Review

MicroRNAs: Important Modulators of oxLDL-Mediated Signaling in Atherosclerosis

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Oxidized low-density lipoprotein (oxLDL) is known to be a major risk factor for the initiation and development of atherosclerosis. It can elicit an array of atherogenic responses in multiple types of cells residing in the arterial wall, such as endothelial cells (ECs), macrophages, dendritic cells (DCs), and vascular smooth muscle cells (VSMCs). Although they have been studied for many years, the detailed mechanisms modulating oxLDL-induced inflammation have not been fully elucidated. Epigenetic mechanisms consist of DNA methylation, histone post-translational modifications (PTMs), and microRNA (miRNA) alterations. Recently, epigenetic factors, especially miRNAs, have emerged as novel components of the gene expression regulating oxLDL-triggered signal transduction. In addition to their regulatory roles in signaling molecules, increasing evidence suggests that the different genetic stability and cross-talk regulation among these epigenetic factors may be particularly important to the sustained inflammation initiated by temporal oxLDL stimulation. Therefore, in this review, we primarily focused on the functional role of miRNAs, as well as other epigenetic factors, on modulating oxLDL-induced signal transduction in different vascular cells, with a special emphasis on the cross-talk interactions between miRNAs and other epigenetic players that help translate transient environment insults into chronic inflammation. Moreover, we extensively discussed the potential applicability of miRNAs as disease biomarkers and therapeutic targets in diagnosing and treating atherosclerosis.

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Introduction

It is well known that atherosclerosis is the underlying mechanism of ischemic heart disease and stroke, which are the leading causes of death in the world today¹⁾. The initiation and progression of atherosclerosis involves decades-long expansion of arterial intima with multiple types of cells, such as macrophages, dendritic cells (DCs), vascular smooth muscle cells (VSMCs), and endothelial cells (ECs), and a diverse set of pro-inflammatory substances, such as adhesion

molecules, cytokines, chemotactic factors, and extracellular matrix (ECM) proteins^{2, 3)}.

Accumulating evidence suggests that oxidized low-density lipoprotein (oxLDL), which exists in multiple forms, characterized by different degrees of oxidation and different mixtures of bioactive components, is the major risk factor for atherosclerosis⁴⁾. A complex interplay between oxLDL and these cells within the arterial wall has been demonstrated in the last few decades, in which several families of membrane-bounded receptors, such as scavenger receptors (SRs), Toll-like receptors (TLRs), and Fcγ receptors are proposed to mediate oxLDL-induced signaling⁵⁾. These receptors are differentially expressed in atherosclerotic lesions, and recognize different subtypes of oxLDLs depending on their distinctive characteristics⁵⁾.

It is reported that oxLDL may elicit an array of atherogenic responses, such as endothelial activation, adhesion molecule expression, monocyte differentia-

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tion, and VSMC migration²⁾. Although there is a well-established network of oxLDL-induced signal transduction pathways responsible for the initiation and progression of atherosclerosis, the complicated mechanisms regulating the expression of these signaling molecules have not been fully understood.

Recently, epigenetic mechanisms, including DNA methylation, histone post-translational modification (PTM), and microRNA (miRNA) alterations, have emerged as important components in the pathogenesis of oxLDL-mediated inflammation. By affecting chromatin structure and gene expression without altering the underlying DNA sequence, these epigenetic factors have been demonstrated to exert both short-term and long-term effects on a diverse set of biological processes and thus play an essential role in modulating gene-environmental interactions⁶⁾.

DNA methylation is a covalent modification at the 5' position of cytosine residues catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor. Hypermethylation of promoter CpG islands is generally associated with transcriptional repression. As one of the most stable epigenetic modifications, it is implicated in many important physical and pathological processes, such as the silencing of repetitive elements, X-inactivation, imprinting, development, and oncogenesis⁶⁾.

In contrast, chromatin histone PTMs, which include lysine acetylation, and methylation of lysine and arginine, have been demonstrated to fine tune gene expression by controlling chromatin access of transcription factors to the cognate cis-elements in promoter and enhancer regions. As a type of relatively more dynamically regulated epigenetic factors, it can elicit either active or repressive gene expression depending on the position, type, and degree of modulation⁶⁾.

In addition, the recent discovery of miRNAs adds an extra layer of epigenetic regulation to further fine tune gene expression in response to environmental stimuli. MiRNAs are a novel class of endogenous small non-coding RNAs, which are extremely dynamically involved in almost all biological processes. They can specifically bind to the 3' untranslated regions (3'-UTR) of target mRNAs, leading to either mRNA degradation or translation inhibition. Each miRNA can regulate multiple targets, not only including signal transduction components, but also the epigenetic factors modulating the signaling cascade. Therefore, miRNAs have been suggested to coordinate different signaling pathways, resulting in a combined biological effect and distinct cellular phenotype⁶⁾.

