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REVIEW ARTICLE

Pathogenic role of microRNAs in atherosclerotic ischemic stroke: Implications for diagnosis and therapy

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Abstract Ischemic stroke resulting from atherosclerosis (particularly in the carotid artery) is one of the major subtypes of stroke and has a high incidence of death. Disordered lipid homeostasis, lipid deposition, local macrophage infiltration, smooth muscle cell proliferation, and plaque rupture are the main pathological processes of atherosclerotic ischemic stroke. Hepatocytes, macrophages, endothelial cells and vascular smooth muscle cells are the main cell types participating in these processes. By inhibiting the expression of the target genes in these cells, microRNAs play a key role in regulating lipid disorders and atherosclerotic ischemic stroke. In this article, we listed the microRNAs implicated in the pathology of atherosclerotic ischemic stroke and aimed to explain their pro- or antiatherosclerotic roles. Our article provides an update on the potential diagnostic use of miRNAs for detecting growing plaques and impending clinical events. Finally, we provide a perspective on the therapeutic use of local microRNA delivery and discuss the challenges for this potential therapy.

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Introduction

Stroke is the second leading cause of death worldwide and is a main cause of permanent disability in adults.[1] When the brain is deprived of oxygen and nutrients either because of artery occlusion or rupture, the neurons near the injured area die, usually leading to irreversible damage, such as disability. According to a report by the World Health Organization,[2] stroke leads to over 5 million deaths and over 40 million disabilities every year. In the United States, ischemic strokes are responsible for approximately 87% of all stroke cases, and the remaining cases are hemorrhagic strokes or result from unknown causes.[3] In China, ischemic strokes are responsible to approximately 70% of all stroke cases. Men are significantly more vulnerable to this disease than women.[4] Cerebral ischemic stroke is an occurrence of neurological injury caused by a lack of cerebral blood flow.[5] As the brain is the largest consumer of oxygen and glucose in the body, the subsequent appearance of dead neural tissue may occur very quickly, usually in a few seconds.[6] The death of tissue in different areas of the brain may lead to disability of diverse degrees after stroke or even to death of the sufferers.

Atherosclerosis, hyperlipidemia, or plaque rupture all result in focal ischemia.[7] Intravascular atheroma is a pathologically deleterious tissue composed of fat, collagen, elastin, macrophages and a thin layer of smooth muscle cells wrapping around the contents. This atheromatous plaque is fragile and easily ruptures or falls off the vascular intima under shear stress caused by the bloodstream and blocks blood flow. Even if the plaque is stably adhered to the intima, it continues to grow (particularly in patients consuming a high-fat diet), and over time, the vessels become narrower and the blood flow decreases. Insufficient oxygen and nutrient delivery are unable to satisfy the huge demand and support the normal function of healthy neural tissues, which leads to the production of reactive oxygen species (ROS). In addition, wastes (e.g., CO_2 and urea) generated by cellular activities are not removed by the plasma in a timely manner; therefore, neuronal cells initiate the inflammatory response and ultimately undergo apoptosis.

The formation of atheromatous plaques involves a series of cellular and vascular events over a long period, usually several years or decades. Although plaques have been studied for many years, the exact mechanism of plaque formation remains unclear. One hypothesis is that some factor induces monocytes to begin to adhere to and enter the endothelium of vessels, leading to focal inflammation. The theory of lipid retention in the atheroprone areas of arteries that leads to inflammation of endothelial cells is also reasonable. Regardless of how atherosclerosis is initiated, this pathological event is mediated by maladaptive inflammation. Monocytes that migrate into the subendothelial space differentiate into macrophages and then proliferate and ingest invading oxidized low-density lipoprotein (oxLDL) from the bloodstream. At the same time, they are equipped with increasingly upregulated lipid uptake receptors to absorb lipids; ultimately, these macrophages become foam cells and die. From the intravascular side, these foam cells form a visible fatty streak covered by a fibrous capsule derived from smooth muscle cells. These smooth muscle cells are originally located in the muscle layer of the vessel wall but migrate to the endothelial surface in response to cytokines secreted by foam cells and damaged endothelial cells. More monocytes are continuously recruited to the lesion, and the plaque continues to grow. Later, calcium, collagen and platelets deposit both under the endothelium and on the surface of the growing plaque. Smooth muscle cells also ingest cholesterol and eventually become foam cells. Together, these cells and structures form a fatty plaque, releasing enzymes that enlarge the artery. Once the expansion of the artery is unable to compensate for the narrowing lumen caused by the atheroma, the blood flow is reduced. However, rupture of the plaque more frequently leads to acute clotting and enlargement of the plaque, which causes a complete obstruction of blood flow and results in ischemia of the brain tissue.[8–10]

MicroRNAs are 22-nucleotide small noncoding RNAs that exist in diverse animals, plants and viruses. They regulate the expression of target genes through complementary binding to messenger RNAs (mRNAs). MicroRNAs belong to the small noncoding RNA family and are not translated. They are originally double-stranded RNAs that are transcribed from target genes but then are cleaved into two single-stranded RNAs, miRNA-3p and miRNA-5p, by an endonuclease, DICER. Each single-stranded RNA is then incorporated into a ribonucleoprotein called the RNA-induced silencing complex (RISC), in which the RNA serves as a template. Once the miRNA template binds to the complementary binding sites in the 3′ untranslated regions (3′UTR) of the free target mRNA, the RISC is activated, and the target mRNA is either degraded or its translation is slowed. By targeting functional genes or genes involved in regulating the expression of functional genes (such as intermediate regulators of signaling pathways), miRNAs confer direct loss-of-function or indirect gain-of-function effects on target genes. In the last 20 years, an increasing number of miRNAs has been shown to be associated with ischemic stroke. These miRNAs are involved in the mechanism of vessel occlusion, in the injury caused by ischemia, and in self-protection and repair after stroke. Among these functions, the role of miRNAs in the formation of atherosclerotic plaques leading to ischemic stroke is a topic of heated discussion. Therefore, in this article, we aimed to thoroughly review the role of microRNAs in the etiology of atherosclerotic ischemic stroke by focusing on 4 specific cell types: liver cells, macrophages, endothelial cells, and vascular smooth muscle cells. We summarized how miRNAs intervene in lipid delivery and deposition, local inflammation, fibrous cap stability, etc., which are all crucial processes involved in atheroma formation.

Various cell types contribute to the etiology of atherosclerosis

Liver cells play an important role in regulating lipoprotein homeostasis. They control both the production and elimination of lipoproteins, including delivering lipids to the tissue by low-density lipoprotein (LDL) and removing lipids

from the tissue by high-density lipoprotein (HDL). The disruption of this homeostasis (such as excessive production or insufficient retrieval of lipids) is one of the main factors inducing hypercholesterolemia, as the number of monocytes is increased 1.5 times and subsequently increases atherosclerosis.[11]

Macrophages are the main inflammatory cells located in atherosclerotic lesions. Upon the initiation of inflammation, they are attracted to the lesion area and activated. Activated macrophages are either proinflammatory (M1 phenotype) or anti-inflammatory (M2 phenotype), depending on the different signals that initiate two opposite activation programs.[12] The M1 phenotype is induced by proinflammatory cytokines such as lipopolysaccharide (LPS), TNF-α and interferon (IFN)-γ produced by Th1 cells, whereas inducers of the M2 phenotype include IL-4, IL-10, IL-13 and tumor growth factor (TGF)-β produced by Th2 cells.[13] Predominant M1 macrophages in the lesion are associated with atheroma progression, whereas M2 macrophages mediate the regression of atheroma. Regardless of the M1 or M2 phenotype, the accumulated macrophages (foam cells) are the dominant component of plaques and are associated with plaque stability.

