MicroRNA in cardiovascular biology and disease

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Abstract

MicroRNAs (miRNAs) are members of a non-coding RNA family. They act as negative regulators of protein translation by affecting messenger RNA (mRNA) stability; they modulate numerous signaling pathways and cellular processes, and are involved in cell-to-cell communication. Thus, studies on miRNAs offer an opportunity to improve our understanding of complex biological mechanisms. In the cardiovascular system, miRNAs control functions of various cells, such as cardiomyocytes, endothelial cells, smooth muscle cells and fibroblasts. The pivotal role of miRNAs in the cardiovascular system provides a new perspective on the pathophysiology of disorders like myocardial infarction, hypertrophy, fibrosis, heart failure, arrhythmia, inflammation and atherosclerosis. MiRNAs are differentially expressed in diseased tissue and can be released into circulation. Manipulation of miRNA activity may influence the course of a disease. Therefore, miRNAs have become an active field of research for developing new diagnostic and therapeutic tools. This review discusses emerging functions of miRNAs in cardiogenesis, heart regeneration and the pathophysiology of cardiovascular diseases.

Key words: microRNA, cardiovascular disease, heart regeneration, heart development

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MicroRNAs (miRNAs) are a class of single-stranded, non-coding RNAs, about 22 nucleotides in length, which negatively regulate gene expression at the post-transcriptional level. They bind to messenger RNA (mRNA) in a complementary way, and cause gene silencing through the inhibition of translation and/or degradation of mRNA.[1] MiRNAs play a role in regulating various biological processes including embryogenesis, cell proliferation and differentiation, apoptosis or tumorigenesis.[2] In the cardiovascular system, miRNAs control cardiomyocyte growth and contractility, the development and maintenance of cardiac rhythm, plaque formation, lipid metabolism and angiogenesis.[2–5] Altered miRNA expression can be found in the blood of patients with various cardiovascular diseases, which makes them attractive candidates for noninvasive biomarkers.[6,7] It has been estimated that miRNAs control the activity of 30–50% of protein-coding genes.[7] Unlike transcriptional regulators, which have a turn-on-and-off function in controlling gene expression, the varied profiles of miRNAs appear to fine-tune the level of protein expression to changes in environmental conditions.[2] A single miRNA may influence the expression of hundreds of genes in a cell, and each mRNA molecule may be regulated by multiple miRNAs that interact or compete with each other.[8] The first miRNA, *lin-4*, was discovered in the nematode *Caenorhabditis elegans* in 1993.[1] To date, about 2500 miRNAs have been identified in the human genome. All known sequences of miRNAs are available in the database at the website www.mirbase.org.

MicroRNA biogenesis and mechanisms of action

MiRNA genes are an evolutionarily conserved integral part of the cell genome. They can be transcribed as independent transcription units in intergenic regions or in the introns and exons of protein-coding genes. MiRNA genes can exist individually or form polycistronic clusters containing multiple miRNA components.[1] MiRNAs are transcribed in the nucleus by RNA polymerase II (RNA Pol II) to primary miRNAs (pri-miRNAs), which can be a few kilobases long. Pri-miRNAs are cleaved by a protein complex containing the RNase III endonuclease Drosha into approximately 70-nucleotide precursor miRNAs (pre-miRNAs) with a hairpin structure.[1] Next, a GTP-dependent protein, exportin-5, recognizes a short stem of 2-3 nucleotides overhanging at the end of the pre-miRNAs and transports them from the nucleus to the cytoplasm.[1] Alternatively, pre-miRNAs can be processed independently of the Drosha complex through the direct splicing of introns.[6]

In the cytoplasm, pre-miRNA is cleaved by the RNase III endonuclease Dicer to approximately 22-nucleotide double-stranded miRNAs. One strand, called a guide strand, is loaded onto the RNA-induced silencing complex (RISC) and becomes mature miRNA, while the other strand, called a passenger strand, is degraded or incorporated into microvesicles and released from the cell.[9] Both mature and pre-miRNAs can be found in microvesicles.[9] The formation of the RISC effector, which contains Argonaute 2 (Ago2) protein, allows miRNAs to bind to target mRNAs.[1,10]

