A New Level of Complexity: The Role of MicroRNAs in Cardiovascular Development

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Abstract: The discovery of the regulatory role of noncoding RNAs, and micro (mi)RNAs in particular, has added a new layer of complexity to our understanding of cardiovascular development. miRNAs regulate and modulate various steps of cardiovascular morphogenesis, cell proliferation, differentiation, and phenotype modulation. miRNAs simultaneously regulate multiple targets, and many miRNAs can bind to the same target, allowing for a complex pattern of regulation of gene expression. miRNA families are continuously added during evolution paralleling the increased complexity of the cardiovascular system in vertebrates compared with invertebrates. Several lines of evidence suggest that the appearance of miRNAs is at least in part responsible for the formation of complex organ systems and stable regulatory mechanisms in vertebrates. We review the current understanding of miRNAs during cardiovascular development. Further progress in this area will help to decipher quantitative changes in gene expression that provide robustness to cellular phenotypes and regulatory options to diseases processes. miRNAs might also provide clues to better understand congenital heart defects, which are the most common birth defects in human newborns. (*Circ Res*. 2012;110:1000-1013.)

Key Words: cardiovascular development ■ miRNA ■ ncRNA ■ cell differentiation

The heart is the first organ to function during vertebrate embryogenesis. The initial steps of cardiac development are relatively simple consisting of the fusion of endocardial and myocardial cell layers in the midline to form the bilayered heart tube that already contracts spontaneously and supports the blood supply of the developing embryo. In mammals, the heart further develops into an increasingly complex structure and eventually into the mature, 4-chambered heart.

A similar increase in complexity is seen during evolution when comparing the blood-pumping organ of crustaceans constituted of a single-chambered sac located into a pericardial chamber with the 4-chambered primate heart composed of a multilayered myocardium lined externally and internally by epicardium and endocardium.1 Clearly, the vertebrate heart is very different from other blood-pumping organs found in metazoans, although a common genetic program appears to underlie the specification and differentiation of...
most if not all cardiac cells. The vertebrate heart is also able to adapt to different conditions, thereby enabling adjustment to different physiological and pathophysiologic requirements. It is evident that the basic genetic program that underlies heart development must be extended, modulated, and fine-tuned to cope with these increasingly complex tasks. The formation of the vascular system in mammals is a similarly intricate process including assembly of mesoderm-derived endothelial precursors (angioblasts) that differentiate into a primitive vascular labyrinth (vasculogenesis), subsequent vessel sprouting (angiogenesis) to create a tubular network that remodels into arteries and veins and recruitment of pericytes, and vascular smooth muscle cells (VSMCs) to form mature vessels. Although vessels are quiescent in adults and rarely form new branches they are subject to complex regulatory signaling networks, which are also of outmost importance for a variety of disease conditions.

Malfunctions of cardiovascular development result in birth defects, which are very common among human beings. Congenital heart disease (CHD), for example, accounts for nearly one-third of all major congenital anomalies and hence represents the most common birth defect in newborns. Germ-line mutations and/or somatic mutations have been identified in several genes, which play important roles in the regulation of cardiovascular development resulting in cardiovascular defects. This collection does also include genes involved in the processing of noncoding RNAs (ncRNAs) such as the DiGeorge syndrome critical region gene 8 protein (DGC8). Moreover, mutations in individual genes representing ncRNAs might cause cardiac defects although convincing data from human patients are still missing. In fact, no known cause can be identified for most congenital heart defects, which might indicate that the complicated regulatory networks that drive cardiovascular development are particularly susceptible not only to genetic components but also to environmental cues.

The Complex World of Micro RNA Processing and Action

Activity of genes is regulated at many different levels and involves epigenetic, transcriptional, and posttranscriptional control including regulation of translation, protein activity, and turnover. A significant degree of this complexity is conferred by regulatory ncRNA molecules, including micro RNAs (miRNAs), endo–small interfering RNAs (endo-siRNAs), PIWI interacting RNAs (piRNAs), and various classes of long ncRNAs. miRNAs are an important subset of endogenous small noncoding RNAs. The 21–24 nucleotide (nt)-long miRNAs are encoded either by distinct genes of endogenous small noncoding RNAs. The 21–24 nucleotide

| Non-standard Abbreviations and Acronyms |
|-----------------|-----------------|
| CHD             | congenital heart disease |
| HUVEC           | human umbilical vein endothelial cells |
| miRNA           | micro RNA |
| ncRNA           | noncoding RNA |
| RISC            | RNA-induced silencing complex |
| SMC             | smooth muscle cell |
| pre-miRNA       | precursor micro RNA |
| VSMC            | vascular smooth muscle cells |

required for processing of a subset of miRNAs, including miRNAs with regulatory roles in the cardiovascular system. Moreover, different well-known signaling pathways regulate maturation of pre-miRNAs to pre-miRNAs. One of the best-studied examples is the TGF/β/BMP signaling pathway, which regulates miRNA processing in VSMCs. miRNA processing is also hormonally regulated via the nuclear estrogen receptor in a p68- and p72-dependent manner. Estrogen signaling appears to affect processing of a specific subset of miRNAs, including miRNAs that have a fundamental role in smooth muscle cell biology although the implications of estrogen signaling on miRNA processing for smooth muscle cell functions have not been investigated in detail. Moreover, the tumor suppressor p53 stimulates posttranscriptional processing of certain miRNAs. Finally, the last step of pre-miRNAs processing, which involves the Drosha/DGCR8 complex to generate hairpin-shaped pre-miRNAs of approximately 60–80 nucleotide length with a 2–3 nt 3’ overhang, might be bypassed. In this case, specific miRNAs (mirtrons) are generated by an alternative pathway directly from intronic sequences without Drosha processing.

