



HHS Public Access

Author manuscript

Circ Res. Author manuscript; available in PMC 2015 March 20.

Published in final edited form as:

Circ Res. 2014 January 3; 114(1): 183–192. doi:10.1161/CIRCRESAHA.114.300645.

microRNA control of HDL Metabolism and Function

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Abstract

Recent discoveries of microRNAs (miRNAs) that control HDL abundance and function have expanded our knowledge of the mechanisms regulating this important lipoprotein subclass. miRNAs have been shown to regulate gene networks that control HDL biogenesis and uptake, as well as discrete steps in the reverse cholesterol transport pathway. Furthermore, HDL itself has been shown to selectively transport miRNAs in health and disease, offering new possibilities of how this lipoprotein may alter gene expression in distal target cells and tissues. Collectively, these discoveries offer new insights into the mechanisms governing HDL metabolism and function, and open new avenues for the development of therapeutics for the treatment of cardiovascular disease.

Keywords

miRNA; lipid metabolism; HDL; post-transcriptional gene regulation

Introduction

Over 60 years ago, an observation was made that would change the way we understood how cholesterol contributes to cardiovascular disease development. In 1951, Barr and colleagues noted that individuals who suffered from atherosclerosis tended to have low levels of plasma α -lipoproteins, now widely known as high-density lipoprotein (HDL)¹. He then put forth the notion that measurement of the levels of these lipoproteins would be a valuable tool for assessing an individual's risk of developing atherosclerosis, and even perhaps aid in its early detection. These observations, combined with the findings of the Framingham Study² set in motion decades of research that would eventually contribute to the “HDL cholesterol hypothesis”, which states that low levels of circulating HDL are a causative factor in the development of cardiovascular disease. Since then, a wealth of epidemiologic studies have demonstrated an inverse correlation between plasma levels of HDL cholesterol and the risk of cardiovascular disease and its thrombotic complications³. This correlation is believed to

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Disclosures K.J.M. is a member of the miR-33 Clinical Advisory Board of Regulus Therapeutics. K.J.M. and K.J.R. have a patent application on the use of miR-33 inhibitors for the treatment of atherosclerosis.

reflect the ability of HDL particles to remove excess cholesterol from peripheral cells, particularly macrophages in atherosclerotic plaques, for return to the liver. Supporting this, a number of studies in animal models have demonstrated that raising the number of HDL particles by either direct infusion of HDL or by over-expressing apoA-I (a major protein component of HDL) can reduce atherosclerosis progression or promote its regression^{4, 5}. As a result of these collective observations, HDL has earned the moniker of the “good” cholesterol⁶. Yet, recent Mendelian randomization studies have shown that certain single nucleotide polymorphisms that raise plasma HDL cholesterol (HDL-C) levels do not lower the risk of myocardial infarction, challenging the concept that HDL is atheroprotective⁷. Compounding these findings, several clinical trials of HDL cholesterol-raising therapeutics, including niacin and inhibitors of cholesterol ester transfer protein (CETP), have failed to show benefit^{8, 9}. These studies have begun to cast doubt on HDL's atheroprotective functions, and spurred further investigations of the molecular mechanisms regulating levels of plasma HDL and its function. One area of rapid growth in this regard, has been the discovery of microRNAs (miRNA) as potent regulators of the gene pathways controlling HDL genesis, cholesterol efflux and reverse cholesterol transport.

Over the last decade, the import of non-coding RNA as regulators of pre-, co-, and post-transcriptional gene expression has emerged¹⁰. Of the various classes of non-coding RNAs, miRNAs are currently the most widely-studied and have established roles in regulating a myriad of biological processes. The human genome contains over 2,500 unique mature miRNAs¹¹, and these are encoded in both intergenic and intronic regions of the genome. A number of reviews describing the transcription and processing of miRNAs have recently been published^{12, 13}. In their mature form, these small, non-coding RNAs of ~22 nucleotides bind to partially complementary sites primarily found in the 3'UTR region of target mRNAs, and inhibit gene expression via induction of mRNA degradation or translational repression. Although the founding work on miRNAs was performed in *C. elegans*^{14, 15}, this mechanism of post-transcriptional gene regulation has been highly conserved throughout evolution. It is now estimated that over 60% of all human genes are regulated by miRNAs^{16, 17}. Studies of miRNA expression and function have demonstrated that miRNAs allow for context-dependent fine-tuning of gene expression¹⁸, for example during embryonic development or environmental stresses (eg. starvation). In this regard, a powerful aspect of miRNAs is their ability to simultaneously repress numerous genes to influence the output of biological gene networks¹⁸. Although the magnitude of a miRNA's effect on any one gene may be modest (often on the order of 10-30%), the ability of miRNAs to repress multiple genes or steps in a pathway can lead to robust inhibition of the output.