Endothelial cells (ECs) function as a docking site for monocytes and play a pivotal role in promoting inflammation. Under hyperlipidemic conditions, ECs (particularly cells located in the predilection area of vessels that are exposed to disturbed blood flow) are prone to injury caused by oxidized LDL. Once insulted by leukocytes, TNF-α released from leukocytes activates signaling cascades that ultimately alter gene expression in endothelial cells.[14] This change initiates inflammation, activates endothelial cells, increases permeability, and upregulates adhesion molecules on the cell surface, which allow leukocytes to adhere to the endothelium, enter the extracellular matrix under the endothelium and induce an inflammatory response.

Vascular smooth muscle cells (VSMCs) are derived from diverse cell types that contribute to atheroma formation, such as collagen-releasing cells and macrophage-like cells. VSMCs also form foam cells by expressing LDL receptors to endocytose lipids.[15] In addition, VSMCs play an important role in inflammation by expressing adhesion molecules such as VCAM-1 and ICAM-1 that facilitate monocyte adhesion and migration.[16] Throughout the progression of atherosclerosis, the proliferation of VSMCs is beneficial, whereas the apoptosis, senescence and differentiation of VSMCs into macrophage-like cells all contribute to atherosclerosis.[17] In advanced lesions, proliferating VSMCs play an important role in protecting plaques from rupture because VSMCs are the main cell component of the fibrous cap, and a thicker fibrous cap indicates a more stable plaque.

MiRNAs play diverse roles in different cell types by inhibiting the expression of specific targets

MiRNAs regulate lipid homeostasis in liver cells

To date, a number of miRNAs have exhibited an ability to regulate lipid homeostasis in the liver. For example, miR-27a/b comprehensively targets the genes involved in cholesterol esterification (acyl coenzyme a-cholesterol acyltransferase, ACAT-1), uptake (LDL and CD36), and efflux (ATP binding cassette transporter A1, ABCA1).[18] A miRNA-122 deficiency results in suppressed expression of various genes encoding cholesterol synthesis-related proteins, such as HMGCR.[19] Notably, miR-223 exerts its atheroprotective effect by suppressing foam cell formation, lipid deposition and proinflammatory cytokine generation. It targets cholesterol synthesis genes such as HMGCS1, SMO, and HDL uptake genes such as SRB1. SRB1 not only mediates the uptake of HDL into liver cells but also removes cholesterol from macrophages in the periphery,[20] indicating its key role in preventing atheroma formation. In addition to miR-223, other miRNAs, such as miR-96, miR-125a, miR-185, and miR-455, also target SRB1, but *in vivo* evidence is currently insufficient to confirm their roles in increasing plasma HDL levels (Fig. 1).

In addition to SRB1, the LDL receptor also controls plasma cholesterol levels through the endocytosis of cholesterol-rich LDL. The miRNAs targeting LDLR include miR-27a/b, miR-128-1, miR-130b, miR-148a, miR-185 and miR-301b, which are proatherosclerotic miRNAs because they increase plasma LDL levels. The inhibition of miR-128-1 and miR-148a upregulates LDLR in liver cells and reduces plasma lipid levels.[21,22] The inhibition of miR-185 *in vivo* significantly increases LDLR expression and decreases the plaque area in mice.[23] Some of these miRNAs also have additional targets to inhibit atheroma formation. Pan S et al found that miR-130b also translationally repressed peroxisome proliferator-activated receptor γ (PPAR-γ).[24] As a multifunctional nuclear receptor, PPAR-γ is associated with adipocyte differentiation and inflammatory cytokine production from monocytes,[25] indicating that miR-130b possesses antiatherosclerotic properties. Pan S et al also found that miR-130b increased the level of miR-378a-3p; however, the latter is implicated in enhanced adipogenesis by antagonizing mitogen-activated protein kinase,[26] but the authors did not determine whether this property of miR-378a-3p has implications for atherosclerosis. Notably, miR-27b appears to have a controversial role in atherosclerosis. Although miR-27b inhibits LDLR and ABCA1, as mentioned above, scientists also found that it did not necessarily influence plasma cholesterol levels.[27] Further studies are needed to identify the explicit role of miR-27b in atherosclerosis.

Apolipoprotein B (apoB) is a protein that carries insoluble lipids in blood vessels and helps deliver lipids to tissues. It is produced in liver cells, wraps around lipids to form chylomicrons or LDLs and is then released into the bloodstream. Overexpression of miR-30c decreases the release of apoB lipoproteins from liver cells by inhibiting lysophosphatidylglycerol acyltransferase 1 (LPGAT1), which is involved in lipid biosynthesis.[28] Moreover, miR-30c also targets microsomal triglyceride transfer protein (MTP) involved in the assembly and secretion of VLDL, ultimately alleviating hypercholesterolemia and atherosclerosis.[28] Likewise, miR-122 decreases the production of very low-density lipoprotein by targeting MTP.[29] Importantly, miR-548 functions by targeting the apoB protein, HMGCR, and ACSL4, which are all involved in VLDL production.[30]

