Role of Flow-Sensitive microRNAs in Endothelial Dysfunction and Atherosclerosis
Mechanosensitive Athero-miRs

Sandeep Kumar,* Chan Woo Kim,* Rachel D. Simmons, Hanjoong Jo

Abstract—Atherosclerosis preferentially occurs in arterial regions exposed to disturbed flow, in part, due to alterations in gene expression. MicroRNAs (miRNAs) are small, noncoding genes that post-transcriptionally regulate gene expression by targeting messenger RNA transcripts. Emerging evidence indicates that alteration of flow conditions regulate expression of miRNAs in endothelial cells both in vitro and in vivo. These flow-sensitive miRNAs, known as mechano-miRs, regulate endothelial gene expression and can regulate endothelial dysfunction and atherosclerosis. MiRNAs such as, miR-10a, miR-19a, miR-23b, miR-17–92, miR-21, miR-663, miR-92a, miR-143/145, miR–101, miR-126, miR-712, miR-205, and miR-155, have been identified as mechano-miRs. Many of these miRNAs were initially identified as flow sensitive in vitro and were later found to play a critical role in endothelial function and atherosclerosis in vivo through either gain-of-function or loss-of-function approaches. The key signaling pathways that are targeted by these mechano-miRs include the endothelial cell cycle, inflammation, apoptosis, and nitric oxide signaling. Furthermore, we have recently shown that the miR-712/205 family, which is upregulated by disturbed flow, contributes to endothelial inflammation and vascular hyperpermeability by targeting tissue inhibitor of metalloproteinase-3, which regulates metalloproteinases and a disintegrin and metalloproteinases. The mechano-miRs that are implicated in atherosclerosis are termed as mechanosensitive athero-miRs and are potential therapeutic targets to prevent or treat atherosclerosis. This review summarizes the current knowledge of mechanosensitive athero-miRs and their role in vascular biology and atherosclerosis. (Arterioscler Thromb Vasc Biol. 2014;34:2206-2216.)

Key Words: atherosclerosis ■ matrix metalloproteinases ■ microRNAs

Atherosclerosis is a chronic inflammatory disease of the vascular wall, which leads to cardiovascular pathologies such as myocardial infarction, ischemic stroke, and peripheral arterial disease. It is a leading cause of morbidity and mortality in developed countries.1–3 Despite the presence of systemic risk factors such as hypercholesterolemia, hypertension, and diabetes mellitus, atherosclerosis preferentially occurs in arterial regions exposed to disturbed flow (d-flow), whereas the arterial regions exposed to stable flow (s-flow) remain healthy.4–10 Recent studies have demonstrated that d-flow induces atherosclerosis in hypercholesterolemic conditions in mouse models.11 D-flow, which typically occurs in branched or curved arteries, is characterized by complex flow patterns with low-magnitude and oscillatory shear stress (OS), whereas s-flow is characterized by high-magnitude, unidirectional laminar shear stress (LS) in straight sections of the vasculature.

The mechanisms by which flow affects endothelial function and atherosclerosis have been reviewed previously4–10 and are also the subject of the accompanying reports in this Arteriosclerosis, Thrombosis, and Vascular Biology In Focus issue. Briefly, d-flow induces and s-flow prevents endothelial dysfunction and atherosclerosis, respectively, in part, due to alterations in gene expression and the epigenetic landscape.12–19 Vascular endothelial cells respond to blood flow through mechanosensors,20 which transduce the mechanical force associated with flow (also known as shear stress) into cell signaling events and, ultimately, changes in gene expression. D-flow promotes and s-flow suppresses atherogenesis, respectively, in part, through differential regulation of proatherogenic and atheroprotective genes.12–19 S-flow upregulates atheroprotective genes such as Klf2,21 Klf4,22 and eNOS,23 whereas d-flow induces many proatherogenic genes. These include vascular cell adhesion molecule-1,11,15 bone morphogenetic protein-4,16 and matrix metalloproteinases,24 which mediate proangiogenic, proinflammatory, prothrombogenic, and proapoptotic responses.5

