miRNAs and Their Emerging Role in Cardiac Hypertrophy

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Abstract MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate gene expression by inhibiting translation or promoting de-gradation of targeted messenger RNAs (mRNAs). More than one-third of human protein-coding mRNAs are predicted as being regulated by miRNAs, which have been implicated in processes as diverse as cancer and muscle biology. Now, recent studies demonstrate that the expression profiles of many miRNAs change during cardiac hypertrophy. Most importantly, both gain- and loss-of-function studies have established a clear functional correlation between miRNAs and cardiac hypertrophy. This previously unrecognized relationship sheds new light onto the regulatory mechanisms underlying the heart's response to pathological stress and suggests the potential of miRNAs as therapeutic targets for cardiovascular disease.

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1 Introduction

Cardiac hypertrophy is the primary cellular response of the heart to stress caused by pathological and physiological hemodynamic overload, abnormal hormonal signaling, and certain inherited disorders involving mutations in particular transcription factors or contractile proteins (Hunter and Chien 1999; Frey and Olson 2003). The hypertrophic growth response sustains cardiac output in the face of such stress and involves numerous changes including enhanced protein synthesis, increased sarcomeric density and increased size of cardiomyocytes. Re-activation of several cardiac fetal genes is also a well-established change accompanying hypertrophic growth. Although cardiac hypertrophy induced by pathological stimuli is considered an adaptive mechanism to sustain cardiac output, prolonged hypertrophy has adverse consequences associated with heart failure and sudden death. Much effort has been expended by the cardiovascular research community to map and understand the complex genetic pathways required for hypertrophy, with the ultimate goal of acquiring new therapeutic strategies to improve both cardiac output and heart patient prognosis.

Recent studies have linked a novel class of small regulatory RNAs, known as microRNAs (miRNAs), to cardiac hypertrophy, and offer new insight into the regulation of this disease process. More than 600 human miRNA genes have been identified, many of which are evolutionarily conserved, and perhaps many more miRNA genes await discovery (Griffiths-Jones 2004; Bentwich et al. 2005; Landgraf et al. 2007). miRNAs are now known to play roles in remarkably diverse processes, but given the prevalence of these molecules, relatively few specific miRNAs have any precise biological functions assigned. Interestingly, the expression profiles for a number of miRNAs change during cardiac hypertrophy. Furthermore, mis-expression of miRNAs, as well as loss-of-function experiments in mice, demonstrate that specific miRNAs can augment or attenuate the hypertrophic growth response and suggest the potential of these molecules as novel therapeutic targets. Here, we review the biology of miRNAs and their emerging roles in cardiac hypertrophy.

2 Biology of miRNAs

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 (Lee et al. 1993; Wightman et al. 1993). 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 (Meister and Tuschl 2004). 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: siRNAs arise from foreign dsRNA, whereas miRNAs are genomically encoded and undergo more extensive post-transcriptional processing than siRNAs (Denli et al. 2004; Gregory et al. 2004). 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, and tumorigenesis, and recently have been linked to cardiac hypertrophy and disease (Ambros and Chen 2007; Chien 2007; Mann 2007).

2.1 miRNA Biogenesis and Mechanism of miRNA Function

Mature miRNAs are approximately 18–24 nucleotides (nt) in length and their biogenesis begins with the transcription of primary-miRNA (pri-miRNA) (Bracht et al. 2004; Lee et al. 2004). RNA polymerase II transcribes pri-miRNAs as 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 (Bartel 2004). In addition to independent transcriptional units, some miRNAs originate from the introns of mRNA transcripts (Bartel 2004). The pri-miRNA 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-nt-long intermediate precursor-miRNA (pre-miRNA) whose hallmark is a stem-loop-like structure and a staggered cut left by microprocessor cleavage at the stem-loop base (Lee et al. 2003; Han et al. 2006). Exportin-5 recognizes the staggered cut and exports the pre-miRNA to the cytoplasm in a Ran-GTP-dependent manner (Yi et al. 2003; Lund et al. 2004). Once the pre-miRNA is cytoplasmic, Dicer, another RNAase III endonuclease, cleaves both stem arms of the pre-miRNA to generate the mature miRNA duplex (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). A single stem of the resulting ~22-nt duplex is incorporated into the RNA-induced silencing complex (RISC), while the other stem arm is presumably degraded. Which stem-arm is incorporated into RISC is thought to be determined, at least in part, by thermal instability: the stem-arm

with the weakest hydrogen bonding at its 5′ end is usually incorporated (Khvorova et al. 2003; Schwarz et al. 2003).

