Functions of microRNA-33a/b and microRNA therapeutics

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A B S T R A C T
Atherosclerosis is a chronic inflammatory disease of the arterial wall. It is characterized by subendothelial accumulation of low-density lipoprotein cholesterol, and its subsequent modification at athero-prone areas leads to further activation of the vascular wall and maintains vascular inflammation. An entirely new level of post-transcriptional gene regulation through microRNA (miR) expression has emerged recently as an important mechanism in the development and progression of numerous diseases, including atherosclerosis. Recently, miR-33a/b have been shown to act as post-transcriptional regulators of lipid metabolism, and their pharmacological inhibition diminished atherosclerosis by raising plasma high-density lipoprotein levels. This review summarizes the current understanding of the functions of miR-33a/b and the progress in miRNA therapeutics for treatment of various diseases, including atherosclerosis.

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Introduction
MicroRNAs (miRNAs; miRs) are endogenous, small (approximately 20–22 nucleotides in length), non-protein-coding RNAs. miRNAs bind to the 3’ untranslated region (UTR) of specific mRNAs according to the complementarity of their sequences and inhibit translation or promote mRNA degradation. miRNAs were initially discovered in Caenorhabditis elegans by Victor Ambros’ group while studying the gene lin-14 [1], and they were later found to be evolutionarily conserved. More than 60% of human protein-coding genes have been shown to be regulated by miRNAs, and so far, approximately 2500 miRNAs have been identified in humans [2]. miRNAs are usually transcribed by RNA polymerase II (Pol II) in the nucleus as longer precursors with a hairpin structure, referred to as pre-miRNAs. These pre-miRNAs are subsequently processed into mature miRNAs by the enzyme Dicer in the cytoplasm. The mature miRNAs then form a complex with Argonaute protein (AGO) and target mRNAs, leading to either translational inhibition or degradation of the mRNAs.

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to as primary miRNAs (Pri-miRNAs), and these are subsequently processed by the Drosha (RNase III)/DGC8 complex to form approximately 70-nucleotide pre-mature miRNAs (Pre-miRNAs). Pre-miRNAs are then translocated to the cytoplasm through exportin 5, where Dicer (an RNA-specific nuclease) cleaves them, resulting in double-stranded miRNAs that form mature miRNAs of 18–25 nucleotides in length. Subsequently, mature miRNAs are incorporated into an RNA-inducing silencing complex (RISC) by associating with Argonaute (Ago) proteins. This complex with the miRNA targets the 3′-UTR of the mRNA, which is then responsible for the decrease in target gene expression, via either degradation of the mRNA or inhibition of protein synthesis [3].

In addition to their existence in various tissues, recent studies have indicated that miRNAs also exist in serum, plasma, urine, and other body fluids in highly stable forms that are protected from endogenous RNase activity by exosomes and other proteins [4]. Circulating miRNA expression profiling has provided strong molecular markers for the detection of various diseases. Altered levels of circulating miRNAs have been observed in acute coronary syndrome [5], heart failure, essential hypertension, and stroke.

Work over the past several years has demonstrated that miRNAs control the expression of most of the genes associated with high-density lipoprotein (HDL) metabolism, including the ATP transporters, ABCA1 and ABCG1, and the scavenger receptor SRB1 [3,6–8]. These findings suggested that miRNAs regulate HDL biogenesis, cellular cholesterol efflux, and HDL cholesterol (HDL-C) uptake in the liver, thus controlling all of the steps of reverse cholesterol transport (RCT). Elucidation of the function of miRNAs may provide novel strategies for the treatment of dyslipidemia. In particular, we have intensively investigated the functions of miR-33a/b in vivo using genetically modified mice [9–12]. The present review summarizes the function of miR-33a/b and current strategies for modulating the function/levels of miRNAs.

microRNA-33a/b regulates high-density lipoprotein cholesterol in vivo

The miR-33 family consists of two intronic miRNAs, miR-33a and miR-33b, which are encoded in the introns of SREBF2 and SREBF1, respectively [13]. The sterol regulatory element-binding proteins (SREBPs) are a family of membrane-bound transcription factors that regulate fatty acid and cholesterol synthesis. SREBF1 encodes SREBP-1a and -1c, which mainly regulate lipogenic genes, such as fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), and acyl-CoA carboxylase 1 (ACCl). SREBF2 encodes SREBP-2, which mainly regulates cholesterol-regulating genes, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCGR), and low-density lipoprotein receptor (LDLR) [14]. In humans, miR-33a and miR-33b are encoded in the introns of SREBF2 and SREBF1, respectively [15], whereas in rodents there is a deletion in the miR-33b encoding sequence and miR-33b cannot be expressed (Fig. 1). Furthermore, miR-33a and miR-33b share the same seed sequence and differ in only three nucleotides. The fact that miR-33a and miR-33b are cotranscribed with their respective host genes suggests that they regulate lipid homeostasis with their host genes.

