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Taking microRNAs to heart

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MicroRNAs (miRNAs) are a class of highly conserved, small non-coding RNAs that regulate gene expression post-transcriptionally. The emerging field of miRNA biology has begun to reveal roles for these regulatory molecules in a wide range of biological processes. Dysregulated miRNA expression has been correlated to diseased hearts in human patients, whereas inhibiting the maturation of miRNAs conditionally in murine hearts has revealed that miRNAs are essential for cardiac development and function. Moreover, genetic studies have identified distinct roles for specific miRNAs during cardiogenesis, cardiac hypertrophy and electrical conduction. These previously unrecognized relationships shed new light on the regulatory mechanisms underlying heart development and pathology and suggest the potential importance of miRNAs as diagnostic markers and therapeutic targets for cardiovascular disease.

MicroRNAs: a new paradigm for cardiac gene regulation

MicroRNAs (miRNAs) (see Glossary) are an evolutionarily conserved class of small regulatory RNAs that have gained status recently as important regulators in cardiac developmental and pathological processes.

They are encoded genomically and are transcribed initially as part of much longer molecules that become processed into a mature approximately 22-nucleotide-long form. miRNAs are generally regarded as negative regulators of gene expression that inhibit translation and/or promote messenger RNA (mRNA) degradation by base pairing to complementary sequences within protein-coding mRNA transcripts [1–3]. Although not yet a well established phenomenon, some evidence suggests miRNAs might also enhance translation under particular circumstances [4]. Hundreds of human miRNA genes have been identified and bioinformatic analyses indicate that miRNAs might regulate the expression of more than a third of human protein-coding genes [5], highlighting the potential magnitude of their influence on gene expression.

Heart development and pathology are linked intimately 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 or 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 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 have an essential role during development [8,9]. miRNA expression-profiling studies demonstrate that expression levels of specific miRNAs change 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

Glossary

3′ untranslated region (UTR): a section of mRNA following the coding region that contains several types of regulatory sequences that affect mRNA stability and translation, including microRNA-target sites.
Apoptosis: a form of programmed cell death.
Arrhythmia: any change in the regular rhythmic beating of the heart.
Cardiac hypertrophy: growth of the heart in response to increased workload by enlarging myocyte size, rather than increasing myocyte number.
Cardiomyopathy: deterioration of the function of the myocardium, often accompanied by an increased risk of arrhythmia and sudden death.
Congenital heart disease: heart disease present at birth and can describe a wide variety of abnormalities affecting the heart.
Cre-LoxP system: a tool for tissue-specific deletion of genes that cannot be investigated in differentiated tissues because of their early embryonic lethality in mice with conventional knockouts. Conventional transgenic mice expressing Cre recombinase are mated to a strain with a gene flanked by LoxP sites ('a floxed gene'). The floxed gene is excised by recombination, and is thus inactivated, in whichever tissues Cre recombinase is expressed.
Dicer: an endonuclease that cleaves double-stranded RNA and that is required for microRNA biogenesis.
Enhancer element: a DNA sequence that, when bound by a specific transcription factor, can enhance the expression level of a nearby gene.
Hyperplasia: abnormal increase in the proliferation of cells within an organ or tissue.
MicroRNA: class of single-stranded RNA molecules of approximately 21–23 nucleotides in length that regulate gene expression post-transcriptionally.
Pericardial edema: accumulation of watery fluid in the pericardial sac of the heart.
Polycistronic: a single RNA transcript encoding two or more gene products.
RNA-induced silencing complex (RISC): a multi-protein complex that uses a bound microRNA to recognize complementary mRNA molecules, which results in translation inhibition and/or degradation.
Single nucleotide polymorphism: a genomic DNA sequence variation occurring at a single nucleotide.
Ventricular septation: formation of the muscular wall that separates the left and right ventricular heart chambers. Defects in ventricular septation are characterized by an abnormal opening between the cardiac ventricles that enables blood to pass directly from the left to the right ventricle, thereby reducing the efficiency of cardiac output.