RISC is the ribonucleoprotein effector complex for miRNA-mediated gene expression regulation and consists of Argonaute protein family members and accessory factors such as R2D2, along with a miRNA and targeted mRNA (Hammond et al. 2000; Filipowicz 2005). Regulation of target gene expression by RISC is facilitated by miRNA complementary base pairing to target sequence(s) within the 3′ UTR of target mRNAs by a mechanism that is not yet fully understood (Doench and Sharp 2004; Pillai et al. 2004). Generally in animals, perfect or near-perfect complementary base pairing between RISC-bound miRNA and targeted mRNA results in immediate mRNA cleavage by Argonaute proteins, the catalytically-active components of RISC. However, the vast majority of miRNAs in animal are imperfectly complementary to their targeted mRNAs, which results in suppression of translation and subsequent mRNA degradation (Bartel 2004; Bagga et al. 2005).

Interestingly, miRNA-mediated translational repression and/or mRNA degradation has been connected to discrete cytoplasmic foci called processing bodies (P-bodies) (Liu et al. 2005a,b; Rossi 2005; Sen and Blau 2005; Chan and Slack 2006). P-bodies are sites of programmed mRNA degradation, where enzymes required for RNA turnover and RNA degradation intermediates accumulate. Argonaute proteins also localize to P-bodies, as well as miRNAs and their targeted mRNAs, indicating that miRNAs may mediate translation suppression and mRNA degradation by directing targeted mRNAs to P-bodies. Disruption of P-bodies in cells decreases the efficiency of RNAi, suggesting that they are an important component of the RNAi pathway (Jakymiw et al. 2005). Moreover, release of mRNAs targeted by miRNAs from P-bodies and subsequent re-expression of those mRNAs suggests that P-bodies may also function as mRNA storage centers (Bhattacharyya et al. 2006). 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.

2.2 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 (Lagos-Quintana et al. 2002, 2003). 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 (Lim et al. 2003; Bentwich et al. 2005). 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 (Mansfield et al. 2004; Shi and Chiang 2005; Wienholds et al. 2005). Recently, a comprehensive sequencing of over 250 small RNA libraries revealed additional new miRNAs and documented the expression patterns of most miRNAs (Landgraf et al. 2007).

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 (Thomson et al. 2004; Baskerville and Bartel 2005; Chen et al. 2006; van Rooij et al. 2006, Tatsuguchi et al. 2007). 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 (Kloosterman and Plasterk 2006), implying that miRNAs may participate in a variety of biological processes.

2.3 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 (Lewis et al. 2003; John et al. 2004; Kiriakidou et al. 2004; Rajewsky and Socci 2004; Krek et al. 2005; Zhao et al. 2005; Grimson et al. 2007). 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 (Zhao et al. 2005; Grimson et al. 2007). 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 (Zhao et al. 2005; Grimson et al. 2007). 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.

3 miRNAs in Cardiovascular Development

The heart is the first organ to form and function during mammalian development (Srivastava and Olson 2000; Olson 2006). Cardiac progenitor cells are derived from the embryonic mesoderm, and they undergo cellular proliferation, differentiation and migration to eventually generate a mature, functional four-chambered heart. The molecular mechanisms that control cardiogenesis are not completely understood. Heart development and pathology are intimately linked to the regulation of complex genetic pathways, and much effort has been expended in attempt to understand these pathways. Most studies have focused on the role of transcription factors and regulatory enhancer sequences required for cardiac gene transcription. The regulation of cardiac gene expression has proved quite complex, with individual cardiac genes being controlled by multiple independent enhancers that direct very restricted expression patterns in the heart. Recent studies of 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.

