miRNAs: roles and clinical applications in vascular disease
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DOI: http://dx.doi.org.ezproxyhost.library.tmc.edu/10.1586/erm.10.103
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Full Text:

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Keywords:
atherosclerosis; biomarker; lipoprotein metabolism; miRNA; therapeutic target; vascular disease; vascular smooth muscle cell

The cardiovascular system is composed of the heart, blood vessels and blood. It is connected intimately with every other organ system, and dysfunction of the cardiovascular system can have devastating downstream effects. The lumen of blood vessels is lined by a monolayer of endothelial cells, which forms the main physical barrier between the blood and vessel wall, controlling the movement of solutes and fluid from the vascular space to the surrounding tissues.[1] Endothelial dysfunction owing to breakdown of the endothelial cell-cell barrier can promote atherogenesis through the increased adherence of leukocytes, monocytes and macrophages, and subendothelial accumulation of cholesterol-bearing lipoproteins.[2,3]. Meanwhile, vascular smooth muscle cells (VSMCs) below the endothelium undergo phenotypic modulation from a contractile phenotype to a proliferative state under the influence of mechanical stress, growth factors, inflammatory mediators, such as low-density lipoprotein (LDL) deposition, and leukocyte or monocyte infiltration.[4]. Aberrant proliferation of VSMCs and the formation of a neointimal lesion are key pathological processes in a variety of proliferative vascular diseases, such as atherosclerosis, coronary heart diseases, post-angioplasty restenosis and transplantation arteriopathy.[5]. During vascular injury, rapid proliferation and growth of vascular cells occur in order to repair the injury, and abnormal repair leads to vascular hyperplasia and neointimal lesion formation; a significant contributor to vascular hyperplasia and neointimal formation is the proliferation of VSMCs.[6,7].

Since the first discovery of miRNAs in 1993, the involvement of miRNAs in different aspects of cardiovascular disease has emerged as an important research field. The dysregulation of many individual miRNAs has been linked to the development and progression of cardiovascular disease. Forced expression or suppression of a single miRNA is enough to cause or alleviate pathological change. The role of miRNAs in the pathogenesis of heart and vascular disease points to a possibility of using miRNAs as potential diagnostic biomarkers and/or therapeutic targets for cardiovascular disease. In this article, the miRNAs that have been associated with different aspects of cardiovascular diseases and their molecular mechanisms are discussed, as well as clinical applications of miRNAs as new biomarkers and/or targets for cardiovascular diseases.

Biogenesis & cellular activity of miRNA

miRNAs are highly conserved, single-stranded, noncoding, small RNAs that control cellular function either by degrading mRNAs or by inhibiting their translation. miRNAs can be located in introns of coding or noncoding genes or in exons. miRNAs that have their own promoters are expressed independently, while miRNAs organized in clusters share the same transcriptional regulation.[8,9]. miRNAs are produced from longer primary RNA precursors (pri-miRNAs) containing stem-loop structures that are transcribed by RNA polymerase II. These pri-miRNAs are cleaved in the nucleus by the complex of the RNase III enzyme Drosha and its partner DGC8/Pasha, to form pre-miRNAs of approximately 70 nucleotides in length.[10-15]. Pre-miRNAs are transported into the cytoplasm by exportin-5 and subsequently processed by the nuclease Dicer into the 20-24-nucleotide mature miRNAs.[16-18].

After processing, the mature miRNA is incorporated into the RNA-induced silencing complex.[19], which directs the miRNA to the target mRNA. This leads either to translational repression or degradation of the target mRNA.[13]. Whereas mRNA degradation requires a high level of miRNA-target complementarity, translational repression is characterized by low miRNA-target complementarity.[20]. It is estimated that approximately a third of all genes are regulated by miRNAs. The complexity of miRNA-dependent gene expression is further extended by the fact that more than one miRNA can cooperatively bind to the same 3′ untranslated region (UTR).[21]. These features of miRNAs facilitate their regulation of important, ubiquitous cellular processes that include apoptosis, senescence and angiogenesis.
Regulation of miRNA biogenesis represents an important site of regulation of miRNA activity, as it involves multiple overlapping interactions between proteins and RNA [22]. For example, similar to protein-coding genes, miRNA genes have promoters that bind transcription factors that regulate their expression. In addition, various co-factors of Dicer, Drosha and their partners augment the miRNA processing machinery; these also regulate the production of miRNAs. Using computational prediction analysis, the tumor suppressors p53, p73 and p63 have been identified as regulators of the miRNA processing machinery, including Drosha-DGCR3, Dicer-TRBP2 and argonaute proteins [23]. Similarly, signaling by the TGF-[beta][P] and bone morphogenetic protein (BMP) family lead to the upregulation of miR-21 and pathologic switching of VSMC into a synthetic phenotype, and this appears to be related to an increase in processing of pre-miR-21 by the Drosha complex [24].

