Biogenesis and Regulation of Cardiovascular MicroRNAs

Johann Bauersachs, Thomas Thum

Abstract: MicroRNAs (miRNAs) are important regulators of gene expression and fundamentally impact on cardiovascular function in health and disease. A tight control of miRNA expression is crucial for the maintenance of tissue homeostasis. However, a comprehensive understanding of the various levels of miRNA regulation is in its infancy. We here summarize the current knowledge about regulation of cardiovascular miRNAs at the transcriptional level by transcription factors, during processing by the Drosha and Dicer complexes and the importance of miRNA modification, editing, and decay mechanisms. As an example, miRNA regulation in diabetic and hypoxic cardiovascular disease conditions is discussed. Better knowledge about regulatory mechanisms of miRNAs in cardiovascular disease will probably lead to improved and novel miRNA-based therapeutic therapies. (Circ Res. 2011;109:334-347.)

Key Words: Dicer ■ Drosha ■ heart failure ■ microRNAs ■ myocardial disease

The recognition of microRNAs (miRNAs) as essential regulators of heart and vessel morphology and function during recent years fundamentally changed our view of the cardiovascular system in physiology and pathology. miRNAs target not only single genes but often functionally related gene networks. The complexity of regulation of gene expression by miRNAs is highlighted by the fact that multiple cardiovascular important miRNAs have been identified, each of which regulating several hundred target genes. Recently published review articles have highlighted the role of miRNAs for cardiovascular development during embryogenesis, for cardiovascular disease such as myocardial infarction, hypertrophy, and fibrosis and as potential targets for cardiovascular important miRNAs have been identified, each of which regulating several hundred target genes. Recently published review articles have highlighted the role of miRNAs for cardiovascular development during embryogenesis, for cardiovascular disease such as myocardial infarction, hypertrophy, and fibrosis and as potential targets for cardiovascular disease prevention, diagnostics, and therapy.1-7 Excellent review articles are available that describe the processing and regulation of miRNAs in a general setting (eg,8-11). However, little is known about the control of miRNAs in the cardiovascular system in physiological states and during disease development and progression. Thus, the main purpose of the present review was to extrapolate general mechanisms and to summarize actual knowledge in the biogenesis and regulation of cardiovascular miRNAs.

Transcriptional Regulation of miRNA Genes

A first regulatory step in the biogenesis of miRNAs occurs at the level of transcription. Most miRNAs are transcribed by the RNA polymerase II and are encoded by introns.12 Interestingly, many miRNAs show a promoter structure similar to that of regular protein coding genes (ie, TATA box, initiator elements, frequencies of CpG islands, transcriptions factor bindings sites, etc.), making them accessible for transcriptional regulation (Figure 1).13 Despite these similarities, a comprehensive understanding of miRNA regulation at the transcriptional level is in its infancy. MiRNAs are processed from primary molecules (pri-miRNAs), which are folded to hairpin structures, followed by an association to the Drosha-DGCR8 complex (Figure 1) and subsequent formation of −70-nt-long precursor hairpin structures (pre-miRNAs).3,14,15 Most pri-miRNAs are processed by the canonical pathway (pre-miRNA production through activities of Drosha and Dicer on pri-miRNAs), but exceptions include so-called mirtrons. Mirtrons represent very short introns of genes, which by splicing activities directly form pre-miRNAs, thus bypassing Drosha (Figure 1). Many miRNAs are regulated at the transcriptional level by transcription factors, which fine tune miRNA expression and often form feedback loops. In the following section, the current knowledge is summarized about transcription factors involved in the regulation of miRNAs important for cardiovascular biology and disease.

Cardiomyocytes and Skeletal Myocytes

The first miRNAs described to be under the control of transcription factors include miR-1-1 and miR-1-2, both of which are specifically expressed in cardiac and skeletal muscle cells. Indeed, miR-1 genes are direct transcriptional targets of muscle differentiation regulators including the serum response factor (SRF), MyoD, and myocyte enhancer factor 2 (MEF2).3 SRF interacts synergistically with myocar-
din (and potentially other transcription factors such as GATA4 and Nkx2.5) to drive miR-1-1 and miR-1-2 expression by binding to CaR\(G\) enhancer elements.\textsuperscript{16} The tight control of miR-1 gene expression is important for the balance between differentiation and proliferation of cardiac progenitor cells during cardiogenesis.\textsuperscript{16} Further supportive evidence for SRF to be a major player in miRNA regulation comes from studies with conditional SRF knockout mice. Knockdown of SRF led to a downregulation of many SRF-dependent miRNAs in the heart including miR-1.\textsuperscript{17} In SRF conditional knockout embryonic mutant hearts about 20 miRNAs, each containing at least 1 conserved CaR\(G\) element (the SRF-binding consensus element) within their promoters, were downregulated in comparison with control hearts. Many other miRNA genes encode binding sites for the SRF transcription factor.\textsuperscript{1,17} YY1 and HOP block SRF-mediated myogenic gene activation.\textsuperscript{18,19} Bioinformatic prediction reveals approximately one third of all mammalian miRNA genes to contain at least one CaR\(G\) element in or close to their respective promoter regions, including miR-1-1, miR-1-2, miR-21, miR-206, miR-214, and others.\textsuperscript{20} Further miRNAs important for the orchestration of cardiac development such as miR-133a1 and miR-133a2 are also regulated by SRF. Indeed, MEF2 and SRF cooperatively regulate the expression of 2 bicistronic miRNA clusters encoding miR-133a-1, miR-1-2, and miR-133a-2/miR-1-1 in cardiac and skeletal muscle.\textsuperscript{16,21,22} In general, miR-133a-1 and miR-133a-2 are identical in sequence, whereas miR-133b, which is expressed in skeletal muscle, differs by only 2 nucleotides at the 3′ terminus (summarized in Thum et al.).\textsuperscript{3} SRF and MEF2 activate miR-133a expression, which directly or indirectly represses genes, involved in many aspects of heart development and function. MiR-133a in turn directly targets SRF and thus provides a negative feedback loop to precisely modulate SRF activity (Figure 2).\textsuperscript{22}