3.1 Global Analysis of miRNA Function During Animal Development

A popular approach to determine the necessity of miRNAs in animal development has been to genetically ablate Dicer function, an endonuclease required for miRNA biogenesis. Dicer is encoded by a single locus in vertebrates and is required to fully process all vertebrate miRNAs to their mature, active form (Hutvagner et al. 2001; Ketting et al. 2001; Bernstein et al. 2003; Wienholds et al. 2003). In mice, abolishing Dicer function resulted in lethality by embryonic day 7.5 (Bernstein et al. 2003). The *Dicer* null mice did not express primitive streak marker *T(brachyury)*, indicating that development was likely arrested before the body plan was configured during gastrulation. Even though Dicer is required for RNAi (Hutvagner et al. 2001; Ketting et al. 2001), it is generally believed that the Dicer loss-of-function phenotype reflects the essential function of miRNAs since little RNAi activity is thought to be involved during embryogenesis. In zebrafish, zygotic *Dicer* mutants lived for almost 2 weeks before dying (Wienholds et al. 2003). Their prolonged survival was attributed to maternally contributed Dicer protein, prompting the creation of maternal-zygotic *Dicer* mutants, which revealed that loss of all miRNAs results in morphogenesis defects during gastrulation and early lethality. Somitogenesis, heart development, and brain formation all proved abnormal in the maternal-zygotic zebrafish *Dicer* mutants (Giraldez et al. 2005). These animal studies of global miRNA function by *Dicer* deletion indicate that miRNAs are important for early development. However, they were unable to address the roles of miRNAs during later development.

To circumvent the early embryonic lethality of the *Dicer* null mouse mutants and study the role of miRNAs in later development, Harfe et al. created a conditional *Dicer* knockout model to study the effect of tissue-specific *Dicer* deletion (Harfe et al. 2005). In this model, *loxP* sites were inserted around an exon encoding the majority of an RNAseIII domain required for miRNA processing. Upon mating the floxed *Dicer* mice to mice expressing Cre recombinase in specific tissues, the floxed exon is excised and miRNA processing inhibited in those tissues (Harfe et al. 2005; Andl et al. 2006; Harris et al. 2006; Yi et al. 2006). The Cre-inducible conditional *Dicer* model has revealed that Dicer function is essential for proper morphogenesis of the vertebrate limb, as well as the skin and lung (Harfe et al. 2005; Andl et al. 2006; Harris et al. 2006; Yi et al. 2006). In addition, Dicer was recently shown to play a critical role in female germline development (Murchison et al. 2007; Tang et al. 2007).

Recently, Zhao and colleagues applied the conditional *Dicer* mouse approach to examine the role of miRNAs in the developing heart (Zhao et al. 2007). Using mice expressing Cre recombinase controlled by the *Nkx2.5* promoter region that directs expression in cardiac progenitors as early as embryonic day (E) 8.5, they report that deletion of *Dicer* specifically in the heart results in death by E 12.5. The *Dicer* mutant mice suffered from cardiac failure and exhibited a range of developmental defects during morphogenesis, including pericardial edema and poorly developed ventricular myocardium. The early lethality in the *Dicer* mutant indicates that miRNAs are required for proper heart development. However, deletion of *Dicer* occurred after gastrulation, leaving questions about the importance of miRNAs in cardiac specification and patterning. Meanwhile, because cardiac-specific *Dicer* mutant mice die during embryogenesis, it is not clear what are the roles of miRNAs in the postnatal and adult hearts.

Collectively, the *Dicer* deletion studies demonstrate that miRNAs play an essential role in animal development. However, since *Dicer* deletion inhibits the processing of all miRNAs, those studies have not provided insight into the precise functions of specific miRNAs. The tissue-specific expression patterns of certain miRNAs, including miRNAs expressed solely in muscle tissues, allude to specific biological roles in those tissues.

3.2 Regulation of Muscle miRNA Expression

Several microRNA genes are specifically expressed or highly enriched in skeletal and/or cardiac muscle, so-called “muscle miRNAs.” These include miR-1, miR-133, miR-206, and miR-208. Thus far, the expression of muscle miRNAs appears largely regulated by well-established muscle transcriptional networks involving SRF, MyoD, Twist, MEF2, and myocardin (Sokol and Ambros 2005; Zhao et al. 2005; Chen et al. 2006; Rao et al. 2006). For example, miR-1 and miR-133 are expressed solely in skeletal and cardiac muscle tissues by two genes that encode polycistronic transcripts for miR-1 and miR-133. That is to say, miR-1-1 and

miR-133a-2 are clustered on mouse chromosome 2, while miR-1-2 and miR-133a-1 are clustered on chromosome 18 (Chen et al. 2006). Promoter analyses demonstrate that both mouse chromosome 2 and chromosome 18 clusters contain upstream enhancers with SRF binding sites, and that myocardin activity increases the expression of those promoters (Zhao et al. 2005; Chen et al. 2006). 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 (Sokol and Ambros 2005; Zhao et al. 2005).

