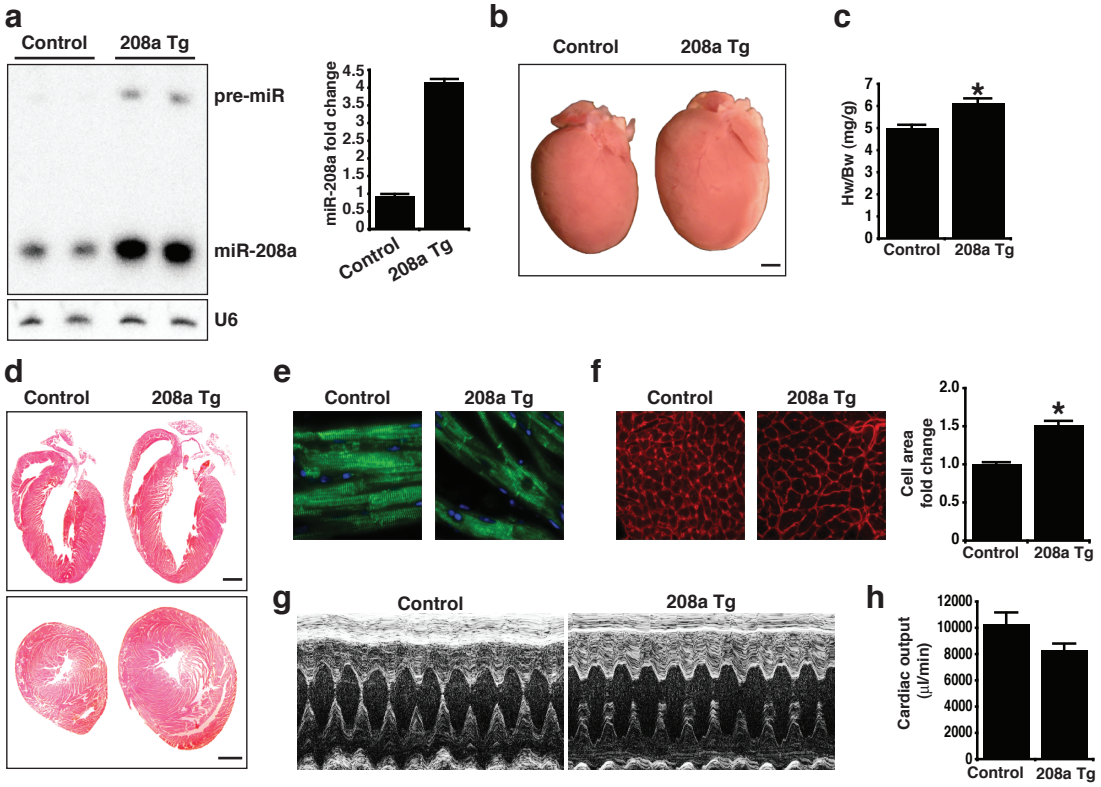


Figure 4.2 Hearts of miR-208a transgenic mice undergo hypertrophic growth. (**a**) Northern analysis showing ~4-fold increase of miR-208a expression in hearts of miR-208a Tg animals compared to control littermates. U6 serves as loading control. (**b**) Gross morphology of miR-208a Tg hearts is enlarged compared to control hearts. Scale bars are 1 millimeter. (**c**) Heart to body weight ratios (Hw/Bw) of 4 month-old miR-208a Tg mice (n = 22) are significantly ($P < 7 \times 10^{-5}$) higher than controls (n = 19). (**d**) Macroscopic view of H&E stained histological sections (*upper*, sagittal; *lower*, transverse) from control and miR-208a Tg hearts. Scale bars are 1 millimeter. (**e**) Sacromeric structure of cardiomyocytes visualized by desmin staining of histological sections. (**f**) Histological sections were stained with wheat germ agglutinin-TRITC conjugate to determine cell size. Mean cardiomyocyte size of miR-208a Tg hearts (n = 930) were significantly larger ($P < 9 \times 10^{-50}$) than control cardiomyocytes (n = 926). (**g**) Representative M-mode echocardiographs from conscious control and miR-208a Tg mice. (**h**) Cardiac output calculated from pressure-volume loop recordings of 3 month-old mice (n = 5 each genotype).

Figure 4.3

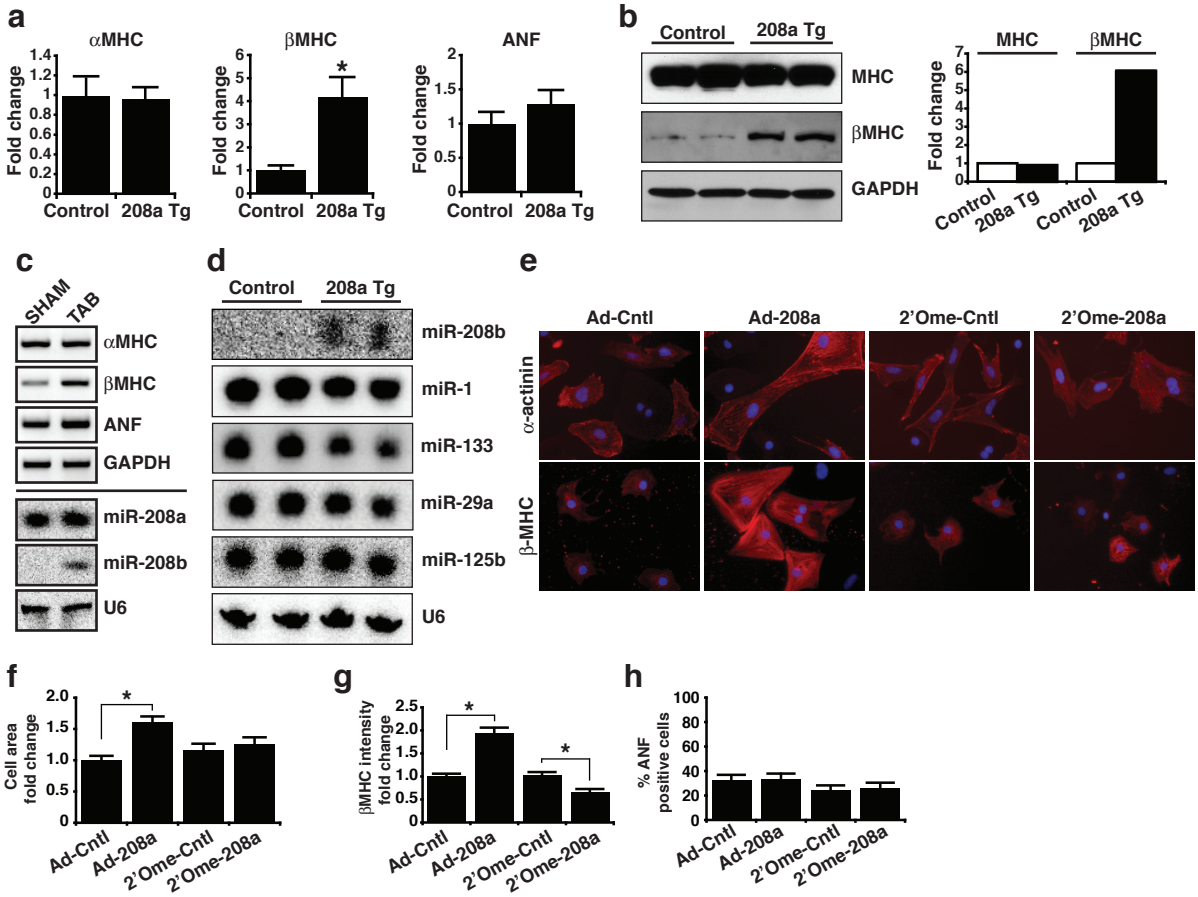


Figure 4.3 miR-208a overexpression induces hypertrophic growth (**a**) Transcripts for αMHC, βMHC and ANF were detected by real-time PCR in 4 month-old hearts from control and miR-208a Tg mice (n = 5 each genotype). Values presented as the fold change in expression ± SEM. *, $P < 0.01$. (**b**) Western blot analysis of total MHC and βMHC protein levels in adult control and miR-208a transgenic hearts. (**c**) *Upper*, αMHC, βMHC and ANF transcripts were detected in wild type hearts following 3 weeks thoracic aortic banding (TAB) or surgical sham hearts as controls by RT-PCR. *Lower*, miR-208a and miR-208b were detected by Northern analysis. U6 serves as loading control. (**d**) Northern analysis of indicated miRNAs using hearts from control and miR-208a Tg mice. (**e-h**) Isolated rat neonatal cardiomyocytes were transduced with miR-208a and control adenoviruses (Ad-208 and Ad-Cntl, respectively), or transfected with oligonucleotides antisense to miR-208a or control oligonucleotides (2'Ome-208a and 2'Ome-Cntl, respectively), as indicated. (**e**) Cardiomyocytes stained for α-actinin or βMHC proteins. (**f**) Mean cell areas ± SEM of a-actinin immunostained cardiomyocytes treated with adenoviruses or oligonucleotides, as indicated (n = 100 cells each condition; *, $P < 4 \times 10^{-12}$). (**g**) Mean fluorescent intensities ± SEM of βMHC immunostained cardiomyocytes treated with adenoviruses or oligonucleotides, as indicated (n = 100 cells each condition; *, $P < 3 \times 10^{-7}$). (**h**) Cardiomyocytes treated with adenoviruses or antisense 2'O-methyl oligonucleotides, as indicated, were scored for ANF staining (n = ~425 cells each condition).

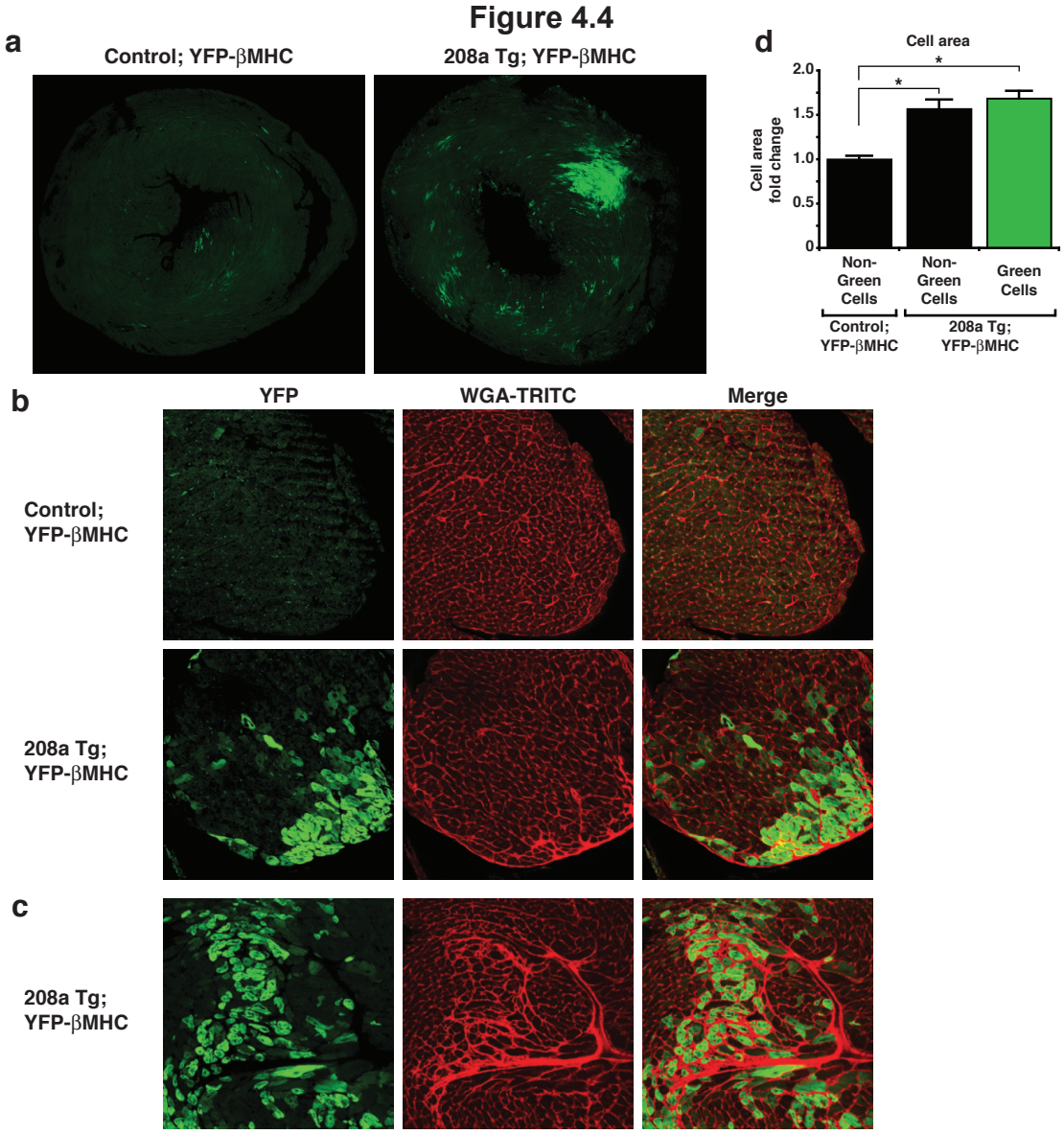
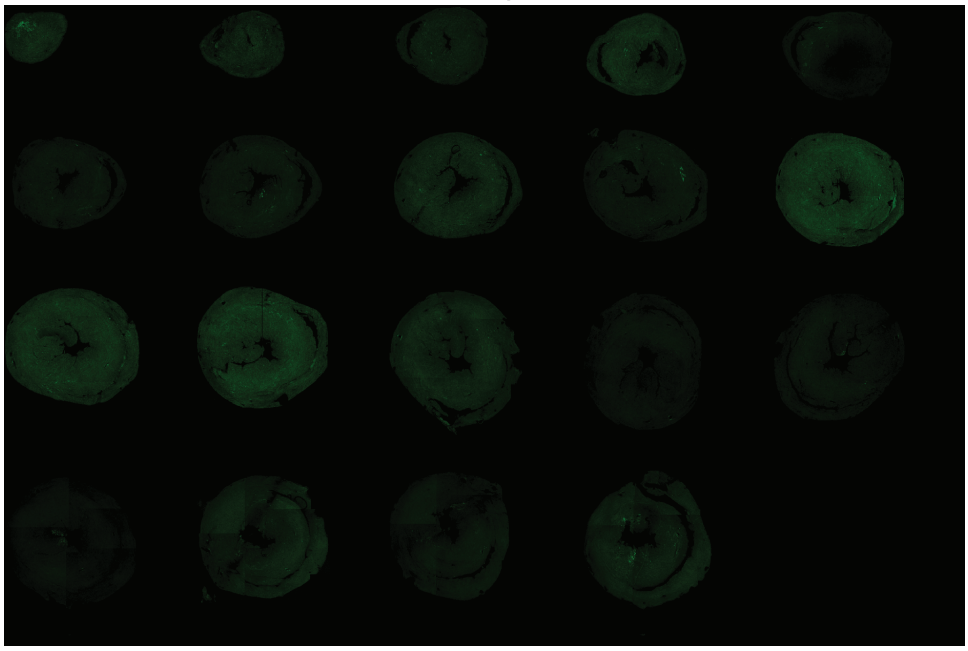