The RISC binding sites are complementary sequences present mainly in the 3'-untranslated region (3'-UTR) of mRNAs. In cases of perfect complementarity of the miRNA-mRNA sequences, Ago2 protein, which has endonuclease activity, cleaves the mRNA, leading to its degradation. Mismatches in the sequence inhibit translation (Fig. 1).[1]

Most miRNAs are localized intracellularly, but some of them are released into the blood in association with proteins (e.g. Ago2, nucleophosmin 1 and HDL) or as a component of cell-derived microvesicles (e.g. exosomes or apoptotic bodies). MiRNAs may be released in response to cell activation stimuli, injury or after cell death.[9] In the circulation, miRNAs are transported to distant sites and interact with cells by fusing with the cell membrane or through receptor-mediated binding, which suggests that miRNAs play a role in cell-to-cell communication.[9,10] For example, in response to tissue damage, miR-126 is transported in endothelial cell-derived apoptotic bodies to vascular smooth muscle cells (VSMCs), where it mediates the synthesis of the CXCL12 chemokine to recruit progenitor cells and provide vascular protection.[5]

The nomenclature of microRNAs

With the exception of a few miRNAs that were discovered early (such as the let family), the nomenclature of mature miRNAs consists of the prefix "miR" and the identifying number, e.g. miR-499. Pre-miRNAs are indicated by italics and the lower case prefix *"mir"*. Three- or four-letter prefixes indicate the species, e.g. hsa-miR-101 in *Homo sapiens*. An additional lower case letter is appended to miRNAs with similar sequences, differing by only one or two nucleotides, e.g. miR-123a or miR-123b.[6] If two pre-miRNAs that are located at different sites in the genome lead to an identical mature miRNA, the miRNA is annotated with an additional hyphen and number, e.g. miR-194-1 or miR-194-2. Two different miRNAs that originate from the same precursor are named according to their location on the hairpin: miR-17-5p (5' arm) or miR-17-3p (3' arm), or based on their level of expression: miR-123 or miR-123*. An asterisk indicates the miRNA strand that is expressed at a lower level.[6]

Controlling microRNA activity

The activity of miRNAs can be modulated by two different approaches based on mimicking miRNA functions

Fig. 1. A schematic representation of the biogenesis of miRNAs and their mechanism of action: a miRNA gene is transcribed by RNA polymerase II (RNA Pol II) to stem-loop primary miRNAs (pri-miRNAs). Within the nucleus, pri-miRNA is processed by the RNase III endonuclease Drosha into hairpin-like precursor miRNAs (pre-miRNAs). Alternatively, pre-miRNAs are processed independently of the Drosha complex, through direct splicing of introns, to form pre-miRNAs called mirtrons. The pre-miRNAs/mirtrons are then transported to the cytoplasm by a GTP-dependent protein transporter, exportin-5. Within the cytoplasm, pre-miRNAs are cleaved by the RNase III endonuclease Dicer to approximately 22-nucleotide double-stranded miRNAs. After unwinding, both miRNA strands can be functional; however, usually one of them, termed the guide strand, is incorporated into the RNA-induced silencing complex (RISC). MiRNA-RISC complexes containing Argonaute 2 (Ago2) protein bind to the 3'-untranslated region (3'-UTR) of target mRNAs and causes gene silencing by inhibiting translation and/or through mRNA degradation