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 (Rodriguez et al. 2004; Baskerville and Bartel 2005; Kim and Kim 2007; van Rooij et al. 2007). Whereas several muscle miRNAs are expressed in both skeletal and cardiac muscle tissues, miR-208 is a heart-specific miRNA that is encoded by an intron of the cardiac muscle α-*myosin heavy chain* (α-*MHC*) gene, whose transcription is largely controlled by thyroid hormone signaling (Lompre et al. 1984; Lagos-Quintana et al. 2003; van Rooij et al. 2007). The expression of miR-208 mirrors the expression pattern of its host gene *α-MHC* during development, suggesting that miR-208 and α-MHC expression are regulated by a common element (van Rooij et al. 2007).

These studies suggest the tissue-specific expression of muscle miRNAs is largely regulated at the transcriptional level. They also suggest that tight temporal and spatial regulation of miRNA expression is important for their function. Interestingly, regulated pre-miRNA processing has been proposed as an alternative mechanism to post-transcriptional control miRNA function (Obernosterer et al. 2006; Thomson et al. 2006). Future studies on both the transcriptional and post-transcriptional mechanisms controlling miRNA expression are needed to provide greater insight into the role of miRNA-mediated regulation in cardiac genetic networks important for human disease. Providing insight into the role of miRNAs during heart development, genetic studies of individual miRNAs expressed specifically in muscle tissues have been undertaken.

3.3 Genetic Studies of Specific miRNAs Reveal Roles in Cardiac Development and Function

Conditional deletion of *Dicer* in the heart revealed a requirement for miRNAs during heart development by downregulating the expression of potentially hundreds of miRNAs. To begin assigning biological roles to individual miRNAs, several groups have undertook gene deletion studies of muscle-specific miRNAs and identified distinct roles for miRNAs in cardiac development and function (Fig. 1)

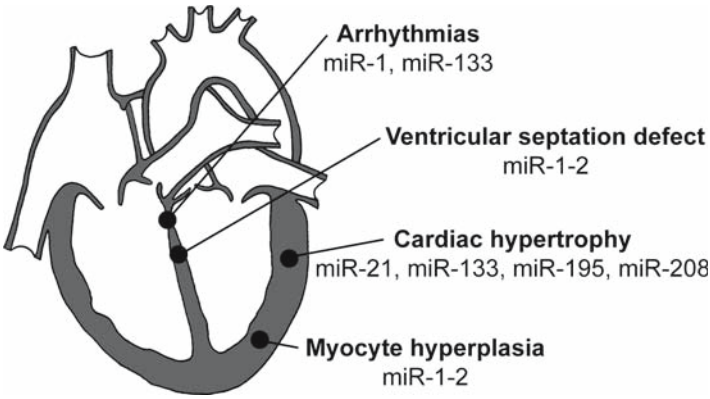

Fig. 1 Roles of miRNAs in heart development and function. The several miRNAs shown are associated with various cardiac abnormalities affecting heart development and function. Arrhythmias relate to electrophysiological defects that result in an irregular heartbeat. Defects in ventricular septation are characterized by an abnormal opening between the cardiac ventricles that allows blood to pass directly from the left to the right ventricle, thereby reducing the efficiency of cardiac output. Cardiac hypertrophy is the primary response of the heart to increased cardiac load and involves increased size of ventricular myocytes. In contrast, the number of myocytes increases during hyperplasia

In *Drosophila,* miR-1 is encoded by a single gene located on chromosome 2. Gene deletion of *Drosophila* miR-1 revealed miR-1 as an essential gene required for viability (Kwon et al. 2005; Sokol and Ambros 2005). Without miR-1, homozygous mutant larvae exhibited decreased locomotion that ultimately progressed to death accompanied by severe gross disruption of the larval musculature (Sokol and Ambros 2005). A subset of severely affected miR-1 null embryos exhibited an enlarged pool of cardiac progenitors, suggesting that miR-1 may modulate differentiation of heart (Kwon et al. 2005). Re-introduction of miR-1 into developing muscle partially rescued the mutant phenotype, strongly supporting a muscle-specific role for miR-1 (Kwon et al. 2005; Sokol and Ambros 2005).

