MicroRNAs and High-Density Lipoprotein Cholesterol Metabolism

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Summary

MicroRNAs (miRNAs) are small non-protein-coding RNAs that negatively regulate gene expression. They bind to the 3'-untranslated region of specific mRNAs and inhibit translation or promote mRNA degradation. Dyslipidemia/hyperlipidemia is a well-accepted risk factor for the development of atherosclerosis. The pathogenesis factors involved in lipid abnormalities are being examined extensively, and there is emerging evidence linking miRNAs to lipid metabolism. Among them, recent studies, including ours, have demonstrated that miRNAs control the expression of genes associated with high-density lipoprotein (HDL) cholesterol (HDL-C) metabolism, including ABCA1, ABCG1, and scavenger receptor class B, type I. Moreover, HDL-C itself was proved to carry miRNAs and deliver them to several different types of cells. In this review, we describe the current understanding of the functions of miRNAs in HDL metabolism and their potential in therapy for treating cardiometabolic diseases. (Int Heart J 2015; 56: 365-371)

Key words: HDL-C, ABCA1, SR-BI, Circulating miRNA

MicroRNAs (miRNAs; miRs) are endogenous, small (approximately 18–25 nucleotides in length), non-protein-coding RNAs, present in almost all organisms. 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.[1,2] miRNAs were initially discovered in Caenorhabditis elegans[3,4] and were later found to be evolutionarily conserved.[5,6] More than 60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs, and so far, approximately 2,500 miRNAs have been identified in humans.[6,7]

miRNAs are usually transcribed as longer primary miRNAs (Pri-miRNAs) by RNA polymerase II (Pol II) and then processed by the Drosha (RNase III)/DGCR8 complex to premature miRNAs (pre-miRNAs) in the nucleus. Pre-miRNAs are exported to the cytoplasm through exportin 5 and are then processed by another ribonuclease enzyme, Dicer, to produce mature miRNAs (Figure 1). It is known that only miR-451 does not require Dicer.[8] Instead, the pre-miRNA becomes loaded into Ago and is cleaved by the Ago catalytic center to generate an intermediate 3' end, which is further trimmed. Mature miRNAs are assembled into an RNA-inducing silencing complex and inhibit post-transcriptionally mRNA expression by binding to the 3'-UTR of their target mRNAs.[9] This association produces mRNA repression either by transcript destabilization, translational inhibition, or both.[10,11]

miRNAs have many functions in physiological and pathological states, and some miRNAs have been shown to have a significant impact on lipid homeostasis.[9,12,13] In the past few years, several groups have demonstrated the roles of miRNAs including miR-33, miR-122, and miR-223 in controlling lipoprotein metabolism.[14-18] Dyslipidemia and related metabolic disorders continue to rise at an alarming rate worldwide and are associated with increased cardiovascular disease risk. A high plasma level of low-density lipoprotein cholesterol (LDL-C) is a major risk factor for coronary artery disease (CAD). Therapy with statins [inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR)], which inhibit cholesterol biosynthesis, effectively reduces the levels of LDL-C,[19] and significantly reduces the risk of CAD, as evidenced by primary and secondary clinical intervention studies.[20,21] However, patients who are treated with high doses of statins are still at considerable risk of cardiovascular disease.[22,23] Thus, we still need another therapeutic option to treat the residual risk.[24-26] Elucidation of the function of miRNAs

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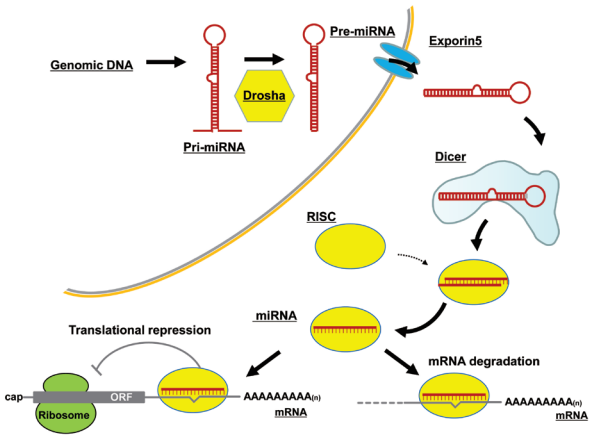

Figure 1. miRNA biogenesis. RISC indicates RNA-inducing silencing complex; and ORF, open reading frame.