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1471-4914/$ – see front matter doi:10.1016/j.molmed.2008.03.006 Available online 3 May 2008

the regulation of key factors important for cardiogenesis, the hypertrophic growth response and cardiac conductance [9,12–16]. Cumulatively, these findings indicate clearly 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.

miRNAs 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 (Figure 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 [17–19]. Dicer deletion in mice caused arrested development during gastrulation before the body plan was fully configured, suggesting that miRNA function is crucial for early development [17]. Similarly, the creation of Dicer zebrafish mutants resulted in abnormal morphogenesis during gastrulation with somitogenesis and heart development both proving abnormal [18,19]. 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 deleted Dicer conditionally from the mouse genome using the Cre-LoxP system have further supported a crucial role for miRNAs in development [8,9,20–23]. Cardiac-specific deletion of Dicer, using Cre-recombinase controlled by the predominately postnatally expressed αMHC (myosin heavy chain) 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 this progressed rapidly 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, reduced 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].

By 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 probably 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 [20], skeletal muscle [21], limb [22] and lung [23]. On a cautionary note, the interpretation that a global loss of miRNAs is solely responsible for the observed Dicer-deletion phenotypes hinges on whether or not Dicer serves any crucial roles outside of miRNA biogenesis.

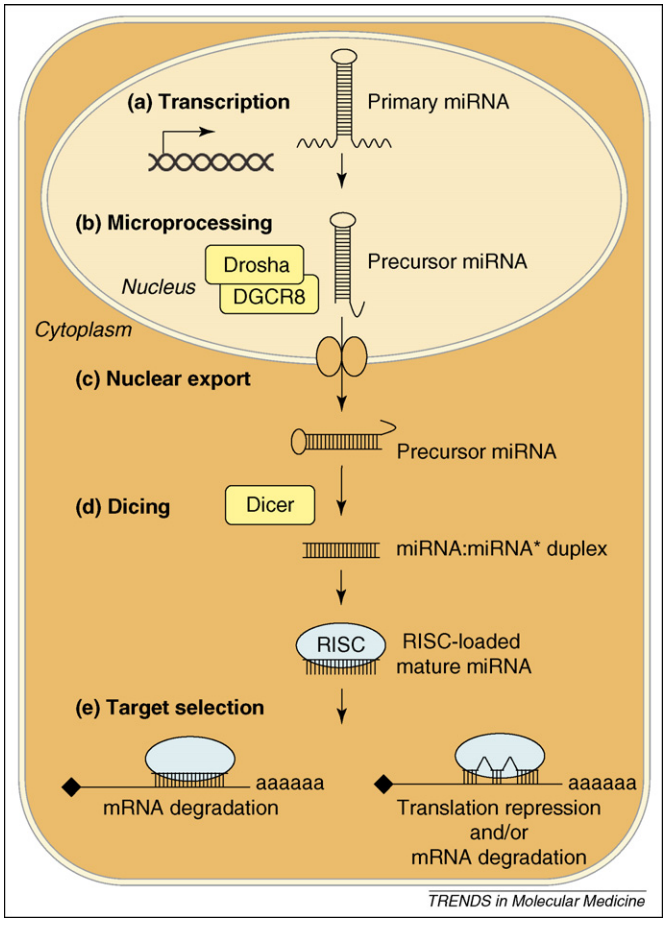

Figure 1. MicroRNA (miRNA) biogenesis and mechanism (see Ref. [54] for a review). **(a)** miRNA biogenesis begins with the transcription of primary-miRNAs by RNA polymerase II from independent transcriptional units with lengths ranging from several hundred to several thousand nucleotides that might encode 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 ~70-nt 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 incorporated selectively into the RNA-induced silencing complex (RISC), whereas 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 suppresses translation and also affects the stability of targeted mRNAs and mediates their degradation.