Pre-miRNAs are exported from the nucleus to the cytoplasm by the nuclear transport receptor exportin 5 and Ran-GTP, followed by further processing by the RNase III Dicer resulting in the formation of duplex RNAs. One strand of the duplex RNA becomes the mature miRNA, whereas the other strand is most often rapidly degraded. The mature single-stranded miRNA together with the argonaute protein forms the RNA-induced silencing complex (RISC) that interacts with miRNAs. However, the latter “passenger” miRNA strand, termed miRNA*, is often found in deep sequencing approaches and can sometimes be included into the RISC thereby functioning as a miRNA. In addition, Dicer not only processes miRNAs but also double-stranded RNA molecules such as siRNAs or endo-siRNAs. The mammalian genome contains several hundreds miRNAs that are catalogued and annotated at the miRBase database. In animals, miRNAs repress the expression of other genes at a posttranscriptional level by imperfect binding to transcripts of protein coding genes most often at 3’UTRs of target miRNAs. The effectiveness by which miRNAs bind targets is affected by the secondary structure of the target miRNA, possible complex formation with RNA-binding proteins, competition with other seed sequences, the number of putative target sites, or the position of a miRNA target site within the 3’ UTR with
The ultimate basis of miRNA-mediated regulatory effects is based on imperfect complementarity of miRNA sequences with respect to the stop codon of the respective ORF. An elegant example of complex interactions that have an impact on miRNA function in regulatory networks is the modulation of miRNA activity by long noncoding RNAs, which act as ceRNAs. Several studies revealed that miRNA families are continually added to bilaterian lineages during evolution resulting in the increased complexity of vertebrate hearts is—at least in part—caused by the advent of additional layers of regulation imposed by newly acquired miRNA regulatory networks. Apparently, miRNA–target interactions are rather stable, which might be promoted by the high number of target miRNAs that are regulated by single miRNAs. Mutations of individual miRNAs will affect regulation of numerous target mRNAs, which most often will have negative consequences for evolutionary fitness. On the other hand, new miRNAs might rapidly evolve to establish additional regulatory circuits after gene duplication allowing an organism to adjust swiftly to evolutionary pressure. Moreover, additional genes can be incorporated into existing regulatory network with relative ease. The location of miRNAs within introns of protein coding genes or within polycistronic primary transcripts does also favor rapid evolvement of new miRNAs. New miRNAs, which are under the control of transcriptional regulators of already existent genes or miRNAs, “only” need to maintain relatively simple structural prerequisites such as hairpin structures combined with small sequence variations. Other mechanisms of miRNA evolution include arm switching, antisense transcription of preexisting miRNA loci, or hairpin shifting. In addition, the regulation of miRNAs by miRNAs is not coupled to the structure and function of the gene product and thus allows more sophisticated interactions. Taken together, the unique properties of miRNAs allow rapid evolvement of new miRNAs providing a molecular playground for evolution of regulatory interactions within the cardiovascular system.

Figure 1. Basic principles of miRNA biology. miRNAs are encoded in the nucleus as intergenic or intronic miRNAs. Blue boxes indicate exons, red and green boxes indicate miRNA coding regions. miRNAs are often clustered and may be transcribed as polycistronic pre-miRNA transcripts. Most miRNAs are transcribed by RNA polymerase II. Expression of miRNAs is regulated by transcriptional regulators and by epigenetic mechanisms in a cell- and differentiation-specific manner. Most miRNAs are processed in the nucleus by a protein complex that contains Drosha, DGCR8, and several other additional proteins such as the RNA helicases p88/p72. miRNA processing is regulated by several signaling pathways such as the TGFβ/SMAD- or estrogen-signaling cascade. Processed pre-miRNAs are exported from the nucleus with the help of Ran-GTP and Exportin5. The hairpin structure of the pre-miRNA is further processed in the cytoplasm by the RNAase III Dicer, and the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), whereas the opposing miRNA* strand is usually rapidly degraded although it sometimes also acts as a miRNA. The mature miRNA in the RISC complex mediates translational repression or decay of the transcript, usually resulting in negative regulation of protein abundance of the primary target. Several modes of action have been described for miRNAs resulting in modulation of protein abundance or complete repression of proteins. The specific mode of action may depend on the abundance and relative stoichiometry of transcript and miRNA. In many cases, feedback loops have been identified in which a particular miRNA regulates its own transcription either directly or indirectly.
to their mRNA targets. Therefore, the primary structure of miRNAs and mRNAs is used to predict potential miRNA targets. Specificity is mainly determined by the “seed” region of the miRNA, which comprises nucleotides 2–8 of the mature miRNA. The other regions of the miRNA are also important for specificity of miRNA binding or for stabilization of miRNA-mRNA binding.\textsuperscript{40,41} Based on sequence similarities between miRNA and mRNA/UTR sequences several algorithms have been developed to predict miRNA targets.\textsuperscript{42–45} Moreover, databases are available offering target predictions for many organisms. To improve reliability of target predictions the conservation of putative miRNA target sites has been implemented assuming that regulatory interactions are conserved. However, predictions of potential targets rely on detailed knowledge of the transcriptome in specific cell types, which is often not available and will yield high numbers of putative targets (usually hundreds to thousands). Comparison of different prediction algorithms or increase of stringency parameters of predicting algorithms has only a limited value for reliable identification of in vivo targets. Appropriate experimental conditions have to be established to overcome the limitations imposed by merely computational predictions of miRNA regulatory interactions (see below).

