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MicroRNAs and atherosclerosis

Julio Madrigal-Matute, Ph.D, Noemi Rotllan, Ph.D, Juan F. Aranda, Ph.D, and Carlos Fernández-Hernando, Ph.D.

Departments of Medicine and Cell Biology, Leon H. Charney Division of Cardiology and the Marc and Ruti Bell Vascular Biology and Disease Program, New York University School of Medicine, New York, NY 10016, USA

Abstract

MicroRNAs (miRNAs) are small (~22nucleotide) sequences of RNA that regulate gene expression at posttranscriptional level. MiRNA/mRNA base pairing complementarity provokes mRNA decay and consequent gene silencing. These endogenous gene expression inhibitors were primarily described in cancer but recent exciting findings have also demonstrated a key role in cardiovascular diseases (CVDs) including atherosclerosis. MiRNAs controls endothelial cell (EC), vascular smooth muscle cell (VSMC) and macrophage functions, and thereby regulate the progression of atherosclerosis. MiRNAs expression is modulated by different stimuli involved in every stage of atherosclerosis and conversely miRNAs modulates several pathways implicated in plaque development such as cholesterol metabolism. In the present review, we focus on the importance of miRNAs in atherosclerosis and we further discuss their potential use as biomarkers and therapeutic targets in CVDs.

Keywords

MicroRNA; atherosclerosis; anti-miRNA therapy; miR-33

INTRODUCTION

MiRNAs are small non-coding sequences of RNA present in the genome of a variety of organisms ranging from viruses, to plants or metazoans. Since their original identification in the nematode *Caenorhabditis elegans* [1] as a developmental regulators, miRNAs have now shown to play key roles in controlling gene expression in most organisms. MiRNAs function as post-transcriptional regulators providing a ubiquitous mechanism for control of gene expression by binding to target mRNAs. So far, more than 1.500 miRNAs have been annotated in the human genome [2, 3] and computational studies estimate that more than 60% of total genes can be regulated by miRNAs [4]. As a consequence, these small endogenous silencers have emerged as epigenetic regulators of a diverse of biological processes such as cell proliferation [5], development [6], angiogenesis [7, 8], cholesterol

*To whom correspondence should be addressed: Carlos Fernández-Hernando Ph.D, 522 First Avenue, Smilow 703, New York, NY 10016, Tel: 212.263.4122, Fax: 212.263.4129, carlos.fernandez-hernando@nyumc.org.

DISCLOSURE

C. Fernández-Hernando has patents on the use of miRNA-33 inhibitors.

metabolism [9], tumorigenesis or cell death [10] among others. In addition to their endogenous physiological role as regulators of gene expression, cells can also passively and/or actively release miRNAs and thus function as paracrine molecules that regulate gene expression in other cells [11–14]. Therefore, it is not surprising that several diseases including cancer, metabolic and brain disorders or atherosclerosis have been recently associated with miRNAs dysfunction. Here, we discuss the recent findings in the field highlighting the therapeutic potential of anti-miRNA oligonucleotides for treating dyslipidemia and cardiovascular diseases.

Biology of miRNAs

MicroRNAs are located within introns of protein coding genes or in intergenic regions. Consequently, they can be co-transcribed with their host gene promoting coordinated co-regulation or they can be transcribed under the control of their own gene promoters [15]. Pri-miRNAs, 500–3000 bp with a stem-loop hairpin, are processed in the nucleus by RNase III Drosha, and a partner protein, Pasha (or DGCR8) [16]. The action of this nuclear complex results in a precursor miRNA (pre-miRNA) stem loop 80–110 nt in length. A GTP Ran/Exportin 5 supports nuclear transport to the cytoplasm where Dicer processes the pre-miRNA into mature miRNA duplex 20–23 nt in length [17]. The guide strand (5p) is loaded into the RNA-induced silencing complex (RISC) targeting the 3′ untranslated region (3′UTR) of mRNA transcripts [18, 19]. It was believed that the passenger strand (3p) was quickly degraded being ineffective, but recent data argues against this traditional view showing important regulatory functions for this strand [20]. After binding to the 3′UTR of their target genes, miRNAs regulate protein expression through mRNA destabilization or/and by inhibition of translation [21]. Recent studies have shown that miRNAs can also repress mRNA targets through binding to other regions, including 5′UTRs or protein-coding exons [22–25].