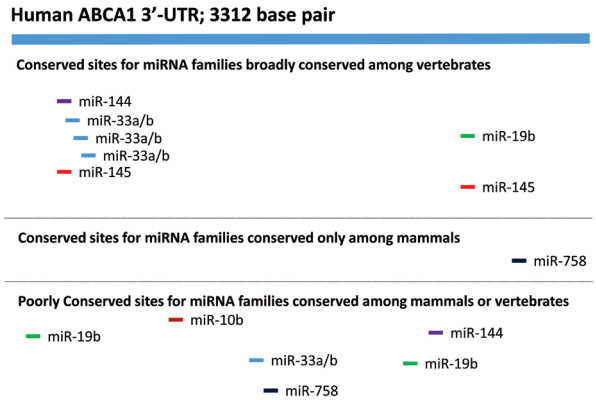

Figure 2. The 3'-untranscribed region of ABCA1 and potential binding sites of micro RNAs. Modified from TargetScan Release 6.2 (http://www.targetscan.org).

may provide avenues to develop a novel treatment for dyslipidemia.

In addition to their existence in 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.[27] Altered levels of circulating miRNAs have been found in acute coronary syndrome,[28,29] stable CAD,[30] heart failure,[31] essential hypertension,[32] and stroke.[33] Interestingly, it has been shown that high-density lipoprotein (HDL) cholesterol (HDL-C) contains miRNAs and delivers them to recipient cells via scavenger receptor class B, type I (SR-BI). Thus, miRNAs are related to lipoprotein metabolism in this context, too.

In the present review, we will focus on how HDL metabolism is regulated by miRNAs and how antagonizing miRNAs could be a potential therapeutic target for the treatment of cardiovascular diseases.

miRNAs That Regulate ABCA1 and/or ABCG1 Expression

The ABCA1 transporter is a unique molecule from the miRNA point of view. The ABCA1 mRNA and protein half-lives are very short (1–2 hours), suggesting that *de novo* transcription and translation are important for controlling its expression in response to environmental stimuli.[34] It is known that post-transcriptional regulation is also important for its turnover.[35] The 3'-UTR of ABCA1 is over 3 kb in length and includes many miRNAs binding sites, including those for miR-33a/b, miR-758, miR-10b, miR-144, miR-145, and miR-19b, among others (Figure 2). Actually, miRNA target prediction algorithms such as TargetScan (http://www.targetscan.org) revealed more than 100 predicted miRNAs that target ABCA1. Because all of these miRNAs have targets other than ABCA1, each miRNA has distinctive roles in lipid homeostasis. In particular, miR-33a and miR-10b regulate not only ABCA1 but also ABCG1 (miR-33a targets ABCG1 only in mice) and this enhances the effect of these miRNAs on HDL-C metabolism and reverse cholesterol transport (RCT).

miR-33a/b: Recent studies have indicated that miR-33a and miR-33b, located in the intron of sterol-regulatory element-binding proteins (SREBPs), control cholesterol homeostasis.[14-16,36,37] In humans, miR-33a and miR-33b are encoded in the introns of SREBF2 and SREBF1, respectively,[15,38] while in rodents, there is a deletion in part of the miR-33b encoding region and miR-33b cannot be expressed (Figure 3). miR-33a and miR-33b share the same seed sequence and differ in only two nucleotides. Thus, most of the targets of these miRNAs are expected to be the same.[39] SREBF1 encodes SREBP-1a and SREBP-1c, which mainly regulate lipogenic genes, such as fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD), and acetyl-CoA carboxylase 1 (ACC1). SREBF2 encodes SREBP-2, which mainly regulates cholesterol-regulating genes, such as HMGCR, and low-density lipoprotein receptor (LDLR).[40-42] miR-33a and miR-33b are considered to be cotranscribed and regulate lipid homeostasis with their host genes.