**Experimental Strategies for miRNA Analysis and Their Limitations**

Expression of miRNAs must be considered in the context of other regulatory processes in a cell or organ. In many cases, changes in the miRNAome of a tissue or organ are the result of alterations of the cellular composition of a tissue or organ together with upregulation or downregulation of miRNAs in individual cell types. During heart development, for example, the ratio between endocardial and myocardial cell changes, which must be taken into account when analyzing effects of miRNA within the developing cardiovascular system. Many methods such as Northern blots, PCR-based methods, microarray hybridization, deep sequencing approaches, or in situ hybridization are available to analyze spatiotemporal expression of miRNAs.\textsuperscript{46} In principle, these methods are trivial but results have to be evaluated with great care as changes in the cellular composition of a tissue might simulate high fold changes of miRNAs. Likewise, it is tempting to use different algorithms and tools to predict putative targets that fit best to a cellular or developmental phenotype, although the regulation of a target mRNA by miRNAs is much more complicated involving additional parameters that cannot be deduced directly from primary sequences. Many attempts were made to refine prediction of targets as for example analysis of miRNA target sequence conservation across species, in vitro modulation of miRNA concentration, the use of artificial reporter genes, and large-scale proteomics, based on the modulation of miRNAs in cellular systems. Yet, all these approaches suffer from several potential pitfalls. Direct approaches aiming to detect miRNA-mRNA complexes by immunoprecipitation of the RISC appear more promising.\textsuperscript{47} An interesting alternative based on the use of bacteriophage MS2 binding protein to affinity-purify MS2-tagged RNA to isolate miRNAs associated to the UTR of a specific gene has also been described recently.\textsuperscript{48} Yet, for the time being, the benefit of such techniques for the analysis of complex in vivo situations remains unclear.

At present, a robust in vivo system under physiological or appropriate pathophysiological conditions appears most suited to reveal the complexity of miRNA-target interactions. Only appropriate in vivo systems will uncover the physiological significance of regulatory interactions. Recent findings emphasized the importance of the dynamics of miRNA-target interaction for regulation of target protein levels\textsuperscript{49} and demonstrated that miRNA effects strongly depend on the concentrations of miRNA or target mRNA.\textsuperscript{50} Hence, identification of physiologically significant interactions remains a challenge of miRNA research. It seems reasonable to assume that only few of the multiple theoretically possible miRNA-target interactions really occur in vivo. Probably even fewer are physiologically significant. Overexpression, that is, gain of function experiments, using miRNAs in an appropriate cellular context is a reasonable approach to understand the function of specific miRNAs but it is of paramount importance to control the concentration of miRNAs and mRNAs. Validation of predicted miRNA:mRNA interactions by ectopic expression of either the mRNA target or the miRNA at nonphysiological levels, may “confirm” an interaction that does not exist in vivo.\textsuperscript{51} The luciferase reporter assay is an example of an experimental set-up with a strong bias since it uses artificial miRNA concentrations in a heterologous cell (−culture) system in combination with nonphysiological concentrations of a hybrid mRNA containing the luciferase open reading frame combined with the UTR of a predicted target. The highly sensitive luciferase reporter assay only proves that a given miRNA:mRNA interaction might occur although this is most often already obvious from target predictions in silico. Luciferase reporter assay can also yield false-negative results if certain cofactors are not present in the particular model system. The use of reporter constructs and mutant controls in vivo using BAC transgenes offers an interesting alternative approach to assess miRNA-target interaction in a much more physiological context.\textsuperscript{52} Temporal knock-down experiments in vitro and in vivo to deplete individual miRNAs in cells or organs\textsuperscript{53,54} represent an attractive alternative but require careful controls since even slight variations of the protocols might yield conflicting results.\textsuperscript{55–58} Knock-down approaches might also cause off-target effects in particular in tissues or cells that do not express the correct miRNA:mRNA combination or have incomplete reduction of miRNAs, which might affect only a subset of miRNA targets.

Constitutive or timely controlled genetic knock-out experiments have distinct advantages compared with knock-down approaches but are also associated with some inherent problems: (1) miRNA targets that are upregulated after loss of a specific miRNA might be hidden among secondarily upregulated genes; (2) compensatory mechanisms, which are activated in particular after prolonged inactivation of a miRNA gene, might mask bona fide miRNA effects; (3) it might be technically challenging to simultaneously inactivate potentially redundant miRNA genes. Despite these restrictions genetic approaches to analyze miRNA, functions in vivo appear to be superior compared with other methods. Genetic approaches are often combined with target prediction algo-
Disruption of the miRNA Processing Machinery Abrogates Normal Heart Development

The importance of miRNA-mediated posttranscriptional regulation for mammalian cardiovascular development has been accessed by generating loss of function mutations of enzymes essential for miRNA biogenesis such as Dicer, Drosha, Ago2, and DGCR8. Knock-out mice individually lacking these key miRNA-processing genes die during early gestation with severe developmental defects preventing analysis of the role of miRNAs in the cardiovascular system. Therefore, Cre-inducible conditional knock-out mice of Dicer and DGCR8 have been generated to get first insights into the role of miRNAs for cardiovascular development. Deletion of Dicer during early heart development (day 8.5 of mouse embryonic development) using a Nkx2.5-Cre leads to pericardial edema and defects in the ventricular myocardium. A slight modification of this approach, using a different Nkx2.5-Cre line revealed that miRNA-mediated regulation of developmental processes is also essential for cardiac outflow tract morphogenesis and chamber septation. Deletion of miRNA signaling in neural crest cells indicates that miRNAs are not required for survival of neural crest cells but are essential for migration and patterning.