### Apoptosis

Numerous miRNAs have been demonstrated to regulate apoptosis experimentally, including the let-7 family, miR-1, miR-1d, miR-7, miR-14, miR-15a, miR-16-1, the miR-17 cluster (miR-17-5p, miR-18, miR-19a, miR-19b, miR-20 and miR-92), miR-21, miR-29, miR-34a, miR-133, miR-146a, miR-146b, miR-148, miR-191, miR-204, miR-210, miR-214, miR-216, miR-278, miR-296, miR-335, miR-Lat and bantam. This list is expected to expand quickly as more studies are performed.

miR-1 promotes oxidative stress-induced apoptosis in cardiomyocytes, mainly by repressing heat-shock protein (HSP)60 and HSP70, and levels of miR-1 are elevated in patients with coronary artery disease [25]. Meanwhile, it has been demonstrated that miR-1 targets both IGFR-1 and IGFR-1 receptor and, in fact, miR-1 and IGFR-1 appear to reciprocally regulate each other [26]. The IGFR signaling pathway is an important modulator of cardiac and skeletal muscle growth and survival, and IGFR-1 has been demonstrated to be antiapoptotic in a variety of cell types [27].

The apoptosis-regulating miRNAs can be divided broadly into two groups: anti- and pro-apoptotic. This distinction is based primarily on experimental results from particular cell types. For example, inhibition of miR-17-5p and miR-20a induces apoptosis in lung cancer cells, indicating that miR-17-5p and miR-20a may protect these cells from apoptosis and, therefore, are termed antiapoptotic [28]. On the other hand, proapoptotic miRNAs, such as miR-15a and miR-16-1, induce apoptosis in chronic lymphocytic leukemia cells [29]. Each miRNA has the potential to regulate over 1000 protein-coding genes that could well be a mixture of some anti- and pro-apoptotic genes [30]. Whether a miRNA is antiapoptotic or proapoptotic may depend upon the cell-specific expression of genes involved in apoptosis. Increasing experimental evidence suggests that the cell context is very important in determining whether a miRNA is anti- or pro-apoptotic, and a miRNA that is antiapoptotic in a particular cell type may become proapoptotic in another cell type.

Zernecke et al. reported the generation of endothelial cell-derived apoptotic bodies during atherosclerosis, which convey paracrine alarm signals to recipient vascular cells, triggering the production of chemokine (C-X-C motif) ligand 12 (CXCL12) [31]. CXCL12 production was mediated by miR-126. Administration of apoptotic bodies or miR-126 limited atherosclerosis, promoted the incorporation of stem cell antigen-1 (Sca-1)-positive progenitor cells, and conferred features of plaque stability in different mouse models of atherosclerosis, indicating that miR-126 has atheroprotective effects [31].

### Senescence

Cellular senescence is a mechanism to inhibit the growth of mammalian cells after oncogenic activation or in response to damage or stress [32]. Increased numbers of senescent endothelial cells are found in mature atherosclerotic plaques, vessels from diabetic patients, postangioplastic restenotic vessels, coronary vessels of patients with ischemic heart disease and in hypertensive patients [33]. One of the candidate miRNAs involved in vascular senescence is miR-34a [34]. miR-34a is highly expressed in primary endothelial cells and its expression increases in senescent human umbilical vein endothelial cells (HUVECs) and the hearts and spleens of older mice. Overexpression of miR-34a was found to induce endothelial cell senescence and also suppress cell proliferation by inhibiting cell cycle progression. Silent information regulator 1 (Sirt1) has been found to be a target of miR-34a. Initially identified as a longevity gene, Sirt1 has recently been implicated as a novel modulator of vascular endothelial cell homeostasis, playing a key role in angiogenesis through the deacetylation of forkhead box O transcription factor 1 (FoxO1) [35,36]. Overexpression of miR-34a inhibits Sirt1 protein expression, while miR-34a knockdown enhances Sirt1 expression. miR-34a triggers endothelial senescence, in part, through Sirt1, since forced expression of Sirt1 blocks the ability of miR-34a to induce senescence. miR-217 also regulates the expression of Sirt1 [37]. Aging is a major risk factor for the development of atherosclerosis and coronary artery disease, and miR-217 is progressively expressed in endothelial cells with aging [38].

### Angiogenesis

Angiogenesis is an important cellular process, both in normal physiology and in pathologic states, such as ischemic heart disease and cancer. miRNAs appear to play a critical role in angiogenesis. This has been demonstrated by genetic silencing of Dicer, the rate-limiting enzyme required for the normal processing of mature miRNA. Silencing of Dicer in vivo reduces angiogenesis, even with stimulation by exogenous VEGF, tumors, limb ischemia and wound healing [39].

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Silencing of Dicer has also been demonstrated to lead to a reduction in markers of angiogenesis in vitro and in vivo; this appeared to be through regulation of the angiogenesis inhibitor thrombospondin-1 (TSP-1) via miRNA let-7 family members [40].

