

# MicroRNAs regulate vascular smooth muscle cell functions in atherosclerosis (Review)

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**Abstract.** Vascular smooth muscle cells (VSMCs) are involved in all stages of the progression of human atherosclerosis (AS). MicroRNAs (miRNAs or miRs) are non-coding, small RNAs that regulate gene expression at the post-transcriptional level through translational repression or messenger RNA (mRNA) decay. Recently, a variety of functions of VSMCs that are involved in AS, including differentiation, migration, proliferation, extracellular matrix (ECM) synthesis and apoptosis, have been found to be regulated by miRNAs. This review provides an overview of the role of miRNAs in the regulation of the functions of VSMCs, as well as their targets and potential implications in AS. The data presented herein suggest that the specific modulation of miRNAs may present an attractive approach for the diagnosis and treatment of AS.

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## 1. Introduction

Cardiovascular disease (CVD) of atherosclerotic origin remain the leading cause of mortality worldwide, accounting for approximately 27% of deaths among males and 32% among females. The initiation and progression of atherosclerosis (AS) involves the chronic expansion of arterial intima with diverse types of cells, including macrophages, dendritic cells (DCs), vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), as well as various sets of pro-inflammatory substances, such as adhesion molecules, cytokines, chemotactic factors and extracellular matrix (ECM) proteins (1).

VSMCs are the most abundant cell type in the arterial wall and are involved in the progression of AS. VSMCs exist in many phenotypes (2,3). Under normal conditions, the predominant phenotype is quiescent/contractile with non-migratory and non-proliferative VSMCs. The loss of ECs due to mechanical forces, including hemodynamic forces and stenting or cell apoptosis, can lead to VSMC migration from the media to the intima, where VSMCs can proliferate to form the neointima and plaque (4-6). VSMCs contribute to the development of AS through a variety of functions, including migration, proliferation, ECM synthesis and apoptosis, as well as inflammation and foam cell formation through cholesterol uptake (7).

MicroRNAs (miRNAs, miRs) are small, non-coding RNAs that can control gene expression by binding to their target messenger RNAs (mRNAs) through translational repression or mRNA decay (8). By binding to target genes and directly degrading mRNA or repressing translation, miRs are able to regulate the expression of hundreds or thousands of genes. The regulation of miRs is involved in a number of physiological and pathological conditions, such as metabolism, differentiation, development, apoptosis, proliferation and oncogenesis (9).

Recent studies have found that miRs play an important role in vascular pathophysiology and AS (10-12). Abnormal miR expression profiles have been reported to be related to CVD in

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Table I. miRs involved in the regulation of the VSMC phenotype.

Function	miRs	Targets	(Refs.)
Promote contractile phenotype	miR-1	KLF4, Pim-1	(19-21)
	miR-31	CREG	(29)
	miR-133	Sp-1, moesin	(3)
	miR-143/145	ELK1, FRA1 ACE, KLF4/5, CALMK, MRTF-B	(35,40)
Promote synthetic phenotype	miR-24	Trb3	(41)
	miR-26a	SMAD1/4	(43)
	miR-221/222	c-kit, p27 (Kip1) p57 (Kip2)	(46)
Dual effect	miR-21	PDCD4, Sp-1	(50-52)

miRs, microRNAs; VSMCs, vascular smooth muscle cells; KLF4, Krüppel-like factor 4; Pim-1, proviral integration site 1; CREG, cellular repressor of E1A-stimulated gene; Sp-1, specificity protein-1; FRA1, Fos-related antigen 1; ACE, angiotensin-converting enzyme; CALMK, calmodulin K; MRTF-B, myocardin related transcription factor B; Trb3, Tribbles-like protein 3; PDCD4, programmed cell death protein 4.

over 400 studies (e.g., 13). Moreover, accumulating evidence suggests a close association between miRs and the functions of VSMCs (14). The identification of the role of miRs in the regulation of VSMCs may lead to an improved understanding of the pathophysiology of AS and is likely to provide novel diagnostic and therapeutic targets.

In this review, we summarize the current knowledge on the role of miRs in the regulation of VSMC functions, including differentiation, proliferation, ECM synthesis, apoptosis and calcification. In particular, we discuss the mechanisms of action of miRs in the regulation of VSMC functions and their possible implications in AS. In addition, we discuss the potential applicability of miRs in the diagnosis and treatment of AS.

## 2. miRNAs regulate VSMC differentiation and phenotype

VSMCs can switch between a differentiated (contractile) state and a dedifferentiated (synthetic) phenotype in response to extracellular stimulation. Spindle-shaped differentiated VSMCs have low rates of proliferation, migration and production of ECM, but high levels of contractile genes, while rhomboid-shaped dedifferentiated VSMCs have increased rates of proliferation, migration and production of ECM, as well as low levels of contractile genes (15). Under pathological conditions, VSMCs can undergo the phenotypic change from the normal contractile state to synthetic, proliferative cells, which have an increased ability to migrate, proliferate and produce ECM (16). In atherosclerotic lesions and neointimal tissue, synthetic VSMC phenotypes are the major cellular types in vascular repair following various injuries. The phenotypic switching of VSMCs depends on numerous local signals, such as cytokines, cell-cell contact, cell adhesion, ECM interactions, injury stimuli, among which platelet-derived growth factor (PDGF)-BB and transforming growth factor- $\beta$  (TGF- $\beta$ )/bone morphogenetic protein (BMP) are the key mediators of VSMC phenotypic switching (17). PDGF-BB significantly promotes