Inhibition of miRNA signaling at later developmental stages indicates an essential role for long-term survival and function of cardiomyocytes. Deletion of Dicer, using an αMHC/Myh6 promoter-driven Cre-recombinase that directs Cre-recombinase expression in cardiomyocytes but not in cardiac fibroblasts, causes depletion of mature miRNAs and accumulation of pre-miRNAs at P0 leading to death of mutant mice between postnatal day 0–4. Hearts of mutant mice are dilated and show a dramatic decrease of fractional shortening and a reduced contraction rate. These phenotypic differences were measured in mutant embryos, which might explain the phenotype but might also result from malformation of the vasculature and subsequent hypoxia. Further, in vitro studies confirmed the requirement of miRNAs for endothelial cell function. Reduction or loss of Dicer expression in endothelial cells leads to misexpression of several genes involved in regulation of endothelial cell function including increased NO release, decrease of endothelial cell growth, and cord formation in matrigel assays, whereas almost no changes in endothelial cell migration was seen. Interestingly, transfection of miR-221 or miR-222, which are strongly expressed in endothelial cells, rescues some of the molecular changes in Dicer depleted cells. In a different in vitro study using human umbilical vein endothelial cells (HUVEC), knock-down of Drosa did not have massive effects on cell viability and tube formation in matrigel. In this study, miR-27b and let-7f were identified to be responsible for some phenotypic effects observed after inactivation of Dicer although multiple miRNAs were downregulated after loss of Dicer or Drosha. Inhibition of miR-27b and let-7f caused impaired sprouting in spheroid-based angiogenesis assays. Human microvascular cells have been used to demonstrate that Dicer is also essential for redox signaling in these cells. Deletion of Dicer in vivo using endothelial specific Tie2-Cre or inducible VE-cadherin-Cre revealed a requirement of miRNAs for postnatal angiogenesis in tumorgenesis, wound healing, and limb ischemia. Loss of Dicer reduces responsiveness to VEGF-induced angiogenesis and VEGF-treatment of endothelial cells stimulates expression of several miRNAs including miRNAs of the miR-17–92 cluster. Apparently, the miR-17–92 cluster plays a major role for vessel sprouting since transfection of miRNAs of the miR-17–92 cluster rescues reduced VEGF-stimulated sprouting in a matrigel assay using HUVEC after loss of Dicer. Surprisingly, no major effects of miRNAs were found in embryonic angiogenesis although theTie2-Cre used to delete Dicer should be active during early embryonic development. This might be explained by residual activity of Dicer in endothelial cells, an incomplete recombination, or reduced requirement of miRNA mediated posttranscriptional regulation at early developmental stages. It will be interesting to analyze the loss of Dicer in endothelial cells at a single cell level.
In contrast, deletion of Dicer in embryonic VSMC is lethal resulting in intraperitoneal bleeding and prenatal death of mutant embryos at E16.5–E17.5, again confirming the fundamental role of miRNA mediated posttranscriptional regulation for embryonic development. Deletion of Dicer using the SM22alpha-Cre-recombinase line abrogates Dicer expression in VSMC and in cardiomyocytes but to a much lower extend in visceral SMCs. Reduction of Dicer in the umbilical cord was monitored at E14.5 although SMC specific miRNAs were not completely lost at E16.5 indicating a high stability of certain miRNAs or incomplete deletion of Dicer in SMCs of the umbilical cord.\(^7\) On a cellular level, loss of Dicer causes incomplete differentiation, reduced proliferation, reduced contractile force, and loss of SMC differentiation markers. Interestingly, expression of several molecular markers was rescued in isolated cells by transfection of miR-145 but not miR-143, miR-21, or miR-221, indicating that loss of miR-145 is a major cause of the observed phenotype. It is likely, however, that other miRNAs than miR-145 have also a strong impact on SMC proliferation and development because loss of miR-145 alone does not cause a major embryonic phenotype (see below). Recently, a second smooth muscle-specific deletion of Dicer was reported using the same Cre-line but a different Dicer allele as before yielding a slightly different outcome.\(^7\) Pan et al reported a smaller size of mutant embryos, which die between E14.5–E15.5 with hemorrhage in different organs including liver, brain, and small intestine. In addition, reduced VSMC proliferation and reduced vascular wall thickness was detected similar to the previous study.\(^7\) The reason for phenotypic differences between the two SM22-Cre mediated deletions of Dicer remains unclear. Postnatal deletion of Dicer in SMCs using a tamoxifen-inducible SM-Myh-Cre results in a dramatic reduction in blood pressure due to significant loss of SMCs, the phenotypes of Dicer knock-out mice is more severe suggesting that addi-}

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**The Role of Individual miRNAs During Cardiac Development**

**Transcriptional Control of Cardiac miRNA Expression**

Among the first miRNAs that were identified to be specifically expressed during cardiac development are the miRNAs miR-1 and miR-133a. In mammals, the genes coding these miRNAs are clustered; interestingly the cluster exists 2 times in the genome, and both clusters are expressed in cardiac and skeletal muscle. The miR-1–1/133a-2 cluster at human chromosome 20 is located at an intergenic position, whereas the miR-1–2/133a-1 cluster at human chromosome 18 is situated within an intron and in an antisense direction relative to the Mibl gene that codes for an E3 ubiquitin ligase. Yet, the miR-1–2/133a-1 cluster has its own promoter and different transcriptional activity as the Mibl gene. The expression of the closely related miR-206/133b cluster is restricted to the skeletal muscle.\(^90,91\) Different studies have shown that the regulation of miR-1/133 clusters early in development is under control of well-known transcriptional networks involving SRF and Mef2.\(^82,83\)

Cardiac and skeletal muscle-specific gene regulatory programs also drive expression of miR-1/133 in vitro.\(^94\) Moreover, miR-1 or miR-133 expression is able to rescue mesodermal gene expression of SRF-deficient ES cells.\(^85\) In addition, downregulation of miR-1/133 has been described in cardiac hypertrophy after transverse aortic arch restriction and in Akt transgenic mice.\(^86\) Intragenic miRNA genes share transcriptional regulatory mechanism with their respective host genes, potentially at the epigenetic level but respond also to transcriptional enhancers or repressors acting on the host genes. An especially elegant example is the control of miR-208a/b expression and the related miR-499 miRNAs that reside in introns of the muscle myosin heavy chain genes. miR-208a resides in the Myh6 gene encoding the myosin heavy chain protein of the adult heart, whereas miR-208b and miR-499 are coexpressed together with the slow myosins Myh7 and Myh7b.\(^87,88\)