Recent studies in the field of lipid metabolism have identified microRNAs as regulators of plasma levels of lipoproteins^{19, 20}, novel intercellular signaling molecules²¹, and plasma biomarkers of physiological status^{22, 23}. As described below, multiple genes affecting HDL and the reverse cholesterol transport pathway have been shown to be under control of these small non-coding RNAs, including those affecting HDL biogenesis, cellular cholesterol efflux, selective cholesterol uptake from HDL and bile transport (Figure 1). These studies have revealed how single miRNAs can target multiple components of this pathway, and also

identified key genes that are under control of multiple miRNAs. These findings have garnered considerable interest not only for their insight into how cells and tissues regulate HDL function, but also for their potential as targets for miRNA-based therapies.

MicroRNAs controlling HDL biogenesis

Nascent HDL is generated in the liver through the efflux of cholesterol and phospholipid across the hepatocyte cell membrane onto newly-synthesized lipid-poor apolipoprotein A-I (apoA-I)³. The ATP-binding cassette transporter A1 (ABCA1) plays a critical role in this process as evidenced by the near-absence of plasma HDL-C in patients with Tangier disease, which results from mutations in the ABCA1 gene²⁴⁻²⁷. The levels of ABCA1 at the plasma membrane controls the rate of cholesterol efflux to apoA-I, and hepatic-specific deletion of ABCA1 results in an ~85% loss of total HDL-C²⁸, with ABCA1 in the adipose and intestine contributing to the residual balance²⁹⁻³². Several miRNAs have recently been identified that target ABCA1 and thus, regulate plasma levels of HDL-C. Among these, miR-33a and miR-33b were the first to be reported^{20, 33} and were intriguing because of their genomic context: miR-33a and b are embedded in intronic regions of the *SREBF2* and *SREBF1* genes, which code for the SREBP2 and SREBP1 transcription factors that control the expression of genes involved in cholesterol and fatty acid synthesis. miR-33a/b are co-regulated with their host genes and act to repress gene programs that oppose SREBP functions, eg. cholesterol efflux and fatty acid oxidation. For example, under low cholesterol conditions that trigger transcription of *SREBF2* and the regulation of genes involved in cholesterol synthesis and uptake, co-transcription of miR-33a acts to inhibit cellular cholesterol export by targeting ABCA1^{20, 33-35}. The 3'UTRs of mouse and human ABCA1 mRNA harbor 4 miR-33a binding sites, resulting in strong repression of ABCA1 mRNA and protein. Furthermore, consistent with the ability of most miRNAs to mediate pathway regulation, miR-33a/b also target other genes that contribute to cholesterol mobilization and efflux from the cell, including *NPC1* and *ABCG1*²⁰. The physiological relevance of miR-33 targeting of *ABCA1* was initially demonstrated using inhibitors of miR-33, which increased cholesterol efflux from hepatocytes to apoA-I *in vitro* and raised levels of plasma HDL-C 30% in mice by 25-30%^{20, 33}. These findings were subsequently confirmed by targeted deletion of miR-33, which resulted in 25 and 40% increases in plasma HDL-C in male and female miR-33 null mice, respectively³⁶.

The two members of the miR-33 family, miR-33a and b, differ by only 2 nucleotides in their mature form, however these nucleotides lie outside the seed region (5' bases 2 to 8 of the mature miRNA) that dictates target recognition. Thus, miR-33a and b are predicted to target a similar subset of genes, and experiments to date have shown comparable repression of ABCA1 and other known targets by these two isoforms³⁷. Nonetheless, differences in their genomic context and evolutionary conservation may influence biological outcomes. For example, the abundance of miR-33a and miR-33b is controlled by factors that regulate their host genes, and the amplitude of the induction of *SREBF2/1*, e.g. levels of SREBP2 mRNA are increased 2-3 fold by sterol depletion, while levels of SREBP1 can be induced over ten times that amount by insulin. Furthermore, the presence of miR-33a within the *SREBF2* locus is highly conserved across species, whereas miR-33b is present in primates, but lacking in rodents and lower organisms³⁸. Such differences would lead to high levels of