Several groups, including ours, reported that miR-33a targets ABCA1 and ABCG1 *in vivo*, using either antisense technology or by generating miR-33a knockout mice.[14-16,36] ABCA1 mediates the efflux of cholesterol to lipid-poor apolipoprotein A-I (apoA-I) and forms nascent HDL, whereas ABCG1 plays a critical role in the process of cholesterol efflux, which exports cellular cholesterol to large HDL particles. As a result, plasma HDL levels increased to 35–50%, without affecting other lipoproteins in anti-miR-33a-treated mice.[15,16,36] miR-33a knockout mice also showed an increase in the levels of ABCA1 and ABCG1 and a 25–40% increase in serum HDL (Figure 4A).[14] We then analyzed the serum lipid profile of miR-33b knock-in (KI) mice, which had miR-33b in the same intron as in humans.[39] In contrast to the results with miR-33a-deficient mice, HDL-C levels in these mice were reduced by almost 35%, even in miR-33b KI heterozygous mice compared with the control mice (Figure 4B). Baldan and colleagues reported that miR-33 also regulates the expression of a number of bile acid transporters, including ABCB11 and ATPB1 that control bile secretion.[43] This finding supports the hypothesis that miR-33s control RCT by affecting HDL-C biogenesis (via ABCA1), cellular cholesterol efflux from peripheral tissues (via ABCA1 and ABCG1), and bile acid secretion (via ATP8B1 and ABCB11).

It has already been proven that antisense inhibition of miR-33a resulted in regression of atherosclerotic plaque in low-density lipoprotein receptor (LDLR)-deficient mice by promoting RCT.[44] Moreover, miR-33a deficiency reduced the progression of atherosclerosis in apoE-deficient mice (Fig-

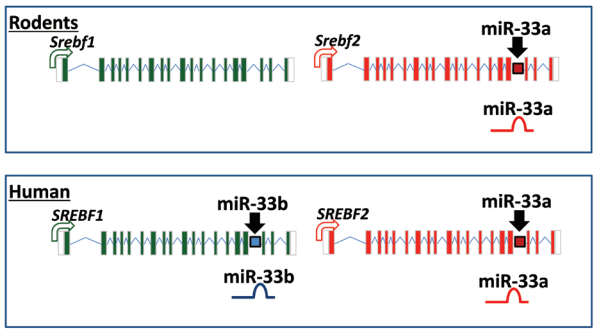

Figure 3. Mice have a single copy of microRNA (miR)-33, but humans have two copies (miR-33a and miR-33b).

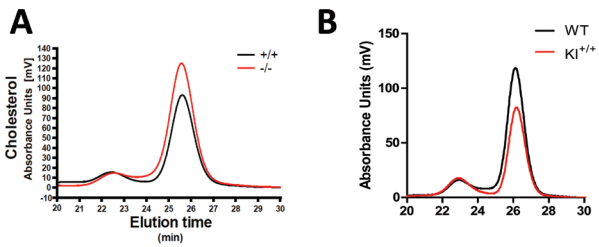

Figure 4. Lipid profiles of wild-type, microRNA (miR)-33a-deficient mice, and miR-33b knock-in (KI) mice. **A:** Representative HPLC analysis of serum cholesterol from male wild-type and miR-33a-deficient mice. **B:** Representative HPLC analysis of serum cholesterol from male wild-type and miR-33b $KI^{+/+}$ mice. (Cited and modified from Horie T, *et al*. Proc Natl Acad Sci U S A 2010; 107; 17321.[14] Horie T, *et al*. Sci Rep 2014; 4; 5312.[39])