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

The conditional deletion of Dicer from the heart presumably down-regulated all cardiac-expressed miRNAs. To understand the contribution of specific miRNAs in cardiac development, several groups have undertaken gain- and loss-of-function studies on individual miRNAs [9,12,16,24]. The outcomes of those studies indicate clearly that single miRNAs are capable of having crucial and specific roles in both cardiac development and function (Figure 2).

miR-1 and miR-133 are highly conserved and are expressed in the musculature of flies, mice and

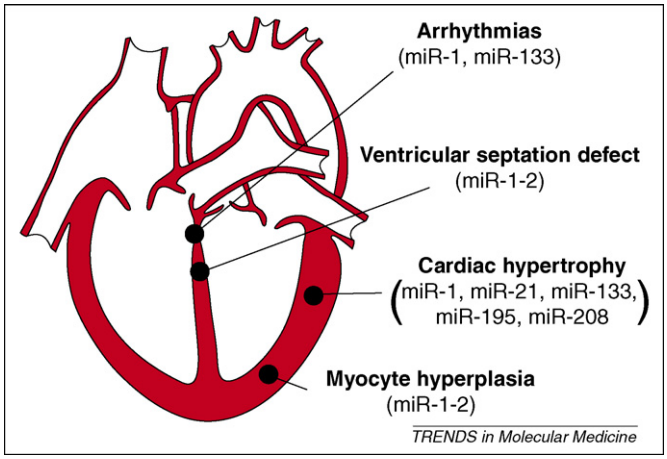

Figure 2. Known roles of miRNAs in heart development and function. Recent studies have demonstrated an association of several miRNAs with various cardiac defects. miR-1 contributes to numerous cardiac abnormalities, including arrhythmias, defective ventricular septation, cardiac hypertrophy and myocyte hyperplasia, whereas miR-133 has arrhythmogenic potential and also has a role in cardiac hypertrophy. Additionally, miR-21, miR-195 and miR-208 are thought to be involved in the control of cardiac hypertrophy. It is expected that more miRNAs will be added to this growing list.

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; for example *miR-1–1* and *miR-133a-2* are clustered on mouse chromosome 2, whereas *miR-1–2* and *miR-133a-1* are clustered on mouse chromosome 18 [24]. Well-known muscle transcriptional networks, consisting of SRF (serum response factor) and myocardin for cardiac muscle expression and MyoD and MEF2 (myocyte enhancer factor 2) for skeletal muscle expression, regulate the expression of these muscle-specific miRNA genes [12,24,25].

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

Complementing the over-production 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 their temporal and spatial expression patterns, which appear largely overlapping [9,12,24,26]. 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* has 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 [24], 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 to be an indispensable guide for advancing miRNA research [5,27,28]. 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 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 over-production in the developing mouse heart [29]. Accordingly, miR-1 over-production reduced Hand2 protein levels, whereas 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, similar to most miRNAs, miR-1 has been predicted to target hundreds of genes. Future studies aimed at determining physiologically relevant targets regulated directly by miRNAs are clearly needed.

Table 1. Experimentally validated targets of cardiac-expressed microRNAs

microRNA	Expression pattern	Validated targets	Refs
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 α 1; Hand2, heart and neural crest derivatives expressed 2; HDAC4, histone deacetylase 4; HSP, heat-shock protein; 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.

miRNA expression during cardiac remodeling

The heart is 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 cardiac hypertrophy [30]. 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 the reactivation of fetal cardiac genes that are normally expressed in the heart before birth. The reactivation of cardiac fetal genes in postnatal cardiomyocytes suggests that the molecular events that control cardiac gene expression during development are redeployed to regulate hypertrophic growth or heart regeneration [31]. 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 [32].

Several groups have implemented microarray technology to analyze the expression of hundreds of miRNA genes simultaneously [10,11,33,34]. 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 [11,33,34]. Profiling studies in human samples reveal that changes in miRNA expression also occur in human failing hearts, including the up-regulation of miRNAs that are expressed normally 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 influence the cardiac hypertrophic growth response both positively and negatively [11,15,16].

miRNAs modulate cardiac hypertrophy

miR-195 is up-regulated during cardiac hypertrophy in both human and mouse hypertrophic hearts and is sufficient to induce hypertrophic growth in cultured rat cardiomyocytes [11]. Furthermore, over-production of miR-195 in mouse hearts induced hypertrophy within several weeks after birth. Continued miR-195 over-production led to dilated cardiomyopathy and heart failure in young mice [11]. The mechanisms underlying miR-195 function are not yet clear because no target genes have yet been verified. miR-214 is also up-regulated during hypertrophy, however, transgenic mice over-expressing *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, are worthy of pursuit.

