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MicroRNAs in the pathobiology of atherosclerosis

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Abstract

MicroRNAs are short non-coding RNAs, expressed in humans and involved in sequence-specific post-transcriptional regulation of gene expression. They have emerged as key players in a wide array of biological processes, and changes in their expression and/or function have been associated with plethora of human diseases. Atherosclerosis and its related clinical complications, such as myocardial infarction or stroke, represent the leading cause of death in the western world. Accumulating experimental evidence has revealed a key role for microRNAs in regulating cellular and molecular processes related to atherosclerosis development, ranging from risk factors, to plaque initiation and progression, up to atherosclerotic plaque rupture. In this review, we will focus on how microRNAs can influence atherosclerosis biology, as well as the potential clinical applications of microRNAs which are being developed as both targets and therapeutics for a growing industry hoping to harness the power of RNA-guided gene regulation to fight disease and infection.

MicroRNA: discovery and function

Since the initial discovery of microRNAs as essential regulators of development in the nematode *Caenorhabditis elegans*,¹ thousands of microRNA genes have been identified in animal and plant genomes.² Around 60% of the human genome and nearly every major gene cascade is predicted to be under microRNA regulation³, suggesting that non-coding RNAs may match or even rival proteins in their regulatory capacity. Because of this, it is not surprising that the disruption of microRNA function contributes to many human disorders, such as cancer, cardiovascular diseases and neurological dysfunctions.⁴ MicroRNAs are processed in a two-step mechanism from primary transcripts (pri-microRNAs) by RNA polymerase II-generated characterized by a hairpin structure with a double stranded stem.⁵ In the nucleus, the RNase III Drosha cleaves the pri-microRNAs at the 5' and 3' end into

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Disclosures

None

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microRNA precursors (pre-microRNAs) that contain a 2-nt overhang at the 3' end. The GTP-dependent Exportin 5 transporter translocates the pre-microRNAs into the cytoplasm, where they are further processed. The RNase III enzyme Dicer recognizes the 2-nt overhang, and cleaves the pre-microRNAs near the terminal loop of the hairpin into 19–24 nt long mature microRNA duplexes. Dicer then assembles with RNA binding proteins and Argonaute (Ago) proteins to form a RNA-induced silencing complexes (RISC) (Table I).⁶ The separation of the microRNA duplexes into corresponding 5' and 3' strands (denoted –5p and –3p, respectively) occurs through a process called unwinding in which the N-terminal end of the Ago proteins wedges into the microRNA double strand, and thereby releases one of the strands (the passenger strand) from the RISC,⁷ thus allowing the mature RISC to bind to target mRNA(s). The canonical target sites interact through complementary pairing with the nucleotides 2–8 at the 5' end of microRNAs (termed the “seed” sequence) and are usually located in the 3' untranslated region (UTR) in the target mRNA. In the final step, the RISC complex recruits the GW182 family of proteins to the 3' UTR region enable the repression of mRNA translation or promote mRNA deadenylation and degradation, leading to the repression of protein expression.⁸ Whether microRNAs remain in the cells that produced them or are transferred to other recipient cells through extracellular vesicles, their predominant biological role is closely linked to their ability to guide RISC complexes in mediating mainly translational repression of specific mRNAs and thus silencing gene expression. MicroRNAs have the unique ability to simultaneously target multiple genes within the same or related pathway, reducing the expression of functionally related genes. MicroRNAs act generally in concert (a mRNA may contain >40 binding sites for different microRNAs), and each microRNA may regulate up to 200 to 300 different mRNAs, thus explaining their ability to control ~60% of the human genome and their involvement in nearly every biological pathway.

Atherosclerosis: a cholesterol-driven inflammatory disease

The development of atherosclerotic vascular disease is promoted by the presence of concomitant risk factors, including dyslipidemia, which drives a chronic inflammatory reaction in the vessel wall. 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 maintain cholesterol homeostasis.⁹ Excess LDL-cholesterol (LDL-C) is retained in the subendothelial space, causing endothelial cells (ECs) to become activated and recruit monocytes into the area to clear away the accumulated cholesterol. Monocyte-derived macrophages engulf the modified LDL (oxidized or aggregated) and become inflammatory foam cells, and recruit further inflammatory cells (T-cells, B-cells) into the growing intimal layer of the vessel. Over time, lesions progress from early fatty streaks to complex vulnerable plaques that are responsible for the clinical consequences of the disease, namely myocardial infarction or stroke. Imbalances that favor the accumulation of cellular cholesterol, such as high levels of LDL-C and low levels of HDL-cholesterol (HDL-C), promote atherosclerosis.

In addition to inflammatory cells, ECs and vascular smooth muscle cells (VSMCs) participate in the atherogenic process. At sites with disturbed laminar blood flow, ECs express adhesion molecules that promote inflammatory cell recruitment.¹⁰ VSMCs

proliferate and engulf lipid and transform into macrophage-like foam cells, further promoting lipid accumulation and lesion expansion. Because the mechanisms and cellular involvement in the development of atherosclerosis are so diverse, microRNAs have unsurprisingly been identified as regulatory players that govern virtually every step in the development of atherosclerosis. We will discuss the major microRNA players below and summarize the pre-clinical findings of these atherosclerosis-targeting microRNAs in Table II.