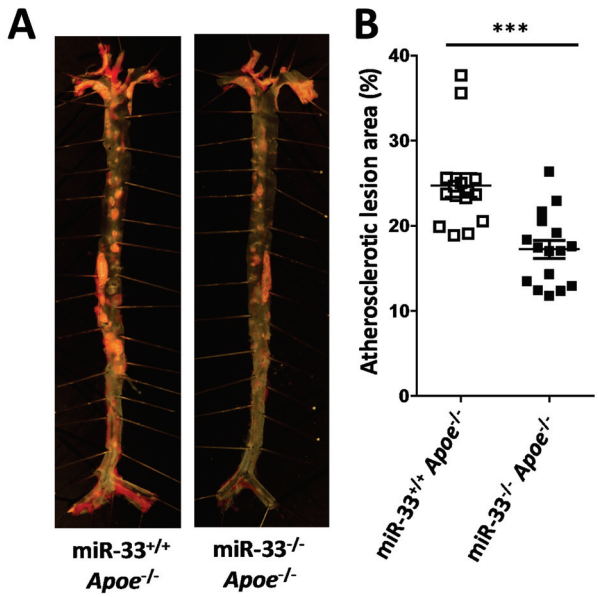

Figure 5. microRNA (miR)-33a deficiency reduced atherosclerosis. **A:** Representative images of the *en face* analysis of the total aorta in miR-$33a^{+/+}$ $Apoe^{-/-}$ and miR-$33a^{-/-}$ $Apoe^{-/-}$ male mice. **B:** Quantification of the atherosclerotic lesion area in *en face* analysis of the total aorta in male mice. Values are the mean ± SE (n = 15–16 each). $^{***}P < 0.001$. (Cited and modified from Horie T, *et al*. J Am Heart Assoc 2012; 1; e003376.[45])

ure 5).[45] In this study, miR-33a deficient mice not only had higher and functional HDL-C but also macrophages, which have a higher cholesterol efflux capacity, resulting in lower lipid accumulation in atherosclerotic areas, and proven in bone marrow transplantation experiments. Other possible beneficial properties of anti-miR-33a therapy include an anti-inflammatory response via upregulation of ABCA1. ABCA1 modulates cell-surface cholesterol levels, inhibits its partitioning into lipid rafts, and decreases the responsiveness of inflammatory signals from innate immune receptors. Furthermore, ABCA1 has been reported to directly act as an anti-inflammatory receptor independent of its lipid transport activities.[46]

miR-758: miR-758 was identified as a miRNA that was repressed by excess cholesterol in mouse peritoneal macrophages.[47] Transfection of human macrophages with miR-758 but not a control miRNA strongly decreased ABCA1 protein expression. Under physiological conditions, a high-fat diet in mice repressed miR-758 both in peritoneal macrophages and in the liver. Moreover, expression levels of pri-miR-758 and miR-758 were significantly reduced in mouse macrophages treated with AcLDL or cholesterol:methyl-β-cyclodextrin. It is interesting that miR-758 is highly expressed in the brain, particularly in astrocytes. The brain accounts for almost 25% of total body cholesterol content, and cholesterol is synthesized by astrocytes and oligodendrocytes in the developed brain.[48] It is known that astrocytes supply cholesterol to other neurons via ABC proteins, such as ABCA1, ABCG1, and ABCG4.[49,50] Cholesterol is required for myelination, dendrite differentiation, and synaptic activity. Disturbances in central nervous system cholesterol homeostasis are implicated in neurodegenerative disorders, including Alzheimer's disease and Huntington's disease.[49] Thus, these findings suggest that miR-758 can help in the retention of neuronal cholesterol by suppression of ABCA1 when cholesterol levels are reduced. Pathological conditions induced by miR-758 dysregulation are still unknown.

Recently, human atherosclerotic plaques obtained by elective carotid endarterectomy were analyzed to detect the changes in miRNA expression levels.[51] As a result, strong and significant upregulations of miR-33b and miR-758 were observed in hypercholesterolemic plaques compared with normocholesterolemic plaques. It is interesting that miR-33a expression was not different between normocholesterolemic and hypercholesterolemic plaques. These results indicated that miR-33b and miR-758 are putative key regulators of ABCA1 within human atherosclerotic plaques.

miR-10b: It has been shown that protocatechuic acid (PCA), a metabolite of cyanidin-3-O-β-glucoside (Cy-3-G), has a remarkable antiatherogenic effect. Wang, *et al*. demonstrated that PCA is an intestinal microbiota metabolite of ingested Cy-3-G. Moreover, they found that expression of miR-10b in macrophages can be reduced by PCA. A putative binding site for miR-10b was found in the 3'-UTR of mouse and human ABCA1 and ABCG1 genes. Functional analyses demonstrated that miR-10 directly represses ABCA1 and ABCG1 and negatively regulates cholesterol efflux from murine- and human-derived macrophages. Further *in vitro* and *ex vivo* analyses indicated that dietary Cy-3-G, through an intestinal microflora and PCA-dependent pathway, promoted an antiatherosclerotic effect via miR-10b-dependent enhancement in ABCA1 and