The MEF2 transcription factor is an essential regulator of muscle development and directly activates transcription of a cistronic primary transcript encoding miR-1-2 and miR-133a-2 via an intragenic muscle specific enhancer located

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**Figure 1. Transcriptional regulation of miRNAs and the Drosha/DGCR8 complex.** Initially, most miRNAs are processed by RNA polymerase II (RNAPII) from independent genes or from introns of protein-coding genes. Many miRNAs have their own promoter, regulated by numerous transcription factors. Transcription factors and miRNAs regularly form negative or positive feedback loops. In a main canonical pathway, primary miRNA precursors (pri-miRNA) are first processed by Drosha, and after export to the cytoplasm by the endonuclease Dicer. Few pre-miRNAs are produced independently (thus bypassing Drosha-mediated processing) from very short introns of genes (mirtrons) by splicing actions. Drosha associates with DGCR8 and many other cofactors to form the Drosha-DGCR8 complex, which processes most pri-miRNAs into smaller (about 70 nt long) hairpin pre-miRNAs. Many factors (protein–protein or RNA–protein interactions) associate with the Drosha/DGCR8 complex, thereby regulating its stability and activity. The p68 forms heterodimers with p72, p53, and Smad factors, whereas further editing of pri-miRNAs occurs by ADARs that affect miRNA accumulation and miRNA target specificity. ARS2 and the heterogeneous nuclear ribonucleoprotein hnRNP A1 additionally regulate Drosha-mediated miRNA processing.

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ADARs</td>
<td>adenosine deaminases that act on RNA</td>
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<tr>
<td>AGO2</td>
<td>argonaute 2</td>
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<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
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<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
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<td>HIF</td>
<td>hypoxia-inducible factor</td>
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<tr>
<td>miRNAs</td>
<td>microRNAs</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<td>Pre-miRNAs</td>
<td>precursor microRNAs</td>
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<td>Pri-miRNAs</td>
<td>primary microRNAs</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
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<td>VEGF</td>
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between miR-1-2 and miR-133a-1 coding regions. Correspondingly, miR-1-1 and miR-133a-2 as well as miR-1-2 and miR-133a-1 miRNA pairs were downregulated in hearts of mice lacking the MEF2c and MEF2d transcription factors.21

More evidence for miR-133 to be regulated by SRF comes from knockdown studies in which siRNA-mediated SRF repression prevented the increase of miR-133 in diabetic cardiomyocytes.23

For miRNAs involved in cardiomyocyte hypertrophy, regulation of miR-23a was recently reported.24 Prohypertrophic stimuli such as isoproterenol or aldosterone increased expression of miR-23a in cultured cardiomyocytes. This was mediated at least in part via a direct transcriptional interaction with the calcineurin downstream effector NFATc3 (Figure 2). Transgenic mice expressing activated calcineurin A (CnA) in the heart show a severe form of hypertrophy; and an initial screen of miRNAs deregulated in this model was published by van Rooij et al.25 A further calcineurin-regulated miRNA is miR-133. Indeed, treatment of cardiomyocytes or animals with cyclosporin A, an inhibitor of calcineurin, prevented miR-133 upregulation during heart failure.26 Applying antisense oligodeoxynucleotides against catalytic subunits of calcineurin and NFAT increased miR-133 expression in cultured primary cardiomyocytes (Figure 2). A reciprocal repression between miRNA-133 and calcineurin in part regulates cardiac hypertrophy, and calcineurin/NFAT signaling regulates miR-133 expression in cardiomyocytes. Recently, miR-199b was identified as a further target gene of the calcineurin/NFAT pathway in vitro and in vivo.27 A 11-kb region upstream of miR199b included conserved cis elements representing NFAT-binding sites. Activation of calcineurin/NFAT during cardiac hypertrophy stimulated miR-199b leading to downregulation of Dyrk1a, contributing to a pathogenic feed-forward mechanism affecting calcineurin-responsive gene expression (Figure 2). Of therapeutic relevance, inhibition of miR199b by specific antagonists blunted cardiac hypertrophy.27

MiRNA-array analysis performed in STAT3 knockout mice and littermate controls identified cardiac upregulation of miR-199a in mice lacking STAT3. MiR-199a targeted the ubiquitin-conjugating enzymes UBe2g1 and UBe2i, which are involved in disruption and biogenesis of sarcomere structures. Interestingly, those targets were markedly reduced in STAT3 knockout hearts. In human failing hearts displaying low STAT3 protein expression miR-199a levels were increased and UBe2g1 expression was decreased, suggesting a conserved regulatory mechanism.28

Another pathway involved in cardiac hypertrophy and remodeling includes Smad-dependent signaling. To investigate miRNAs regulated by Smad3, a miRNA microarray approach was performed in hearts of Smad3 knockout animals and littermate controls. Of 55 miRNAs identified to be differentially expressed, 10 miRNAs were predicted to bind genes that regulate extracellular matrix production including miR-25 and miR-29a.29

A further transcription factor involved in cardiac hypertrophy is GATA4.30 Analysis of a promoter region of the miR-144/451 cluster identified a binding site for this transcription factor.31 Indeed, overexpression of GATA4 in adult rat cardiomyocytes led to an upregulation of the miR-144/451 cluster, whereas downregulation of endogenous GATA4 by siRNA decreased expression. These data were confirmed by luciferase gene reporter assays in which cotransfection of GATA4 strongly augmented luciferase activity of respective miRNA constructs.31
Fibroblasts