The majority of miRNAs have been found to be downregulated in endothelial cells [40]. In HUVECs, meanwhile, 15 miRNAs have been found to be highly expressed and are thought to regulate several angiogenic factors, including Flt-1, Nrp-2, Fgf-R and c-Met, based on prediction algorithms [38]. HUVECs are useful for studying angiogenesis because of their formation of tubes, which are structures that resemble capillaries. It has been demonstrated in HUVECs that miR-221 and miR-222 modulate angiogenesis through their interaction with the 3´UTR of c-kit, which is a receptor for stem cell factor (SCF), itself being a proangiogenic factor [38]. miR-221 has been implicated in inhibiting endothelial cell tube formation and migration; this appears to be mediated by increased expression of the antiangiogenic homeobox gene GAX, through the regulation of ZEB2 by miR-221 [41].

The pathologic angiogenesis involved in tumor formation has also been investigated. Recently, miR-132 has been proposed to be an angiogenic switch in tumor formation; this miRNA is highly upregulated in conditions of angiogenesis but is absent in normal endothelium, and miR-132 appears to suppress p120RasGAP, leading to increased Ras activity and neovascularization. This pattern was also observed in human tumors in comparison with normal tissue. Meanwhile, administration of anti-miR-132 reversed these effects [42]. miR-126 has also been found to modulate angiogenesis in a positive way; its deletion leads to vascular leakage, hemorrhage and early death in mice; these effects appear to be mediated through repression of Spred-1, which is a negative regulator of MAPK signaling [43]. miR-126 appears to increase the response of endothelial cells to VEGF, promoting angiogenesis in vivo and in vitro in zebrafish [44]. Meanwhile, administration of VEGF to human vascular endothelial cells has been demonstrated to increase the level of several miRNAs in human tumors, including miR-155, miR-191, miR-21, miR-18a, miR-17-5p and miR-20a; these have been associated with tumor growth, survival and angiogenesis [39].

Several authors have reviewed the literature on all angiogenesis-related miRNAs, including miR-27b, miR-126, miR-17-92, let-7, miR-17-92 cluster, miR-130a, miR-210, miR-378 and miR-296, which are thought to have potential angiogenic effects; meanwhile miR-221/222, miR-328, miR-92a and miR-214 have been found to be antiangiogenic [45,46]. The effects of these miRNAs on endothelial biology and angiogenesis have been identified in a variety of settings, both in cultured endothelial cells in vitro and in ischemia-induced angiogenesis in vivo, using limb ischemia and myocardial infarction animal models [47,48].

Atherosclerosis

Atherosclerosis is a pathological process in which deposits of fatty compounds, cholesterol, cellular waste products, calcium and other substances accumulate in the inner lining of an artery, forming plaques. It usually affects large- and medium-sized arteries. Plaques can grow large enough to significantly reduce the blood flow through an artery. The most serious damage may occur when plaques become fragile and rupture. Plaque rupture can trigger vascular thrombosis, which can block blood flow, or plaques can embolize, leading to myocardial infarction, stroke or acute or chronic limb ischemia. The major risk factors for atherosclerosis include elevated levels of cholesterol and triglyceride in the blood, high blood pressure, tobacco smoking, diabetes, obesity and physical inactivity. The first major event in the progression of the early atheroma is the loss of endothelial integrity. Endothelial dysfunction then facilitates the subendothelial accumulation of cholesterol-bearing lipoproteins and compromises vasodilation.

In a recent study, individuals with atherosclerosis, as defined by coronary artery disease (CAD), showed a significantly higher expression of miR-221 and miR-222 in endothelial progenitor cells (EPCs) compared with non-CAD individuals [49]. EPCs play an important role in the maintenance of vascular integrity. Atorvastatin treatment in CAD groups increased EPC numbers and decreased miR-221/222 levels, indicating that miR-221/222 levels were inversely related to EPC levels, as CAD individuals had significantly lower EPC numbers [49].

Inflammatory environment in atherosclerosis

A key step in the development of chronic inflammatory atherosclerotic disease is the migration of circulating monocytes into the subendothelial space and their differentiation into macrophages. A recent study showed that miR-125a-5p mediates lipid uptake and decreases the secretion of some inflammatory cytokines, including IL-2, IL-6, TNF-[alpha] and TGF-[beta] in oxidized LDL-stimulated monocyte-derived macrophages [50]. The target gene of miR-125a-5p has been found to be ORP9, which has diverse roles in the regulation of lipid metabolism, including vesicle transport, as well as cell cycle regulation and differentiation [51,52]. Meanwhile, miR-10a has been implicated in contributing to the proinflammatory endothelial phenotype observed in atherosclerosis formation. In swine, atherosusceptible regions of the arterial tree had lower levels of endothelial miR-10a than elsewhere. Homeobox A1 (HOXA1) is a known downstream target of miR-10a, and it was found to be upregulated in the same areas. Meanwhile, miR-10a knockdown in endothelial cells led to an increase in inflammatory biomarkers. Furthermore, miR-10a was found to interact with the 3´UTRs of MAP3K7 and...
miR-33 is an intronic miRNA located within the gene encoding sterol-regulatory element-binding factor-2, a transcriptional regulator of cholesterol synthesis, and miR-33 modulates the expression of genes involved in cellular cholesterol transport. miR-33 appears to be regulated by dietary cholesterol in vivo and have several roles in cholesterol homeostasis. It appears to regulate both HDL biogenesis in the liver and cellular cholesterol efflux. miR-33 targets the 3'UTR of the adenosine triphosphate-binding cassette (ABC) transporter ABCA1 in mouse peritoneal macrophages and in human cells; as a result, atherogenic cholesterol efflux to apolipoprotein A1 is reduced. Similarly, in a mouse model, lentiviral delivery of miR-33 represses ABCA1 expression in the liver, leading to a reduction in circulating HDL levels, while mice expressing anti-miR-33 in vivo demonstrate increased plasma HDL levels; however, there have been conflicting results regarding the effect of miR-33 silencing on hepatic expression of ABCA1, with one investigation showing an increase in hepatic ABCA1 and another showing...