Several groups have shown that miR-33a targets genes involved in cholesterol export, such as ATP-binding cassette transporter A1 (Abca1) and Abcg1 in vivo, using either antisense technology or by generating miR-33a-deficient (knockout) mice [9,13]. ABCA1 promotes the movement of excess free cholesterol out of the cell. In the liver, it is essential for efflux of cholesterol to lipid-poor apolipoprotein A-I (apoA-I) and forms nascent HDL. In peripheral cells, ABCA1 and ABCG1 efflux excess cholesterol to apoA-I and HDL particles for delivery to the liver for excretion in the bile and feces. Therefore, ABCA1 is an essential molecule for HDL biogenesis and RCT. The ABCA1 mRNA and protein half-lives are short (1–2 h), suggesting that de novo transcription and translation are important for controlling its expression in response to stimuli. The 3′-UTRs of mouse and human ABCA1 and mouse ABCG1 contain binding sites for miR-33a/b, and transfection of miR-33a/b strongly repressed ABCA1 and ABCG1 mRNA and protein in a variety of cells. It was shown that mice treated with antisense oligonucleotides or anti-miR-33a lentivirus exhibited increased ABCA1 expression in the liver and ABCA1 and ABCG1 expression in macrophages. Furthermore, inhibition of endogenous miR-33a/b in both these cell types promotes an increase in the expression of ABCA1 and ABCG1 protein and cholesterol efflux to apoAI and HDL-C, indicating the physiologically relevant role of this miRNA in regulating ABCA1 and ABCG1. Notably, in an anti-miR-33a-treated mouse model of atherosclerosis, plasma HDL levels increased by 35–50% without affecting other lipoproteins [13], miR-33a knockout mice also showed a significant increase in the expression of ABCA1 and ABCG1 and a 25–40% increase in serum HDL-C [9] (Fig. 2A). Anti-miR-33a therapy has been shown to contribute to enhance the RCT pathway not only by increasing HDL-C through ABCA1 and ABCG1 upregulation but also by increasing bile secretion through upregulation of ABCB11 and ATP8B1, which are the other targets of miR-33a/b [16]. Using an in vivo assay to measure the efficiency of RCT, it was shown that the HDL-C generated by miR-33a inhibition was functional and increased the transport of cellular radiolabeled cholesterol to the plasma, liver, bile acid, and feces. Interestingly, in contrast to the results with miR-33a-deficient mice, HDL-C levels in miR-33b knock-in (KI) mice, which has miR-33b in the same intron as in humans [12], were reduced by almost 35%, even in miR-33b KI heteromice compared with the control mice (Fig. 2B).

microRNA-33 inhibition has a beneficial effect on atherosclerosis

The function of miR-33a inhibition in animal models of atherosclerosis has been examined by several groups. It has already been proven that antisense inhibition of miR-33a resulted in regression of atherosclerotic plaque in LDLR-deficient mice by promoting RCT [17]. This promotion of RCT was shown to be achieved in two ways: by directly increasing HDL-C biogenesis in the liver, and by increasing cellular cholesterol efflux from plaque macrophages. Moreover, miR-33a-deficient mice reduced the progression of atherosclerosis in apo-E-deficient mice (Fig. 3) [10]. In our study, miR-33-deficient mice not only had higher and functional HDL-C but also had macrophages with higher cholesterol efflux capacity, resulting in the lower lipid accumulation in atherosclerotic areas [10]. Other possible beneficial properties of
anti-miR-33a/b therapy include an anti-inflammatory reaction via upregulation of ABCA1. ABCA1 decreases cell-surface cholesterol levels, inhibits its partitioning into lipid rafts, and reduces the responsiveness of inflammatory signals from innate immune receptors. Furthermore, ABCA1 has been reported to act directly as an anti-inflammatory receptor independent of its lipid transport activities [18].