the VSMC phenotype switch from a differentiated, contractile state into a dedifferentiated, synthetic state, thus promoting VSMC proliferation and migration, while TGF- $\beta$  and BMP help to maintain the VSMC contractile phenotype (18,19). Abnormal regulation of this phenotype switching leads to the development and progression of AS and restenosis (20). By preventing the VSMC phenotypic modulation from a contractile state to a synthetic state, neointimal progression in AS may be controlled. Previous studies have identified many miRs in the regulation of VSMCs phenotypic modulation (Table I and Fig. 1).

## 3. miRNAs and pro-contractile phenotype

miR-1 levels are significantly higher in more differentiated and less proliferative VSMCs and downregulated during neointima formation (21). miR-1 has been proven to regulate the differentiation of VSMCs by targeting Krüppel-like factor 4 (KLF4) and proviral integration site for Moloney murine leukemia virus 1 (Pim-1) (21-23). KLFs are a subclass of evolutionarily conserved transcription factors (24), and KLF4 has been demonstrated to significantly repress the expression of multiple genes in VSMCs during the phenotypic switching of SMCs in response to vascular injury, TGF- $\beta$  or PDGF-BB (25-27). For instance, KLF4 can downregulate the dedifferentiation marker gene, non-muscle myosin heavy chain B (Smemb) (28). Pim-1, an oncogenic serine/threonine kinase, has been reported to induce neointimal hyperplasia formation and promote the proliferation of cultured VSMCs (29).

miR-31 is upregulated in contractile VSMCs and downregulated in proliferative VSMCs, the proliferation of which was induced by PDGF (58). In addition, miR-31 levels have been shown to be higher in the serum of patients with coronary artery disease (CAD) with restenosis compared to patients with CAD without restenosis (30). miR-31 has been proven to promote the VSMC contractile phenotype by repressing cellular repressor of E1A-stimulated genes (CREG) expres-

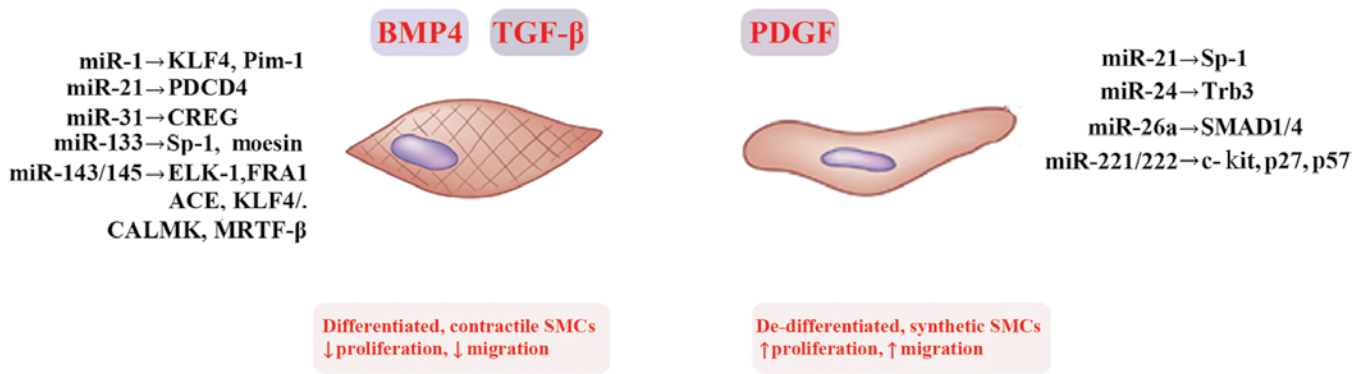


Figure 1. Regulation of vascular smooth muscle cell (VSMC) proliferation and migration by microRNAs (miRs) and their targets. miR-1, miR-21, miR-31, miR-133 and miR-143/145 inhibit bone morphogenetic protein (BMP)4 and transforming growth factor- $\beta$  (TGF- $\beta$ )-induced VSMC proliferation and migration. miR-21, miR-24, miR-26, and miR-221/222 increase PDGF-induced VSMC proliferation and migration.

sion. It has been reported that the overexpression of CREG maintains the differentiated phenotype in cultured VSMCs, whereas shRNA-mediated CREG knockdown promotes VSMC dedifferentiation, implicating a critical role for CREG in maintaining the phenotypic switching of VSMCs (31). However, the exact mechanism of action of CREG in regulating VSMC phenotypic modulation remains unclear.

miR-133 is downregulated in proliferating VSMCs but upregulated when VSMCs are back to the quiescence phenotype (32). Furthermore, the circulating levels of miR-133 are also downregulated in humans with CAD (33). miR-133 has been shown to inhibit the VSMC switch to the synthetic phenotype by inhibiting the transcription factor, specificity protein-1 (Sp-1) (32). Sp-1 is a transcription factor activated by a phenotypic switch that promotes stimuli and is upregulated in animal models of vascular injury (34,35). Activated Sp-1 in turn increases the activity of KLF4, which can repress myocardin and therefore downregulates the expression of most of the genes in VSMCs (36).