1. MicroRNAs and cholesterol homeostasis

Recent clinical and preclinical studies have shown that microRNAs control LDL and HDL abundance and function, thus improving our understanding of the regulatory circuits governing plasma lipoprotein levels. Thanks to their ability to modulate expression and function of transcription factors, enzymes and receptors governing lipoprotein metabolism, microRNAs control the genesis and metabolism of lipoproteins. Recently, a genome-wide association study investigated the association between single nucleotide polymorphisms (SNPs) and abnormalities of plasma lipids and found an interesting association with 69 microRNAs and blood lipids.¹¹ Several of them (eg, miR-148a, miR-128-1, miR-130b, and miR-301b) were identified as effective modulators of key genes in lipoprotein metabolism, such as LDL-receptor (LDLR) and ATP-binding cassette A1 (ABCA1). LDLR expression in the liver promotes the clearance of circulating LDL particles and is a major determinant of plasma cholesterol levels, whereas ABCA1 mediates unidirectional efflux of phospholipids and cholesterol to lipid-free apolipoproteins and HDL.¹² Notably, miR-148a is predominantly expressed in liver and able to regulate both expression and function of these gene *in vivo*.^{11,13} Experimental inhibition of miR-148a by antisense oligonucleotides resulted in an increase in hepatic LDLR and ABCA1 expression with marked modification in plasma lipoprotein profile (decreased LDL-C levels and increased HDL-C) and without abnormalities in markers of liver damage, suggesting that altered expression of this microRNA may contribute to dyslipidemia.¹³ MiR-223, whose expression is dependent on intracellular cholesterol levels, has also been found to play a role in lipid metabolism.¹⁴ Indeed, miR-223 inhibits cholesterol synthesis via posttranscriptional regulation of 3-hydroxy-3-methylglutaryl(HMG)-CoA synthase 1 (HMGs1) and methylsterol mono-oxygenase 1, and by affecting cholesterol efflux through repression of scavenger receptor class B member 1 (SR-BI) and up-regulation of ABCA1, whereas miR-223^{-/-} mice display increased HDL-C levels, as well as hepatic and plasma total cholesterol.¹⁴ Moreover, miR-223 is also transferred from cells to HDL particles via an SR-BI-dependent process. Its levels are higher in patients with familial hypercholesterolemia and in ApoE- and LDLR-deficient mice under high-fat diet, and might play a role as intercellular communication system regulating liver gene expression.¹⁵

The liver plays a major role in both the production and the clearance of lipoproteins, and many hepatic-enriched microRNAs have been identified as regulators of lipoprotein metabolism. MiR-122, which account for about 70% of the total liver microRNAs, was shown to regulate plasma triglycerides and cholesterol levels, as the inhibition of miR-122 by both anti-sense interference or genetic deletion in transgenic mice leads to a strong reduction of both lipid pools.¹⁶⁻¹⁸ Targets of this miR-122 include HMGCoA reductase

(HMGCR), the rate-limiting enzyme for intracellular cholesterol biosynthesis, and microsomal triglyceride transfer protein (MTTP), a protein required for the hepatic assembly of VLDL and ApoB-containing lipoproteins.^{16,18} Notably, miR-122 function seems to be broadly required for the expression of hepatocyte-specific genes, rather than the specific targeting of lipid metabolism pathways¹⁹. In contrast, miR-27b and miR-30c act as direct post-transcriptional regulators of cholesterol and lipoprotein metabolism networks.^{14,20,21} MiR-27b is a cholesterol-responsive hepatic microRNA that represses many targets involved in lipid metabolism and lipoprotein remodeling, such as ANGPTL3 and GPAM.²⁰ Finally, miR-30c was shown to have a potent effect on the production of VLDL and LDL. Indeed, miR-30c reduces de novo lipogenesis by targeting lysophosphatidylglycerol acyltransferase 1 (LPGAT1) responsible for de novo hepatic lipid biosynthesis, and miR-30c overexpression reduced hyperlipidemia and attenuated atherosclerosis in ApoE^{-/-} mice by targeting MTTP.²¹ A large body of literature has focused on the role of microRNAs in the regulation of HDL biogenesis and cholesterol efflux, which control levels of plasma HDL-C and the reverse cholesterol transport pathway through which excess cholesterol is removed to the liver for excretion. Indeed, epidemiological studies have shown an atheroprotective role for HDL, which is thought to be associated with its ability to mediate reverse cholesterol transport. Among the relevant effectors, ABCA1 plays a central role in these processes by controlling cholesterol efflux across the cell membrane onto lipid-poor apoA1 to mediate both hepatic HDL biogenesis and the removal of excess cholesterol from peripheral cells, particularly macrophages in atherosclerotic plaques.¹² Of note, the sequence of the ABCA1 3'UTR is among the longest in the human genome and include numerous conserved binding sites for microRNAs including miR-33,^{22–24} miR-758,²⁵ miR-128,¹³ miR-144,²⁶ miR-148a,¹¹ miR-106,²⁷ and others.²⁸ Of these, inhibition of miR-33, miR-144, miR-128, and miR-148a has also been tested *in vivo* and shown to increase plasma levels of HDL-C in mice or monkeys. The final step of reverse cholesterol transport is the selective uptake of HDL cholesterol into the liver, and is mediated by SR-BI. In this context, miR-223,²⁹ miR-455-5p,³⁰ miR-96,²⁹ miR-185,²⁹ and miR-125a³⁰ have been demonstrated to repress hepatic SR-BI, providing an additional mechanism that modulates HDL cholesterol transport and may drive atherosclerosis development.

