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## MicroRNA regulation of atherosclerosis

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### Abstract

Atherosclerosis and its attendant clinical complications such as myocardial infarction, stroke, and peripheral artery disease, are the leading cause of morbidity and mortality in western societies. In response to biochemical and biomechanical stimuli, atherosclerotic lesion formation occurs from the participation of a range of cell types, inflammatory mediators, and shear stress. Over the past decade, microRNAs have emerged as evolutionarily conserved, non-coding small RNAs that serve as important regulators and “fine-tuners” of a range of pathophysiological cellular effects and molecular signaling pathways involved in atherosclerosis. Accumulating studies reveal the importance of miRNAs in regulating key signaling and lipid homeostasis pathways that alter the balance of atherosclerotic plaque progression and regression. In this review, we highlight current paradigms of microRNA-mediated effects in atherosclerosis progression and regression. We provide an update on the potential use of miRNAs diagnostically for detecting increasing severity of coronary disease and clinical events. Finally, we provide a perspective on therapeutic opportunities and challenges for miRNA delivery in the field.

### Keywords

MicroRNAs; atherosclerosis; vascular; lipoprotein

### Subject Terms

Atherosclerosis; Mechanisms; Pathophysiology; Vascular Biology

### Introduction

Atherosclerosis represents a chronic inflammatory disease of the arterial wall initiated by endothelial injury and subendothelial lipoprotein retention, particularly at sites of disturbed blood flow.<sup>1</sup> The pathogenesis of atherosclerotic lesion formation is a multistage process

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involving both immune and non-immune cellular constituents of the vessel wall. Research over the last 3 decades has uncovered key signaling and molecular regulatory pathways involved in the initiation and progression of atherosclerotic plaques. The recent emergence of microRNAs (miRNAs) as important regulators of pathophysiological processes such as cellular adhesion, proliferation, lipid uptake and efflux, and generation of inflammatory mediators has provided novel molecular insights into their impact on these pathways in atherosclerosis, and identified new therapeutic targets. In addition, the appreciation that miRNAs can be detected extracellularly including in circulating blood raises the potential for their use as biomarkers for diagnosis, prognosis, or in response to cardiovascular therapeutics. This review highlights the role of miRNAs implicated in regulating critical aspects of atherosclerotic lesion formation and regression. We also review the potential use of miRNAs diagnostically for detecting increasing severity of coronary disease and clinical events.

### Brief Primer on MicroRNAs

MiRNAs were first identified in *C. elegans* in 1993, however, their functional roles in human disease were not appreciated for nearly a decade later.<sup>2</sup> MiRNAs are evolutionarily conserved, small (average ~18–24 nucleotides), single-stranded noncoding RNAs that regulate gene expression at the post-transcriptional level by typically binding to the 3'-untranslated region (3'-UTR) of specific target mRNA sequences (utilizing a conserved ~7–8 nucleotide seed sequence), thereby leading to reduced protein expression by blocking mRNA translation and/or by promoting mRNA degradation.<sup>3–8</sup> It is estimated that >60% of all protein-coding genes are directly regulated by miRNAs.<sup>9</sup> Furthermore, a given miRNA may bind to and regulate more than one target, sometimes as part of the same signaling pathway. Conversely, a given mRNA may harbor several distinct miRNA binding sites within its 3'-UTR, adding multiple levels of regulation. As such, miRNAs are “fine-tuners” of gene expression patterns in response to pathophysiological stimuli.

Intergenic miRNAs are located within the genome between gene-coding regions and are transcribed by their own promoters. By contrast, intronic miRNAs are found within intronic sequences of protein-coding genes and often share the same upstream promoters and transcriptional regulation with their host genes. MiRNA biogenesis proceeds in response to a well-defined series of cellular events.<sup>2, 3, 5</sup> MiRNAs are transcribed primarily by RNA polymerase II (more rarely by polymerase III) to generate the primary miRNA transcript, termed pri-miRNAs that harbor a canonical hairpin structure including a 5' cap and 3' poly-A tail.<sup>4</sup> The pri-miRNAs are converted to ~70 nt-long hairpin precursor miRNAs (pre-miRNAs) by the Drosha/DGCR8 (Di George Syndrome Critical Region Gene 8) complex. Subsequently, the pre-miRNA is exported from the nucleus to the cytoplasm by Exportin 5 where it undergoes further processing by the RNase III enzyme complex Dicer/TRBP (TAR RNA-binding protein) producing a mature miRNA duplex.<sup>10</sup> This double-stranded product is composed of the mature miRNA guide strand and the miRNA passenger strand (miRNA-5p and miRNA-3p, respectively). The guide strand is typically incorporated in the RNA-induced silencing complex (RISC) that contains Argonaute2 (Ago2), which facilitates the miRNA binding to the target mRNA leading to mRNA degradation or translation inhibition.<sup>11</sup> Accumulating studies indicate that both miRNA-3p and miRNA-5p strands can

be loaded into the RISC complex to target mRNA expression. Finally, mature miRNAs (and pre-miRNAs) can be released from the cell and packaged into a range of microvesicles (ie. exosomes, apoptotic bodies, microparticles) that are detectable in peripheral circulation (sometimes in association with RNA-binding proteins such as Ago2) and may also be taken-up within tissues by cell-to-cell communication.

## MicroRNA regulation of lipoprotein homeostasis

Cholesterol homeostasis is essential for cellular physiology, and altered levels of cellular or systemic cholesterol are associated with metabolic diseases. In the circulation, cholesterol is carried on lipoproteins, which can both deliver [eg. low density lipoprotein (LDL)] and remove [eg. high density lipoprotein (HDL)] cholesterol from cells and tissues to mediate cholesterol homeostasis. Imbalances that favor the accumulation of cellular cholesterol, such as high levels of LDL cholesterol (LDLc) and/or low levels of HDL cholesterol (HDLc), promote atherosclerosis. The recent discoveries of miRNAs that control LDL and HDL abundance and function have greatly expanded our understanding of the regulatory circuits governing plasma lipoprotein levels.

The liver plays a major role in both the production and clearance of lipoproteins, and a number of hepatic-enriched miRNAs have been identified that functionally regulate lipoprotein metabolism. miR-122 was the first miRNA implicated in lipoprotein metabolism and its expression is highly enriched in the liver<sup>12</sup>. Loss-of-function experiments in both mice and non-human primates identified miR-122 as a crucial regulator of cholesterol and fatty acid synthesis, and thus lipoprotein homeostasis<sup>12, 13</sup>. Notably, miR-122 function appears to be broadly required for the expression of hepatocyte-specific genes, rather than the specific targeting of lipid metabolism pathways<sup>14</sup>. By contrast, miR-223 and miR-27b act as key post-transcriptional regulatory hubs controlling networks of cholesterol and lipoprotein metabolism genes<sup>15, 16</sup>. miR-223 represses genes involved in cholesterol biosynthesis (*HMGSI*, *SC4MOL*) and HDL uptake (*SRBI*), and *Mir223*<sup>-/-</sup> mice display increased HDL-C levels, as well as hepatic and plasma total cholesterol<sup>15</sup>. miR-27b is a cholesterol-responsive hepatic miRNA that represses a number of targets (*PPARG*, *GPAM*, *ANGPTL3* and *NDST1*) involved in lipid metabolism and lipoprotein remodeling<sup>16</sup>. In addition to these gene network-regulating miRNAs, miR-30c was shown to have a potent effect on the production of apoB-containing lipoproteins (VLDL, LDL). miR-30c targets the microsomal triglyceride transfer protein (*MTP*), a protein essential for the lipidation of nascent apoB, and also reduces de novo lipogenesis by targeting lysophosphatidylglycerol acyltransferase 1 (*LPGATI*)<sup>17</sup>. Lentiviral overexpression of miR-30c in mice reduces the assembly and secretion of apoB-lipoproteins<sup>17</sup>, leading to decreased levels of plasma total and LDL cholesterol. Furthermore, studies in apolipoprotein E (apoE)-deficient mice (*ApoE*<sup>-/-</sup>) mice showed that miR-30c overexpression mitigated hyperlipidemia and atherosclerosis without inducing steatosis<sup>17</sup>, an undesirable side effect associated with conventional MTP inhibitors.