miR-143/145, encoded by a bicistronic gene cluster, are highly conserved and abundantly expressed in normal VSMCs, regulating VSMC differentiation. miR-143/145 are downregulated in animal models of AS (37,38). Furthermore, the circulating levels of miR-143/145 are downregulated in humans with CAD (33). The transfer of miR-143 and miR-145 from ECs to VSMCs promotes the contractile VSMC phenotype and attenuates AS (39). Aged miR-143/145<sup>-/-</sup> mice develop spontaneous atherosclerotic lesions in the femoral artery in the absence of hypercholesterolemia, and the overexpression of miR-145 reduces AS in Apoe<sup>-/-</sup> mice (40,41). A network of transcription factors, such as KLF4, KLF5, ELK1, versican, several actin remodeling proteins and angiotensin-converting enzyme (ACE), have been identified as the targets of miR-143/145 (37,42). These targets function as transcription and translation factors, receptors, phosphatases, kinases, growth factors, RNA binding proteins, and so forth, and are involved in different cellular processes in addition to regulating VSMC plasticity. This represents an example of how a single miRNA can regulate a number of related or unrelated cellular processes.

#### 4. miRNAs and pro-synthetic phenotype

In response to PDGF-BB, the expression of miR-24 is upregulated and its target, Tribbles-like protein 3 (Trb3), is downregulated (43). Trb3 has been reported to interact with type-II BMP receptor (BMPRII) and promote the degradation of Smad ubiquitin-regulatory factor-1 (Smurf1), a negative regulator of BMP and TGF- $\beta$  Smad-dependent signalling (44). Trb3 has been proven to be essential for the PDGF-BB-mediated induction of the VSMC synthetic phenotype. In addition, VSMC proliferation and the repression of Trb3 coincides with the reduced expression of Smad proteins and a decrease in BMP and TGF- $\beta$  signalling, which promotes a synthetic phenotype in VSMCs.

It has been demonstrated that miR-26a has multiple roles in VSMCs, including the promotion of VSMC proliferation, migration and the inhibition of apoptosis. miR-26a is significantly upregulated in synthetic VSMCs (45). The overexpression of miR-26a inhibits the differentiation of VSMCs and helps maintain the balance of cells in the synthetic and contractile states and governs phenotypic shifting by directly targeting SMAD1 and SMAD4 (45). SMAD1 and SMAD4 are two TGF- $\beta$ - and BMP-related pro-differentiation factors, in which SMAD-1 is primarily an element of BMP-responsive pathways, while SMAD-4 is in the common pathway for BMP and TGF- $\beta$  (45).

miR-221 and miR-222 are clustered on the X chromosome and share a common seed sequence. Both are significantly upregulated *in vivo* in VSMCs following the balloon injury of the vessel (46). miR-221 is upregulated in VSMCs upon the PDGF-BB-mediated VSMC phenotypic switch into the synthetic phenotype (47). miR-221/222 can regulate multiple functions of VSMCs, including the reduced expression of contractile genes, and increased proliferation and migration. miR-221/222 regulate multiple functions through multiple targets, including p27 (Kip1), p57 (Kip2) and c-kit (48). p27 and p57 are cyclin-dependent kinase inhibitors (CKIs), negatively regulating cell proliferation. The overexpression of p27 or p57 inactivates the cyclin-CDK complexes and leads to cell cycle arrest, inversely correlates with VSMC proliferation (49).

Other than the cell cycle control, p57 has been reported to be involved in cell differentiation in chondrocytes (50). c-kit, a proto-oncogene, is involved in multiple cellular functions, such as cell survival, proliferation, differentiation, adhesion, homing and migration (51). However, to the best of our knowledge, the role of p27, p57 and c-kit in VSMC differentiation has not been investigated to date.

miR-21 regulates multiple functions of VSMCs, including phenotypic changes, proliferation and apoptosis. However, its role in the phenotypic modulation and differentiation of VSMCs remains controversial. In some studies, miR-21 has been proven to inhibit VSMC differentiation, promote proliferation and reduce apoptosis through Sp-1 (52,53). However, another study demonstrated that miR-21 increases the biosynthesis of contractile protein in VSMCs by targeting programmed cell death protein 4 (PDCD4) (54). PDCD4, a tumor suppressor gene, has been reported to promote the differentiation of acute myeloid leukemia (AML) cells and female germline stem cells (55,56). However, to our knowledge, the role of PDCD4 in VSMC differentiation has not been investigated to date. The dual effect of miR-21 may be due to the diverse target genes of miR-21 in regulating VSMC differentiation. Further studies are required to elucidate the exact role of miR-21 in the phenotypic regulation of VSMCs.