MiR-33 is the most extensively investigated microRNA in the regulation of ABCA1 and ABCG1. In humans, miR-33a and miR-33b are encoded within introns of the sterol regulatory element-binding protein (SREBP) 2 and SREBP1 genes, the main transcription factors controlling cholesterol and fatty acid metabolism.³¹ Thus, the concomitant transcription of SREBP2, which promotes intracellular cholesterol synthesis and LDL uptake, and miR-33a, which represses cholesterol efflux via down-regulation of ABCA1 (in humans and mice) and ABCG1 (in mice), plays a synergistic effect in increasing intracellular cholesterol levels in macrophages and hepatocytes.³¹ As a result, *in vivo* short term antagonism of miR-33 in rodents and in non-human primates provides a significant increase in plasma HDL and enhanced cholesterol transport from macrophages to the plasma, liver, and feces by up to 80%.^{23,24,31} This increase in reverse cholesterol transport is likely the combined effects of de-repression of ABCA1 and additional miR-33 targets such as *ABCB11* and *ATP8B1* that promote cholesterol excretion into bile.³² In fact, miR-33 has become one of the most attractive microRNA targets for drug development to treat

dyslipidemia and atherosclerosis (Figure 1). Although initial studies showed that inhibition of miR-33 reduced atherosclerosis in LDL receptor $-/-$ or apoE $-/-$ mice,^{24,33} other studies have reported that inhibition of this microRNA did not attenuate the progression of atherosclerosis in LDLR $-/-$ mice.^{34,35} Indeed, in some mouse models, long-term treatment with anti-miR-33 in mice fed a high-fat diet was associated with increased plasma triglycerides levels and moderate liver steatosis.³⁶ These outcomes could be explained by the concomitant overexpression of genes involved in fatty acid synthesis, namely acetyl-CoA carboxylase and fatty acid synthase, though this was not observed in non-human primates or in some mouse models.^{34,36} Therefore, although miR-33 represents a promising target for dyslipidemia and atherosclerosis, the potential side-effects of long-term treatment mandate further investigation.

Although miR-33 has been the most extensively studied *in vivo*, several other microRNAs have also been shown to modulate cholesterol efflux in macrophages and other cell types. Recently, miR-302a was found to target ABCA1, and blocking miR-302a using antagomirs reduced atherosclerosis progression in mice.³⁷ MiR-10b also targets ABCA1 and ABCG1 and thus reduces cholesterol efflux from lipid-loaded macrophages.³⁸ MiR-26 can also target ABCA1, as well as other genes involved in cholesterol mobilization, such as ADP-ribosylation factor-like 7, an intracellular transport protein that acts in concert with ABCA1 to mobilize cholesterol to the cell surface for removal.³⁹ Recently, coenzyme Q10 was demonstrated to reduce miR-378 expression, which increased the expression of ABCG1 in mouse and human macrophages, thus facilitating macrophage cholesterol efflux *in vitro* and *in vivo*.⁴⁰ Taken together, the available evidence strongly supports a relevant contribution of microRNAs in lipid metabolism and shed light on new potential therapeutic targets for treatment of dyslipidemia and for cardiovascular prevention.

2. MicroRNAs in initiation of atherosclerosis

MicroRNAs in endothelial activation

When dyslipidemia occurs, especially under disturbed patterns of blood flow, the endothelium becomes activated by pro-inflammatory cytokines and by oxidized lipoproteins. The activation of ECs promotes leukocyte recruitment from the blood into extravascular tissues, thus contributing to the pathogenesis of atherosclerosis.⁴¹ Several signaling pathways participate in this process, prominently promoting the transcription factor NF- κ B. In the endothelium, NF- κ B signal transduction modulates the expression of numerous pro-inflammatory genes involved in cell adhesion such as E- and P-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1), as well as various chemokines and cytokines.^{42,43} As one of the most highly expressed microRNAs in ECs, miR-126-3p interferes with this process by inhibiting the expression of VCAM-1 and hampering *in vitro* leukocyte adhesion to ECs (Figure 1).⁴⁴ MiR-31 and miR-17-3p directly target E-selectin and ICAM-1 expression, respectively, thus acting as a brake on TNF α -induced EC activation.⁴⁵ Finally, miR-155 and miR-221/222 inhibit the angiotensin II-induced inflammatory response in ECs by targeting the transcription factor Ets-1 and its downstream genes, such as VCAM-1 and monocyte chemoattractant protein-1.⁴⁶ MiR-10a is highly expressed at predilection sites of the aorta exposed to disturbed flow, and has