Atherosclerosis and miRNAs

Endothelial dysfunction—Atherosclerosis is now defined as an inflammatory disease in which hypercholesterolemia [26] plays a major role in the origin and development of the pathology. Early stages of atherosclerosis are characterized by the infiltration of Low-density lipoproteins (LDLs) in the arterial wall. LDLs are retained in the subendothelial space and oxidized. Oxidized LDLs (oxLDLs) stimulate the expression of endothelial adhesion molecules (e.g. ICAM-1, VCAM-1) and secretion of chemotactic factors (e.g. CCL2), increasing leukocyte (monocytes, lymphocytes or neutrophils) grip and migration towards the intima. Several miRNAs are involved in the modulation of endothelial dysfunction such as miR-10a, which inhibits a number of pro-inflammatory genes in ECs including VCAM-1, E-selectin or the NF- κ B pathway [27]. This signaling pathway is also modulated by miR-181b, which targets directly the importing subunit α -4 (KPNA4), a protein required for NF- κ B nuclear translocation [28]. MiR-126, miR-31 and miR-17-3p also regulate vascular inflammation by controlling the expression of the adhesion molecules VCAM-1, ICAM-1 and E-SEL [29, 30]. In addition to pro-inflammatory cytokines, shear stress also regulates endothelial cell activation through miRNAs. In this regard, atheroprotective laminar shear flow downregulates miR-92a and consequently increases the expression of well-known targets of this miRNA such as Kruppel-like factor 2 (KLF2) [31]

or KLF4 [32]. Some other miRNAs have important roles in endothelial aging such as miR-146a delaying EC senescence [33], or miR-217 and miR-34a promoting EC senescence [34, 35].

Cholesterol homeostasis—Cholesterol is a key player in every stage of atherosclerosis development, and many other diseases result from perturbations in lipid homeostasis, including metabolic syndrome and type II diabetes [36]. Thus, it seems reasonable that a fine-tuning system that controls cholesterol levels must exist to impede the initiation of any lipid-related disease. To supervise the membrane levels of sterols, the cell essentially employs a feedback system that is mainly regulated at the transcriptional level by endoplasmic reticulum (ER)-bound sterol regulatory element-binding proteins (SREBPs) [37]. Under low cholesterol levels, SREBPs are transported to the Golgi where are proteolytically processed. The active peptides generated may enter to the nucleus and activate the transcription of target genes. There are two *Srebp* genes, *Srebp2* and *Srebp1*, which encode three different transcripts. *SREBP2* and *SREBP1a* activate the transcription of cholesterol-related genes expression including 3-hydroxy-3-methylglutaryl coenzyme A reductase (*Hmgcr*) or low-density lipoprotein receptor (*Ldlr*) [37, 38], while *SREBP1c* enhances the expression of fatty acid metabolism-related genes, such as fatty acid synthase (*Fas*) [39, 40]. Under high cholesterol levels SREBP is unable to exit the ER and as a consequence the transcription of target genes is reduced [41]. Interestingly, in a recent breakthrough study, our group among others, identified *miR-33a* and *miR-33b* as intronic miRNAs located within the *Srebp2* and *Srebp1* genes respectively. Both miRNAs are co-transcribed with their host genes and regulate cholesterol and fatty acid metabolism [9, 42, 43]. Other miRNAs, such as miR-122, are also involved in the regulation of cholesterol metabolism. MiR-122 is the most abundant miRNA in the liver accounting for more than 80% of total miRNA content in this organ. Inhibition of miR-122 levels in the liver results in a significant reduction of plasma cholesterol levels in mice and non-human primates [44]. Similar results have been recently reported in miR-122 deficient mice [45]. Gene expression analysis revealed that genes involved in cholesterol synthesis such as 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*), *HMGCR*, 7-dehydrocholesterol reductase (*DHCR7*) and squalene epoxidase (*SQLE*) were downregulated in mice treated with miR-122 inhibitors. However, these genes are not direct targets of miR-122 and the mechanism of regulation remains unknown. MiR-370 also regulates lipid metabolism by controlling cholesterol, fatty acid synthesis and fatty acid β -oxidation. Interestingly, miR-122 antisense oligonucleotides inhibit miR-370 effects on lipid metabolism suggesting that miR-370 controls lipid homeostasis by regulating miR-122 levels. Thus, miRNAs seem to play an important role in modulating cholesterol and fatty acid metabolism.