### 5. miRNAs regulate VSMC proliferation

Aberrant VSMC proliferation significantly contributes in neointimal plaque formation during the progression of AS. VSMC proliferation has been proven to be solely responsible for in-stent restenosis (57). As mentioned above, VSMCs can switch between two phenotypes, a differentiated/contractile phenotype and a dedifferentiated/synthetic phenotype. VSMCs with the dedifferentiated/synthetic phenotype have an increased ability to migrate, proliferate and produce ECM, which contributes to the development of AS. Thus, the proliferation of VSMCs is closely associated with the phenotypic regulation of VSMCs. Accumulating evidence has demonstrated that miRs play an important role in the mediation of VSMC proliferation through the regulation of the post-transcriptional expression of several genes (Table II). Some miRs have been found to promote the proliferation of VSMCs, while others have been reported to inhibit the proliferation of VSMCs. By preventing VSMC proliferation, neointimal progression in AS may be controlled.

### 6. miRNAs promote the proliferation of VSMCs

miR-21 is one of the most upregulated miRs in the vascular wall following balloon injury. The inhibition of miR-21 can decrease the proliferation of cultured VSMCs in injured rat carotid arteries (53).

In addition to phenotypic regulation, miR-26a can also promote VSMC proliferation and aortic SMCs deficient in miRNA-26a show a significant reduction in proliferation (45).

As mentioned above, miR-31 can modulate the VSMC phenotype by targeting CREG (30). It can also regulate the proliferation of VSMCs. The expression of miR-31 is significantly increased in proliferative VSMCs. miR-31 exerts a pro-proliferative effect on VSMCs by targeting large tumor

Table II. miRs involved in the regulation of VSMC proliferation.

Function	miRs	Targets	(Refs.)
Promote proliferation	miR-21	PTEN, Bcl-2	(51)
	miR-26a	SMAD-1/4	(43)
	miR-31	LATS2	(56)
	miR-130a	GAX	(58)
	miR-146a	KLF4	(66)
	miR-208	p21	(67)
	miR-221/222	PTEN, Bcl-2, p27, p57	(44,45)
Inhibit proliferation	miR-1	Pim-1	(13)
	miR-15a	?	(39)
	miR-133	Sp-1	(17)
	miR-143/145	KLF5, ELK1, myocardin	(19)
	miR-152	DNMT1	(42)
	miR-155	-	(44)
	miR-181a	-	(45)
	miR-195	Cdc42, CCND1, FGF1	(46)
	miR-424/322	Cyclin D1, calumenin	(47)
	miR-490-3p	PAPP-A	(48)
	miR-638	NDR1	(49)
	let-7d	KRAS	(50)
let-7g	LOX-1	(51)	

miRs, microRNAs; VSMCs, vascular smooth muscle cells; LATS2, large tumor suppressor homolog 2; GAX, growth arrest-specific homeobox, KLF4, Krüppel-like factor 4; Pim-1, proviral integration site 1; Sp-1, specificity protein-1; DNMT1, DNA methyltransferase 1; PAPP-A, pregnancy-associated plasma protein A; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.

suppressor homolog 2 (LATS2) (58). LATS2 has been proven to play a major role in cell proliferation and apoptosis and is an important regulator of tissue and organ development (59).

miR-130a is upregulated in the remodeled aorta and arteries with hypertension. The overexpression of miR-130a has been shown to significantly promote the proliferation of VSMCs by inhibiting the expression of growth arrest-specific homeobox (GAX) (60). GAX has been proven to be downregulated in some vascular diseases, such as balloon injury (61), pulmonary hypertension (62), or by certain growth factors including Ang-II (63) and PDGF (64). GAX can significantly inhibit the proliferation, differentiation and migration of VSMCs (65,66).

The expression of miR-146a is significantly upregulated in atherosclerotic arteries compared with non-atherosclerotic arteries (38). miR-146a promotes VSMC proliferation in cultured VSMCs by targeting KLF4. KLF4 exerts an anti-proliferative effect on VSMCs through the upregulation of p21, which is a member of the cyclin-dependent kinase (CDK)-inhibitory protein family (67). The transfection of antisense miR-146a