Although these preclinical studies of miR-33a inhibition in mice are encouraging, it has been shown that obesity and hepatic steatosis were observed in miR-33a-deficient mice at the age of 50 weeks or when fed a high-fat diet for 12 weeks [11]. We found that Srebf1 is a good target of miR-33a, and enhanced expression of SREBP-1 by miR-33a indicated the existence of a more direct and fine regulatory mechanism between SREBP1. It was also shown that chronic inhibition of miR-33a by antisense oligonucleotides increased the expression of genes involved in fatty acid synthesis such as ACC1 and FAS in the livers of mice [19]. It was also reported that anti-miR-33a therapy enhanced the expression of nuclear transcription Y subunit gamma (NFYC), a transcriptional regulator required for DNA binding and full transcriptional activation of SREBP-responsive genes, including ACC and FAS.

It is also of note that mice lack miR-33b, which is present in the Srebf1 gene of medium and large mammals, and that extrapolating the findings in rodents to humans is complicated. This difference between mice and humans may be particularly relevant under conditions in which the transcription of Srebf1 is upregulated, such as feeding after fasting and hyperinsulinemia. Because such a condition may lead to greater downregulation of cellular cholesterol efflux and plasma HDL-C levels, not complete but partial inhibition of miR-33a/b, which avoids the induction of further Srebf1, would be beneficial for the increase of HDL-C and prevention of atherosclerosis.

Other functions of microRNA-33a/b

miR-33a/b are known to have several other target genes including AMP-activated kinase (Ampkα1) and Cpt1a, which are involved in the regulation of lipid and glucose metabolism [20]. However, no significant difference in these target genes has been observed in miR-33a-deficient and miR-33b-KI mice compared with their levels in control mice [11,12].

It has been suggested that miR-33a/b regulates stem cell self-renewal via downregulation of p53 [21]. p53 has two putative miR-33a/b binding sites in the 3′-UTR, and miR-33s overexpression
repressed p53 expression and p53-mediated apoptosis. This study suggested that miR-33s may promote the repopulation capacity of hematopoietic stem cells. It is known that SREBP-1 and cellular cholesterol levels have also been shown to regulate cell-cycle progression. Therefore, miR-33a/b may cooperate with their host genes in regulating cell-cycle progression.

**MicroRNA modifications and chemistry**

The ability of miRNAs to regulate multiple genes in signal transduction pathways makes them attractive therapeutic targets. For this reason, miRNA therapeutics may be superior to other therapies that target a single protein or use small molecules.

miRNA therapeutic approaches can be divided into two categories: miRNA inhibition (blockade) to reduce the expression of miRNAs upregulated in the setting of a pathological condition, and miRNA replacement to restore the expression of miRNAs repressed in the setting of a disease (Fig. 4).

**Inhibition of microRNA function**

Current strategies to inhibit miRNAs include the use of miRNA inhibitors and miRNA sponges. An ideal miRNA inhibitor needs to have several functions, including high affinity to the target genes, low toxicity, high specificity, resistance to exonucleases, and low cost for synthesis.

**Antisense oligonucleotides**

miRNA inhibitors are chemically modified single-stranded antisense oligonucleotides (ASOs) that are complimentary to the mature miRNA. To date, antagomiRs (target-single miRNAs conjugated with cholesterol), antimiRs (target-single miRNAs), and tiny antimiRs (target miRNA families) have been synthesized (Fig. 4A). These ASOs can reduce the levels of pathogenically expressed miRNAs. A number of chemical modifications have been used to improve the pharmacodynamics and pharmacokinetics of ASOs. The modifications that are most commonly used are 2’-O-methoxyethyl (2’-MOE), 2’-O-methyl ribose-modified RNA (2’-OMe), 2’-fluoro (2’-F), and locked nucleic acid (LNA) modifications. In order to see the effect of miR-33a/b inhibition, 2’-F/MOE-modified phosphorothioate-backbone-modified antisense miR-33 was injected into African green monkeys [22]. It was reported that increased hepatic expression of ABCA1 and sustained increase in plasma HDL-C levels were observed over 12 weeks. Addition of LNAs to the ASOs increased the binding specificity. LNA-modified oligonucleotides contain one or more nucleotides in which an extra methylene bridge fixes the ribose moiety in the 3’-endoconformation [23]. This modification possesses high affinity and results in significant increases in melting temperature of up to several degrees per LNA residue and leads to the formation of a thermodynamically strong duplex with complementary RNA. In addition, this character of LNA oligonucleotides allows for the generation of ‘tiny LNAs’ that bind only to the seed region of target miRNAs. Tiny LNAs are 7–8 nucleotides long and can inhibit a family of miRNAs at the same time, an advantage over antagomiRs that can only target individual miRNAs. Rottiers et al. demonstrated that pharmacological inhibition of the miR-33 family by a subcutaneous delivered 8-mer LNA-modified antimiR into obese and insulin-resistant nonhuman primates, which resulted in derepression of miR-33 targets, such as ABCA1, and increases in circulating HDL-C [24].