Lipoprotein metabolism

Lipids, such as triglycerides and cholesterol, are insoluble in circulating plasma. In order to carry these lipids through plasma, proteins called apolipoproteins bind with lipids to form lipoproteins, such as LDL and high-density lipoprotein (HDL). The primary functions of lipoproteins are the delivery of neutral lipids, such as triglycerides and cholesterol, to peripheral cells; they also remove excess cellular cholesterol by the reverse cholesterol transport pathway. As such, lipoproteins are critical mediators of atherosclerosis formation. miR-33 appears to regulate both HDL biogenesis in the liver and cellular cholesterol efflux. miR-33 is an intronic miRNA located within the gene encoding sterol-regulatory element-binding factor-2, a transcriptional regulator of cholesterol synthesis, and miR-33 modulates the expression of genes involved in cellular cholesterol transport. miR-33 appears to be regulated by dietary cholesterol in vivo and have several roles in cholesterol homeostasis. It appears to regulate both HDL biogenesis in the liver and cellular cholesterol efflux. miR-33 targets the 3'UTR of the adenosine triphosphate-binding cassette (ABC) transporter ABCA1 in mouse peritoneal macrophages and in human cells; as a result, atherogenic cholesterol efflux to apolipoprotein A1 is reduced. Similarly, in a mouse model, lentiviral delivery of miR-33 represses ABCA1 expression in the liver, leading to a reduction in circulating HDL levels, while mice expressing anti-miR-33 in vivo demonstrate increased plasma HDL levels; however, there have been conflicting results regarding the effect of miR-33 silencing on hepatic expression of ABCA1, with one investigation showing an increase in hepatic ABCA1 and another showing...

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miR-122 also has a direct role in cholesterol metabolism. The expression of miR-122 is highly restricted to the liver. Overexpression of miR-122 with adenovirus increases the expression of cholesterol-synthesis genes (Hmgrcs1, Sqle and Dhcr7) in the liver, which in turn increases cholesterol synthesis. Silencing of miR-122 expression in hepatocytes downregulates genes implicated in cholesterol biosynthesis and triglyceride metabolism, increasing hepatic fatty acid oxidation and reducing plasma cholesterol, hepatic fatty acid and cholesterol synthesis. However, the direct targets of miR-122 that confer these effects on the cholesterol biosynthetic and metabolic pathways are still unknown. Meanwhile, miR-125a-5p has been found to mediate lipid uptake in oxidized LDL-stimulated monocyte-derived macrophages.

**Human endothelial nitric oxide synthase regulation**

Human endothelial nitric oxide (NO) synthase (eNOS) is an enzyme that is constitutively expressed, mainly in endothelial cells. eNOS, in the form of homodimers, catalyzes L-arginine oxidation to generate L-citrulline and NO in the presence of several cofactors. NO, meanwhile, is an antiatherogenic molecule. While a large amount of data exist regarding the regulation of eNOS enzyme activity, relatively little information is available about the role of miRNAs in regulating eNOS expression.

Shear stress is an important activator of eNOS expression and has been demonstrated to increase the expression of 13 miRNAs in HUVECs. Among them, the upregulation of miR-21 was highest. Western blot analysis demonstrated that PTEN, a known target of miR-21, was downregulated in HUVECs exposed to shear stress or transfected with pre-miR-21. Importantly, HUVECs overexpressing miR-21 demonstrated decreased apoptosis and increased eNOS phosphorylation and NO production. These data demonstrate that shear stress regulates the expression of miR-21 in endothelial cells, which, in turn, influences endothelial biology by decreasing apoptosis and activating the NO pathway.

Ginsenoside-Rg1 is one of the active components of ginseng, and it is an angiogenesis inducer of eNOS expression. This effect appears to be mediated by a ginsenoside-Rg1-induced downregulation of miR-214, leading to increased eNOS expression. Meanwhile, computational prediction has revealed that miR-214 may potentially target eNOS, as sequence alignment showed that miR-214 is partially complementary to the RNA sequence extending from nucleotide 168 to 189 within the 3′-UTR of eNOS mRNA. In addition, transfection of a miR-214 precursor mimic (pre-miR-214) repressed expression of eNOS in HUVECs, while transfection of an antagoniRNA antisense to mature miR-214 (AS-miR-214) enhanced eNOS expression. Overall, these data strongly suggest that miR-214 is involved in Rg1-induced eNOS expression and angiogenesis in HUVECs.