In this review, we summarize the functional role of epigenetic factors, especially miRNAs, that modu-

late oxLDL-induced signaling cascades in different vascular cells. Moreover, we focused on the relationship among these epigenetic components, and proposed that cross-talk interactions between miRNAs and other epigenetic players may be particularly important in modulating disease onset by translating transient environment insults into chronic inflammation. In addition, we discuss the potential applicability of miRNAs as disease biomarkers and therapeutic targets in diagnosing and treating atherosclerosis.

Epigenetic Factors Modulate oxLDL-Induced Signaling in ECs

ECs form the innermost line of the vascular wall and are essential for regulation of vascular tone and homeostasis. OxLDL accumulation in the vessel wall has been suggested to trigger endothelial inflammation, resulting in adhesion molecule expression and vasoconstriction^{7, 8)}. Endothelin (ET)-1 is one of the most potent and long-lasting vasoconstrictive peptides, which can be up-regulated by oxLDL stimulation and plays multiple roles in the development of atherosclerosis⁹⁾. Although it is reported that the synthesis of ET-1 requires the expression of precursor protein preproET-1¹⁰⁾, little is known about the mechanisms governing ET-1 posttranscriptional regulation. The first evidence for the involvement of miRNAs in regulating vasoconstrictive gene expression was provided by Li *et al.*¹¹⁾ who demonstrated that both miR-125a-5p and miR-125b-5p, which share an identical 'seed sequence', were highly expressed in ECs and synergistically inhibited oxLDL-induced ET-1 production by targeting the 3' untranslated region (3'-UTR) of preproET-1. Moreover, the study revealed that endogenous miR-125a/b-5p were decreased in the aorta of stroke-prone spontaneously hypertensive rats, associated with enhanced preproET-1 expression, suggesting that miR-125a/b-5p are important epigenetic regulators in modulating the threshold of *in vivo* preproET-1 expression. Of note, miR-125a/b-5p were differentially regulated by oxLDL, in which the expression of miR-125a-5p was significantly increased by oxLDL, which may represent a feedback mechanism in ET-1 regulation, whereas miR-125b-5p was constitutively decreased after oxLDL stimulation. The authors pointed out that this opposing effect of oxLDL on the different tendencies of miR-125a/b-5p expression was due to their different mechanisms of transcriptional regulation since they were harbored in totally different genomic locations. However, further investigation is needed to determine whether miR-125a/b-5p has a direct influence on vasomotor, vascular inflammation, and other athero-

sclerotic processes by targeting ET-1 both *in vitro* and *in vivo* (Fig. 1).

In addition to endothelial inflammation, oxLDL has also been proposed to induce endothelial apoptosis at the initial stage of atherosclerosis. The first study revealing the involvement of epigenetic mechanisms in oxLDL-induced endothelial apoptosis was conducted by Mitra *et al.*¹²⁾, who suggested that the transgenerational inheritance of the altered susceptibility to oxLDL-induced apoptosis was regulated by DNA methylation at specific gene promoters in ECs. In this study, the author demonstrated that oxLDL exposure evoked a dose-dependent increase in apoptosis in the first passage of human umbilical vein endothelial cells (HUVECs), which was completely abrogated by LOX-1-neutralizing antibody. In addition, oxLDL-induced apoptosis was associated with the up-regulation of proapoptotic genes (*LOX-1*, *ANXA5*, *BAX*, and *CASP3*) and down-regulation of other antiapoptotic genes (*BCL2* and *cIAP-1*), accompanied with reciprocal changes in the methylation of their promoter regions in the first generation. Moreover, subsequent passages of cells displayed an attenuated apoptotic response to repeat oxLDL challenge with blunted gene expression and exaggerated promoter methylation in those proapoptotic genes. Furthermore, treatment of cells with LOX-1 antibody before initial oxLDL treatment prevented both gene-specific promoter methylation and expression changes, and reduction of apoptotic response to repeat oxLDL challenge in subsequent generations. Collectively, these data suggest that exposure of HUVECs to oxLDL induces resistance to apoptosis in subsequent generations through LOX-1-mediated DNA methylation at some apoptosis-related gene promoters (Fig. 1).