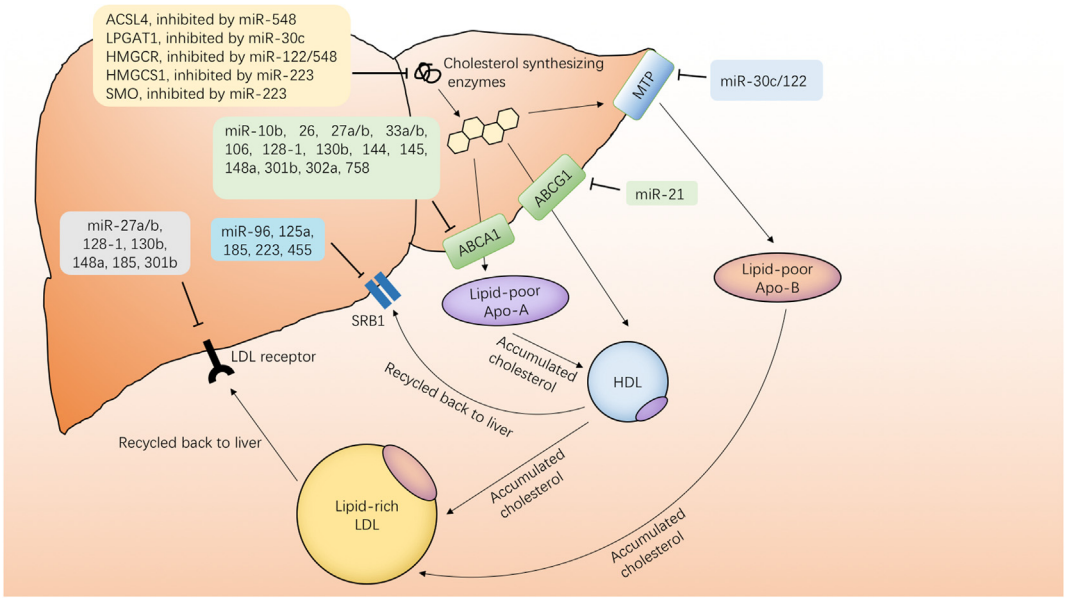

Figure 1 The important miRNAs in lipid metabolism in the liver. Note that the flat arrow indicates the inhibitory effect of miRNAs on the target. The primary mechanism by which miRNAs regulate lipid homeostasis is through targeting lipid transporters and lipid receptors and regulating lipid biosynthesis. Some lipid output processes in this figure are also applicable to macrophages because some transporters (such as ABCA1 and ABCG1) are commonly expressed in macrophages and liver cells. However, the transport of cholesterol from macrophages to the blood prevents hypercholesterolemia and atherosclerosis. Therefore, although the miRNAs targeting these transporters are expressed in both macrophages and liver cells, they exert different functions in inducing atherosclerosis. Abbreviations: ACSL4, acyl CoA synthetase long chain family member 4; LPGAT1, lysophosphatidylglycerol acyltransferase 1; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; SMO, sterol-C4-methyl oxidase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; ABCA1, ATP binding cassette transporter A1; MTP, microsomal triglyceride transfer protein; SRB1, scavenger receptor class B type 1.

MiRNAs regulate macrophage behaviors in atherosclerosis

MiRNAs regulate the recruitment and activation of macrophages. Several miRNAs are involved in regulating the inflammatory response of macrophages to stimuli by enhancing or attenuating signaling pathways. For example, by regulating the intracellular Ca^{2+}-activated PKC-oxLDL-LOX-1 pathway, miR-let-7g attenuates macrophage recruitment and migration in the subendothelial region.[31] By targeting programmed cell death 4 (PDCD4), which is implicated in the secretion of proinflammatory cytokines important for macrophage activation, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, miR-16 inhibits macrophage activation; miR-16 is also known to regulate proinflammatory MAPK and NF-κB signaling.[32] MiR-21 is a multifunctional atheroprotective miRNA involved in various aspects of atherosclerosis, such as vascular inflammation, foam cell formation, cell apoptosis and plaque necrosis. A miR-21 deficiency in macrophages increases the level of mitogen-activated protein kinase kinase 3 (MKK3), which activates p38/JNK/ERK signaling. This signaling pathway increases macrophage apoptosis and aggravates inflammation.[33] In addition to miR-21, miR-147 has also been shown to counteract macrophage apoptosis and attenuate inflammation (Fig. 2).[34]

The NF-κB signaling pathway is well known to regulate the inflammatory response in macrophages, endothelial cells and vascular smooth muscle cells. Various proteins, such as TNF-α, IL-α and TLRs, activate NF-κB signaling.[35] Upon ligand binding, PI3K and PKC are activated and then activate NADPH oxidase, which is responsible for producing ROS, an activator of IκB kinase. Subsequently, activated IκB, an inhibitor of NF-κB signaling, is degraded, and the NF-κB protein is released.[36] Activated NF-κB enters the nucleus and induces the expression of numerous genes. These genes are involved in inducing the production of cytokines such as TNF-α and adhesion molecules such as E-/P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule (ICAM-1).[37] Cytokines amplify the inflammatory response, while adhesion molecules mediate chemoattraction between ECs and leukocytes. However, the outcomes of NF-κB activation in different cells are diverse. For example, in endothelial cells, NF-κB signaling promotes inflammation, while in macrophages, it alleviates inflammation.[37] One of the targets of NF-κB is miR-146a that is involved in a feedback loop of the NF-κB pathway in macrophages. During the last few decades, its roles have been extensively exploited and clearly illustrated by numerous scientists. In terms of the regulation of inflammation in macrophages, miR-146 binds to TNF receptor-associated factor 6 (TRAF6) and IL receptor-associated kinase 1 (IRAK1), which are both upstream adaptors of NF-κB signaling, to reduce NF-κB signaling and attenuate inflammation.[38] A miR-21 deficiency coincides with the activation of NF-κB, indicating

