MicroRNAs: novel regulators in cardiac development and disease

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MicroRNAs (miRNAs) are endogenous, small ribonucleotides regulating the translation of target messenger RNAs that have been shown to be involved in orchestrating growth, development, function, and stress responses of various organs, including the heart. Muscle miRNAs are mainly controlled by a network of myogenic transcription factors, and throughout cardiac development they fine-tune regulatory protein levels in a spatiotemporal manner. Recent profiling studies revealed that miRNA expression patterns are derailed in both human cardiac disease and animal models of cardiac hypertrophy and failure. Modulation of miRNA expression in vitro as well as in vivo has revealed an important role of miRNAs in regulating heart function, particularly cardiac growth and conductance. Here, we overview the recent findings on miRNAs in cardiac development and disease and report the latest advances in the identification and validation of miRNA targets, which are important for a comprehensive understanding of cardiac miRNA function. Finally, we focus on the development and use of miRNA antagonists (antagomirs) to target miRNAs in vivo, which may translate into novel therapeutic strategies for heart disease in the future.

1. Introduction

MicroRNAs (miRNAs) are natural, endogenous, single-stranded molecules consisting of approximately 22 non-coding nucleotides that regulate target genes.1 There are approximately 500–1000 different mammalian miRNA genes; a complete list and details about the nomenclature of the miRNAs identified thus far can be viewed at Sanger miRBase 10.1 (http://microrna.sanger.ac.uk/sequences/). The first miRNA assigned to a specific function was lin-4, which targets lin-14 during temporal pattern formation in Caenorhabditis elegans.2 Since then, a variety of miRNAs have been discovered. Each miRNA regulates dozens to hundreds of distinct target genes, thus fundamentally impacting on cell biology. However, an understanding about the diverse functions of individual miRNAs is still in its infancy. Alterations of miRNA expression have been associated with the deregulation of developmental processes in plants, Drosophila, zebrafish, mice, and humans, but also with certain disease states, such as cardiac hypertrophy and failure. With regard to their physiological importance, miRNAs regulate stem cell differentiation, neurogenesis, haematopoiesis, insulin and other hormone secretion, immune response, as well as skeletal and cardiac muscle development and function.3–9

The processing and maturation of miRNAs has been previously described.10 In brief, miRNAs are transcribed as parts of longer molecules (approximately 2 kb in length; pri-miRNAs) that are processed in the nucleus into hairpin RNAs by the RNase III-type enzyme Drosha11 and the double-stranded RNA-binding protein DGCR812 to form pre-miRNAs (approximately 70 nucleotides in length) (Figure 1). Subsequently, the pre-miRNAs are transported to the cytoplasm via exportin-5 and are further cleaved by the ribonuclease Dicer to form approximately 22-nucleotide complexes.13

One strand of the mature miRNA is incorporated in the so-called miRNA-induced silencing complex (miRISC). Interaction with miRNA recognition elements that are mainly located in the 3′-untranslated region of target messenger RNAs leads to the degradation or translational inhibition with subsequent protein repression.1,14,15 Argonaute proteins seem to play a crucial role, as they are located in the heart of RISC complexes.13

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been named ‘mirtrons’ and function mainly as translational repressors. Intriguingly, miRNAs may also lead to an upregulation of gene expression. However, the exact mechanism is currently unknown but may be the result of direct effects, such as chromatin remodelling, or indirect effects, e.g. suppression of transcriptional repressors.

2. MicroRNAs in cardiac development

2.1 MicroRNA expression in cardiac development

Tissue development depends on the correct spatiotemporal expression of particular miRNAs. Indeed, cell- and/or tissue-specific modulation of miRNA expression levels has been demonstrated to determine the fine-tuning of specific targets that need to be activated or silenced at a certain time.

Relatively few miRNAs are detectable during early embryogenesis, e.g. let-7b, miR-130b, and miR-367 in the chick epiblast, and miR-430 in early zebrafish. An analysis of the miRNAs expressed in undifferentiated mouse embryonic stem cells and differentiating cardiomyocytes was recently published. A selection of miRNAs that are enriched during the differentiation of mouse embryonic stem cells to cardiomyocytes is given in Table 1. Interestingly, a considerable number of miRNAs upregulated in differentiating cardiomyocytes are also enriched in human foetal heart tissue (Table 1). Indeed, the number of detectable miRNAs increases rapidly in tissues derived from all three germ layers (endoderm, ectoderm, and mesoderm). Thus, miRNAs appear to play important roles in orchestrating organogenesis as well as in early embryonic patterning processes. Among miRNAs detected in early chick heart development, as well as in myotomal skeletal muscles are