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Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>d-flow</td>
<td>disturbed flow</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<td>IL</td>
<td>interleukin</td>
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<td>LS</td>
<td>laminar shear stress</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<td>OS</td>
<td>oscillatory shear stress</td>
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<td>s-flow</td>
<td>stable flow</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<td>XRN1</td>
<td>exoribonuclease-1</td>
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Small noncoding RNAs, microRNAs (miRNAs), have been implicated in the regulation of gene expression primarily as post-transcriptional repressors.25 miRNAs interact with the 3′-untranslated region of specific target mRNAs in a sequence-specific manner, resulting in mRNA degradation or translational inhibition.26 It has been previously demonstrated that LS and OS differentially regulate the expression of miRNAs in endothelial cells. The majority of flow-sensitive miRNAs (mechano-miRs) has been identified and characterized using cultured endothelial cells that were subjected to LS or OS conditions. Of these, a select few were subsequently validated in vivo. Therefore, only a limited number of direct linkages to atherogenesis have been established. Here, we review and briefly summarize our current knowledge of mechano-miRs and their role in endothelial dysfunction and atherosclerosis.

Mechano-miRs and Their Effect on Endothelium and Atherosclerosis

Emerging evidence indicates that mechano-miRs are responsible for the overall flow-dependent control of endothelial dysfunction and atherosclerosis. Current literature divides mechano-miRs into 3 classes according to their putative proatherogenic and antiatherogenic effects: (1) antiatherogenic mechano-miRs, (2) proatherogenic mechano-miRs, and (3) dual-role mechano-miRs (Table 1). Antiatherogenic mechano-miRs include miR-10a, 19a, 23b, 101, 143, and 145, all of which are either upregulated by s-flow/LS or are downregulated by d-flow/OS. Proatherogenic mechano-miRs include miR-17–92, 92a, 663, 712, and 205, all of which are either upregulated by d-flow/OS or are downregulated by s-flow/LS. Dual-role mechano-miRs include miR-21, 155, and 126. Dual-role mechano-miRs have been implicated in either proatherogenic or antiatherogenic events, but with inconsistent flow sensitivity. Table 1 also shows the flow/shear-dependent responses of each mechano-miR, the experimental system in which the miRNA was studied, its validated target genes, and its role in endothelial dysfunction and atherosclerosis. Figure shows a short summary of the role of mechanosensitive miRNAs tested in atherosclerosis in vivo.

Chien and colleagues were the first to report mechano-miRs (miR-19a and 23b) in cultured endothelial cells in vitro, whereas Davies and colleagues reported miR-10a as the first mechano-miR identified directly from the endothelium in vivo. Although the majority of mechano-miRs have been identified using endothelial cells in vitro, it is important to validate them in vivo because numerous mechanosensitive genes identified in vivo are known to be either dysregulated or lost during endothelial cell culture.15,27 Currently, only a few studies have examined miRNA expression profiles in vivo, in part, due to technical difficulties collecting endothelial-specific RNAs in these studies.27,28

Although most of the mechanosensitive miRNAs have been identified from in vitro studies using cone-and-plate viscometers or flow chambers, miR-712 and miR-10a are 2 mechanosensitive miRNAs that have been identified directly from in vivo studies.27,28 The expression of cells in vivo is intricately regulated by intracellular signaling components, paracrine factors from neighboring cells, and circulating humoral factors. Although cells may continue to survive and expand in vitro, the gene and miRNA expression profiles are altered during the process of adaptation from the in vivo environment to the culture dish. This becomes evident on careful review of various reports comparing the gene expression of newly extracted cells to cells expanded ex vivo, including hematopoietic progenitor cells, adipocytes, neutrophils, natural killer cells, and other hematopoietic cells.29–38 This phenomenon has been observed in endothelial cells as well; however, there are limited studies comparing the gene and miRNA expression changes between newly isolated endothelial cells and established endothelial cell lines.15,27,39 To study the effect of shear stress on gene expression changes in endothelial cells, it is extremely important to correlate the in vitro and in vivo gene and miRNA expression changes. Therefore, methodologies to directly isolate endothelial cells (ECs) from arterial regions exposed to d-flow or s-flow provide direct insight into the mechanosensitivity of a particular gene or miRNA. Whether these mechanosensitive genes or miRNAs retain or lose their response to shear stress after adapting to in vitro culture and how the sensitivity is lost or dysregulated is important to validate whether in vitro findings are relevant to that of in vivo.

