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MicroRNAs in Cardiac Development

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Abstract The transcriptional regulation of cardiovascular development requires precise spatiotemporal control of gene expression, and heterozygous mutations of transcription factors have frequently been implicated in human cardiovascular malformations. A novel mechanism involving post-transcriptional regulation by small, noncoding microRNAs (miRNAs) has emerged as a central regulator of many cardiogenic processes. We are beginning to understand the functions that miRNAs play during essential biologic processes, such as cell proliferation, differentiation, apoptosis, stress response, and tumorigenesis. The identification of miRNAs expressed in specific cardiac and vascular cell types has led to the discovery of important regulatory roles for these small RNAs during cardiomyocyte differentiation, cell cycle, conduction, and vessel formation. Here, we overview the recent findings on miRNA regulation in cardiovascular development. Further analysis of miRNA function during cardiovascular development will allow us to determine the potential for novel miRNA-based therapeutic strategies.

Keywords Cardiac development · Gene regulation · MicroRNAs

During the last decade, animal studies and advances in human genetics have highlighted the need for precise regulation of key molecular pathways during embryonic development. This is particularly true for the cardiovascular system, in which haploinsufficiency typically causes human disease [59]. The dosage of cardiogenic pathways can be controlled at numerous levels, some of which have been well studied. In particular, the transcriptional regulation of cardiomyocyte differentiation and cardiac morphogenesis is highly conserved across species, and heterozygous mutations of transcription factors have frequently been implicated in human cardiac malformations [46]. Recently, post-transcriptional regulation by small noncoding RNAs, such as microRNAs (miRNAs), has emerged as a central regulator of many cardiogenic processes.

miRNAs are a large class of evolutionarily conserved, small, noncoding RNAs, typically 20–26 nucleotides (nt) in length, that primarily function post-transcriptionally by interacting with the 3′ untranslated region (UTR) of specific target mRNAs in a sequence-specific manner (reviewed in Zhao and Srivastava [57]) The first animal miRNA was described in 1993 as a regulator of developmental timing in *Caenorhabditis elegans* [27, 52], but it was not until 2001 that miRNAs were recognized to be widespread in all eukaryotes, including vertebrates [26, 37, 39]. More than 650 miRNAs are encoded in the human genome, and each is assumed to target >100 mRNAs, resulting in mRNA degradation or translational inhibition. Interactions between miRNAs and mRNAs are thought to require sequence homology in the 5′ end of the miRNA; however, significant variance in the degree of complementation in the remaining sequence allows a single miRNA to target a wide range of mRNAs, often regulating multiple genes within a common pathway. As a result, more than one third of mRNAs in the mammalian genome are believed to be regulated by one or more miRNAs [8].

Despite advances in miRNA discovery, the role of miRNAs in physiologic and pathophysiologic processes is just emerging. It has become clear that miRNAs play

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diverse roles in fundamental biologic processes, such as cell proliferation, differentiation, apoptosis, stress response, and tumorigenesis. Identification of miRNAs expressed in specific cardiac cell types has led to the discovery of important regulatory roles for these small RNAs during cardiomyocyte differentiation, cell cycle, and conduction, as well as during stages of cardiac hypertrophy in adults, indicating that miRNAs may be as important as transcription factors in controlling cardiac gene expression.

Here, we review the basic mechanisms by which miRNAs function, with a focus on the role of miRNAs during development of the heart and vessels. It appears that a network of miRNAs can be superimposed on well-described signaling and transcriptional networks with considerable intersection between the two. Ultimately, knowledge of the function and regulation of specific miRNAs and their mRNA targets in the heart will lead to a deeper understanding of cardiac cell-fate decisions and morphogenesis and ultimately could result in the development of novel therapeutic or preventive approaches for heart disease.