After this study, another group also linked oxLDL-triggered miRNA alteration to endothelial apoptosis. Using microarray analysis and quantitative real-time PCR (qRT-PCR), Qin *et al.*¹³⁾ identified 15 differentially expressed (4 up- and 11 down-regulated) miRNAs in response to oxLDL exposure, in which miR-365 was up-regulated by nearly 3-fold. Further bioinformatics analysis suggested that anti-apoptotic protein B-cell CLL/lymphoma 2 (*Bcl-2*) is a potential target gene of miR-365. Since previous studies have demonstrated that oxLDL-induced endothelial apoptosis was accompanied by a decrease in *Bcl-2* expression^{14, 15)}, the functional role of miR-365 on *Bcl-2* expression was further elucidated. It was shown that transfection of miR-365 inhibitor partly restored *Bcl-2* expression at both mRNA and protein levels, leading to a reduction of oxLDL-mediated apoptosis in HUVECs; however, whether *Bcl-2* is a direct target of miR-365

remains unclear¹³⁾ (Fig. 1).

MiRNAs Modulate oxLDL-Induced Signaling in Macrophages

The formation of foam cells characterized by redundant cholesterol loading in macrophages is the hallmark of early atherosclerosis. After monocytes have transmigrated into the subendothelial space where they subsequently differentiate into macrophages and take up oxLDL to transform into foam cells, these macrophage-derived foam cells can in turn express many inflammatory molecules, including adhesion molecules, pro-inflammatory cytokines, chemotactic factors, and SRs, to further promote atherosclerosis¹⁶⁾. Although they have been studied for many years, little is known regarding the complex upstream regulators of gene expression and translation involved in these responses (Fig. 2).

It was not until a recent study by Chen *et al.*¹⁷⁾ who found, for the first time, that five miRNAs (miR-125a-5p, miR-9, miR-146a, miR-146b-5p, miR-155) were upregulated in oxLDL-stimulated THP-1 cells, that we began to understand the involvement of epigenetic factors in regulating monocyte/macrophage pathology after exposure to oxLDL. In this study, miR-125a-5p, which exhibited the highest fold change obtained by both microarray analysis and qRT-PCR, was further studied to determine its potential biological function. It was shown that miR-125a-5p inhibitor significantly increased cholesterol loading in oxLDL-treated THP-1 cells, associated with enhanced expression of some SRs (*LOX-1* and *CD68*, but not *CD36*) and pro-inflammatory cytokines (*IL-2*, *IL-6*, *TGF- β* , *TNF- α*). Furthermore, the same research group also revealed that miR-125a-5p repressed *ORP9* transcript and protein expression by directly targeting the 3'-UTR of *ORP9* mRNA. Given that *ORP9* has been proposed to be closely related to lipid metabolism¹⁸⁾ and membrane transport¹⁹⁾, the author concluded that decreased lipid uptake may be the direct consequence of miR-125a-5p-mediated *ORP* inhibition. All these data indicate that miR-125a-5p may partly provide post-transcriptional regulation on inflammatory response, lipid uptake, and SR expression in oxLDL-stimulated monocytes/macrophages, and may therefore play a protective role against the development of atherosclerosis. Although the precise role of miR-125a-5p in regulating the expression of SRs and cytokines remains unclear, this research is particularly important since it revealed several potential miRNA candidates regulating oxLDL-induced inflammation in monocytes/macrophages for future

Fig. 1. Epigenetic regulation in oxLDL-stimulated endothelial cells (ECs).

❶ Both miR-125a-5p and miR-125b-5p were reported to inhibit ET-1 production in ECs by targeting its 3'-UTR; however, they exhibited reciprocal changes when stimulated by oxLDL. ❷ Exposure to oxLDL induced apoptosis in the first generation of HUVECs through LOX-1-mediated DNA methylation changes at the promoters of some apoptosis-related genes. ❸ miR-365 was up-regulated upon oxLDL stimulation and promoted apoptosis of HUVECs by decreasing anti-apoptotic Bcl-2 expression. ND: not determined.

studies (**Fig. 2**).

On the other hand, Yang *et al.*²⁰⁾ reported an opposing result in their microarray analysis in which miR-146a was down-regulated in oxLDL-stimulated macrophages. Moreover, they demonstrated that exogenous miR-146a synchronously reduced cholesterol loading and the secretion of inflammatory factors (IL-6, IL-8, MCP-1, CCL2, and MMP-9) in oxLDL-stimulated macrophages by directly down-modulating TLR4 and inhibiting the activation of TLR4-dependent signaling molecules, including Src family kinases (Yes, Fyn, Fgr, Lck, Hck, and Lyn), FAK, JNK, Pyk2, paxillin, p38 MAPK, ERK1/2, JNK pan, and NF- κ B²⁰⁾. Previously, the TLR4/Src/FAK/JNK axis and phosphorylation of Pyk2 and paxillin have been associated with macropinocytosis and lipid uptake triggered by oxLDL²¹⁻²⁴⁾, and p38 MAPK, JNK, and ERK1/2 have been associated with inflammation processes following TLR4 activation²⁵⁾. In addition, given that both miR-146a and miR-146b have been proposed to directly inhibit IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) in the TLR4/MyD88 signaling pathway²⁶⁾, which are upstream regulators of I κ B kinase (IKK)-mediated NF- κ B acti-

vation²⁷⁾, and that NF- κ B can transactivate both miR-146a/b genes²⁶⁾ as well as many inflammatory genes²⁸⁾, miR-146a/b seem to control TLR-mediated inflammation through a negative feedback regulation loop. Taken together, miR-146a is a key multifaceted regulator that helps to fine tune oxLDL-triggered TLR signaling in macrophages and therefore inhibits atherogenesis (**Fig. 2**).