Another miRNA strongly increased during cardiac hypertrophy and remodeling is miR-21.32 MiR-21 is enriched in cardiac fibroblasts and regulates cardiac fibrosis.33 Down-regulation of miR-21 by specific antagonists attenuated cardiac fibrosis and improved cardiac function in both preventive and therapeutic approaches.33 The role of miR-21 in organ fibrosis and the antifibrotic action of miR-21 inhibitors were confirmed in a bleomycin-induced pulmonary fibrosis model.34 Recently, Patrick et al investigated the role of miR-21 in pathological cardiac remodeling by injecting miR-21 inhibitors in mice exposed to transverse aortic constriction (TAC), which displayed enhanced cardiac miR-21 expression.35 The used short (8 nt) oligonucleotides against miR-21 did not block the remodeling response of the heart to stress, suggesting different effects of miRNA inhibitor chemistries at least in the case of miR-21.36 In addition, miR-21 null mice displayed cardiac hypertrophy and fibrosis in response to cardiac stress, which may be explainable by genetic compensatory mechanisms over the course of development. This suggests that the role of miR-21 in cardiovascular diseases is not entirely clear and more information, eg, about its regulation under physiological and pathophysiological conditions, is needed. Indeed, several studies now have been performed to identify regulation of miR-21; recently the transcription factor p53 was shown to piggy-back onto NF-κB/RelA complexes by utilization of the κB motif at a cis-regulatory region to control miR-21 expression (Figure 2).37 Also in dilated and failing human hearts with miR-21 upregulation, the P53-RelA complex was associated with this cis element of the miR-21 promoter. Using high-throughput sequencing, the genome-wide binding sites for the P53-RelA complex in diseased and control human hearts were analyzed and a significant overrepresentation of the STAT3 motif was identified.37 Next to this putative P53-binding site a gene structure study of miR-21 identified a promoter sequence and highly conserved region approximately 2.5 kb upstream of the putative P53 binding site.38 P53 requires and cooperates with NF-κB/RelA, and indeed transfection of P53 alone did not up-regulate miR-21 luciferase activity but significantly augmented the activity induced by NF-κB/RelA. In addition, the previously described miR-21 promoter was shown to be regulated through conserved AP-1 and PU.1 binding sites.39 This promoter, however, was regulated neither by P53 nor NF-κB/RelA, indicating that P53/NF-κB regulated miR-21 via a putative P53-binding site but not the previously described miR-21 promoter sequence. Chromatin-immunoprecipitation (ChIP) using anti-RelA and anti-P53 antibodies demonstrated that the putative P53 region was occupied by both RelA and P53 in vivo. Indeed, in end-stage cardiomyopathic hearts a nuclear accumulation of RelA in both myocytes and nonmyocytes was identified, whereas a significant P53 activation was only detectable in nonmyocytes such as cardiac fibroblasts. This may explain the selective upregulation of miR-21 in cardiac fibroblasts as reported by others.33,40,41 MiR-21 expression also is partly STAT3-mediated, and 2 conserved STAT3 binding sides lie upstream of miR-21.42 Indeed, STAT3 is required for p53-RelA-mediated miR-21 gene expression. Thus, a tight cooperation between at least 3 independent transcription factors is important to regulate miR-21 during heart failure.37 Further information about the regulation of miR-21 and its role in cancer and cardiovascular diseases was recently reviewed.32

Additional miRNAs important in fibroblast biology include those of the miR-29 family. MiR-29 targets many miRNAs that encode proteins involved in fibrosis such as collagens and is repressed after myocardial infarction.43 Regulation of miR-29 is less clear, but the promoter region of miR-29b shows at least 4 different binding sites for NF-kappaB; and NF-kappaB signaling, via ligation of Toll-like receptors, repressed miR-29b expression in malignant cells.44 Thus activation of NF-kappaB signaling post infarction may be causally related to the observed repression of miR-29 in the heart.

Endothelial Cells

Information about the regulation of angiogenic miRNAs is sparse. Recently, a therapeutic relevance of some members of the miR-17～92 cluster has been reported.45 This cluster is located in an intergenic region and its expression is controlled tightly by its own promoter. This group of miRNAs is expressed from a single transcription unit forming a polycistron. Transcriptional activators of this miR-17～92 cluster include E2F and cyclin D1 as well as AML-1 (Figure 3).46–49 The hypoxia-inducible factor (HIF) is a heterodimer consisting of an oxygen-sensitive alpha subunit (HIFα) and a constitutively active beta subunit (HIF1β) and has 3 isoforms.50 The hypoxia sensitive miR-210 is regulated in part
by the HIF1α but not HIF2α. In the absence of hypoxia, HIF1α overexpression was sufficient to induce miR-210 expression (Figure 3). Other miRNAs induced by HIF1α include miR-23, miR-24, miR-26, miR-37, and miR-107. The E26 transformation-specific sequence (ETS) factors interact with a core GGAAT consensus sequence. ETS factors are expressed in endothelial cells and Ets-1 and Ets-2 control expression of miR-126 by targeting a genomic region between −71 and −100 bp upstream of the miR-126 transcriptional start site (Figure 3).

Smooth Muscle Cells
A regulation of miRNAs involved in smooth muscle cell biology including miR-145 and miR-143 has recently been described. Those miRNAs are direct transcriptional targets of SRF, myocardin, and Nkx2-5. A 4.2-kb genomic region upstream of miR-143/miR-145 is highly conserved between human and mouse. Deletion studies of this miR-143/145 enhancer identified a 0.9-kb region to be sufficient for miR-143/145 cardiac and smooth muscle expression. Within these regions, cis elements are highly conserved among human, mouse, and zebrafish and represent potential binding sites for essential cardiac transcription factors such as SRF and Nkx2-5. Although SRF only weakly activated this enhancer upstream of a luciferase reporter, cotransfection of myocardin synergistically activated luciferase activity. Nkx2-5 also independently activates this enhancer, and the combination of SRF, myocardin, and Nkx2-5 has additive effects suggesting that those 3 factors are essentially involved in regulation of the miR-143/145 cluster. In vivo, mutations of the SRF binding site disrupted lacZ expression in the outflow tract and aorta, whereas disruption of the Nkx2-5 binding site diminished expression of those miRNAs in ventricles and atria. Within hearts of Nkx2-5 mutant mouse embryos, miR-143 and miR-145 expression was decreased.