miRNA Organization, Biogenesis, and Target Recognition

miRNAs regulate gene expression at the post-transcriptional level through mRNA degradation, translational repression, or miRNA-mediated mRNA decay. Mature miRNAs are formed in a multistep biologic process involving critical endonucleases (Fig. 1). miRNAs are initially transcribed from the genome into long (several kilobases) 5′ capped, polyadenylated (poly(A)) primary transcripts (primiRNAs) by RNA polymerase II [7]. Some miRNAs interspersed among repetitive DNA elements, such as Alu repeats (5′ AG/CT 3′), can also be transcribed by RNA polymerase III [5]. The miRNA-encoding portion of the pri-miRNA forms a hairpin structure that is recognized and cleaved in the nucleus by a microprocessing complex. This complex consists of the double-stranded RNA-specific nuclease DROSHA and its cofactor, DiGeorge syndrome critical region 8 (DGCR8) [25]. The resulting approximately 70-nt hairpin precursor miRNA (pre-miRNA) is exported to the cytoplasm by the RAN-GTP–dependent nuclear transport receptor, exportin-5, which acts by recognizing a 2- to 3-base pair overhang of the pre-miRNA stem-loop structure [4, 56]. A complex of the RNAse III-like ribonuclease, Dicer, and the transactivator RNA-binding protein then cleaves the pre-miRNA to release the mature miRNA duplex.

An asymmetry in the relative thermodynamic stability of the 5′ ends of the miRNA duplex results in preferential loading of the less stable approximately 22-nt strand into the RNA-induced silencing complex (RISC); the other strand is degraded, although in some cases both strands are incorporated into the RISC [22, 40, 43]. The RISC helps mediate miRNA–mRNA interactions and subsequent mRNA repression or destabilization [19]. miRNAs typically bind to the 3′ UTRs of their mRNA targets with imprecise complementarity. Typically, the degree of Watson–Crick base-pairing between bases 2 and 7 (the "seed region") at the 5′ end of the miRNA is critical for binding mRNA targets [38, 48] and mediating repression. RISC-bound miRNAs may also be sequestered away from translational machinery in processing bodies, which act by recruiting poly(A) nucleases to help modulate deadenylation of mRNA and thereby prevent translation [16, 23, 29].

miRNAs can be found in exons or introns of noncoding transcripts with independent enhancer regulation and in the introns and 3′ UTRs of protein-coding transcripts. They can also overlap with either an exon or an intron, depending on the alternative splicing pattern. In flies and worms, some miRNAs in intronic regions bypass Drosha processing and enter the miRNA biogenesis pathway as pre-miRNAs [41]. In many cases, miRNAs are clustered near other miRNAs, and they are transcriptionally coregulated and share cooperative regulatory roles.

Several algorithmic databases have been designed for miRNA target prediction that rely, for the most part, on the following criteria: (1) conservation across species, (2) complementarity of the 5′ miRNA "seed match" to the 3′ UTR (approximately 7 nt) [28, 38, 59], (3) G:U wobbles in the seed [6], (4) the thermodynamic context of target mRNA-binding sites (i.e., mRNA targets located in regions of high free energy and unstable secondary structure are favored) [58, 59], and (5) multiple miRNA-binding sites in 3′ UTR [14]. These computational programs are continuously updated to integrate new knowledge from validated miRNA–mRNA interactions (reviewed in Sethupathy [44]).

One approach that has showed the importance of miRNAs during vertebrate development has been to create mutations in Dicer, the enzyme required to process miRNAs into their active form. *Dicer* is encoded by a single locus in vertebrates. Zebrafish lacking maternal and endogenous *Dicer* die from defects in gastrulation, brain morphogenesis, somitogenesis, and heart development [18, 51]. In mice, targeted deletion of *Dicer* causes lethality at embryonic day 7.5 (E7.5), before body axis formation [2]. Cardiac-specific deletion of *Dicer* using Cre recombinase expressed under the control of the endogenous Nkx2.5 regulatory elements resulted in embryonic lethality at E12.5 [59]. The *Nkx2.5-Cre* is active from E8.5 during heart patterning and differentiation but only after initial commitment of cardiac progenitors [34]. It will be important to determine whether *Dicer* is required for earlier steps of cardiogenesis (before E8.5), such as cardiac lineage