miR-33b in insulin-resistant states in humans, and thus repression of ABCA1 and plasma HDL, that would not be observed in mice. To determine whether the findings of miR-33 inhibition in mice were translatable to primates, a study was undertaken in African green monkeys using 2'F/MOE anti-miR-33 oligonucleotides designed to inhibit both miR-33a and miR-33b. Anti-miR33 treatment increased hepatic expression of ABCA1 and plasma HDL-C in monkeys fed both a chow diet and a high-carbohydrate diet designed to increase levels of *SREBF1* and thus miR-33b³⁹. Notably, these effects of miR-33 inhibition were accompanied by increases in the number of large HDL particles and apoA-1 in the circulation, attributes that have been shown to be atheroprotective in studies of other HDL-raising therapies^{40, 41}. These studies solidified the notion that miR-33 represses hepatic *ABCA1* expression and thus, dampens plasma HDL-C levels, in a model highly relevant to humans, and highlighted its potential as a therapeutic target to raise HDL.

Although the majority of miR-33 studies have focused on the 5p strand, a recent report indicates that the miR-33 passenger strand or * strand may also be active in certain cell types or tissues⁴². Based on the low abundance of passenger strands of many mature miRNAs, these *strands were thought to be degraded. Yet an increasing number of miRNA* sequences with abundant expression have been reported to act as guide miRNAs⁴³, prompting renewed interest in their function. Goedeke et al showed that miR-33a* and miR-33b* accumulate under steady state conditions in various mouse, monkey and human tissues⁴², and other groups have noted the specific regulation of miR-33a* in endothelial cells subjected to hypoxia⁴⁴ and in M2-polarized macrophages⁴⁵. The miR-33a* and miR-33b* strands are highly conserved across species, suggesting a conserved function. Interestingly, miR-33a* and miR-33b* were shown to target a similar subset of lipid metabolism genes (*NPC1*, *CROT*, *IRS2*, *SRC3*, *NFYC*, *RIP140*, *ABCA1* indirectly) as their sister strands⁴², implying that both strands of the miR-33 locus may work in concert to regulate cellular cholesterol metabolism. MiR-33a* and miR-33b* have different seed sequences from miR-33a and miR-33b, and thus are predicted to bind different sites in the 3'UTR of their target genes. This dual targeting of lipid metabolism genes by both strands of the miR-33 duplex would result in strong repression of their targets. Future studies examining the regulation of miR-33 and miR-33* abundance will be important to understand the factors that support their accumulation in the cell. The mechanisms of miRNA strand selection and loading into the RISC complex remain obscure, but are thought to be related to the thermodynamic stability of each strand of the duplex. Interestingly a recent study reported stabilization of strands by their target mRNAs⁴⁶, suggesting a scenario in which miR-33* may be stabilized to help miR-33 regulate genes involved in cholesterol efflux and/or fatty acid oxidation. The functional effects of miR-33* on HDL and/or triglyceride levels *in vivo* have yet to be examined, but are likely to be relevant to the design of current therapeutic strategies to inhibit miR-33 for the treatment of atherosclerosis, as targeting of one arm of the duplex would not necessarily inhibit the functionality of the other, and may in fact lead to stabilization of the non-targeted strand.

ABCA1 has an uncommonly long 3'UTR of >3.3kb, rendering it particularly susceptible to post-transcriptional regulation by miRNAs. Indeed, shortly after the discovery of miR-33, other miRNAs were found to repress *ABCA1* and cholesterol efflux *in vitro*, including

miR-758⁴⁷, miR-26⁴⁸ and miR-106b⁴⁹. Recently, two groups reported that miR-144, an intergenic miRNA present in a bicistronic cluster with miR-451, also targets ABCA1 in the liver and modulates plasma HDL cholesterol levels^{50, 51}. Interestingly, miR-144 expression is regulated by two members of the nuclear hormone receptor family, the farnesoid X receptor (FXR) and the liver X receptors (LXR)^{52, 53}, providing fine-tuning of ABCA1 expression under specific biological contexts. These ligand-activated transcription factors contribute to the regulation of cholesterol homeostasis through transcriptional regulation of lipid associated genes: FXR controls hepatic sterol and bile acid levels, and LXR controls components of the cellular cholesterol efflux pathway in the liver and macrophages, including ABCA1. FXR induction of miR-144 transcription may channel cholesterol to the bile for excretion by repressing hepatic ABCA1, and thus HDL biogenesis. While this serves to reduce plasma HDL-C levels, this effect of miR-144 may be favorable overall by promoting reverse cholesterol transport, as has been observed with probucol treatment⁵⁴. On the other hand, LXR upregulation of miR-144 during cholesterol excess may function as a feedback mechanism to prevent uncontrolled LXR-induced cholesterol efflux through ABCA1. In both studies, anti-miR inhibition of miR-144 in mice resulted in increased hepatic ABCA1 expression and plasma HDL-C levels^{50, 51}. However, further studies of the effect of miR-144 inhibition on reverse cholesterol transport will be required to determine its impact on biliary cholesterol excretion.