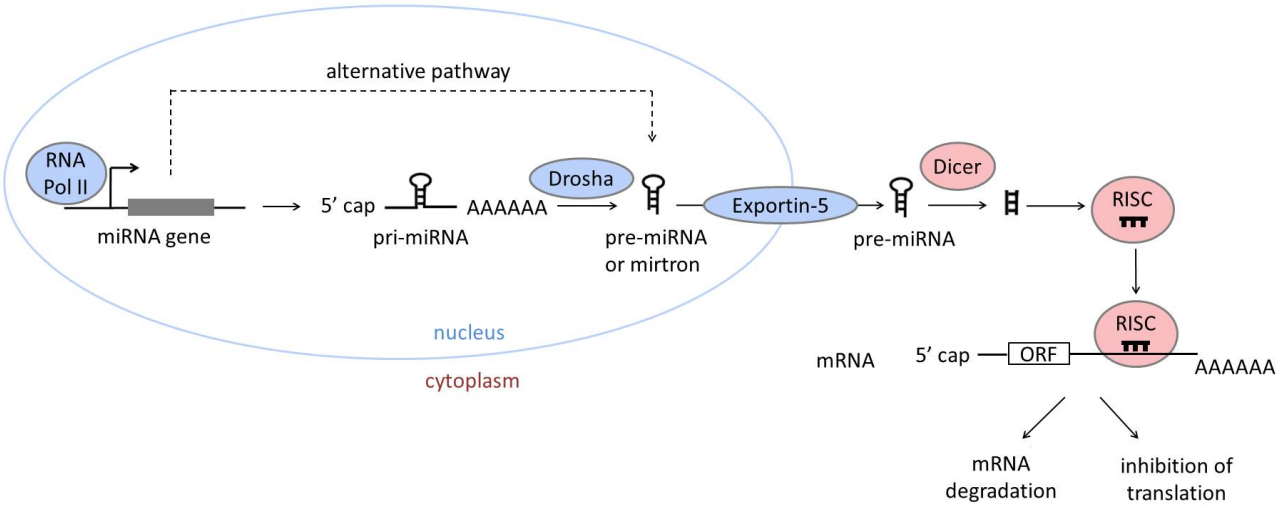
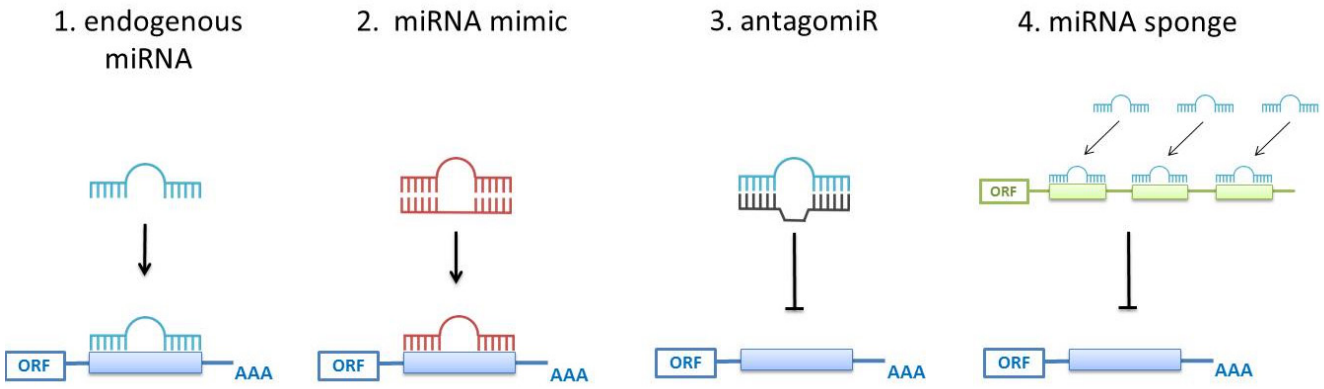