**Arterial remodeling**

miR-222 has been demonstrated to be involved in inflammation-mediated vascular remodeling. miR-222 targets STAT5A and negatively correlates with STAT5A expression in human endothelial cells from advanced neovascularized atherosclerotic lesions. Furthermore, upregulation of STAT5A due to IL-3/basic FGF (bFGF)-induced downregulation of miR-222 controls endothelial cell proliferation and migration and, therefore, facilitates intraplaque neovascularization during atherosclerosis. In fact, in advanced lesions, there was an increased proliferation rate of endothelial cells lining vessels, which correlated with a diminished expression of miR-222. Thus, miR-222 acts as a negative regulator of neovascularization through post-transcriptional regulation of STAT5A.

**miRNA as a biomarker for coronary artery disease**

Levels of circulating miRNAs are being studied extensively as a novel class of biomarker for various diseases, especially cancer. miRNA in clinical plasma samples is stable with respect to freezing and rethawing and appears to be protected from endogenous RNase activity. miRNA has been found to circulate in the plasma in microvesicles, platelets and peripheral blood mononuclear cells (PBMCs), and in healthy human subjects, it has been determined that over 250 miRNAs can be detected in the circulation. However, the lack of standardized control values for circulating miRNA levels is presently a limitation in the interpretation of data in which miRNA is investigated as a biomarker. In addition, in this emerging field, small sample sizes often limit the immediate translational applicability of the data. Nonetheless, these issues should be addressed shortly, and at present, exciting data exist to suggest that miRNAs may prove to be valuable biomarkers in the detection of cardiovascular disease.

Expression of miR-146a is upregulated in the PBMCs of patients with acute coronary syndrome (ACS), including stable angina and acute myocardial infarction (AMI). Overexpression of miR-146a positively correlates with the plasma concentrations of pro-inflammatory cytokines, such as IFN-γ and NF-κB p65, a critical transcription factor in atherosclerosis. Overexpression of miR-146a in PBMCs significantly upregulates the function of Th1 cells.
cells and is related to the onset and progression of ACS. By contrast, miR-146a inhibitor reduced this effect \[80] . Thus, miR-146a may represent a novel regulatory factor in ACS patients and could be a new therapeutic target for atherosclerosis and ACS.

Several other recent studies have examined levels of circulating miRNAs in patients with AMI \[79,81-83\]. Expression of miR-1, which was found previously to be upregulated in ischemic cardiac tissue, was found to be significantly higher in plasma from AMI patients compared with non-AMI subjects; the level of miR-1 dropped to normal upon discharge \[79,81,82\]. The levels of miR-208a, miR-133a and miR-499 were also elevated in AMI patients in comparison with non-AMI patients, and there was no detectable miR-208 in healthy subjects \[83,84\]. However, of the four miRNAs, only miR-208a appears to be expressed in a cardiac-specific manner \[83,85\]; plasma elevations of miR-1, miR-133a and miR-499 may occur after skeletal muscle injury, which may limit their use as biomarkers for AMI \[83\]. Meanwhile, miR-499 has been described in other investigations to be produced almost exclusively in the heart \[84\]. In addition, it has been demonstrated that miR-1 and miR-133a, while highly expressed in skeletal muscle, do not increase in a mouse model of skeletal muscle damage due to acute hind-limb ischemia \[79\]. In light of these findings, it has been hypothesized that under conditions of cardiac injury, specific miRNAs in the systemic circulation may reflect myocardial damage and prove to be valuable biomarkers for AMI. However, again, these studies will first need to be extended to larger populations and the cardiac specificity of the individual miRNAs delineated clearly in order to achieve clinical significance.

Levels of circulating miRNAs have also been examined in patients with stable CAD in comparison with healthy subjects. Most vascular-derived miRNAs, including miR-126 and the miR-17-92 cluster, were actually downregulated in patients with CAD; this may be due to uptake into atherosclerotic lesions within the vasculature of these patients. Meanwhile, in the same patients, there was a trend towards increased levels of skeletal/cardiac muscle-derived miRNAs, such as miR-133a and miR-208a. While these patients were not losing active myocardial tissue, as evidenced by baseline troponins, the elevation in muscle-derived miRNAs may be due to a "low grade of cardiac myocyte injury" \[86\].

**Stroke**

Stroke is the second most common cause of death and consumes approximately 2-4% of total healthcare costs worldwide. The majority of strokes are ischemic and may result from thrombosis, embolism or global hyperperfusion. A search for biomarkers to aid in the diagnosis of stroke has commenced as well. Through array analysis of 836 miRNAs in blood samples in stroke patients, eight miRNAs (let-7f, miR-126, miR-1259, miR-142-3p, miR-15b, miR-186, miR-519e and miR-768-5p) were poorly expressed across the three subtypes of stroke (large artery, small artery and cardioembolic stroke), while 17 miRNAs (let-7e, miR-1184, miR-1246, miR-1261, miR-1275, miR-1285, miR-1290, miR-181a, miR-25*, miR-513a-5p, miR-550, miR-602, miR-665, miR-891a, miR-933, miR-939 and miR-923) were highly expressed in these subtypes \[87\]. However, it is not clear whether these miRNAs will be useful as biomarkers for stroke.