Figure 4.4 Distribution of βMHC-YFP fusion protein in miR-208a transgenic hearts. (**a**) Confocal fluorescent images of coronal sections from control and miR-208a Tg hearts. (**b**) Papillary muscle from control and miR-208a Tg hearts imaged with a 20x objective for βMHC-YFP (green) expression and wheat germ agglutinin-TRITC staining (red). (**c**) Representative fluorescent images of βMHC-YFP expression in (green) an area of interstitial fibrosis (red) in miR-208a Tg hearts. (**d**) Mean cell areas ± SEM of cardiomyocytes from miR-208a Tg; βMHC-YFP versus control; βMHC-YFP hearts. Cells measured for area were also scored for presence or absence of βMHC-YFP expression (n = 100 each genotype; *, $P < 0.001$).

Figure 4.5

Control; YFP-βMHC



miR-208a Tg; YFP-βMHC

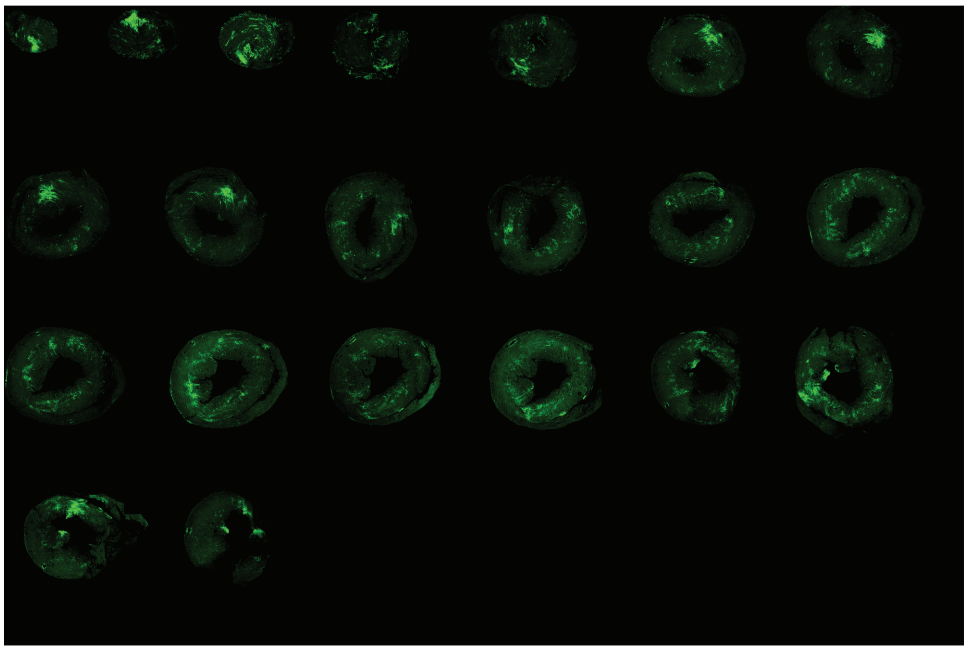


Figure 4.5. Confocal microscopy for YFP detection on serial coronal sections from control; YFP-βMHC and miR-208a Tg; YFP-βMHC hearts (from left to right, top to bottom: apex to the top of the ventricles).

Figure 4.6

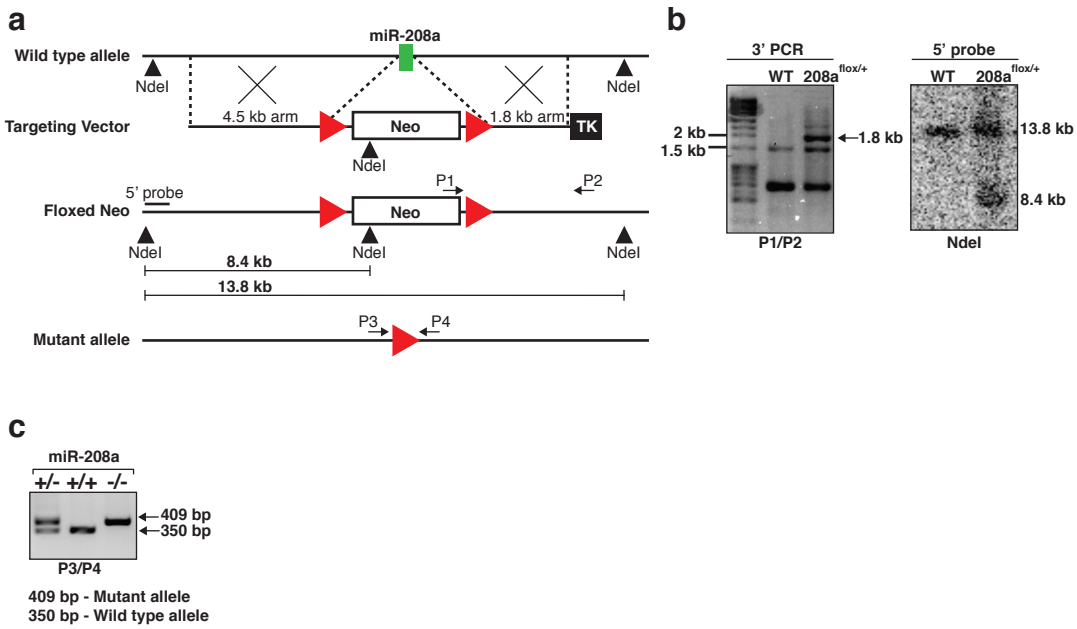


Figure 4.6 Deletion of miR-208a from mouse genome. (**a**) Strategy to delete miR-208a from intron 31 of the αMHC gene by homologous recombination. The miR-208a coding sequence (green bar) was replaced by a neomyocin selection cassette (Neo) flanked by loxP sites (red triangles). The selection cassette was excised from the germline by mating to mice that ubiquitously express Cre recombinase, creating a mutant allele that contained a single loxP sequence in place of miR-208a. Genotyping PCR primers and 5' probes as marked. (**b**) The occurrence of the intended recombination event in mouse embryonic stem cells was confirmed by PCR and Southern analyses. (**c)** The increased length of the mutant allele was the basis for a PCR-based genotyping strategy.

Figure 4.7

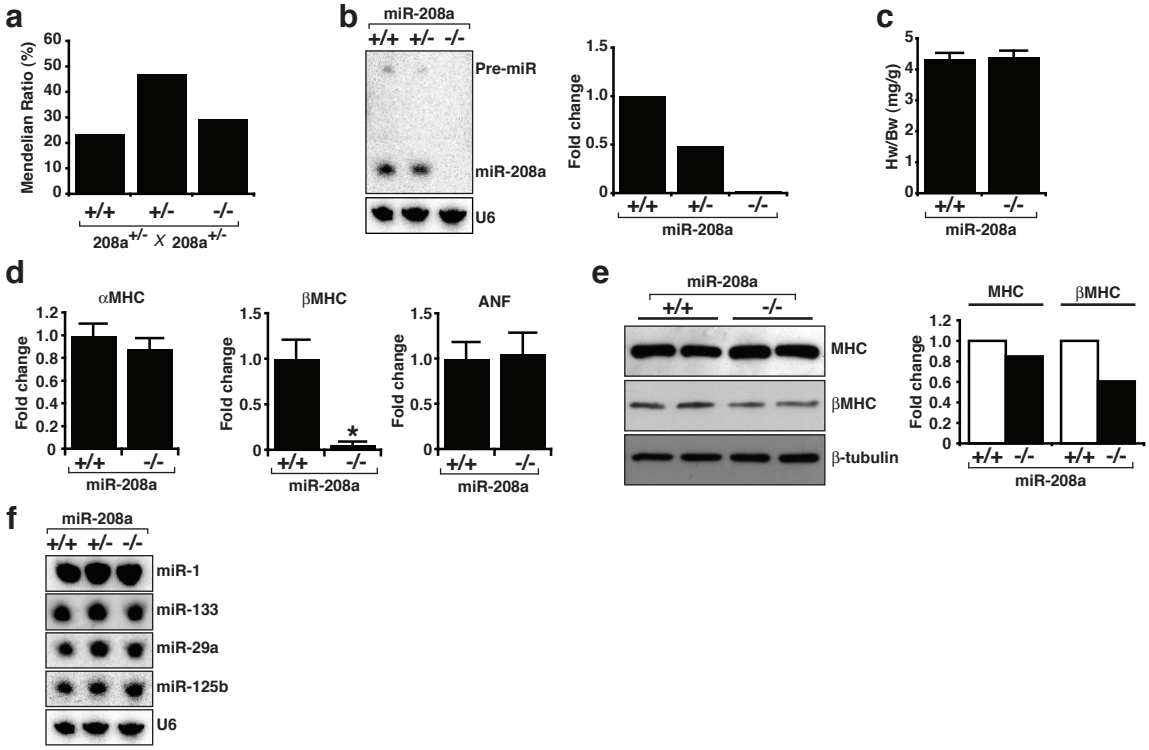


Figure 4.7 Expression of βMHC is decreased in 208a$^{-/-}$ hearts. (**a**) Genotypes of progeny from mating miR-208a$^{+/-}$ mice were born in Mendelian ratio (n = 128). (**b**) Northern analysis for miR-208a expression in hearts from wild type (miR-208$^{+/+}$), miR-208$^{+/-}$ and miR-208$^{-/-}$ mice. (**c**) Heart weight to body weight ratios of 4 month-old wild type and miR-208$^{-/-}$ mice (n = 25 each genotype). (**d**) Transcripts for aMHC, βMHC and ANF were detected by real-time PCR in hearts from wild type and 208$^{-/-}$mice (n = 5 each genotype). Values presented as the fold change in expression ± SEM. *, $P <$ 0.01. (**e**) Western analysis of total MHC and βMHC protein levels in hearts from wild type and miR-208$^{-/-}$ mice. (**i**) Northern analysis of indicated miRNAs using hearts from wild type, miR-208$^{+/-}$ and 208$^{-/-}$ mice.