Figure 1 Processing of microRNAs (miRNAs). MiRNAs are transcribed as parts of longer molecules (pri-miRNAs) that are processed in the nucleus into hairpin RNAs by the protein Drosha. These pre-miRNAs are transported to the cytoplasm via exportin-5 and are further processed by the ribonuclease Dicer. Mature miRNAs are then incorporated in the RNA-induced silencing complex (RISC) and interfere with the regulation of messenger RNA (mRNA) translation by targeting miRNAs with the resulting mRNA degradation or by translational inhibition with the resulting target repression.

Table 1 Summary of regulated microRNAs (miRs) in cardiac development and disease

<table>
<thead>
<tr>
<th>Downregulated miRs</th>
<th>Upregulated miRs</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td>Disease</td>
<td>Development</td>
</tr>
<tr>
<td>1, 7a/b, 10a/b, 26a/b, 29a/b/c, 30a-3p/a-5p/b/c/d/e/f, 30e, 93, 126-5p, 133a/b, 139, 149, 150, 151, 155, 181b, 185, 187, 194, 218, 292-5p, 373, 378, 451, 466, 486</td>
<td>1, 18, 20, 23b, 24, 26a, 30c, 133, 143, 182, 183, 200a/b, 292-3p, 293, 295, 335</td>
<td>Mouse</td>
</tr>
<tr>
<td>10b, 15b, 17-5p, 19b, 22, 23b, 24, 27a, 30a-5p/b/c/d/e-5p, 107, 126, 130b, 135a, 136, 148a, 150, 182, 186, 192, 199a, 218, 299-5p, 302b*, 302c*, 325, 339, 342, 452/<em>, 494, 495, 497, 499, 507, 512-5p, 515-5p, 520d</em>/h, 520, 523, 526/b/*</td>
<td>1, 20, 21, 66a, 92, 127, 129, 130a, 199b, 200a, 335, 424</td>
<td>10b, 15b, 17-5p, 18b, 19a/b, 20b, 21, 23a/b, 24, 25, 27a/b, 29a, 31, 103, 106a, 107, 125b, 126, 127, 140/<em>, 142-3p, 146, 153, 154, 195, 199a/a</em>/b, 200a, 208, 210, 211, 214, 217, 218, 221, 222, 330, 341, 351, let-7b/c, 424</td>
</tr>
</tbody>
</table>

Upregulated (enriched) miRNA during mouse cardiac development as described in the recent work of Srivastava and colleagues. Those authors determined which miRNAs are enriched during differentiation of mouse embryonic stem (mES) cells into cardiomyocytes. They used a mES cell line carrying a green fluorescent protein (GFP) transgene under control of the β-myosin heavy chain promoter, which is uniquely expressed in differentiated cardiomyocytes. RNA was isolated from GFP+ and GFP− cells by fluorescence-activated cell sorting after 13 days of embryonic body differentiation and miRNA expression was profiled by microarray analysis (see upregulated miRNAs, development, mouse). Data on upregulated (enriched) miRNA in human cardiac development were obtained from our laboratory (and unpublished results). We provide data about miRNAs enriched in fetal human heart tissue (10–12 weeks of gestation) (see upregulated miRNAs, development, human). Data about altered miRNAs in heart failure and animal models of heart disease originate from different results published earlier. Note that in some cases results were not consistent between the different laboratories as outlined in the discussion of this review.
miR-1 and miR-133,27 miR-1 and miR-133 play key roles in skeletal muscle proliferation and differentiation.9 miR-1 promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression, whereas miR-133 enhances myoblast proliferation by repressing serum response factor (SRF), which in turn is required for the transcriptional activation of various other miRNAs (see below). miR-1 also regulates cardiac differentiation.8,28–30 Indeed, loss-of-function of miR-1 in Drosophila resulted in embryonic/larval lethality with most of the mutant flies displaying altered sarcomeric gene expression and, in a subset of embryos, an increased number of undifferentiated muscle progenitors.28 In contrast, miR-1 gain-of-function led to 100% embryonic fly lethality because of disrupted patterning of cardiac and skeletal muscle with insufficient numbers of cardioblasts. Altogether, these results reveal a tight control of spatiotemporal miR-1 expression for proper cardiac and/or skeletal muscle development. miR-1 controls heart development in mice by regulating gene expression and, in a subset of embryos, an increased number of undifferentiated muscle progenitors.28 In contrast, miR-1 gain-of-function led to 100% embryonic fly lethality because of disrupted patterning of cardiac and skeletal muscle with insufficient numbers of cardioblasts. Altogether, these results reveal a tight control of spatiotemporal miR-1 expression for proper cardiac and/or skeletal muscle development. miR-1 controls heart development in mice by regulation of the cardiac transcription factor Hand2.8 Likewise, genetic ablation of Hand2 leads to a similar failure in ventricular myocyte expansion.31 Additional miRNA targets that mediate cardiogenesis will be discussed later in this review.