Recently, two different research groups successively demonstrated that miR-155, another aberrantly expressed miRNA previously being suggested in Chen's research¹⁷⁾, was also involved in negative feedback regulation of oxLDL-induced inflammation in macrophages via different signaling pathways^{29, 30)}. As shown from previous research, MyD88 is a universal adapter protein used by Toll-like receptors to activate NF- κ B signaling and hence mediates inflammatory responses³¹⁾. It has also been reported that MyD88 is a direct target of miR-155³²⁾. Huang *et al.*²⁹⁾ first found that miR-155, which was markedly up-regulated after oxLDL stimulation, could impair the secretion of several cytokines (IL-6, IL-8, and TNF- α) by attenuating MyD88-dependent NF- κ B activation. Meanwhile, miR-155 also down-regulated lipid uptake by sup-

Fig. 2. Epigenetic regulation in oxLDL-stimulated macrophages.

① MiR-125a-5p was up-regulated in oxLDL-stimulated THP-1 macrophages, which in turn inhibited SR associated cytokine secretion and lipid uptake. ORP9 was one of its direct targets. ② MiR-146a formed a negative feedback loop to inhibit the activation of several TLR4-dependent signaling molecules. ③ MiR-155 was found to inhibit SR-mediated lipid uptake as well as the expression of adhesion molecule in macrophages. In addition, SCG2 and MyD88 were demonstrated to be the direct targets of miR-155. SR*: miR-125a-5p was reported to only inhibit LOX-1 and CD68, but not CD36, although contrasting inhibitory effects of miR-155 on SRs were reported by two research groups, with one group suggesting LOX-1, CD68, and CD36, and the other suggesting LOX-1 and SRA, with CD36 not being suppressed in macrophages. ND: not determined.

pressing some SRs (LOX-1, CD36, and CD68) critical to cholesterol loading in oxLDL-stimulated THP-1 cells²⁹). After this study, another research group extended knowledge about the roles of miR-155 in oxLDL-induced inflammation³⁰. It was showed that miR-155 not only decreased lipid uptake through inhibiting SRs, but also suppressed the expression of adhesion molecules (VCAM-1 and ICAM-1) and chemotactic factors (CCL19, CCR21, and CCR7) in both macrophages and DCs. Of note, the expression of SRs was differentially regulated by miR-155, with LOX-1 and SRA, but not CD36, being suppressed in macrophages and LOX-1 and CD36 being suppressed in DCs³⁰), which is partly different from Huang's study²⁹). The author attributed these phenomena to the different cell models and the disparate sensitivity of those receptors. Furthermore, SCG2, a key AP-1 regulatory protein and a direct angiogenic cytokine, was found to be the direct target of miR-155. They also demonstrated that the change of SCG2 expression caused by miR-155 subsequently resulted in altered

expression of adhesion molecules³⁰); therefore, despite the conflicting data and the unidentified mechanisms underlying these effects, the above two researches give several interesting clues about miR-155 as an important epigenetic regulator of multiple oxLDL-induced signaling pathways in both macrophages and DCs (Fig. 2, 3).

MiRNAs Modulate oxLDL-Induced Signaling in DCs

DCs, a class of antigen-presenting cells mainly residing in the arterial wall, are important modulators of T cell-mediated inflammatory responses in atherosclerotic lesions. Having been sufficiently activated by pathogens or modified autoantigens, such as oxLDL, via membrane-bounded receptors such as SRs and/or TLRs, the mature DCs can express chemotactic factors, adhesion molecules, and cytokines to attract and then activate effector T cells, which can in turn kill plaque-resident cells and damage the plaque structure,

Fig. 3. Epigenetic regulation in oxLDL-stimulated dendritic cells (DCs).

① MiR-155 was reported to inhibit SR-mediated lipid uptake and the expression of both adhesion molecules and chemokine markers in DCs. ② MiR-146a repressed the positive feedback loop of CD40 by inhibiting the expression of its ligand CD40L during oxLDL-mediated DC maturation. ③ MiR-29a was up-regulated by oxLDL stimulation and in turn decreased cytokine secretion and SR-mediated lipid uptake in DCs. In addition, LPL was proved to be the direct target of miR-29a. SR*: miR-29a was reported to inhibit LOX-1, SRA, and CD36, whereas miR-155 was shown to repress LOX-1 and CD36 in DCs. ND: not determined.

leading to decreased plaque stability¹⁶. Therefore, DC maturation plays an important role in the development of atherosclerosis (**Fig. 3**).