Unlike miR-195 and miR-214, miR-1 and miR-133 are down-regulated during hypertrophy [11,15,35]. Their matching expression patterns are not surprising because miR-1 and miR-133 are both transcribed together from the same polycistronic genes. Over-expression of *miR-1* or *miR-133* inhibited hypertrophic growth in an *in vitro* model of cardiac hypertrophy using primary cardiomyocytes [15,35]. 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]. Although *miR-1* expression is down-regulated during cardiac hypertrophy [11,15,35], 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 the expression of growth-related genes [12,15,35], suggesting that these miRNAs might act as growth suppressors that are relieved during cardiac hypertrophy. Intriguingly, a recent report suggested that miR-1 and miR-133 might also have a distinct role in the regulation of cardiomyocyte apoptosis: miR-1 seems to be pro-apoptotic, whereas miR-133 appears anti-apoptotic [36]. Clearly, understanding how miRNAs and their regulatory targets integrate into relevant genetic pathways will be the crux of future studies.

miR-208 is expressed specifically in the heart and was deleted recently from the mouse genome by van Rooij and colleagues [16]. 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 two months of age and a continued reduction in cardiac function in later life. Although miR-208 does not appear to be necessary for cardiogenesis, a requirement for miR-208 in the cardiac hypertrophic growth response was identified. miR-208 null mice fail to undergo hypertrophic growth in response to activated calcineurin signaling or cardiac pressure-overload-induced stress, and they also fail to induce βMHC expression in response to hypothyroidism [16]. These results suggest that the genetic pathways coordinating cardiac hypertrophy share a common component that is regulated by miR-208. One such candidate proposed is thyroid hormone receptor-associated protein 1 (Thrap1), a co-factor of the thyroid hormone nuclear receptor, which can influence transcription positively and negatively. The 3′ untranslated region (3′UTR) of Thrap1 is targeted directly by miR-208 and Thrap1 protein levels were elevated in miR-208 null hearts, suggesting that miR-208 might function, at least in part, by regulating the expression of a thyroid hormone signaling pathway component [16]. These 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 [37–39], is up-regulated in response to agonist-induced cardiac hypertrophy in cell-culture experiments and in pressure-overload-induced hypertrophy *in vivo* [11,33–35]. However, the exact nature of miR-21 function remains unclear. Inhibition of miR-21 using antisense oligonucleotides suppresses agonist-induced hypertrophic growth in primary cardiomyocytes [33]. Inhibition of miR-21 using locked nucleic acid-modified miR-21 antisense oligonucleotides stimulated hypertrophic

growth *in vitro* [34]. Although 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 [38], whereas it was also reported to activate apoptosis and to inhibit cell proliferation [37,39]. 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-208, 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.

miR-1 and miR-133 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 might 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 as also 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 over-production of miR-1 in both normal and infarcted rat hearts slowed cardiac conduction and led to arrhythmias. Those effects appear to be mediated, at least in part, through post-transcriptional repression of the 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 the 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 [11,15,35], however, miR-133 is elevated in a rabbit model of diabetes [13]. The elevated miR-133 levels occurred concurrently with lowered protein levels but without a reduction in mRNA levels of ether-a-go-go (ERG), a cardiac potassium-ion channel that is important for myocyte repolarization and is associated with congenital arrhythmias. A target site partially complementary to miR-133 was identified within the 3′UTR of ERG, indicating that miR-133 might regulate ERG expression directly. In support, introduction of miR-133 into isolated cardiomyocytes reduced ERG expression post-transcriptionally and, accordingly, delayed myocyte repolarization. Collectively, an emerging portrait has become apparent in which muscle miRNAs have a much larger and broader role in the regulation of the cardiovascular system, including cellular proliferation and differentiation, apoptosis, cardiomyocyte hypertrophy and cardiac conduction.

miRNAs 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 [40]. Over the past decade, clinical studies have identified several congenital heart diseases that are associated with mutations in specific genes, with the majority of those reported mutations affecting cardiac transcription factors and structural proteins [41,42]. 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 correlate with 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 [43]. 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 [44,45]. 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 [43]. Although 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 might identify allelic mutations in miRNA genes and/or their target sites associated with human disease [46].