Figure 4.8

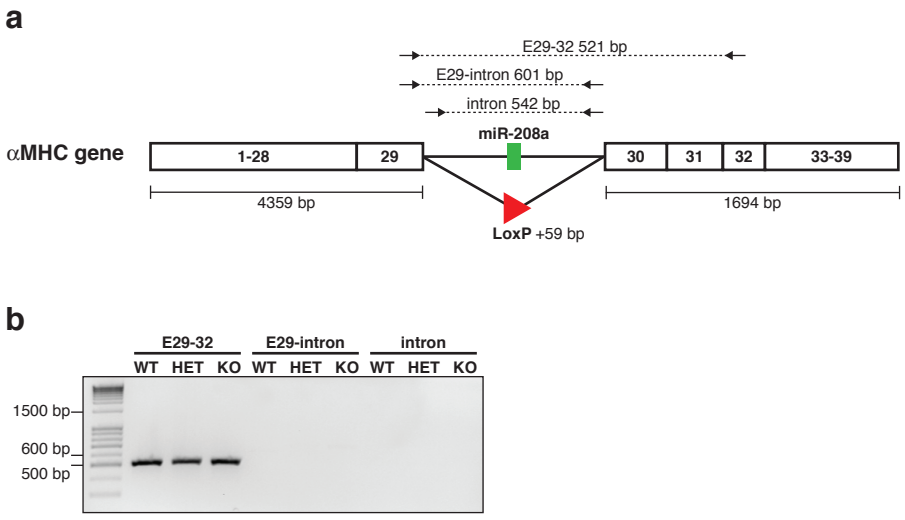


Figure 4.8. Splicing of αMHC transcript containing mutant miR-208a allele is undisturbed. (**a**) Diagram of αMHC gene showing the proper splicing pattern of the intron that encodes the miR-208a allele or a mutant allele that carries a loxP site instead. Location of primers and regions amplified marked by arrows and dashed lines.(**b**) Results of PCR analysis using genomic DNA from wildtype (WT), miR-208$^{+/-}$ (Het) or miR-208$^{-/-}$ (KO) animals and the primer sets as marked.

Figure 4.9

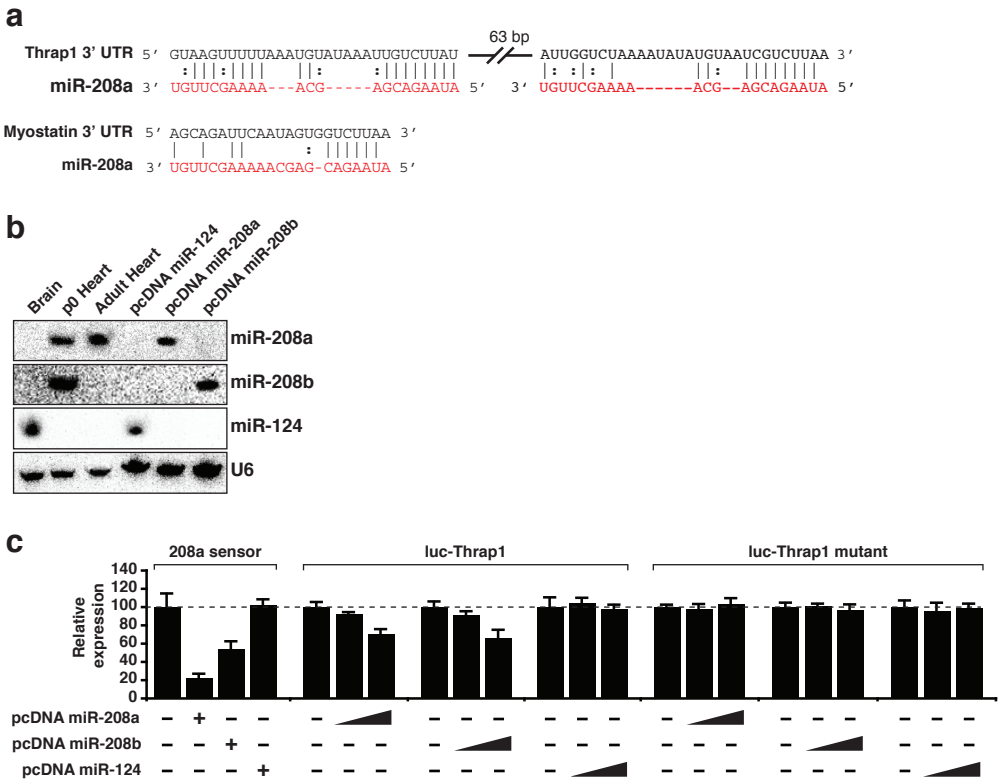


Figure 4.9 miR-208a and miR-208b target Thrap1 and myostatin. (**a**) Sequence alignment between miR-208a and candidate binding sites in the 3' UTR of Thrap1 or Myostatin. (**b**) Northern analysis demonstrating miR-208a, miR-208b and miR-124 expression plasmids produce mature miRNAs when transfected into 293T cells. Total RNA from mouse brain, neonatal and adult hearts included as controls. U6 serves as loading control. (**c**) 293T cells were transfected with a luciferase reporter designed to detect miR-208a expression (208a sensor), along with the indicated miRNA expression plasmids. A Thrap1 3' UTR (luc-Thrap1) and a mutated Thrap1 3' UTR (luc-Thrap1 mutant) were also tested. Values are luciferase activity ± SD relative to the luciferase activity of reporters co-transfected with empty expression plasmid.

Figure 4.10

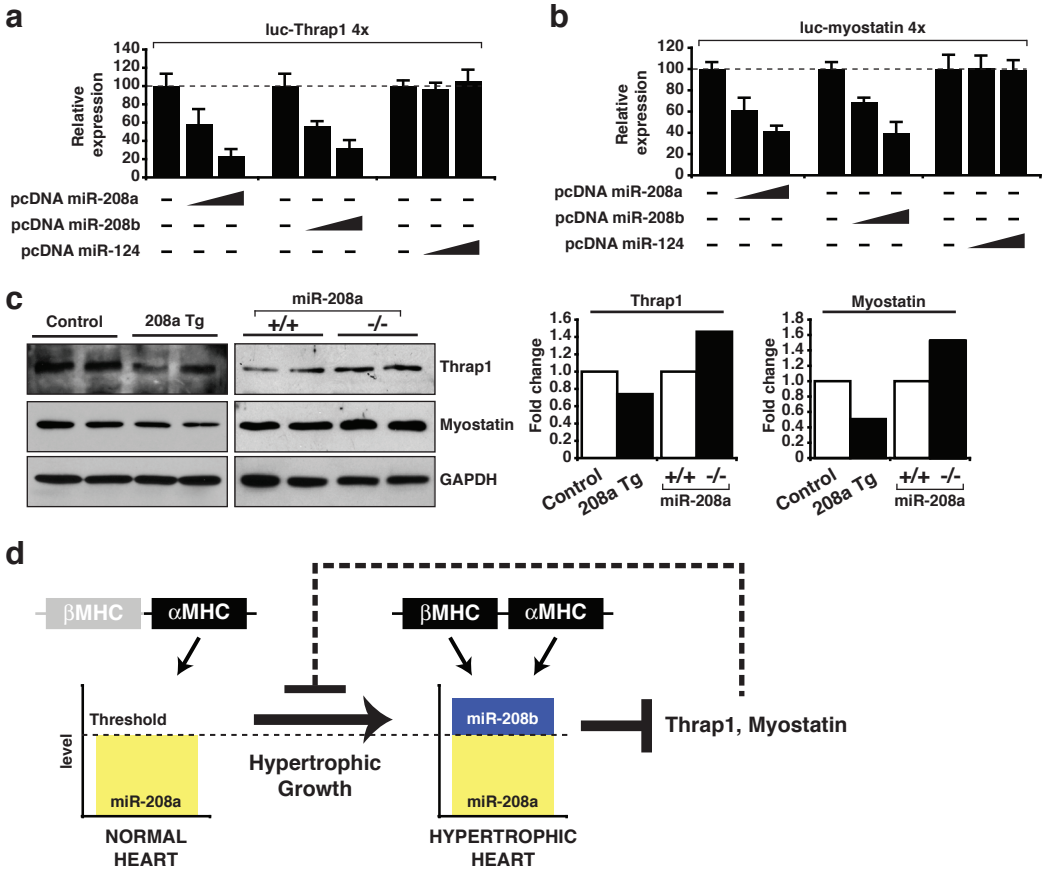


Figure 4.10 miR-208a and miR-208b repress the expression of Thrap1 and myostatin. (**a**) A luciferase reporter with duplicated Thrap1 binding sites (luc-Thrap1 4x) was co-transfected with indicated miRNA expression plasmids and luciferase activity determined. (**b**) A luciferase reporter with four repeats of the putative myostatin binding site was co-transfected with indicated miRNA expression plasmids and luciferase activity determined. (**c**) Western blot analysis for Thrap1 and myostatin protein levels in hearts from 4 month-old miR-208a Tg versus control animals and miR-208 null versus wild type animals. GAPDH serves as loading control. (**d**) A model for miR-208 family function during normal and stress-induced hypertrophic growth conditions. In the normal adult heart, miR-208a is the predominant miR-208 member and helps fine-tune the expression of anti-hypertrophy genes Thrap1 and myostatin. During hypertrophic growth, bMHC is induced and accompanied by increased miR-208b expression, which raises the level of total miR-208 family members above the normal threshold for maintenance of Thrap1 and myostatin expression levels. The additive effect of miR-208a and miR-208b on repressing expression of anti-hypertrophy genes further promotes hypertrophic growth in feed-forward manner.

Figure 4.11

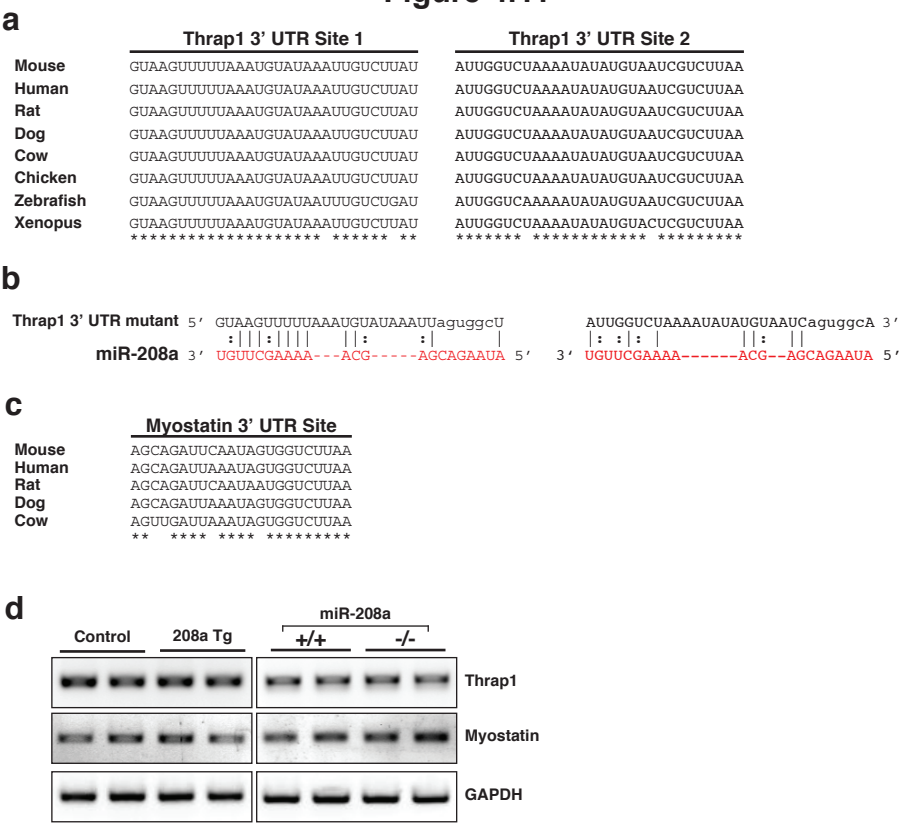


Figure 4.11 (**a**) Target sites for miR-208a in the 3' UTR of Thrap1 show a high level of cross-species sequence conservation. (**b**) Alignment of miR-208a with mutated Thrap1 target sites. Lower case lettering indicates mutant nucleotides. (**c**) Target site for miR-208a in the 3' UTR of myostatin shows a high level of cross species sequence conservation. (**d**) RT-PCR analysis for Thrap1 and myostatin transcript levels in hearts from 4 month-old miR-208a Tg versus control animals and miR-208 versus wild type (+/+) animals. GAPDH serves as loading control.

Table 4.1. Echocardiography of Dimensions and Function of 3 month-old miR-208a trangenic mice.

	Control n = 10			208a Tg n = 10		
BW (g)	25.0	±	1.14	26.1	±	1.24
LV mass index (mg)	107	±	1.45	163	±	6.33**
LV mass index/BW (mg/g)	3.94	±	0.09	6.04	±	0.24**
HR (bpm)	713	±	8.9	697	±	12.3
IVSTD (mm)	0.93	±	0.01	1.11	±	0.04**
IVSTS (mm)	1.55	±	0.02	1.73	±	0.04*
PWTD (mm)	0.85	±	0.03	1.00	±	0.03*
PWTS (mm)	1.60	±	0.03	1.48	±	0.06
LVEDD (mm)	3.2	±	0.03	3.5	±	0.09**
LVESD (mm)	1.48	±	0.03	2.0	±	0.07**
FS%	51.7	±	0.77	41.2	±	1.16**

Transthoracic echocardiography on unanesthetized mice. Data are mean ± SEM. BW, body weight; LV, left ventricular; HR, heart rate; IVSTD, interventricular septal thickness in diastole; IVSTS, interventricular septal thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension. LV mass index was calculated as (external LV diameter in diastole^3 – LV end-diastolic dimension^3) x 1.055. Fractional shortening (FS) was calculated as (LV end-diastolic dimension – LV end-systolic dimension)/LV end-diastolic. *, $P < 0.001$; **, $P < 0.0001$.