Antiatherogenic Mechano-miRs

These miRNAs were either increased by s-flow/LS or decreased by d-flow/OS in endothelial cells and were shown to be antiatherogenic (Table 1).

miR-10a

MiR-10a expression in the endothelium is decreased by d-flow in the atheroproner, lesser curvature region of the porcine aortic arch when compared with that of the atheroprotected, thoracic aorta.28 Mechanistically, miR-10a is an anti-inflammatory miRNA that inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation by targeting MAP3K7 and JTRC, both of which promote 1kB degradation and p65 translocation.28

miR-19a and miR-23b

Initial miRNA expression analysis studies used cultured human umbilical vein endothelial cells (HUVECs) to determine the flow sensitivity of miRNAs.40 After 12 hours of LS...
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<td>NT</td>
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(Continued)
(12 dyn/cm²), 35 miRs were upregulated and 26 miRs were downregulated when compared to the static control cells. Among these, LS increased expression of miR-19a, which targets cyclin D1, thereby inducing endothelial quiescence.40 Later studies identified 8 upregulated and 13 downregulated miRNAs in response to 24 hours of pulsatile LS (12±4 dyn/cm²) when compared to the static no-flow condition.41 One of the upregulated miRNAs was miR-23b, which suppressed endothelial proliferation by reducing E2F1 expression and Rb phosphorylation.41

miR-101 and miR-143/145

MiR-101 is upregulated in response to LS and was reported to target the mammalian target of rapamycin gene, thus

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AA indicates aortic arch; δ-TRC, δ-transducin repeat-containing gene; BCL2, B-cell lymphoma 2; BCL6, B-cell lymphoma 6; CAMK2δ, calcium/calmodulin-dependent protein kinase type II delta chain; d-flow, disturbed flow; Dik1, delta-like 1 homolog; EC, endothelial cell; FL1, Follicular lymphoma, susceptibility to 1; FOXO3, Forkhead box 03; IRS1, insulin receptor substrate 1; ITGAS, integrin alpha 5; KLF2 and 4, Krüppel-like factor 2 and 4; LS, laminar shear stress; MAP3K7, mitogen-activated protein kinase kinase 7; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; MYLK, myosin light chain kinase; NAV2, neuron navigator; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NT, not tested; OS, oscillatory shear stress; PHACTR4, phosphatase and actin regulator 4; PPARα, peroxisome proliferator-activated receptor alpha; PTEN, phosphatase and tensin homolog; Rb, retinoblastoma protein; SIRT1, Sirtuin 1 (Silent mating type Information Regulation 2); SLCL7AS5, solute carrier family 7 member 5; SMC, smooth muscle cell; SOCS-1, suppressor of cytokine signaling 1; SSH2, slingshot homolog 2; and Timp3, tissue inhibitor of metalloproteinase 3.

Figure. Role of mechanosensitive microRNAs (miRNAs) in atherosclerosis. The role of the following mechano-miRs in atherosclerosis has been tested in vivo. Expression of miR143/145 is increased under laminar shear stress condition in a Krüppel-like factor (KLF) 2-dependent pathway. Endothelial-derived miR143/145 is transported to the medial smooth muscle cells (SMCs) in extracellular vesicles. This vesicle-mediated delivery of miR143/145 has an antiatherogenic effect by preventing SMC dedifferentiation. Low shear stress upregulates miR-92a and induces endothelial inflammation and atherosclerosis through upregulation of delta-like 1 homolog. Expression of miR-10a was lower in the atherosusceptible regions of the inner aortic arch when compared with other regions in the porcine arterial system. miR-10a induces a proinflammatory endothelial phenotype by targeting mitogen-activated protein kinase kinase 7 and δ-transducin repeat-containing gene. D-flow induces expression of miR-712 in an exoribonuclease-1 (XRN1)-dependent manner. D-flow also induces expression of miR-205. Both miR-712 and miR-205 target tissue inhibitor of metalloproteinase 3 (TIMP3). Loss of TIMP3 leads to the activation of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs), which result in endothelial inflammation and hyperpermeability and, ultimately, atherosclerosis. miR-712 and miR-205 were also increased in circulation and were also increased in the medial SMCs and circulating immune cells where they affected SMC migration and leukocyte–EC interactions, respectively, further contributing to atherosclerosis. Systemic treatment with anti-miR-712 prevented atherosclerosis in murine models of atherosclerosis. CAMK2δ indicates calcium/calmodulin-dependent protein kinase II delta; d-flow, disturbed blood flow; ECM, extracellular matrix; ELK, ETS domain-containing protein; EV, extracellular vesicle; miR, microRNA; MO, macrophage; PHACTR4, phosphatase and actin regulator 4; s-flow, stable flow; SSH2, slingshot protein phosphatase 2; and VSMC, vascular smooth muscle cell.
inducing cell cycle arrest. MiR-143/145 levels were increased by LS in an AMPKε2-dependent manner. A subsequent study showed that LS increased the expression of miR-143/145 by a KLF2-dependent pathway. Furthermore, it was demonstrated that endothelial-derived miR-143/145 can be transferred to medial smooth muscle cells (SMCs) via extracellular vesicles and prevent atherogenesis by preventing SMC dedifferentiation.