**The Function of Specific miRNAs in Cardiac Development**

In differentiating ES cells, miR-1 and miR-133 are under the transcriptional control of the regulatory network that controls differentiation of cardiomyocytes. Expression of both miRNAs facilitates mesodermal gene expression and disturbs and suppresses endodermal or neuronal differentiation. However, miR-1 appears to promote cardiac or skeletal muscle cell formation during terminal differentiation, whereas miR-133 counteracts cardiac differentiation.\(^85\) In contrast, it has also been shown that miR-1 overexpression inhibits myocardial differentiation in a different setting by targeting cdk9, which is required to activate cardiac specific genes.\(^89,90\)

Drosophila miR-1 is regulated by SRF\(^91\) and by the Nkn2.5-orthologue timnan.\(^92\) miR-1 affects drosophila heart and muscle development by regulation of the notch ligand delta\(^91\) and the Rho GTPase Cdc42.\(^92\) Modulation of miR-1 expression during drosophila development revealed a role of miR-1 in the formation of cardiac (dorsal vessel) cell polarity.\(^93\) miR-1 might have a similar function in mammals, which would explain cardiac patterning defects after miR-1 deletion in mice, although the exact mechanisms are enigmatic and await further studies.

miRNA-1–1 and miRNA-1–2 are the most abundant miRNAs in the mouse heart, accounting for almost 40% of all reads in deep sequencing approaches of small RNAs. No
other single miRNA reached a similar abundance not even the clustered miR-133a, which indicates differential processing or differential stability of these miRNAs. Deep sequencing of small RNAs from isolated cardiomyocytes revealed that other miRNAs might reach a higher abundance under certain conditions (Schweisgut, Boettger, and Braun, unpublished data). In any case, the vast amount of miRNA molecules in physiological conditions (Schweisgut, Boettger, and Braun, unpublished data). In any case, the vast amount of miRNA molecules in the heart is composed of only a few abundant miRNAs. Some of these miRNAs are not expressed in a cell type–specific manner such as the ubiquitously expressed let7/miR-98 family, which is the second most abundant miRNA family in the heart.70 The abundance of let7 family miRNAs in the heart suggests that not all effects observed in Dicer mutant mice are due to the loss of heart specific miRNAs.

Zhao et al approached the function of miR-1 in mammals by overexpression of miR-1. Myh7/BMHC-miR-1 transgenic animals contain a decreased number of proliferating cardiomyocytes in the developing heart at E13.5 resulting in reduction of the compact layer of the developing heart. This has been ascribed to down-regulation of Hand2, which represents a conserved target of miR-1.82 Deletion of 1 of the 2 copies of miR-1 (miR-1–2) in a knock-out model resulted in developmental alterations including ventricular septum defects at E15.5 and pericardial edema formation. However, this phenotype appeared only with a partial penetrance and was not seen in another miR-1–2 knock-out, raising concerns about potential effects of neighboring genes such as Mib1. Developmental alterations in miR-1–2 mutant hearts were attributed to increased expression of the Hand2 protein, although it is unclear whether other miR-1–2 regulated genes like Gata6 do also contribute to the phenotype.61 Surviving homozygous mutant miR-1–2 animals suffer from sudden cardiac death with changes in QRS complex length and morphology, again with variable penetrance. It has been proposed that the absence of miR-1–2 alters expression of the homeodomain transcription factor Irx5 leading to secondary changes of the expression of the IRX5 target Kcnq2, which is involved in cardiac repolarization.61,84 MiR-1 as well as the clustered miR-133 are downregulated in different models of cardiac hypertrophy.86,95 Because miR-1 overexpression in vivo and in vitro attenuates aspects of isoproterenol-induced cardiac hypertrophy and overexpression of miR-1 negatively regulates Mef2a and calmodulin expression in isolated neonatal rat cardiomyocytes, it seems possible that miR-1 restricts cardiac hypertrophy by reducing expression of Mef2a and calmodulin (Table).86,95

The function of miR-133 during heart development has been investigated in several overexpression and knock-down experiments. For example, knock-down of miR-133a via administration of miR-133a antagonirs resulted in cardiac hypertrophy, which might be mediated by the potential miR-133a targets RhoA and Cdc42.86 However, these results were not confirmed in a subsequent study, in which the miR-133a genes were deleted.86 Reduced expression of miR-133a in mice lacking either miR-133a-1 or miR-133a-2 did not cause an apparent phenotype. Deletion of both miR-133a genes resulted in lethal ventricular-septal defects in some double mutant embryos. Surviving double-mutant animals
developed a dilative cardiomyopathy after birth, which was characterized by ectopic expression of smooth muscle marker genes and abnormal proliferation of cardiomyocytes. These changes were attributed to increased expression of the miR-133a targets cyclinD2 and Srf. SRF might directly stimulate expression of smooth muscle genes and cyclinD2 might contribute to increased cell proliferation.86 Interestingly, in an affinity purification approach miR-133 together with miR-1 was found to bind to the UTR of Hand2 mRNA. The regulation of Hand2 protein expression by miR-133a was also verified in miR-133a knock-out mice,48 indicating that the clustered and coexpressed miRNAs miR-1 and miR-133 cooperatively target the same pathways and genes.