Reverse cholesterol transport—Although eukaryotic cells obtain cholesterol by endogenous synthesis and lipoprotein uptake [46, 47], cholesterol cannot be degraded and it must be effluxed and transported to the liver, where it can be reused and excreted, a process named reverse cholesterol transport (RCT) [48]. Cellular cholesterol efflux is regulated by ATP transporters including ABCA1 and ABCG1. ABCA1 efflux cholesterol to poor-lipidated APOA1, while ABCG1 is mostly involved in the lipidation of mature HDL particles. ABCA1 also regulates the HDL biogenesis in the liver and its deficiency causes

the Tangier disease, which is characterized by absence of circulating HDL particles resulting in an increase of cardiovascular disease risk [49–51]. Interestingly, *Abca1* and *Abcg1* are targeted in the 3'UTR by miR-33a/b [9], although *Abcg1* silencing is only significant in murine cells. MiR-33 inhibits cellular cholesterol efflux to ApoA1 and mature HDL and reduces circulating HDL-cholesterol (HDL-C) levels in mice and non-human primates. Importantly, genetic deletion of miR-33 in mice increases plasma HDL-C levels and reduces the progression of atherosclerosis [52]. ABCA1 has a very long 3'UTR and several reports have shown that can be regulated by multiple miRNAs including miR-758, miR-26, miR-106b or miR-10b [53–56]. However, only the inhibition of miR-33 *in vivo* has been shown to increase circulating HDL-C levels. Therefore, further studies are necessary to determine the physiological relevance of miR-758, miR-26, miR-106b or miR-10b in regulating plasma lipids and in preventing the progression of atherosclerosis.

Plaque development—Inside the intima, monocytes/macrophages and VSMCs are loaded with modified LDLs through scavenger receptors and LDLR giving rise to foam cells and activating VSMCs. Lipid uptake and inflammatory responses in monocytes/macrophages are regulated by miRNAs such as miR-155 [57] or miR-125a-5p [58]. As a result, neointimal accumulation of foam cells and fatty streaks can be reduced which are a main determinant of plaque development and instability. Nonetheless, data regarding miR-155 are ambiguous since it has been also shown that miR-155 in macrophages stimulates CCL2 expression and NF- κ B binding activity [59]. The evolution from fatty streaks to a fibrous atheroma is promoted by VSMCs proliferation in the neointima. Vascular differentiation and apoptosis is regulated by transforming growth factor- β (TGF- β), which is a known target of miR-26a [60]. Metalloproteinases (MMP) expression, such as MMP2/9, controls VSMCs proliferation. MMPs expression is regulated by DNMT3b, a methyltransferase enzyme that silences the expression of these genes. Interestingly, Chen and colleagues have recently shown that miR-29b is upregulated in VSMCs treated with ox-LDLs and targets MMP2 thereby reducing VSMC migration [61]. The vascular wall surrounding foam cells and extracellular lipid droplets (lipid core) is characterized by a switch from a contractile to a secretory phenotype in VSMCs. This phenotype switch might be modulated by miR-145 favoring a contractile phenotype through upregulation of KLF4 and myocardin [62]. Furthermore, oxLDLs overload in vascular cells can be accompanied with transdifferentiation to a macrophage-like phenotype. The fibro-atheroma evolves to a more complicated plaque, often characterized by calcification that is regulated in VSMCs by miR-125b through targeting the osteoblast transcription factor SP7 (Osterix) [63].

Neoangiogenesis—Normal vessels nurture through oxygen diffusion from the lumen of adventitial vasa vasorum but as intima wall thickens, the oxygen effective diffusion distance is impaired, and vasa vasorum proliferates in the inner layers of the vessel wall [64]. Cholesterol-loaded macrophages are in part responsible for the production of cytokines that promotes neovessels formation. Neovessel formation is regulated by miR-222/221 and miR-155 by targeting endothelial nitric oxide synthase (eNOS) [65, 66] and by miR-22, which silences signal transducer and activator of transcription 5A (STAT5A) [67]. In fact, there is a negative correlation between miR-22 and STAT5A in ECs from advanced neovascularized atherosclerotic plaques [67]. Another interesting miRNA that regulates

angiogenesis is the microRNA cluster miR-17-92. MiRNA-17-92 expression is regulated by vascular endothelial growth factor (VEGF) and targets thrombospondin-1 (TSP-1), an anti-angiogenic molecule. However, the role of miR-17-92 cluster in regulating angiogenesis appears to be complex and several reports have shown contradicting results [7]. Finally, MiR-27a/b targets SEM6A thereby regulating endothelial cell adhesion and angiogenesis [68, 69].