**Proatherogenic Mechano-miRs**

These miRNAs were either induced by d-flow/OS or decreased by s-flow/LS in endothelial cells and were shown to induce endothelial dysfunction and proatherogenic responses.

**miR-17–92 and miR-92a**

The miR-17–92a cluster comprises several miRs, including miR-17, 18a, 19a 19b, 20a, and 92a. The miR-17–92 cluster is regulated by shear stress in that some members (miR-17, miR-19b, miR-20a, and miR-92a) were downregulated by 24 hours of pulsatile LS. Subsequent studies showed that miR-92a was downregulated by LS and was upregulated by OS. These in vitro findings are consistent with in vivo studies showing that ECs in the atherosusceptible porcine aortic arch have increased miR-92a levels when compared with those of the atheroresistant thoracic aorta. Further studies demonstrated that miR-92a induced endothelial inflammation by targeting KLF2 and KLF4.

**miR-663**

From a miRNA array study using HUVECs exposed to 24 hours of LS or OS, miR-663 was identified as one of the most highly upregulated mechano-miRs in OS conditions. Furthermore, miR-663 overexpression induced endothelial inflammation, thus suggesting its potential proatherogenic role. Consistent with this report, miR-663 was upregulated in HUVECs exposed to proatherogenic oxidized phospholipids and was found to play a permissive role in the induction of VEGF (vascular endothelial growth factor) and activation of ATF4 (activating transcription factor 4) branch of unfolded protein response. Interestingly, miR-663 in vascular SMCs induced a contractile phenotype and forced overexpression of this human-specific mechnano-miR with an adenaloviral construct reduced neointimal formation in mice with complete carotid ligation. This shows that miR-663 may have cell type-dependent responses which ultimately determine its role in atherosclerosis.

**miR-712 and miR-205 Family**

Recently, 2 mechnano-miRs, miR-712 and miR-205, were identified from a mouse model of flow-induced atherosclerosis, known as the partial carotid ligation model. In this model, 3 out of 4 of the caudal branches of the left common carotid artery are ligated, thus inducing d-flow along the entire length of the straight section of the artery. As the contralateral right common carotid artery is left undisturbed, it continues to be exposed to s-flow. This model directly demonstrates the causal relationship between d-flow and atherosclerosis, as the left common carotid artery develops atherosclerosis within 2 weeks of surgery, while the right common carotid artery remains plaque-free. Additionally, by carefully extracting endothelial-enriched RNA from mouse carotid arteries after ligation, we were able to identify >500 mechnanosensitive genes. Using the same mouse model, 2 mechnano-miRs, miR-712 and 205, were identified and their function was established in vivo in 2 different animal models of atherosclerosis. We found that d-flow induced expression of miR-712, and that it contributed to endothelial dysfunction, and thus atherosclerosis. Additionally, treatment with anti-miR-712 prevented plaque development. The proinflammatory and proatherogenic effects of miR-712 were mediated by its target, tissue inhibitor of metalloproteinase-3. Loss of tissue inhibitor of metalloproteinase-3 by miR-712 resulted in activation of matrix metalloproteinases and a disintegrin and metalloproteinases, ultimately leading to endothelial inflammation, hyperpermeability, and atherosclerosis. Interestingly, miR-712 is murine-specific, and miR-205 is a homolog in human and other vertebrates. MiR-205 (1) shares the same seed sequence with miR-712, (2) its expression is upregulated by d-flow, and (3) it also targets tissue inhibitor of metalloproteinase-3 in a flow-dependent manner.