Since macrophages and DCs have been suggested to share some critical characteristics in atherogenesis³³, it is not surprising that similar epigenetic mechanisms may be shared in both of the cells, similar to miR-155 discussed in the previous section. Recently, miR-146a, another macrophage-derived miRNA, has also been found to exhibit specific effects in DC pathology. Chen *et al.*³⁴ demonstrated that exogenous miR-146a, which was transfected before oxLDL stimulation, decreased the expression of only some surface co-stimulatory molecules, such as CD40, CD80, and CD86, but not CD209 and HLA-DR, indicating that it partly regulated the maturation of oxLDL-treated DCs. Furthermore, miR-146a was shown to repress CD40L-mediated cytokine secretion (IL-6 and TNF- α) by directly targeting CD40L mRNA³⁴. CD40L is the ligand of CD40 which, in concert with other surface costimulatory molecules expressed by activated DCs, is crucial for the induction of adaptive immune responses³⁵. It has been well-established that the interaction between CD40L and CD40, which leads to the

recruitment of TRAF6 and the subsequent activation of NF- κ B, can enhance the expression of CD40 itself as well as a variety of pro-inflammatory cytokines, such as IL-6 and TNF- α ³⁶; therefore, miR-146a-mediated inhibition of CD40L may be particularly important to cease this key positive feedback loop during DC maturation (**Fig. 3**).

Recently, miR-29a, another multifaceted miRNA widely studied in tumors³⁷ and cardiovascular diseases^{38, 39}, has also been shown to be differentially expressed during DC maturation. Chen *et al.*⁴⁰ later showed that miR-29a, which was up-regulated by oxLDL stimulation, decreased pro-inflammatory cytokine secretion (IL-6 and TNF- α) and SR expression (LOX-1, SRA, and CD36) by specifically targeting lipoprotein lipase (LPL). Moreover, it was shown that overexpression of miR-29a decreased cholesterol loading through LPL inhibition, whereas suppression of miR-29a did not achieve the opposite effect, suggesting partial regulation of miR-29 in LPL-mediated lipid uptake in DCs. These results are consistent with and may explain the results of a previous study conducted on THP-1 macrophages, in which oxLDL stimulation inhibited LPL, resulting in decreased cyto-

kin secretion and lipid uptake and increased SR expression⁴¹). Of note, since oxLDL has been proposed to have both pro- and anti-inflammatory effects depending on the degree of oxidation and the stage of oxLDL exposure⁵), oxLDL-triggered miR-29a up-regulation may be an underlying epigenetic mechanism partly responsible for the protective role of oxLDL. Taken together, miR-29a plays an important role against DC maturation in atheroma formation (**Fig. 3**).

Epigenetic Factors Modulate oxLDL-Induced Signaling in VSMCs

The migration and subsequent transformation of VSMCs are vital processes for atheroma formation and plaque stability, in which matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, have been suggested to play a pivotal role in both early and advanced stages of neointimal formation by promoting ECM degradation and VSMC migration⁴²). Although evidence shows that MMP-2/MMP-9 can be epigenetically regulated in carcinogenesis, little is known about their epigenetic regulation in the context of atherogenesis.

Chen *et al.*⁴³) proved for the first time that miR-29b, which was up-regulated by oxLDL stimulation, orchestrated VSMC migration through epigenetic modifications of the MMP-2/MMP-9 genes. They demonstrated that DNMT3b, which is one of the three DNMTs responsible for maintaining the methyl status of the human genome, was suppressed by oxLDL stimulation in primary human aortic smooth muscle cells (HASMCs), resulting in reduced DNA methylation and increased expression of MMP-2/MMP-9. In addition, miR-29b was found to directly inhibit DNMT3b expression and indirectly reduce DNA methylation, resulting in enhanced MMP expression and HASMC migration. In addition, LOX-1-mediated reactive oxygen species (ROS) generation was shown to be involved in the oxLDL-up-regulated miR-29b pathway. In a more recent study also conducted by Chen *et al.*⁴⁴), oxLDL was found to only activate the miRNA-29b-1/miRNA-29a cluster gene on chromosome 7 but not the other distinct microRNA-29b gene located on chromosome 1. The LOX-1/Ca²⁺/ROS/ERK/c-Fos signaling pathway was further proved to be responsible for transcriptional induction of the gene cluster. Moreover, oxLDL-induced histone PTMs were found to modulate the transcriptional induction of the miR-29b gene, in which the up-regulation of H3K9 acetylation (H3K9ac) and H3K4 methylation (H3K4me), two marks of transcription activation, in addition to the down-regulation of H3K9me, a mark