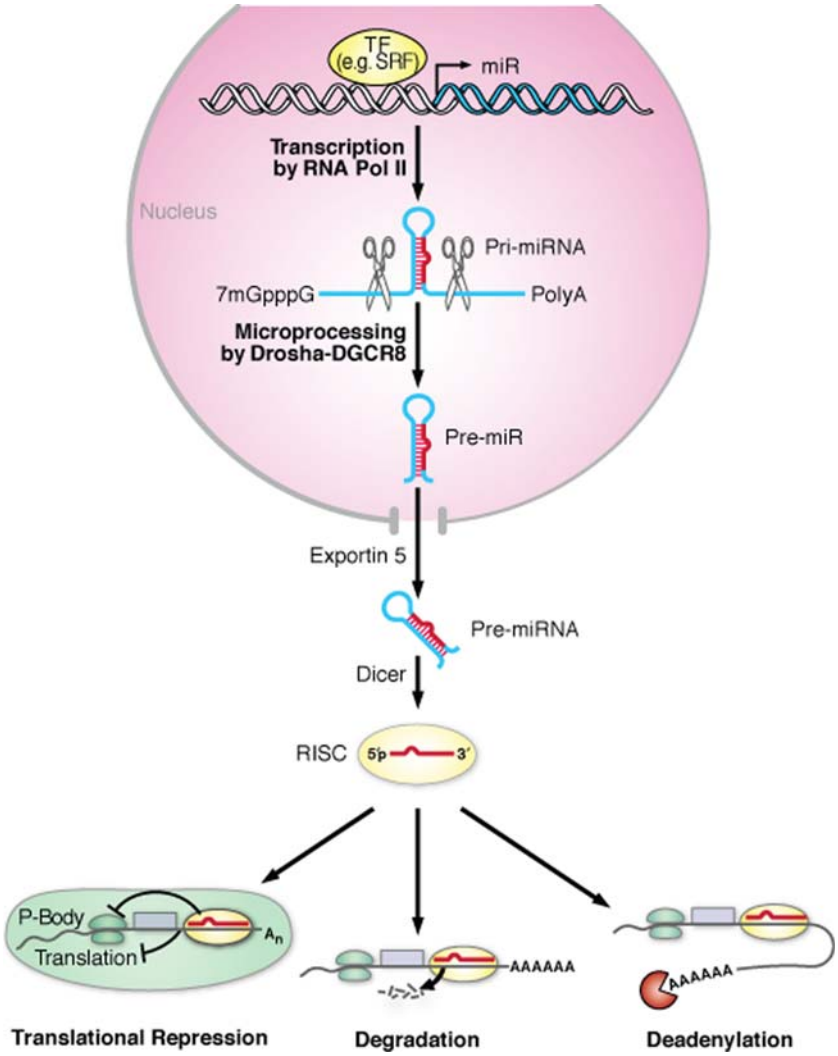

Fig. 1 Schematic representation of miRNA biogenesis and function. Transcription of miRNA genes is typically mediated by RNA polymerase II (pol II) and can be controlled by various transcription factors (TF). The initial transcripts, termed "primiRNAs," can range from a few hundred nucleotides to several kilobases long. The primiRNA has a characteristic stem-loop structure that can be recognized and cleaved by the RNase III endonuclease Drosha, along with its partner DGCR8 (DiGeorge syndrome critical region 8 gene; also known as Pasha). The cleavage product, an approximately 70-nt stem-loop pre-miRNA, is exported from the nucleus by Exportin 5. In the cytoplasm, another RNase III enzyme, Dicer, further cleaves the pre-miRNA into a double-stranded mature miRNA (approximately 21 nt), which is incorporated into the RISC, thus allowing preferential strand separation of the mature miRNA to repress mRNA translation or destabilize mRNA transcripts through cleavage or deadenylation (adapted from Zhao and Srivastava [57])

specification, because *Dicer* is required for embryonic stem cell differentiation [21, 35]. Deletions of Dicer in specific heart populations will show the importance of miRNA function in distinct aspects of heart development.