**Mechano-miRs With Dual Role in Atherosclerosis**

Some mechnano-miRs, such as miR-21, 126, and 155, have been implicated in both anti- and proatherogenic responses. This may reflect the fact that a single miRNA can target numerous target miRNAs, some of which mediate proatherogenic responses while others act in an opposite manner. The overall response of the cell depends on cellular context, cell type, or environment.

**miR-21**

miR-21 was upregulated by LS in HUVECs and prevented apoptosis by targeting phosphatase and tensin homolog. However, another study showed that OS upregulated miR-21 in a time-dependent manner inhibited peroxisome-proliferator-activated receptor-α, thus leading to the enhanced expression of the proinflammatory vascular cell adhesion molecule-1. It is interesting that miR-21 was upregulated by both LS and OS when compared to static conditions, suggesting that the mechnanosensitivity of miR-21 is a transient adaptive response to imposed shear. Interestingly, miR-21 was increased in arterial endothelium exposed to d-flow when compared with that of s-flow in the mouse partial carotid ligation model. Whether miR-21 plays a pro- or antiatherogenic role still remains to be determined.

**miR-126**

The effect of flow on the expression of miR-126 and its role in atherosclerosis is also currently confusing. MiR-126 (also referred to as miR-126-3p) and miR-126* (also known as miR-126-5p) are highly expressed in ECs, and have been shown to regulate vascular integrity and angiogenesis as well as inflammation. The secretion of miR-126-3p into conditioned media, but not its intracellular expression per se, was decreased by LS and increased by OS, respectively, in HUVEC. Endothelial-derived miR-126-3p regulated SMC turnover in an EC–SMC coculture system. Furthermore, genetic knockout of miR-126 inhibited neointimal formation in a complete carotid ligation model, whereas local reintroduction of miR-126 in the knock-out mice enhanced neointimal formation. This suggests that
miR-126 is a proatherogenic miRNA. By contrast, a recent study showed that d-flow decreased expression of both miR-126-5p and 126-3p; however, treatment with miR-126-5p mimic, and not miR-126-3p, reduced atherosclerotic lesion formation. The antiatherogenic effect of miR-126-5p was mediated by targeting the Notch1 inhibitor, delta-like 1 homolog, which promoted the proliferative potential of endothelial cells.60 Given the well-known effect of d-flow on increased endothelial proliferation in atheropron regions, however, the underlying mechanisms by which miR-126-5p inhibited atherosclerosis remain to be seen. Another interesting report from the same group showed that miR-126-3p present in apoptotic bodies had an atheroprotective effect in a murine carotid cuff model of atherosclerosis.57 Together, these results suggest that the role of miR-126-5p and miR-126-3p still needs further clarification.

miR-155
miR-155 expression is increased by LS in HUVECs and is abundantly expressed in the intima of the thoracic aorta, which is naturally exposed to s-flow in vivo, thus suggesting it is an antiatherogenic mechano-miR.58 Consistent with this idea, hematopoietic deficiency of miR-155 induced atherosclerosis and decreased plaque stability by increasing myeloid inflammatory cell recruitment to the plaque regions.59 However, other studies showed evidence suggesting that miR-155 mediates proatherogenic responses.60,61 MiR-155 was shown to directly target eNOS mRNA in HUVECs and impair endothelium-dependent vascular relaxation in human arteries.62 Leukocyte-specific miR-155 deficiency reduced plaque size and lesional macrophage count in the partial carotid ligation model of atherosclerosis.60 Another study showed that genetic knockdown of miR-155 ameliorated atherosclerosis in ApoE−/− mice by reducing inflammatory responses of macrophages and increasing macrophage cholesterol efflux.61 Also, tissue-specific genetic knockdown of miR-155 in bone marrow-derived cells suppressed atherosclerosis in ApoE−/− mice,61 demonstrating its role as proatherogenic miRNA.