ABCG1 mediated cholesterol efflux. Moreover, Cy-3-G consumption promoted macrophage RCT and regressed atherosclerotic lesion in a gut microbiota-dependent pathway in Apoe$^{-/-}$ mice.

It is known that human DNA represents < 10% of the total DNA within each of us. Every mucosal surface harbors a diverse and distinct microbial composition. The above study reinforces the importance of the microflora as an important participant at the nutrient-host interface.

miR-144: The bile acid receptor farnesoid X receptor (FXR), which regulates many aspects of lipid metabolism, is expressed in the liver, intestine, kidney, and the adrenal gland.[52] Activation of FXR regulates genes controlling the synthesis, secretion, and resorption of bile acids.[53] Although activated FXR functions to directly induce target genes, FXR can also result in reduced expression of many genes via indirect mechanisms.[53] Therefore, it was hypothesized that FXR may enhance miRNA production that in turn could negatively regulate specific genes. Synthetic FXR agonist enhanced the expression levels of miR-144 and miR-451, which are cotranscribed as a bicistronic cluster from an intergenic region on mouse chromosome 11,[54,55] in wild-type mice but not in Fxr$^{-/-}$ mice.[56] It was reported that expression of the locus encoding miR-144 and miR-451 is strictly dependent on Argonaute 2 and is required for erythroid homeostasis.[57] Mice deficient in the miR-144/451 cluster display a cell autonomous impairment of late erythroblast maturation, resulting in erythroid hyperplasia, splenomegaly, and a mild anemia.

There are two conserved putative miR-144 binding sites in the 3'-UTR of ABCA1 mRNA. miR-144 overexpression in a human hepatoma cell line reduced ABCA1 protein levels and reduced cholesterol efflux to apoA-I. Moreover, overexpression of miR-144 in mice decreased hepatic ABCA1 and plasma HDL-C levels and silencing of miR-144 in mice caused an increase in ABCA1 protein and plasma HDL-C levels.

It is known that activated FXR results in induction of SR-BI, resulting in increased uptake of plasma HDL-C.[58] Therefore, it is possible that the combined effect of inducing hepatic SR-BI and repressing ABCA1 by miR-144 enhances the channeling of plasma HDL-C into bile for subsequent excretion.

miR-145: Experiments to identify miRNAs that regulate ABCA1 expression revealed that miR-145 can regulate both ABCA1 protein levels and cholesterol efflux function in HepG2 cells.[59] The human ABCA1 3'-UTR contains two binding sites for miR-145. In murine islets, an increase in miR-145 expression decreased ABCA1 protein expression, increased total islet cholesterol levels, and decreased glucose-stimulated insulin secretion. It was shown that miR-145 expression was induced after serum starvation in human colorectal carcinoma-116 and -8 cell lines.[60] Increased glucose levels in media significantly decreased miR-145 levels in cultured pancreatic β cells.[59] Therefore, the physiological role of miR-145 regulation of ABCA1 may be a response to the presence of nutrients. The decreased expression of miR-145 caused by glucose can then increase the expression of miR-145 target genes involved in insulin secretion such as ABCA1.