of transcription inhibition, were collectively identified at the promoter of the miR-29b gene. Furthermore, the knockdown of c-Fos recovered the reduced expression of histone deacetylase 1 (HDAC1), which can remove acetyl groups from histones and thus induce gene silencing, and significantly abolished histone PTM changes at H3K9 in response to oxLDL stimulation, suggesting that c-Fos can modulate the miRNA-29b-1/miRNA-29a gene cluster through both direct transcriptional and indirect posttranscriptional regulation. Taken together, the above data suggest that oxLDL-mediated LOX-1 activation can cause both epigenetic modifications and signaling regulation on the miRNA-29b gene located on chromosome 7, which can in turn target DNMT3b to down-regulate DNA methylation at the MMP-2/MMP-9 gene promoter, resulting in increased gene expression and enhanced VSMC migration (**Fig. 4**).

In addition to the above-mentioned miR-29b, the same research group also demonstrated that the production of let-7g was involved in LOX-1-mediated signal transduction in HASMCs. Previously, OCT-1, a ubiquitously expressed transcriptional regulator either enhancing or repressing the expression of a wide range of cellular genes, was indicated to induce LOX-1 promoter activation in oxLDL-treated HASMCs⁴⁵). Recently, both *in vitro* and *in vivo* experiments conducted by Chen *et al.*⁴⁶) revealed that OCT-1 was up-regulated in response to oxLDL stimulation via the LOX-1/Ca²⁺/PKC pathway, which in turn repressed let-7g gene expression. Moreover, it is suggested that let-7g mimics inhibited oxLDL-enhanced cell proliferation and migration through directly targeting 3'-UTR of LOX-1 mRNA. Therefore, OCT-1 seems to promote LOX-1 expression through both direct transcriptional regulation of the LOX-1 gene and indirect posttranscriptional modulation mediated by let-7g. Furthermore, considering that the levels of serum let-7g were reduced in subjects with hypercholesterolemia and that a decreasing let-7g level was noticed in normal subjects, hypercholesterolemic subjects and stroke subjects with hypercholesterolemia, the author suggested that let-7g levels were associated with the severity of cholesterol-induced atherosclerosis. Interestingly, stroke subjects without hypercholesterolemia had a similar let-7g level to normal controls, implying that, in humans, let-7g may play an important role in dyslipidemia-induced atherosclerosis (**Fig. 4**).

Fig. 4. Epigenetic regulation in oxLDL-stimulated vascular smooth muscle cells (VSMCs).

① Multiple signaling pathways have been shown to be responsible for the up-regulation of miR-29b, which in turn inhibited its direct target DNMT3b, leading to decreased DNA methylation at MMP-2/MMP-9 gene promoter and eventually resulting in increased expression of the MMP-2/MMP-9 gene and enhanced VSMC migration. ② LOX-1 was demonstrated to be the direct target of let-7g; however, the expression of let-7g was suppressed when stimulated by oxLDL through LOX-1-mediated signaling pathways. M histone methylation; A histone acetylation.

Cross-Talk Interactions between miRNAs and Other Epigenetic Players Help Translate Transient Environment Insults into Chronic Inflammation

The differing genetic stability among different types of epigenetic factors is another important characteristic in addition to their regulatory roles in oxLDL-induced signaling transduction. In the previous section, we summarized that transgenerational inheritance of resistance to oxLDL-induced endothelial apoptosis may be mediated by DNA methylation changes at the promoters of some pro- and anti-apoptotic genes¹², which supports the notion that DNA methylation is the most long-lasting epigenetic modification. However, recent evidence also suggests that H3Kme-regulated longevity can be genetically transferred through generations⁴⁷, implying that histone methylation may play an important role in passing on parental features.

In addition, metabolic memory, also known as the legacy effect, which is defined as the continuous progression of diabetic complications even after blood glucose normalization, has recently been found to be mediated by sustained alteration in histone methylation at the promoters of key inflammatory genes⁴⁸. These results collectively imply that transient exposure to environmental stimuli may induce long-lasting epigenetic modifications, especially DNA and histone methylation, which further leads to sustained alterations in chromatin structure and gene expression, and results in continued functional and morphological changes in the first and subsequent generations, even if the initial stimuli have long been removed. Considering that atherosclerosis has a chronic nature of inflammatory responses in which lipid reduction and normalization through both persistent lifestyle change and drug therapy, such as statin administration, can only delay, halt or, in some cases, reverse plaque size

progression but are unable to completely eliminate already-existing lesions⁴⁹⁾, it is possible that sustained inflammation in atherosclerotic lesions, similar to metabolic memory, may also be the very result of long-lasting epigenetic modifications initiated by temporal environmental insults, such as hypercholesterolemia and oxLDL stimulation.