Fig. 2. Different approaches to targeting miRNA activity. (1) Endogenous miRNA (blue) binds to a complementary sequence known as a seed sequence (blue box) present in the 3'-untranslated region (3'-UTR) of mRNA. (2) A miRNA mimic (red) is a chemically synthesized double-stranded RNA molecule. This structure imitates endogenous miRNA and targets complementary mRNA. (3) An antagomiR (black) is a synthetic oligonucleotide that is complementary to a particular miRNA. This oligonucleotide binds to miRNA and inhibits its action. (4) MiRNA sponge (green) contains several seed sequences for a particular miRNA. Delivery to the cell results in binding with the target miRNAs and reduces the number of free and active miRNAs

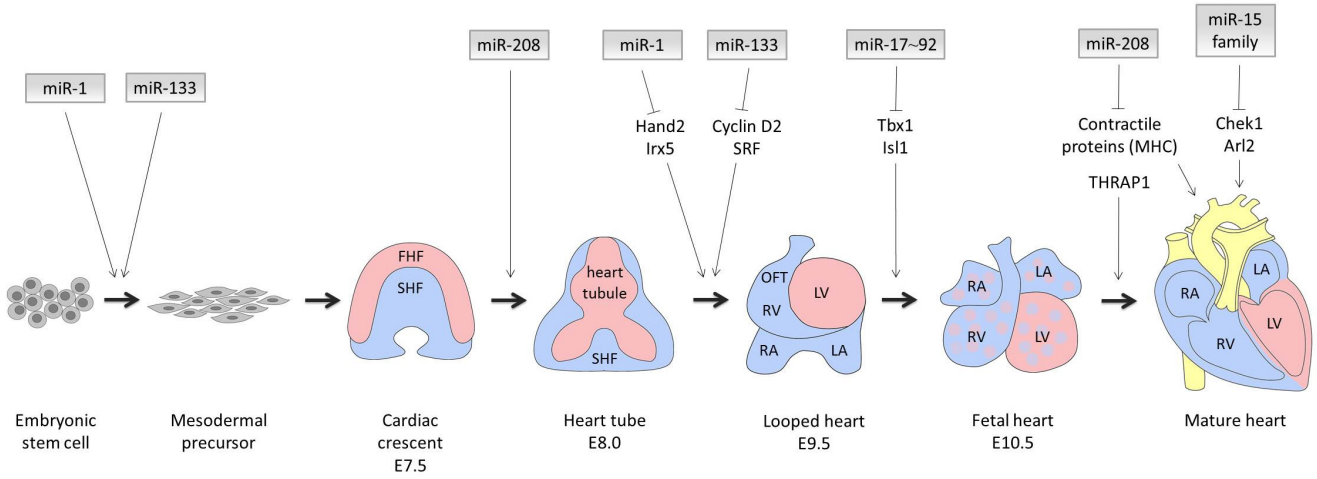


or silencing its action (Fig. 2).[3] In cases of compromised miRNA levels, exogenous miRNAs can be administrated in vivo. MiRNA mimics are small, chemically synthesized double-stranded RNAs that imitate endogenous miRNAs and cause gene silencing. One strand of this molecule is identical to the native form of miRNA, the other is complementary. The double-stranded structure is required so that the RISC can recognize miRNA mimics accurately.[11]

AntagomiRs are synthetic RNA molecules used to silence aberrantly expressed miRNAs. AntagomiRs function by binding to miRNAs and inhibiting their actions. They are complementary to the sequence of a full-length sequence or a seed sequence (a 28-nucleotide-long mRNA binding site) of a specific miRNA. They are chemically modified to improve cellular uptake, in vivo stability and affinity to miRNA.[11] MiRNA sponges are another approach to reducing miRNA levels. MiRNA sponges are transcripts that contain multiple complementary regions for miRNAs that have the same target site. Delivering miRNA sponges to a cell results in their binding with the target miRNAs and reduces the number of free and active miRNAs.[11]

The highly conserved miRNA sequence facilitates the adaptation of results from studies in animal models to