Collectively, the studies of miR-33 and miR-144 have begun to illuminate the intricate network that fine-tunes ABCA1-dependent cholesterol efflux from the liver to regulate plasma HDL-C. It is likely that numerous other microRNAs will be identified to act in concert to regulate ABCA1 expression in the liver, with their individual and combined contributions determined by factors that regulate their expression and abundance under specific conditions. The identification of such metabolic rheostats will no doubt provide new opportunities for therapeutic manipulation of plasma HDL-C levels and reverse cholesterol transport.

MicroRNAs controlling cellular cholesterol mobilization

The efflux of excess cholesterol from peripheral tissues, particularly macrophages in the artery wall^{29, 31}, is essential for maintaining cholesterol homeostasis. At the cellular level, this requires that cholesterol first be mobilized from internal stores via cooperation of the lysosome, lipid droplets, neutral cholesteryl ester hydrolase, and the autophagy machinery⁵⁵. The final step of cholesterol efflux from the plasma membrane is mediated by ABCA1 to lipid poor ApoA-I (ApoE in the brain) and through the related transporter, ABCG1, to mature HDL particles. This ability of HDL and ApoA-I to act as acceptors of excess cholesterol from cells is thought to be central to their protective functions and this constitutes the first step in the reverse cholesterol transport (RCT) pathway through which HDL ferries cholesterol back to the liver for excretion. Through the coordination of these cholesterol mobilization pathways, net cholesterol balance in the arterial wall is maintained and pro-inflammatory responses by arterial cholesterol-loaded macrophages are reduced. As each of these steps represents potential points of microRNA control, the complexity of microRNA regulation of cholesterol efflux is likely to be much greater than originally anticipated.

As an example, autophagy, which regulates the availability of free cholesterol for efflux⁵⁶⁻⁵⁹ and contributes prominently to macrophage RCT *in vivo*⁵⁷, is a complex process that requires multiple sequential membrane remodeling and trafficking events, orchestrated by a small army of autophagy-related gene (ATG) proteins. This pathway has recently been shown to be regulated by a number of miRNAs, including miR-18a, miR-20a, miR-30a, miR-30d, miR-101, miR-106b, miR-132, miR-181a, miR-196, miR-212, miR-221, miR-222, miR-376b, miR-502⁶⁰⁻⁶⁹, which act by targeting ATG proteins (ATG2, ATG4, ATG5, ATG12)^{62, 63, 65, 68} or their upstream effectors (BECN1, mTOR, ULK1)^{61, 63, 64, 66, 68}. To date, the majority of these miRNAs regulating autophagy have been characterized in different types of cancer (breast cancer,^{60, 62} hepatocellular carcinoma,⁶⁷ chronic myelogenous leukemia,⁶⁹ colon cancer,^{64, 70} melanomas⁶¹), as well as in cardiac hypertrophy,^{71, 72} Parkinson's disease,⁷³ and Crohn's disease.^{74, 75} While the role of these miRNAs in regulating lysosomal trafficking of cholesterol has yet to be investigated, they are likely to have a major impact on cholesterol efflux, RCT and HDL function. As the activation of autophagy in macrophages has been shown to suppress foam cell formation and atherogenesis in mice⁵⁶, therapeutic targeting of microRNAs that limit this pathway may provide new therapeutic targets for enhancing cholesterol flux.