Role of Nonmechanosensitive Athero-miRs
From early endothelial dysfunction to fully developed atherosclerosis, there are multiple cell types in the vessel wall that play a critical role in the development and progression of atherosclerosis. These include endothelial cells, medial SMCs, adventitial fibroblasts and adipocytes, platelets, and immune cells. Additionally, lipid metabolism, which regulates the circulating levels of triglycerides, cholesterol, and oxidized low-density lipoprotein, also affects the process of atherogenesis. miRNAs have been shown to regulate vascular inflammation and atherosclerosis by affecting various cell types in the vasculature. Several studies have recently shown the importance of specific miRNAs in atherosclerosis that are not necessarily flow sensitive (nonmechanosensitive athero-miRs). These nonmechanosensitive miRNAs can be divided into 2 classes according to their putative pro- or antiatherogenic roles: (1) antiathero-miRs and (2) proathero-miRs (Table 2).

Antiathero-miRs
The following miRNAs have been experimentally validated as antiatherogenic miRNAs through in vitro and in vivo studies.

**miR-1, 146a, 181b, and 195**
Inhibition of miR-1 using antago-miR enhanced the endothelial hyperpermeability effect seen under hypercholesterolemic conditions and overexpression using miR-1 mimic prevented endothelial barrier dysfunction.63 MiR-146 negatively regulates inflammation in that overexpression of miR-146a blunts endothelial activation, whereas in vitro inhibition of miR-146a/b or deletion of miR-146a in mice aggravates endothelial inflammation.64 Mechanistically, miR-146 represses the NFκB and MAP (mitogen-activated protein) kinase pathways through downregulation of human antigen R, which promotes endothelial activation in the vessel wall.64 Similarly, systemic delivery of miR-181b reduced atherosclerosis through inhibition its target gene, importin-α3, which mediates NFκB nuclear translocation specifically in the vascular endothelium.55,66 Also, adenoviral delivery of miR-195 reduced neo-intimal formation in a balloon-injury carotid artery model by inhibiting vascular smooth muscle cell proliferation, migration, and interleukin (IL)-1β, IL-6, and IL-8 synthesis.67

**miR-30c, 144, and 467b**
These 3 miRs play an important role in the regulation of plasma cholesterol level, a critical risk factor of atherosclerosis. Overexpression of miR-30c in the liver reduced the hyperlipidemia seen in Western diet-fed mice by decreasing lipid synthesis and the secretion of triglyceride-rich ApoB-containing lipoproteins, which in turn prevented atherosclerosis in ApoE−/− mice.68 Also, miR-467b reduces plasma cholesterol and atherosclerosis. Specifically, miR-467b inhibition by using antago-miR-467 enhanced the progression of atherosclerosis by increasing lipid accumulation and inflammatory cytokine secretion.69 Additionally, miR-144 regulates hepatic expression of the ABCA1 protein, thereby high-density lipoprotein cholesterol as well, but its contribution to the development of atherosclerosis is unknown.70

Proathero-miRs
**miR-342**
Macrophage-derived miR-342-5p increases atherosclerosis and inflammatory stimulation of macrophages through inhibition of Akt1.71 Suppression of Akt1 by miR-342-5p indirectly induces Nos2 and IL-6 in macrophages through miR-155. Therefore, systemic miR-342-5p inhibition reduced atherosclerotic plaque development in the aorta of ApoE−/− mice.

**miR-33**
MiR-33 plays a critical role in the suppression of inflammation and atherosclerosis. Multiple studies show that silencing of miR-33 prevents inflammation and atherosclerosis.72–75 Anti-miR-33 treatment or genetic knockout of miR-33 in mice promotes reverse cholesterol transport and inhibition of atherosclerosis through regulation of ABCA1.72,74 Also, anti-miR-33 increased hepatic expression of ABCA1 and induces a sustained increase in plasma high-density lipoprotein in a nonhuman primate model,73 thus validating its antiatherogenic role. However, a recent report showed that long-term silencing of miR-33 with anti-miR-33 failed to demonstrate the antiatherogenic effects...
The inconsistent responses reported in these studies may reflect different target genes and roles of miR-33 in various cells and tissue types. Although miR-33 may control its major gene target ABCA1 in some cell types such as hepatocytes, it may regulate other less well-characterized target genes or off-targets in other cell types leading to complex responses in vivo. Thus, these observations raise an important concern.