Importantly, it should be noted that cross-talk regulation between miR-29b and DNMT3b, which results in DNA demethylation activation of the MMP-2/MMP-9 gene and subsequent VSMC migration⁴³⁾, as discussed previously, resembles the epigenetic mechanism in the control of metabolic memory, in which up-regulated miR-125b in diabetic *db/db* mice directly targets histone methyltransferase Suv39h1 to cause sustained histone demethylation activation of multiple inflammatory genes and the diabetic proinflammatory phenotype, even after blood glucose normalization^{48, 50)}. This similarity strongly implies that oxLDL-induced miRNAs may also be interface regulators that interpret dynamically changing environmental stimulations into long-lasting epigenetic modifications and subsequent chronic inflammatory responses during atherogenesis. However, whether such “legacy effects” caused by DNA methylation and/or histone methylation also hold true in hypercholesterolemia- and oxLDL-mediated atherosclerosis still need to be further verified. Nonetheless, these experiments give interesting clues that cross-talk regulation between dynamically-changed miRNAs and long-lasting histone PTMs and DNA methylation may play a key role in modulating sustained inflammation and atheroma formation.

In addition, although not as commonly studied as the above-mentioned factors, other epigenetic mechanisms, such as histone deamination, isomerization, phosphorylation, and ubiquitination, may also have great potential to influence oxLDL-mediated signaling. Increasing evidence has suggested that these histone PTMs can act in concert with histone methylation and acetylation of lysine and arginine to further fine tune gene expression by controlling the chromatin access of transcription factors⁶⁾. Hence, their specific assisting roles in atherogenesis are also worthy of continuous exploration. Further researches should place as much stress on the collective role of epigenetic mechanisms as on their respective roles in the pathogenesis of atherosclerosis.

Diagnostic and Therapeutic Prospectives of miRNAs

During recent decades, it has been well estab-

lished that inflammatory responses play a crucial role in all stages of atherosclerosis. Large-scale studies have reported that the majority of patients with coronary heart disease have one or more inflammation-associated risk factors, such as hypertension, diabetes, hyperlipidemia, and cigarette smoking, predisposing the onset of atherosclerosis^{51, 52)}. Furthermore, several other studies demonstrated that most atherosclerotic manifestations could be prevented if these risk factors were eliminated⁵³⁾. Therefore, identification of potential diagnostic biomarkers and therapeutic targets to monitor and ameliorate inflammatory states is becoming increasingly important.

Although several invasive and non-invasive imaging modalities, such as optical coherence tomography (OCT), intravascular ultrasound (IVUS), multislice computed tomography (MSCT), and magnetic resonance imaging (MRI), are currently used in clinical practice⁵⁴⁾, all of these techniques require the plaque to reach a certain visually distinguishable size; hence, novel methods capable of finding lesions at a much earlier stage would be of great value.

Previously, the conventional view of intercellular communication was limited to cell-to-cell adhesion conduits (e.g. gap junctions) or secreted signals (e.g. hormones and neurotransmitters); however, new studies have demonstrated that plasma-membrane-derived vesicles, namely exosomes and microvesicles, can transfer proteins, mRNAs, and miRNAs from donor cells to recipient cells. Furthermore, it has been shown that these circulating miRNAs are relatively stable in plasma and possess functional targeting capabilities when delivered into recipient cells⁵⁵⁾. Ever since their discovery, circulating miRNA profiles have been associated with a range of diseases, such as tumor, stroke, and heart diseases⁵⁶⁾. Notably, Kasey *et al.*⁵⁷⁾ recently revealed that high-density lipoprotein (HDL) also helped to deliver sufficient miRNAs to directly alter gene expression in recipient cells. Many of these genes have a role in lipid metabolism, inflammation, and atherosclerosis; therefore, circulating miRNAs have great potential as disease biomarkers for monitoring the initiation and development of atherosclerosis. In addition, these discoveries certainly raise an intriguing possibility that other non-cellular plasma particles (e.g. LDL) may also participate in the transport and delivery of inflammation- and atherosclerosis-related miRNAs.

On the other hand, since atherosclerosis involves a chronic inflammatory condition which is difficult to reverse with the currently available drugs⁴⁹⁾, novel approaches with improved preventive and therapeutic effects are badly needed.