Regulation of miRNAs by Other miRNAs and Kinases
Next to transcription factors there is now also evidence for inter-miRNA regulation, eg, miRNAs can regulate other miRNAs. A first example was shown for miR-208a, which within the adult heart is encoded in the MYH6 (α-MHC) gene. MiR-208a regulates the expression of 2 slow myosins and their intrinsic miRNAs MYH7 (β-MHC)/miR-208b and MYH7b/miR-499, respectively. Interestingly, certain miRNAs such as 208a and miR-208b are expressed together with their host genes and accordingly are regulated in part by the host gene promoter. For instance, within the adult heart, miR-208a is coexpressed with the Myh6 gene, whereas miR-208b is within the Myh7 gene.

Other important regulators of miRNAs include various kinases. Transgenic mice with enhanced cardiomcyocyte phosphoinositide 3-kinase (PI3K) activity subjected to myocardial infarction showed differences in miRNA expression. The PI3K transgenic animals had a preserved cardiac function after myocardial infarction, whereas the animals overexpressing a mutated form depicted impaired function. Insulin-like growth factor-1 (IGF-1) stimulated-PI3K had beneficial effects in mouse models of pressure overload. Generally, it is accepted that the PI3K pathway regulates physiological hypertrophy and cardiac protection. MiRNAs differentially expressed in hearts of mice where PI3K has been modulated were recently identified: miR-222, miR-31, miR-27a, miR-221, and miR-103 were all up-regulated in hearts overexpressing a dominant negative form of PI3K. In contrast, those miRNAs were down-regulated in hearts with an overexpression of constitutively active PI3K. Further downstream of this kinase is the Akt pathway. Transgenic mice with a selective cardiac overexpression of a constitutively active mutant of the Akt kinase show cardiac hypertrophy and reductions in miR-1 and miR-133 levels. Likewise, overexpression of a constitutively active Akt markedly reduced miR-199a-5p in cardiac myocytes associated with robust increases in HIF1 and SIRT1 that were reversed by overexpression of miR-199a-5p. Insulin receptor–stimulated activation of the Akt pathway attenuated down-regulation of miR-199a-5p and the up-regulation of its targets. In contrast, β-adrenergic receptor activation in vitro and in vivo induced miR-199a-5p expression. Overall, activation of β-adrenergic signaling counteracts the survival effects of the Akt pathway via up-regulation of miR-199a-5p.

Regulation of miRNA Processing
miRNA Processing by the Drosha Complex
Long primary miRNA transcripts are often several thousand nucleotides long and undergo a first cleavage within the nucleus by the RNase III enzyme Drosha (Figure 1). At the base of the miRNA stem loop, Drosha binds and cleaves a 60- to 100-nt-long hairpin premiRNA with a 2-nt overhang at the 3’ end. However, recombinant Drosha in vitro fails to generate pre-miRNA, suggesting that other cofactors must be important for the catalytic activity of Drosha. This is further supported by the fact that Drosha is at least associated with >20 distinct polypeptides forming the Drosha microprocessor complex. The best-known cofactor is DGCR8, which promotes pre-miRNA cleavage in vitro and stabilizes Drosha protein levels. Drosha expression and activity itself can be regulated. For instance, in certain cancers, Drosha expression is increased, whereas limited information about Drosha regulation in cardiovascular diseases is available so far. Surprisingly, a homologous chromosomal deletion of the region where Drosha-cofactor DGCR8 is located leads only to a moderate decrease in expression of about 60 miRNAs. However, a cardiomycyte-specific genetic shutdown of DGCR8 results in dilated cardiomyopathy and premature lethality, suggesting an important role of selected DGCR8-dependent miRNAs in cardiac biology. The Drosha microprocessor complex can also cleave hairpin structures from protein coding genes that are not further processed by Dicer. This allows Drosha to modulate protein coding genes independent of miRNA production.

Additional Drosha-regulating proteins include the DEAD-box RNA helicases p68 and p72, which are associated with DGCR8 (Figure 1). Interestingly, p68/p72 null embryos are embryonically lethal and display no specific degeneration in heart development but suffer from malformation of blood vessels, suggesting roles of p68/p72 in vascular biology.
Moreover, in fibroblasts of p68–p72 null embryos the association of Drosha with miR-199a is diminished, suggesting that the helicases p68 and p72 promote Drosha-mediated processing of certain miRNA subsets.68 As miR-199a plays an important role in cardiomyocyte hypertrophy and ischemia,28,63 it will be of interest to test the involvement of p68/p72 in scenarios of cardiac hypertrophy and myocardial infarction. The p68 also forms heterodimers with p53, Smad factors, and the estrogen receptor alpha (Figure 1), all of which have an important role in the cardiovascular system. For instance, TGF-beta and bone morphogenetic protein 4 (BMP4) are crucial in the differentiation of vascular smooth muscle cells via up-regulation of miR-21.69 Interestingly, the Smad-mediated regulation of miR-21 only occurs at the posttranscriptional level because pri-miR-21 levels were unaltered. Indeed, TGF-beta stimulation leads to the inclusion of Smad in a complex together with Drosha, p68, and the pri-miR-21 hairpin.69 The SMAD nuclear interacting protein 1 (SNIP1) likewise interacts with Drosha and p68, and SNIP1 reduction results in lower miR-21 levels at least in Arabidopsis.69 Whether Smad factors and p68 regulate miR-21 expression in other cardiovascular cells such as fibroblasts or endothelial cells remains to be investigated.