The Role of Intrinsic miRNAs in Postnatal Cardiac Development

miR-208a, miR-208b, and miR-499, which are encoded in introns of Myh6 (αMHC), Myh7 (βMHC, “slow” heavy chain of cardiac myosin) and Myh7b, probably represent the best-studied examples of miRNAs in cardiac development and disease so far. The expression of Myh6 and Myh7 is inversely regulated during cardiac development and varies in a species-specific manner. In mice, Myh7/miR-208b is expressed in the embryonic heart and Myh6/miR-208a is predominantly found at postnatal stages, whereas in humans, ventricular expression of Myh7 is continued into adulthood. Under cardiac stress conditions or hypothyroidism, Myh7 is reexpressed in the adult mouse heart. In contrast, Myh7b and miR-499 are expressed in the embryonic as well as in the adult heart.97 In principle, expression of miR-208a and miR-208b parallels expression of their respective host genes during development,87 premiR-208a and Myh6 expression are strongly correlated under experimental conditions, indicating that these miRNAs follow the same transcriptional cues as the host gene.88 This setting allows a tight coordination of a major structural protein together with a miRNA that is able to alter the cellular environment. Genomic deletion of miR-208a without impairing Myh6 expression leads to ectopic expression of fast skeletal muscle markers in the adult heart, demonstrating that the Myh6 intron-encoded miR-208a affects cardiac differentiation.89 Loss of miR-208a does not influence expression of Myh7/miR-208a or Myh7b/miR-499 in the embryonic heart, probably because miR-208b expression in the embryonic heart rescues Myh7b/miR-499 expression. However, in the adult mouse heart miR-208a is essential for Myh7b/miR-499 expression97 and for expression of the miR-208b containing Myh7.87,88 In addition, miR-208a is indispensable for mediating some aspects of the cardiac stress response during pressure overload after transaortic constriction in mice. In particular, hypertrophic growth, fibrosis, and the concomitant upregulation of Myh7 appear to be under the control of miR-208a, whereas other stress responses such as increased ANP and BNP expression were not reduced but even exaggerated after loss of miR-208a.88 The effects of miR-208 and miR-499 in the developing and adult heart are in part mediated via the miR-208 targets Thrap1, a component of the thyroid hormone receptor complex, and myostatin.87,88 miR-208a expression is also essential for some electrophysiological properties of the heart,87 which might involve the miR-208a target Gata4. Remarkably miR-208a/b as well as miR-499 appear not to have a major role during heart development, possibly reflecting the fact that these miRNAs are part of a robust network that compensates for certain malfunctions. Hence, the described miR–208–Thrap1 interaction might be nonessential for early cardiac development.

Reexpression of miR–499 in miR–208a knock-out animals rescues several aspects of the miR–208 phenotype including Myh7/208b expression in hypothyroid animals and the suppression of some ectopic skeletal muscle markers although miR–499 expression is not required for Myh7 expression in hypothyroid animals.97 This led to the conclusion that miR–499 acts downstream of miR–208a at least at some stages of development. An interesting aspect of miR–208/miR–499 signaling is that some but not all regulatory interactions in the heart are also active in slow skeletal muscle fibers, again demonstrating that specific functions of a miRNA can only be understood in its precise physiological context.97 In larger animals, including human beings, Myh7 is expressed in the adult heart. Therefore, it has been suggested that miR–208b, which has the same seed sequence as miR–208a, might exert the same functions in humans as miR–208a does in rodents.97 miR–499 also supports myogenic differentiation in vitro in cardiomyocyte progenitor cells isolated from human embryos, which might at least in part be mediated by Sox6.98 A similar function was disclosed in differentiating human ES cells, in which lentiviral overexpression of miR–499 promotes differentiation of ES cells to the ventricular cardiomyocyte lineage, including upregulation of Myh7/Myh6 and troponin T.99 In ischemic heart, miR–499 regulates mitochondrial dynamics after myocardial infarction. Thus, overexpression of miR–499 might inhibit cardiomyocyte apoptosis via targeting calcineurin and dynamin-related protein–1. Interestingly, it has also been shown that p53 negatively regulates miR–499 expression during ischemia independent of its host gene Myh7b, which suggest a host-independent regulation of miR–499 under certain conditions.100

The miR–17–92 Cluster Is Essential for Embryonic Cardiac Development

The miR–17–92 cluster, as well as the paralog clusters miR–106a–363 and miR–106b–25, is broadly expressed in different tissues of the body. Recent loss-of-function studies have addressed the functions of these miRNAs in different tissues.101–103 Of note, deletion of the miR–17–92 cluster causes ventricular-septum defects in mice and severe lung hypoplasia101 leading to perinatal death, whereas loss of the paralogous clusters did not affect viability of mutant animals. In a more thorough analysis of the cardiac phenotype of the miR–17–92 mutants, this cluster has been identified as part of a signaling cascade in the second heart field that is essential for myocardial differentiation in the cardiac outflow tract. It has been proposed that BMP signaling promotes transcription of the miR–17–92 cluster through conserved Smad-binding sites. Upregulation of the miR–17–92 cluster might directly downregulate the cardiac progenitor genes Isl1 and Tbx1, thereby facilitating myocardial differentiation.102
A Role of the miR-15 Family in Postnatal Cardiac Development

The miRNA family of miR-15–related miRNAs comprises 6 miRNAs (miR-15a/b, miR-16–1/2, miR497/-195) with identical seed sequences. These miRNAs, which are located on human chromosome 13 (miR-15a/miR-16–1), 3 (miR-15b/miR-16–2), and 17 (miR-497/miR-195), represent another example of clustered and duplicated miRNAs. Members of this miRNA family show dynamic regulation during postnatal cardiac development and disease, miR-19 and the clustered miR-497 are upregulated during early postnatal cardiac development of the mouse between postnatal day 1 and postnatal day 10. The upregulation coincides with the exit of cardiomyocytes from cell proliferation and subsequent binucleation. Overexpression of miR-195 in the embryonic heart causes precocious cell cycle arrest and repression of mitotic genes. The checkpoint kinase 1 (Chek1) appears to be a major primary target of miR-195, mediating some effects of miR-195 on cell cycle regulation. In addition, miR-15 family members have been identified in a screen for miRNAs that regulate cellular ATP levels in rat neonatal cardiomyocytes. Neutralization of miR-15b by transfection of a miR-15b decoy vector in vitro changes mitochondrial morphology, which appears to involve the ADP-ribosylation factor-like 2 gene. Interestingly, miR-15 family members are also upregulated after myocardial infarction in cardiac tissue. Inhibition of miR-15 under hypoxic conditions increases cardiomyocyte survival in vitro and reduces ischemia-induced impairment of cardiac function in mice. Comparison of predicted miR-15 targets with genes upregulated after miR-15 inhibition identified pyruvate dehydrogenase kinase 4 (PDK4), a regulator of mitochondrial function and SGK1.