Table 1. Epigenetic factors target oxLDL-induced signaling molecules

Cell types	Epigenetic mechanisms	OxLDL-Rs [§]	Epigenetic regulators	Targets (direct or putative)	Ref.
ECs	miRNA targeting	ND	miR-125a-5p	ET-1	11
		ND	miR-125b-5p	ET-1	11
		ND	miR-365	Bcl-2 ^{§§}	13
	DNA methylation	LOX-1	ND	LOX-1, ANXA5, BAX	12
		LOX-1	ND	CASP3, Bcl-2, cIAP-1	12
Macrophages	miRNA targeting	ND	miR-125a-5p	ORP-9	17
		TLR4	miR-146a	TLR4; IRAK1, TRAF6	17; 26
		ND	miR-155	MyD88; SCG2	29, 32; 30
DCs	miRNA targeting	ND	miR-155	SCG2	30
		CD40	miR-146a	CD40L	34
		ND	miR-29a	LPL	40
VSMCs	miRNA targeting	LOX-1	miR-29b	DNMT3b	43
		LOX-1	Let-7g	LOX-1	46
	Histone PTM	LOX-1	HDAC1	miR-29b gene	44
	DNA methylation	ND	DNMT3b	MMP-2/MMP-9 genes	43

[§]OxLDL-Rs: specific oxLDL receptors responsible for regulating their downstream epigenetic factors upon oxLDL stimulation.

^{§§}Bcl-2 was shown to be a potential target of miR-365; however, evidence supporting its direct targeting remains unclear.

ND: not determined.

Taking into consideration that environmental factors (e.g. high-fat diet) influence the occurrence of atherosclerosis^{51, 52} and that miRNAs serve as interface regulators translating dynamic environmental stimuli into long-lasting inflammation-related epigenetic modifications⁴³, we speculate that circulating miRNAs might be an ideal therapeutic target to prevent atherosclerosis from its onset by inhibiting and/or reversing the chronic inflammatory condition through altering the DNA methylation and/or histone methylation states of crucial inflammatory genes.

Moreover, since epigenetic factors are proposed to mediate the expression of clusters of genes, drugs targeting these factors may potentially offer higher therapeutic efficacy than classical agents with single targets⁵⁸. In addition, it should be noted that, since miRNAs can be specifically transported to recipient cells at a distant location through blood circulation⁵⁵, there is a possibility that the manifestations of atherosclerosis may originate from foci other than the lesion sites. miRNA is therefore an important objective worthy of further research.

Previous studies have demonstrated that artificial microparticles could specifically transport and deliver exogenous miRNAs or small interfering RNAs (siRNAs) to recipient cells to further perform their functions^{57, 59}. With this methodological advance, it is possible to deliver specific exogenous miRNA mimics

and antisense RNAs to certain types of tissues and cells, and thus improve the safety and effectiveness of antisense and antigene therapies.

In addition, peptide nucleic acids (PNAs) have recently been suggested to be excellent candidates to modulate miRNAs. PNAs are DNA/RNA mimics extensively used for pharmacological regulation of gene expression in a variety of cellular and molecular systems. They can directly targeting endogenous miRNAs and modify their biological roles within cells. Considering the increasingly noted involvement of miRNAs in human pathologies, PNA-mediated targeting of miRNAs could become a novel therapeutic approach for epigenetic therapies.

Taken together, modifying endogenous or delivering exogenous miRNAs by target-specific delivery of miRNA mimics, antisense RNAs, and/or PNAs could be employed as a promising method to prevent and treat atherosclerosis in the future.

However, as we are still in the early stage of research and development, there are still many limitations of epigenetic therapy. For instance, determining absolute amounts of miRNA has not been well-established because of the lack of standardized control values for circulating miRNA levels, especially under disease conditions. In addition, recent researches have also shown complications while using drugs to modulate epigenetic factors^{60, 61}. Therefore, further studies,

with regard to the detailed mechanisms, possible side effects, as well as monitoring and delivering techniques, need to be carefully performed before clinical application to treat atherosclerosis.

Conclusions

In this review, we have summarized the regulatory roles of epigenetic factors in modulating different oxLDL-triggered signaling pathways in multiple types of vascular cells (**Table 1**). Notably, the different genetic stability and cross-talk regulation among these epigenetic factors may be particularly important contributors to the sustained inflammation initiated by temporal dyslipidemia. Furthermore, since miRNAs serve as interface regulators translating environment-gene interactions through modifying other epigenetic factors with more stable hereditary characteristics, circulating miRNAs are expected to have great diagnostic and therapeutic potential in preventing the disease onset. However, it should also be noted that almost all of the current understanding about oxLDL-mediated signaling cascades focuses on the regulatory roles of miRNAs rather than epigenetics as a whole and that the specific oxLDL receptors responsible for the upregulation of these epigenetic factors upon oxLDL stimulation remain mostly undetermined (**Table 1**). Therefore, the underlying epigenetic mechanism involved in oxLDL-mediated signaling which modulates the initiation and development of atherosclerosis is a promising subject needing further exploration in the future.

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Conflict of Interest (COI)

We declare no conflicts of interest.

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