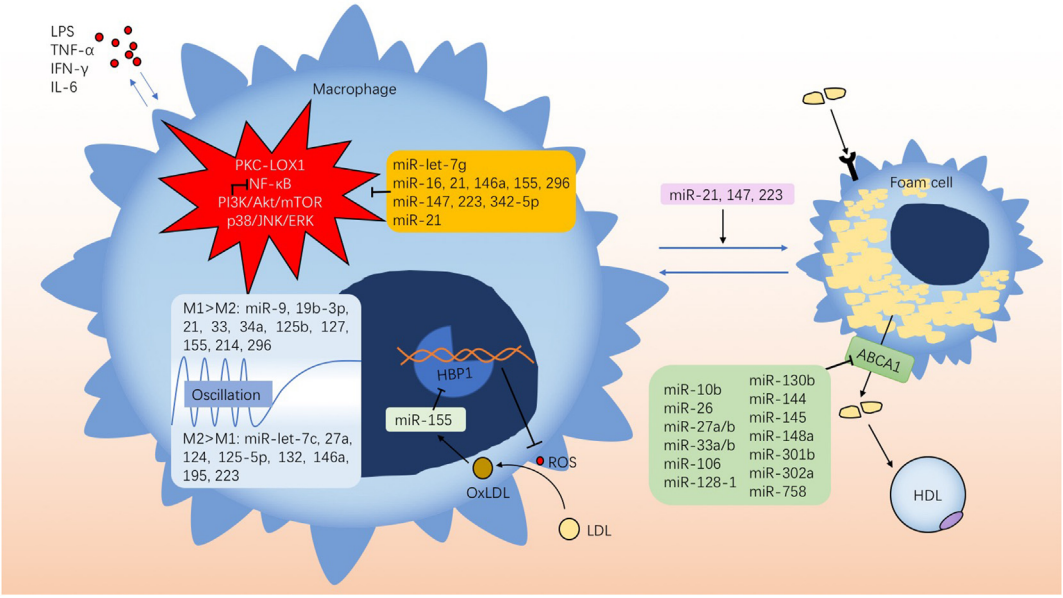

Figure 2 The miRNAs involved in biological processes that occur when macrophages transform into foam cells. Note that the flat arrow indicates the inhibitory effect of miRNAs on the target, while the normal arrow indicates the effect of miRNAs on increasing the expression of the target. The large cell on the left represents a macrophage, and the small cell on the right represents a foam cell. We chose the miRNAs associated with macrophage polarization, inflammation, foam cell transformation and lipid output and present these miRNAs in this figure. Once macrophages are exposed to proinflammatory signals, such as LPS, TNF-α, IFN-γ and IL-6, they experience substantial M1-M2 oscillations. MiR-9,16b-3p, 21, 33, 34a, 125b, 127, 155, 214, and 296 drive macrophages toward the proinflammatory M1 phenotype, and miR-let-7c, 27a, 124, 125-5p, 132, 146a, 195, and 223 drive macrophages toward the anti-inflammatory M2 phenotype. The M1 macrophages induce inflammation, which is mediated by several signaling pathways, such as the PKC-LOX1, NF-κB, PI3K/Akt/mTOR, and p38/JNK/ERK signaling pathways. These pathways are also targeted by distinct miRNAs. MiR-let-7g targets PKC-LOX1; miR-16, 21, 146a, 155, and 196 target NF-κB; miR-147, 223, and 342-5p target PI3K/Akt/mTOR; and miR-21 targets p38/JNK/ERK. Note that PI3K/Akt/mTOR signaling inhibits NF-κB signaling. At the same time, these activated macrophages produce more inflammatory cytokines and act on ECs, VSMCs, and themselves. This signaling undoubtedly forms a positive feedback loop that aggravates inflammation. ROS are also inflammatory factors, but their upregulation does not propagate inflammation; instead, ROS participate in a negative feedback loop. ROS are responsible for transforming LDL to oxLDL, and the latter upregulates miR-155. MiR-155 suppresses HBP1, a transcriptional repressor, which ultimately inhibits ROS production. Disordered lipid metabolism and inflammation gradually drive macrophages to transform into foam cells. MiR-21, 147, and 223 may facilitate and accelerate this process. ABCA1 is probably the most important transmembrane lipid pump expressed in macrophages. A list of miRNAs that inhibit its expression is shown in the figure. Abbreviations: NF-κB, nuclear factor-κ light chain enhancer of activated B cells; HBP1, HMG box-transcriptional protein 1.

that miR-21 negatively regulates inflammation in macrophages.[39] In contrast, miR-296 targets Numb 1, which inhibits NF-κB, thereby enhancing inflammation.[40]

Another predominant miRNA that regulates atherosclerosis, miRNA-155, is also a downstream target of NF-κB signaling. However, the role of miR-155 in atherosclerosis is still unclear. Some researchers found that miR-155 promotes atherosclerosis, while others reported conflicting results. For example, Nazari-Jahantigh M et al argued that miR-155 targets B-cell lymphoma 6 (Bcl6), a transcription factor that suppresses NF-κB signaling.[41] Therefore, miR-155 appears to promote NF-κB signaling through a positive feedback mechanism. Additionally, miR-155 knockout in mice decreases the expression of CCL2, which is important in recruiting monocytes.[41] Moreover, miR-155 suppresses the expression of negative regulators of inflammatory mediators, such as suppressor of cytokine signaling (SOCS1, which is also the target of several other miRNAs mentioned later) and Src homology 2 domain-containing inositol-5-phosphatase-1 (SHIP1).[42,43] This evidence suggests that miR-155 plays a crucial role in local inflammation. However, in other studies, completely opposite results were obtained. For example, Huang RS et al showed that miR-155 inhibition promoted NF-kB activation and the release of several proinflammatory cytokines.[44] Moreover, Li X et al suggested that miR-155 alleviates inflammation by repressing calcium-regulated heat stable protein 1 (CARHSP1) expression.[45] Obviously, the exact role of miR-155 is still a topic of heated discussion, and more evidence is needed to verify its function.

PI3K/Akt/mTOR signaling is another well-known proinflammatory pathway that has been proven to be a negative regulator of TLR4/NF-κB signaling.[46] Likewise, this pathway is targeted by a few miRNAs, such as miR-147 and miR-223.[47–49] The expression of miR-223 is predominantly upregulated in the lesional area, and this overexpression is associated with decreased foam cell formation, lipid accumulation and proinflammatory cytokine production, indicating that miR-223 reduces atherosclerosis through

many pathways.[49] The mechanism underlying these functions may be that miR-223 activates the PI3K/AKT pathway, hence attenuating inflammation.[49] Notably, miR-342-5p is a prominent enhancer of inflammation in macrophages that targets Akt1. Akt1 suppresses miR-155 expression, and the expression of miR-155 is associated with nitric oxide synthase 2 (Nos2) and IL-6 induction (both are proinflammatory cytokines of macrophages). During early atherosclerosis, miR-342-5p is upregulated and targets Akt1, resulting in increased Nos2 and IL-6 production.[50]

Another mechanism by which miRNAs regulate macrophage behavior in the pathogenesis of atherosclerosis is by modulating macrophage polarization. Various miRNAs are implicated in regulating the balance between M1 and M2 macrophages, including miR-let7c, miR-9, miR-19, miR-21, miR-27a, miR-33, miR-34a, miR-124, miR-125a-5p, miR-125b, miR-127, miR-132, miR-146a, miR-155, miR-195, miR-214, miR-223, and miR-296. Among these miRNAs, some have M2-promoting and M1-preventing functions. For example, miR-let7c is believed to maintain M2 traits, and overexpression of miR-let7c in M1 macrophages favors the loss of M1 traits.[51] Additionally, miR-27 promotes the M2 gene expression program and stimulates the secretion of IL-10, a well-known inflammation inhibitor.[52] By targeting the C/EBP-α-PU.1 pathway, miR-124 favors the M2 phenotype.[53] Moreover, miR-125a-5p also favors M2 polarization and inhibits the M1 phenotype, probably by targeting Krueppel-like factor 13 (KLF13).[54] MiR-195 significantly reduces the production of cytokines needed for M1 polarization and impairs smooth muscle cell migration; therefore, it is an atheroprotective miRNA.[55] IL-4 stimulates the expression of miR-223, whereas LPS exposure reduces miR-223 expression. A deficiency in miR-223 leads to a phenotype that is more similar to M1 macrophages, probably through targeting Pbx/knotted 1 homeobox (Pknox1), but more evidence is needed to prove the role of Pknox1 in M1 polarization.[56]

Other miRNAs promote the M1 gene expression program. For example, peroxisome proliferator-activated receptor δ (PPARδ) is suggested to be involved in the M1 proinflammatory program, which coincides with the upregulation of miR-9.[57] SOCS1 is a target of miR-19b-3p, and the inhibition of SOCS1 leads to the M1 phenotype.[58] MiR-21 upregulates TNF-α and IL-6 and downregulates IL-10, facilitating M1 polarization.[59] MiR-33 drives macrophages toward the M1 phenotype by promoting aerobic glycolysis, which provides energy to M1 macrophages. In addition, miR-33 suppresses lipid oxidation, which fuels M2 macrophages.[60] MiR-34a is upregulated in response to TNF-α and is associated with the M1 phenotype.[56] MiR-125b targets interferon regulatory factor 4, thus enhancing the response of activated M1 macrophages to IFN-γ.[61] MiR-155 not only promotes the M1 phenotype but also represses the M2 phenotype. By targeting SOCS1, miR-155 increases proinflammatory IFN signaling by releasing the inhibition of its adaptor STAT1 and simultaneously decreasing the level of IL-13 that drives the M2 phenotype.[62] Similarly, miR-296 targets STAT5A, which is responsible for inducing SOCS-2, a negative regulator of M1, thereby inducing the M1 phenotype.[40] MiR-214 is a target of NF-κB that is implicated in increasing TNF-α and IL-6 production, thus playing an important role in the positive feedback loop that amplifies M1 signals.[63]