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**Table 2.** Nonmechanosensitive Athero-miRs That Induce Inflammation and Atherosclerosis

**Notes:**
- ABCA1 indicates ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette sub-family G member 1; CCND1, cyclin D1; CDC42, cell division control protein 42 homolog; CPT1A, carnitine palmitoyltransferase 1A; CROT, carnitine O-octanoyltransferase; FGF1, fibroblast growth factor 1; HADHB, hydroxyacyl-CoA dehydrogenase/3-ketobacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit; HDL, high-density lipoprotein; HuR, human antigen R; LDL, low-density lipoprotein; LPL, lipoprotein lipase; MAP, mitogen-activated protein; MLCK, myosin light-chain kinase; MTP, microsomal triacylglycerol transfer protein; NF<sub>x</sub>B, nuclear factor kappa-light-chain-enhancer of activated B cells; NOS2, nitric oxide synthase 2; NT, not tested; and VLDL, very-low-density lipoprotein.
regarding the off-target effects of systemically delivered miRNAs, anti-miRs, or antago-miRs in animal studies and clinical use.

**Circulating miRNAs and Their Transport via Vesicle-Dependent and Independent Pathways**

miRNAs serve as messengers between the ECs and SMCs and are critical for homeostasis and maintenance of the vasculature. Alterations in EC–SMC communication have been implicated in the pathogenesis of atherosclerosis as well as in the formation of aneurysms. While knowledge of circulating miRNAs in cardiovascular disease is emerging, fundamental questions still remain as to which cells produced them by which mechanisms and how they act on the ECs and SMCs as well as other cell types. In a previous report, extracellular vesicles enriched in miR-143/145 are secreted in response to overexpression of the mechanosensitive transcription factor KLF2 in HUVECs. It was also shown that endothelial-derived miR-143/145 can be transferred to SMCs via extracellular vesicles and prevent atherogenesis by preventing SMC dedifferentiation.44 In another study, miR-126 was shown to be secreted by ECs, and transported via RNA and RNA-protein complexes, independently of vesicular or DNA components. This was followed by uptake by cocultured SMCs, which promoted SMC proliferation, cell cycle progression, and apoptosis.55 Exposure of endothelial cells to LS inhibits miR-126 secretion, which in turn abrogates the atherogenic actions of miR-126 on the cocultured SMCs. However, the mechanisms that control the specific loading of miRNA species into extracellular vesicles (exosomes) remain unknown. Recently, the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) was identified as a miRNA sorting protein that specifically binds exosomal miRNAs through the recognition of specific motifs and controls their loading into the vesicles.77 We recently reported that miR-712 and miR-205 are increased in medial SMCs and circulating immune cells, which serve to regulate SMC migration and leukocyte–EC interaction, respectively.78,79 but whether the miRNAs are transported via a vesicle-dependent or-independent mechanism is not yet clear. Nevertheless, these reports suggest that circulating miRNAs, regardless of the mechanism of cellular export, can regulate communication between endothelial cells and SMCs, which in turn can control the overall outcome on endothelial inflammation and atherosclerosis.

**Biogenesis of Mechano-miRs: Atypical Pathway**

Typically, miRNAs are generated as primary miRNA (pri-miRNAs) transcripts by RNA polymerase II from genes.78 These pri-miRNA transcripts are initially processed within the nucleus by the RNase III enzyme Drosha and its cofactor DGCR8 into small 60- to 100-nt-long hairpin premiRs.79–81 Pre-miRNAs are then exported into the cytosol where they are further processed into mature miRNAs by Dicer enzyme. Mature miRNAs are then incorporated into the miRNA-induced silencing complex where they bind to the complementary 3′-untranslated region of the respective target gene(s), resulting in mRNA decrease or translation inhibition.82 In addition to the aforementioned canonical biogenesis pathway, some atypical miRNAs, such as mirtrons and simtrons, can be generated by noncanonical pathways that do not require the microprocessor components Drosha and DGCR8. Mirtrons represent short introns of genes that form premiRNAs directly after splicing, thus bypassing Drosha, whereas simtrons are splicing machinery-independent mirtron-like miRNAs.83