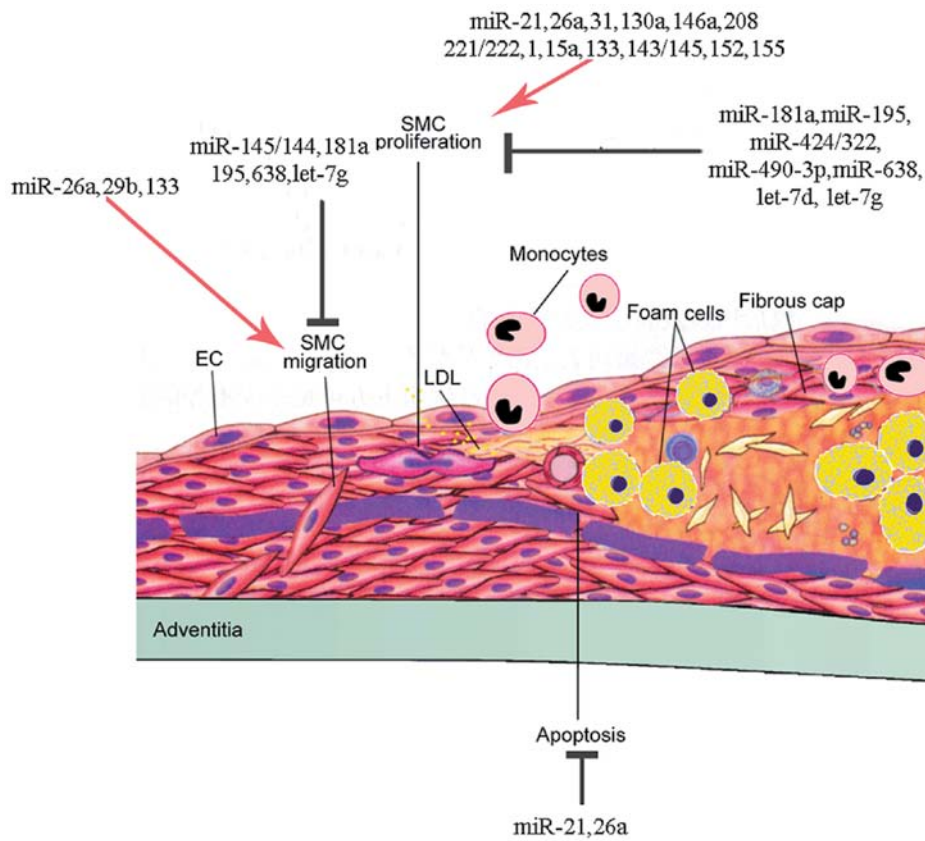


Figure 2. Regulation of vascular smooth muscle cell (VSMC) phenotypic switch by microRNAs (miRNAs) and their targets. VSMCs switch between the contractile phenotype and the synthetic phenotype in response to several external stimuli. Deregulation of switching between these phenotypes is associated with vascular disorders and atherosclerosis (AS). Under normal conditions, platelet-derived growth factor (PDGF) induces multiple aspects of the synthetic VSMC phenotype, characterized by increased rates of proliferation and migration. Inversely, transforming growth factor- $\beta$  (TGF- $\beta$ ) and the related factor bone morphogenetic protein 4 (BMP 4) promote the contractile VSMC phenotype, characterized by decreased rates of proliferation and migration. miRNAs reported to modulate the VSMC phenotype switching are shown. KLF4/5, Krüppel-like factor 4/5; Pim-1, proviral integration site 1; CREG, cellular repressor of E1A-stimulated gene; SP-1, specificity protein-1 transcription factor; ELK1, ELK1 members of ETS oncogene family; FRA1, Fos-related antigene 1; ACE, angiotensin-converting enzyme; CALMK, calmodulin K; MRTF-B, myocardin related transcription factor B; Trb3, Trbbles-like protein-3; p27, cyclin-dependent kinase inhibitor 1C (CDKN1C); PDCD4, programmed cell death protein 4.

oligonucleotide into balloon-injured rat carotid arteries has been shown to markedly decrease neointimal hyperplasia (68).

miR-208 regulates insulin-induced VSMC proliferation through the downregulation of its potential target, p21. However, as previously demonstrated, an miR-208 inhibitor alone had no effect on VSMC proliferation (69).

As mentioned above, miR-221/222 regulates multiple functions of VSMCs, such as reducing the expression of contractile genes and increasing proliferation and migration. The expression of miR-221/222 is significantly increased in proliferative VSMCs. The knockdown of miR-221/222 has been shown to decrease the proliferation of cultured VSMCs and neointima formation in balloon-injured rat arteries (46). miR-221 and miR-222 have been proven to regulate VSMC proliferation by targeting p27 and p57 (46,47). p27 and p57 are CKIs, negatively regulating cell proliferation (Fig. 2).

### 7. miRNAs inhibit the proliferation of VSMCs

As mentioned above, miR-1 expression is significantly higher in more differentiated and less proliferative VSMCs and is downregulated during neointima formation. miR-1 expression

is higher in VSMCs treated with myocardin, which can strongly inhibit the proliferation of VSMCs. The introduction of miR-1 into VSMCs inhibits their proliferation by directly targeting Pim-1 (21). Pim-1 has been proven to encode an oncogenic serine/threonine kinase, which is required for injury-induced neointima formation and SMC proliferation (29). miR-15a is upregulated in VSMCs treated with KLF4, which can strongly inhibit the proliferation of VSMCs. Therefore, miR-15a contributes to the anti-proliferative behaviors of KLF4, which has a strong inhibitory effect on the proliferation of VSMCs (70).

In addition to the phenotypic regulation of VSMCs mentioned earlier, miR-133 expression is decreased in proliferating VSMCs and following vascular injury. The overexpression of miR-133 decreases VSMC proliferation and migration, while anti-miR-133 can lead to an increased proliferation and migration by specifically suppressing the expression of the transcription factor, Sp-1 (32).