2.2 Specific microRNA signature expression patterns

Many miRNAs are enriched in a tissue-/cell-specific manner,32 miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, and the let-7 family are abundantly but not exclusively expressed in adult cardiac tissue (Table 2). In addition to cardiomyocytes, the heart contains many other 'non-cardiomyocyte' cell types, such as endothelial cells, smooth muscle cells, fibroblasts, and immune cells, which may have completely distinct miRNA expression profiles. Indeed, (skin) fibroblasts mainly express miR-16, miR-21, miR-22, miR-23a, miR-24, miR-27a, and others (Table 2), an expression pattern that is highly different from that of cardiomyocytes (see above and Table 2). Cardiac and skin fibroblasts probably have a comparable miRNA expression profile with approximately 10–30-fold higher expression of miR-21 compared with adult cardiomyocytes (unpublished observations; Table 2). Other miRNAs, such as the let-7 family, miR-126, miR-221, and miR-222, are highly expressed in human endothelial cells.33,34 In addition, miRNA expression profiles can change during cardiac development, and many miRNAs that are only normally expressed at significant levels in the foetal human heart are re-expressed in cardiac disease, such as heart failure.25,35 Finally, also miRNAs whose expression is not restricted to the heart may have important cardio-specific functions. However, this needs to be tested in the future.

2.3 Transcriptional regulation and biogenesis of microRNAs

MiRNAs are transcribed in the nucleus and are, as other genes, regulated by transcription factors. SRF is a cardiac-enriched transcription factor responsible for the regulation of organized sarcomeres in the heart.36 Expression of SRF follows a restrictive pattern during mouse development and finally becomes confined to the heart tube and mesenchymal somites.30,37 SRF target genes contain single or multiple copies of the SRF-binding consensus element (known as CArG box), which are usually found in the promoters of genes regulating contractility, cell movement, and growth signalling.38,39 The activity of SRF is controlled by various interactions with other tissue-specific regulatory cofactors, such as GATA4, Nkx2.5,40 and myocardin41 (Figure 2). YY1 and HOP appear to block SRF-mediated myogenic gene activation.42,43 Recently, several miRNAs have been shown to be regulated by SRF.44 Indeed, approximately one-third of all mammalian miRNA genes contain at least one CArG element in or close to their promoter regions, including miR-1-1, miR-1-2, miR-21, miR-206, miR-214, and