Plaque rupture—Neoangiogenesis and hemorrhage intra-plaque drive blood components to the atherosclerotic lesion, promoting plaque vulnerability due to enrichment in proinflammatory, prooxidant and proteolytic factors. This pro-inflammatory scenario might be promoted by miR-146a driving peripheral blood mononuclear cells (PBMCs) towards a Th1 response [70]. The loss of collagen, endothelial and VSMCs due to the high expression of proteolytic enzymes and apoptosis favors plaque instability and further rupture. At this regard, miR-29 inhibits the expression of Col3A1, and elastin (ELN), thus reducing vascular integrity. Interestingly, miR-29 expression has been shown to be upregulated in aortic aneurysms from fibulin-4 knockout and fibrillin transgenic mice [71, 72]. miR-365 can function as a pro-thrombotic factor by stimulating endothelial apoptosis [73], whereas miR-21 protects VSMCs from H₂O₂-induced apoptosis [74]. miR-221/222 have opposite effects in VSMCs and ECs, protecting from apoptosis or promoting cell death respectively [75]. The clinic manifestation of atherosclerosis is a result of the destabilization, rupture and formation of a further thrombus and miRNAs seem to be main actors playing in this scenario, although we are just beginning to understand their potential relevance in CVDs.

Circulating miRNAs

Whether circulating miRNAs can be considered as good biomarkers, main players of a disease or both, is still a matter of debate. A biomarker could be defined as “marker reflecting or integrating one or several biological activities” and it must be detectable and quantifiable [76]. Circulating miRNAs have enormous potential as novel disease biomarkers since differential plasma miRNA profiles have been described for many diseases, including fatty liver [77], atherosclerosis [78, 79] and cancer [80]. For example, miR-146a levels are increased in patients with acute coronary syndrome (ACS) [70]. miRNAs are detected in secreted circulating exosomes derived from plasma membranes of donor cells [81, 82], but also in HDL particles [14] and in lipid-free protein complexes such as AGO2-miRNAs [83]. HDL particles are also able to deliver miRNA to recipient cells. Interestingly, the miRNA profile of the HDL particles, and the subsequent gene expression in target cells, is different in healthy people than in subjects with familial hypercholesterolemia [14]. Circulating extracellular miRNAs may also be biologically active as have been demonstrated *in vitro*, altering gene expression in recipient cells [84, 85]. Therefore, miRNAs could be considered as newly described forms of intercellular communication. As a meaningful example, extracellular vesicles secreted by shear-stressed Human Umbilical Vein Endothelial Cells (HUVECs) enriched in miR-143/145 are engulfed by VSMCs targeting thereby gene expression in host cells [11]. Conversely, ECs can be targeted by exogenous miRNAs secreted by other cells from the vasculature such as monocytes. As a result, there is an increase proliferation in endothelial cells due to miR150 enrichment in the microvesicles

(MVs) of monocytic origin, particularly in those from atherosclerotic patients [12]. Similarly, apoptotic bodies enriched in miR-126 are taken by HUVECs and promote atherosclerosis regression by inducing CXCL12 expression through CXCR4 [13]. There is a tremendous interest in the study of the mechanism leading miRNA into exosomes, delivery, targeting and recognition machinery. In this regard, it is likely that future research in secreted miRNA will open new therapeutic approaches for cardiovascular diseases treatment and their use as potential biomarkers.

MiRNA as therapeutic targets in atherosclerosis

Since a number of miRNAs are involved in the modulation of several key processes in every stage of plaque development, regulation of miRNA expression could have beneficial outputs in the treatment of atherosclerosis. Different approaches have been undertaken to examine the potential of miRNAs therapeutics.