**Pulmonary hypertension**

Severe pulmonary arterial hypertension (PAH) is characterized by significant increases in pulmonary artery pressures to levels present in the systemic circulation. PAH is a major determinant of morbidity and mortality in cardiopulmonary disease. The pathogenesis of PAH revolves around excessive vasoconstriction and/or abnormal pulmonary vascular remodeling. Recent experimental evidence has linked the pulmonary vascular disease present in PAH to an abnormal proliferative vascular cell phenotype, which is also characterized by resistance to endothelial and/or VSMC apoptosis \[88\]. Caruso et al. screened and profiled miRNA signatures in the rat lung, and compared them longitudinally with rats exposed to either chronic hypoxia or monocrotaline injuries at 2, 7 and 21 days. Monocrotaline, a pyrrolizidine alkaloid and a toxic plant constituent, causes PAH, right ventricular hypertrophy and pathological changes in the pulmonary vasculature. The study showed that the expression of Dicer during the onset of PAH after hypoxia was reduced. miR-22, miR-30 and let-7f were downregulated, whereas miR-322 and miR-451 were upregulated significantly during the development of PAH in both hypoxic and monocrotaline models. miR-21 and let-7a were significantly reduced only in monocrotaline-treated rats \[89\]. Chen et al. investigated the involvement of miR-759 in chronic thromboembolic pulmonary hypertension (CTEPH). One of the leading causes of severe pulmonary hypertension, CTEPH is characterized by persistent pulmonary embolism that increases pulmonary vascular resistance, resulting in pulmonary hypertension and subsequent right ventricular heart failure. The 3′ UTR of FGA was found to interact with miR-759, and a 28-bp deletion polymorphism at this site was found to be more frequent in patients with CTEPH \[90\]. Fibrinogen, also called factor I, plays an important role in blood clotting, fibrinolysis and inflammation \[91\].

**Neointimal hyperplasia & vascular response to injury**

Neointimal lesion formation is a common pathological event seen in diverse proliferative vascular diseases, such as atherosclerosis, coronary heart disease, post-angioplasty restenosis and transplantation arteriopathy. Neointimal hyperplasia has also generally been accepted as the main feature of vascular repair responses to various injuries and several investigations have suggested that miRNAs are important mediators of this process.
Post-angioplasty stenosis

Multiple miRNAs are differentially expressed in rat carotid arteries demonstrating neointimal growth after angioplasty. Among the aberrantly expressed miRNAs, miR-21 expression underwent a more than fivefold increase in balloon-injured arteries compared with normal control vessels. Meanwhile, knockdown of miR-21 in balloon-injured rat carotid arteries significantly inhibited the growth of neointimal lesions. Antiproliferative and proliferative effects on VSMCs are thought to be the major mechanisms whereby miR-21 mediates vascular neointimal growth in cultured VSMCs in vitro and in rat carotid arteries in vivo. miR-21 targets mainly PTEN and PDCD4 when exerting its effect on VSMCs. In addition, reactive oxygen species (ROS) contribute to the pathogenesis of atherosclerosis and restenosis after angioplasty. miR-21 is highly expressed in VSMCs treated with ROS, such as hydrogen peroxide. In the rat carotid artery balloon-injury model, neointimal hyperplasia of injured artery was significantly repressed when antisense oligonucleotide-mediated miRNA depletion led to downregulation of miR-21.

Following vascular injury, miR-221 and miR-222 increase in the balloon-injured carotid arteries and localize primarily in VSMCs of the vascular wall. Knockdown of miR-221 and miR-222 can inhibit VSMC proliferation and neointimal thickening in rat carotid artery after vascular injury. In another vascular-injury model, miR-143 and miR-145 expression was significantly decreased in ligated carotid arteries compared with control arteries. Furthermore, miR-145 was decreased to nearly undetectable levels in mouse arteriosclerotic lesions. Meanwhile, miR-143/145-mutant mice showed a significant increase in neointimal lesion formation with large amounts of VSMCs, macrophages and deposits of amorphous collagen I in the femoral arteries. Several investigations have shown that introduction of miR-145 or miR-143/145 into injured rat carotid arteries via adenovirus-mediated gene transfer leads to a significant reduction in neointimal lesion formation; when miR-145 was administered, the reduced lesion formation was accompanied by upregulation of VSMC differentiation markers. The effect of miR-143 and miR-145 on neointimal hyperplasia is also reported in a mouse model of vascular injury. Taken together, these data indicate that miR-143 and miR-145 play pivotal roles in the control of the contractile phenotype of VSMCs and the response of the vascular wall to injury, which has important therapeutic implications.