MicroRNA regulation of plasma levels of LDLc through targeting of the LDL receptor (LDLR) has also been reported. LDLR expression in the liver promotes the clearance of circulating LDL particles, and is a major determinant of plasma cholesterol levels. Two

recent studies identified miR-148a as a negative regulator of LDLR expression and activity, and showed that inhibition of miR-148a in mice could increase the clearance of circulating labeled LDL and decrease plasma LDLc levels<sup>18, 19</sup>. Notably, single-nucleotide polymorphisms (SNPs) in the promoter region of miR-148A are associated with altered LDLc in humans<sup>19</sup>, suggesting that altered expression of this miRNA may contribute to dyslipidemias. Indeed, analysis of genome-wide association study (GWAS) data identified 3 other miRNAs predicted to target the LDL-R (miR-128-1, miR-130b and miR-301b) that lie in close proximity to human SNPs associated with abnormal levels of plasma lipids<sup>19</sup>. Like miR-148a, inhibition of miR-128-1 with locked nucleic acid (LNA) anti-sense oligonucleotides increased hepatic LDLR expression and LDL clearance in mice<sup>19</sup>. In addition to LDLR, miR-148a and miR-128-1 also target additional genes involved in lipid and energy metabolism (miR-148a: ABCA1, AMPKa1, CPT1a and SIK1; miR-128-1: ABCA1, SIRT1 and IRS1)<sup>19</sup>, suggesting important roles for these miRNAs in regulating metabolic pathways.

miRNAs have also been identified to act as critical regulators of HDL biogenesis and cholesterol efflux. These pathways control levels of plasma HDLc and the reverse cholesterol transport pathway through which excess cholesterol is removed to the liver for excretion. The ATP-binding cassette transporter ABCA1 plays a central role in these processes by controlling cholesterol efflux across the cell membrane onto lipid-poor apoA1<sup>20</sup> to mediate both hepatic HDL biogenesis and the removal of excess cholesterol from peripheral cells, particularly M $\phi$  in atherosclerotic plaques. A number of miRNAs have been identified that target ABCA1 to reduce cholesterol efflux to apoA1 *in vitro*, including miR-33<sup>21–27</sup>, miR-758<sup>28</sup>, miR-26<sup>29</sup>, miR-106<sup>30</sup>, miR-144<sup>31, 32</sup>, as well as the above mentioned miR-128-1<sup>18</sup> and miR-148a<sup>19</sup>. Of these, inhibition of miR-33<sup>21–27</sup>, miR-144<sup>31, 32</sup>, miR-128-1<sup>18</sup> and miR-148a<sup>19</sup> has also been tested *in vivo* and shown to increase plasma levels of HDLc in mice or monkeys. Circulating levels of HDLc are also regulated by hepatic clearance via the scavenger receptor BI (SR-BI), which has been shown to be targeted by miR-223<sup>33</sup>, miR-455-5p<sup>34</sup>, miR-96<sup>33</sup>, miR-185<sup>33</sup>, and miR-125a<sup>34</sup>. However, only miR-223 has been manipulated *in vivo* and shown to impact plasma HDLc levels<sup>15</sup>.

Epidemiologic studies have shown an atheroprotective role for HDL, which is thought to be associated with its ability to mediate reverse cholesterol transport. Of the miRNAs targeting the HDL/reverse cholesterol transport pathway, the miR-33 family has been the most extensively studied *in vivo* using pre-clinical animal models. miR-33a and b are intronic miRNAs that are co-expressed with their host genes, *SREBF2* and *SREBF1*, which code for transcription factors that regulate cholesterol and fatty acid synthesis/uptake<sup>23–25</sup>. Thus, transcription of *SREBF2* and *SREBF1* also results in expression of miR-33a and b, which cooperate with their host genes to balance cellular lipid levels by repressing genes that oppose SREBP-regulated pathways, such those involved in cholesterol efflux (ABCA1, ABCG1)<sup>23–25</sup> and fatty acid oxidation (HADHB, CROT, CPT1A, PRKAA1)<sup>21, 28, 35</sup>. Inhibition of miR-33 using modified anti-sense or LNA oligonucleotides increased hepatic *ABCA1* expression and plasma HDLc levels in both mice and monkeys by 40–50%<sup>23–25, 27, 36, 37</sup>, and enhanced cholesterol transport from macrophages to the plasma,

liver and feces by up to 80%<sup>27</sup>. This increase in reverse cholesterol transport is likely the combined effects of derepression of ABCA1 and additional miR-33a/b targets such as *Abcb11* and *Atp8b1* that promote cholesterol excretion into bile<sup>38</sup>. Importantly, HDL isolated from monkeys treated with anti-miR-33 was shown to retain its anti-inflammatory properties, particularly its ability to promote macrophage cholesterol efflux and to protect endothelial cells from cytokine induced inflammation<sup>27</sup>.

Multiple studies of miR-33 inhibition or deletion have now been conducted in mouse models of atherosclerosis. Targeted deletion of *Mir33* increased plasma levels of HDL and reduced atherosclerotic plaque size in *ApoE*<sup>-/-</sup> mice<sup>39</sup>. Furthermore, treatment of *Ldlr*<sup>-/-</sup> mice with established atherosclerosis with a miR-33 inhibitor for 4 weeks, in conjunction with a chow diet, increased plasma levels of HDL-C and markedly regressed atherosclerosis<sup>27</sup>. Notably, this intervention study was designed to simulate a clinical scenario where plasma LDLc levels were lowered in conjunction with anti-miR-33 treatment. However, two subsequent studies evaluating the effects of miR-33 inhibitors in *Ldlr*<sup>-/-</sup> mice on a Western diet showed no effects on HDLc levels, presumably due to decreased hepatic expression of SREBF2/miR-33 under these conditions<sup>40, 41</sup>. Despite this, Rottlan et al observed reductions in aortic atherosclerotic lesion area after 8 weeks of anti-miR-33 treatment<sup>40</sup>, suggesting that miR-33 inhibition may have atheroprotective effects beyond raising HDL. This paradox may be explained by a recent report that miR-33 can also instruct macrophage inflammatory polarization by altering the balance of cellular aerobic glycolysis and mitochondrial oxidative phosphorylation<sup>42</sup>. Targeted deletion or inhibition of miR-33 in macrophages increases oxidative respiration - a metabolic program characteristic of alternatively activated macrophages (AAM) - and induces the expression of genes that define AAM or M2 macrophage polarization. Notably, inhibition of miR-33 also increased macrophage expression of the retinoic acid-producing enzyme *Aldh1a2* and retinal dehydrogenase activity both *in vitro* and *in vivo*. Retinoic acid can foster the differentiation of atheroprotective regulatory T cells, and consistent with this, *Ldlr*<sup>-/-</sup> mice treated with miR-33 inhibitors for 8 weeks on western diet showed the accumulation of M2 macrophages and FoxP3+ T regulatory cells in plaques, and reduced atherosclerosis progression in the absence of changes of plasma HDL<sup>42</sup>. This study suggests that antagonism of miR-33 is atheroprotective, in part, by promoting M2 macrophage and regulatory T cell polarization to suppress plaque inflammation.

Despite the beneficial effects of miR-33 inhibition on plasma levels of HDLc and atherosclerosis, some studies of genetic deletion<sup>43</sup> or antagonism of miR-33<sup>44</sup> in conjunction with western diet (WD) feeding have reported an increase in circulating triglycerides and hepatosteatosis. Not all studies have observed these effects of miR-33 inhibitors in mice<sup>45</sup>, and increases in triglycerides or hepatic lipid levels have not been reported in studies of non-human primates treated with anti-miR33 for up to 16 wks<sup>(26, 37)</sup>. These conflicting findings suggest that more studies of miR-33 are warranted, particularly to understand if effects on triglycerides and hepatosteatosis are species specific or due to potential differences of chemical modifications, delivery, or time of administration of anti-miR-33 oligonucleotides. Nonetheless, these studies highlight the potential utility of miRNA mimics and inhibitors in the treatment of dyslipidemias (Figure 1).

## MicroRNA regulation of endothelial cell inflammation and plaque progression

Sustained hyperlipidemia and altered shear stress predispose the vessel wall to atherosclerotic lesion formation. In response to biochemical and biomechanical stimuli, endothelial cells undergo a series of molecular and cellular conformational changes that promote atherogenesis. For example, early induction of adhesion molecule expression such as vascular adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, and E-selectin facilitates leukocyte recruitment to the vessel wall and may be among the earliest hallmarks associated with nascent plaques.<sup>46</sup> Several miRNAs have been implicated in atherogenesis by virtue of their ability to directly target the 3'-UTRs of these molecules such as miR-17-3p (targets ICAM-1) and miR-31 (targets E-selectin).<sup>47</sup> However, the functional role of both these miRNAs in experimental atherosclerosis remains unknown. Nuclear Factor (NF)- $\kappa$ B signaling is a major pathway that activates not only these pro-adhesive molecules, but also a range of other pro-inflammatory and pro-thrombotic factors. MiR-181b and miR-146a are two cytokine-responsive miRNAs that regulate distinct components of NF- $\kappa$ B signaling and are atheroprotective<sup>48</sup> (Figure 2).

### Cytokine-responsive miRNAs that regulate atherosclerosis

#### MiR-181b

Accumulating studies in mice and human subjects highlight a critical role for miR-181b as an inhibitor of endothelial inflammatory responses by targeting NF- $\kappa$ B signaling in both acute (e.g. sepsis) and chronic (e.g. atherosclerosis) vascular disease states.<sup>49, 50</sup> MiR-181b, an intergenic miRNA,<sup>51</sup> inhibits NF- $\kappa$ B signaling uniquely in endothelial cells but not leukocytes by targeting importin- $\alpha$ 3, a protein important for NF- $\kappa$ B cytoplasmic-nuclear translocation. In leukocytes, the dominant isoform that mediates NF- $\kappa$ B nuclear import is importin- $\alpha$ 5, which miR-181b does not target thereby leaving leukocyte NF- $\kappa$ B signaling intact. Nonetheless, upon systemic intravenous delivery, miR-181b decreased endothelial NF- $\kappa$ B activation, an effect that was sufficient to markedly suppress leukocyte recruitment and atherosclerotic lesion formation in atherosclerotic-prone ApoE<sup>-/-</sup> mice. Importantly, the miR-181b anti-atherosclerotic effects were independent of any changes in lipid profiles. These findings are consistent with endothelial-specific NF- $\kappa$ B (IKK $\gamma$ ) knockout and dominant-negative I $\kappa$ B $\alpha$  transgenic mice that also exhibit protection against atherosclerotic lesion formation.<sup>52</sup> A range of pro-inflammatory stimuli (e.g. TNF- $\alpha$ , LPS) reduce miR-181b expression in endothelial cells in vitro. In vivo, miR-181b expression is reduced by 53% in the vascular endothelium and by ~40% in plasma of mice after just 4 weeks of high cholesterol diet in ApoE<sup>-/-</sup> mice, suggesting that loss of homeostatic control of miR-181b expression may predispose to inflammation in the vessel wall. In line with these observations, miR-181b expression is also reduced in plasma of human subjects with angiographically defined coronary artery disease (CAD) compared to those without CAD.<sup>50</sup> Therapeutically, the cell-specific miR-181b inhibitory effects on NF- $\kappa$ B in the vascular endothelium, and not in myeloid cells, may be advantageous in order to maintain optimal protection in response to infectious pathogens. Indeed, miR-181b delivery also inhibits endothelial inflammation and confers protection against sepsis in mice.<sup>49</sup> Collectively, these

findings provide cogent evidence that miR-181b serves as an important regulator of NF- $\kappa$ B signaling in the vascular endothelium in response to diverse stimuli, and provide new opportunities for anti-inflammatory “replacement” therapy.