Other auxiliary proteins involved in Drosha regulation include the arsenite-resistance protein 2 (ARS2)70 and the heterogeneous nuclear ribonucleic protein hnRNP A1, which is involved in processing of miR-18a,24 a member of the miR-17 to 92 cluster. This information may also be of importance for vascular biology because high levels of miR-18a inhibit 3-dimensional spheroid sprouting of endothelial cells in vitro.72 Other regulatory proteins interacting with the loop region during Drosha-mediated miRNA processing include the KH-type splicing regulatory protein (KSRP). This factor targets G-rich regions of a set of pri-miRNAs during processing, and KSRP silencing leads to reductions in mature let-7a and miR-206 expression.73 Such a process may contribute to the pathogenesis of different diseases, because for instance reduced let-7a levels in lung tissue were found in pulmonary hypertension models,74 or in the case of miR-206 in muscle-related diseases.75 Let-7 in turn interacts with LIN-28 to prevent miRNA processing by Drosha/DGCR8.76 KSRP is also involved in the posttranscriptional regulation of miR-155,77 which may play a role in inflammatory heart diseases.

**miRNA Maturation by Dicer and Related Proteins**

After processing of pri-miRNAs by the Drosha/DGCR8 complex, pre-miRNAs are transported via exportin 5 into the cytoplasm awaiting further modifications (Figures 1 and 4). Here, most pre-miRNAs are processed by the ribonuclease Dicer into small 20- to 23-nucleotide-long miRNA duplexes. In vertebrates, Dicer is encoded within a single locus and appears to be essential for processing of most miRNAs, although the existence of alternative miRNA-processing enzymes is likely but rather unexplored. There are also examples of Dicer-independent miRNA processing; for instance, miR-451 requires cleavage by argonaute 2 (AGO2), but is independent of Dicer and its 3’end is generated by exonucleolytic trimming (Figure 4).80 The miR-451 is involved in erythropoiesis,35 but also has functions in cancer,81 pulmonary hypertension,74 and the cardiovascular system.31 Dicer-deficient animals fail to synthesize new miRNAs, resulting in embryonic lethality in zebrafish82 and mice.83 More specifically, cardiac Dicer knockout animal studies assessed the global role of miRNAs for cardiac development and function: Zhao and colleagues deleted a floxed Dicer allele in mice using Cre recombinase, which was under the control of the endogenous Nkx2.5 regulatory region.84 This resulted in embryonic death at E12.5, and the corresponding hearts displayed pericardial edema and failed to develop sufficient ventricular myocardium for proper function (Figure 4). Deleting Dicer reduced the availability of functional miRNAs during cardiac development, resulting in disturbed development of 4-chambered, dual outflow cardiac functionality in mammals.85 To analyze the role of Dicer postnatally, a tamoxifen-inducible Cre recombinase model was used to investigate the effects of Dicer depletion in young and adult mice.86 Specifically, mice homozygous for Dicer-floxed alleles and transgenic α-MHC-CreMer mice were crossed to generate double-transgenic (α-MHC-MCM/Dicer-flox/flox) mice. Dicer loss in cardiomyocytes of young mice resulted in sudden cardiac death due to arrhythmias and a mild form of heart failure, whereas Dicer knockdown in older animals led to development of severe heart failure (Figure 4). When the miRNA expression profiles were compared between cardiomyocyte-specific Dicer knockout and wild-type mice, not only down-regulated miRNAs were found as expected but also many highly up-regulated miRNAs after Dicer depletion. It is likely that this higher expression of certain miRNAs results from non-cardiomyocytes in which Dicer has not been deleted as observed for the fibroblast-enriched miR-21.33,86 Another group investigated the effects of cardiac-specific Dicer knockdown by generating Dicer floxed and Cre double heterozygous mice by crossing Dicer-flox/flox with MHC-Cre/+ mice.87 Here, there was a strong reduction of most miRNAs investigated in Dicer knockout hearts.87 It is likely that changes in alpha myosin heavy chain (MYH6) expression during cardiac development and the postnatal period may explain the differences reported in 2 such studies.88 Dicer-dependent control of miRNA expression likewise plays an important role in the regulation of vascular function: in vitro, Dicer knockdown in endothelial cells leads to reduced capillary sprouting and proliferation.89,90 Surprisingly, the effect of Drosha knockdown on impairment of angiogenesis was only modest,90 pointing to additional alternative Drosha-independent pathways of miRNA biogenesis. Stress by serum withdrawal resulted in Dicer down-regulation in human umbilical vein endothelial cells (HUVECs) and subsequent miRNA-mediated increased caspase-3 and decreased nitric oxide synthase (NOS) 3 expression, resulting in endothelial apoptosis. In contrast, overexpression of Dicer in HUVECs markedly reduced apoptosis on serum withdrawal in vitro.91 To assess the role of endothelial Dicer in vivo, 2 mouse models that were homozygous for the conditional floxed Dicer allele and expressed Cre-recombinase under the regulation of Tie2 promoter/enhancer or Tamoxifen (TMX)-inducible expressed Cre-recombinase
(Cre-ER\textsuperscript{T2}) under the regulation of vascular endothelial cadherin promoter (VEcad) were generated (Figure 4).\textsuperscript{92} The reduction of endothelial miRNAs by conditional inactivation of Dicer reduced postnatal angiogenic responses to a variety of stimuli, including exogenous VEGF, tumors, limb ischemia, and wound healing.\textsuperscript{92,93} These studies show endothelial miRNAs to be required for an appropriate angiogenic response in vivo. Interestingly, deletion of Dicer by Tie2-Cre, which is expressed not only in endothelial but also hematopoietic cells, blocked development and maturation of CD1\textsuperscript{a} days-restricted invariant natural killer T (iNKT) cells in the thymus and led to additional severe immunologic problems.\textsuperscript{94}

Interestingly, Dicer is down-regulated in failing human hearts,\textsuperscript{87} suggesting changes of Dicer regulatory mechanisms in cardiac disease. There are several proteins associated with Dicer such as the TAR RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT), both of which stabilize Dicer stability and processing activity (Figure 4).\textsuperscript{95}