inhibition by antagomirs enhances NF- κ B– dependent adhesion molecule expression in ECs.⁴⁷ Moreover, let-7g has been shown to act as a suppressor of endothelial inflammatory activation through inhibition of both TGF- β and SIRT1, whereas ECs acquire a pro-inflammatory-activated phenotype in its absence.⁴⁸ Likewise, hyperlipidemic patients have lower let-7g levels with increased circulating levels of PAI-1, a marker of endothelial dysfunction and thrombogenic activation.⁴⁸ Studies in mice and humans demonstrated that miR-181b acts as a suppressor of EC inflammatory responses in vascular disease states by targeting Importin- α 3, a protein required for nuclear translocation of NF- κ B, thus inhibiting a set of NF- κ B-responsive genes including VCAM-1 and E-selectin.^{49,50} Systemic delivery of miR-181b mimic reduces NF- κ B activity and atherosclerotic lesion development in mice, which was in part due to decreased numbers of lesional macrophages and CD4+ T cells.⁴⁹ MiR-146a plays also an important role in dampening endothelial activation by promoting eNOS expression through targeting the RNA-binding protein HuR, and by repressing the induction of adhesion molecules through targeting TRAF6 and IRAK1/2, once again acting as a negative feedback loop for NF κ B.⁵¹ Atheroprotective flow patterns are associated with a reduced level of miR-92a and an increase in KLF-2, which promotes an anti-inflammatory phenotype in ECs, thus supporting a pivotal role for miR-92a in regulating ECs response to shear stress.⁵² On the other hand, KLF-2 is able to regulate the expression of specific microRNAs, such as miR-145, which can repress the expression of junctional adhesion molecule-A and thus inhibits monocyte adhesion to endothelium.⁵³

MicroRNAs in endothelial proliferation

Even if ECs growth occurs rather sporadically in the adult vasculature, it has recently been established that microRNAs can influence endothelial proliferation. Increased ECs turnover mainly takes place at predilection sites of atherosclerosis in response to increased levels of stress-induced ECs death and detachment.^{54,55} In this setting, miR-126 has been shown to regulate endothelial proliferation. Although both strands of this microRNA are present and functional in ECs, only the miR-126-5p strand impacts ECs proliferation improving re-endothelialization after vascular injury by targeting Delta-like 1 homolog (DLK1).⁵⁶ Reduced miR-126-5p levels at predilection sites upregulated DLK1 expression, thus controlling and limiting ECs proliferation. In contrast, at non-predilection sites, laminar blood flow protects EC function and limits EC turnover and provides a higher proliferative capacity by inducing miR-126-5p expression.⁵⁶ Oxidized LDL has been shown to inhibit ECs replication *in vitro* and hyperlipidemia significantly reduces ECs proliferation at predilection sites *in vivo*.^{56,57} Under hyperlipidemic conditions, the DLK1-mediated anti-proliferative response turns out disadvantageous at the predilection sites and results in decreased EC regeneration, whereas at the non-predilection sites, high levels of miR-126-5p reduce DLK1 expression, thereby enabling EC regeneration.⁵⁶ Indeed, miR-126-5p mimic therapy under these condition was shown to improve EC proliferative reserve at predilection sites, thereby limiting atherogenesis.⁵⁶ MiR-495 is also able to promote ECs proliferation by targeting CCL2, but additional studies need to be performed to elucidate his potential role during atherogenesis⁵⁸. MiR-92a, which is preferentially up-regulated by the combination of disturbed flow and oxidized LDL, was also shown to be able to modulate endothelial proliferation in atheroprone regions and mouse models.⁵⁹ Indeed, *in vivo* blockade of miR-92a expression reduced endothelial inflammation, decreased atherosclerotic plaque size

and promoted a more stable lesion phenotype. Hence, anti-miR-92a therapy may serve as another atheroprotective therapeutic strategy.

MicroRNAs in endothelial senescence

As proliferating cells, ECs can also react to stress by undergoing a phenotypically distinctive and permanent form of growth arrest called “cellular senescence” which is a driver of vascular dysfunction. MiR-217 was shown to negatively regulate the expression of silent information regulator 1 (SirT1), an atheroprotective flow-induced anti-inflammatory deacetylase that activates eNOS and inactivates NF- κ B.⁶⁰ Indeed, overexpression of miR-217 in young ECs induced a premature senescent-like phenotype and decreased angiogenesis, whereas its inhibition in old ECs reduced senescence and increased angiogenesis. SirT1 can also be targeted by miR-34a, inducing EC senescence and suppressing cell proliferation by inhibiting cell cycle progression.⁶¹ On the other hand, aging, which is related to an increase of reactive oxygen species (ROS) and promoted senescence, decreases the expression of miR-146 in ECs, which negatively regulate the expression of NOX4, the main endothelial isoform of the NADPH oxidases complex.⁶² Thus, miR-146 overexpression appears to be a novel therapeutic tool for vascular disorders by targeting the production of ROS and therefore regulating senescence. That being said, all these studies were performed *in vitro* and further *in vivo* studies are needed to determine microRNAs ability to regulate endothelial senescence and potentially impact atherogenesis through this path.