Organization and Regulation of *miR-1* and *miR-133*

Two widely conserved miRNAs that display cardiac muscle- and skeletal muscle-specific expression during development and in the adult human are *miR-1* and *miR-133a* (Fig. 2), which are derived from a common precursor transcript (bicistronic) [9, 58]. Two highly conserved loci encode the mature *miR-1* (miR-1-1 and miR-1-2) and *miR-133a* (*miR-133a-1*, *miR-133a-2*) transcripts, which appear to be genetically redundant [24]. The mature forms of miR-1 derived from the distinct loci are identical, as are the miR-133a forms.

Cardiac transcription of *miR-1/miR-133* bicistronic precursors is directly regulated by myocyte enhancer factor-2 (Mef2) and serum response factor (SRF) (Fig. 2a) [58]. SRF binds to CArG motifs in promoters and enhancers of muscle-specific genes that regulate hundreds of miRNAs [36], although only a limited number have been assigned target mRNAs. In the heart, SRF binds and activates the enhancer regions of *miR-1/miR-133* in vitro and in vivo through a serum-response element conserved from fly to human [58]. Concordant with their common cis- and trans-regulation, both *miR-1* and *miR-133* are coexpressed in cardiac and skeletal muscle throughout mouse development (Fig. 2b) and are robustly expressed in the adult human [9, 30, 58].

Function of *miR-1* and *miR-133a* During Cardiogenesis

miR-1 expression is detectable by E8.5 in mouse and increases throughout development. Overexpression of *miR-1* under the control of the β-MHC promoter diminishes the pool of proliferating ventricular myocytes by inducing a

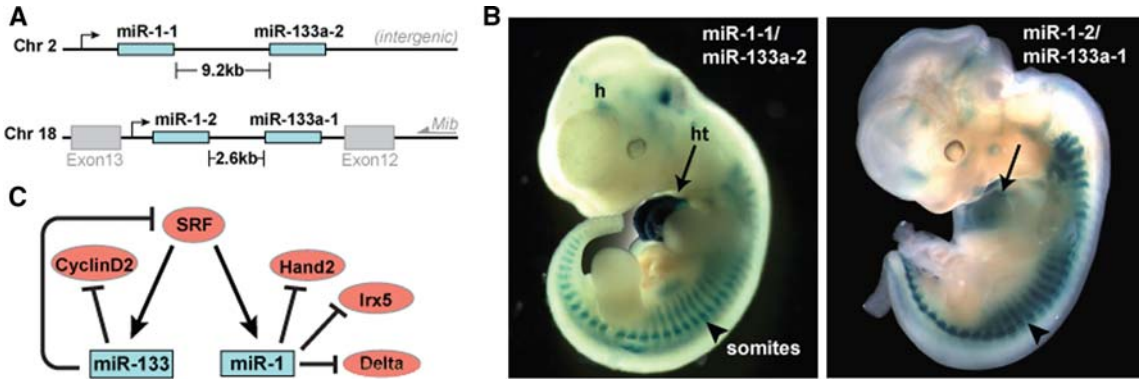

Fig. 2 Summary of *miR-1* and *miR-133* genomic organization, regulation, and expression during mouse cardiogenesis. **a** Chromosomal locations of mouse *miR-1* and *miR-133a*. The *miR-1/133a* clusters are transcribed as bicistronic transcripts. **b** LacZ directed by an upstream enhancer of the *miR-1-2/miR-133a-2* and *miR-1-1 miR-133a-1* clusters, respectively, shows expression in the heart (ht) and somites (*arrowhead*) at mouse embryonic day 11.5. **c** Cardiac expression of *miR-1* and *miR-133* is regulated by SRF. Targets of *miR-1* and *miR-133* in cardiac muscle are shown