Table 4.2. Echocardiography of Dimensions and Function of 7 month-old miR-208a transgenic mice.

	Control n = 5			miR-208a Tg n = 5		
BW (g)	28.7	±	1.58	33.7	±	2.97
LV mass index (mg)	104	±	4.10	169	±	10.1***
LV mass index/BW (mg/g)	3.67	±	0.24	5.60	±	0.22**
HR (bpm)	666	±	15.1	672	±	18.7
IVSTD (mm)	0.89	±	0.03	1.16	±	0.10**
IVSTS (mm)	1.42	±	0.08	1.79	±	0.10**
PWTD (mm)	0.89	±	0.02	1.17	±	0.09**
PWTS (mm)	1.39	±	0.06	1.55	±	0.11
LVEDD (mm)	3.2	±	0.04	3.4	±	0.11*
LVESD (mm)	1.62	±	0.04	1.9	±	0.10**
FS%	49.5	±	0.71	41.495	±	0.33***

Transthoracic echocardiography on unanesthetized mice. Data are mean ± SEM. BW, body weight; LV, left ventricular; HR, heart rate; IVSTD, interventricular septal thickness in diastole; IVSTS, interventricular septal thickness in systole; PWTD, posterior wall thickness in diastole; PWTS, posterior wall thickness in systole; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension. LV mass index was calculated as (external LV diameter in diastole^3 – LV end-diastolic dimension^3) x 1.055. Fractional shortening (FS) was calculated as (LV end-diastolic dimension – LV end-systolic dimension)/LV end-diastolic. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$.

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CHAPTER 5

MIR-208A IS A REGULATOR OF CARDIAC CONDUCTION

Abstract

The highly coordinated passage of electrical impulses through the cardiac conduction system is essential for proper heartbeat rhythm. Functional defects in the cardiac conduction system result in arrhythmias that affect millions of people and are associated with sudden cardiac death. Despite intensive investigation, the molecular mechanisms that underlie the development and maintenance of the cardiac conduction system are not well understood. In this chapter, we investigate the role of miR-208a in the cardiac conduction system. We find that overexpression of miR-208a induces cardiac arrhythmias in the adult mouse. Conversely, we demonstrate miR-208a is required to maintain proper cardiac conduction using a mouse model in which miR-208a was genetically deleted. Molecular analyses reveal that miR-208a gain- or loss-of-function results in dysregulated expression of key cardiac conduction system components. Furthermore, miR-208a appears to directly regulate the expression of the calcium ion channel subunit CACNB2. Our results provide genetic evidence for miR-208a as an important regulator of the cardiac conduction system and reveal a novel role for the miR-208 family in the adult mouse heart.

Introduction

The rhythmic heartbeat is the result of highly coordinated electrical impulses that are propagated throughout the myocardium by a specialized network of cells collectively known as the cardiac conduction system (1). The electrical impulses that pace the heart originate at the sinoatrial (SA) node and are transmitted throughout the atria then converge on the atrioventricular (AV) node. The transmission of the electrical impulses from the atria to the ventricles are briefly delayed by slow conducting myocytes in the AV node to provide sufficient time for ventricular filling between the sequential contractions of the atria and ventricles. Past the AV node, electrical impulses are rapidly conducted throughout the ventricles via the His bundle and fascicular branches of the Purkinje fibers, which results in coordinated ventricular contraction. Functional defects in the conduction system affects millions of people and results in arrhythmias, which may occur from congenital disorders and often accompany heart disease (2). While familial arrhythmias are linked to numerous sequence variants in cardiac ion channels, sequence variants in cardiac transcription factors are also linked to arrhythmogenesis and point to the importance of gene expression regulation for proper cardiac conduction (3). However, our understanding of the genetic networks that direct development and function of the cardiac conduction system is incomplete.

MicroRNAs (miRNAs) are a class of small RNA molecules that post-transcriptionally regulate gene expression and their recent discovery has added a new regulatory paradigm to our understanding of genetic networks (4). Roles for

heart-expressed miRNAs are now known in cardiogenesis, the hypertrophic growth response, as well as for proper cardiac conduction (5-10).

We and others previously found that the miR-208 family of microRNAs (miRNAs) is an important regulator of cardiac hypertrophy (see Chapter 4 and (10)). The miR-208 family consists of miR-208a and miR-208b, which are respectively encoded within an intron of the α- and β-myosin heavy chain genes (α- and βMHC). The expression patterns of miR-208a and miR-208b are developmentally regulated: miR-208a is predominately postnatally expressed, while miR-208b expression occurs mostly during development but becomes up-regulated in the adult mouse heart during hypertrophy. The miR-208 family regulates hypertrophic growth by fine-tuning the expression of anti-hypertrophy genes during normal and pathological conditions (see Chapter 4).

In this chapter, I demonstrate that the miR-208a is a regulator of the cardiac conduction system. I find that overexpression of miR-208a in adult mouse hearts induces AV conduction block defects, while its genetic deletion leds to SA conduction block defects. Molecular analyses reveals that modification of miR-208a levels results in dysregulated expression of key cardiac conduction system components, including cardiac transcription factors and ion channels. Furthermore, miR-208a directly regulates the expression of the calcium ion channel subunit CACNB2. Collectively, this study demonstrates that miR-208a is an important regulator of the cardiac conduction system and suggests important pathophysiological functions for miR-208a, in addition to cardiac hypertrophy.

Materials and Methods

Surface electrocardiograms

All procedures were approved by and performed in accordance with the University of North Carolina Institutional Animal Care and Use Committee. The miR-208a transgenic and miR-208a knockout mouse lines were described previously (see Materials and Methods in Chapter 4). For the transgenic mouse studies, single transgenic animals genotyped αMHC-tTA (referred to as 'control' throughout this study) were compared to double transgenic littermates genotyped αMHC-tTA/TRE-miR-208 (referred to as 'miR-208a Tg'). Mice were anesthetized with 1-2% isoflurane in 700 ml 0_2/minute via facemask (following induction chamber containing 5% isoflurane). Rectal temperature was monitored and maintained at 37C using a heat pad and heat lamp. Baseline Lead I electrocardiograms were recorded for about 3 minutes at 2k Hz from needle electrodes inserted subcutaneously into the each limb. Recordings were analyzed using the ECG module of Chart5 software (ADInstruments) and corrected QT (QTc) intervals were calculated using a murine formula (11).

Transcript analyses, immunoblotting, and immunostaining

RNA analyses by quantitative RT-PCR were essentially performed as described (12-14). Immunoblotting and immunostaining was essentially performed as described (12) using antibodies against connexin-40 (36-4900, Zymed), connexin-43 (C8093, Sigma), Hsp70 (SPA-815, Stressgen), GATA4 (sc-1237, Santa Cruz), Hop (a kind

gift from Dr. Eric Olson, University of Texas South Western), βtubulin (C4585, Sigma) and GAPDH (AB374, Chemicon).

Constructs and luciferase assays

Expression plasmids encoding miR-208a, and miR-208b were described previously (see Materials and Methods in Chapter 4). A modified pGL3-control vector (pGL3cm) for 3' UTR-luciferase reporter assays was described previously (12). The luc-CACNB2 4x reporter construct was generated by annealing oligionucleotides encoding two mouse miR-208 target sites separated by 10 bp and cloning them in tandem downstream of the luciferase gene. To confirm miRNA expression in the reporter assays, we employed miRNA sensor constructs consisting of perfectly complementary sequences to miR-208a directly downstream of the luciferase gene. Reporter assays were conducted using human embryonic kidney 293T cells in 24 well plates. All experiments were repeated in triplicate at least three times. Transfections were performed with 50 ng of reporter and 50, 100, 150 ng of miRNA plasmids (Fugene6, Roche). A CMV-lacZ reporter was used as an internal control to normalize for transfection efficiencies, and total amount of DNA per well was kept constant by adding the corresponding amount of empty expression vector.

Statistics

Values are reported as means ± SEM, unless indicated otherwise. The two-tailed Mann-Whitney test was used for comparing two means (Prism; GraphPad). Values of $P < 0.05$ were considered statistically significant.

Results

miR-208a overexpression induced atrioventricular conduction blocks

We overexpressed miR-208a specifically in the heart under the control of the α-myosin heavy chain (αMHC) promoter using a bigenic system consisting of a transgene encoding miR-208a downstream of a tetracycline-responsive promoter (*TRE-miR-208a*) and a second transgene encoding the tetracycline-controlled transactivator protein driven by the αMHC promoter (*αMHC-tTA*) (see Chapter 4 and (15)). In this study, heterozygous mice carrying the *αMHC-tTA* and *TRE-miR-208a* transgenes (simply referred to hereafter as 'miR-208a Tg') were compared to mice heterozygous for *αMHC-tTA* (referred to hereafter as 'control'). Northern blot analysis previously showed miR-208a levels are ~4-fold higher in miR-208a Tg hearts compared to control hearts (see Chapter 4, Fig. 4.2).

We previously observed that miR-208a overexpression induced hypertrophic growth accompanied by decreased cardiac function (see Chapter 4, Fig. 4.2). To determine whether miR-208a overexpression disturbed other aspects of heart physiology, we recorded surface electrocardiograms (ECGs) of 1, 4, and 6-month old miR-208a Tg and control mice (**Table 5.1**). Analysis of the ECG recordings showed significantly prolonged PR intervals in miR-208a Tg mice compared with control mice at all time points (**Table 5.1** and **Fig. 5.1a**). No significant differences were detected in other ECG parameters, such as QRS, QT, or QTc intervals (**Table 5.1**). The PR interval is the period of time between the onset of atrial depolarization and the onset of ventricular depolarization; abnormal prolongation of the PR interval is clinically termed first-degree AV block. Interestingly, approximately 30% of the

miR-208a Tg mice suffer Mobitz II second-degree AV blocks in which one or more of the electrical impulses from the atria unexpectedly fail to pass through the AV node to the ventricles, causing failures in ventricular contraction (**Fig. 5.1b,c**). Second-degree AV blocks appear on the ECG tracings as P-waves (atrial depolarizations) without subsequent occurrence of QRS complexes (ventricular depolarizations) (**Fig. 5.1b**). Taken together, the development of progressive heart blocks in miR-208a Tg mice demonstrates that miR-208a overexpression causes cardiac conduction abnormalities and suggests miR-208a regulates cardiac conduction system components.

miR-208a is necessary for normal cardiac conduction

We and others previously found that genetic deletion of miR-208a does not affect viability or cause any readily apparent gross morphological heart defects, but is required for stress-dependent heart growth (Chapter 4 and (10)). To determine whether miR-208a is also required during normal conditions, we monitored heart function of 4-month old miR-208$^{-/-}$ and wild type littermates by surface ECGs and found that miR-208a is necessary for proper cardiac conduction. Similar to miR-208a Tg animals, surface ECG analysis revealed prolonged PR intervals in miR-208a$^{-/-}$ mice compared with wild type animals (**Table 5.2**). However, approximately 80% of the miR-208a$^{-/-}$ mice suffered partial and complete SA blocks accompanied by junctional escape rhythms (**Fig. 5.2a-c**). The junctional escape rhythm is a protective mechanism whereby a secondary pacemaker maintains heartbeat rhythm. The SA blocks appears on the ECG tracings as missing P-waves preceding QRS complexes

(**Fig. 5.2a,b**). We also recorded and analyzed surface ECGs using 1-month old animals and obtained similar results (data not shown). Collectively, the ECG analyses of miR-208a transgenic and null mice demonstrate that miR-208a is an important component of the cardiac conduction system.

miR-208a regulates expression of cardiac connexins

Normal conduction is mediated by the orderly propagation of electrical impulses from one cardiomyocyte to the next. The connexin proteins are gap junction proteins required for this propagation and their altered expression is a common feature in a variety of chronic human heart diseases associated with increased risk of arrhythmias and sudden death (16-19). Mouse models have demonstrated that deficiencies in either connexin 43 (Cx43) or connexin 40 (Cx40) results in cardiac conduction defects (20). Cx43 is expressed in cardiomyocytes throughout the heart, whereas Cx40 expression is restricted to the atria and the specialized cardiomyocytes that constitute the His bundle and its branches as well as the Purkinje fibers (20). We hypothesized that abnormal connexin protein expression might account for, at least in part, the cardiac conduction defects induced by altered miR-208a levels.