Consistent with the role for miR-1 in muscle differentiation, over-expression of miR-1 in the developing mouse heart resulted in reduced ventricular myocyte expansion and decreased the number of proliferating myocytes (Zhao et al. 2005). This phenotype was explained in part by the presence of a miR-1 target site in the 3′ UTR of the Hand2 cardiac transcription factor (Zhao et al. 2005), whose genetic ablation in the mouse produced a similar failure in ventricular myocyte expansion (Srivastava et al. 1997). In agreement with this notion, over-expression of miR-1 in the mouse heart decreased Hand2 protein levels (Zhao et al. 2005). Similarly, introduction of miR-1 into *Xenopus* embryos interfered with heart development (Chen et al. 2006).

Further extending the analysis of miR-1 function in vertebrates, Zhao and colleagues targeted mouse miR-1-2, one of two miR-1 genes expressed in skeletal and cardiac muscle (Zhao et al. 2007). miR-1-1 and miR-1-2 are encoded by separate genes and appear to target the same mRNAs, but some questions seem to remain regarding the temporal and spatial overlap of their expression patterns (Wienholds et al. 2005; Zhao et al. 2005, 2007; Chen et al. 2006).The authors report that half of miR-1-2 null animals die by weaning age and some suffer from incomplete ventricular septation, which is 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 indicates that miR-1-2 plays non-redundant roles with miR-1-1 in the heart despite their overlapping expression patterns (Zhao et al. 2007). Interestingly, miR-1-2 deletion did not appear to affect skeletal muscle development by gross morphological analysis. It is intriguing to speculate that the different requirement for miR-1 in cardiac versus skeletal muscle development reflects distinct target genes affected in those two muscle tissues.

miR-208 is expressed specifically in the heart and was recently deleted from the mouse genome by van Rooij et al. (2007). miR-208 null animals were viable and appeared normal without any apparent gross developmental defects. However, the miR-208 null animals exhibited a slight reduction in contractility at 2 months of age and a continued reduction in cardiac function in later life. Further analyses revealed a requirement for miR-208 in the cardiac hypertrophic growth response. Although miR-208 does not appear to be necessary for cardiogenesis, the physiological defects exhibited in the absence of miR-208 during adulthood indicate that miR-208 plays distinct roles in the adult heart. Collectively, compelling genetic evidences have established that miRNAs are indeed an emerging class of molecules important for cardiac muscle development (Table 1).

4 miRNAs in the Hypertrophic Growth Response

Cardiac myocytes proliferate rapidly during embryogenesis, but adult cardiac myocytes lose their proliferative capacity, and they respond to mechanical and pathological stimuli by hypertrophic growth, defined by an increase in myocyte size and/or myofibrillar volume without a change in myocyte number (Frey and Olson 2003). Cardiac hypertrophy is accompanied by the activation of a set of fetal cardiac genes that are normally expressed in the heart only before birth. The reactivation of cardiac fetal genes in post-natal cardiomyocytes in response to hypertrophic signals suggests that the molecular events that control cardiac gene expression during development may be redeployed to regulate hypertrophic cardiac growth or heart regeneration (Heineke and Molkentin 2006). Recent studies indicate that a change in the expression of miRNAs also accompanies cardiac hypertrophy and also indicate that miRNAs are novel regulators of the hypertrophic growth response.

Table 1 microRNAs implicated in the cardiac hypertrophic growth response

microRNA	Expression pattern	Expression change during hypertrophy	Validated targets	References
miR-1	Heart, skeletal muscle	Downregulated	Cdk9, Delta, Fibronectin, GJA1, Hand2, Irx5, KCNJ2, HDAC4, KCNE1, RasGAP, Rheb	Kwon et al. (2005), Zhao et al. (2005), Chen et al. (2006), Rao et al. (2006), Luo et al. (2007), Yang et al. (2007), Zhao et al. (2007)
miR-21	Heart, spleen, small intestine, colon	Upregulated	PTEN, TPM1	van Rooij et al. (2006), Cheng et al. (2007), Meng et al. (2007), Tatsuguchi et al. (2007), Zhu et al. (2007)
miR-133	Heart, skeletal muscle	Downregulated	Cdc42, ERG, KCNQ1, WHSC2, SRF, RhoA	Chen et al. (2006), Rao et al. (2006), Care et al. (2007), Luo et al. (2007), Xiao et al. (2007)
miR-195	Heart, lung, kidney, skin	Upregulated	None reported.	Lagos-Quintana et al. (2003), van Rooij et al. (2006)
miR-208	Heart	Not reported	Thrap1	van Rooij et al. (2007)