Fig. 3. A schematic representation of mouse cardiac morphogenesis and the role of miRNAs in this process. The colors represent the contribution of different precursor pools to the forming chambers. The heart is of mesodermal origin. At embryonic day 7.5 (E7.5), 2 populations of cells, termed the first heart field (FHS) and the second heart field (SHF), build up the cardiac crescent. Subsequently, the cells migrate and the linear heart tube is formed at E8.0. Shortly thereafter, the heart tube starts spontaneous contractions and supports the blood supply of the developing embryo. Finally, the process of cardiac looping and a series of morphological changes contribute to the formation of the four-chambered heart by E10.5. The progressive septation of the atria, ventricles and the common outflow track then takes place



Inhibition of the miR-15 family promotes myocyte proliferation and ameliorates cardiac functions in adults after myocardial infarction.[23] MiR-133 is another molecule regulating the cell cycle of cardiomyocytes. Following resection of 20% of the zebrafish ventricular apex, proliferation of the remaining cardiomyocytes occurs, which leads to complete heart regeneration. Studies have noted reduced expression of miR-133 in regenerating zebrafish heart. Upregulation of miR-133 diminishes its regenerative potential, whereas decreasing the level of miR-133 with specific miRNA sponges promotes regeneration.[24] To sum up, the miR-15 and miR-133 families attenuate the regenerative capacity of the heart by inhibiting cardiomyocyte proliferation.

MiR-199 and miR-590 have been found to induce cell cycle re-entry in cardiomyocytes. Intracardiac administration of these molecules into the infarct border zone stimulates cardiomyocyte proliferation in adult mice. MiR-199 and miR-590 have been shown to promote regeneration of the myocardium and to improve cardiac function.[25]

Stem cell-based therapies represent an attractive approach for treating cardiovascular diseases. However, regenerative therapy based on delivering stem cells into the heart has not yet been successful in clinical trials. Nowadays, many studies aim to improve the effects of this therapy by increasing the survival of cells transplanted into the infarcted region and enhancing the differentiation of stem cells into cardiomyocytes.[26] MiR-1 and miR-133 have been found to induce the differentiation of stem cells and heart progenitor cells into cardiomyocytes.[14] Moreover, transplanting stem cells overexpressing miR-1 into the infarcted zone increases cardiomyocyte differentiation, promotes regeneration and improves cardiac function.[27] Similar results are achieved by transplanting c-kit+ cardiac progenitor cells overexpressing miR-499.[28]

Cardiac fibrosis following myocardial infarction, resulting from excessive fibroblast activation, reduces regeneration, leads to pathological remodeling, impairs systolic function and increases susceptibility to arrhythmias. It has been shown that the expression of a suitable combination of transcription factor genes (*Gata4*, *Mef2c*, *Tbx5*) could convert resident cardiac non-myocyte fibroblasts into contractile cells by direct reprogramming.[29] A similar effect can be obtained when miRNAs are used. Lentiviral-mediated delivery of miR-1, miR-133, miR-208 and miR-499 into the infarct border zone has been found to induce direct fibroblast reprogramming into cardiomyocytes in situ.[30] Reprogrammed cardiomyocytes express cardiac markers and sarcomeric organization, and have electrophysiological properties characteristic of mature ventricular cardiac myocytes. Moreover, reprogramming is associated with an improvement in fractional shortening, suggesting the functional recovery of the damaged myocardium.[31]

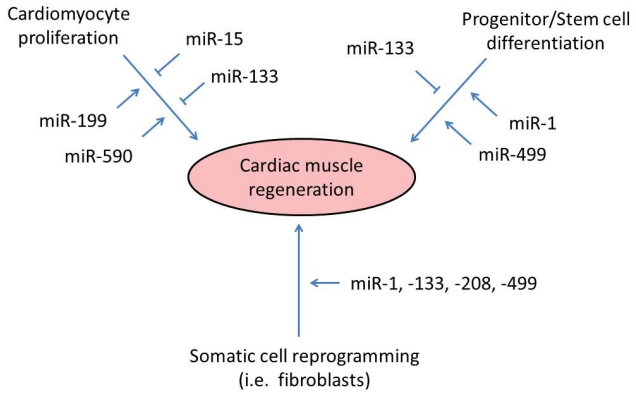
Fig. 4 summarizes the role of miRNAs in cardiac regeneration: regulation of cardiomyocyte proliferation, stem or progenitor cell differentiation and direct reprogramming of fibroblasts.