We evaluated the expression of Cx43 and Cx40 in hearts from 4-month old miR-208a Tg and miR-208a$^{-/-}$ mice. Western blot analysis of hearts from miR-208a Tg mice showed increased Cx43 and Cx40 protein levels compared with control hearts (**Fig. 5.3a**). Conversely, miR-208a$^{-/-}$ hearts showed decreased Cx43 and Cx40 protein levels compared with wild type hearts (**Fig. 5.3b**). Transcript analysis

by real-time PCR did not reveal any readily apparent changes to Cx43 transcript levels in either miR-208a Tg hearts or miR-208a$^{-/-}$ hearts, nor were Cx40 transcript levels affected in miR-208a Tg hearts (**Fig. 5.3c,d**). However, Cx40 transcript levels were markedly decreased in miR-208$^{-/-}$ hearts compared with wild type (**Fig. 5.3d**), indicating that miR-208a is required for Cx40 transcription. We also observed Cx43 expression by fluorescent immunohistochemistry and found Cx43 proteins localized to the cell membrane in both miR-208a Tg and miR-208a$^{-/-}$ hearts (**Fig. 5.4a,b**). The complementary phenotypes of increased or decreased connexin protein levels resulting from respective increased or decreased miR-208a levels indicates that miR-208a is required to maintain proper levels of connexin proteins in the adult mouse heart.

Loss of miR-208a abolished expression of Hop in the heart

The transcription factor homeodomain-only protein (Hop) is highly expressed within the adult murine conduction system and its genetic deletion resulted in postnatal conduction defects accompanied by a loss of Cx40 expression (21). Therefore, we speculated that the decreased Cx40 expression observed in miR-208a$^{-/-}$ hearts might partially stem from reduced Hop expression. We evaluated Hop transcripts levels by real-time PCR using hearts from 4-month old animals and found Hop expression abolished in miR-208a$^{-/-}$ mice (**Fig. 5.5a**). Accordingly, Hop protein was also undetectable in miR-208a$^{-/-}$ hearts (**Fig. 5.5b**). While decreased Hop levels in miR-208a$^{-/-}$ hearts might account for decreased Cx40 expression, the genetic deletion of Hop does not reportedly affect Cx43 expression (21). Furthermore, Cx40

and Cx43 expression levels increased in miR-208a Tg hearts without any accompanying changes to Hop expression levels (**Figs. 5.3a,c, 5.5c** and data not shown). Taken together, these observations indicate that Hop is not directly targeted by miR-208a and suggests that an undetermined miR-208a regulatory target exists upstream of Hop, Cx43, and Cx40.

miR-208a regulates GATA4 expression

The cardiac transcription factor GATA4 is expressed within the cardiac conduction system of the adult heart and was previously shown to transactivate the promoter of Cx40 (23, 24). The 3' untranslated region (3'UTR) of GATA4 mRNA contains a predicted miR-208a target site (25), thus we predicted that the miR-208a gain- and loss-of-function phenotypes might partially result from irregular GATA4 protein expression. In support, the protein levels of GATA4 in 4-month old hearts from miR-208a Tg compared with control mice appeared decreased, while no changes in transcript level were observed (**Fig. 5.5b**). Conversely, GATA4 protein levels were increased in hearts from miR-208$^{-/-}$ mice compared with wild type mice (**Fig. 5.5d**). No changes in GATA4 transcript levels were observed in miR-208a Tg or miR-208-/- mice, indicting that the changes in GATA4 expression occurs post-transcriptionally (**Fig. 5.5a,c**). Together, these observations indicate that GATA4 is post-transcriptionally regulated by miR-208a. Further analyses are needed to confirm whether this regulation is conferred through the predicted miR-208a target site located within the 3'UTR of GATA4. GATA4 and Nkx2.5 were previously shown to synergistically activate Cx40 expression, while Tbx5 suppresed such interaction

(24). Therefore, it would also be interesting to test whether the counterintuitive increase in Cx40 expression that is accompanied by decreased GATA4 expression in those miR-208a Tg animals could be explained by simultaneous expression changes in GATA4 cofactors or repressors.

Increased miR-208a levels decreased Hsp70 expression in the heart

The cytosolic heat shock protein 70 (Hsp70) facilitates degradation of Cx43 by the proteosome in cardiomyocytes under normal and stress conditions (22). To determine whether miR-208a overexpression might alter Hsp70 expression, real-time PCR analysis was applied and found Hsp70 transcript levels significantly decreased in miR-208a Tg hearts compared with controls (**Fig. 5.5c**). Accordingly, western blot analysis showed Hsp70 protein levels down-regulated in miR-208a Tg hearts compared with controls (**Fig. 5.5d**). This data suggests that increased Cx43 and Cx40 protein levels in miR-208a Tg animals might stem from reduced protein turnover as the result of decreased Hsp70 levels. In agreement with deficient post-transcriptional regulation, no significant changes to Cx43 or Cx40 transcript levels were detected by real-time PCR analysis of miR-208a Tg hearts compared with controls (**Fig. 5.3c**). No changes to Hsp70 transcript or protein levels were detected in miR-208$^{-/-}$ hearts compared with wild type hearts (**Fig. 5.5a,b**), indicating that miR-208a is not required to maintain Hsp70 expression levels.

miR-208a directly regulates L-type calcium ion channel CACNB2 expression

L-type calcium ion channels are heterotetrameric complexes that allow for depolarization induced calcium influx into the cytosol during excitation-contraction coupling (26). During excitation-contraction coupling in cardiomyocytes, voltage-gated L-type calcium channels are opened in response to transient action potentials and allow calcium ions to enter the cell. This increase in intracellular calcium occurs at localized regions near calcium-sensitive ryanondine receptors, which become activated and release large amounts of calcium stored within the sacroplasmic reticulum into the cytoplasm. The resulting surge of cytosolic calcium binds troponin-C resulting in a conformational that allows actin-myosin binding and sarcomeric contraction. Thus, L-type calcium channels are responsible for providing the 'trigger' calcium required for excitation-contraction coupling.

L-type calcium channels are composed of at least three subunits: α_1, β, and α_2/δ. The β subunit encoded by CACNB2 modulates calcium channel activity in the heart and enables trafficking of the pore forming α subunit to the cell surface by masking an endoplasmic retention signal (27). Recently, a sequence variant in CACNB2 was clinically linked to an inherited arrhythmia associated with sudden cardiac death (28). The 3' UTR of CACNB2 harbors a predicted miR-208a target site (25), therefore we predicted that miR-208a might fine-tune CACNB2 expression and that overexpression or deletion of miR-208a might result in aberrant CACNB2 levels and lead to arrhythmogenesis.

The predicted miR-208a target site is evolutionarily conserved in mammals and is located approximately 600 bp downstream of the CACNB2 stop codon (**Fig.**

5.6a). We previously cloned genomic fragments encoding miR-208a, miR-208b, and miR-124 into plasmids for overexpression in cultured cells (see Chapter 4). We hypothesized that similar sequence and identical seed region of miR-208a and miR-208b would enable them to repress similar sets of genes, while miR-124 is a brain-specific miRNA and served as a control miRNA for specificity. In order to directly test whether miR-208a could repress the expression of CACNB2, we constructed four repeats of the CACNB2 target sequence downstream of a luciferase gene (luc-CACNB2 4x) and co-transfected with miRNA expression plasmids. Consistent with our prediction of miR-208a-mediated regulation, co-transfection of either miR-208a or miR-208b represses luc-CACNB2 4x luciferase activity (**Fig. 5.6b**). Co-transfection of miR-124 and luc-CACNB2 4x plasmids resulted to no decrease in luciferase activity, confirming that miR-208a and miR-208b specifically target the CACNB2 3' UTR (**Fig. 5.6b**).

Discussion

In this chapter, we show that miR-208a is an essential regulator of the cardiac conduction system. Our experiments demonstrate that miR-208a is sufficient to induce cardiac arrhythmias, while miR-208a is also required to maintain proper cardiac conduction. Increased Cx43 and Cx40 expression accompany the arrhythmias induced in the adult heart by miR-208a overexpression. Conversely, deletion of miR-208a resulted in decreased Cx43 and Cx40 expression, providing genetic evidence that miR-208a is an important regulatory component of the cardiac conduction system.

An important part of our study is the finding that Hop expression is abolished in miR-208a$^{-/-}$ hearts. Genetic deletion of miR-208a partially phenocopies reports of Hop$^{-/-}$ mice in that adult hearts from both knockout lines appear structurally normal but suffer arrhythmias accompanied by decreased Cx40 transcript and protein levels (21). However, differences also exist between those two knockout lines: approximately half of the Hop$^{-/-}$ mice are embryonic lethal and deletion of Hop did affect Cx43 expression, while miR-208a mice are fully viable and have reduced Cx43 expression. The arrhythmias that present also differ: the conduction defect in Hop$^{-/-}$ mice occurs below the AV node, while the miR-208a$^{-/-}$ conduction defect appears to occur above the AV node. How the arrhythmias in Hop$^{-/-}$ and miR-208a$^{-/-}$ animals manifest might reflect their differential expression of connexin proteins.

We and others previously found that miR-208a is both sufficient and necessary for cardiac hypertrophy (see Chapter 4 and (10)). Our present finding that miR-208a is required to maintain Hop expression may help to explain the blunted hypertrophic growth response of miR-208a$^{-/-}$ hearts (10). Unlike most homeobox transcription factors, Hop does not bind DNA directly. Instead, Hop recruits histone deacetylase 2 (HDAC2) and inhibits the transcriptional activity of serum response factor (SRF) in cardiomyocytes (29-31). Interestingly, Hop overexpression was reported to induce cardiac hypertrophy and is proposed to inhibit an antihypertrophy gene program in the adult heart (31). A potential explanation for the inability of miR-208a$^{-/-}$ hearts to undergo hypertrophic may stem from the lack of Hop protein available to repress this antihypertrophy gene program. Indeed, we and others have proposed that miR-208a fine-tunes the expression of so-called antihypertrophy

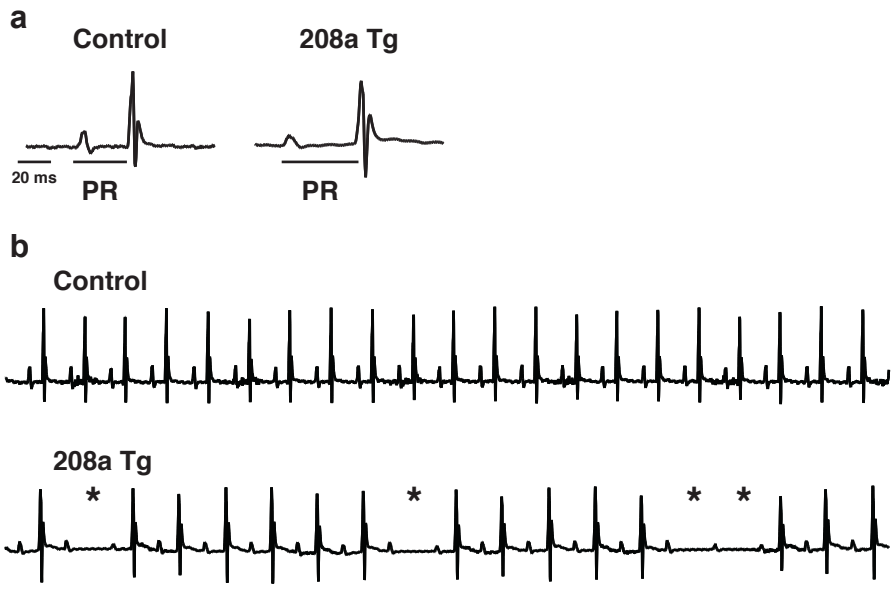
genes, which may include miR-208a targets Thrap1 and myostatin (see Chapter 4 and (10, 32)). Further studies to understand how miR-208a regulates the expression of Hop and connexin proteins will likely shed light on the biology of cardiac hypertrophy and conduction.

Our findings suggest that miR-208a post-transcriptionally represses the expression of CACNB2, a cardiac subunit of the L-type calcium ion channel (**Fig. 5.5b**). L-type calcium channels are critical for excitation-contraction coupling and defects affecting their function have been clinically linked to arrhythmias (28, 33). We speculate that modification of miR-208a levels directly affects CACNB2 expression, which participates in the arrhythmogenesis occurring in the miR-208a gain- and loss-of-function animals. While experimental validation of the predicted miR-208a target within the CACNB2 3' UTR using an *in vitro* reporter assay is useful, the confirmation of such repression in a whole animal system remains untested. While we have previously used miR-208a Tg and miR-208$^{-/-}$ mice to support direct miR-208a regulation of Thrap1 and myostatin (see Chapter 4), presently no antibodies of sufficient quality against CACNB2 are commercially available at present to enable such analysis. It is our hope to develop or acquire this regent in the future to determine whether miR-208a-mediated regulation of CACNB2 is relevant *in vivo*.

Recent studies have pointed to two miRNAs, miR-1 and miR-133, which are implicated in cardiac development, muscle proliferation and differentiation, as regulating components of the cardiac conduction system and having the potential to induce arrhythmias (7, 8). Our results, which provide genetic evidence that miR-

208a is an important regulatory component of the cardiac conduction system, adds to the growing cardiovascular genetic network in which microRNAs are becoming known as critical players for the regulation of cardiomyocyte hypertrophy and cardiac conduction. Whatever the precise molecular mechanisms underlying the miR-208a conduction defect or its role in the hypertrophic growth response turns out to be, it's clear that miR-208a is a component of very complex genetic network work that will take much effort to fully understand.