Several miRNAs also regulate macrophage lipid deposition and control the foam cell phenotype transition by targeting genes involved in lipid deposition in macrophages. For example, ABCA1, ABCB11, and ATP8B1 are transmembrane cholesterol pumps expressed on both liver cells and macrophages. They transport cholesterol from the cytosol to apolipoprotein A-I (apoA-I), the main carrier of lipids in the circulatory system. Since ABCA1 is located on both liver cells and macrophages, an ABCA1 deficiency might lead to different atherosclerotic outcomes in different cells; in macrophages, a deficiency in this protein remarkably increases the risk of atherosclerosis, whereas in the liver, this deficiency alleviates hyperlipidemia and atherosclerosis. A growing number of miRNAs have been shown to target ABCA1, including miR-10b, miR-26, miR-27a/b, miR-33a/b, miR-106, miR-128-1, miR-130b, miR-144, miR-143/145, miR-148a, miR-301b, miR-302a and miR-758.[18,21,37,64–67] A decrease in the level of ABCA1 on the cell membrane of macrophages traps the absorbed lipids in macrophages and eventually drives these macrophages to transform into foam cells, leading to the growth of atherosclerotic lesions. Notably, some miRNAs, such as miR-27a/b, miR-128-1, miR-148a, and miR-223, regulate both lipid uptake and lipid output, as mentioned above, which requires further investigation of the exact pro- or anti-atherosclerotic outcome. In addition to ABCA1, ABCG1 is also a critical transporter involved in lipid efflux in macrophages that is increasingly degraded in cells lacking miR-21.[33]

As discussed above, miR-155 itself in fact plays a controversial role in atherosclerosis. One line of evidence supporting the antiatherosclerotic role of miR-155 is that a miR-155 deficiency promotes lipid deposition in macrophages.[68] This finding may be explained by miR-155-mediated inhibition of HMG-box transcription factor 1 (HBP1), a transcriptional repressor known to repress macrophage inhibitory factor (MIF) and $p47^{phox}$ expression,[69,70] consequently decreasing oxLDL uptake and ROS production in macrophages.[71] As stated above, further studies are needed to elucidate the role of miR-155 in atherosclerosis. However, not all miRNAs exacerbate lipid deposition. For example, both miR-21 and miR-147 counteract the foam cell phenotypic transition.[34] Moreover, miR-223 overexpression is associated with the amelioration of lipid deposition by targeting TLR4.[49]

MiRNAs regulate EC behaviors in atherosclerosis

The primary mechanism by which miRNAs control atherosclerosis progression is to regulate the inflammatory process of ECs. MiR-126 is one of the most dominant miRNAs expressed in ECs and has long been a research hotspot. In the past few years, scientists found that miR-126 was upregulated concomitantly with the blockade of PI3K/Akt/NF-κB signaling.[72] In addition, miR-126 is suggested to reduce the production of ROS and the expression of TNF-α by targeting TRAF7. TRAF7 is responsible for binding to the TNF receptor and inducing ROS production; therefore, TRAF7 inhibition protects ECs from oxidative stress and

subsequent inflammation and apoptosis.[73] Similarly, miR-10a significantly inhibits inflammation in ECs. Knock out of miR-10a significantly increases the phosphorylation of IκBα and aggravates inflammation. In addition, a miR-10a deficiency leads to upregulated expression of monocyte chemoattractant protein (MCP)-1, IL-6, IL-8, VCAM-1 and E-selectin. Moreover, miR-10a suppresses the expression of two promotors of IκBα degradation, mitogen-activated kinase kinase kinase 7 (MAP3K7) and β-transducin repeat-containing gene (βTRC).[74] Notably, miR-92a attenuates endothelial inflammation by targeting KLF2/4 and SOCS5 (Fig. 3).[75]

In addition to its role in macrophages, miR-146 also functions as a potential inhibitor of EC activation and inflammation. We have mentioned several times that miR-146a represses NF-κB signaling pathways. The inhibition of HuR, a mediator of NF-κB signaling, by miR-146 may also support the atheroprotective role of miR-146. Additionally, miR-146 suppresses both mitogen-activated protein kinase (MAPK) and the JNK/AP-1 pathway.[76] In contrast to its obscure role in macrophages, miR-155 produced by ECs clearly exerts a protective effect. In ECs, myosin light chain kinase mediates the opening of cell—cell junctions between ECs and increases the permeability of ECs in response to proinflammatory factors, which incapacitates the endothelial barrier and promotes monocyte migration. However, a deficiency in myosin light chain kinase caused by miR-155 targeting reduces atherosclerosis, proving the atheroprotective role of miR-155 in mice.[77]

Based on considerable evidence, miR-181 is another predominant atheroprotective miRNA. In mice, miR-181b inhibits NF-κB signaling and reduces atheromas; *in vitro*, various proinflammatory factors reduce miR-181b expression in endothelial cells. Generally, both miR-181-5p and miR-181-3p reduce the expression of genes involved in inflammation, such as adhesion molecules and inhibitors of the inflammatory signaling pathway. They also suppress the recruitment of macrophages into lesions. Previous studies have suggested that miR-181b inhibits importin-α, which is important in the translocation of the transcription factor NF-κB (whereas overexpression of miR-181b in leukocytes has no effect on NF-κB signaling).[78] However, a recent study by another group showed that miR-181-5p and miR-181-3p cooperatively inhibit NF-κB signaling by binding to TGF-β-activated kinase 1-binding protein (TAB2) and NF-κB essential modulator (NEMO), respectively.[79]

VCAM, ICAM and E-selectin are all adhesion molecules mediating adhesion between leukocytes and endothelial cells. Their expression in ECs and monocytes is strictly controlled by multiple miRNAs. For example, miR-17-3p and miR-31 suppress ICAM-1 and E-selectin expression, respectively, miR-126-3p inhibits VCAM-1 expression, and miR-221/222 target ICAM-1, VCAM-1 and monocyte chemotactic protein 1 (MCP-1).[80—82] Moreover, miR-296 targets ICAM-1