3. MicroRNAs regulation of inflammatory pathways

Inflammation is a major contributor to atherosclerosis. In this context, macrophages are crucial in maintaining vessel-wall lipid homeostasis and coordinating inflammatory responses, thus playing a central role in the pathophysiology of atherosclerosis.⁶³ MicroRNAs are key players in macrophage biology by regulating their inflammatory capacity which can profoundly affect plaque evolution.⁶⁴ Several studies revealed an essential role for microRNAs in regulating macrophage polarization, a crucial component of the inflammatory response (Figure 1). For example, miR-124 inhibits macrophage activation and polarization from a pro-inflammatory M1 toward an anti-inflammatory M2 phenotype, by targeting the transcription factor C/EBP- α .⁶⁵ Similarly, miR-223 was shown to have a suppressive effect on macrophage pro-inflammatory activation by targeting the protein Pknox1, and miR-223-deficient macrophages exhibited increases in M1 and decreases in M2 polarization biomarkers.⁶⁶ MiR-33 is also involved in fate of atherosclerotic macrophages as miR-33 inhibition in hypercholesterolemic mice was shown to result in accumulation of inflammation-suppressing M2 macrophages, thus reducing atherosclerosis progression.⁶⁷ MiR-342-5p, expressed in lesional macrophages, is the most prominently induced microRNA during early atherosclerosis. Notably, miR-342-5p enhances pro-inflammatory macrophage mediators, such as inducible nitric oxide synthase and IL-6 via up-regulation of miR-155, and his inhibition was shown to reduce atherosclerotic lesion formation in ApoE^{-/-} mice.⁶⁸ MiR-155, is induced in macrophages in atherosclerotic regions of ApoE^{-/-} mice where it targets B cell leukemia/lymphoma 6 (BCL6), a transcription factor that attenuates NF- κ B activation.⁶⁹ ApoE^{-/-} mice with hematopoietic miR-155 deficiency

showed a reduction in macrophage inflammatory responses, increased macrophage cholesterol efflux, and reduced lesion size.⁷⁰ In contrast, bone marrow miR-155 deficiency in LDLR^{-/-} mice enhanced atherosclerosis by recruiting more myeloid inflammatory cells to the plaque and inducing a more pro-inflammatory macrophage phenotype.⁷¹ Together, these data reflect the stage-specific and lipid-dependent roles for endogenous miR-155 in regulating the inflammatory signaling pathways in atherosclerotic macrophages, which can differ in the different mouse models of atherosclerosis. Other microRNAs have been shown as regulators of macrophage response to inflammatory stimuli. For example, overexpression of miR-125b potentiates the responsiveness of macrophages to interferon (IFN- γ) via the miR-125b suppression of IFN regulatory factor 4.⁷² The related microRNA, miR-125a-5p, diminished the M1 phenotype but promoted M2 marker expression induced by IL-4.⁷³

Because lipoprotein uptake by macrophages is one of the earliest pathogenic events in nascent atherosclerotic plaques to induce the formation of foam cells, miR-125a-5p and miR-146a may play a protective role against the development of atherosclerosis as they were found to decrease lipid uptake and cytokine release in oxLDL-stimulated macrophages, notably through the targeting of oxysterol binding protein-like 9 and TLR4, respectively.^{74,75} Likewise, miR-155 overexpression reduced lipid uptake in monocytic cell lines and primary monocyte-derived dendritic cells *in vitro*,⁷⁶ but in primary macrophages from atherosclerotic ApoE^{-/-} mice, miR-155 was found to enhance oxLDL-induced foam cell formation by targeting HMG box-transcription protein 1, implying that miR-155 also has cell-specific functions.⁷⁷ Anti-miR-155 injection in ApoE^{-/-} mice effectively decreased lipid-laden macrophage accumulation in lesions as well as the formation of aortic atherosclerotic plaques.⁷⁷ However, as mentioned above, it is possible that miR-155 exerts stage-specific effects during atherogenesis, depending on which microRNA targets are expressed. Finally, miR-155 expression was significantly higher in CD14⁺ monocytes from patients with coronary artery disease than from healthy controls, pointing this microRNA as a potentially relevant myeloid target for atherosclerosis. Indeed, miR-155 was shown to mediate inflammatory mediators in macrophages to promote atherosclerotic plaque formation and rupture via the SOC1S-STAT3-PDCD4 axis.⁷⁸

Other immune cells, such as dendritic cells and T cells, are present and play an important role during atherosclerosis development, and microRNAs were shown to regulate their function. miR-181a targets the c-Fos transcription factor, and attenuates the oxLDL-induced inflammatory response by blocking the secretion of pro-inflammatory cytokines (TNF- α , IL-6), and reducing dendritic maturation cell surface molecules CD40 and CD83.⁷⁹ The activation of the T cell receptor increases the expression of miR-146a, which protects T cells by reducing apoptosis by targeting the pro-apoptotic factor Fas-associated death domain (FADD) and the NF- κ B mediators TRAF6/IRAK1.⁸⁰ Indeed, T cells lacking miR-146a are hyperactivated by antigen stimulation, however, more studies will be necessary to understand the role of dendritic- and T cell specific microRNA-mediated effects in the context of atherosclerosis.