As mentioned earlier, miR-143/145 expression is down-regulated following both vascular injury and PDGF-BB treatment. The restoration of the expression of miR-143/145 inhibits neointima formation in the rat carotid artery following

balloon injury (37,71,72). The adenovirus-mediated gene transfer of miR-145 in rat balloon-injured carotid arteries has been proven to inhibit neointimal lesion formation (71).

miR-152 is downregulated in lipopolysaccharide (LPS)-treated VSMCs. The overexpression of miR-152 decreases cell proliferation in LPS-treated VSMCs. miR-152 exerts its effects by downregulating DNA methyltransferase 1 (DNMT1) and subsequently decreasing the methylation of ER $\alpha$  gene promoter region (73). DNMT1 is the most abundant DNA methyltransferase in mammalian cells and is the key maintenance enzyme for hemimethylated DNA during DNA replication of various types of cancer (74).

miR-155 is specifically expressed in atherosclerotic plaques, where it is induced by oxidized low-density lipoprotein (ox-LDL) (75). However, the circulating levels of miR-155 have been shown to be significantly downregulated in patients with CAD compared to the controls (33). Therefore, *in vivo* studies on the role of miR-155 in AS are conflicting. miR-155 is considered to contribute to the suppression of certain members of the matrix metalloproteinase (MMP) family (76). MMPs are involved in the atherosclerotic plaque formation by participating in ECM production and cell migration. In particular, MMPs can destruct the existing collagen that lends strength to the protective fibrous cap of atherosclerotic plaques, which may lead to lethal thrombosis and stenosis in AS.

miR-181a is downregulated by Ang-II, which plays a central role in the pathogenesis of AS. miR-181a inhibits the adhesion of VSMCs to collagen in response to Ang-II, which is essential for migration and proliferation (77). The overexpression of miR-181a inhibits Ang-II-mediated osteopontin (OPN) expression, which is a pro-inflammatory molecule and is found in abundance in atherosclerotic plaques.

miR-195 expression is altered in ox-LDL-treated VSMCs. miR-195 inhibits ox-LDL-induced VSMC proliferation, migration and synthesis of IL-1 $\beta$ , IL-6, and IL-8. In addition, the administration of miR-195 has been shown to substantially reduce neointima formation, and thus plays a potential therapeutic role (78). miR-195 can repress the expression of the Cdc42, cyclin D1 (CCND1) and fibroblast growth factor 1 (FGF1) genes, which are functionally related in the same signalling pathway and are involved in cell cycle control, migration and proliferation. Cdc42 is a downstream effector of phosphoinositide 3-kinase (PI3K) and can promote CCND1 (78) and FGF1 (80) expression. FGF1 has been reported to be an important angiogenic factor that can induce VSMC proliferation and migration (81,82).

miR-424, the ortholog of rat miR-322, is upregulated in proliferative VSMCs and after vascular injury. However, increased levels of miR-424/322 inhibit VSMC proliferation *in vitro* and in injury-induced remodeling *in vivo* (83). In addition, the early and ample introduction of miR-424/322 *in vivo* in balloon-injured vessels has been shown to markedly reduce neointima formation in a rat model of restenosis. Cyclin D1 and calumenin have been confirmed to be the direct target genes of miR-424/322. Cyclin D1 has been proven to be a direct target of miR-424 in other tissues (84). It has been demonstrated that calumenin, a Ca<sup>2+</sup>-binding protein, is a regulator of SERCA2a, which is able to inhibit its activity in cardiac myocytes (85). miR-424/322 can inhibit calumenin protein expression. This decrease can improve SERCA2a activity and allow better Ca<sup>2+</sup> cycling in VSMCs.

miR-490-3p is significantly downregulated in ox-LDL-treated VSMCs and human AS plaques. miR-490-3p inhibits the proliferation of VSMCs induced by ox-LDL by targeting pregnancy-associated plasma protein A (PAPP-A) (86). PAPP-A, a metalloproteinase, is abundantly expressed in VSMCs. PAPP-A can activate local insulin-like growth factor (IGF). However, whether PAPP-A is pro- or anti-atherosclerotic protein remains controversial since the role of IGF in AS has not yet been fully elucidated.

miR-638, which is enriched in human aortic VSMCs, is significantly downregulated in proliferative VSMCs. miR-638 inhibits PDGF-BB-induced VSMC proliferation and migration by targeting neuron-derived orphan receptor 1 (NOR1) (87). NOR1 is a critical regulator implicated in proliferative vascular diseases (88).

Let-7d is downregulated in proliferating VSMCs. The overexpression of let-7d in VSMCs reduces VSMC growth by targeting KRAS (89). KRAS, a member of the small GTPase superfamily, regulates the pathway involved in proliferation, differentiation and programmed cell death (90,91).

The human serum levels of let-7g are lower in subjects with hypercholesterolemia compared with normal controls. A lower level of let-7g has been observed in mice fed a high-fat diet than in mice fed a normal chow diet. The transfection of let-7g into VSMCs has been shown to significantly inhibit VSMC proliferation and migration induced by ox-LDL by targeting lectin-like oxidized-low-density lipoprotein receptor-1 (LOX-1) (92). The role of LOX-1 in the pathogenesis of vascular disorders has been well documented (93). LOX-1 can be activated by oxLDL and stimulates the expression of endothelial pro-inflammatory genes and superoxide radical formation, which is believed to play an active role in atherogenesis (94) (Fig. 2).