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Cardiomyocyte- and fibroblast-specific microRNAs (miRNA)</th>
</tr>
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<tbody>
<tr>
<td><strong>(a)</strong></td>
<td>Heart</td>
</tr>
<tr>
<td>hsa-mir-126</td>
<td>281</td>
</tr>
<tr>
<td>hsa-mir-143</td>
<td>76</td>
</tr>
<tr>
<td>hsa-let-7a-1, -7a-2, -7a-3</td>
<td>37</td>
</tr>
<tr>
<td>hsa-mir-16-1, -16-2</td>
<td>34</td>
</tr>
<tr>
<td>hsa-let-7b</td>
<td>28</td>
</tr>
<tr>
<td>hsa-mir-30d</td>
<td>18</td>
</tr>
<tr>
<td>hsa-mir-1-1, -1-2</td>
<td>15</td>
</tr>
<tr>
<td>hsa-let-7c</td>
<td>12</td>
</tr>
<tr>
<td>hsa-mir27b</td>
<td>11</td>
</tr>
<tr>
<td>hsa-mir-451</td>
<td>11</td>
</tr>
<tr>
<td>hsa-let-7f-1, -7f-2</td>
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</tr>
<tr>
<td>hsa-mir-22</td>
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</tr>
<tr>
<td>hsa-mir-26a-1, -26a-2</td>
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</tr>
<tr>
<td>hsa-mir-30a</td>
<td>7</td>
</tr>
<tr>
<td>hsa-mir-133a-1, -133a-2, -133b</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>(b)</strong></th>
<th>Fibroblasts</th>
<th>Heart</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-16-1, -16-2</td>
<td>51</td>
<td>34</td>
<td>1.5</td>
</tr>
<tr>
<td>hsa-mir27a</td>
<td>47</td>
<td>4</td>
<td>11.8</td>
</tr>
<tr>
<td>hsa-mir-21</td>
<td>33</td>
<td>1</td>
<td>33.0</td>
</tr>
<tr>
<td>hsa-mir-24-1, -24-2</td>
<td>32</td>
<td>2</td>
<td>16.0</td>
</tr>
<tr>
<td>hsa-mir-29b-1, -29b-2</td>
<td>31</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>hsa-mir-22</td>
<td>20</td>
<td>7</td>
<td>2.9</td>
</tr>
<tr>
<td>hsa-mir-23a</td>
<td>14</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>hsa-mir-31</td>
<td>13</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>hsa-mir-7a-1, -7a-2, -7a-3</td>
<td>12</td>
<td>37</td>
<td>0.3</td>
</tr>
<tr>
<td>hsa-let-7b</td>
<td>12</td>
<td>28</td>
<td>0.4</td>
</tr>
<tr>
<td>hsa-mir-29a</td>
<td>11</td>
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<td>2.8</td>
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<tr>
<td>hsa-let-7f-1, -7f-2</td>
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<tr>
<td>hsa-mir-221</td>
<td>10</td>
<td>1</td>
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</tr>
<tr>
<td>hsa-mir-143</td>
<td>9</td>
<td>76</td>
<td>0.1</td>
</tr>
<tr>
<td>hsa-mir27b</td>
<td>8</td>
<td>11</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Information in this table was extracted from Landgraf et al.32 The results are based on miRNA microarray analysis. For quantification of the specificity of the expression of in the various cell types, first the hierarchical tissue classification scheme was used to determine the clone count of each miRNA for each tissue class. miRNA counts were then normalized within each sample and a ‘normalized tissue enrichment’ for each miRNA was calculated as the ratio of the normalized clone count for one tissue type relative to the total clone count.32 The top 15 miRNAs with high expression in the human heart (a) or human fibroblasts (b) are shown. miRNAs more than five-fold expressed in one cell type than in the other are given in bold.
Transcriptional regulation of microRNAs (miRs) is best known for miR-1/miR-133. Serum response factor (SRF) interacts synergistically with myocardin (and potentially with other transcription factors such as Nkx2.5 and GATA4) to activate miR-1-1 and miR-1-2 by binding to the upstream CArG enhancer elements. The importance of the SRF binding site between miR-1-2 and miR-133a-1 is less known. The bicistronic primary transcripts miR-1-1/133a-2 and miR-1-2/133a-1 are downregulated in the hearts of mice lacking MEF2 expression. In addition, a MEF2-dependent enhancer upstream of the miR-1133a-2 locus was shown to regulate cardiac and skeletal muscle expression in vivo. Finally, MEF2 also activates transcription of the bicistronic precursor RNA encoding miR-1-2 and miR-133a-1 via an intragenic muscle-specific enhancer. This probably is also the case for miR-1-1/133a-2.

miR-133 is regulated by SRF, as small-interfering RNA (siRNA)-mediated SRF knockdown prevents the increase of miR-133 in diabetic cardiomyocytes. MEF2, a critical regulator of muscle development, additionally activates transcription of a bicistronic primary transcript encoding miR-1-2 and miR-133a-1 via an intragenic muscle-specific enhancer located between the miR-1-2 and miR-133a-1 coding regions. In somite myotomes and in all skeletal muscle fibres, during embryogenesis and adulthood, MEF2 together with myocardin and potentially other transcription factors and cofactors synergistically induces respective miRNAs. In general, regulation of striated muscle miRNA expression appears to be controlled by a network of myogenic transcription factors, including SRF, MyoD, MEF2, and myocardin.

MicroRNAs in the heart

3. MicroRNAs and cardiac hypertrophy and failure

The heart is very sensitive to physiological stimuli or pathological states, and even slight perturbations can lead to severe cardiac remodelling, eventually with detrimental outcomes. As a major response to these stimuli, the heart undergoes extensive tissue remodelling known as physiological or pathological hypertrophic growth, which is defined as an augmentation of ventricular mass because of increased size of cardiomyocyte. Functionally, cardiac hypertrophy is characterized by an initial compensatory process that helps the heart in sustaining cardiac output. However, this process is only an initial ‘adaptive’ response, and chronic exposure to stress signals eventually leads to impaired inotropic/lusitropic function that, in many cases, progresses to cardiac failure. This maladaptive change is accompanied by a switch in the gene programme that leads to re-expression of foetal type genes, an event correlating with cardiac failure. The involvement of miRNAs in this pathological process has been recently recognized. A selection of deregulated miRNAs in cardiac disease is given in Table 1.