MicroRNAs in smooth muscle cell phenotype

VSMCs play an important role in atherosclerosis development, notably through their shift from a “contractile” to a “synthetic” phenotype. Indeed, beside the higher rates of migration and proliferation, synthetic VSMCs express higher proportions of lipoprotein and scavenger receptors that could promote pathological lipid uptake.⁸¹ These last few years, microRNAs were shown to be involved in the phenotypic switch of VSMCs, especially the miR-143/-145 cluster which can direct VSMCs fate and regulate their quiescent versus proliferative phenotype (Figure 1).⁸² MicroRNA-143/-145 are abundantly expressed in VSMCs and can also be taking up by them from ECs-derived microvesicles.^{82,83} Following VSMC cholesterol loading, miR-143/-145 was shown to be involved in their conversion into a dysfunctional macrophage-like phenotype.⁸⁴ MiR-143/-145 effects on VSMCs differentiation and response to injury are a consequence of targeting several genes, such as KLF-4, KLF-5, or ACE.^{82,85,86} However, the role of miR-143/-145 in atherosclerosis is still under ongoing debate, as both overexpression and genetic deletion of miR-143/-145 reduces neointima formation and prevents atherosclerosis.^{86–88} As miR-143/-145, miR-133a prevents VSMCs from the switch toward a synthetic phenotype and its levels are decreased after vascular injury.⁸⁹ Moreover, miR-663 has been shown to promote VSMCs differentiation by targeting the transcription factor JunB and its downstream molecules such as myosin light chain 9 and matrix metalloproteinase 9, while local overexpression of miR-663 reduces neointima formation after carotid artery ligation injury.⁹⁰ Finally, miR-15b and miR-16 have also been described as important players in maintaining the contractile phenotype of VSMCs through targeting of the oncogene yes-associated protein.⁹¹ On the other hand, some microRNAs can also promote “dedifferentiation” of VSMCs. Indeed, miR-26a induces both VSMCs proliferation and migration by interfering with the TGF- β signaling pathway,⁹² while after a vascular injury, miR-21 contributes to neointima formation by promoting VSMCs proliferation through the targeting of Phosphatase and TENsin homolog protein and Bcl-2.⁹³ Similarly, miR-221 and miR-222 increase the proliferative rate of VSMCs by silencing Kip1 and Kip2. In fact, inhibition of miR-221 and miR-222 results in decreased VSMCs proliferation and reduced neointima formation after angioplasty.⁹⁴

4. MicroRNAs in atherosclerotic plaque rupture

Rupture-prone vulnerable plaques are typically associated with the presence of highly inflammatory cell content and a large necrotic core covered by a thin fibrous cap.⁹⁵ Accumulating evidence demonstrate various roles for microRNAs in processes related to the risk of atherosclerotic plaque rupture. Due to the fact that animal models poorly mimic the process of plaque rupture in humans, it has been difficult to directly assess the contribution of individual microRNAs in this process. Nonetheless, each step in the formation of a stable plaque is affected by microRNAs.

Fibrous cap thinning

Thickness of the fibrous cap is determined by the balance between collagen synthesis and breakdown. VSMCs are likely the main source of collagen and, as discussed above, microRNAs are key regulators of VSMCs phenotype with effects on atherosclerosis

progression. Moreover, several microRNAs are able to regulate collagen synthesis and fibrosis, including miR-133a, miR-145, miR-192, miR-21, and miR-29b.^{87,96–99} In addition to its ability to regulate VSMCs differentiation, overexpression of miR-145 enhances collagen type I and III expression in human aortic VSMCs and drives atherosclerotic plaques toward the acquisition of features typical of stable phenotype, such as increased collagen content and fibrous cap area.⁸⁷ As a result, miR-143/145 may lead to decreased VSMCs proliferation in during atherosclerotic plaque initiation, but also stabilize the fibrous cap in advanced plaques. Overexpression of miR-21 inhibits ROS-induced VSMCs apoptosis and death,¹⁰⁰ while delivery of vascular miR-126 in mice promoted an increase in the number of intimal VSMCs, higher collagen content, and reduced apoptotic cells, consistent with a more stable plaque phenotype.¹⁰¹ Notably, miR-29 might play a role in plaque stability as it was shown to directly suppress IFN- γ production by targeting IFN- γ transcript, which normally block the expression of pro-collagens by VSMCs.¹⁰² Collagens are stable proteins and their degradation relies on the activity of matrix-metalloproteinases (MMPs). In atherosclerotic plaques, macrophages are able to synthesize an array of MMPs including MMP-1, -2, -3, -8, -9, -13, -14.¹⁰³ MMP9 is one of the most highly expressed MMPs in unstable plaques and was shown to be targeted by miR-133a, but also indirectly upregulated by miR-21 via repression of its inhibitor, RECK, in macrophages.^{104,105} Moreover, anti-miR-24 therapy was shown to enhance MMP-14 proteolytic activity, thus promoting atherosclerotic plaque progression and characteristics associated with plaque instability, such as decreased collagen content and increased macrophage infiltration.¹⁰⁶ In summary, therapeutic stabilization of existing plaques could be achieved through direct targeting VSMC-derived microRNAs to prevent VSMC death, reduction in collagen production and breakdown in the fibrous cap.