Regulation of TRBP and PACT during cardiovascular diseases is currently not known. In addition, Dicer is alternatively spliced in different tissues,\textsuperscript{96} but the specific roles of various Dicer isoforms especially in cardiovascular tissues await clarification. Dicer may also be regulated by several cellular signaling pathways. Indeed, the ERK-MAP kinase promotes TRBP phosphorylation.\textsuperscript{97} As ERK-MAP kinase activity is altered in cardiac hypertrophy\textsuperscript{98} and fibrosis,\textsuperscript{33} this might have consequences for further Dicer complex–mediated miRNA processing in certain cardiovascular diseases. A further example may be the regulation of the smooth muscle cell–enriched miRNAs miR-143 and miR-145, whose mature expression is reduced in cancer, whereas the expression of the respective pre-miRNAs is not altered, suggesting inhibition of Dicer processing during cancer.\textsuperscript{99} Currently, it is unknown whether this type of Dicer regulation also exists in cardiovascular diseases.

Dicer associates with other proteins to form the RISC-loading complex, in which the cleavage of the pre-miRNA by...
Dicer results in the production of an unstable dsRNA built up by an active guide strand (miRNA) and the passenger strand (miRNA*), which mostly undergoes degradation (Figure 4). There are 4 argonaute (AGO) proteins in the human genome, of which AGO2 is the only protein with cleavage activity in mammals and plays a crucial role in miRNA-mediated mRNA silencing. AGO2 stabilizes Dicer, whereas AGO2 deletion leads to reduced levels of mature miRNAs. Likewise, AGO2 can be phosphorylated, affecting small RNA binding. As certain growth factors regulate AGO2 stability, this may have consequences also for miRNA processing during cardiac hypertrophy and fibrosis.

Modification of miRNAs, Editing and Decay Mechanisms
Most miRNAs bear a 7-methyl-guanylate cap at the 5’-end and a poly (A) tail at the 3’-end as shown for protein-coding mRNA genes (see Figure 4). The 3’-end of certain miRNAs can undergo modifications that result in alterations of stability. For instance, adenosine deaminases that act on RNA (ADARs) promote the conversion of adenosine to inosine in dsRNA molecules thus affecting stability of many pri- and premiRNAs (Figure 1 and 4). A certain splicing variant of ADAR2 is enriched in cardiac tissue, but its biological role is currently unclear. Likely, miRNA editing mechanisms finally leading to altered mature miRNA stability are of importance in cardiovascular biology. Alterations in miRNA expression and stability are also the consequence of changes in miRNA decay. Surprisingly, the half-life of some miRNAs in cells and organs such as the heart is relatively long. For instance, miR-208, which is involved in stress-dependent cardiac growth, was shown to have a half-life of >12 days in cardiac cells. In contrast, a relatively high turnover of certain miRNAs was shown in neural tissue (eg, for miR-128 and miR-206 expression is dependent on the MEK1/2 pathway and the transcription factor SRF (Figure 2 and Table 1).

Exemplary Descriptions of miRNA Regulation in Cardiovascular Disease
Impact of Diabetes, Hormones, and Growth Factors on miRNA Regulation
Cardiomyocytes and Fibroblasts
Diabetes is a major cardiovascular risk factor. Mechanistically, high glucose levels initiate and lead to the progression of cardiovascular disease by direct proapoptotic effects on cardiomyocytes as well as by the induction of endothelial and endothelial progenitor cell dysfunction. Exposure of cardiomyocytes to high glucose concentrations in vivo and in vitro significantly increases in miR-1 and miR-206 levels that in turn reduce expression of heat shock protein 60 by posttranscriptional inhibition. The glucose-mediated miR-1 and miR-206 expression is dependent on the MEK1/2 pathway and the transcription factor SRF (Figure 2 and Table 1).

A further pathway involved in miRNA-mediated apoptosis of cardiomyocytes in response to high glucose levels includes IGF-1 signaling. H9C2 cells exposed to high glucose showed increased miR-1 expression levels and higher number of apoptotic events. On glucose challenge, the miR-1-target IGF-1 was silenced. Interestingly, miR-1 overexpression blocked the capacity of IGF-1 to prevent glucose-induced apoptosis. A reciprocal regulation of miRNA-1 and IGF-1 signaling cascade in cardiomyocytes has recently been reported. Here the authors have shown in neonatal cardiomyocytes treated with IGF-1 that miR-1 expression was significantly repressed through modulation of the FoxO3a transcription factor. This could be validated in human samples suggesting the involvement of the miR-1/IGF-1 regulatory loop in controlling human cardiac hypertrophy. MiR-1 silences IGF-1 expression, leading to a negative feedback loop via FoxO3a, which silences miR-1. Identification of this interplay helps to understand the importance of a tight control in IGF-1-mediated effects on cardiac morphology and function. Treatment with the antidiabetic peroxisome proliferator-activated receptor (PPAR)–gamma agonist pioglitazone reduced myocardial infarct size, but the exact mechanism has not been elucidated. Pioglitazone treatment resulted in cardiac down-regulation of miR-29a and miR-29c levels. In line, antagonists against miR-29a or miR-29c reduced myocardial infarct size as well as apoptosis in hearts subjected to ischemia–reperfusion injury by targeting the antiapoptotic Bcl2 member Mcl-1. In vivo, diabetic rabbits show a strong overexpression of miR-133 in parallel with increased expression of SRF, which is a known transactivator of miR-133. Increased miR-133 expression repressed the ion channel ERG and contributed to repolarisation slowing and thereby QT prolongation and associated arrhythmias in diabetic hearts.