3.1 Deregulation of microRNAs in cardiac remodelling

Evidence for a deregulation of miRNA expression in cardiac remodelling was initially reported in an array study based on two mouse models of pathological hypertrophy: transverse aortic constriction (TAC), an in vivo model of hypertrophy induced by left ventricular pressure-overload, and calcineurin transgenic mice, a calcium-dependent model of maladaptive response. Of over 180 miRNAs analysed, a specific group of miRNAs characterized by a peculiar expression profile was similarly deregulated in both the models. MiR-23a, miR-23b, miR-24, miR-195, miR-199a, and miR-214 were all found to be upregulated during cardiac hypertrophy, and their over-expression in cardiomyocytes in vitro was sufficient to induce hypertrophic growth. Intriguingly, miR-24, miR-125b, miR-195, miR-199a, and miR-214 were similarly upregulated in the tissue of patients with end-stage failing human hearts. More specifically, the in vitro observations,
first proving that a single miRNA can induce pathological cardiac hypertrophy and failure, could be recapitulated in a transgenic mouse model over-expressing miR-195. Despite the interesting phenotype of this model, neither targets nor mechanisms underlying miR-195’s way of action have been unravelled. In contrast to miR-195, in vitro overexpression of miR-150 and miR-181b, which were downregulated during hypertrophy, resulted in reduced cardiomyocyte cell size.

Within the last year, several groups have published miRNA expression profiles in different experimental settings of in vitro and in vivo cardiac hypertrophy, both in human and murine hearts. Altogether, hundreds of miRNAs have been identified that are either upregulated, downregulated, or unchanged (see for review). Consistent with the reaction of the foetal gene programme during pathological forms of hypertrophy, an impressive similarity has been found between the miRNA expression pattern occurring in human failing hearts and that seen in the hearts of 12-14-week-old fetuses. Indeed, approximately over 80% of the analysed miRNAs were found to be similarly regulated in both failing adult and foetal human hearts compared with adult normal heart tissue. The most consistent changes were upregulation of miR-21, miR-29b, miR-129, miR-210, miR-211, miR-212, miR-423 and downregulation of miR-30, miR-182, and miR-526. In addition, gene expression analysis of the same samples revealed that most of the upregulated genes were characterized by the presence of a significant number of predicted binding sites for downregulated genes and vice versa. Mechanistically, transfection of cardiomyocytes with a set of foetal miRNAs induced cellular hypertrophy as well as changes in gene expression comparable to the failing heart. This suggests a novel mode of regulation for the transcriptional changes seen in cardiac failure. Reactivation of the foetal miRNA programme seems to substantially contribute to the alterations of gene expression, triggering pathological changes in the myocardium associated with progressive dysfunction.

MicroRNA expression profiling studies are important for revealing novel miRNA-based pathways underlying the cardiac diseases. However, in some cases contradictory microRNA data have been reported under comparable experimental conditions. For example, in a microRNA array analysis performed at different time-points after TAC in mice, miR-21 was highly upregulated 1–2 weeks post-TAC but decreased to a normal level after 3–4 weeks. In contrast, other studies reported an upregulation that was maintained over time. In a similar time-course analysis after TAC in mice, identified miR-1 as one of the most downregulated microRNAs, while expression of miR-133a/b was unchanged. The miR-1 level was reduced already after 1 day of TAC, reaching a minimum at 1-week post-TAC and returning to near normal levels by day 14. On the other hand, in a similar TAC model, downregulation of miR-133a/b was reported by van Rooij et al., while no changes in miR-1 expression was found. Similarly, only miR-133a/b, but not miR-1, was found to be deregulated by Cheng et al. as well as Yang et al. report miR-1 upregulation in dilative cardiomyopathy and patients with coronary artery disease, which is in line with miR-1 upregulation in cardiomyocytes upon oxidative stress.

It is likely that the microRNA expression profile changes during the transition of cardiac hypertrophy to failure with marked consequences for target expression levels. Indeed, although the exact disease state (e.g. NYHA class, severity of aortic stenosis, current drug treatment) was not described. In strong contrast to others in the field, these authors did not find an increase in miR-21 expression in the heart tissue of the investigated patient collective. Therefore, more microRNA expression studies with myocardial specimens of patients with different cardiovascular diseases are required that focus on the effects of the underlying aetiology, effects of drug treatment, age, gender, and other potential regulators on changes of microRNA expression patterns.