miRNAs 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. First, miRNA expression changes have been documented in diseased hearts, making miRNAs probable biomarkers or diagnosis indicators for cardiovascular disease. Second, miRNAs are small molecules, making their *in vivo* delivery feasible [47,48]. Third, 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 concert to control a common pathway and/or biological function. This feature is likely to make miRNAs efficient tools for targeting a particular disease pathway or process. Yet, this feature of miRNAs could be a

Box 1. Outstanding questions

- Which microRNAs are necessary for heart development, function and pathology?
- Which mRNAs are regulated directly by microRNAs?
- In what instances do microRNAs enhance, rather than repress, the expression of targeted genes?
- How are microRNAs and their targeted genes integrated into complex genetic pathways that are important for heart disease?
- Can specific expression patterns of microRNAs be used as diagnostics for cardiovascular disease?
- What are the most effective approaches for delivering therapeutic microRNAs and microRNA inhibitors?

two-edged sword that brings about 'off-target' side effects. For example, miR-133 is thought to repress cardiac hypertrophy, raising the possibility of a therapeutic application in which synthetic miR-133 molecules are introduced into patients to control pathological hypertrophy [15]. However, the over-production of miR-133 induces arrhythmias [13]. Clearly, caution and future studies directed at understanding the pathways regulated by cardiac miRNAs are needed before clinical treatments can be considered seriously.

Concluding remarks

The biology of miRNAs is a young research area and, as an emerging field, there are many more questions than answers (Box 1). 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 failing human hearts. The expression signatures of miRNAs in disease might 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 are a prerequisite for the development of miRNAs as potential therapeutic targets. 'Taking miRNAs to heart', we face big challenges but with the big promise that miRNAs might provide us with powerful tools to battle cardiovascular disease.

Acknowledgements

Research in D-Z.W.'s laboratory is supported by the March of Dimes Birth Defects Foundation, NIH and Muscular Dystrophy Association. T.E.C. is a Predoctoral Fellow and D-Z.W. is an Established Investigator of the American Heart Association.

References

1 Kiriakidou, M. *et al.* (2007) An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell* 129, 1141–1151
2 Bagga, S. *et al.* (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122, 553–563
3 Humphreys, D.T. *et al.* (2005) MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc. Natl. Acad. Sci. U. S. A.* 102, 16961–16966
4 Vasudevan, S. *et al.* (2007) Switching from repression to activation: microRNAs can up-regulate translation. *Science* 318, 1931–1934
5 Lewis, B.P. *et al.* (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20
6 Olson, E.N. (2004) A decade of discoveries in cardiac biology. *Nat. Med.* 10, 467–474
7 Olson, E.N. (2006) Gene regulatory networks in the evolution and development of the heart. *Science* 313, 1922–1927
8 Chen, J.F. *et al.* (2008) Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *Proc. Natl. Acad. Sci. U. S. A.* 105, 2111–2116
9 Zhao, Y. *et al.* (2007) Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* 129, 303–317
10 Thum, T. *et al.* (2007) MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* 116, 258–267
11 van Rooij, E. *et al.* (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. U. S. A.* 103, 18255–18260
12 Zhao, Y. *et al.* (2005) Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* 436, 214–220
13 Xiao, J. *et al.* (2007) MicroRNA miR-133 represses HERG K^+ channel expression contributing to QT prolongation in diabetic hearts. *J. Biol. Chem.* 282, 12363–12367
14 Yang, B. *et al.* (2007) The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. *Nat. Med.* 13, 486–491
15 Care, A. *et al.* (2007) MicroRNA-133 controls cardiac hypertrophy. *Nat. Med.* 13, 613–618
16 van Rooij, E. *et al.* (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* 316, 575–579
17 Bernstein, E. *et al.* (2003) Dicer is essential for mouse development. *Nat. Genet.* 35, 215–217
18 Wienholds, E. *et al.* (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat. Genet.* 35, 217–218
19 Giraldez, A.J. *et al.* (2005) MicroRNAs regulate brain morphogenesis in zebrafish. *Science* 308, 833–838
20 Andl, T. *et al.* (2006) The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr. Biol.* 16, 1041–1049
21 O'Rourke, J.R. *et al.* (2007) Essential role for Dicer during skeletal muscle development. *Dev. Biol.* 311, 359–368
22 Harfe, B.D. *et al.* (2005) The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc. Natl. Acad. Sci. U. S. A.* 102, 10898–10903
23 Harris, K.S. *et al.* (2006) Dicer function is essential for lung epithelium morphogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 103, 2208–2213
24 Chen, J.F. *et al.* (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* 38, 228–233
25 Rao, P.K. *et al.* (2006) Myogenic factors that regulate expression of muscle-specific microRNAs. *Proc. Natl. Acad. Sci. U. S. A.* 103, 8721–8726
26 Wienholds, E. *et al.* (2005) MicroRNA expression in zebrafish embryonic development. *Science* 309, 310–311
27 Krek, A. *et al.* (2005) Combinatorial microRNA target predictions. *Nat. Genet.* 37, 495–500
28 Griffiths-Jones, S. *et al.* (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res.* 36, D154–D158
29 Srivastava, D. *et al.* (1997) Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. *Nat. Genet.* 16, 154–160
30 Hunter, J.J. and Chien, K.R. (1999) Signaling pathways for cardiac hypertrophy and failure. *N. Engl. J. Med.* 341, 1276–1283
31 Heineke, J. and Molkentin, J.D. (2006) Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat. Rev. Mol. Cell Biol.* 7, 589–600
32 Frey, N. and Olson, E.N. (2003) Cardiac hypertrophy: the good, the bad, and the ugly. *Annu. Rev. Physiol.* 65, 45–79
33 Cheng, Y. *et al.* (2007) MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? *Am. J. Pathol.* 170, 1831–1840
34 Tatsuguchi, M. *et al.* (2007) Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy. *J. Mol. Cell. Cardiol.* 42, 1137–1141