Proliferative signal transduction pathways

Different types of signal transduction pathways are involved in miRNA-mediated effects on the vascular system. One example is the previously mentioned involvement of TGF-[beta]P signaling pathways during the transition of VSMCs from a contractile to a synthetic phenotype. Modulation of the VSMC phenotype from a quiescent contractile phenotype to a proliferative synthetic phenotype has been implicated in vascular injury repair, as well as in the pathogenesis of vascular proliferative diseases. BMP and TGF-[beta]P signaling pathways promote a contractile phenotype, while the PDGF-BB signaling pathway promotes a switch to the synthetic phenotype. PDGF-BB can induce miR-24, which, in turn, leads to downregulation of Tribbles-like protein-3 (Trb3). Repression of Trb3 coincides with the reduced expression of Smad proteins and a decrease in BMP and TGF-[beta]P signaling, promoting a synthetic phenotype in VSMCs. Inhibition of miR-24 by antisense oligonucleotides abrogates the downregulation of Trb3 as well as the prosynthetic activity of the PDGF signaling pathway. Both miR-21 and miR-24 have been implicated in the pathways whereby PDGF and TGF-[beta]P control the VSMC phenotype.

miRNAs as therapeutic targets

Ongoing research shows that miRNAs are involved in almost all types of cellular processes, making them ideal targets for therapeutic use. Multiple miRNAs are involved in the development of both human and animal vascular diseases as they regulate vascular cell differentiation, migration, proliferation and apoptosis through their target genes. Several in vivo animal studies have revealed promising results in treating vascular disease. Thus, miRNAs may represent both new biomarkers and new therapeutic targets for diverse vascular diseases.

Vascular diseases are complex, multifactorial diseases, in which many genes are involved. Meanwhile, one miRNA can have multiple targets, and one gene can be regulated by several miRNAs. Keeping this in mind, miRNA-based therapy may have both advantages and disadvantages. miRNAs that have only a single target gene should be easy to suppress using anti-miRNA technology, which represents an advantage. However, suppression of miRNAs that have multiple target genes will affect several genes and might induce some unexpected side effects; this, then, could be a disadvantage. Since miRNAs are endogenous, restoration of aberrantly expressed miRNAs, both upregulated and downregulated, to physiological levels should not have major unexpected side effects. Therefore, strategies for miRNA-based therapies are based on either the restoration of suppressed genes by reducing or inhibiting specific miRNAs or the delivery/overexpression of specific miRNAs to suppress the target gene responsible for disease formation.

As we are still in the early stages of developing miRNA-based therapy, further studies need to be performed carefully before it can be used in the clinic. First, the critical miRNAs responsible for the development of vascular diseases should be identified definitively. Second, the detailed cellular and molecular mechanisms of these critical miRNAs in the
prevention and treatment of vascular diseases should be elucidated. Third, in addition to the biological effects of these miRNAs on endothelial cells and VSMCs, their effects on other vascular disease-related cellular processes should be identified. Fourth, although methods are available to downregulate miRNA in vivo, technology for upregulating miRNA in the vascular walls in vivo requires further development. Finally, the potential side effects of miRNA-based therapy should be studied before application in patients.

There are several strategies by which miRNAs can be deactivated or destroyed effectively. The fact that miRNAs bind to their target mRNAs by Watson-Crick base pairing indicates that the usage of an oligonucleotide that is complementary to the miRNA and effectively competes with the mRNA target (i.e., anti-miRNAs) represents a potentially effective way of inactivating pathological miRNAs; thus, downregulation of important target genes that promote gene expression could be avoided \cite{66-68}. Alternatively, miRNA mimics (synthetic, non-natural nucleic acids that can bind to the unique sequence of target miRNAs in a gene-specific manner) may induce target downregulation and thereby diminish gene expression; however, this approach has not been tested in vivo \cite{99,100}. The use of anti-miRNAs in cultured cells has been successful. Chemical modification of miRNA inhibitors is key in the development for use in vivo.

Three different types of chemical modifications have been carried out to enact the inhibition of miRNA function in vivo. The first class of anti-miRNAs is conjugated to cholesterol (anti-miRNA) to facilitate cellular uptake. Other classes use oligonucleotides with locked nucleotides acid (anti-miRNAs) or 2′-O-methoxymethyl phosphorothioate modifications. Antagonism of miR-122 in mouse liver using these three classes of anti-miRNAs in three independent studies revealed that miR-122 antagonism led to reduced plasma cholesterol levels in cardiovascular diseases, including hypertrophy, heart failure, cardiac injury, arrhythmia and atherosclerosis \cite{66-68}. Therefore, these approaches to manipulate the expression of miRNAs, either positively or negatively, can potentially be used for therapeutic purposes. For those miRNAs that are downregulated in disease states, a forced miRNA re-expression strategy may be used to recover miRNA expression; conversely, an anti-miRNA strategy may be employed to suppress the expression of upregulated miRNAs. Although the targeting of miRNAs represents a promising therapeutic strategy, substantial preclinical work still needs to be conducted in order to identify precise targets among the many possible targets in vascular disease.