premature exit from the cell cycle. This negatively regulates cardiac growth, in part by inhibiting translation of the heart and neural crest derivative-2 protein, Hand2 [58], a basic helix-loop-helix protein involved in ventricular myocyte expansion. In mice, *Hand2* is initially expressed throughout the linear heart tube and then becomes restricted to the developing atrial and ventricular myocardium with highest expression in the right ventricle. Mice that lack Hand2 die at E10.5 from right ventricular hypoplasia and decreased trabeculation in the left ventricle [47, 49, 55]. In mice overexpressing *miR-1*, trabeculation is also decreased, consistent with the *Hand2* mutant phenotype, corroborating *Hand2* as a direct target of *miR-1* [58]. Mice lacking miR-1 have an increase in Hand2 protein, providing yet further evidence of Hand2 as a direct target of miR-1.

In *Drosophila*, *miR-1* functions to pattern the dorsal vessel (i.e., aorta/heart tube). Moreover, deletion of the single *miR-1* gene (*dmiR-1*) results in a muscle-differentiation defect [24, 45]. In a subset of *dmiR*-1-null flies, muscle progenitors are arrested in a proliferative state and accumulate ectopically. *Drosophila hand* does not seem to be a target of *miR-1* because the fly *hand* ortholog lacks *miR-1*-binding sites in its 3′ UTR, suggesting that miRNA–mRNA interactions may differ somewhat between species. Instead, *dmiR-1* targets transcripts encoding the Notch ligand, *Delta*, which regulates the expansion of cardiac and muscle progenitor cells [24], suggesting that *miR-1* promotes muscle differentiation through downregulation of the Notch signaling pathway. This is consistent with the known function of the Notch/Delta–signaling pathway during developmental cell-fate decisions, including those involving cardiac specification [1].

In cultured myoblasts, *miR-1* promotes myoblast differentiation, whereas *miR-133* stimulates myoblast proliferation [9]. Chen et al. showed in myoblast culture that *miR-1* targets the histone deacetylase 4 (*HDAC4*) mRNA, a transcriptional repressor of Mef2-dependent activation of muscle-specific gene expression, suggesting that translational repression of *HDAC4* by *miR-1* enhances gene activation of Mef2-dependent promoters. They also showed that *miR-133* targets SRF, which is important in muscle proliferation, differentiation, and activation of the *miR-1/miR-133* transcript, thus creating a negative-feedback regulatory loop (Fig. 2c). When rat ventricular cells are subjected to oxidative stress, *miR-1* and *miR-133* have opposing effects on apoptosis: *miR-1* targets the anti-apoptotic heat-shock proteins HSP60 and HSP70 and is apoptotic, whereas *miR-133* represses caspase-9, a regulator of mitochondria-mediated apoptosis [54], and is anti-apoptotic.

Targeted deletion in mice will be invaluable for investigating the functional role of individual miRNAs. Surprisingly, disruption of just one of the two *miR-1* family members, *miR-1-2*, results in a range of abnormalities, including cell-cycle dysregulation, heart malformations, and postnatal electrophysiological defects. Heterozygous *miR-1-2*-null mice survive to reproduce, but 50% of *miR-1-2* homozygous null mice die between E15.5 and birth from ventricular septal defects and cardiac dysfunction. These defects can occur from dysregulation of a multitude of events during cardiogenesis, and it is likely that *miR-1-2* regulates numerous genes during this process. Precise dosage of *Hand2* is crucial for normal cardiomyocyte proliferation and development, and increased levels of *Hand2* may contribute to the ventricular septal defects (Fig. 2c) [59].

miR-1-2-null mice that survive until birth often die suddenly [59]. Electrophysiologic testing showed a spectrum of cardiac arrhythmias in mutant mice, which may be caused in part by increased levels of the transcription factor Iroquois homeobox 5 (Irx5). Irx5 regulates the cardiac ventricular repolarization gradient by negatively regulating the

expression of potassium channel genes, such as *Kcnd2* [13]. The 3′ UTR of *Irx5* contains a well-conserved *miR*-1–binding site and is a direct target of *miR-1* (Fig. 2c) [59]. In the *miR-1*-2–null hearts, *Irx5* transcripts were upregulated, and its target gene, *Kcnd2*, was correspondingly downregulated.