Gene therapy—To our knowledge there is only one article regarding microRNA overexpression and gene therapy. In this work, miR-145 overexpression in VSMCs promotes a reduction in atherosclerotic plaque size in the most common sites of plaque formation such as aortic sinuses, ascending aortas, and brachiocephalic arteries. Furthermore, mir-145 favors plaque stability through an increase in the number of VSMCs, collagen content and fibrous cap area associated with a decrease in the number of macrophages and necrotic area [62]. These data emphasize the beneficial effects of specific up-regulation of atheroprotective miRNAs while conversely; suppression of proatherogenic miRNAs could be also of potential interest in atherosclerosis treatment. Regardless, safety for prospective patients should be evaluated before clinical translation of gene therapy.

Pharmacological compounds—The impending benefits of gene therapy such as off-site effects counteract with troubles arisen from difficult and expensive techniques and the biohazards for patients. Thus, therapeutic compounds have been developed to study, not only the functionality of miRNAs *in vivo* but also its possible use as tools for treating atherosclerosis.

The use of microRNA antisense oligonucleotides (ASOs) has become a useful tool in the study of miRNA functionality despite its risks of unintended targeting on other RNA species and the lack of accuracy methods to test ASOs efficacy [86]. There are different types of ASOs depending on the 2' sugar and backbone modification; 2'-O-methyl (2'-OMe), 2'-O-methoxyethyl (2'-MOE), 2'-fluoro (2'F) and locked nucleic acid (LNA). Systemic delivery *in vivo* of these ASOs has been tested with efficacy and safety in different organisms ranging from mice [87–89] to non-human primates [90, 91]. Pharmacological inhibition of miR-33 using 2'F/MOE ASOs has been carried out in mice and non-human primates with slight differences may be due to the lack of *miR-33b* in the murine *Srebp1* gene. In the mice model ABCG1 was targeted and there was an increase in RCT along with a reduction in the atherosclerotic plaque. In addition, the atherosclerotic plaques from anti-miR-33 treated mice showed less macrophage infiltration and lipid accumulation, and increased features of plaque stability [92]. Nevertheless, in a recent study, prolonged silencing of miR-33 did not prevent the progression of atherosclerosis in *Ldlr*^{-/-} mice [93]. In the non-human primates

model there was a significant reduction in plasma VLDL levels as a result of an increased expression of genes involved in the oxidation of fatty acids (*Crot*, *Cpt1a*, *Hadhb*, *Ampk1a*) and decreased expression of target genes involved in fatty acid synthesis (*Srebf1*, *Fasn*, *Acy*, *Acaca*) [91]. Nonetheless, antagonism of miR-33 increases hepatic ABCA1 expression and circulating HDL cholesterol levels. Pharmacological inhibition of miR-122 has been also studied in mice and non-human primates using 2'-O-methoxyethyl-phosphorothioate-modified ASOs and LNAs, respectively with no apparent side-toxic effects [44, 89]. In both animal models, inhibition of miR-122 decreased circulating cholesterol levels [89, 44]. However, the decrease of cholesterol levels is not only reflected in the desirable reduction in LDL levels but also in HDL levels [44, 45, 89]. As a consequence of these last data, the lack of accurate mechanistic knowledge about miR-122 targeting and a possible relationship with hepatocellular carcinoma the strength of miR-122 as an attractive therapeutic target has been lessened [94, 95].

Thus, specific modulation of miRNAs by pharmacological compounds is an attractive therapeutic approach but it is of major importance to be aware about the dosage and the potential side effects. Studies in non-human primates and future clinical studies should provide key data to evaluate the prospective use of miRNA modulation in human diseases.

CONCLUSION

MiRNA levels are modulated along the pathological scenario of atherosclerosis development. This could reflect the changes in intracellular miRNA levels but also can potentially modify its extracellular/circulating levels. In the field of atherosclerosis considerable efforts have been committed to examine the intracellular function of miRNAs as well as their role as paracrine molecules, including their potential role as biomarkers. Their biological roles have been shown to be of major importance for CVD diseases and thus miRNAs are emerging as significant therapeutic targets to strengthen vascular defenses and delay atherosclerosis development.

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Figure 1. MicroRNAs and atherosclerosis

A) Atherosclerotic plaque showing some of the relevant factors involved in atherosclerosis development: cell types (leucocytes/macrophages, SMCs, ECs, RBCs, platelets), cholesterol particles (VLDL, LDL and HDL), collagen, necrotic and lipid core. B–D) Table of the some relevant cell types involved in atherosclerosis, microRNAs and the processes these miRNAs modulate in the given cell. E) Table of the cell communication established by different cell types involved in atherosclerosis.