Figure 5.1

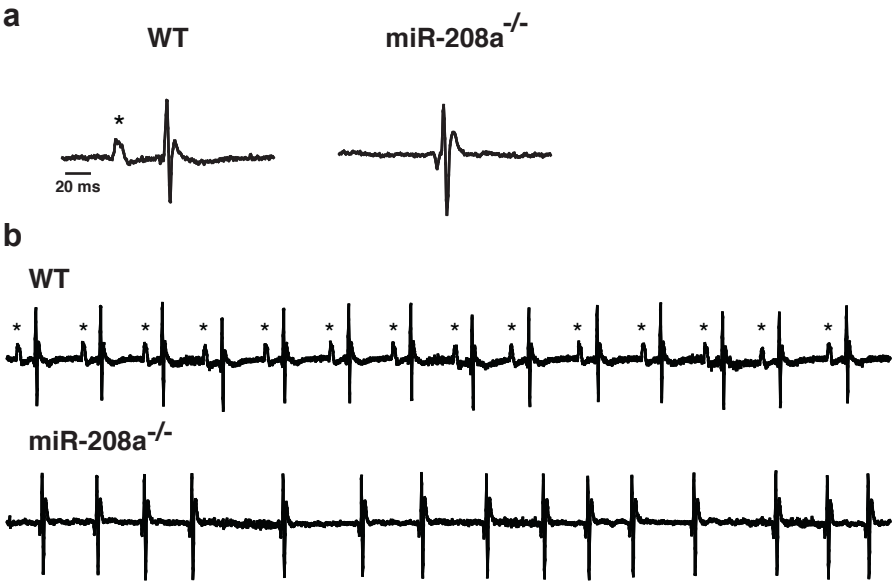


c

Second Degree AV Block Penetrance	
	Percent
1 Month	
Control	0% (n=16)
208a Tg	33% (n=21)
4 Month	
Control	0% (n=15)
208a Tg	35% (n=23)
6 Month	
Control	0% (n=7)
208a Tg	22% (n=9)

Figure 5.1 miR-208a overexpression is sufficient to induce arrhythmias. (**a**) Representative waveforms in lead I indicate the location and relative duration of PR intervals in 4-month old miR-208a transgenic and control mice. (**b**) Representative electrocardiograms in lead I of 4-month old miR-208a transgenic and control mice. Asterisks mark missing QRS complexes and indicate occurrences of second-degree atrioventricular block. (**c**) The number and percentage of second-degree atrioventricular (AV) blocks that were detected in 1, 4, and 6-month old miR-208a transgenic and control mice.

Figure 5.2



c

SA Block Penetrance	
	Percent
4 Month	
WT	0% (n=8)
miR-208a$^{-/-}$	80% (n=10)

Figure 5.2 miR-208a is necessary for proper cardiac conduction. (**a**) Representative waveforms in lead I from miR-208a null (-/-) and wild type (+/+) mice indicate the normal position of the P-wave. (**b**) Representative electrocardiograms in lead I from 4-month old miR-208a null (-/-) and wild type (+/+) mice. Asterisks mark presence of P-wave. (**c**) The number and percentage of 4-month old miR-208a null (-/-) and wild type (+/+) mice with second-degree sinoatrial (SA) blocks during electrocardiographic screening.

Figure 5.3

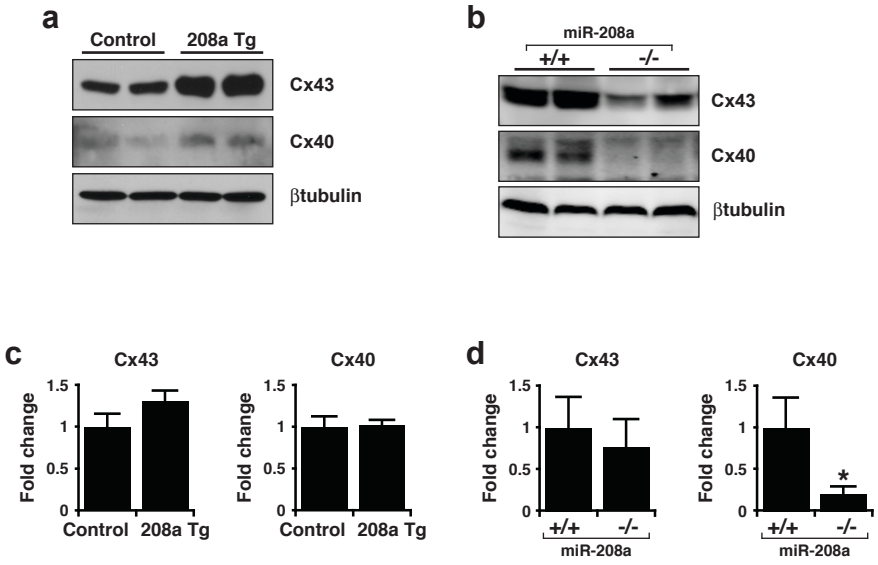


Figure 5.3 miR-208a regulates expression of cardiac connexins. Western blot analyses of connexin 43 (Cx43) and connexin 40 (Cx40) proteins using hearts from 4-month old (**a**) miR-208a transgenic and control mice or (**b**) wild type (+/+) and miR-208a null (-/-) mice. βtubulin serves as loading control. Transcripts for Cx43 and Cx40 were detected by real-time PCR in hearts from (**c**) miR-208a transgenic and control mice (n = 5 each genotype) or (**d**) wild type (+/+) and miR-208a null (-/-) mice (n = 5 each genotype). Values presented as the fold change in expression ± SEM. *, $P < 0.01$.

Figure 5.4

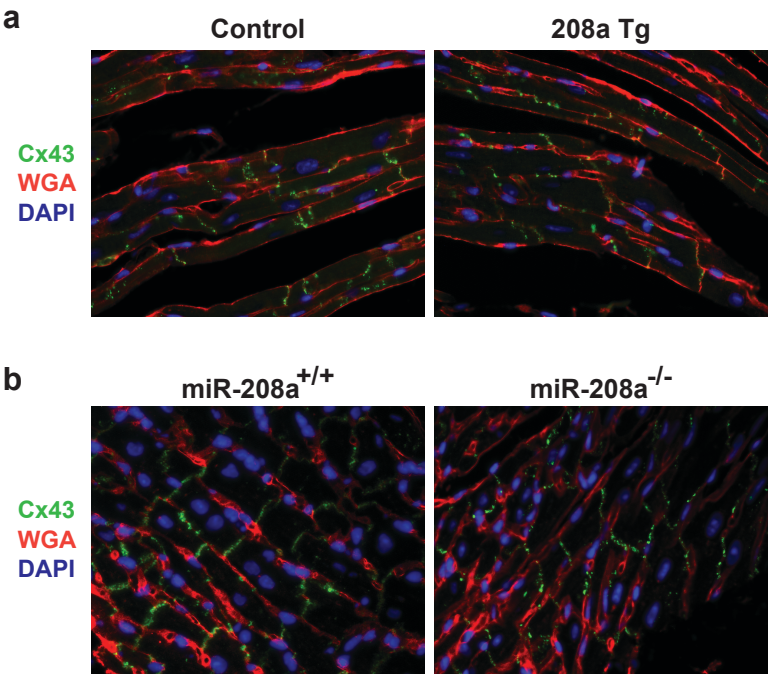


Figure 5.4 Histological sections were stained for Cx43 proteins (green) and WGA-TRITC (red) was used to visualize cell borders in (**a**) miR-208a transgenic and control mice or (**b**) wild type (+/+) and miR-208a null (-/-) mice. DAPI staining (blue) used to visualize nuclei.

Figure 5.5

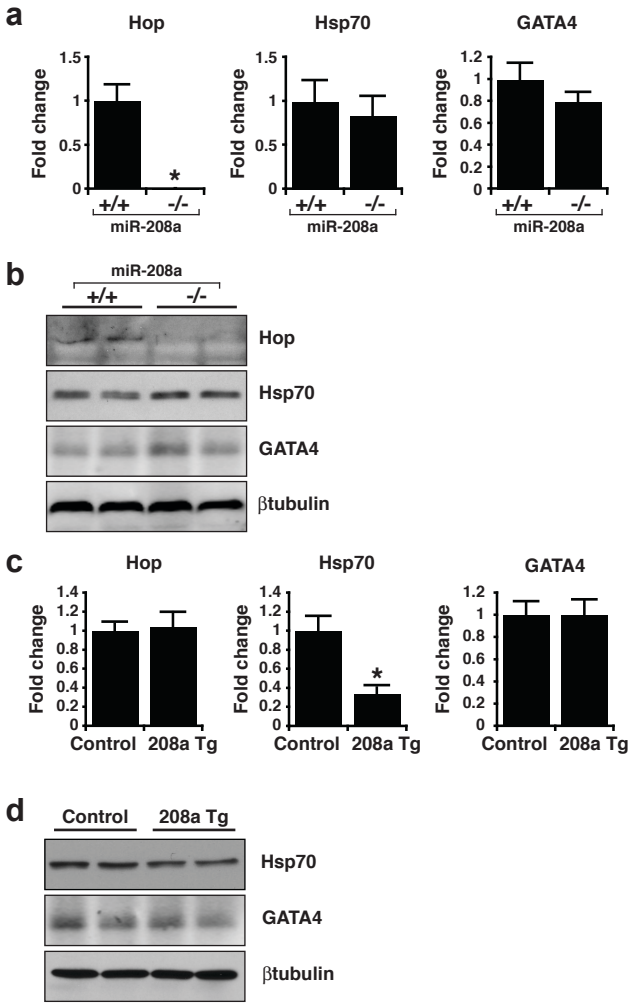


Figure 5.5 miR-208a overexpression and deletion causes aberrant cardiac gene expression (**a**) Transcripts for Hop, Hsp70, and GATA4 were detected by real-time PCR in hearts from wild type (+/+) and miR-208a null (-/-) mice (n = 5 each genotype). Values presented as the fold change in expression ± SEM. *, $P < 0.01$. (**b**) Western blot analyses of Hop, Hsp70, and GATA4 proteins using hearts from 4-month old wild type (+/+) and miR-208a null (-/-) mice. βtubulin serves as loading control. (**c**) Transcripts for Hop, Hsp70, and GATA4 were detected by real-time PCR in hearts from miR-208a transgenic and control mice (n = 5 each genotype). Values presented as the fold change in expression ± SEM. *, $P < 0.01$. (**d**) Western blot analyses of Hsp70 and GATA4 proteins using hearts from 4-month old miR-208a transgenic and control mice. βtubulin serves as loading control.

Figure 5.6

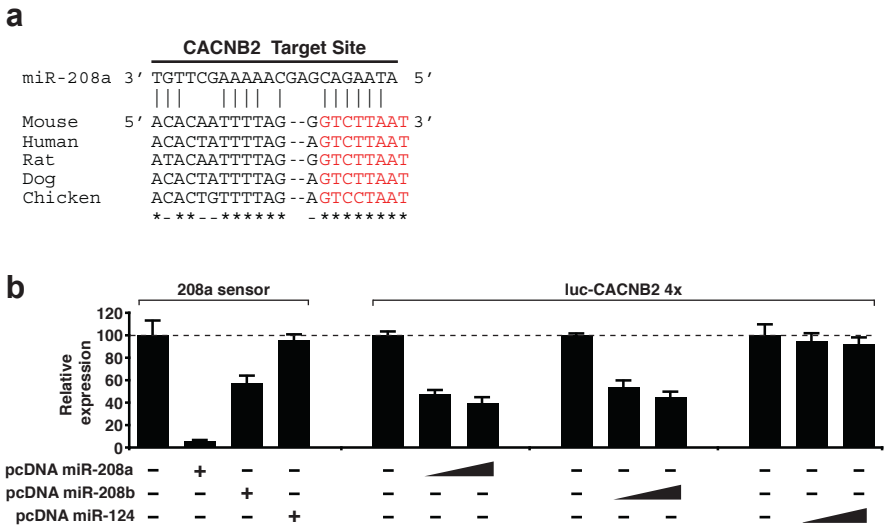


Figure 5.6 miR-208a and miR-208b repress the expression of CACNB2. (**a**) Sequence alignment between miR-208a and evolutionarily conserved candidate binding site in the 3' UTR of CACNB2. Asterisks denote sequence conservation. (**b**) 293T cells were transfected with a luciferase reporter designed to detect miR-208a expression (208a sensor), along with the indicated miRNA expression plasmids and luciferase activity determined. A luciferase reporter with four repeats of the putative CACNB2 binding site was also co-transfected with indicated miRNA expression plasmids and luciferase activity determined. Values are luciferase activity ± SD relative to the luciferase activity of reporters co-transfected with empty expression plasmid.