MicroRNAs in heart diseases

Myocardial infarction and cardiac remodeling

Ischemia/reperfusion injury associated with myocardial infraction leads to remodeling in the myocardium, which is regulated by various miRNAs. Activation of

Fig.4. MiRNA activity in the process of myocardial regeneration. Three approaches to cardiac muscle regeneration are presented: regulation of cardiomyocyte proliferation; stem or progenitor cell differentiation; and direct reprogramming of fibroblasts



stress signaling pathways triggers changes in miRNA expression; miR-24, miR-320 and miR-29 are downregulated in myocardial infarction. MiR-24 inhibits translation of Bim, a proapoptotic protein. Restoration of miR-24 to physiological levels by specific miRNA mimics attenuates apoptosis and decreases scar size.[2] Proapoptotic properties are attributed to miR-320, which negatively regulates heat shock protein 20 (HSP20), which functions as a cell protector after an ischemic injury.[2] MiR-29 controls genes encoding collagen (*COL1A1*, *COL1A2*, *COL3A1*) and extracellular matrix proteins, including fibrillin (*FBN1*) and elastin (*ELN1*). Low expression of miR-29 after myocardial infarction results in scar formation.[32] In addition, miR-199 is downregulated in cardiac myocytes during oxygen deprivation. This induces its target genes: hypoxia-inducible factor-1α (*HIF-1α*) and sirtuin 1 (*Sirt 1*), and subsequent activation of hypoxia-triggered pathways. Restoration of physiological miR-199 levels inhibits *HIF-1α* expression and its stabilization of p53, a tumor supressor responsible for sustaining the genome integrity, which leads to a reduction in apoptosis.[33]

Expression of the miR-15/16 family and miR-499 increases after myocardial infarction.[4,34] The miR-15/16 family regulates cardiomyocyte proliferation and survival in response to injury, and its inhibition protects cardiomyocytes from apoptosis.[34] MiR-499 influences cardiomyocyte apoptosis by downregulating calcineurin and dynamin-related protein 1 (Drp1), which are involved in mitochondrial fission. According to the literature, the upregulation of miR-499 reduces apoptosis and infarct size, while miR-499 knockdown has the opposite effect.[4] In contrast, another report showed that miR-499 overexpression in the heart can lead to cardiomyocyte hypertrophy and cardiomyopathy, suggesting discrepancies that may be caused by acute or chronic modulation of miRNA.[4] MiR-214, which increases in mouse and human tissue after myocardial infarction, exerts a protective effect during ischemia/reperfusion. It reduces calcium overload and promotes cardiomyocyte survival through inhibition of the sodium/calcium exchanger (NCX1) and Bim. Deletion of miR-214 increases injury and mortality following myocardial infarction.[4]

At the molecular level, cardiac remodeling is accompanied by a gene expression switch from the adult α–*MHC* isoform to the fetal β-*MHC*. MiR-208a, which is encoded in the intron of the α-*MHC* gene, has been shown to be involved in this process, inducing cardiomyocyte hypertrophy, fibrosis and increasing β-*MHC* expression. Deletion of miR-208a protects the heart from pathological remodeling under stress conditions. Thyroid-hormone-receptor-associated protein 1 (*THRAP1*) is considered a target gene for miR-208a.[3,34]