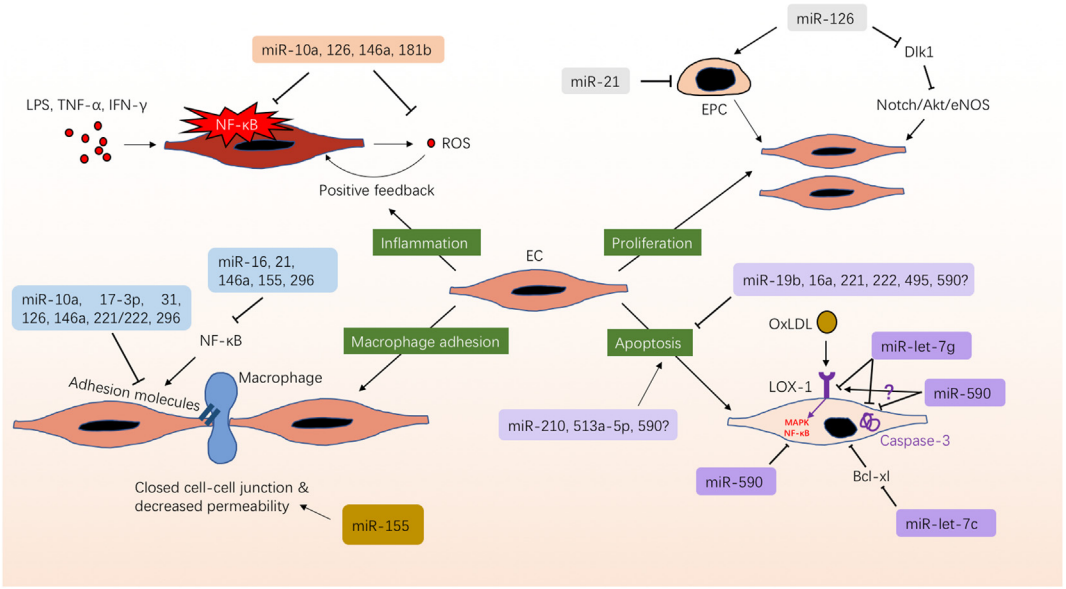

Figure 3 The role of miRNAs in regulating EC activities involved in initiating atherosclerosis, including inflammation, macrophage adhesion, proliferation and apoptosis. Proinflammatory cytokines activate ECs and induce the production of ROS by ECs, forming a positive feedback loop that amplifies inflammation. This process is targeted by miR-10a, 126, 146a and 181b. Macrophage—EC adhesion requires the expression of adhesion molecules on the surface of ECs and macrophages and requires separated ECs to allow macrophages to enter the intima. Many adhesion molecules are targets of NF-kB signaling, and their expression is either directly or indirectly regulated by miRNAs. EC proliferation is regarded as a protective activity that restores an integral endothelium and prevents atherosclerosis. MiR-21 inhibits this process by inhibiting the proliferation of EPCs, while miR-126 promotes EC proliferation by activating Notch/Akt/eNOS signaling. EC apoptosis is a proatherosclerotic process that exacerbates inflammation. MiRNAs regulate EC apoptosis by targeting the receptor LOX-1, downstream MAPK/NF-κB signaling, the antiapoptotic factor Bcl, and the protease Caspase-3.

and CX3C chemokine receptor 1 (CX3CR1) and subsequently suppresses monocyte adhesion.[40] KLF2/4 are transcription factors induced by ECs under high shear stress. Both of these proteins help maintain tight intercellular junctions, reduce permeability and attenuate inflammation in ECs. However, miR-103, the direct suppressor of KLF2/4 expression, enhances monocyte adhesion to ECs.[83] CXC ligand 1 (CXCL1) is one of the chemokines required for macrophage adhesion that is secreted by ECs upon activation, and miR-103 increases CXCL1 expression and aggravates atherosclerosis.[83] MiR-146 targets HuR, which inhibits endothelial nitric oxide synthase (eNOS) responsible for producing NO that prevents leukocyte-EC adhesion.[76] In addition, miR-146 indirectly inhibits the expression of adhesion molecules by inhibiting NF-κB signaling.[76] Notably, miR-146 itself potently and directly decreases adhesion molecule levels.[76] Since both E-selectin and VCAM-1 are target genes of the transcription factor NF-κB, miRNAs that inhibit the nuclear translocation of NF-κB or target other adaptor proteins in the NF-κB signaling pathway all prevent monocytes from adhering to ECs.

The proliferation of ECs is one of the most crucial processes in the repair of vascular injury and ameliorating the progression of atherosclerosis. To date, an increasing number of miRNAs have been shown to regulate EC proliferation, and miR-126 is the most frequently discussed miRNA. Indeed, hyperlipidemic conditions impair the proliferation of ECs and the resulting insufficient endothelial repair exacerbate atherosclerosis. Fortunately, miR-126-5p counteracts this process and promotes EC proliferation by suppressing delta-like 1 (Dlk1), as suggested by Schober A et al.[84] Dlk1 inhibits Notch/Akt/eNOS signaling associated with EC proliferation. This finding explains the role of miR-126-5p in maintaining vascular homeostasis and protecting vessels from atherosclerosis. Schober A et al also showed that miR-126-5p knockout mice exhibit impaired endothelial repair, further proving the antiatherosclerotic effect of miR-126-5p.[84] Additionally, increased miR-126 expression activates endothelial progenitor cells (EPCs), which are derived from mature ECs and contribute to endothelial repair and proliferation.[85] EPCs are the main cellular source of EC regeneration and vessel recovery. The TGF-β/BMP signaling pathway plays a vital role in the differentiation of EPCs but suppresses their proliferation. Importantly, miR-21 downregulates WW domain-containing protein 1 (WWP1) and activates the TGF-β/BMP signaling pathway, ultimately inhibiting EPC proliferation and contributing to the initiation and progression of atherosclerosis.[86]

The accumulation of oxLDL in endothelial cells drives their apoptosis through diverse mechanisms involving multiple signaling pathways. Apoptotic ECs are new proinflammatory stimuli for the endothelium that exacerbate endothelial injury. OxLDL-induced EC apoptosis also contributes to plaque rupture and coagulation, accelerating the progression of atherosclerosis and leading to ischemic stroke. The receptor for oxLDL on ECs is lectin-like LDL receptor-1 (LOX-1). Once bound to oxLDL, LOX-1 transduces signals to downstream mediators, including MAPK and NF-κB, leading to endothelial activation, dysfunction and ultimately apoptosis. LOX-1 deficiency has been implicated in decreased atherosclerosis.[87] Several miRNAs have been found to regulate the expression of LOX-1. For example, miR-let-7g targets LOX-1 and caspase-3 (a key protease involved in apoptosis that initiates the cleavage and activation of many other proteases) and inhibits EC apoptosis.[88,89]