Although most mechano-miRs are regulated by the canonical pathway, some miRNAs, such as miR-712 and miR-663, are regulated by atypical pathways. Recently, we found that the murine-specific miR-712 and the human-specific miR-663 are the most highly upregulated miRNAs in conditions of d-flow, as determined from 2 completely independent array studies.47 These intriguing results suggested that there may be a common mechanism between the 2 mechanosensitive miRNAs. We have found that both miRNAs are synthesized from an unexpected source, preribosomal RNA, derived from the RN45S genes. Human and mouse RN45S genes are present in ≥5 different chromosomes, each containing 30 to 40 repeats, and they are not officially annotated yet. A RN45S unit is polycistronic, coding for 3 rRNAs with 2 intervening internal transcribed spacer (ITS) sequences called ITS1 and ITS2 (18S rRNA—ITS1—5.8S rRNA—ITS2—28S rRNA). Through sequence alignments, we found that miR-712 is derived from the ITS2 region of the murine RN45S gene, whereas miR-663 is derived from the ITS1 region of the human RN45S gene.77 This is the first report that demonstrates that miRNAs can be derived from the spacer regions of the rRNAs. Interestingly, these spacer regions of ribosomal RNA contain species-specific nucleotide sequences that are used as phylogenetic markers. Typically, under normal conditions, these spacer regions are rapidly degraded by exoribonuclease-1 (XRN1), following the transcription of rRNAs. However, d-flow decreases the expression of XRN1, but not the canonical miRNA processors DGCR8/DROSHA, in both human and mouse endothelial cells.27 This indicates that there may be a common role of XRN1 in the expression of human miR-663 and murine miR-712. Knockdown of XRN1 significantly increased both miR-712 and miR-663 expression, whereas knockdown of DGCR8 did not affect their expression. These results indicate that miR-712 induction by d-flow is dependent on XRN1 but independent of the canonical miRNA processors. At present, it is not known whether XRN1 is directly or indirectly responsible for biogenesis of these atypical miRNAs and this needs to be determined. In addition to flow, miR-712 expression is also regulated by angiotensin-II, suggesting that miR-712 and possibly other ITS-derived miRNAs such as miR-663 can be regulated by nonmechanical, humoral stimuli as well.84 Because 2 of the most flow-sensitive mechano-miRs are produced from the ITS regions of the ribosomal RNA gene in a similar atypical mechanism, it would be interesting to determine whether there are more miRNAs generated in this manner and the general role of miRNAs processed in such a manner.

**Summary and Perspectives**

Here, we summarized the role of flow-sensitive miRNAs (mechano-miRs) in the regulation of endothelial function and atherosclerosis (athero-miRs). Taken together, these results demonstrate that mechano-miRs are crucial mediators...
of endothelial function and atherosclerosis. However, our knowledge of miRNAs, especially mechano-miRs and athero-miRs, their regulation, and their functions is still in its infancy. Further investigation is required to identify other RNAs and explore their biological roles, mechanisms, and potential therapeutic applications. There have been some promising results from recent phase II clinical trials indicating the safety, feasibility, and therapeutic potential of anti-miRs for the treatment of hepatitis. In animal studies, some anti-miRs targeting mechano-miRs, such as miR-712, miR-205, or miR-155, and athero-miRs such as miR-33 have demonstrated their potential as antiatherogenic therapies. This encourages the development of miRNA therapeutics for the treatment of atherosclerosis in humans. Despite these encouraging results, the development of miRNA therapeutics must overcome several major challenges. First, the current methods to inhibit miRNAs include anti-miR/antagomir and miR-sponge, both of which directly bind to miRNAs, thereby affecting all of their target genes (hundreds of genes) indiscriminately, potentially causing undesirable effects. Therefore, to minimize the potentially undesirable effects of miRNA inhibition, better strategies should be developed to deliver these inhibitors either specifically to cells of interest or to design more specific inhibitors that can specifically block a unique but desired mRNA–miRNA interaction without affecting the expression of off-target genes. Second, targeting ubiquitously expressed miRNAs will lead to unintended side effects in other cells or tissues. Therefore, the use of cell type–specific delivery strategies such as nanotechnology-based platforms should be explored. Finally, the biology of miRNAs is still emerging, and identification of specific target genes of miRNAs in each cell type and organs continues to be a major challenge in understanding the role of miRNAs. In summary, expression of miRNAs is robustly regulated by different flow conditions in endothelial cells. These mechano-miRs are critical regulators of vascular function and atherosclerosis, thereby serving as diagnostic and therapeutic targets of the vascular disease.

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Disclosures

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