Postnatal mouse cardiomyocytes terminally exit the cell cycle after the first 10 days of life. However, *miR-1*-2–null adult mice have an increase in mitotic cardiac myocytes along with cardiac hyperplasia. These abnormalities could reflect the effect of miR-1 on Notch signaling and the derepression of Hand2 (Fig. 2c), which promotes myocyte expansion. In addition, genome-wide profiling of *miR-1-2* mutant adult mouse hearts suggests a broad upregulation of positive regulators of the cell cycle and downregulation of tumor suppressors, indicating a shift in the "threshold" of cells to re-enter the cell cycle [59]. Whether this change promotes cardiac repair after injury remains to be determined.

Similarly, targeted deletion of miR-133a in mice has helped to elucidate its role during cardiac development [31]. Unlike miR-1, single mutations of either *miR-133a-1* or *miR-133a-2* caused no overt phenotype. However, combined deletion of both miRNAs led to partially penetrant ventricular septal defects and decreased survival at birth. Adult compound mutant mice developed dilated cardiomyopathy and lethal heart failure. miR-133a was found to suppress smooth-muscle gene expression and cardiomyocyte proliferation, in part by way of repression of SRF and cyclin D2 (Fig. 2c).

During early cell fate decisions of mouse and human embryonic stem (ES) cells, *miR-1*, and *miR-133* are expressed just as mesoderm emerges and function in concert to promote mesoderm induction while suppressing differentiation into the ectodermal or endodermal lineages (Fig. 3) [20]. However, *miR-1* and *miR-133* have antagonistic effects on further adoption of muscle lineages: *miR-1* promotes differentiation of mouse and human ES cells toward a cardiac fate, whereas *miR-133* inhibits differentiation into cardiac muscle. *miR-1* appears to exert this effect, in part, by translationally repressing the mammalian orthologue of *delta*, *Delta-like-1* (*Dll-1*), similar to the repression seen in the fly (Fig. 2c) [20]. Thus, the bicistronic *miR-1/miR-133* transcript encodes distinct mature miRNAs that likely share common targets yet complement each other by balancing the differentiation and proliferation of cardiac and skeletal muscle lineages.

miR-143/145 Regulate Smooth-Muscle Plasticity

Vascular smooth muscle cells have the unique capacity to oscillate between a contractile phenotype and a less differentiated, more synthetic state in response to external queues. This phenotypic modulation is a major component of the vascular repair process and certain diseases, such as atherosclerosis. Not surprisingly, it involves dramatic changes in the smooth-muscle gene program, which are heavily influenced by particular miRNAs.

Among the most highly enriched microRNAs in the vascular smooth muscle are miR-143 and miR-145. These two microRNAs are cotranscribed from a single locus under the transcriptional control of SRF, myocardin, and Nkx2.5 [12]. Interestingly, their expression is strongly downregulated in injured or atherosclerotic vessels, which are comprised of synthetic smooth muscle cells [10, 12, 15, 53]. Indeed, miR-143 and miR-145 target a network of transcription factors, including Klf4, Klf5, myocardin,