Cdc42 Cell division cycle 42; *Cdk9* cyclin-dependent kinase 9; *ERG* ether-a-go-go postassium channel; *GJA1* gap junction protein alpha 1; *Hand2* heart and neural crest derivatives expressed 2; *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; *PTEN* phosphatase and tensin homolog; *RasGAP* Ras GTPase-activating protein; *Rheb* Ras homolog enriched in brain; *RhoA* Ras homolog A; *SRF* serum response factor; *Thrap1* thyroid hormone receptor associated protein 1; *TPM1* tropomyosin 1; *WHSC2* Wolf-Hirschhorn syndrome candidate 2

4.1 miRNA Expression is Dynamically Regulated in Response to Cardiac Hypertrophy

Using miRNA microarrays, the global miRNA expression profile is regulated during cardiac hypertrophy in both in vitro and in vivo model systems, suggesting that miRNAs are involved in this disease process (van Rooij et al. 2006; Cheng et al. 2007; Tatsuguchi et al. 2007). Olson and colleagues are among the first to report the change of miRNA expression in thoracic aortic-banded hearts (TAB) as well as the calcineurin over-expression transgenic mice, two animal models of pathological cardiac hypertrophy (van Rooij et al. 2006). In their report, the

expression of only a relatively small fraction of miRNAs were changed in response to cardiac hypertrophy (van Rooij et al. 2006), which is consistent with similar reports from other groups (Ji et al. 2007; Tatsuguchi et al. 2007). In contrast, another report documented differential expression of over 100 miRNAs during cardiac hypertrophy (Cheng et al. 2007). The cause of those differences is not known. Additional experiments, in particular those using alternative approaches, such as northern blotting and real-time RT-PCR, seem necessary to verify some of the prior results. Regardless of those differences, an emerging picture has convincingly demonstrated that miRNAs are a new class of previously unrecognized regulators for cardiac hypertrophy. Furthermore, dysregulated miRNA expression has been shown in human patients with failing hearts (van Rooij et al. 2006; Yang et al. 2007). One of the surprising observations from those miRNA profiling studies is that there were more miRNAs found upregulated than downregulated in response to cardiac hypertrophy. It is not clear what this means, although one intriguing explanation is that the translation/expression of many proteins could be inhibited during cardiac hypertrophy, given that miRNAs are repressors of gene expression. Functional analyses using both gain- and loss-of-function approaches 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 (Table 1) (van Rooij et al. 2006, 2007; Care et al. 2007; Cheng et al. 2007; Sayed et al. 2007; Tatsuguchi et al. 2007).

4.2 Genetic Studies Reveal that miRNAs Can Modulate Pathological Hypertrophy

Muscle-specifically expressed miR-1 and miR-133 play a critical role in skeletal muscle proliferation and differentiation (Chen et al. 2006). miR-1 was further shown as essential for heart development (Zhao et al. 2007). Interestingly, expression of miR-1 and miR-133 are both downregulated during cardiac hypertrophy. Downregulation of these two miRNAs is proposed to be necessary for the expression of targeted growth-related genes and induction of hypertrophic growth (Care et al. 2007; Sayed et al. 2007). In support, ectopic expression of miR-1 or miR-133 inhibited target gene expression and the hypertrophic growth response in a tissue-culture model of cardiac hypertrophy (Care et al. 2007; Sayed et al. 2007). Conversely, blocking endogenous miR-133 function in isolated cardiomyocytes augmented agonist-induced hypertrophy (Care et al. 2007). Furthermore, prolonged inhibition of miR-133 in vivo using chemically-modified oligonucleotides antisense to miR-133, delivered by an osmotic minipump implanted into the heart, caused a marked hypertrophic response (Care et al. 2007). It should be pointed out that while the expression of miR-1 appears dysregulated in cardiac hypertrophy (Sayed et al. 2007), there is not yet direct genetic evidence supporting a role for this miRNA in the regulation of hypertrophy. Instead, miR-1 was found to have arrhythmogenic

potential when overexpressed in adult rat hearts (Yang et al. 2007), suggesting that miR-1 may play an essential role in cardiac electrophysiology, in addition to its role in heart development.