The Role of Specific miRNAs During Vascular Development: Endothelial Cells

The first hint that miR-126 is specifically expressed in the cardiovascular system dates back to 2002. After development of methods such in situ detection that allowed precise spatial resolution of miRNA expression it became clear that miR-126 is specifically expressed in the cardiovascular system and more precisely in endothelial cells. It was found that miR-126 is expressed in endothelial cell lines and in the endothelial lineage of differentiating ES cells, where miR-126 is among the most strongly upregulated miRNAs. Although miR-126 appears not to be involved in endothelial lineage commitment, manipulation of the miR-126 levels in human umbilical vein endothelial cells (HUVEC) modulates the phenotype of these cells and in particular the response to VEGF- and bFGF-induced migration. Inhibition of miR-126 in zebrafish embryo by morpholino injection revealed no gross malformation of vascular patterning of the early zebrafish embryo, but blood circulation was disturbed probably due to collapsed lumen and compromised endothelial tube formation between 36–72 hpf. miR-126 targets the sprouty-related protein Spred1, a regulator of MAP kinase signaling downstream of VEGF and FGF signaling, and PK3R2a, a regulator of PI3 kinase signaling. Knock-down of SPRED1 or PIK3B2/p85β partially rescues the miR-126 knock-down phenotype in HUVEC and increased expression of SPRED1 thereby mimicking effects of miR-126 knock-down in the vascular system of zebrafish embryos with respect to vascular integrity. In mice, deletion of the intronic miR-126 leads to vascular abnormalities with failure of cranial vessel growth and endothelial leakage at reduced penetrance. Moreover, endothelial cells of miR-126 knock-out mice show impaired outgrowth and reduced response to FGF2 in vitro. Using microarray analysis combined with target prediction, Spred1 was also identified in the mouse as an important miR-126 target mediating crucial functions of miR-126. In support of this hypothesis, virus-mediated overexpression of miR-126 restored the migratory activity of miR-126 knock-out endothelial cells. miR-126 might also have additional functions in endothelial cells on top of its role in VEGF receptor signaling because it also targets other interesting molecules such as Vcam-1 involved in leukocyte–endothelial cell interaction.

Another elegant example of the role of miRNA controlled regulatory circuits in angiogenesis is the slit-miR-218-robo system described in 2 recent studies in mouse and zebrafish. The slit ligands and their robo receptors have crucial functions in axon guidance but these molecules also instrumental for vascular development. The slit-2 and slit-3 genomic loci both encode miRNA-218 miRNAs. Hence, miR-218 is expressed in the brain as well as in organs containing large numbers of endothelial cells. Interestingly, miR-218 can target robo1 and robo2 receptors (in slit expressing cells) as well as components of the heparin sulfate proteoglanucan synthesis system, which is involved slit-robo signaling. Manipulation of miR-218 concentrations in vitro modulates endothelial cell migration and knock-down of miR-218 in the developing retina results in anomalous development of the vascular plexus of the retina suggesting a role of miR-218 in this process. Unfortunately, the molecular mechanism and the precise role of cell-cell interactions in this model are not completely clear. A knock-down of miR-218 in zebrafish did not reveal a function of miR-218 in development of the vascular system although slit-miR-218-robo signaling modulates VEGF signaling in endocardial cells. Future genetic studies using tissue specific loss of function mutations will help to improve our understanding of the role of miR-218 and its impact on slit-robo signaling for development of the cardiovascular system.

MicroRNA-92a as well as other members of the miR-92–92 cluster are also expressed in endothelial cells and manipulation of these miRNAs in vitro modulates properties of endothelial cells. miR-92 is upregulated in ischemia and overexpression in vitro in endothelial cell based assays as well as in zebrafish embryogenesis impairs angiogenesis and vessel formation. Inhibition of miR-92 enhances functional recovery of ischemic tissues in mice, which supports a negative regulatory role in angiogenesis. A recent study of miR-92 did also uncover a function of miR-92 in endoderm formation. Overexpression of miR-92 inhibited endoderm development and depletion of miR-92 promoted endoderm formation, effects that were in both cases associated with defects in endoderm dependent developmental processes. However, deletion of the miR-17–92 cluster did
not cause an obvious vascular phenotype, whereas only additional deletion of the paralogous miR-106b–25 cluster results in embryonic lethality before E15 with edema and vascular congestion although the ultimate cause of this phenotype remained unclear.101

The miRNAs miR-23, miR-24, and miR-27, which are also highly expressed in endothelial cells, are encoded in 2 clusters. The miR-23a–27a–24 to 2 cluster is positioned on chromosome 19, and the miR-23b–27b–24 to 1 cluster on human chromosome 9. miR-23 is essential for proper cardiac valve formation during zebrafish development, particularly in formation of endocardial cushions from the endocardium. An important primary target of miR-23 is the hyaluronic acid synthase 2 (Has2), which is directly involved in remodeling of the extracellular matrix of the endocardial cushions and valves.121 Inhibition of miR-23 in zebrafish leads to ectopic expression of Has2, ectopic depositions of hyaluronic acid, and aberrant development of the cardiac cushions. miR-23 overexpression in vitro in mouse endothelial cells inhibits TGF-β-induced endothelial to mesenchymal transition.122 In addition, knock-down of miR-23 or miR-27 in HUVEC results in reduced capillary tube formation in matrigel assays. Knock-down of miR-27 reduced VEGF-induced proliferation and migration of HUVEC cells. Moreover, Sprouty2, Sema6A, and Sema6D were identified as potential primary targets of miR-23/27–mediating effects on angiogenesis.123 Several other miRNAs are regulated during endothelial cell differentiation,110,111 and miRNAs have been categorized in proangiogenic and antiangiogenic, based on different types of experiments although the developmental function of most of miRNAs in endothelial cells still is not understood. Thus far, most actions of miRNAs were linked to regulatory molecules that are well known to play major roles for angiogenesis. It seems likely that additional functions exist that are not necessarily associated with already established signaling pathways.