### **MiR-146a**

MiR-146a is another cytokine-responsive miRNAs that confers atheroprotective properties in the vessel wall. Cytokines such as TNF- $\alpha$  and IL-1 $\beta$  induced expression of miR-146a and miR-146b in a delayed kinetic manner in ECs that coincided with the resolution of inflammatory gene expression.<sup>53</sup> MiR-146a overexpression inhibited cytokine responsiveness in ECs suggesting it may participate in a negative feedback mechanism to limit EC inflammatory signaling. Interestingly, miR-146a expression is also increased in human and mouse atherosclerotic plaques.<sup>54</sup> Indeed, miR-146a repressed both NF- $\kappa$ B and MAPK signaling pathways by directly targeting HuR, an RNA-binding protein that exerts inhibitory effects on endothelial nitric oxide synthase (eNOS). In addition, miR-146a represses the induction of EC adhesion molecules by targeting upstream adaptor proteins TRAF6 and IRAK1/2.<sup>53</sup> In contrast to the more selective inhibitory role of miR-181b on EC NF- $\kappa$ B signaling, miR-146a inhibits NF- $\kappa$ B signaling in both ECs and macrophages.<sup>55</sup> Indeed, overexpression of ApoE in ApoE<sup>-/-</sup> macrophages induced miR-146a expression to reduce macrophage and systemic delivery of miR-146a mimics markedly reduced atherosclerotic lesion progression. The specific cell subsets (e.g. endothelial cells or leukocytes) and mechanisms (e.g. NF- $\kappa$ B-dependent or -independent) by which miR-146a confers these favorable effects in experimental atherosclerosis will require future study. However, given the anti-inflammatory role of miR-146a in regulating a range of immune cells (macrophages, dendritic cells, and T cells,<sup>56</sup> it may participate more broadly to limit inflammatory stimuli. Nevertheless, miR-146 is another important cytokine-responsive miRNA that may serve to dampen EC inflammation in a negative feedback manner.

### **Mechano-sensitive EC miRNAs that regulate atherosclerosis**

Atherosclerotic lesions preferentially develop at arterial branch points, bifurcations, and the lesser curvature of the aorta in mice and human subjects.<sup>57-59</sup> These findings are explained, in part, by the presence of disturbed flow in these regions which increases endothelial permeability (e.g. by altering the expression and localization of intracellular junctional proteins) and proinflammatory signaling (e.g. NF- $\kappa$ B activation, adhesion molecule expression), thereby promoting leukocyte accumulation and lesion formation.<sup>60-63</sup> In contrast, laminar flow confers an anti-inflammatory, anti-adhesive, and anti-thrombotic properties in the vessel wall. A surprisingly large number of miRNAs have been identified as shear stress-responsive by either disturbed flow (d-flow) or laminar flow (L-flow).

### **miR-92**

MiR-92a, a member of the miR-17~92 cluster, is expressed highly in endothelial cells and dynamically regulated by shear stress both in vitro and in vivo.<sup>64, 65</sup> Upon exposure of ECs to pulsatile L-flow in vitro, miR-92a expression is reduced, whereas D-flow increases its expression; in addition, miR-92a bound to the 3'-UTR of KLF2, a flow-responsive transcription factor, suggesting a direct role for regulating laminar flow.<sup>64</sup> Similarly, in vivo

miR-92a expression is highly induced in athero-prone areas of the aortic arch compared to athero-resistant regions.<sup>66</sup> In vitro studies suggest that miR-92a overexpression suppresses the 3'-UTRs of KLF2 and KLF4. In contrast, miR-92a-mediated inhibition of TNF- $\alpha$ -induced cytokines and leukocyte adhesion can be rescued, in part, by KLF4 siRNAs, indicating partial KLF4 dependency.<sup>66</sup> In a separate microarray profiling study, Loyer et al. identified that upon exposure of ECs to oxLDL and low shear stress, expression of miR-92a and pro-inflammatory markers (MCP-1 and IL-6) are increased in a STAT3-dependent manner.<sup>65</sup> Overexpression of miR-92a in ECs reduced expression of KLF2 and KLF4, flow-responsive transcription factors, whereas miR-92a antagonism reduced EC inflammation as reflected by lower phospho-p65 expression. Mechanistically, miR-92a also targets SOCS5 in ECs in the presence of oxLDL and low shear stress conditions.<sup>65</sup> While the in vivo role of SOCS5 in atherosclerosis remains unclear, siRNA-mediated knockdown of SOCS5 increased MCP-1 and IL-6 expression in ECs without affecting expression of eNOS, KLF2, or KLF4. In addition, neutralization of miR-92a in LDLR $^{-/-}$  mice decreased EC inflammation and suppressed the progression of atherosclerotic lesion formation. Moreover, mice harboring a genetic deletion of miR-92a in Tie2-expressing endothelial cells were protected from neointimal formation after mechanical arterial injury, attributed, in part, to enhanced re-endothelialization.<sup>68</sup> Given the protective role of miR-92a-deficiency in other processes including re-endothelialization after mechanical arterial wire injury<sup>67, 68</sup> and angiogenesis after myocardial or peripheral ischemia,<sup>69, 70</sup> targeting this miR-92a may hold promise for a range of cardiovascular relevant disease states.

### miR-126

miR-126 is among the most abundantly expressed miRNAs in ECs and has been implicated in regulating both inflammation and angiogenesis in a flow-dependent manner. MiR-126 was initially described in vitro to bind to the 3'-UTR of VCAM-1 to limit leukocyte adhesion.<sup>71</sup> Subsequent studies revealed that miR-126-deficient mice exhibit impaired vascular integrity and defects in EC proliferation and angiogenesis.<sup>72</sup> Indeed, miR-126 may be induced by KLF2 to control flow-dependent angiogenesis.<sup>73</sup> In the context of atherosclerosis, Zerneck A et al. demonstrated that miR-126 was the most abundant miRNA expressed in EC-derived apoptotic bodies where it induced CXCL12 expression by targeting RGS16, a negative regulator of SDF-1/CXCR4 signaling and progenitor cell mobilization.<sup>74</sup> Consistent with this pathway, intravenous delivery of endothelial apoptotic bodies mobilized progenitor cells in the circulation, and enhanced their incorporation into aortic plaques, an effect that suppressed atherosclerotic progression in a miR-126-dependent manner. Within the vascular endothelium, Schuber et al. elegantly identified a functional role for the passenger strand, miR-126-5p, as a flow-responsive miRNA that is suppressed by D-flow, an effect that facilitates lesion formation through induction of a negative regulator of EC proliferation termed delta-like 1 homolog (Dlk-1).<sup>75</sup> Systemic delivery of miR-126-5p mimics rescued EC proliferation at vulnerable sites and inhibited lesion progression.<sup>75</sup> Because the pro-inflammatory transcription factors Ets-1 and Ets-2 also induce miR-126 expression,<sup>76</sup> the miR-126 duplex may mechanistically serve to limit pro-inflammatory factors such as TNF- $\alpha$  and Ang-II in a negative feedback manner in the vascular endothelium. However, ECs exposed to L-flow induced KLF2-dependent expression of pri-miR-126 but not miR-126-3p. Moreover, miR-126-5p expression, but not



miR-126-3p expression, was lower at predilection sites of ApoE<sup>-/-</sup> aortas. In addition, delivery of miR-126-5p antagomiRs, but not miR-126-3p antagomiRs, increased lesion formation after EC denudation in ApoE<sup>-/-</sup> mice, suggesting that miR-126-3p may not be implicated in flow-responsive vascular protection. Finally, atheroprotective L-flow increases the release of miR-126 bound to Ago2 into extracellular microvesicles that serves as a mediator to increase VSMC turnover by targeting genes (e.g. FOXO3, BCL2, and IRS1) implicated in maintaining an atheroprotective VSMC contractile phenotype.<sup>77</sup> In this study, miR-126 deficiency inhibited neointimal formation of mouse carotid arteries induced by cessation of blood flow, an effect rescued by miR-126 mimics or by conditioned media from static EC monocultures. These latter findings raise the possibility that despite its atheroprotective in ECs, miR-126 may thin SMCs of the fibrous plaque leading to destabilization. Future long-term studies will be required to assess the relative role of the miR-126-5p/miR-126-3p duplex in response to a range of important biochemical and biomechanical stimuli and in different phases of atherosclerosis.

### **MiR-712/miR-205**

While miRNA biogenesis typically utilizes the canonical Drosha/DGCR8 and Dicer miRNA pathway, accumulating studies also highlight an important role for non-canonical pathways for miRNA maturation.<sup>78, 79</sup> miR-712 (human homologue miR-205) was identified as a d-flow responsive miRNA derived from non-canonical pre-ribosomal RNA (using the *RN45S* gene).<sup>80</sup> Functionally, d-flow-induced miR-712 targets TIMP3, an effect that increases MMPs and ADAMs to stimulate pro-inflammatory EC responses. Indeed, neutralization of miR-712 de-represses TIMP3 expression and ameliorates atherosclerotic progression in mice.<sup>80</sup> Future studies will be required to determine whether the *in vivo* neutralization of miR-712 directly affects other relevant cell types, such as immune subsets and vascular smooth muscle cells.