3.2 Insight from microRNA mouse models: modulation of cardiac hypertrophy and failure

Very recently, studies using both gain- and loss-of-function approaches, functionally corroborated the aforementioned array findings and highlighted the important role of stress-regulated microRNAs in determining cardiac hypertrophic growth response.

As mentioned above, an elegant gain-of-function approach was used by van Rooij et al., who described hypertrophy and failure of a mouse model over-expressing miR-195. The same authors also generated transgenic mice with cardiac-specific overexpression of miR-214 and miR-24, which were both upregulated during hypertrophy. While no phenotype was obtained by miR-214 overexpression, miR-24 overexpression resulted in embryonic lethality. Despite the lack of extensive characterization as well as identification of miRNA target genes, this study indicates that specific microRNAs can play determinant roles in the cardiac hypertrophy programme.

Unlike miR-195, miR-214, and miR-24, expression levels of both miR-1 and miR-133 were downregulated during cardiac hypertrophy with an expected similar reduction in the expression levels as both the microRNAs belong to same transcription unit. Notably, miR-1 and miR-133 expression was consistently downregulated both in pathological and physiological hypertrophy as demonstrated in mice subjected to TAC. Akt-overexpressing transgenic mice or exercise-trained wild-type mice, respectively. Thus, both these microRNAs are regulated during cardiac hypertrophy irrespective of whether the underlying cause is pathological or physiological.

To determine functional changes by downregulation of miR-1 and miR-133, gain- and loss-of-function studies were performed both in vitro and in vivo. In vitro, transduction of cardiomyocytes with a miR-133-expressing viral vector blunted the hypertrophic response of cardiomyocytes stimulated with the hypertrophic agonist, phenylephrine. In vivo, a single infusion of miR-133-antisense oligonucleotides (antagomir) was sufficient to obtain persistent downregulation of the endogenous miR-133 expression level, leading to a significant induction of the cardiac hypertrophic response accompanied by foetal gene re-activation. Importantly, several putative targets were found and validated as main effectors involved in the underlying mechanism (see below).
Using a different loss-of-function approach, Zhao et al. developed a mouse model with a genomic deletion of the miR-1-2 isoform. Knockout of miR-1-2 resulted in partial embryonic lethality characterized by profound septal defects. In addition, in mice that survived to adulthood, thickening of chamber walls was observed, probably because of hyperplasia extended until adult life. Interestingly, Irx5 (Iroquois family of homeodomain-containing transcription factor), a protein involved in the regulation of cardiac repolarization, was identified and validated as a target of miR-1 (see below). Indeed, there is a strong relationship between altered cardiac conduction and deregulation of miRNAs.

Van Rooij et al. generated mice deficient of miR-208, which is encoded by an intron of the α-MHC gene and thus specifically expressed in the heart. MiR-208 knockout mice are viable and exhibit no apparent gross developmental defects. However, upon induction of TAC pressure-overload, the mice had ablated hypertrophic and fibrotic response. As expected, foetal gene expression was increased, except for β-MHC expression, which was unchanged. In addition, α-MHC expression, which is normally downregulated during cardiac hypertrophy, was upregulated.

4. Cardiac microRNA targets

4.1 Complexity in microRNA target identification

For a comprehensive understanding of miRNA function and potential therapeutic use in heart disease, identification and validation of miRNA targets is of fundamental importance. Several bioinformatic methods have been developed to predict miRNA targets based on the assumption that the 5′-nucleotides of miRNAs are most critical for target recognition. Such methods easily result in the prediction of hundreds of potential miRNA targets, which are difficult to validate one by one. Target accessibility is not only an important factor for effective antisense oligonucleotide- and siRNA-mediated silencing, but also for miRNA target repression. Virtually all miRNA binding sites in 3′-UTRs of target mRNAs are located in unstable regions as calculated on the basis of free energy predictions and RNA structure. Based on this background knowledge, a novel miRNA target identification tool has recently been developed, which also includes target accessibility by evaluation of energy states of sequences flanking the miRNA target. However, it remains to be determined whether this stringent approach may identify less false-positive targets without missing others.