Other established targets of miR-145 include OCT4, SOX2, and KLF4, and miR-145 represses pluripotency in human embryonic stem cells (hESC).[61] Increased miR-145 expression inhibits hESC self-renewal, represses the expression of pluripotency genes, and induces lineage-restricted differentiation. Loss of miR-145 impairs differentiation and elevates OCT4, SOX2, and KLF4 expression levels. Furthermore, the miR-145 promoter is bound and repressed by OCT4 in hESCs. Thus, there is a double-negative feedback loop involving OCT4, SOX2, KLF4, and miR-145. This work revealed a direct link between the core reprogramming factors and miR-145.

miR-19b: Several reports indicated that miR-19b contributes to atherosclerosis progression. miR-19b is upregulated in human atherosclerotic plaques and rat abdominal aneurysms.[62,63] The human ABCA1 3'-UTR has 3 miR-19b binding sites, including one conserved binding site and two poorly conserved binding sites.[64] It was shown that miR-19b directly regulated the expression levels of endogenous ABCA1 in foam cells derived from human THP-1 macrophages and mouse peritoneal macrophages.[64] The excretion of ^{3}H-cholesterol originating from cholesterol-laden mouse peritoneal macrophages into feces was decreased in mice overexpressing miR-19b. Moreover, the miR-19b precursor reduced plasma HDL-C levels and increased aortic plaque size and lipid content. In contrast, treatment with miR-19b antisense oligonucleotides prevented and reversed these effects.

Previous reports indicated that miR-19b positively regulates NF-kB signaling by targeting the inhibitory regulators of NF-kB signaling including A20/Tnfaip3, Rnf11, Fbxl11/Kdm2a, Zbtb16, and Cyld.[65] Therefore, the anti-atherosclerotic effects of miR-19b antisense oligonucleotide may be partly due to its anti-inflammatory properties.

miRNAs That Regulate SR-BI Expression

Circulating lipoproteins, particularly HDL, deliver cholesteryl esters (CEs) to cells via a selective CE pathway.[66,67] SR-BI is a physiologically relevant HDL receptor that binds HDL particles and mediates selective uptake of HDL CE, which is a pivotal step in RCT.[68,69] SR-BI is expressed in the liver and other organs such as adrenal glands, ovary, and testis, where SR-BI delivers cholesterol substrate for steroidogenesis.[68] Although considerable evidence supports trophic hormone (ACTH and gonadotropins)-mediated transcriptional regulation of steroidogenic SR-BI, not much is known about its post-transcriptional regulation or the potential post-transcriptional regulators of SR-BI.[70]

miR-125a/b and miR-455: miR-125a-5p and miR-455 have recently been shown to regulate SR-BI expression by direct targeting.[71] Transfection of pre-miRNA-125a or pre-miRNA-455 resulted in the suppression of SR-BI at both the transcript and protein levels and reduced selective HDL CE uptake and HDL-stimulated progesterone production. Moreover, *in vitro* treatment of primary rat granulosa cells and MLTC-1 cells with cyclic AMP (cAMP) or *in vivo* treatment of rat adrenals with adrenocorticotropic hormone decreased the expression of miR-125a, miR-125b, and miR-455 and reciprocally increased SR-BI expression. It is known that trophic hormone and cAMP upregulate SR-BI expression and function by promoting transcriptional induction.[70,72] Thus, these agents upregulate SR-BI by both transcriptional and post-transcriptional mechanisms.

miR-223: miR-223 was previously found to control monocyte differentiation and regulate multiple inflammatory genes.[73,74] In monocytes and macrophages; however, it was suggested that miR-223 also regulates many genes associated with cho-

lesterol homeostasis.[75] Interestingly, miR-223 promoter activity and mature miR-223 expression levels were linked to cellular cholesterol states in murine macrophages and human hepatoma cells.[18] Hypercholesterolemia was associated with increased hepatic miR-223 levels in Apoe$^{-/-}$ mice. Mechanistically, miR-223 was found to regulate HDL-C uptake through direct suppression of SCARB1 and to inhibit cholesterol synthesis through targeting 3-hydroxy-3-methlglurtaryl-CoA synthase 1 (HMGCS1) and methylsterol monooxygenase 1 (SC-4MOL) in humans. Additionally, miR-223 indirectly promotes ABCA1 expression though Sp3, thereby enhancing cellular cholesterol efflux. Genetic loss of miR-223 resulted in increased HDL-C levels and particle size, as well as increased hepatic and plasma total cholesterol levels. Thus, miR-223 has a role in systemic cholesterol regulation by direct and indirect regulation of multiple genes in cholesterol biosynthesis, uptake, and efflux.