## 8. miRNAs regulate VSMC migration

In the native vessel, VSMCs are maintained in a quiescent/contractile and non-migratory and non-proliferative state, surrounded by a complex, highly structured ECM. In response to vascular injury, such as platelet aggregation on the lining of the vascular wall, or mechanical injury (ballooning with or without stenting, VSMCs can migrate from the medial wall to the intimal space, where they can proliferate and deposit ECM components, converting fatty streak into mature fibrofatty atheroma. Therefore, the migration of VSMCs plays a central role in the growth of AS lesions. Similar to their proliferation, the migration of VSMCs is regulated by growth promoters and inhibitors. PDGF-BB is one of the most potent stimuli for the migration of VSMCs. VSMCs form a structure known as podosomes when they migrate and invade. In fact, the proliferation and migration of VSMCs are closely associated. An increasing number of studies have found that miRs are involved in this process (Table III and Fig. 1).

## 9. miRNAs promote the migration of VSMCs

As mentioned earlier, miR-26a has multiple effects on VSMC functions. We have discussed its role in phenotypic regulation and proliferation. miR-26a can also promote the migration of VSMCs. As previously shown, cells deficient in miR-26a are

Table III. miRs involved in the regulation of VSMC migration.

Function	miRs	Targets	(Refs.)
Promote migration	miR-26a	SMAD1/4	(26)
	miR-29b	DNMT3b	(52)
	miR-133	Sp-1	(17)
Inhibit migration	miR-143/145	KLF5, ELK1, myocardin	(41)
	miR-181a	-	(45)
	miR-195	Cdc42, CCND1, FGF1	(46)
	miR-638	NDR1	(49)
	Let-7g	LOX-1	(51)

miRs, microRNAs; VSMCs, vascular smooth muscle cells; DNMT3b, DNA methyltransferase 3b; Sp-1, specificity protein-1; KLF5, Krüppel-like factor 5; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.

Table IV. miRs involved in the regulation of VSMC apoptosis, ECM synthesis and calcification.

Function	miRs	Targets	(Refs.)
Inhibit apoptosis	miR-21	PDCD4, Sp-1	(30-32)
	miR-26a	SMAD1/4	(26)
Inhibit ECM synthesis	miR-145	-	(60)
Inhibited calcification	miR-29a/b	ADAMTS-7	(62)
	miR-125b	Sp-7	(63)

miRs, microRNAs; VSMCs, vascular smooth muscle cells; ECM, extracellular matrix; PDCD4, programmed cell death protein 4, Sp-1, specificity protein-1.

less able to migrate towards a growth factor/serum gradient compared with the control group (45).

The miR-29 family (i.e., miR-29a, miR-29b and miR-29c) has been reported to be overexpressed in AS-related diseases (95,96). More specifically, miR-29b expression is upregulated in VSMCs treated with ox-LDL. miR-29b promotes VSMC migration by directly targeting DNA methyltransferase 3b (DNMT3b) and epigenetically regulating the expression of MMP-2/MMP-9 genes (97). The MMP-2 and MMP-9 genes, which are involved in ECM remodeling, can promote VSMC migration and contribute to neointima formation (98).

### 10. miRNAs inhibit the migration of VSMCs

As mentioned earlier, the overexpression of miR-133 results in decreased VSMC proliferation and migration, while anti-miR-133 can lead to an increased proliferation and migration (32).

miR-143/145 inhibit podosome formation, which is an important morphological feature of VSMC migration *in vitro* (99,100). miR-143/145 are downregulated in VSMCs treated with PDGF-BB, which has been proven to induce VSMC migration through podosome formation (42,72).

As mentioned above, miR-181a inhibits the adhesion of VSMCs to collagen in response to Ang-II, which is essential for migration and proliferation (77). miR-195 can inhibit VSMC proliferation, migration and the synthesis of IL-1 $\beta$ , IL-6 and IL-8 induced by ox-LDL (78). miR-638, which is significantly downregulated in proliferative VSMCs, inhibits VSMC migration in response to PDGF-BB by targeting the NOR1/cyclin D1 pathway (87). Let-7g can significantly inhibit the oxLDL-induced LOX-1 and OCT-1 expression in VSMCs, as well as their proliferation and migration (101).

### 11. miRNAs regulate VSMC apoptosis

Apoptosis, or programmed cell death, is a part of normal development, senescence and other biological processes. The apoptosis of VSMCs in the fibrous cap causes the thinning of the fibrous cap and the expansion of the underlying necrotic core. Therefore, the apoptosis of VSMCs plays a critical role in determining plaque stability and thus, the most important consequence of AS, i.e., plaque rupture. The apoptosis of VSMCs is a tightly regulated process that contributes to atherosclerotic plaque rupture and restenosis (102). Myocardial infarction mainly occurs due to the uneven thinning and rupture of the fibrous cap and is often initiated at the shoulders of the lesion where cell apoptosis may occur (103). The rupture sites of the atherosclerotic plaque lack VSMCs and are rich in macrophages and inflammatory cells (104). The apoptosis of VSMCs has been detected in the shoulder regions of atherosclerotic plaques, which are most likely to rupture (105). VSMC apoptosis may be a potential critical target for the development of therapies for unstable AS. However, studies on the role of miRs in the regulation of VSMC apoptosis in AS are relatively limited (Table IV).