In addition to LOX-1, other molecules important for modulating EC apoptosis are also potential targets of miRNAs. For example, miR-let-7c is suggested to enhance apoptosis in ECs by inhibiting the expression of Bcl-xl.[90] By targeting 3′-phosphoinositide-dependent kinase-1 (PDK1), miR-210 also enhances EC apoptosis.[91] TNF-α-induced EC apoptosis is initiated by miR-513a-5p through its inhibition of X-linked inhibitor of apoptosis (XIAP) expression.[92] Xue Y et al suggested that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a common target of miR-19b, miR-221 and miR-222, and the deficiency of these miRNAs leads to ROS accumulation and ultimately EC apoptosis.[93] However, in the same year, Qin B et al observed that E-Twenty-Six-1 (Ets-1) and its downstream target p21 are also possible targets of miR-221/222.[94] This evidence indicates that miR-221/222 inhibit EC apoptosis through more than one pathway. By inhibiting these two targets, miR-221/222 attenuate atherosclerosis.[94] Likewise, miR-590 has multiple targets to reduce EC apoptosis. For example, miR-590 reduces the oxLDL-induced expression of pro-apoptotic factors such as p53 and Bax.[95] By reducing the activation of caspase-3, miR-590 upregulates Bcl-2, a well-known cell apoptosis inhibitor.[95] Additionally, miR-590 decreases MAPK activation and NF-κB translocation.[95] Moreover, upregulated miR-590 reverses the overexpression of ROS and upregulates LOX-1, which are all proatherosclerotic assaults.[95] MiR-26a is another suppressor of EC apoptosis that directly targets transient receptor potential cation channel 6 (TRPC6).[96] MiR-495 not only suppresses EC apoptosis but also induces proliferation by targeting C—C ligand 2 (CCL2).[97]

MiRNAs regulate the proliferation and migration of VSMCs in atherosclerosis

Under normal conditions, VSMCs are contractile and nonproliferative cells. After exposure to an insult, they begin to proliferate and migrate, which contributes to atherosclerosis. Previous studies have shown that VSMC proliferation exerts adverse effects on atherosclerosis, but in recent years, scientists have gradually recognized the role of VSMC proliferation in preventing the progression and rupture of atherosclerotic plaques.[17] Although researchers are still debating whether the proliferation of VSMCs is beneficial or harmful, VSMC proliferation undeniably improves the stability of plaques to some extent. For example, the repeatedly mentioned miR-21 promotes the proliferation of VSMCs. Downregulated miR-21 levels are accompanied by unstable plaques.[98] In turn, the delivery of miR-21 mimics to the lesion area leads to a thicker fibrous cap and increased plaque stability, probably because miR-21 directly targets repressor element-1 (RE-1) silencing transcription (REST), a suppresser of VSMC proliferation.[98]

The proliferation of VSMCs is often accompanied by migration. Some miRNAs that promote or inhibit the proliferation of VSMCs also exert the same effect on VSMC migration. For example, the expression of miR-34a is

elevated in response to PDGF-BB and TGF-α.[99] Both factors are proinflammatory signals important for inducing inflammation in macrophages, ECs and VSMCs in the pathogenesis of atherosclerosis. The upregulated miR-34a in turn inhibits both VSMC proliferation and migration by targeting Notch1, completing a negative feedback loop that attenuates atherosclerosis.[100] Other similar examples are listed in Table 1.

MiRNAs critically regulate plaque stability

The integrity of plaques depends primarily on the stiffness of the fibrous cap, which is critically maintained by the extracellular matrix (ECM). The molecules contributing to the fibrous cap mainly include collagen and elastin. A balance of ECM synthesis and degradation is critically important to maintain the integrity of this cap. In the intima, VSMCs synthesize ECM and macrophages are responsible for secreting various enzymes (such as matrix metalloproteinases and MMP) that degrade ECM.[129] Both insufficient production and overdegradation of ECM would lead to a weakened fibrous cap. Therefore, the proliferation, activation, senescence and apoptosis of macrophages, ECs and VSMCs all influence plaque stability. These unstable plaques are usually characterized by a thin fibrous cap, a necrotic core rich in extracellular lipids, macrophage infiltration and excess inflammation, increased calcification, and local

Table 1 MiRNAs involved in regulating VSMC proliferation and their targets.

MicroRNA	Role in VSMC proliferation (and probably migration)	Target	References
21	positive	REST, PTEN	98,101
22-3p	negative	HMGB1	102
24	negative	HMGB1	103
34a	negative	Notch1	100
34c	negative	SCF	104
124	negative	S100A4	105
126-3p	negative	LRP6	106
126-5p	positive	Dlk1	84
129-5p	negative	Wnt5a	107
132	negative	LRRFIP	108
133a	positive	IGF-1R	109
135b-5p	positive	MEF2C	110
136	positive	PPP2R	111
138	positive	SIRT1	112
141	negative	PAPP-A	113
143/145	positive	Klf4, myocardin, Elk-1	114
146a/b	positive	SMAD4	115
147b	positive	YY1	116
148b	positive	HSP90	117
155	negative	ATR1	118
185	negative	STIM1	119
214	negative	NCKAP1	120
221/222	positive	p27(Kip1), p57(Kip2)	121
362-3p	negative	ADAMTS1	122
365b-3p	negative	ADAMTS1	123
378a-5p	positive	CDK1	124
379	negative	IGF-1	125
448	positive	MEF2C	126
499a-3p	positive	MEF2C	110
503	negative	INSR	127
599	negative	TGFB2	128

Some of these miRNAs are also associated with VSMCs migration, such as miR-22-3p, 24, 34a, 135b-5p, 138, 147b, 148b, 178a-5p, 362-3p, 365b-3p, 379, 448, 499a-3p, 503, and 599. Those miRNAs important in both VSMCs proliferation and migration tends to have same effect on both processes, either promoting or suppressing. Abbreviations: REST, RE1-silencing transcription factor; PTEN, phosphatase and tensin homolog; HMGB1, high mobility group box-1; SCF, stem cell factor; S100A4, S100 calcium-binding protein A4; LRP6, lipoprotein receptor related protein 6; Dlk1, Notch1 inhibitor delta-like 1 homolog; LRRFIP, leucine-rich repeat (in Flightless 1) interacting protein-1; IGF-1R, insulin-like growth factor-1 receptor; MEF2C, myocyte enhancer factor 2C; PPP2R2A, protein phosphatase; SIRT1, sirtuin silent information regulator 1; PAPP-A, pregnancy-associated plasma protein A; Klf4, Kruppel-like factor 4; YY1, Yin Yang 1; HSP90, heat shock protein 90; ATR1, angiotensin II 1 type receptor; STIM1, stromal interaction molecule 1; NCKAP1, NCK associated protein 1; ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motifs 1; CDK1, cyclin-dependent kinase; MEF2C, myocyte-enhancer factor 2; INSR, insulin receptor; TGFB2, transforming growth factor B2.

vascular degeneration.[130] These unstable plaques most commonly rupture at the shoulder area of the plaque, which contains fewer VSMCs and more macrophages.[131]

Several miRNAs regulate plaque stability through diverse mechanisms. Matrix metalloproteinase (MMP) is the main degrader of ECM and increases the risk of plaque rupture. However, ECM degradation also makes room for proliferating VSMCs, which facilitates VSMC proliferation and migration to some extent.[31] Therefore, a reasonable hypothesis is that some MMPs promote plaque stability, while others render plaques prone to rupture. For example, overexpressed MMP-2 and MMP-9 may be associated with unstable plaques, but MMP-14 may lead to stable plaques in individuals with advanced atherosclerosis.[132] This difference may be because MMP-14 promotes VSMC proliferation, which thickens the fibrous cap and stabilizes the plaque.[133] Knock out of miR-24 significantly upregulates MMP-14 expression in foam cells and enlarges the plaque size.[134] MiR-133a suppresses the expression of MMP-9 in ECs, indicating its role in increasing plaque stability.[135] MiR-181b negatively regulates tissue inhibitor of metalloproteinase-3 (TIMP-3) and elastin production, in turn increasing collagen degradation and leading to plaque instability.[136] MiR-497 is indirectly associated with decreased MMP-9 levels because of its negative effect on MAP kinase kinase 1 (MEK1), a key member of the MAPK/ERK family that is an important regulator of MMP-9 expression.[137]