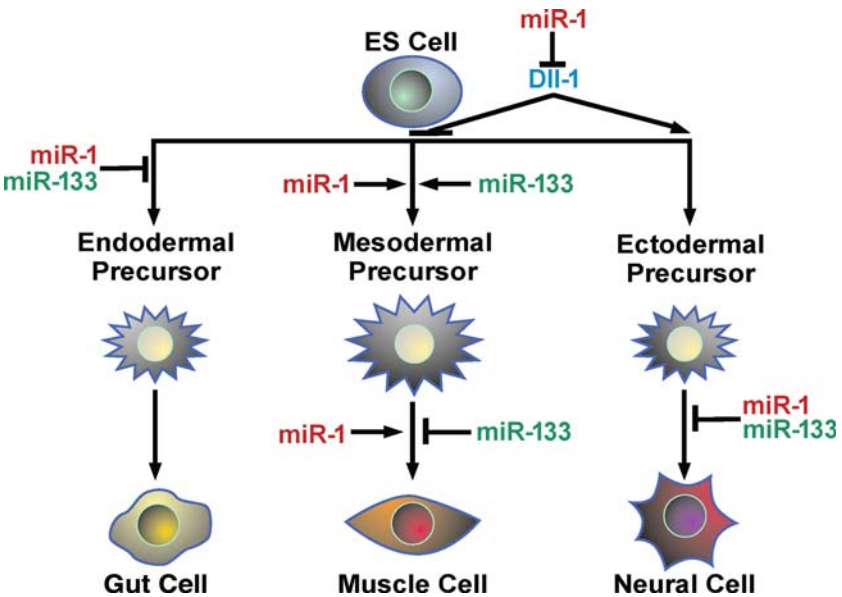

Fig. 3 Model of miR-1/miR-133 effects during embryonic stem-cell differentiation. miR-1 and miR-133 promote differentiation of mesoderm and inhibit endoderm and ectoderm differentiation at specific stages as indicated and have opposing effects in later steps of muscle differentiation. miR-1 inhibition of Dll-1 translation, along with yet unknown targets, likely contribute to the observed effects of miR-1 (from Ivey et al. [20])

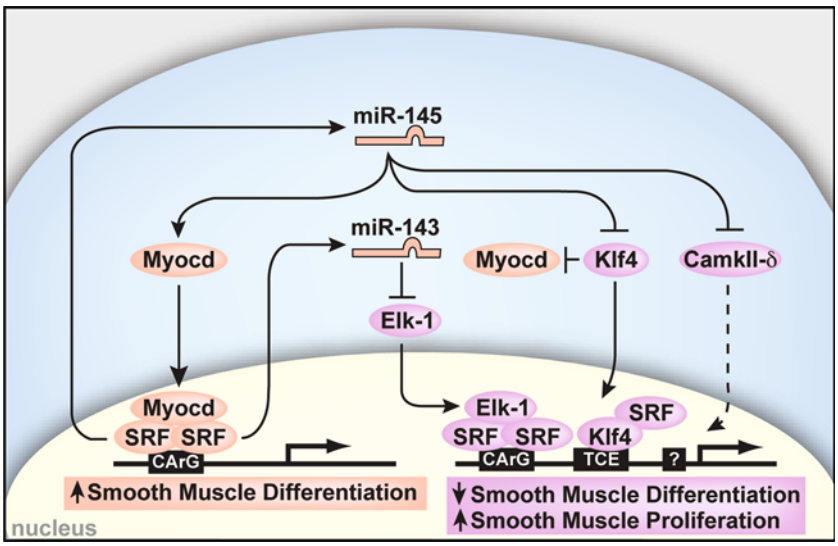

Fig. 4 miR-143 and miR-145 are transcriptionally regulated by SRF and repress multiple factors that normally promote the synthetic smooth-muscle phenotype (*lavender*). These include Klf4, which interacts with SRF and also represses Myocd. miR-145 has a positive effect on Myocd activity to concurrently promote the contractile smooth-muscle phenotype (peach), thereby also reinforcing miR-145 and miR-143 expression (from Cordes et al. [12])