Whereas miR-1 and -133 are downregulated during cardiac hypertrophy, miR-195 is upregulated and was found sufficient to induce hypertrophic growth in cultured cardiomyocytes as well as in transgenic mice (van Rooij et al. 2006). In contrast, transgenic mice over-expressing miR-214, a miRNA also upregulated during hypertrophy, caused no detectable phenotypic effect in the heart (van Rooij et al. 2006). Those studies indicate that some miRNAs, but not others, are sufficient to induce cardiac hypertrophy. It will be interesting to investigate whether they are necessary for the hypertrophic response using a loss-of-function approach. Several growth-related genes have been identified as specific mRNA targets for miR-1, miR-133, and miR-195 (Care et al. 2007; Meng et al. 2007; Sayed et al. 2007; Zhu et al. 2007), but how those miRNAs integrate into relevant genetic pathways to modulate the hypertrophic response is unclear.

Although genetic ablation did not identify a critical role for miR-208 in the developing mouse, a striking postnatal role for miR-208 was revealed (van Rooij et al. 2007). Loss of miR-208 protects mice against cardiac hypertrophy and upregulation of β-MHC induced by hypothyroidism, activated calcineuron signaling and cardiac pressure-overload-induced stress (van Rooij et al. 2007). These results suggest that the genetic pathways coordinating cardiac hypertrophy share a common component regulated by miR-208. One 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. Expression of Thrap1 mRNA is targeted by miR-208, and Thrap1 protein levels are elevated in miR-208 mutant hearts, suggesting that miR-208 may function, at least in part, by regulating the expression of a thyroid hormone signaling pathway component (van Rooij et al. 2007).

miR-21, a miRNA implicated in tumor-related cell growth and apoptosis (Chan et al. 2005; Cheng et al. 2005; Si et al. 2006), is upregulated in response to agonist-induced cardiac hypertrophy in cell culture experiments and in pressure-overload-induced hypertrophy in vivo (van Rooij et al. 2006; Cheng et al. 2007; Sayed et al. 2007; Tatsuguchi et al. 2007). Inhibition of miR-21 by transfection of locked nucleic acid (LNA)-modified antisense oligionucleotides induced hypertrophic growth in rat neonatal cardiomyocytes, as assessed by cardiomyocyte cell size and expression of hypertrophic markers such as ANF, α-actinin, and skeletal muscle α-actin (Tatsuguchi et al. 2007). A role for miR-21 in hypertrophy was further supported by a gain-of-function approach where ectopic miR-21 expression slightly decreased cardiomyocyte size and reduced expression of hypertrophic marker genes (Tatsuguchi et al. 2007). In contrast, the inhibition of miR-21 by transfection of 2′*O*-Methyl antisense oligonucleotides attenuated global protein synthesis and cell size in an agonist-induced model of hypertrophy in isolated cardiomyocytes (Cheng et al. 2007). Interestingly, other reports on miR-21 function also appear contradictory: while one study documented that miR-21 inhibition provoked cell growth in HeLa cells (Cheng et al. 2005), others showed that miR-21

inhibition led to activation of apoptosis and decreased cell proliferation (Chan et al. 2005; Si et al. 2006). Clearly, the identification of the miR-21 regulatory targets and further analysis of the molecular pathways modulated by miR-21 in different biological systems are needed to better understand the biological function of this miRNA.

Collectively, these studies support miR-1, miR-21, miR-133, miR-195, and miR-208 as important and newly identified regulators of cardiac hypertrophy. In addition, identification of the hypertrophic miRNA expression signature has yielded many hitherto unrecognized candidate genes involved in cardiac hypertrophy, and those candidates are awaiting closer examination. Given the complexity of the cardiac remodeling occurring during hypertrophy, the identification of specific targets of miRNAs involved in the hypertrophic response will provide more insight into the molecular mechanisms underlying this disease process.

5 Conclusions and Future Directions

It is becoming more and more evident that miRNAs are important regulators of normal and pathological processes, including cardiac hypertrophy. These findings that miRNAs participate in heart formation and function offers a new paradigm for the post-transcriptional regulation of gene expression in cardiovascular biology. However, we are still left to answer many basic questions regarding miRNAs: how many miRNAs exist, where are they expressed, and which genes do they target? Are many miRNAs important for cardiac function or just a handful of them? And how are those miRNAs integrated into the complex genetic networks that regulate specific processes in the heart? Understanding the role of miRNAs in the heart will provide more insight into the molecular basis of cardiovascular disease, and it is very intriguing to speculate that miRNAs might one day be exploited as therapeutic targets.

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