Another hormone involved in myocardial remodeling is aldosterone. A recent study showed that hypertrophic stimulation by aldosterone or isoproterenol leads to a decreased miR-9 expression level in cultured cardiomyocytes (Table 1). MiR-9 suppressed the translational activity of myocardin by targeting NFATc3. Administration of miR-9...
attenuated aldosterone-mediated cardiomyocyte hypertrophic responses. Thus, aldosterone treatment can result in cardiac hypertrophy by a miRNA-dependent pathway. Next to direct effects of aldosterone on cardiomyocytes this hormone leads to significant increases in extracellular matrix production and cardiac fibrosis. Indeed, changes of the extracellular matrix additionally result in deregulation of myocardial expression of miRNAs. For instance, matrix metalloproteinase 9 (MMP-9) knockout mice showed a decreased expression of miR-376b and an increase in expression of miR-1, miR-26a, miR-30 days, and miR-181c. Most of those miRNAs have putative candidate mRNAs to be involved in MMP-9–mediated cardiac dysfunction and may play roles in the regulation of G protein coupled receptors as well as in calcium handling–dependent development of cardiac hypertrophy. Whether other calcium-handling proteins such as SERCA2a are regulated by miRNAs remains to be investigated. Thioredoxin-1 (Trx1) is a small redox protein involved in many important biological processes, including detoxification of reactive oxygen species and cardiac biology. Trx1 was shown to up-regulate members of the let-7 family, including miR-98, in the heart. Overexpression of miR-98 in cardiomyocytes reduced cell size, whereas its knockdown augmented angiotensin II-induced cardiac hypertrophy, suggesting endogenous miR-98/let-7 to mediate antihypertrophic effects of Trx1. Whereas most studies focused on direct transcriptional changes of miRNAs by modulation of transcription factors, little is known about the role of hormones and cytokines in posttranscriptional miRNA processing. An example is the cardio-protective estrogen receptor alpha that forms a heterodimer with the helicase p68, thus influencing selective Drosha-mediated pri-miRNA processing (see above and Figure 1). Vascular Cells and Progenitor Cells Exposure of the vascular endothelium to high glucose concentrations leads to endothelial dysfunction, decreased production of nitric oxide, and to atherosclerosis. When human umbilical vein endothelial cells (HUVECs) were treated with high levels of glucose, miR-221 was strongly increased, whereas c-kit (the receptor for stem cell factor) was reduced. In this study the inhibitory effects of high glucose on endothelial cell migration were prevented when glucose-stimulated miR-221 expression was blocked by miR-221 inhibitors. MiR-221 antagonism also restored c-kit protein expression even under high levels of glucose. In smooth muscle cells both miR-221 and miR-222 regulate proliferation and are involved in neointimal hyperplasia. Platelet-derived growth factor (PDGF) stimulated expression of both miRNAs in smooth muscle cells, whereas knockdown of miR-221 and miR-222 resulted in decreased cellular proliferation, suggesting miR-221/-222 to be promising therapeutic targets in vascular diseases especially in diabetic conditions (Table 1).

The growth hormone vascular endothelial growth factor (VEGF) regulates the expression of several miRNAs, including the up-regulation of components of the c-Myc oncogenic cluster miR-17 to 92. Transfection of endothelial cells with VEGF-induced miRNAs of the miR-17-92 cluster rescued the induced expression of thrombospondin-1 and the defect in endothelial cell proliferation and morphogenesis initiated by the loss of Dicer. The growth factor IGF-1 improves function of circulating angiogenic progenitor cells in aged individuals by an increase in nitric oxide bioavailability, potentially contributing to improved vascular homeostasis. Likely, growth factors or or cytokines play a crucial role in the regulation of miRNA expression levels in the vascular system.

Cardiovascular progenitor cells contribute to regeneration of the cardiovascular system during disease. Identification of endogenous or exogenous molecules that regulate miRNA expression may be of interest to control and maybe improve function in such cells. Treatment of individuals with the granulocyte colony-stimulating factor (G-CSF) leads to an up-regulation of miR-10, miR-126, and miR-155 in mobilized circulating CD34 positive progenitor cells. As miR-126 plays a fundamental role in regulation of endothelial cell function, it is of interest that G-CSF up-regulates this miRNA in hematopoietic progenitor cells. Nitric oxide plays an important role in vascular homeostasis and regulates function of angiogenic progenitor cells. Thus modulation of nitric oxide bioavailability may also have an impact on miRNA expression. Indeed the nitric oxide synthase inhibitor asymmetrical dimethylarginine (ADMA) results in significant deregulation of miRNAs in angiogenic progenitor cells (Table 1). Up-regulation of miR-21 by ADMA leads to a down-regulation of the superoxide dismutase 2 as well as the endogenous ERK MAP kinase inhibitor sprouty2 (Table 1). In this study, inhibition of miR-21 blocked the detrimental effects of ADMA on angiogenic progenitor cell function, suggesting inhibition of this miRNA to be of therapeutic interest in cardiovascular diseases with impaired function of vascular progenitor cells.

Hypoxia, Ischemia/Reperfusion Injury and Cardiac Remodeling Changes of environmental conditions that occur during cardiovascular diseases likewise affect miRNA expression. Local tissue hypoxia leads to miRNA deregulations by either direct or indirect mechanisms. In vitro, hypoxia alters expression of many miRNAs. In vivo, miRNAs are also significantly deregulated by cardiac ischemic insult such as down-regulation of miR-29 family members. Importantly, targeting of miRNAs deregulated after cardiac ischemia has been shown to provide therapeutic benefit. A variety of hypoxia-regulated miRNAs play critical roles in cancer and angiogenesis. Hypoxic conditions increased miR-424 levels in endothelial cells by RUNX-1 and c/EBPα and subsequent PU.1 transactivation (Figure 3). Increased miR-424 could be blocked by specific miRNA inhibitors in vitro and in vivo. MiR-424 plays an important physiological role in postischemic vascular remodeling and angiogenesis. Targeting increased miR-424 levels after myocardial infarction might be an interesting therapeutic approach in the future. Other hypoxia-induced miRNAs, such as miR-210...
and others, have been described above. Recently the hypoxia-sensitive miR-24 was shown to critically regulate vascularization after myocardial infarction and thus serves as a new target to prevent cardiac ischemia.129