MRTFB, and Elk-1, which is consistent with a role for these miRNAs in regulating the quiescent versus proliferative phenotype of smooth muscle (Fig. 4) [12, 53]. Further, targeted deletion of these miRNAs in mice showed their importance in achieving vascular tone and responding to vascular injury [3, 15, 53] and led to the identification of the angiotensin-converting enzyme as a target of miR-145 [3]. miR-145 was also found to be necessary for smooth-muscle conversion from fibroblasts and sufficient to differentiate smooth muscle cells from multipotent neural crest stem cells, further implicating microRNAs as regulators of cell-fate decisions [12]. Similarly, miR-221 and miR-222 regulate smooth-muscle cell proliferation and modulate the deleterious vascular response after angioplasty [32]. Therefore, these miRNAs represent potential targets for the diagnosis and therapy of vascular disease.

miR-138 Regulation of Cardiac Patterning

Intricate transcriptional networks establish chamber-specific gene expression, and these patterning events are highly conserved across species, from zebrafish to human [46]. Zebrafish are useful models to study cardiac patterning events because of their simple two-chambered heart, which consists of a single atrium and ventricle separated by the atrioventricular canal (AVC). The atrial and ventricular chambers express unique myosin genes, whereas the AVC expresses distinct genes, such as *cspg2*, encoding versican, *notch1b*, and *tbx2* [11, 42]. miR-138 is a highly conserved miRNA found in many parts of the embryo, but within the zebrafish heart it is specifically expressed in the ventricular chamber [33]. Disruption of miR-138 function led to expansion of AVC gene expression into the ventricle and failure of ventricular cardiomyoctyes to fully mature. miR-138 normally restricts AVC gene expression by directly repressing *cspg2* in the ventricle. This event is reinforced by ventricular repression of retinoic acid dehydrogenase, resulting in decreased retinoic acid, which is a positive regulator of *cspg2* [33]. It is likely that other region-specific miRNAs will reinforce known signaling and transcriptional networks that establish patterns of gene expression throughout the developing heart tube.

miR-126 Regulation of Angiogenesis

In addition to miRNA regulation of cardiomyocytes and smooth muscle, two recent reports regarding the endothelial-specific miRNA, miR-126, illustrate what will likely be a broader function of tissue-specific miRNAs during cardiovascular development [17, 50]. miR-126, which is located in the intron of an endothelial-specific gene, Egfl7, regulates endothelial cell migration and integrity of capillary tubes in vitro [17]. In zebrafish embryos, miR-126 was similarly required for vascular integrity with vascular collapse and extensive hemorrhage observed on disruption of miR-126 function [17]. This function was conserved in mice, in which targeted deletion of miR-126 resulted in cranial hemorrhages and defects in angiogenesis [50]. miR-126 appears to function in part to promote vascular endothelial growth factor (VEGF) signaling by directly targeting multiple repressors of VEGF signaling, including Spred1 [17, 50] and Pik3r2 [17]. Mice lacking miR-126 had an impaired ability to generate new blood vessels in response to cardiac injury. Thus, miR-126-mediated regulation of angiogenesis may be a valuable therapeutic target to promote new blood vessel formation in ischemic conditions as well to inhibit angiogenesis during tumor growth.

Summary

The function of miRNAs in cardiovascular development reviewed here likely foreshadows a much broader role of dozens of miRNAs in regulating most aspects of cardiovascular development. Through their ability to post-transcriptionally regulate mRNA levels, and thus manage protein dosage, miRNAs provide finer regulation within the complex molecular networks that regulate cardiogenesis. The importance of this fine regulation is highlighted by the recognition that most known genetic causes of heart malformations in humans result from haploinsufficiency or heterozygous point mutations. The field of miRNA biology is growing rapidly, and new tools and mechanisms are becoming available. A critical hurdle will be to efficiently identify direct miRNA targets and to integrate cardiovascular enriched targets with previously described regulatory networks. With further characterization, elucidating the function of cardiac-enriched miRNAs may provide us with new diagnostic, prognostic, and therapeutic targets for many forms of cardiovascular disease.

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