Necrotic core

Death of foam cells can lead to sub-endothelial accumulation of cell debris, lipoproteins and cholesterol crystals, thus forming a lipid-rich area with low cell content, termed the necrotic core. Indeed, defective apoptotic cell clearance through impaired efferocytosis leads to apoptotic cell accumulation, delayed resolution of inflammation, and expansion of the necrotic core. MiR-155 inhibits efferocytosis by targeting BCL6, thus miR-155 deficiency reduces necrotic core formation and the deposition of apoptotic cell debris,¹⁰⁷ making the interaction between miR-155 and BCL6 as a promising target to inhibit atherosclerosis progression. By contrast, elevated macrophage miR-21 was shown to promote efferocytosis and suppress the innate immune response.¹⁰⁸ Interestingly, ABCA1 and ABCG1 are also involved in preserving macrophage cell viability during efferocytosis,¹⁰⁹ thus suggesting that macrophage microRNAs involved in the regulation of these transporters might affect efferocytosis and impact atherosclerosis evolution in advanced stages. Finally, in addition to breaching the fibrous cap in the necrotic core, cholesterol crystals also activate the NLRP3 inflammasome that induces the release of active IL-1 β and other cytokines to further promote the instability of atherosclerotic plaque.¹¹⁰ In this context, miR-223 negatively regulates the inflammatory response by blocking NLRP3 inflammasome and IL-1 β production.¹¹¹

5. Other risk factors for atherosclerosis & microRNA targeting

In addition to their role in cholesterol homeostasis, microRNAs are also involved in regulation of other risk factors that are linked to the development of atherosclerosis. Indeed, since the discovery of microRNAs, an increasing number of them have been found to be involved in diabetes mellitus pathogenesis, and their dysregulation can lead to profound impairment of glucose metabolism.¹¹² MicroRNAs can particularly affect pancreatic β -cells through the regulation of cell survival and apoptosis, proliferation, differentiation, or insulin secretion.¹¹³ Besides, they are also implicated in the insulin resistance regulation by targeting insulin receptors and their substrates.¹¹³ MicroRNAs have been also shown to be important regulators of hypertension and blood pressure. Notably, a murine models of Dicer deletion displayed a drop in blood pressure,¹¹⁴ as well as miR-143/-145 KO mice,¹¹⁵ and plethora of microRNAs were shown to be involved in hypertension in both human studies and animal models.¹¹⁶ Taken together, these studies demonstrated that microRNAs can regulate atherosclerosis development through a lot of different ways by affecting and regulating numerous related-diseases and risk factors.

6. Potential clinical applications of microRNAs

Unique and reliable biomarkers for atherosclerosis and plaque stability are greatly needed. MicroRNAs, especially circulating microRNAs, are currently investigated as biomarkers in a wide range of cardiovascular conditions including atherosclerosis. Indeed, circulating microRNAs are remarkably stable and easy to detect in body fluids. Moreover, changes in their expression are often tissue- or disease-specific. For instance, several studies revealed a distinct microRNA expression signature in the blood of patients with acute coronary syndrome.^{117,118} Combining multiple microRNAs in a microRNA profile may provide greater accuracy than assessment of a single one, thus making large-scale studies indispensable to establish the real potential of circulating microRNAs as biomarkers for atherosclerosis-related diseases.

A huge jump forward was made in the field of RNA therapeutics with the recent Food and Drug Administration approval of the Mipomersen, an antisense oligonucleotide drug targeting apoB to treat familial hypercholesterolemia.¹¹⁹ This will likely pave the way for other oligonucleotide-based drugs, including microRNAs therapies. The multiplicity of microRNA targets enables them to regulate several steps in a disease pathway by fine-tuning downstream signaling mediators in parallel. Thus, regulating specific stages of atherosclerosis through delivery of a pool of microRNA mimics or inhibitors might be an attractive therapeutic approach. Therapies based on microRNA inhibitors or microRNA mimics are now being developed to repress pathological microRNAs or over-express protective microRNAs, respectively. Indeed, numerous microRNAs therapeutics are in pre-clinical development, with two of them that have reached clinical trials. The first is a locked nucleic acid directed against miR-122 (Miravirsen), which targets hepatitis C virus RNA.¹²⁰ In non-human primates miR-122 inhibition resulted in long-lasting suppression of hepatitis C virus viremia with no evidence of viral resistance or side effects.¹²¹ In a human phase II study, Miravirsen demonstrated dose-dependent antiviral activity. Remarkably, in several patients treated with the highest doses of Miravirsen, hepatitis C virus RNA became

undetectable during the study.¹⁷ The second microRNA therapeutic in clinical trial is a double-stranded microRNA mimic of miR-34 (MRX34), which has entered a clinical Phase I trial in patients with advanced liver cancer. MRX34 is a miR-34 mimic encapsulated in a liposomal nanoparticle which acts as a tumor suppressor by inhibiting multiple oncogenic pathways and stimulating antitumor immune responses.¹²² Systemic delivery of microRNA-derived therapeutics are mostly taken up in the liver, the kidney, and the spleen,¹²³ but many additional peripheral tissues, such as vessels and heart, have also been successfully targeted using currently available delivery methods.⁴⁹ MicroRNA mimetic therapy using synthetic microRNAs offers the ability to reconstitute a microRNA that is downregulated during disease or to decrease gene pathways involved in disease pathology, but the delivery of therapeutic microRNA molecules faces many challenges because of 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. Conversely, single-stranded anti-miR oligonucleotides can be formulated in saline for subcutaneous or intravenous delivery and do not require lipid-based delivery systems. However, some of the current challenges associated with microRNAs therapeutics include the ability to target the drug to a specific tissue, and the potential requirement of multiple doses to achieve sustained target repression or microRNAs enrichment.