35 Sayed, D. *et al.* (2007) MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ. Res.* 100, 416–424
36 Xu, C. *et al.* (2007) The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes. *J. Cell Sci.* 120, 3045–3052
37 Chan, J.A. *et al.* (2005) MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res.* 65, 6029–6033
38 Cheng, A.M. *et al.* (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res.* 33, 1290–1297
39 Si, M.L. *et al.* (2006) miR-21-mediated tumor growth. *Oncogene* 26, 2799–2803
40 Rosamond, W. *et al.* (2008) Heart disease and stroke statistics–2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 117, e25–e146
41 Mandel, E.M. *et al.* (2005) Transcriptional mechanisms of congenital heart disease. *Drug Discov. Today* 2, 33–38
42 Ransom, J. and Srivastava, D. (2007) The genetics of cardiac birth defects. *Semin. Cell Dev. Biol.* 18, 132–139
43 Clop, A. *et al.* (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat. Genet.* 38, 813–818
44 Lee, S.J. (2004) Regulation of muscle mass by myostatin. *Annu. Rev. Cell Dev. Biol.* 20, 61–86
45 Schuelke, M. *et al.* (2004) Myostatin mutation associated with gross muscle hypertrophy in a child. *N. Engl. J. Med.* 350, 2682–2688
46 Mardis, E.R. (2008) The impact of next-generation sequencing technology on genetics. *Trends Genet.* 24, 133–141
47 Krutzfeldt, J. *et al.* (2005) Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature* 438, 685–689
48 Esau, C. *et al.* (2006) miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab.* 3, 87–98
49 Kwon, C. *et al.* (2005) MicroRNA1 influences cardiac differentiation in *Drosophila* and regulates Notch signaling. *Proc. Natl. Acad. Sci. U. S. A.* 102, 18986–18991
50 Sokol, N.S. and Ambros, V. (2005) Mesodermally expressed *Drosophila* microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes Dev.* 19, 2343–2354
51 Boutz, P.L. *et al.* (2007) MicroRNAs regulate the expression of the alternative splicing factor nPTB during muscle development. *Genes Dev.* 21, 71–84
52 Meng, F. *et al.* (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 133, 647–658
53 Zhu, S. *et al.* (2007) MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J. Biol. Chem.* 282, 14328–14336
54 Filipowicz, W. *et al.* (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* 9, 102–114