Ischemia/Reperfusion Injury of the Heart
Ischemic reperfusion injury leads to alterations of a variety of miRNAs, including miR-1, miR-21, miR-29, miR-92a, miR-133, miR-199a, and miR-320.130 Another miRNA downregulated after murine ischemia–reperfusion injury and in human myocardial infarction is miR-494. Cardiac-specific overexpression of this miRNA leads to improved recovery of contractile performance during ischemia–reperfusion injury. Likewise, myocardial infarct size was significantly reduced in transgenic hearts on ischemia–reperfusion injury when compared with wild-type hearts. Thus miR-494 might constitute an interesting target for the treatment of ischemic heart disease.131 A global miRNA expression profile in murine hearts subjected to ischemia–reperfusion injury showed a significant down-regulation of miR-320 both in vivo and ex vivo. Overexpression of miR-320 enhanced cardiomyocyte death and apoptosis, whereas its knockdown was cytoprotective.132 In line, transgenic mice with cardiac-specific overexpression of miR-320 showed an increased extent of apoptosis and infarction size on ischemia–reperfusion injury, whereas in vivo treatment with an antagonim against miR-320 reduced infarct size.132

MiR-199a is immediately down-regulated in cardiomyocytes on a decline in oxygen tension.133 This reduction is required for the rapid up-regulation of its target Hif-1α (Figure 2). Knockdown of miR-199a during normoxia results in the up-regulation of Hif-1α and Sirt1, and mimics hypoxic preconditioning.133 Ischemic preconditioning may also alter miRNA expression in stem cells used for regeneration of the infarcted heart. For instance, preconditioning of mesenchymal stem cells results in an increase of miR-210. Inhibition of Hif-1α or of miR-210 abrogated the cytoprotective effects of preconditioning. Indeed, preconditioning of mesenchymal stem cells before transplantation to hearts with myocardial infarction improved stem cell survival after engraftment via modulation of miR-210.134

In general, preconditioning may prevent severe cardiac injury. Interestingly, ischemic preconditioning resulted in up-regulation of miR-21 that may protect cardiomyocytes against ischemia–reperfusion injury through its target PDCD4 (Figure 2).135 In this study, miRNA array profiling identified 40 miRNAs differentially expressed in rat hearts subjected to ischemic preconditioning. In vivo, ischemic preconditioning that mediated cardiac protection against ischemia–reperfusion injury was inhibited by knockdown of cardiac miR-21. Next to early preconditioning, late preconditioning may lead to a regulation of miRNAs. MiRNAs induced after ischemic preconditioning in the heart may create a preconditioned phenotype through up-regulation of protective proteins. Ischemic preconditioning resulted in increases of miR-1, miR-21, and miR-24, and injection of miRNAs isolated from preconditioned hearts reduced infarct size in comparison with miRNAs isolated from nonischemic controls. Thus miRNAs increased by ischemic preconditioning may trigger cardioprotection potentially by up-regulating eNOS and several heat shock proteins.136 Another strategy to induce protective genes is heat shock therapy. Indeed, mice subjected to cytoprotective heat shock also showed a significant increase in miR-1, miR-21, and miR-24 expression in the heart. MiRNAs isolated from heat shock–treated mice and injected in nonheat shock–treated mice significantly reduced infarct size after ischemia–reperfusion injury.137

Cardiac Remodeling
The miR-21 is also highly expressed in cardiac fibroblasts, where it improves cell survival, leading to enhanced cardiac fibrosis.138 During chronic cardiac remodeling, inhibition of miR-21 via specific antagonors attenuated fibrosis development and improved cardiac function. Another study identified up-regulation of miR-21 after myocardial infarction and identified miR-21 to specifically localize to the infarct region of the heart with ischemia–reperfusion injury. Immunohistochemistry data showed that miR-21 was specifically localized in cardiac fibroblasts. Modulation of miR-21 regulated expression of matrix metalloprotease 2 via a PTEN pathway, further contributing to cardiac fibrosis (Figure 2).139 Others have shown that miR-21 expression is significantly down-regulated in infarcted areas130 but up-regulated specifically in the border areas of infarcted rat hearts both at 6 hours and 24 hours after acute myocardial infarction.130 Cardiomyocyte-specific overexpression of miR-21 did not change effects of pressure overload,131 but was described to result in smaller infarct size after myocardial infarction.132

MiRNAs are also regulated during pressure/volume overload of the left ventricle as shown by many authors (for a review, see Small et al and Cattalucci et al12). A recent study compared differences in miRNA expression on increased preload and afterload. Seven days after increase of load by transaortic constriction (pressure overload) or shunt surgery (volume overload), 13 miRNAs were differentially regulated. The only significantly regulated miRNA that was increased by both afterload and preload was miR-21,140 suggesting important pathophysiological relevance of this miRNA during cardiac remodeling.

Conclusion and Outlook
The discovery of miRNAs and their role in the orchestration of signaling pathways added a new level of complexity to our understanding of biology. Fundamental observations with respect to miRNA-mediated mechanisms have been made, putting miRNAs in the forefront of new target-based therapeutic strategies in cardiovascular medicine. However, many unsolved issues remain, and by large an understanding of the regulation of individual miRNAs in physiology and pathophysiology is in its infancy. In many cells and tissues, upstream as well as downstream signaling is different for miRNAs as both miRNA-regulator and miRNA-target availability often differs in cells and especially during disease initiation/progression. MiRNAs are influenced at the transcriptional level by many transcription factors but are also regulated during further downstream processing. Here, the regulation of the Drosha and Dicer complexes plays a dominant role in the control of miRNA expression and
stability. Many posttranscriptional regulatory mechanisms of miRNA processing have been identified, but their translation into cardiovascular science and the identification of a potential role in cardiovascular diseases is rather unexplored. Our understanding about the regulation of miRNA-dependent mechanisms needs to grow, hopefully resulting in the development of clinically relevant and improved cardiovascular therapeutic strategies in the future.

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