### **miR-143/miR-145**

An emerging paradigm in cell-to-cell communication is the ability of miRNAs to be transferred from one cell to another within tissues. For example, in response to L-flow or KLF2 overexpression in ECs, extracellular microvesicles containing miR-143 and miR-145 are released that confer atheroprotective properties in adjacent VSMCs. Intravenous delivery of these extracellular vesicles also blocked atherosclerotic lesion progression in a miR-143/145-dependent manner.<sup>81</sup> Interestingly, miR-143/miR-145, which are expressed higher in VSMCs compared to ECs under basal conditions *in vitro*, may also participate in VSMC to EC communication via intercellular tubes called tunneling nanotubes (TNTs) *in vitro*.<sup>82</sup> In support of this VSMC-to-EC passage, SMC-specific deletion of the miR-143/miR-145 cluster in mice effectively blocked the induction of miR-143/miR-145 expression in coronary artery endothelial cells in response to trans-aortic constriction-induced pressure overload, an effect that may be mediated by activation of the TGF- $\beta$  signaling pathway and the miR-143/miR-145 targets hexokinase II and integrin- $\beta$ 8.<sup>82</sup> Collectively, while it remains to be determined whether bi-directional extracellular miRNA passage occurs between SMC and EC under atherosclerotic conditions, these findings raise the provocative question of skewed, preferential cell-to-cell “miRNA shifts” that may emerge under atherosclerotic progression or regression, or in response to pharmacologic therapies.

**Others: miR-10a, miR-663, miR-155, miR-30-5p**

Several other mechano-sensitive microRNAs, such as miR-10a, miR-663, and miR-155, have been identified using a variety of profiling approaches. However, the functional role of these three miRNAs in regulating experimental atherosclerosis in mice has not yet been validated (miR-10a, miR-663) or remains unclear (miR-155). Nonetheless, they may figure prominently at predilection sites in the macrovasculature. For example, miRNA microarray profiling of ECs from the inner aortic arch of pig, an atherosusceptible region, and ECs from the descending thoracic aorta, miR-10a expression was identified as significantly reduced in the atherosusceptible regions.<sup>83</sup> Mechanistically, miR-10a targets the 3' UTRs of MAPK kinase kinase 7 and the  $\beta$ -transducin repeat-containing gene, two key regulators of I $\kappa$ B $\alpha$  degradation. Indeed, miR-10a overexpression inhibits canonical NF- $\kappa$ B signaling in ECs in vitro.<sup>83</sup> Therapeutic manipulation of miR-10a in atherosclerotic-prone mice will be required to assess its relative contribution to EC function at predilection sites and lesion pathogenesis. Because miR-10a's family member, miR-10b, which may also bind to similar consensus sites in target genes, has been implicated in promoting tumor invasion and metastasis, delivery of miR-10 mimetics may require close scrutiny for therapeutic gain. Another miRNA, miR-663, was identified by 2 groups as a D-flow-induced miRNA in ECs.<sup>80, 84</sup> Interestingly, human miR-663, like miR-712, may also be derived from the same ribosomal RNA gene, RN45S. Because the XRN1 exonuclease can rapidly degrade the spacer regions from where this rRNA is derived, Son et al examined whether silencing XRN1 may affect miR-663 and miR-712 expression. Indeed, D-flow reduced XRN1 expression in predilection regions in the mouse carotid and aortic arch, and XRN1 deficiency in ECs in vitro significantly increased both miR-663 and miR-712 expression, suggesting another level of regulation and therapeutic modulation of atypical mechano-sensitive miRNAs in the vascular endothelium.<sup>80</sup> Finally, miR-155 is increased by L-flow in ECs and in the thoracic aorta compared to the lower aortic arch.<sup>75</sup> Mechanistic studies indicate that miR-155 may bind to both anti-inflammatory targets (e.g. eNOS) and pro-inflammatory targets (e.g. MYLK, RhoA, SOCS-1, and Bcl6).<sup>75, 85-87</sup> Studies examining the role for miR-155 in experimental atherosclerosis in mice highlight both pro- and anti-atherosclerosis effects depending on the context. For example, LDLR $-/-$  mice harboring bone marrow with miR-155 deficiency exhibited increased atherosclerosis with reduced plaque stability.<sup>88</sup> In contrast, miR-155 deficiency in macrophages reduced atherosclerotic plaque size in ApoE $-/-$  mice, an effect thought to be mediated by miR-155's ability to target BCL6 and, in turn, reduced CCL2-mediated macrophage recruitment.<sup>87</sup> Moreover, miR-155 delivery in vivo reduced atherosclerotic lesion formation, an effect that may be mediated by targeting MAP3K10.<sup>89</sup> Because miR-155 also targets myosin light chain kinase (MYLK) in endothelial cells,<sup>75</sup> and MYLK-deficiency in ApoE $-/-$  mice reduces atherosclerosis by improving endothelial barrier dysfunction and monocyte migration<sup>90</sup>, EC-derived miR-155 may be viewed as a potentially protective miRNA in the vessel wall. In line with this hypothesis, miR-155 expression is significantly higher in the thoracic aorta, an area associated with unidirectional shear stress, than in the lower curvature of the aortic arch, a region associated with low shear stress.<sup>90</sup> These findings raise nuanced questions for miR-155 in vascular inflammation and future studies will be required to define the EC- and macrophage-specific roles of miR-155 in atherogenesis. Finally, laminar flow shear stress or KLF2 overexpression in ECs induce members of the miR-30-5p family that, in turn,

decrease anti-inflammatory markers such as VCAM-1 and ICAM-1 by targeting angiopoietin-2.<sup>91</sup> However, a role for miR-30-5p family members in regulating endothelial inflammation and atherosclerosis in mice will require future investigation.

## miRNAs regulating leukocyte recruitment and activation in atherosclerosis

Immune responses critically shape atherogenesis. One of the earliest pathogenic events in atherosclerosis is the recruitment of monocytes from the circulation to the artery wall in areas of endothelial dysfunction and lipoprotein retention. Upon differentiation into macrophages, these cells play central roles in the pathophysiology of atherosclerosis by maintaining lipid homeostasis in the vessel wall and secreting inflammation-promoting mediators that act on both immune and non-immune cell types in the artery wall<sup>92</sup>. Lipoprotein uptake by macrophages in the nascent plaque results in the formation of lipid-laden macrophage foam cells that are hallmarks of atherosclerosis<sup>92</sup>. For reasons that are poorly understood, these macrophage foam cells persist in the artery wall, setting off a maladaptive immune response that promotes the formation of plaques. These macrophages are a source of inflammatory mediators, including cytokines and chemokines that mediate the recruitment and/or activation of other immune cells, thereby chronically sustaining the inflammation that fuels plaque progression. miRNAs can impact each of these key macrophage processes to influence the progression of atherosclerosis.

Macrophage cholesterol homeostasis is maintained by the balance between cholesterol uptake, endogenous synthesis, esterification/hydrolysis and efflux. A number of miRNAs have been implicated in macrophage cholesterol metabolism. miR-27a/b, discussed above with regard to lipoprotein homeostasis, can regulate macrophage cholesterol homeostasis by targeting genes involved in cholesterol esterification (ACAT1), uptake (LDL, CD36) and efflux (ABCA1)<sup>93</sup>. miR-125a-5p and miR-146a decrease lipid uptake and cytokine release in oxLDL-stimulated macrophages, in part by targeting the genes oxysterol binding protein-like 9 and TLR4, respectively<sup>94, 95</sup>. In addition, miR-155, may also regulate macrophage foam cell formation by targeting the transcriptional repressor HMG box-transcriptional protein 1 (HBP1), which negatively regulates macrophage inhibitory factor (MIF), a protein known to increase the uptake of oxLDL by macrophages<sup>96</sup>. Furthermore, numerous miRNAs have now been identified that promote foam cell formation by inhibiting macrophage cholesterol efflux via ABCA1, including miR-26<sup>29</sup>, miR-33<sup>23–25</sup>, miR-106<sup>30</sup>, miR-144<sup>31, 32</sup>, miR-128-1<sup>18</sup>, miR-130b<sup>18</sup>, miR-148a<sup>18</sup>, miR-301b<sup>18</sup>, miR-302a<sup>97</sup> and miR-758<sup>28</sup>. This high degree of miRNA targeting of ABCA1 points to the need for careful fine tuning of macrophage cholesterol efflux to maintain cholesterol homeostasis.