A potential relationship between altered miRNA expression and changes in messenger RNA expression profiles in failing human left ventricles has recently been explored. Computational prediction identified multiple potential target genes with at least one binding site for highly upregulated miRNAs during heart failure. In contrast, in the transcriptome analysis conducted in parallel, more theoretically predicted target genes were upregulated, demonstrating no obvious preponderance of gene repression. It is likely that multiple binding sites for upregulated miRNAs work in concert to drive gene regulation. The identification of more binding sites for upregulated miRNAs in repressed genes and vice versa in the failing human myocardium, suggests that a considerable amount of targets have to be regulated during heart failure by altered miRNA activation and/or silencing.

4.2 MicroRNA targets during cardiac development

Using luciferase-reporter assays and miR-1 overexpressing transgenic mice, the transcription factor Hand2 was identified as a miR-1 target. Hand2 is a cardiac-specific transcription factor that regulates cardiomyocyte expansion, and indeed embryonic overexpression of miR-1 in vivo resulted in a cardiac phenotype with thin-walled ventricles because of premature cellular differentiation and early withdrawal from the cell cycle. In contrast, miR-1 knockout mice displayed thickened chamber walls because of enhanced hyperplasia. MiR-1 also targets histone acetylase 4 (HDAC4), thus inhibiting muscle differentiation and skeletal muscle gene expression, mainly by repressing MEF2C, which is an essential muscle-related transcription factor. HDAC4 inhibits cardiomyogenesis by downregulation of GATA4 and Nkx2.5 expression in P19 embryonic carcinoma stem cells. Its effect on cardiac differentiation in vitro remains to be elucidated.

4.3 MicroRNA targets in cardiac disease

Targeted deletion of miR-1-2 in mice resulted in 50% lethality mainly because of large ventricular wall defects. In addition, a great portion of surviving animals displayed arrhythmias leading to sudden death. This could be linked to upregulation of Irx5, which is a direct target of miR-1. Uptregulation of miR-1 repressed the KCNJ2 and GJA1 channels responsible for the inward rectifier K+ current (I_{Ks}) and the gap junction chloride current (I_{Cl}), respectively, and eliminated arrhythmias in the mice. Interestingly, Yang et al. found miR-1 to be overexpressed in cardiac tissue of patients with coronary artery disease that are at high risk to develop arrhythmias. Likewise, overexpression of miR-1 in infarcted rat hearts exacerbates, whereas elimination of miR-1 by antisense inhibitors relieved arrhythmias. These data are in agreement with a recent study reporting up to three-fold higher expression of miR-1 in left ventricular tissue of patients with end-stage heart failure. In line with this, miR-1 is upregulated in cultured H9c2 cells upon oxidative stress, leading to reduction of the heat-shock proteins HSP60 and HSP70 and subsequently to apoptosis. In contrast, Ikeda et al. reported repressed levels of miR-1 in cardiac tissue of patients with DCM or aortic stenosis. Differences may be explained by variations of diseases, different biopsy locations, technical differences, or altered cellular composition of the biopsies. Further, miR-1 targets have recently been summarized including Ras GTPase-activating protein (RasGAP), cyclin-dependent kinase 9 (Cdk9), and Cdk2, which are essential for cycling and cell cycle progression.

MiR-133 targets involved in cardiac hypertrophy have been identified in vitro and in vivo, including Cdc42 (implicated in cytokskeletal modifications during cardiac remodeling), Rho-A (a GTP–GDP-binding molecule, also critical for hypertrophy), and NELF-A/WHSC2 (a nuclear factor involved in heart genesis). While Rho-A and Cdc42 have already been established as fundamental factors for cell growth, cytoskeletal reorganization, and regulation of contractility in cardiomyocytes, the role of NELF-A/WHSC2 in cardiac hypertrophy has not yet been defined. Transduction of cardiomyocytes both in vitro and in vivo with an adenoviral vector containing a Whsc2 transgene resulted in protein synthesis inhibition, but induced the foetal gene programme and upregulation of Rho-A, corroborating the idea that WHSC2 could play a selective role in hypertrophy.
miR-208, was found to be changed. Further studies are there-
occur pre-miR-208 level, but not the mature and active hypertrophy as well as in human failing hearts only the pre-
knockout mice. However, in both mouse models of cardiac tributary to a blunted response to pressure overload in miR-208 responsive element, respectively. This shift in expression con-
positive and negative thyroid hormone response element delayed rectifier potassium channel. There is additional evidence for miR-1 and miR-133 to regulate the cardiac receptor (THR)-associated protein 1. During cardiac hyper-
delayed rectifier potassium channel. 