Also, whether therapeutic manipulation of microRNA may represent an efficient and safe atherosclerosis treatment in humans is still, however, an open debate. Although it is obvious that several microRNAs have an important regulatory impact on atherosclerosis *in vivo*, it is unclear what the contribution of individual microRNAs may be. Moreover, in most animal studies so far, the phenotypic effects of microRNAs therapeutics have only been studied in the targeted tissue of interest, and this might omit potential off-target effects of microRNAs in different tissues, stages, or environment. Indeed, because microRNAs can simultaneously alter expression of multiple target genes and often disrupt entire signaling networks, resulting in efficient changes in the activity and phenotype of target cells, this broad functionality can be a liability that results in difficult-to-control off-target effects and toxicities. Lentiviruses (LVs), adenoviruses (AVs), and adeno-associated viruses (AAVs) can transfer microRNA sequences,¹²⁴ which after viral entry into target cells are transcribed and processed into mature microRNA. Despite efficient cellular delivery, viral vectors raise concerns over safety of genomic integration of LVs, which may trigger expression of oncogenes, or excessive immunogenicity and the transient nature of microRNA expression in the case of AVs and AAVs. Nonviral methods of microRNA delivery instead rely on lipid and polymeric nanoparticles to protect the microRNA from degradation by nucleases and increase their half-life in the circulation, but the cell-specific delivery is still a major concern. Recently, a new strategy was developed for the targeted delivery of microRNA mimics. The tumor suppressor let-7g microRNA mimic was conjugated in the form of an unformulated oligonucleotides to an aptamer specific for the oncogenic tyrosine kinase receptor, Axl, which is expressed by various types of solid tumors.¹²⁵ The resulting aptamer-microRNA conjugate retained the cellular specificity, binding affinity, and inhibitory function and was able to successfully target let-7g in target tissues.¹²⁶ Although this has not yet been extended to chronic and multi-cellular diseases like atherosclerosis, the

combined targeting of a disease-specific microRNA with a cell-specific receptor shows the promise of this approach.

Conclusion

In conclusion, there is little doubt that microRNAs are an important biological player in the development and progression of atherosclerosis. To enable to therapeutic path forward, it will be critical to understand the full complement of targets of specific microRNAs and understand what microRNA pathways may cross-talk. As RNA-based therapeutics move into the clinic, targeting microRNAs for chronic diseases like atherosclerosis remain not far off the horizon.

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Summary

Atherosclerosis is a multifactorial and slowly progressing disease responsible for most of the cardiovascular morbidity and mortality in industrialized countries. MicroRNAs are non-coding RNAs that are predicted to regulate translational repression of ~60% of the human genome, and thus involved in regulation of every cellular processes.

Accumulating studies unveiled a prominent role for microRNAs in regulating atherogenesis, making them promising candidate for microRNAs-based therapies and clinical applications.

Figure 1.

Overview of the major microRNAs and their impacts on the cellular mechanisms that drive atherosclerosis. Arrow (—>) denotes promotion and line (—|) denotes inhibition.

EC=endothelial cell, SMC=smooth muscle cell, LDL=low-density lipoprotein, MMP=matrix metalloproteinases,

Table I

Summary of important microRNA-related terms.

3'UTR	The 3' UTR controls many aspects of mRNA metabolism, such as transport, localization, efficiency of translation and stability. 3' UTRs can extend over several kilobases and generally contain binding sites for various regulatory proteins and microRNAs allowing dynamic and combinatorial regulation
MicroRNA-3p	The mature miRNA strand derived from the 3' arm of the precursor duplex.
MicroRNA-5p	The mature miRNA strand derived from the 5' arm of the precursor duplex.
RISC	A ribonucleoprotein complex that consists of a small RNA guide strand bound to an Argonaute protein. RISC mediates all RNA-silencing pathways, and it can also include auxiliary proteins that extend or modify its function
Seed sequence	Nucleotides at position 2–8 at the 5' end of microRNAs, which interact through complementary pairing with the target mRNA in the 3' UTR
Antagomirs	Oligonucleotides of about 22 nucleotides that are complementary to a given microRNA sequence and can specifically inhibit microRNA activity

Table II

Summary of microRNA-based therapies tested in pre-clinical models of atherosclerosis.

microRNA-based therapies	Effect(s)	References
Anti-miR-148a	Reduction of LDL-C, and increase of LDLR, ABCA1 and HDL-C levels in transgenic mice	13
Anti-miR-122	Reduction of plasma triglycerides and cholesterol levels in mice	16
MiR-30c overexpression	Reduction of hyperlipidemia and atherosclerosis development in ApoE ^{-/-} mice	21
Anti-miR-33	Increase in HDL levels, enhancement of cholesterol efflux, and reduction of atherosclerosis in LDLR ^{-/-} and ApoE ^{-/-} mice and primates.	23, 24, 31, 33
Anti-miR-33	No alteration of atherosclerosis development in LDLR ^{-/-} mice	34
MiR-126-5p overexpression	Increase of endothelial cell proliferation and reduction of atherosclerosis in ApoE ^{-/-} mice	56
Anti-miR-92a	Reduction of endothelial inflammation and atherosclerosis development in LDLR ^{-/-} mice	59
Anti-miR-33	Increase of anti-inflammatory M2 macrophages, and reduction of atherosclerosis in LDLR ^{-/-} mice	67
Anti-miR-155	Reduction of atherosclerotic lesion formation in ApoE ^{-/-} mice	68–70
Anti-miR-155	Enhancement of atherosclerotic development in LDLR ^{-/-} mice	71