Table 5.1

Summary of miR-208a Transgenic Surface ECG Findings

	HR (bpm)	PR (ms)	QRS (ms)	QT (ms)	QTc (ms)
1 Month					
Control (n=6)	453 ± 26	34 ± 1	9 ± 1	51 ± 1	44 ± 1
208a Tg (n=7)	405 ± 27	49 ± 3**	11 ± 1	53 ± 1	43 ± 2
4 Month					
Control (n=11)	500 ± 24	38 ± 1	9 ± 0	48 ± 2	43 ± 1
208a Tg (n=16)	460 ± 17	46 ± 2**	9 ± 1	50 ± 2	43 ± 1
6 Month					
Control (n=6)	425 ± 25	40 ± 2	10 ± 1	54 ± 2	45 ± 2
208a Tg (n=7)	436 ± 15	51 ± 3*	11 ± 1	59 ± 2	50 ± 2

*$P < 0.05$, **$P < 0.01$

Table 5.2

Summary of miR-208a Knockout Surface ECG Findings

	HR (bpm)	PR (ms)	QRS (ms)	QT (ms)	QTc (ms)
4 Month					
WT (n=8)	491 ± 22	37 ± 1	9 ± 0	53 ± 1	48 ± 1
$208a^{-/-}$ (n=10)	503 ± 22	49 ± 1*	11 ± 1	50 ± 2	54 ± 2

*$P < 0.001$

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation describes original research focused upon roles for transcription factors and microRNAs in coordinating cardiac gene expression during heart development and disease. Genetically engineered mouse models were developed and characterized using diverse molecular, biochemical, histological, and physiological approaches, and protein structure-function and gene expression studies were conducted. The key findings include discovery of a signaling pathway that regulates myocardin transactivation of cardiac genes and the identification of a microRNA sufficient to induce pathological cardiac remodeling and necessary for proper cardiac conduction. Conclusions and potential future directions to extend those findings are summarized below.

BMP SIGNALING AND MYOCARDIN

The BMP signaling transduction pathway, where Smad1 plays an important role in transmitting signals from the plasma membrane to the nucleus, is essential for cardiac development (1). Since Smad proteins are ubiquitously expressed, it is not clear how the BMP signaling pathway specifically regulates cardiac development. As described in Chapter 2, my studies demonstrate myocardin transactivation of cardiac gene expression is modulated by BMP signaling through a protein-protein interaction between myocardin and BMP downstream effector Smad1, providing another novel mechanism in which myocardin is integrated into an important signaling pathway to regulate gene expression. Intriguingly, BMP signaling was also able to induce expression of myocardin, suggesting a potential positive feedback mechanism.

These results suggest myocardin may serve as a cardiac-specific cofactor of Smad1 to convey the BMP signaling pathway to cardiac lineages

Key Results

- Smad1 enhances myocardin transactivation of cardiac gene expression.
- SRF binding site CArG is necessary for such interaction, whereas Smad Binding Elements are not.
- Myocardin and Smad1 directly interact, and such physical interaction is important for their synergistic activation of cardiac promoters.
- BMP treatment induces myocardin expression in cardiomyocytes.

Future Directions

In order to better define the molecular nature of myocardin-Smad1 interaction, systematic mapping of the regions/domains of myocardin and Smad1 mediate their association could achieved using a combination of coimmunoprecipitation and GST fusion protein pull-down assays. Site-directed mutagenesis could also be applied to further define the essential domains/residues required for these protein-protein interactions.

The role of Smad1 phosphorylation in the interaction between Smad1 and myocardin is another important issue for further investigation. Smad1 is phosphorylated directly by BMP receptors on serine residues in its carboxy-terminus and becomes transcriptional active. Mutations of those serine residues are reported to prevent Smad1 accumulation in the nucleus and loss of its transcriptional activity (2). Does Smad1 phosphorylation play a role in the synergistic activation of cardiac

genes by myocardin and Smad1? To address this question, phospho-defective Smad1 mutants could be tested for functional and/or physical interaction with myocardin using coimmunoprecipiation and luciferase reporter assays.

It is not yet known whether the Smad1 and myocardin interaction is BMP-responsive. This might be determined through coimmunoprecipation assays that assess whether the endogenous physical interaction between Smad1 and myocardin is increased by BMP treatment in cardiomyocytes.

Finally and most crucially, the biological relevance of the molecular interaction between myocardin and Smad1 is unknown. This issue could be resolved in mouse models using gene replacement strategies to introduce specific mutations into myocardin and/or Smad1 at sites found necessary for their physical and functional interaction *in vitro*. If myocardin is indeed a tissue-specific interpreter of BMP signaling important for cardiac gene expression during cardiogenesis, such genetic dissection of the Smad1-myocardin interaction would provide much-needed insight into the mechanisms underlying how extracellular signals initiated by cytokines or growth factors can activate gene expression programs in a tissue/organ-specific manner.

MIR-208 AND ITS REGULATORY ROLES IN THE HEART

miRNAs are an evolutionarily conserved class of small noncoding RNAs known to regulate translation of target messenger RNAs in animals (3). Hundreds of miRNA genes exist within the mammalian genomes and roles for miRNAs are suggested in remarkably diverse biological processes, however most miRNAs remain

uncharacterized. As described in Chapters 4 and 5, gain- and loss-of-function mouse models were developed to study the role of miR-208a in the heart. Analyses of these mice suggest the miR-208 family act as a fulcrum in the balance between normal and hypertrophic growth. Furthermore, we found miR-208a necessary for maintenance of cardiac conduction in the adult mouse heart.

Key Points

- The miR-208 family, consisting of miR-208a and miR-208b, are differentially expressed during heart development, paralleling the expression of their respective host genes αMHC and βMHC. The expression of miR-208a and miR-208b expression are co-regulated with their host genes in response to hormonal signaling and pathological stress.
- Cardiac overexpression of miR-208a induced hypertrophic growth and cardiac arrhythmias, while its genetic deletion showed miR-208a necessary for proper cardiac conduction.
- Overexpression of miR-208a induced βMHC and miR-208b expression, as well as gap junction proteins Cx40 and Cx43. Loss of miR-208a resulted in lowered βMHC, Cx40, and Cx43 expression, providing complementary genetic evidence that miR-208a is a regulator of those genes.
- Both miR-208a and miR-208b share similar sequence identity and appear to repress the translation of the same regulatory targets, which include Thrap1, myostatin, and potentially GATA4 and L-type calcium ion channel subunit CACNB2.

- Taken together, these studies indicate miR-208a is an important regulator of gene expression in the adult heart and is required for proper heart function.

Future Directions

Does the miR-208 family have a developmental function?

A burning question remaining for the miR-208 family is whether it has a role in the developing heart. In the mouse heart, αMHC and βMHC expression starts during formation of the linear heart tube, but during αMHC expression becomes restricted to atria during morphogenesis while βMHC is expressed throughout the embryonic heart (4). Thus, it is not entirely surprising these studies found miR-208b is the predominant miR-208 family member expressed in the developing heart (**Fig. 6.1**). Gene targeting studies that delete miR-208b from its host intron within the βMHC gene should be undertaken to identify its potential roles during development. Is miR-208b necessary for proper heart development? In the adult heart miR-208a is required for stress-induced cardiac remodeling but is not required under basal conditions. It's difficult to propose a firm hypothesis for miR-208b function from those results since the gene expression programs and dynamic processes occurring in developing versus adult hearts are different (5, 6). The proposed roles for miRNAs in gene expression regulation include fine-tuning the expression of targeted mRNAs that produce protein products important for a particular tissue and for reducing transcriptional noise by helping turning over 'mis-expressed' mRNAs (7-10). Microarray analysis for mRNA expression using miR-208a null hearts by the Olson laboratory found particular skeletal muscle genes were up-regulated in cardiac

tissue in the absence of miR-208a (11). The mRNAs of those up-regulated genes don't harbor predicted miR-208a binding sites, indicating the up-regulation of those genes is a secondary effect. This result suggests miR-208a is responsible for fine-tuning expression of a particular transcriptional network component important for differentiating between skeletal versus cardiac gene expression in the adult heart. It would be interesting to identify that targeted component and also test whether miR-208b plays a similar role in the developing heart.

How does miR-208a regulate βMHC expression?

An interesting finding from the studies described within this dissertation was the up-regulation of βMHC expression in the hypertrophic miR-208a transgenic hearts, but not of the cardiac hormone ANF, which is often up-regulated in hypertrophic hearts. The specific up-regulation of βMHC expression suggests miR-208a is involved in a regulatory network specific for cell growth, rather than activating a more general hypertrophic pathway involving ANF. The basal level of βMHC expression was found reduced in miR-208a null hearts, providing further convincing genetic evidence that miR-208a is important for βMHC expression. Furthermore, one of the direct targets identified for miR-208a is a component of the thyroid hormone signaling pathway, which is known to negatively regulate βMHC gene expression through cis-acting promoter elements.

Is Thrap1 the critical miR-208a target responsible for changes in βMHC expression? One might address this using a gene replacement strategy that destroys the two miR-208a binding sites in the Thrap1 3' UTR. If Thrap1 is a critical

regulator of βMHC expression and is 'fine-tuned' by miR-208a, then deletion of miR-208a binding sites should have the same effect as miR-208a deletion. Our current model predicts that deleting the miR-208a binding sites in Thrap1 would raise Thrap1 protein levels and decrease βMHC expression. Destruction of those binding sites is also expected to relieve Thrap1 repression observed in miR-208a Tg hearts and result in normal βMHC expression levels.

If the hypothesis that Thrap1 is the critical mediator of miR-208a-mediated βMHC expression regulation is shown false, then an unidentified regulator of βMHC and miR-208a target likely exists. The identification of biologically relevant targets is a big issue facing the miRNA field as most researchers currently rely on bioinformatic predictions, which contain many false positives but also might miss many relevant target sites as the current algorithms search only 3' UTRs instead of the full-length mRNA sequences. The utility of mRNA microarrays to identify targets is also somewhat limited as miRNAs may repress the translation of, rather than mediate the degradation of, their targeted mRNAs. As discussed in Chapter 3, it's hoped a direct and facile method to identify miRNA target genes, possibly employing a proteomics-based strategy or from functional screening of cDNA libraries composed of 3' UTRs of regulatory target genes, will become available and advance the miRNA field.

Why does βMHC up-regulation in the miR-208a transgenic hearts occur predominately in regions of fibrosis? The correlation of βMHC expression and fibrosis was recently described in other animal models of cardiac hypertrophy, thus the phenomenon is not unique to miR-208a-induced hypertrophy. The molecular

mechanisms and physiological significance of this correlation is unclear. Chronic βMHC transgene expression in adult cardiomyocytes alone does not led to fibrosis (12), while stretch-induced stress of isolated cardiomyocytes is sufficient to stimulate βMHC in the absence of fibrosis (13). The localized structural remodeling caused by fibrosis that surrounds cardiomyocytes changes how those cardiomyocytes are normally stretched during contraction and might help explain the stimulus for βMHC up-regulation in fibrotic areas. The observation that βMHC expression occurred in specific regions within miR-208a transgenic hearts suggests that βMHC up-regulation is a secondary effect as a result of hypertrophic growth and the accompanying fibrosis (**Fig. 4.4**). On the other hand, genetic deletion of miR-208a decreased the basal level of βMHC expression (**Fig. 4.10**), and argues that miR-208a regulation of βMHC does not result solely from fibrosis. Furthermore, overexpression of miR-208a in isolated cardiomyocytes increased βMHC expression in the absence of fibrosis (**Fig. 4.3**). It appears that, at least *in vivo*, ubiquitous miR-208a overexpression is sufficient to induce hypertrophic growth, but not βMHC expression, throughout the heart. This hypertrophic growth results in fibrotic lesions and the cardiomyocytes in those areas consequentially up-regulate βMHC expression because of mechanical stress. Although miR-208a overexpression alone is insufficient to ubiquitously up-regulate βMHC expression *in vivo*, miR-208a is required to maintain basal βMHC expression in the adult mouse as demonstrated by miR-208a null hearts (**Fig. 4.10**). Future studies, perhaps utilizing laser dissection to facilitate the molecular analysis of individual cardiomyocytes specifically in fribrotic

regions, are clearly needed to better characterize the βMHC-fibrosis phenomenon and may provide additional insight into miR-208a regulation of βMHC expression.

Do miR-208 expression levels and distribution allow for a threshold model?