The Role of Specific miRNAs During Vascular Development: SMCs
The developmental origin of VSMCs is diverse and several independent cell lineages have been identified to contribute SMCs. SMCs are derived from the neural crest, somites, the second heart field, and other cell lineages. SMCs coordinately activate a common set of smooth muscle marker genes despite of their heterogenous origin, indicating the presence of a common developmental program essential for smooth muscle differentiation. Marker genes of SMC differentiation include smooth muscle α-actin, transgelin, smooth muscle calponin, and smooth muscle myosin heavy chain. SMCs from different origins differ substantially aside of their core program and respond differently to various stimuli.124 In addition, differentiated SMCs exhibit a wide range of phenotypic variations reflecting their functional diversity. Some SMCs are primarily involved in regulation and maintenance of blood pressure and perfusion, whereas others are part of repair, adaptation, and reconstruction processes. miRNAs are part of the developmental programs leading to the formation of VSMCs, as has been shown by the use of smooth muscle specific knock-out of Dicer. Several miRNAs are highly enriched in VSMCs, suggesting a more specific function in this cell type. The most prominent miRNAs in this respect are the miRNAs miR-143 and miR-145. These miRNAs are clustered in the genome and both respond to common regulatory stimuli, resulting in an SMC-specific expression pattern through most parts of embryogenesis and adult life. Similar to other SMC markers, the miR-143/145 cluster is also expressed in the early developing heart. A conserved SRF binding site (CArG box) in the 5′ enhancer region of the miR-143/145 locus is essential for SMC-specific expression.125,126 miR-143/145 are downregulated in vessels after vascular injury or arteriosclerosis reflecting the acquisition of a synthetic phenotype or a change in the cellular composition within the lesions.125,127,128 In vitro overexpression of miR-145/143 was sufficient to promote differentiation of multipotent neural crest stem cells into SMCs, and miR-145 was necessary for myocardin-induced reprogramming of fibroblasts into SMCs.215 However, miR-143 and miR-145 as well as a combination of both are dispensable for specification of SMCs in vivo. Instead, the loss of miR-145 or the miR-143/145 cluster results in a modulation of the phenotypic properties of VSMCs126,129 (Figure 2). Several molecules that are known to be involved in regulation of the contractile and synthetic phenotype of SM were identified as possible targets of miR-145 or miR-143. For example Klf4, Klf5, Srgap2, adducin-3, and Mrtf-B are possible target genes of miR-143 or miR-145. In most cases, ultimate proof that single target
genes mediate the loss of contractile properties of SMCs is lacking, which highlights the fact that miRNAs usually target several genes that synergistically influence biological processes at different levels. Unbiased screening for miRNA regulated molecules on the RNA and protein level revealed that several predicted targets of miR-143 and miR-145 are upregulated in miR-143/145-deficient VSMCs in vivo. Angiotensin-converting enzyme (ACE), a major regulator of blood pressure and VSMC contractility, was identified as a major miR-145 target, and clear target-phenotype relations were established. However, the role of other target genes such as Wif1 and Nmt1, not formerly known to play a role in SMC biology, is still unclear. Analysis of miR-143/145 knock-out mice revealed impaired blood pressure regulation. Surprisingly, miR-143/145 knock-out mice develop spontaneous arteriosclerotic lesions but fail to show injury-induced neointima formation. This apparent paradox might be explained by the hypothesis that miR-143/145 enable responsiveness to external stimuli to allow contraction and tissue remodeling. According to this model, loss of the ability to respond to external signals (ie, loss of miR-143/145) results in a signaling-refractive, cell autonomous phenotype, which prevents signaling-dependent neointima formation but favors development of SMC-dependent arteriosclerosis. Such a model fits nicely to the finding that inhibition of the miR-145 target ACE in knock-out animals by ACE inhibitors partially rescues the impaired contractility of isolated vessels and restores responsiveness to external cues and decreased collateral vessel remodeling in a hind limb ischemia model (Boettger and Braun, unpublished).

Conclusions and Outlook
The discovery that miRNAs play a pivotal role in cardiovascular development has added an additional layer of complexity to our understanding of molecular circuits controlling morphogenesis, cell proliferation, and differentiation of the cardiovascular system. Clearly, we are only beginning to understand the role of miRNAs for complex regulatory decisions. The finding that miRNAs are continuously added to the metazoan genome during evolution in particular in complex and organ-bearing nephrozoans argues for a decisive role of miRNAs for development of higher vertebrates. miRNAs and ncRNAs in general enable additional regulatory processes and thus might allow mammalians to build complex organs with essentially the same number of genes than more primitive organisms. Complex systems have the ability to buffer imbalances and cope with adverse situations. Hence, regulatory molecules that modulate complex systems are prone to exert subtle effects. Inactivation of individual miRNAs in the cardiovascular system and in other organs often does not give strong phenotypes despite the fact that miRNA sequences are under intense selection. Deletion of a single miRNA sometimes may cause a delicate phenotype that is not always obvious and therefore might escape detection in superficial forward genetic screens but may be strongly disadvantageous in a natural environment or under disease conditions. It is of paramount importance to obtain data that overcome the insensitivity of phenotypic analysis under laboratory conditions and to apply standardized disease conditions to enable accurate comparisons between different laboratories. Finally, we must better understand the potential redundancy in the regulatory architecture that might support developmental robustness. The rapid progress in miRNA biology that we witnessed in recent years raises hopes that we are on the right track, although the road to comprehensive understanding might be long and winding.

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None.

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