MiR-21 promotes myocyte hypertrophy and fibrosis by repressing the Sprouty2 transcription factor, which controls the pro-fibrotic extracellular signal-regulated kinase-mitogen-activated protein kinase (ERK-MAPK) pathway. Specific antagomiR-mediated inhibition of miR-21 blocks this cascade and results in a reduction in both hypertrophy and fibrosis.[13] However, genetic deletion of miR-21 does not alter the pathological cardiac response to pressure overload.[34] This discrepancy indicates that miR-21 plays a complex role in the pathophysiology of heart diseases, which requires further investigation. Another possibility may be the existence of a compensatory mechanism revealed under a permanent miR-21 knockdown.[34]

Heart failure

Impaired cardiac contractile function caused by disrupted calcium handling is a hallmark of heart failure. Recently, Cai et al. demonstrated that miR-765 is overexpressed in failing hearts and is involved in contractile regulation. MiR-765 contributes to increased protein phosphatase 1 (PP-1) activity and the subsequent dephosphorylation of key calcium cycling proteins by silencing its endogenous inhibitor-1.[35] Likewise, miR-25 is upregulated in failing hearts and controls myocyte contractile function by repressing the sarcoplasmic reticulum calcium uptake pump, SERCA2a. Anti-miR-25 delivery restores cardiac function and improves survival.[36] Also, miR-24 regulates calcium homeostasis through Junctophilin-2 repression, which results in decreased efficiency of excitation-contraction (E-C) coupling in cardiomyocytes.[37]

In a recent paper, Melman et al. provided evidence that increased cardiac miR-30d expression has a significant impact on responses to cardiac resynchronization therapy (CRT), and that plasma levels of miR-30d may correlate with responsiveness to CRT in heart failure patients. MiR-30d is regulated by mechanical stretch, and is released in exosomes by cardiomyocytes. It protects cardiomyocytes from TNF-α-elicited inflammation and cell

dothelial activation and subsequent vessel injury. Moreover, low serum levels of Let-7g have been associated with increased circulating plasminogen activator inhibitor-1 (PAI-1).[45]

Vascular injury triggers phenotypic changes (de-differentiation) in VSMCs. An altered phenotype is characterized by improper contractility, increased proliferation and migration, which result in restenosis.[13] Upon arterial injury, miR-143 and miR-145 are downregulated, whereas miR-21 is upregulated. Restoration of physiological miRNA levels protects VSMCs from de-differentiation and restenosis.[13] MiR-145 controls neointimal lesion formation by silencing Kruppel-like factor 5 (KLF5) and its downstream molecule, myocardin.[3]. MiR-21 function is mediated by a tumor suppressor known to negatively regulate Akt/PKB signaling pathway PTEN and anti-apoptotic protein Bcl-2. MiR-221 promotes VSMC proliferation by repressing the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and reduces expression of contractile genes α-smooth muscle actin (SMA), smooth muscle calponin (CNN), and SM22α ($p27^{Kip1}$-independent mechanism). Similar effects are caused by miR-26, which downregulates extracellular signal transducers Smad1 and Smad4 in the bone morphogenetic protein (BMP) signaling pathway.[2]

Zhao et al. reported the miR-143/145 expression in SMCs is induced by ECs. MiR-145 targets TGF-β receptor II (TGFBR2) and regulates TGF-β signaling in a selective manner: MiR-145 diminishes expression of matrix genes, while smooth muscle differentiation genes remain unaffected.[46] On the other hand, when stimulated by cell-to-cell contact, EC-derived TGF-β mediates miR-143 and miR-145 transfer from SMCs to ECs through membrane protrusions known as tunneling nanotubes. This decreases the ability of ECs to form capillary-like structures and lowers their proliferation index, which leads to vessel stabilization. MiR-143 and miR-145 target hexokinase II (*HKII*) and integrin β 8 (*ITGβ8*) genes, respectively.[47]