In response to microenvironmental signals, macrophages can initiate different activation programs, including the 'classical' pro-inflammatory phenotype (also called M1) and the 'alternatively' activated M2 phenotype associated with an anti-inflammatory profile. Atherosclerosis progression is associated with the predominance of an M1 macrophage phenotype in the plaque, whereas plaques undergoing regression are enriched in M2 macrophages<sup>92</sup>. A growing list of miRNAs is implicated in regulating the balance between the M1 and M2 phenotypes, including miR-let7a<sup>98</sup>, miR-19a<sup>99</sup>, miR-21<sup>100</sup>, miR-27a<sup>101</sup>,

miR-33<sup>42</sup>, miR-124<sup>102</sup>, miR-125a<sup>103</sup>, miR-146a<sup>104</sup>, miR-155<sup>105</sup>, miR-214<sup>106</sup>, miR-223<sup>107</sup>. However, few of these have been investigated in the context of atherosclerosis. Of note, miR-33, which plays a central role in regulating cholesterol efflux, also regulates macrophage cellular metabolism to alter the cell's inflammatory phenotype. miR-33 reduces fatty acid oxidation, the metabolic program that fuels M2 macrophages, and promotes aerobic glycolysis, which in turn sustains the inflammatory M1-like macrophage phenotype<sup>42</sup>. Inhibition of miR-33 metabolically reprograms plaque macrophages to the M2 phenotype involved in resolving inflammation and tissue repair, which in turn promotes the accumulation of atheroprotective T regulatory cells. miR-155, which as described above has a controversial role in atherosclerosis, can also reprogram macrophages from the M2 to M1 phenotype, and thus may play a role in the accumulation of M1 macrophages<sup>105</sup>. miR-155 expression is significantly higher in CD14+ monocytes from patients with CAD than from healthy controls, and is induced by oxidized forms of LDL in macrophages<sup>96</sup>. miR-155 acts to repress negative regulators of inflammatory cytokine signaling, such as suppressor of cytokine signaling 1 (SOCS1), Src homology 2 domain-containing inositol-5-phosphatase-1 (SHIP-1), or B cell lymphoma 6 (BCL6), and thereby promoting the release of pro-inflammatory mediators<sup>75, 85–87</sup>. Conversely, miR-223, which regulates lipid metabolism-related genes in the liver<sup>15</sup>, can suppress M1 pro-inflammatory pathways and enhance alternative activation, in part by targeting *Pknox1*<sup>107</sup>. Finally, miR-27a, which also plays a role in macrophage foam cell formation, can promote markers of M2 macrophages (eg. CD206 and DC-SIGN) and secretion of IL-10<sup>101</sup>.

Other miRNAs have been implicated in enhancing or reducing macrophage responses to inflammatory stimuli. For example, miR-147 and miR-21 attenuate TLR-associated signaling events in macrophages to limit inflammation<sup>108, 109</sup>. Likewise, miR-146a/b are induced in macrophages in an NFκB-dependent manner and are involved in inflammation resolution by limiting Toll-like receptor and cytokine signaling. Recently, expression of miR-146a was shown to be induced in macrophages by apolipoprotein E (apoE), a protein with anti-atherosclerotic properties, and to suppress macrophage inflammatory responses in vitro and in vivo<sup>55</sup>. Furthermore, the transcription factor Krüppel-like factor 2 (KLF2), whose expression in macrophages protects from atherosclerosis, downregulates expression of proatherosclerotic chemokines (eg. CCL2 and CXCL1) by increasing expression of miR-124a and miR-150<sup>110</sup>. During early atherogenesis, one of the most prominently induced miRNAs in lesional macrophages is miR-342-5p<sup>111</sup>. This miRNA enhances the production of macrophage inflammatory mediators such as iNOS and IL-6 by suppressing Akt1-mediated suppression of miR-155. Accordingly, inhibition of miR-342-5p in *ApoE*<sup>-/-</sup> mice reduces atherosclerosis progression<sup>111</sup>.

Other immune cells, such as dendritic cells and T cells, also participate in atherogenesis and the regulation of plaque inflammation. By sensing and presenting antigens in the plaque, DCs are positioned at the crossroad of innate and adaptive immune responses. Furthermore T-cell (Th1, Th2, Th17, Treg) and B-cell subpopulations can modulate atherosclerosis development. Thus, miRNAs that regulate immune cell differentiation and/or function would be expected to have wide-ranging effects on plaque evolution. An atlas of miRNA expression patterns in human T and B cell subsets was recently completed<sup>112</sup>, and their roles

in regulating T and B cell differentiation, activation and function were recently reviewed<sup>113</sup>. However to date, miRNA regulation of these immune cell subsets in atherosclerosis has been underexplored compared to macrophages.

Notably, several miRNAs shown to regulate atherogenic processes in macrophages have also been implicated in dendritic cell function. For example, miR-155 is required for efficient DC maturation by targeting the transcription factor c-Fos<sup>114</sup>, and its expression increases TLR/IL-1 and type I IFN signaling pathways in human monocyte-derived DCs required to promote antigen-specific T-cell activation<sup>115-117</sup>. Furthermore, like its role in macrophages, miR-146a reduces TLR signaling and cytokine production in DCs, thereby regulating DC activation<sup>118</sup>. Moreover, miR-148/152 inhibit the production of pro-inflammatory cytokines (eg. IL-12, IL-6, TNF $\alpha$ ) by targeting CaMKII $\alpha$ , consequently reducing DC-triggered antigen-specific T cell proliferation<sup>119</sup>. By regulating activation of DCs and their interactions with T and B cells in plaques, these miRNAs are likely to impact the inflammatory milieu of the plaque and atherosclerosis progression.

## miRNAs implicated in Vascular Smooth Muscle function and atherosclerosis

VSMCs contribute to the maintenance of vascular wall function by assuming a differentiated, contractile phenotype. In response to vascular injury, VSMCs undergo a switch to a synthetic phenotype, an effect that induces signals that promote migration, proliferation, and inflammation.<sup>120</sup> Several miRNAs have been implicated in regulating important VSMC nodal regulators such as the transcription factors (serum-response factor (e.g. SRF/KLF4), co-activators (e.g. myocardin), TGF- $\beta$  signaling effectors (e.g. Smads), or cytokines/growth factors (e.g. PDGF). While the traditional view of VSMCs in vascular injury states is that they “dedifferentiate” to assume a synthetic, proliferative state,<sup>121</sup> recent studies have broadened this concept to suggest that VSMCs may adopt a reprogrammed “transdifferentiated” macrophage phenotype.<sup>121, 122</sup> While the role of miRNAs have emerged as important players in the former, their role in VSMC-to-macrophage transdifferentiation remains unexplored. We summarize below major miRNA regulators implicated in VSMC and atherosclerotic lesion formation (Figure 3).

### MiR-143/miR-145

The miRNAs miR-143 and miR-145 are co-transcribed as a single primary-miR transcript due to their close proximity and among the highest expressed miRNAs in VSMCs and the medial layer of the vessel wall.<sup>123</sup> Indeed, these miRNAs are reduced in the vessel wall in response to vascular injury or the presence of atherosclerosis.<sup>124, 125</sup> A series of gain- and loss-of-function studies suggest that miR-143/miR-145 are major regulators of VSMC contractile function. Indeed, miR-143/miR-145-deficient mice exhibit VSMCs with reduced SMC contractile marker expression and function, impaired actin stress fibers and cytoskeletal dynamics, and decreased vessel wall medial thickness.<sup>124-126</sup> These miR-143/miR-145-deficient mice also had reduced blood pressure at baseline and in response to vasopressor challenge, an effect that the authors attributed to reduced expression of the target gene angiotensin converting enzyme (ACE).<sup>127</sup> Conversely, delivery of miR-145 in

ApoE<sup>-/-</sup> mice using a lentivirus under the control of the SMC-specific SM22 $\alpha$  promoter significantly reduced atherosclerotic plaque size and promoted a more favorable plaque composition with increased fibrous cap area and plaque collagen content, and reduced necrotic core area and macrophage accumulation.<sup>128</sup> Consistent with miR-145's known role in promoting a contractile phenotype in VSMCs, these atherosclerotic stabilizing effects were mechanistically related to reduced KLF4 and increased myocardin expression in the vessel wall of ApoE<sup>-/-</sup> mice.<sup>128</sup> Analogous protective effects of miR-145 have been observed in response to neointimal vascular lesion formation.<sup>129</sup> In addition to KLF4 and myocardin, miR-143/miR-145 have been shown to target several other key transcriptional regulators implicated in modulation of VSMC differentiation, such as ELK-1 and KLF5.<sup>124, 125, 130</sup> Because upstream control of miR-143/miR-145 expression is governed in part by serum response factor (SRF), Nkx2.5, and myocardin, miR-143/miR-145 may contribute to 'fine-tuning' a SMC-specific transcriptional program important in regulating the contractile VSMC state.

### miR-221/miR-222

In contrast to miR-143/miR-145, the miRNAs, miR-221 and miR-222, are increased in response to injury in neointimal lesions.<sup>131</sup> In vitro studies have implicated miR-221/miR-222 in regulating PDGF-mediated VSMC proliferation. PDGF induced miR-221/miR-222 in VSMCs, an effect leading to reduced expression of its target genes c-Kit and p27Kip1 and decreased SMC-specific contractile gene expression.<sup>132</sup> Using miR-221/miR-222 knockdown approaches, in vivo studies demonstrated that miR-221 and miR-222 deficiency reduced VSMC proliferation and neointimal lesion formation after mechanical injury by targeting p27(Kip1) and p57(Kip2).<sup>131</sup> In human atherosclerotic lesions, reduced expression of miR-221 and miR-222 was noted in the shoulder of plaques from patients undergoing carotid endarterectomy (CEA) due to an acute neurological event occurring within 5 days of the CEA. While this study demonstrated a compelling association of reduced miR-221/miR-222 with unstable lesions, it also revealed that the target gene p27(Kip1) was significantly increased.<sup>133</sup> Interestingly, miR-222 expression in isolated human ECs decreased from early lesions to advanced plaques.<sup>134</sup> Collectively, these studies provide accumulating evidence that miR-221/miR-222 may play a destabilizing role in atherosclerotic lesions.