In addition to its role in regulating phenotypic changes and the proliferation of VSMCs, miR-21 has an anti-apoptotic effect in cultured VSMCs. The inhibition of miR-21 has been proven to increase apoptosis in cultured VSMCs, as well as *in vivo* in injured rat carotid arteries (53). Phosphatase and tensin homolog (PTEN) and Bcl-2 are targets of miR-21. PTEN has been proven to regulate VSMC apoptosis through the inhibition of Akt phosphorylation/activation (106). Bcl-2, an anti-apoptotic protein, inhibits apoptosis by preventing the release of cytochrome *c* from the mitochondria and plays a role in the initiation and progression of vascular cell apoptosis (107).

In addition to the regulation of the phenotype switch, and the proliferation and migration of VSMCs, miR-26a can also regulate the apoptosis of VSMCs. Cells deficient in miR-26a display significantly increased rates of apoptosis, indicating that miR-26a may inhibit the apoptosis of VSMCs (45). miR-26a has several possible target genes associated with apoptosis, including Bcl-2-antagonist/killer 1 (BAK1), p21 protein (Cdc42/Rac)-activated kinase 2 (PAK2) and sulfatase 1 (SULF1). However, further studies are required to specify its target gene.

## 12. miRNAs regulate ECM synthesis by VSMCs

Under pathological conditions, VSMCs may undergo the phenotypic change from the normal contractile state to a synthetic state, in which the cells an increased ability to migrate, proliferate and produce ECM proteins. Synthetic VSMCs can synthesize ECM consisting of fibrillar collagens and elastins and matrix-modifying enzymes that remodel the ECM. ECM production and remodeling are necessary to form the neointima (108). However, studies on the effects of miRs on ECM synthesis by VSMCs are limited (Table IV).

Lysyl oxidase (Lox), which can crosslink adjacent collagen triple helices, confer tensile strength to collagen fibrils and contribute to neointimal growth through the chemotactic activity of VSMCs and monocytes (109,110), is negatively regulated by miR-145. ApoE, which can inhibit ECM gene expression, reduces the levels of Lox by increasing the expression of miR-145 (111).

## 13. miRNAs regulate VSMC calcification

Vascular calcification is defined as the deposition of calcium phosphate mineral in the vessel wall. The exact role of vascular calcification in plaque stability remains unclear. VSMCs have been proven to significantly contribute to vascular calcification, which is a prominent feature of AS (112). miRs have been reported to be involved in the regulation of VSMC calcification (Table IV).

In addition to the role of miR-29b in promoting VSMC migration, miR-29a/b is downregulated in calcifying VSMCs. miR-29a/b inhibits VSMC calcification by suppressing the expression of a disintegrin and metalloproteinase with thrombospondin motifs-7 (ADAMTS-7) (113). In injured vessels, ADAMTS-7 has been proven to mediate the degradation of cartilage oligomeric matrix protein (COMP) (114,115), which can inhibit VSMC calcification *in vitro* and *in vivo* by interfering with BMP 2 expression and preventing the osteochondrogenic transdifferentiation of VSMCs (116).

miR-125b is significantly downregulated in calcified vessels. miR-125b expression may be increased during atherosclerotic inflammation processes since an increased expression of miR-125b has been observed in ApoE<sup>-/-</sup> mice fed a high-fat diet (117). Furthermore, the inhibition of endogenous miR-125b promotes the transdifferentiation of VSMCs into osteoblast-like cells. miR-125b has been proven to be involved in vascular calcification by targeting SP7 (osterix) (118). SP7 is a zinc finger transcription factor expressed by osteoblasts is necessary for osteoblast differentiation in mice (119).

## 14. Implications in the management of AS

The regulation of specific miRs during neointima formation in AS and restenosis suggests that miRs are key determinants of the changes in the function of VSMCs. Altered circulating levels of miRs in patients with AS may provide a novel approach for the detection of CAD. For example, as the circulating levels of miR-145 are lower in the serum of patients with CAD (33), it may serve as a biomarker for CAD. However, large-scale studies are required to confirm the use of miRs as biomarkers for the diagnosis of disease. Furthermore, miRs may also

serve as potential targets for therapeutic strategies since their expression can be altered through genetic approaches. Since the modifications of miRs are reversible, it is possible to use specific miRs to reduce VSMC proliferation, migration and apoptosis, in order to prevent neointima formation in AS and restenosis. For example, the adenovirus-mediated gene transfer of miR-145 in rat balloon-injured carotid arteries has been proven to inhibit neointimal lesion formation. The knockdown of miR-221 and -222 in vessels reduces VSMC proliferation and intimal thickening in response to vascular injury (46). However, *in vivo* data on the role of miRs in the regulation of VSMC functions in AS and restenosis are limited. Further studies are required to elucidate the underlying mechanisms of action of miRs and their contribution to VSMC functions and AS *in vivo*.

## 15. Conclusions

In this review, we summarized the roles of miRs in the function of VSMCs and their contributions to AS. miRs critically affect VSMC functions, including differentiation, proliferation, migration, ECM synthesis, calcification and apoptosis, all of which play critical roles in the pathogenesis of AS and restenosis. We also discussed the changes in miR expression patterns in humans and animal models associated of AS. We focused on the different functions of VSMCs to elucidate the mechanisms through which miRs regulate each specific cellular function. miRs exert their functions by regulating a number of target genes, such as KLF4, p27 and Sp-1. The discovery of miR interference provides a promising insight into the understanding of the regulation of gene expression.

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