The miR-208 regulation of hypertrophy threshold model presented in Chapter 4 proposed that miR-208a fine-tunes anti-hypertrophy gene expression during basal conditions, and that βMHC and its hosted gene miR-208b increases during cardiac hypertrophy and assists in the repression of anti-hypertrophy genes (**Fig. 4.10**). The threshold model stipulates that miR-208a and miR-208b cooperatively promote hypertrophic growth by repressing anti-hypertrophy gene expression. Two conditions that determine whether this threshold model is viable are 1) the ratio of miR-208 molecules to target mRNA molecules and 2) whether the miR-208 molecules and target mRNA molecules are present in the same cells. Regarding the first issue, the average number of miR-208a or miR-208b molecules per heart cell can be estimated by quantitative RT-PCR, as could the number of mRNAs of a particular target gene (14). However, determining how many genes are targeted is remains difficult since the miRNA field currently relies on imperfect prediction algorithms to locate potential miRNA target sites, as discussed in Chapter 3. It's also worth noting that most predicted target genes targeted by multiple miRNAs. Despite those confounding combinatorial issues, one might predict that if miR-208a molecules targets Thrap1 and several other genes under basal conditions, miR-208a is likely expressed at a higher copy number than any single targeted mRNA. Because miR-208a is co-expressed with a core component of the sarcomere (αMHC), it's not

surprising our northern blot analyses show abundant miR-208a expression in the adult heart during basal conditions. The high expression level suggests miR-208a is present in sufficient quantity to repress or 'fine-tune' expression of many target genes. During hypertrophy, the increase of miR-208b levels is relatively low compared to pre-existing miR-208a levels, however it's formally possible this increase is sufficient to 'tip the balance' of miR-208a and miR-208b molecules to mRNA target molecules towards repression rather than maintenance. The second issue for the threshold model mentioned above is whether miR-208 molecules and target mRNA molecules are present in the same cells. The issue is important since the heart is not a homogeneous organ. αMHC and therefore miR-208a are ubiquitously expressed throughout cardiomyocytes in the adult mouse heart. In contrast, we observe that βMHC and miR-208b expression occurs in discrete regions associated with fibrosis during hypertrophy, rather than ubiquitously throughout the myocardium. Since hypertrophic growth occurs throughout the myocardium, this focal expression pattern of miR-208b argues against the threshold model since total miR-208 levels are not raised to enable cooperative repression of anti-hypertrophy gene expression in all hypertrophic cardiomyocytes. Instead of the threshold model, does miR-208a instead act to buffer anti-hypertrophy gene expression during basal conditions? Interestingly, mouse hearts that lack miR-208a are unable to respond hypertrophic stimuli (11), suggesting that post-transcriptional regulation by miR-208a is critical for repression of the anti-hypertrophy program. Given that mature miRNAs are a component of a multi-protein complex, it would be interesting to test whether miR-208a activity is subject to hypertrophic signaling; i.e.

does hypertrophy stimuli increase the ability of pre-existing miR-208a to repress target gene expression?

Could miR-208a be therapeutically targeted to modulate βMHC expression?

The up-regulation of βMHC that occurs during cardiac disease reduces contractile performance and is thought be a maladaptive response (12, 15, 16). The shift towards βMHC is reversible under particular conditions that are associated with improved cardiac performance, including the regression of hypertrophy and in human patients that respond favorably to beta-blocker therapy (17-21). Thus it is intriguing to speculate reducing miR-208 family levels may inhibit the maladaptive features of hypertrophy, like βMHC expression, and improve function of the diseased heart. In support of this notion, the Olson laboratory reported miR-208a null hearts fail to undergo hypertrophic growth or up-regulate βMHC expression in response to pressure overload-induced hypertrophy (11). A remaining question is whether reducing miR-208 family levels reverses the hypertrophic growth process and two potential strategies are conceivable to answer it. The first strategy might inhibit endogenous miR-208 family members with chemically-modified oligonucleotides antisense to miR-208a and/or miR-208b delivered through an osmotic minipump implanted into the mouse heart. This approach was previously reported for miR-133 inhibition in the mouse heart (22). In this manner, whether sustained delivery of miR-208 inhibitors regresses hypertrophic growth could be tested in a banded heart hypertrophy model. A second strategy might employ an inducible gene deletion system in which the miR-208a and/or miR-208b alleles are immediately flanked by

LoxP sequences. Introduction of these floxed miR-208 alleles into a drug-inducible Cre recombinase mouse line would create a power genetic tool to test the requirement for miR-208 in sustaining hypertrophic growth. Evidence from either strategy demonstrating a requirement for miR-208 in hypertrophic growth maintenance would prompt further investigation of miR-208 as a novel therapeutic target in the fight against heart disease.

How does miR-208a regulate of cardiac conduction?

As discussed in Chapter 5, I identified miR-208a as an important regulatory molecule necessary for proper cardiac conduction in the adult mouse heart. miR-208a modulates the expression of gap junction proteins Cx40 and Cx43, whose misexpression are associated with cardiac arrhythmias. However, the precise molecular mechanisms underlying miR-208a mediated regulation of cardiac conduction system components are not yet clear. I believe the answer will likely be found by identifying cardiac transcription factors whose expressions are post-transcriptionally fine-tuned miR-208a. Indeed, we already found GATA4 and Hop, well-known regulators in the cardiac conduction system, are respectively up-regulated and down-regulated in miR-208a$^{-/-}$ hearts. The 3'UTR of GATA4 harbors a predicted miR-208a binding site and further studies are needed to confirm whether miR-208a directly targets that site. In support of miR-208a-mediated post-transcriptional regulation, we found GATA4 protein levels, but not transcript levels, are down-regulated in miR-208a Tg hearts. Interestingly, Hop appeared transcriptionally down-regulated in miR-208a$^{-/-}$ hearts, as both transcript and protein

levels were lower compared with wild type hearts. Analysis of Nkx2.5, a well-known cardiac conduction system transcriptional regulator, may explain the change in Hop expression (23). Genetic deletion of Nkx2.5 in the adult mouse heart results in AV block and down-regulation of Hop expression (24). Nkx2.5 binds the Hop promoter and transactivates its expression (25). Although Nkx2.5 is not a predicted direct miR-208a target, Nkx2.5 transcription is directly regulated by GATA4 (26), suggesting that miR-208a might indirectly fine-tune Nkx2.5 expression via post-trancriptional repression of GATA4. This implies that miR-208a post-transcriptional regulates GATA4 expression, which in turn affects Nkx2.5 and Hop expression. In this way, altering the expression of miR-208a would affect the delicate regulation of potent cardiac transcription factors and result in the dramatic phenotypes observed in the miR-208 transgenic and knockout mouse lines. Additional studies are needed to test and further define this intriguing possibility.

Why does miR-208a-mediated repression of Hop expression not fully recapitulate the $Hop^{-/-}$ phenotype? Inactivation of mouse Hop resulted in partially penetrant embryonic lethality with developmental cardiac defects (25). Mice that survive to adult display conduction defects with decreased Cx40 expression, whereas Cx43 expression is unaffected (27). In $miR\text{-}208a^{-/-}$ mice, no embryonic lethality is observed, Hop expression is absent in adult hearts, conduction defects are present and both Cx40 and Cx43 are down-regulated. What might account for these differences? Hop is expressed throughout the developing heart, but is restricted to the cardiac conduction system in the adult heart (25, 27). As described in Chapter 4, miR-208b is highly expressed in the developing heart, while miR-208a

expression is activated shortly after birth. Thus, presumably deletion of miR-208a would not affect Hop expression during development, which helps to account for the lack of embryonic lethality in miR-208a$^{-/-}$ mice. In adult mouse hearts, Hop and Cx40 are expressed in the specialized myocytes of the cardiac conduction system, while αMHC/miR-208a and Cx43 are expressed throughout the myocardium. Thus, the loss of miR-208a might affect both Hop and Cx40 in the cardiac conduction system and Cx43 in the working myocardium, further accounting for the phenotypic differences observed between Hop and miR-208a mice. It would be interesting to test whether genetic deletion of miR-208b recapitulates the embryonic phenotype observed in Hop$^{-/-}$ mice.

What's the consequence of reduced Hsp70 expression in miR-208a transgenic hearts? Hsp70 proteins are important for protein quality control, including monitoring proper protein folding and mediating protein degradation (28). Hsp70 transcript and protein levels are reduced in adult miR-208a transgenic hearts (**Fig. 5.3**). Hsp70 was previously shown to mediate connexin protein turnover, therefore reduced Hsp70 expression may partially account for the increased connexin protein levels observed in miR-208a transgenic heart (29). However, genetic deletion of miR-208a lowered connexin protein levels without affecting Hsp70 expression, providing complementary genetic evidence that miR-208a is a regulator of connexin expression. It's presently not clear whether decreased Hsp70 levels is an obligate component of increased connexin expression in miR-208a transgenic hearts, but this might be addressed by simultaneously over-expressing Hsp70 and miR-208a in the adult mouse heart. Is reduced Hsp70 expression in miR-208a transgenic hearts

responsible for the hypertrophic phenotype? Although adult Hsp70$^{-/-}$ mice do present a mild hypertrophic phenotype (30), miR-208a transgenic hearts suffer a more severe hypertrophic phenotypic with a relatively minor reduction in Hsp70 expression. This indicates reduced Hsp70 expression is not the critical factor for the hypertrophic growth observed in miR-208a transgenic hearts. Hsp70 is cardioprotective during ischemic injury (28), so it might be interesting to test whether miR-208a overexpression and the subsequent reduction in Hsp70 expression confers more susceptibility to ischemic injury. Given the growing interest within the biomedical research community to pursue miRNAs as therapeutic targets, knowledge of such potential 'off-target' effects will become very important.

How many roles for miR-208 in the heart?

Although the research presented in this dissertation focused on miR-208 in cardiac hypertrophy and cardiac conduction, and reported that miR-208 post-transcriptionally regulates the expression of Thrap1, myostatin, and CACNB2, it's very likely that miR-208a regulates the expression of other genes and is involved in other physiological processes. In support of miR-208a having additional roles, bioinformatic algorithms predict target sites for miR-208a targets in over 140 genes (31, 32). To gain insight into the potential roles for miR-208a using those predictions, I first removed genes of unknown function and excluded genes exclusively expressed outside of muscle tissue from the pool of predicted targets. The remaining 90 predicted target genes are associated with diverse fundamental processes including gene expression, metabolism, and signal transduction (**Fig.**

6.1). Strikingly, 35% of the miR-208a predicted genes are linked to transcription and include the cardiac transcription factors GATA4 and SP3. It is intriguing to speculate that miR-208a may influence the expression critical transcription factors to invoke potent biological responses. The Wang laboratory is currently closely examining the predicted pool of miR-208a targets and experimentally determining which are biologically relevant. Whatever the precise molecular mechanisms underlying miR-208a function, it's clear that miR-208a is a component of very complex genetic network work important for proper heart function and will take much effort to fully understand.

Figure 6.1

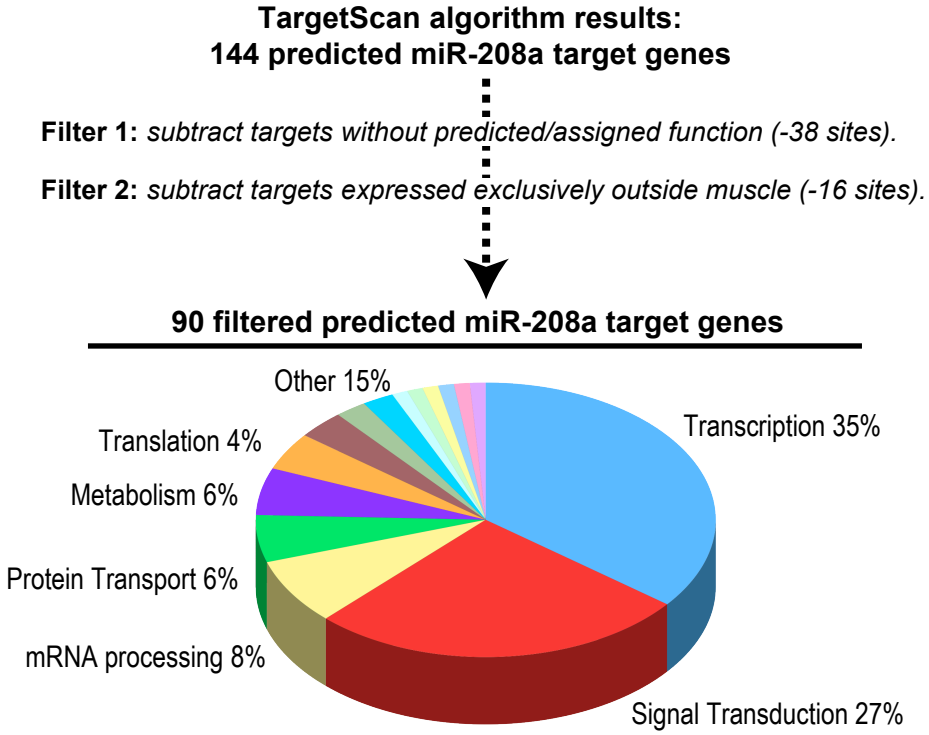

Figure 6.1. Target predictions suggest diverse roles for miR-208a. Predicted target genes for miR-208a were obtained electronically from the TargetScan database. The 144 predicted target genes were classified by gene ontology (GO) using the Open Biomedical Ontologies database and expression patterns were found in published literature or determined using the National Center for Biotechnology Information gene expression omnibus (GEO) database. The predicted target gene pool was filtered by two criteria: 1) 38 predicted target genes were excluded because they lacked assigned or predicted function. 2) 16 predicted target genes were excluded because they are reportedly expressed outside of muscle tissue and presumably play no role in the heart. The remaining 90 predicted target genes are represented in the pie chart by gene ontology classification.

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