It was also shown that miR-185, miR-96, and miR-223 repressed SR-BI by targeting the 3'-UTR in a coordinated manner.[76]

Circulating miRNAs

Mature miRNAs were found to be present in cell-free blood plasma and serum in 2008 by several independent groups.[27,77,78] The mechanism that makes it possible for miRNAs to be nuclease resistant outside cells proved to be encapsulation into membrane-vesicles such as exosomes, microvesicles, and apoptotic bodies.[79-81] It was also shown that miRNAs bound to AGO family protein are also stable in protease- and nuclease-rich environments.[82]

Interestingly, some miRNAs were found in purified fractions of HDL from human plasma.[83] Among them, miR-223 is one of the most abundant. HDL particles were able to deliver miR-223 to recipient cells and mediate a significant reduction in miR-223 target gene expression via SR-BI. HDL-derived miR-223 can be also transferred to endothelial cells and inhibit intracellular adhesion molecule 1 (ICAM-1), thereby reducing monocyte adhesion and inflammation.[84] This finding might explain in part the anti-inflammatory effects of HDL-C.

On the other hand, it was shown that the analyzed HDL-miRNAs constituted only a small proportion of circulating miRNAs.[85] This report which analyzed HDL-bound miRNAs isolated from human blood revealed that the concentration of the most abundant HDL-bound miRNA was miR-223 and that it contributed only 8% of total miR-223 in the circulation. Additional studies are necessary to address the importance of miRNAs loaded in HDL-C.

Conclusions: miRNAs have emerged as powerful and important regulators of lipoprotein metabolism. Previous reports indicated that miRNAs control most of the genes that regulate HDL-C metabolism, such as ABCA1, ABCG1, and SR-BI (Figure 6). Therefore, miRNAs are important in HDL-C biogenesis, cholesterol efflux, and HDL-C uptake in the liver for the control of RCT. These findings indicate that modulation of miRNAs may help in the treatment of dyslipidemia and atherosclerosis in the future.

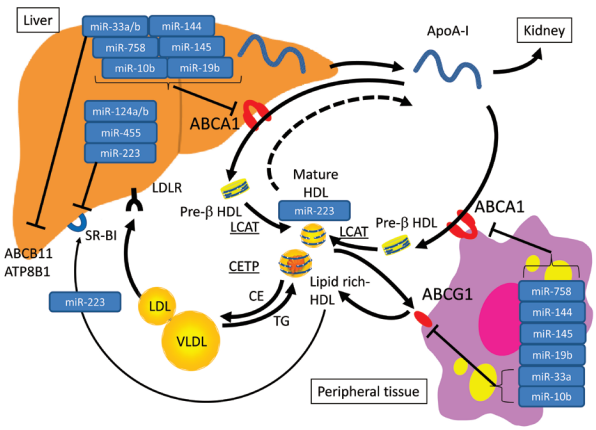

Figure 6. Mechanisms of microRNA (miRNA) regulation of high-density lipoprotein (HDL) cholesterol (HDL-C) metabolism. The liver is the site of HDL biogenesis, where ABCA1-mediated lipidation of lipid-poor apolipoprotein A-I (apoA-I) generates nascent HDL. Free cholesterol on nascent HDL particles is esterified to cholesteryl esters (CE) by lecithin-cholesterol acyltransferase (LCAT). In turn, ABCG1 mediates cholesterol transfer to mature HDL. CE transfer protein (CETP) mediates CE transfer from HDL to apolipoprotein B-containing lipoproteins (very low density lipoprotein [VLDL]/low density lipoprotein [LDL]) in exchange for triglycerides (TG), promoting plasma cholesterol clearance by the uptake of VLDL/LDL lipoproteins through the low density lipoprotein receptor (LDLR) pathway. Hepatic scavenger receptor class B, type I (SR-BI) mediates the removal of FC and CE from HDL, through the selective uptake pathway, and excess cholesterol is excreted from the liver into the bile. Both the ABCB11 and ATP8B1 transporters promote hepatic clearance.

Disclosures

Conflict of interest: Koh Ono; none, Takahiro Horie; none, Osamu Baba; none, Yasuhide Kuwabara; none, Takeshi Kimura; none.

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