### MiR-21

While miR-21 has been implicated in promoting VSMC proliferation in response to a range of vascular mechanical injury models, its role remains to be defined in atherosclerotic lesion formation. Neutralization of miR-21 reduced neointimal lesion formation in response to mechanical balloon injury.<sup>129</sup> In keeping with its role in regulating VSMC contractile phenotype, miR-21 expression increased significantly in isolated 'dedifferentiated' VSMCs compared to mature differentiated VSMCs. Consistent with these findings for miR-21 in rodent vascular injury, delivery of an anti-miR-21-coated stent into balloon-injured human internal mammary arteries using a humanized rat model also revealed protective effects on neointimal lesion formation.<sup>135</sup> Mechanistically, miR-21's proliferative properties may be due to its ability to target PTEN in VSMCs and indirectly increasing the anti-apoptotic gene Bcl-2.<sup>129</sup> Furthermore, miR-21 mediates the induction of the VSMC contractile phenotype

by TGF- $\beta$  and BMP signaling in a unique post-transcriptional processing step implicating that Smad proteins may control DROSHA-regulated maturation of miRNAs.<sup>136</sup> Future studies will be required to assess whether the anti-proliferative effects of miR-21 neutralization also reduce atherosclerotic progression in non-mechanically injury vessels.

## MiRNAs as diagnostic markers of atherosclerotic disease severity

Accumulating studies implicate miRNAs as potential diagnostic or prognostic markers in a range of disease states. Because circulating miRNAs can be detected in peripheral blood, saliva, and urine, their expression may be harbingers of various stages of coronary artery disease from subclinical atherosclerotic disease to acute coronary syndromes. Herein, we summarize the profiling of several studies linking specific miRNAs to atherosclerotic disease burden as primarily diagnostic markers. Future studies will be required to further define these miRNAs for their prognostic significance in coronary artery disease (CAD).

### Stable CAD

One of the earliest studies that analyzed miRNAs from plasma of eight stable CAD subjects and healthy controls examined candidate miRNAs based on their putative cell-selective expression patterns. In the CAD group, expression of EC-enriched miRNAs (miR-126, miR-17, and miR-92a), VSMC-enriched miRNAs (miR-145), and inflammatory cell enriched miRNAs (miR-155) were all reduced. In contrast, cardiomyocyte-enriched miRNAs (miR-133 and miR-208a) were increased.<sup>137</sup> Other groups have identified reduced expression of miRNAs (miR-19a, -484, -155, -222, -145, -29a, -378, -342, -181d, -150, -30e-5p) from whole blood of patients with angiographically defined stable CAD (at least one epicardial vessel with >50% stenosis) compared to healthy subjects.<sup>138</sup> Interestingly, when this cassette of 11 miRNAs were compared to another group of subjects with at least 2 cardiac risk factors, but without angiographically defined CAD, there were no differences observed suggesting that this cassette of miRNAs may also be associated with subclinical atherosclerosis.<sup>138</sup> Surprisingly, reduced expression of 9 of the miRNA cassette was also associated with reduced expression in CAD or at-risk CAD patients taking ACE inhibitors or ARB antagonists compared to CAD/at-risk CAD patients not taking ACEI/ARBs. No associations were observed in this same cohort for treatment of statins vs no-statins. Another study by Zhu et al identified miR-155 expression in peripheral blood mononuclear cells (PBMCs) or plasma in patients with increasing severity of coronary syndrome and found that miR-155 was lower in patients with unstable angina or acute myocardial infarction than in patients with chest pain syndrome.<sup>139</sup> MiR-155 expression was further reduced in patients with 2 or 3 diseased vessels compared to patients with 0 or 1 diseased vessels. However, miR-155 also negatively correlated with a range of traditional cardiac risk factors. In addition, D'Alessandra et al. identified increased expression of miR-337-5p, miR-433, and miR-485-3p out of 178 miRNAs profiled in patients with CAD. Specifically, miR-1, miR-122, miR-126, miR-133a/b, miR-199a, miR-485-3p, and miR-377-5p were increased in subjects with stable angina or unstable angina. Using only miR-1, miR-126, and miR-485-3p, this classified correctly subjects with stable angina compared to controls, whereas miR-1, miR-126, and miR-133a classified patients correctly with unstable angina vs controls in greater than 87% of cases. However, no combination could correctly discriminate

between unstable angina and stable angina, suggesting that these markers likely reflect atherosclerotic burden present in both UA and SA patients.<sup>140</sup> Moreover, using PBMCs, a higher ratio of miR-135a to miR-147 was used to help discriminate patients with or without CAD.<sup>141</sup> In addition, reduced expression of miR-181a in PBMCs of obese patients is associated with prevalence of CAD.<sup>142</sup> In contrast, increased expression of miR-146a or miR-146b in patients with CAD was associated with an increased risk of atherosclerosis. Interestingly, the rs2910164 polymorphism in the miR-146a locus is associated with an increased risk of CAD in a Chinese Han population.<sup>143</sup> Finally, miR-146a expression was significantly increased in a Japanese cohort of 66 patients with CAD compared to no CAD. Furthermore, 12-months of statin therapy with an ACEI or ARB indicated that miR-146a/b and its regulated targets (IRAK, TRAF6, and TLR4) were significantly decreased.<sup>144</sup>

### Unstable angina

While the diagnosis of acute myocardial infarction can be facilitated using myocardial injury biomarkers such as cardiac troponin I or T, the diagnosis of unstable angina (UA) in patients with normal troponin values can be elusive. Because unstable angina is associated with a higher risk of cardiovascular events, identification of a set of ischemic biomarkers may be useful for diagnosis, risk stratification, and therapeutic decision-making.<sup>145</sup> Only a few miRNAs have been uniquely linked to unstable angina compared to stable CAD. However, these findings have not been replicated in larger clinical data sets. For example, expression of miR-134, miR-198, and miR-370 from peripheral blood mononuclear cells was enriched higher in 25 patients with UA compared to 25 with SA. However, these findings were not verified in a separate cohort of 19 UA and 34 SA patients.<sup>140</sup> When the comparator group was non-coronary chest pain patients (instead of SA patients), a different cassette of miRNAs (miR-132, miR-150, and miR-186) was found to be increased in UA patients.<sup>146</sup> Collectively, these findings suggest that rigorous phenotyping of patients with UA compared to other distinct patient sub-groups (e.g. AMI, SA, and non-coronary chest pain) will be required for appropriate discrimination of this unique and important UA group with elevated cardiac risk.

### Acute Myocardial Infarction

While cardiac troponin is used to assist with the diagnosis of acute myocardial infarction (AMI), it may serve as a poor discriminator between AMI subtypes.<sup>145</sup> In particular, additional biomarkers are desperately needed to further define Type I MI – ischemic myocardial necrosis due to atherosclerotic-plaque rupture (ACS) vs. Type II – ischemic myocardial not due to ACS, but typically due to supply/demand mismatch.<sup>145</sup> Emerging studies indicate that different miRNAs may be released into the peripheral circulation in response to distinct pathophysiological stimuli, suggesting that identification of a cassette of miRNAs may possess improved discriminatory power to diagnose MI subtype or possibly lead to an earlier diagnosis. For example, the cardiac-specific miRNA, miR-208b, is detected in circulation by 3 hrs after AMI and may persist with an elevated expression over 90 days.<sup>147, 148</sup> Several other groups validated the potential clinical usefulness of miR-208b as an early biomarker for AMI. Interestingly, miR-208 expression levels were increased in all n=33 patients 4 hrs post-MI compared to 85% for cardiac troponin I. Compared to other cardiac-enriched miRNAs (miR-1, miR-133a, miR-499), use of miR-208b conferred more



favorable receiver operating characteristic curve with higher sensitivity and specificity to diagnose AMI compared to non-coronary heart disease patients.<sup>149</sup> Similarly, miR-208b and miR-499 were significantly increased in AMI (n=32) compared to controls (n=36) that correlated with plasma cTnT.<sup>150</sup> However, in another study that used a larger number of AMI patients (n=224), the diagnostic value for miR-208b (as well as miR-499 and miR-320a) in AMI was significantly lower than that of cardiac troponin I or T.<sup>151</sup> Furthermore, while miR-208b and miR-499 expression were increased higher in patients with ST elevation myocardial infarction (STEMI) compared to non-STEMI (NSTEMI) and correlated with hs-cTnT and CK-MB in the plasma 1 hr after onset of chest pain, their diagnostic value was comparable to hs-TnT.<sup>152</sup> Nonetheless, in a more focused patient cohort of NSTEMI geriatric subjects (mean age 82.6 years), Olivieri et al. examined plasma levels of miR-1, miR-21, miR-133a, miR-208a, miR-423-5p, and miR-499-5p. Remarkably, miR-499-5p was equivalent to cardiac troponin T in discriminating NSTEMI compared to healthy controls patients or those with acute congestive heart failure (CHF). Furthermore, the diagnostic accuracy of miR-499-5p was *higher* than either conventional or high sensitivity-troponin T in discriminating NSTEMI and acute CHF (AUC = 0.86 for miR-499-5p vs. cTnT AUC = 0.68 or hs-cTnT AUC=0.70).<sup>153</sup> Finally, the bicistronic miRNA cluster of miR-1/miR-133 has also been implicated in AMI,<sup>154-156</sup> Despite detection of these 4 miRNAs, miR-208b, miR-499-5p, miR-1, and miR-133a, in the plasma after AMI, these cardiac-enriched miRNAs did not improve diagnostic accuracy of AMI compared to cTnT.<sup>157</sup> These findings may reflect the well-established ability of high sensitivity troponins to be released into circulation after generalized myocardial injury.