MiR-145 has been also implicated in the pathophysiology of atherosclerosis due to its effect on VSMC proliferation and phenotypic changes. In endothelial cells miR-143/145 expression is mediated by shear-responsive transcription factor - Kruppel like factor 2 (KLF2). ECs release extracellular vesicles (EVs) containing miR-143/145, which are absorbed by SMCs to control target genes and act as atheroprotective molecules. Administering EC-derived EVs enriched with miR-143/145 results in a reduction in atherosclerotic lesion formation in the aortas of apolipoprotein E-deficient mice.[4] An increase in miR-145 expression reduces plaque size in the aortic sinuses, diminishes the necrotic core and promotes collagen synthesis; these phenomena lead to plaque stabilization.[3] On the other hand, neoangiogenesis and atherosclerotic plaque hemorrhage increase the subject's susceptibility to plaque rupture and clot formation, and miR-222/221, the miR-155 family and the miR-17~92 family are involved in the process.[3] Macrophages are the main effector cells in atherogenesis, as they promote inflammatory response, degrade lipoproteins and phagocyte cell debris. Cholesterol-loaded macrophages produce VEGF, a proangiogenic cytokine, and the miR-155 family, the miR-17~92 family and miR-222/221 regulate this process. Moreover, miR-342--5p activates macrophages by inhibiting Akt1 kinase.[3]

Considering the important role of cholesterol in the pathophysiology of atherosclerosis, it is worth mentioning miR-122 and miR-33, which have been described as regulators of lipid homeostasis.[3] MiR-122 is highly expressed in the liver, where it is involved in fatty acid oxidation and lipid synthesis. Downregulation of miR-122 results in decreased levels of both HDL and LDL cholesterol.[4] MiR-33a and miR-33b are encoded in the introns of the sterol regulatory element-binding protein genes *SREBP1* and *SREBP1,* respectively. MiR-33a targets ATP-binding cassette transporter A1 (*ABCA1*) and inhibits cellular cholesterol export. Because 3'UTR mouse and human *ABCA1* genes possess several miR-33a binding sites, mRNA repression is strong. In addition, miR-33a/miR-33b regulate *NPC1* and *ABCG1*, which are also involved in cholesterol trafficking[48]; as well as *CROT, CPT1a*, *HADHB* and *AMPKa,* which are engaged in fatty acid oxidation.[4] Inhibition of miR-33a/b upregulates ABCA1 expression in hepatocytes and macrophages, and leads to increased total cholesterol and HDL levels in serum.[4] Interestingly, both strands of the miR-33 locus act together in lipid metabolism regulation, since miR-33a* and miR-33b* repress genes similar to those targeted by miR-33a/b.[48]

Conclusions

The discovery of miRNA has changed our understanding of the regulation of gene expression. In the cardiovascular system, miRNAs control the proliferation and differentiation of stem and progenitor cells, and the function of cardiac myocytes, pacemaker cells, endothelial cells and smooth muscle cells. MiRNAs play a crucial role in cardiac development and regeneration. They are involved in cardiovascular pathophysiology and their expression is altered in various cardiovascular diseases. Modulation of miRNA expression may indeed change the course of a disease. The encouraging results of miRNA applications in experimental settings and reports of negligible toxicity to healthy tissues suggest that these molecules have considerable therapeutic potential.[3]

Currently, only two chemically modified oligonucleotides have been used in clinical trials. An antagomiR directed against miR-122 has completed the second phase of clinical trials. This oligonucleotide is used to treat hepatitis C virus (HCV). MiR-122 is specific to liver cells and is required for HCV replication. Delivering the antisense inhibitor of miR-122 reduces the number of viral copies

without evidence of treatment resistance.[49] Another molecule, MRX34, which mimics miR-34, has recently (at the time of writing) entered phase I clinical trials for the treatment of primary liver cancer. MiR-34 inhibits multiple oncogenic pathways and induces apoptosis in tumor cells.[50]

MiRNA studies represent an attractive and promising field of investigation. Identifying and understanding the role of miRNAs is an important step in the development of new therapeutic and diagnostic tools.

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