Recently, another kind of miRNA inhibitor has been developed. These are referred to as ‘blockmirs,’ which target the miRNA-binding site in a specific target gene (Fig. 4B) [25]. They are designed to bind to the seed region with additional nucleotides to increase specificity. The unique feature of blockmirs is that they target specific mRNA while still allowing the miRNA to regulate other target genes.

**miRNA sponges**

A recent clinical trial indicated that five weekly injections of an antimiR were sufficient to achieve inhibition for at least 6 weeks in humans. However, repeated injection may be necessary to allow for longer inhibition. To overcome these barriers, ‘miRNA sponges’ were developed to inhibit miRNA activity (Fig. 4C). miRNA sponges contain multiple miRNA-binding sites, which act as competitive

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Fig. 4. microRNA (miRNA) manipulation strategies. Modification of miRNAs can be achieved by several strategies. Red lines indicate the methods to increase target gene expression and blue lines indicate approaches to reduce them. (A) AntagomiRs and antimiRs for miRNA inhibition. (B) Blockmirs for specific target inhibition. (C) miRNA sponges for miRNA inhibition. (D) miRNA mimics for miRNA replacement.
inhibitors for miRNA binding. This technology mainly utilizes viral gene transfer vectors to deliver the miRNA sponges into cells/tissues. However, nonviral delivery systems can also be used, such as liposomes, polysaccharides, and artificial polymers. miRNA sponges have been used to regulate miRNAs specifically in skeletal muscle [26].

Restoration of microRNA levels

Although efforts to inhibit overexpressed miRNAs have been a major focus over the past years, several successful strategies have been employed to express depleted miRNAs in diseased cells/tissues. miRNA levels are restored using oligonucleotide-based miRNA mimics, or by the use of viruses to drive expression of a given miRNA. miRNA mimics are double-stranded synthetic oligonucleotides that are processed into a single-stranded miRNA to regulate target genes in the cells (Fig. 4D). Montgomery et al. restored the functional miR-29 mimic to target fibrotic disease [27]. The miR-29 mimic was conjugated to cholesterol to facilitate cellular uptake and contained mismatches to prevent it from acting as an inhibitor. There are several reports that miRNA mimics were successfully used in clinical trials and the therapeutic potential of miRNA mimics has been discussed [28].

Nonviral delivery

Nonviral delivery of miRNAs is achieved by conjugation-based methods, liposomes, nanoparticles, or antibody-based methods. Lipid-conjugated dsRNAs, including palmitic acid, lauric acid, and cholesterol, in which these lipids modified the 5′-end of the sense strand, have been used to facilitate cellular uptake and to add a potent gene silencing effect for the delivery of siRNAs or miRNAs [29]. Liposome nanoparticles have been utilized for miRNA delivery applications. Nanoparticles can be targeted to specific cells and tissues by adding cell-surface receptor, ligands, and antibodies [30].

Viral delivery

Retrovirus, lentivirus, adeno-associated virus (AAV), and adenovirus have been used for miRNA loss and gain of function research. An AAV vector that delivers miRNAs driven by tissue-specific promoters allows for specific regulation in the cell type of choice. However, there is a concern in using these viral vectors because of safety and efficacy issues. Pre-existing immunity against viral vectors may lead to rejection responses.

Conclusion

miRNAs are able to directly regulate the expression of transcription factors and signaling molecules and play critical roles in numerous diseases. Given the role of miR-33a/b in repressing cholesterol efflux and atheroprotective effects, pharmacological targeting of miR-33a/b may be a promising strategy for the treatment of atherosclerosis. Recent reports have demonstrated the ability of exogenously administered miRNA inhibitors or miRNA mimics to modulate these pathological processes, thereby ameliorating disease, which opens the door for novel therapeutic approaches in the future.

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Conflict of interest

The author has no conflict of interest to disclose.

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