Atherosclerotic-based biomarkers that may reflect AMI or increased predisposition to plaque rupture or erosion would help discriminate MI subtype. In keeping with this notion, non-cardiomyocyte specific miRNAs may provide further refinement. For example, increased circulating levels of the miR-663 family member, miR-663b (a potential endothelial-enriched and flow-sensitive miRNA implicated in atherosclerosis (see above section on “Mechano-sensitive EC miRNAs that regulate atherosclerosis”) exhibited a high sensitivity (95%), specificity (90%), and accuracy (92.5%) to discriminate AMI (n=20 subjects) compared to controls (n=20 subjects).<sup>158</sup> Examination of different subtypes of ischemic injury may also be informative for use of miRNA to assist in diagnostic discrimination of related disease conditions. For instance, plasma levels of miR-21-5p and miR-361-5p were significantly increased in AMI patients (n=17), whereas miR-519e-5p was reduced with comparable diagnostic accuracy to cTnI. In contrast, these three miRNAs were all increased in patients with ischemic stroke (n=9) or pulmonary embolism (n=8), whereas decreased miR-519e-5p was only detected in AMI.<sup>159</sup> Collectively, these findings indicate that miRNAs may be useful to discriminate AMI subtype, and raise the possibility of incorporating miRNAs as a tool for linking pathophysiological events, prognosis, and stage-specific therapy.<sup>160</sup>

Taken together, these findings indicate that miRNAs may be associated with subclinical atherosclerosis. Furthermore, caution should be required for interpretation of similar miRNA profiling studies based upon baseline characteristics of medical treatment.

## Therapeutic opportunities and challenges

Nucleic acid-based therapies represent a new frontier in the treatment of human diseases. Several classes of RNA therapeutics are currently under clinical development, including anti-sense oligonucleotides (ASO), small interfering RNA (siRNA) and miRNA mimetics and inhibitors. The field of RNA therapeutics saw a huge leap forward with the recent FDA approval of the first anti-sense oligonucleotide drug, Mipomersen, an antisense oligonucleotide (ASO) drug targeting apoB to treat homozygous familial hypercholesterolemia<sup>161–164</sup>. This will likely pave the way for other oligonucleotide-based drugs, including miRNA replacement and inhibitor therapies. Notably, pharmacological targeting of miRNAs in disease moves beyond the “one-drug-one-target” mode of treatment offered by siRNAs, and this has both benefits and drawbacks. The ability of miRNAs to target gene networks provides a unique approach for the treatment of disease by modulating gene pathways, but also opens the door to potential unwanted effects on additional target genes. Yet, this type of approach may prove to be more effective at improving complex diseases like atherosclerosis, which involve many pathways and would benefit from a multi-modal therapeutic.

Anti-sense oligonucleotides offer the ability to silence miRNAs to fine tune specific pathways or to reduce miRNAs expression dysregulated by disease. A number of approaches have been used to chemically modify anti-miR oligonucleotides to enhance target affinity, stability and tissue uptake<sup>165</sup>. These include: (i) locked nucleic acids (LNAs) which have high binding efficiencies and improved stability with the addition of a methylene link between the 2'-oxygen and the 4'-carbon resulting in a “locked” position and reducing the flexibility of the ribose ring<sup>166</sup>, (ii) ribose 2'-OH group modifications such as 2'-methoxyethyl (MOE) and 2'-4'-constrained 2'-O-ethyl (cEt), and (iii) phosphorothioate (PS) modification. Careful evaluation of these chemical modifications will be required to minimize potential side effects and unanticipated toxicities. For example, phosphorothioate modification may facilitate nucleotide-based drugs to bind and activate platelets eliciting thrombus formation in response to carotid injury, pulmonary thromboembolism, and mesenteric artery injury in mice.<sup>167</sup> Unlike their double-stranded counterparts, single stranded anti-miR oligonucleotides can be formulated in saline for subcutaneous or intravenous delivery and do not require lipid-based delivery systems. Upon systemic delivery, these compounds rapidly leave the plasma and are taken up by multiple tissues, most prominently liver, spleen, kidney, adipose tissue and bone marrow<sup>168, 169</sup>. Once taken up by cells, the anti-miR forms a stable, high-affinity bond with the miRNA reducing the availability of the endogenous miRNA for binding to the 3'UTR of the mRNA target. Preclinical studies in non-human primates using naked anti-miR oligonucleotides have shown promise, particularly in targeting miRNA expression in the liver (eg. miR-122, miR-33)<sup>13, 26</sup>. Cholesterol analogs have been added to anti-miRs in an attempt increase cellular uptake, and this promotes their incorporation into low and high density lipoproteins (LDL and HDL)<sup>170–172</sup>. Another approach for miRNA inhibition involves the use of miRNA sponges or decoy transcripts<sup>173</sup>, which act as competitive inhibitors of the miRNA of interest. miRNA sponges contain multiple binding sites that are complementary to the seed sequence of a miRNA of interest, which “mops up” the miRNA and inhibits its

function. miRNA sponges can be delivered using viral vectors, and their expression can be made to be inducible in a specific cell type or developmental stage by using specific promoters. Notably, when vectors containing miRNA sponges were transfected into cells, miRNA target genes were as derepressed as when anti-miR oligonucleotides were used<sup>174</sup>.

miRNA mimetic therapy using synthetic miRNAs (miRNA mimics) offers the ability to reconstitute a miRNA that is downregulated during disease, or to decrease gene pathways involved in disease pathology. For example, patients with CAD exhibit decreased plasma levels of miR-181b, which is also expressed in ECs,<sup>50</sup> and miRNA mimics could be used to restore its expression. The delivery of therapeutic miRNA molecules *in vivo* faces many of the same challenges as siRNAs due to their double-stranded nature. Drug delivery vehicles such as liposomes, polymeric micelles and lipoprotein-based drug carriers are being developed to deliver these oligonucleotides to cells. Notably, miRNAs were recently shown to be associated with endogenous HDL particles and to deliver their miRNA cargo to other cells<sup>175</sup>, offering the potential to use HDL infusion as a miRNA delivery vehicle. Some of the current challenges associated with miRNA replacement technology include the ability to target miRNAs to a specific tissue, and the potential requirement of multiple doses of a miRNA mimetic to achieve sustained target repression. Viral vectors can be used as gene delivery carriers for miRNAs, including short hairpin RNAs that can be processed in the target cell into the mature miRNA, a approach that has been used in multiple preclinical studies<sup>17, 25, 176</sup>. However, viral delivery systems will require careful scrutiny for clinical use.

While miRNA mimics and oligonucleotide inhibitors are taken up more efficiently in the liver, we and others have demonstrated success at penetrating the vascular endothelium of the vessel wall and peripheral blood mononuclear cells<sup>49, 50</sup>. Because only a minority of miRNAs are tissue-specific, delivery of miRNA mimics or inhibitors in a targeted cell- or tissue-specific rather than a systemic manner may represent a novel opportunity to prevent the development or progression of atherosclerosis. Recent approaches to tailor miRNA delivery to specific tissues include incorporating target sites for tissue-specific miRNAs, such as liver specific miR-122 and miR-192<sup>177</sup>, which would silence the vector in the liver, but allow it to be functional in other tissues.

A number of miRNA based therapeutics are currently in preclinical development, and two have reached clinical trials. The first is a LNA directed against miR-122 (Miravirsen), which targets hepatitis C virus (HCV) RNA<sup>178</sup>. Studies in non-human primates showed that miR-122 inhibition resulted in long-lasting suppression of HCV viremia with no evidence of viral resistance or side effects<sup>179</sup>. In a human phase 2 study Miravirsen demonstrated dose-dependent antiviral activity when given as a 4-week monotherapy that was maintained for more than 4 weeks after the end of therapy<sup>180</sup>. Notably, HCV RNAs in four of nine patients treated with the highest doses of Miravirsen became undetectable during the study<sup>181</sup>, demonstrating the potential of anti-miR therapeutics. The second miRNA therapeutic in clinical trial is a double-stranded miRNA mimic of miR-34, which acts as a tumor suppressor by inhibiting multiple oncogenic pathways and stimulating anti-tumor immune responses<sup>182</sup>. MRX34 is a miR-34 mimic encapsulated in a liposomal nanoparticle formulation that is being tested in patients in a range of advanced solid tumors and

hematological malignancies. The results of these first clinical trials of a miRNA mimic and inhibitor are eagerly awaited.

Over the last several decades, significant progress has been achieved to treat atherosclerosis. Most notably, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) have been widely used to treat patients in the primary and secondary prevention of coronary artery disease. While statins effectively reduce LDL levels and cardiovascular events, a considerable residual burden of coronary artery disease remains even in patients treated with statins. Novel complementary therapeutic approaches will likely be necessary for treating disease states such as atherosclerosis involving complex signaling networks. In this regard, because miRNAs target multiple genes often in the same regulatory network, miRNAs may have tremendous effects on biological pathways, cell function, and homeostasis in the vessel wall, liver, and periphery. Delivery of a cassette of miRNA mimics or inhibitors may thereby offer an attractive therapeutic approach to facilitate “fine-tuning” specific stages of atherosclerotic disease and in the management of its complications.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-standard Abbreviations and Acronyms

<b>AMI</b>	acute myocardial infarction
<b>ASO</b>	anti-sense oligonucleotides
<b>Ago2</b>	Argonaute2
<b>ApoE</b>	apolipoprotein E
<b>CAD</b>	coronary artery disease
<b>D-flow</b>	disturbed flow
<b>eNOS</b>	endothelial nitric oxide synthase
<b>HDLc</b>	HDL cholesterol
<b>GWAS</b>	genome-wide association study
<b>ICAM-1</b>	intracellular adhesion molecule 1
<b>L-flow</b>	laminar flow
<b>LDLc</b>	LDL cholesterol
<b>LDLR</b>	LDL receptor