To date, more than 800 human miRNAs have been identified (miBase) and work continues in the search for new miRNAs. miRNAs as important regulators of gene expression represent a relatively recent discovery. It appears that miRNAs are key regulators in almost all cellular processes and in many human diseases. In this article, we have summarized the role of miRNAs in the regulation of vascular disease and the possibilities of using miRNA-directed therapies as a treatment option for vascular diseases associated with the aberrant expression of miRNAs. The biggest obstacle to the development of miRNA-based clinical therapy is how to efficiently deliver miRNA mimics and inhibitors to target organs or cells. With rigorous fundamental and clinical studies, a clearer understanding about miRNAs as biomarkers and targets for cardiovascular disease will develop.

**Expert commentary**

With the recent surge of research into miRNAs, our understanding of the role of miRNAs in vascular biology is rapidly evolving and several strategies have been developed to suppress or overexpress specific miRNAs. The functions of aberrantly expressed miRNAs in the vascular system and their mechanisms of actions, particularly their target genes and signaling pathways, need to be studied in detail in order to efficiently develop biomarker applications and therapeutic approaches. The combination of computational analysis, bioinformatics and detailed molecular mechanism analysis using *in vitro* cell culture and *in vivo* knockout analysis in animals will be needed for the identification of miRNA target genes. Of note, again, one miRNA may have several target genes or one gene might be regulated by several miRNAs. Therefore, targeting a specific miRNA may have unexpected effects in the experimental system. Several strategies, including anti-miRNAs, miRNA mimics, miRNA inhibitors and miRNA sponges, have been developed to suppress pathological miRNAs. However, stringent research is needed to efficiently deliver these miRNA inhibitors to the specific target organs or cells. Finally, substantial preclinical work still needs to be conducted to identify precise target genes among the many predicted target genes in vascular diseases.

**Five-year view**

Results from recent studies demonstrate a broad role of miRNAs in cardiovascular development and disease formation. Determining miRNA regulatory circuits and defining the mechanisms whereby miRNAs control the expression of target genes will be key to future miRNA-based therapeutic applications towards cardiovascular disease prevention. The specific recognition of a miRNA to its target 3′ UTR is highly regulated; yet, the mechanisms that regulate this recognition are still under investigation. One important research direction is the impact of genomic variation in miRNA genes on miRNA function and the contribution of this individual variability to disease formation. Currently, more than 800 miRNAs have been discovered and many new miRNAs are being discovered. Therefore, it is certain that many new and unanticipated roles of miRNAs in the control of cardiovascular functions will be discovered and will assist clinicians and researchers in developing potential therapeutic applications.

**Key issues**
miRNAs are endogenously expressed, small (22 nucleotides long), noncoding RNAs that mainly regulate gene expression at the post-transcriptional level.

miRNAs are highly expressed in the vascular wall and their expression is found to play important roles in angiogenesis, atherosclerosis, lipoprotein metabolism, acute coronary artery syndrome, neointimal lesion formation, hypertension, arterial remodeling, senescence, cell migration, proliferation and apoptosis.

Circulating miRNAs may represent a novel class of biomarkers for myocardial infarction.

Owing to critical roles of miRNAs in the vascular system, specific miRNAs may represent new therapeutic targets for diverse vascular diseases.

Further studies are required to address the contribution of specific miRNAs and their targets in vascular disease formation in animal models and clinical trials.

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* Describes the expression and function of miR-145 in vascular smooth muscle cells in vitro and in vivo.


* Describes human Drosha, a component of two multiprotein complexes that mediate the genesis of miRNA from the primary miRNA transcript.


* Summarizes basic information about miRNAs.


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*ã¢â€œ Describes the prediction of regulatory target genes for conserved vertebrate miRNAs.


*ã¢â€œ Describes a method for identifying miRNA binding sites and their corresponding heteroduplexes.


*â€* Describes miR-217 as an endogenous inhibitor of SirT1, which promotes endothelial senescence.


*â€*â€ Describes the approaches and current experimental evidence for the involvement of miRNAs in the angiogenic process; potential therapeutic applications for vascular diseases associated with abnormal angiogenesis are also addressed.


*â€*â€ Describes the expression of the miR-17-92 cluster in human endothelial cells and the role of miR-92a in angiogenesis.


*â€*â€ Describes miR-10a, which targets HOX1 and is involved in unique signaling pathways, including the regulation of inflammation.


*â¢*â¢ Describes miR-145 and miR-143 function in regulating the quiescent versus proliferative phenotype of vascular smooth muscle cells.


*â¢*â¢ Describes the roles of miR-221 and miR-222 in regulating vascular smooth muscle cell proliferation and neointimal hyperplasia.


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*â¢*â¢ Describes a novel class of chemically engineered oligonucleotides, termed 'âantago-miRNAs'â.


*áç Describes the expression and function of miR-21 in neointimal lesion formation.


*ã¢â††ã¢â€† Describes the limitations and possibilities of locked nucleotide acid-modified siRNA and its possible use in therapeutic applications.


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Financial & competing interests disclosure

This work was partially supported by a NIH grant (R01HL083471 to Changyi Chen) and by the Michael E DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX, USA. Sarah M Weakley and Lidong Zhang were supported by a NIH training grant (T32HL083774). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Source Citation  (MLA 8th Edition)

Gale Document Number: GALE|A266807877