Theoretically, the apoptosis of macrophages, foam cells, VSMCs, and ECs all contributes to the expansion of the necrotic core and softens the plaque. The vitality and normal activities of VSMCs are the key to maintaining a tough plaque. In this respect, the previously mentioned miRNAs prevent the apoptosis of VSMCs, and miRNAs inducing proliferation of VSMCs significantly protect the plaque from rupture. For example, the previously mentioned study by Jin H et al reported an important role for miR-21 in stabilizing the fibrous cap of an atheroma. In unstable areas, the concentration of miR-21 is significantly decreased.[85] In addition, knock out of miR-21 leads to insufficient VSMC proliferation, and local delivery of miR-21 reverses this situation.[85] These miRNAs increase VSMC survival and prevent plaque rupture. For example, by targeting adenomatous polyposis coli (APC, a tumor suppressor), miR-210 regulates Wnt signaling and ensures VSMC survival, ultimately stabilizing advanced plaques.[138]

However, not all evidence of miRNAs regulating plaque stability has fully explained their clear mechanisms. For example, the increased $p27^{Kip1}$ expression caused by a loss of miR-221/222 may result in plaque rupture, as suggested by Bazan HA et al.[139] MiR-92a and miR-155 have been reported to reduce plaque stability, while 143/145 and miR-494 stabilize plaques.[68,75,130,140] These findings require an in-depth investigation to further clarify the role of miRNAs in regulating atherosclerosis, specifically plaque stability.

The prospect of miRNAs in clinical use as biomarkers and preventive targets of imminent atherosclerotic ischemic stroke

Numerous studies have indicated simultaneous changes in miRNA levels, such as miR-21, miR-27b, miR-130a, miR-210, and miR-221, after plaque rupture in either the lesional area or serum or both.[130] This result suggests that miRNAs are potential biomarkers of atherosclerotic ischemic stroke. Unfortunately, to date, most studies have only reported synchronous changes in the levels of several miRNAs upon plaque rupture but have failed to define the sequential order and logical relations between these two events. Therefore, we are unable to equate the upregulation or downregulation of these miRNAs with imminent atherosclerotic ischemic stroke. Scientists must also further clarify which miRNAs are atheroprotective and which induce rupture. Although we have provided some examples in this article, more evidence is definitely required since some miRNAs have multiple and sometimes contradictory roles, such as miR-155.

Indeed, miRNA-based therapy is a current hotspot of drug development in various areas. After all, miRNAs play vital roles in both the initiation of atherosclerotic ischemic stroke and processes occurring after injury, such as pathological changes in neurons, rescue of the penumbra zone and subsequent neural tissue repair. Obviously, miRNAs have great potential as therapeutic targets. However, only a few drugs have been approved by the Food and Drug Administration (FDA) to date. One example is mipomersen sodium an antisense oligonucleotide drug effective at reducing LDL-C, apoB and total cholesterol levels that was approved in 2013, and it has been proven to be effective in patients with homozygous familial hypercholesterolemia.[141] This treatment represents a good start, but we are still far from developing miRNA mimics or inhibitors specifically targeting those proteins that are only important for initiating atherosclerosis and plaque rupture. As described in this review, many miRNAs have more than one protein target, and each protein has its own role in different gene networks that are involved in diverse cell activities. This situation inevitably results in side effects and becomes an obstacle to the clinical application of miRNA mimics or inhibitors. In conclusion, miRNAs represent potential biomarkers of impending atherosclerotic ischemic stroke. Delivery of miRNA mimics or inhibitors may be a prospective treatment for preventing atherosclerotic ischemic stroke.

Author contributions

Contributions of each author to the manuscript:
Study design: J.X. and Q.J.
Data Collection & Analysis: Q.J. and Y.L.
Manuscript preparation: Q.J.
Manuscript modification: J.X., Q.Z. and Q.W.

Conflict of interests

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work. No professional or other personal interest of any nature or kind in any product, service and/or company exists that could be construed as influencing the position presented in, or the review of, the manuscript.

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Data availability statement

All data have been provided. We do not have any experimental data or pictures due to the article type of this review.

Abbreviations

ABCA1 ATP binding cassette transporter A1
ACAT-1 acyl coenzyme a-cholesterol acyltransferase
APC adenomatous polyposis coli
Apo apolipoprotein
Bcl6 B-cell lymphoma 6
βTRC β-transducin repeat-containing gene
CARHSP1 calcium-regulated heat stable protein 1
CCL2 C—C ligand 2
CX3CR1 CX3C chemokine receptor 1
CXCL1 CXC ligand 1
Dlk1 delta-like 1
ECM extracellular matrix
ECs endothelial cells
eNOS endothelial nitric oxide synthase
Ets-1 E-Twenty-Six-1
EPCs endothelial progenitor cells
HBP1 HMG-box transcription factor 1
ICAM-1 intercellular adhesion molecule
IFN interferon
IL interleukin
IRAK1 IL receptor-associated kinase 1
KLF13 Krueppel-like factor 13
LOX-1 lectin-like LDL receptor-1
LPGAT1 lysophosphatidylglycerol acyltransferase 1
LPS lipopolysaccharide
MAPK mitogen-activated protein kinase
MAP3K7 mitogen-activated kinase kinase kinase 7
MCP-1 monocyte chemoattractant protein-1
MEK1 MAP kinase kinase 1
MIF macrophage inhibitory factor
MKK3 mitogen-activated protein kinase kinase 3
MMP matrix metalloproteinases
MTP microsomal triglyceride transfer protein
NEMO NF-κB essential modulator
Nos2 nitric oxide synthase 2
OxLDL oxidized low-density lipoprotein
PDCD4 programmed cell death 4
PDK1 3′-phosphoinositide-dependent kinase-1
PGC-1α peroxisome proliferator-activated receptor γ coactivator 1α
Pknox1 Pbx/knotted 1 homeobox
PPAR-γ peroxisome proliferator-activated receptor γ
REST repressor element-1 (RE-1) silencing transcription
RISC RNA-induced silencing complex
SHIP1 Src homology 2 domain-containing inositol-5-phosphatase-1
SOCS suppressor of cytokine signaling
TAB2 TGF-β-activated kinase 1-binding protein
TGF-β transforming growth factor-β
TIMP-3 tissue inhibitor of metalloproteinase-3
TNF tumor necrosis factor
TRAF6 TNF receptor-associated factor 6
TRPC6 transient receptor potential cation channel 6
VCAM-1 vascular cell adhesion molecule-1
VSMCs vascular smooth muscle cells
WWP1 WW domain-containing protein 1
XIAP X-linked inhibitor of apoptosis

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