<b>miRNA</b>	microRNA
<b>NF-<math>\kappa</math>B</b>	Nuclear Factor $\kappa$ B
<b>PBMCs</b>	peripheral blood mononuclear cells
<b>RISC</b>	RNA-induced silencing complex
<b>SA</b>	stable angina
<b>SiRNA</b>	small interfering RNA
<b>SNPs</b>	single-nucleotide polymorphisms
<b>SR-BI</b>	scavenger receptor BI
<b>STEMI</b>	ST elevation myocardial infarction
<b>TRBP</b>	TAR RNA-binding protein
<b>UA</b>	unstable angina
<b>UTR</b>	untranslated region
<b>VCAM-1</b>	vascular adhesion molecule 1

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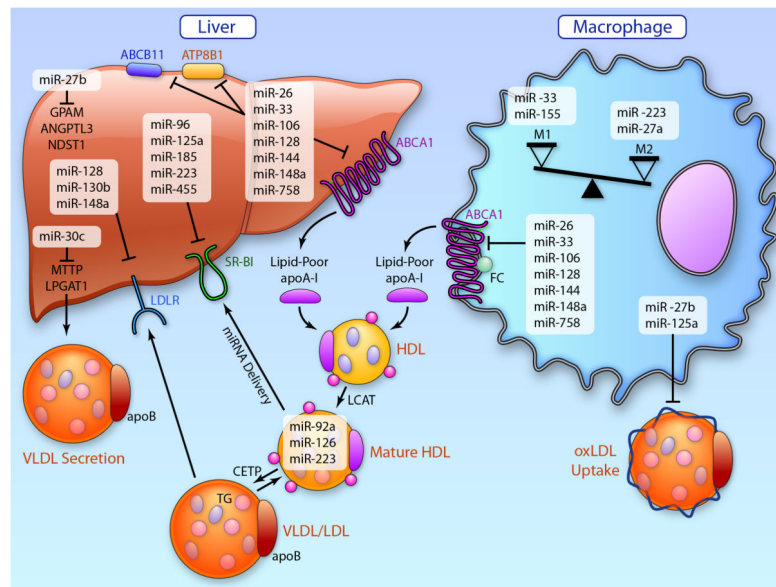
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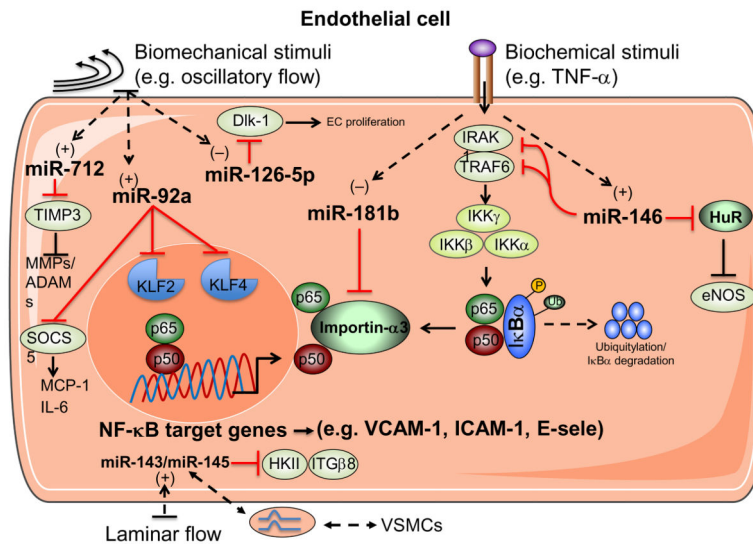
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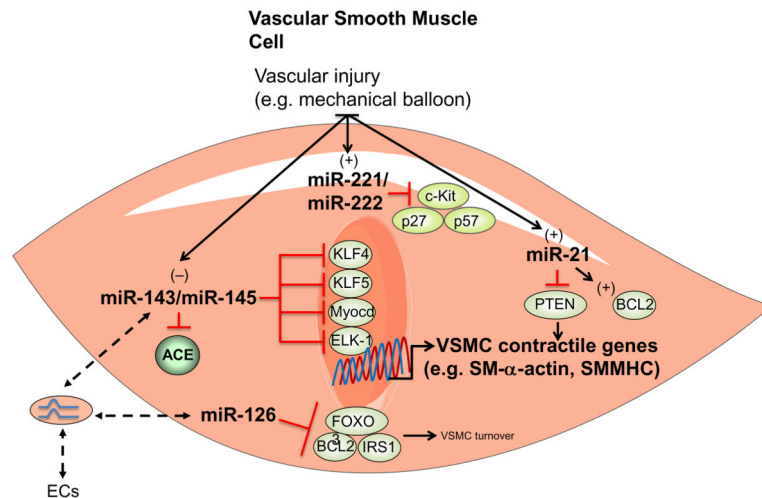
**Figure 1. microRNA orchestration of cholesterol homeostasis and macrophage activation in atherosclerosis**

In the liver, miRNAs repress the expression of genes involved in lipoprotein packaging and secretion (eg. miR-30c, miR-27b), uptake (eg. miRNAs targeting *LDLR* and *SR-B1*), and cholesterol efflux (eg. miRNA targeting *ABCA1*). miRNA repression of *ABCA1* decreases cholesterol efflux to lipid-poor apolipoprotein A-I (apoA-I), and biogenesis of HDL. As the nascent HDL particle mediates free cholesterol (FC) uptake and remodels due to lecithin-cholesterol Acyltransferase (LCAT) conversion of FC to cholesterol ester (CE), secreted microRNAs, such as miR-223, miR-92a and miR-126 are detected on the mature HDL particles. These HDL carried and may mediate extracellular signaling by repressing genes in target tissues and HDL interaction with macrophages and endothelial cells may also result in miRNA exchange (ie. pick-up or delivery via scavenger receptor B1 (*SR-B1*)). MiRNA targeting of *SR-B1* and the *ABCB1* and *ATP8B1* transporters reduce selective cholesterol uptake by the liver and excretion, respectively. In macrophages, miRNA targeting of *ABCA1* reduces cholesterol efflux and reverse cholesterol transport back to the liver. In addition, miRNAs regulate the polarization of macrophages toward classical M1 (eg-33, miR-155) or alternative M2 (eg. miR-223, miR-27a) inflammatory activation, and also regulate lipoprotein uptake and foam cell formation. (Illustration Credit: Ben Smith).



### Figure 2. Endothelial microRNAs regulate vascular inflammation

In response to biochemical and biomechanical stimuli, microRNAs regulate specific targets in endothelial cells (ECs) that alter the balance of pro- or anti-inflammatory signaling pathways. Biochemical stimuli such as tumor necrosis factor (TNF)- $\alpha$  reduces the expression of miR-181b, while it increases the expression of miR-146. MiR-181b targets importin- $\alpha$ 3 in ECs, an effect that prevents cytoplasmic-to-nuclear translocation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) family members, p50 and p65, thereby reducing NF- $\kappa$ B-responsive gene expression such as adhesion molecules vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), or E-selectin. Induction of miR-146 targets the 3'-UTR of TNF-receptor-associated factor 6 (TRAF6), IRAK1, to regulate upstream NF- $\kappa$ B signaling and targets HuR which regulates endothelial nitric oxide synthase (eNOS) expression. The expression of miR-92a and miR-712 are increased in response to disturbed flow (d-flow), whereas miR-126-5p expression is reduced. miR-92a targets the transcription factors Kruppel-like factor 2 (KLF2) and 4 (KLF4), and suppressor of cytokine signaling (SOCS5) expression, an effect that decreases anti-inflammatory pathways and increases monocyte chemoattractant protein (MCP)-1 and IL-6 that further promote EC activation. miR-712 suppresses tissue inhibitor of metalloproteinase-3 (TIMP3) thereby increasing the expression of matrix metalloproteinases (MMPs) and A Disintegrin and MMP (ADAMs). In contrast, d-flow reduces expression of miR-126-5p thereby de-repressing its target gene delta-like 1 homolog (Dlk-1), a negative regulator of endothelial cell proliferation. Lamellar flow induces the expression of the miR-143/miR-145 cluster, which may be packaged and released extracellularly in microvesicles and taken-up by neighboring vascular smooth muscle cells (VSMCs). Conversely, ECs may enable the passage of miR-143 and miR-145 released from VSMCs through specialized membrane protrusions known as tunneling nanotubes. HKII, hexokinase II. ITG $\beta$ 8, integrin  $\beta$  8.



### Figure 3. MicroRNA regulation of vascular smooth muscle cell phenotype

In response to vascular wall injury or atherosclerosis, the expression of the miR-143/miR-145 cluster is markedly reduced in vascular smooth muscle cells (VSMCs). MiR-143 and miR-145 target the transcriptional regulators KLF4, KLF5, myocardin, and ELK-1 important for VSMC phenotypic switching from a contractile, mature, and differentiated cell type to a de-differentiated synthetic, and proliferative cell type. In addition, miR-143/miR-145 target genes important to the regulation of blood pressure such as angiotensin converting enzyme (ACE). In contrast, vascular wall injury increases expression of miR-221 and miR-222, an effect that decreases the expression of the cell cycle regulator c-Kit, p27(Kip1), and p57(Kip2). Induction of miR-21 expression targets phosphatase and tensin homolog (PTEN), thereby increasing the anti-apoptotic regulator B-cell lymphoma 2 (Bcl-2). Microvesicles or exosomes released by neighboring endothelial cells, and carrying microRNAs such as miR-143/miR-145 or miR-126 (bound to Argonaute2 (Ago2)), may be taken up by VSMCs enabling suppression of target genes and altering VSMC functional responses. FOXO3, Forkhead Box O3. IRS1, insulin receptor substrate 1.