An anti-apoptotic role for miR-133 in cultured rat ventricu-
lar cells owing to silencing of caspase-9 protein expression has also been described. 

MiR-133 additionally effects cardiac conductance. In the diabetic heart, strong upregulation of miR-133 expression resulted in the downregulation of protein expression of the ether-a-go-go-related gene (ERG), which encodes the rapid delayed rectifier potassium channel. There is additional evidence for miR-1 and miR-133 to regulate the cardiac 
pace-maker channel genes HCN2 and HCN4, and interest-
ingly, miR-1-2 knockout mice display about 20% reduction in heart rate. 

Figure 3 
Cardiac microRNA targets. Validated targets of miR-1, miR-133, and miR-208 with various effects on cardiac function are presented.

Targets of miR-208 include thrap1 [thyroid hormone receptor (THR)-associated protein 1]. During cardiac hyper-
trophy, a reduction in the expression level of α-MHC results in reduced miR-208 transcript levels, which in turn, abolishes its negative effects on thrap1. The resulting increased THRAP 1 protein expression affects the THR-regulated expression of α-MHC and β-MHC, which are inversely regulated through a positive and negative thyroid hormone response element responsive element, respectively. This shift in expression con-
tributed to a blunted response to pressure overload in miR-208 knockout mice. However, in both mouse models of cardiac hypertrophy as well as in human failing hearts only the pre-
cursor pre-miR-208 level, but not the mature and active miR-208, was found to be changed. 

We wish to point out that many potential miRNA targets have been proposed but just a few have been extensively validated by multiple techniques. This is an important task in the future. It is also likely that not only miRNAs but also their targets are highly tissue-specific and that miRNAs regulate targets in a particular tissue but not in others. An overview of the identified and validated miRNA targets in cardiac disease is shown in Figure 3.

5. MicroRNA modulators as potential therapeutics in cardiovascular diseases

The identification of miRNAs as important regulators not only for single genes but whole gene networks has enormous therapeutic implications. Recently, chemically engineered oligonucleotides, termed ‘antagomirs’ have been developed and proven to be efficient and specific silencers of endogenous miRNAs in mice. Chemical modifications and cholesterol conjugations have been shown to stabilize and facilitate intravenous delivery of antagomirs. This approach resulted in a marked reduction of the corresponding miRNAs in different organs such as liver, lung, kidney, skin, bone marrow, skeletal and cardiac muscle. The silencing effect was considerably sustained over time probably because of a long half-life of endogenous miRNAs. The mechanism of oligonucleotide-mediated miRNA silencing is still unknown. However, antagomirs interact with miRNAs in the cytoplasm upstream of P-bodies (Figure 1) and lead to specific miRNA downregulation when injected systemically or locally. Hypothetically, antagomirs may cleave miRNAs within the RISC with the antagomir acting as the guide strand. However, Kruitzfeld et al. recently demonstrated Argonaute 2-mediated cleavage unlikely to be involved in this process (Figure 1). Antagomirs may bind to mature miRNAs in the cytoplasm before, during, or after RISC loading with subsequent miRNA degradation (Figure 1). The exact mechanism remains to be shown in the future. There is a high target specificity of antagomirs, as certain point mutations in an antagonist result in the loss of downregulation. Specificity of drug-like oligonucleotides is of great importance to minimize off-target effects and to specifically discriminate between different miRNAs that often differ by a single nucleotide. In addition, induction of stable loss-of-function phenotypes for specific miRNAs by lentiviral-mediated antagomir expression has recently been described. However, this approach needs to be tested and validated in future in vivo scenarios.

6. Conclusion

Recent studies provide clear evidence that miRNAs modulate a diverse spectrum of cardiac functions with developmental, (patho)physiological, and clinical implications. Understanding miRNA regulation and identification of tissue-specific miRNA targets employing transgenic/knockout models and/or modulating oligonucleotides will improve our knowledge in cardiac biology and disease. MiRNA antagonists such as antagomirs can be a safe and dose-dependent fashion to study miRNA targets and may be valuable as potential therapeutics. However, future tasks include improvements in the understanding of the underlying mechanisms, the delivery, specificity, reversibility, and potential toxicity of such miRNA modulators. Hopefully, the development of novel antagomir designs and delivery strategies will allow to specifically treat single tissues or even specific cell types by conjugation with specific antibodies or other tissue-specific homing signals. In conclusion, unravelling the regulatory circuits of miRNAs is a great challenge, but may provide attractive targets for mechanism-based treatment of heart diseases.

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