Although macrophage RCT does not significantly alter total plasma HDL-C levels^{76, 77}, it's contribution is critical to atheroprotection. Indeed, Khera et al showed that the efflux capacity of HDL is an independent and robust predictor of atherosclerosis in humans, which is not simply explained by levels of HDL-C in the circulation⁷⁸. As ABCA1 and ABCG1 control the terminal steps of cholesterol efflux to nascent and mature HDL from extrahepatic cells, microRNAs that target these genes would be predicted to inhibit reverse cholesterol transport. In mice, miR-33 targets both ABCA1 and ABCG1 (ABCG1 is not a target in humans), and miR-33 inhibitors enhance macrophage cholesterol efflux to apoA-1 and *HDL in vitro*^{20, 33, 35}. Furthermore, parenteral delivery of anti-miR33 oligonucleotides in mice increased RCT from labeled macrophages *in vivo*^{79, 80}, and directly upregulated ABCA1 in atherosclerotic plaque macrophages to reduce plaque cholesterol content⁷⁹. Although miR-33 has been the most extensively studied *in vivo*, several other microRNAs have been shown to regulate ABCA1 in macrophages and other cell types, including miR-758, miR-26, miR-106 and miR-144⁴⁷⁻⁴⁹. Like miR-33, miR-26 also downregulates other genes involved in cholesterol mobilization in addition to ABCA1, such as ADP-ribosylation factor-like 7 (ARL7), an intracellular transport protein that moves cholesterol to the membrane for removal by ABCA1⁴⁸. Expression of miR-26 is suppressed by LXR, and thus would be predicted to be downregulated under conditions of cholesterol excess during which increased levels of ABCA1 would be needed. On the other hand, miR-144 would be induced by LXR to target ABCA1 under similar conditions⁵⁰, and thus further studies of the temporal expression and abundance of these two microRNAs will be needed to resolve their relative contribution to cholesterol efflux control. Finally, miR-758 and miR-106b have been found to be highly enriched in the brain where ABCA1 plays a key role in effluxing excess cholesterol to apoE, the predominant apolipoprotein in the brain^{81 49}. Notably, ABCA1-dependent cholesterol efflux appears to reduce the accrual of amyloid- β in the brain, and in accordance with this, overexpression of miR-106b in neuronal cells increased the accumulation of amyloid- β in these cells⁴⁹. Thus, while multiple miRNAs can mediate post-

transcriptional regulation of ABCA1, their individual impact on cholesterol efflux and RCT will be influenced by factors such as their relative tissue enrichment, transcriptional regulation, as well as miRNA cooperation and/or competition.

Recent studies have highlighted the complex interplay of dietary nutrients and intestinal microbiota composition in influencing cardiometabolic diseases^{82, 83}. Dietary anthocyanins, such as the cyanidin-3-O-B-glucoside (Cy-3-G) polyphenol commonly found in fruits, berries and red wine, have been associated with reduced risk of cardiovascular disease. This has now been linked in a series of studies to the actions of a Cy-3-G metabolite, protocatechuic acid (PCA), which reduces levels of miR-10b, a newly identified repressor of ABCA1 and ABCG1^{84, 85}. Studies in antibiotic-treated and germ-free mice established that dietary Cy-3-G conversion to PCA, and its downstream enhancement of RCT, was dependent on the gut microbiota⁸⁵. Using physiological concentrations of PCA achieves with Cy-3-G dietary supplementation, the authors showed that PCA treatment of macrophages reduced miR-10b, causing derepression of its target genes ABCA1 and ABCG1, and increasing cholesterol efflux capacity. In *ApoE*^{-/-} mice treated for 4 weeks with dietary Cy-3-G or PCA, these observed changes in miR-10b, macrophage cholesterol efflux and RCT were associated with a reduction in atherosclerotic plaque size. This study underscores the complex interaction of the gut microbiome with risk factors for cardiovascular disease, and highlights a new mechanism through which miRNAs that regulate cholesterol homeostasis could be modulated. Future studies investigating how other gut-microbiota derived compounds, such as the recently identified plasma metabolite trimethylamine N-oxide (TMAO) that promotes macrophage cholesterol accumulation and atherosclerosis⁸³, might also alter cholesterol-associated microRNAs will no doubt reveal new mechanistic links between metabolism and host-microbial interactions.

MicroRNAs targeting hepatic HDL uptake and excretion

Transport of HDL-C to the liver for bile acid synthesis and excretion is the final step of RCT and can occur either directly via the scavenger receptor B-I (SR-BI), or after transfer to apolipoprotein B-containing lipoproteins by the cholesteryl ester transfer protein (CETP) present in humans³. MicroRNAs targeting these pathways are just beginning to be explored, and represent exciting new therapeutic targets to influence the route of delivery of HDL's cargo. SR-BI is a plasma membrane glycoprotein structurally similar to CD36 that is most highly expressed in liver and steroidogenic tissues, where it delivers HDL-derived cholesterol for excretion and steroid hormone synthesis. SR-BI-mediated selective uptake of HDL-C is considered a beneficial pathway, as it both increases the rate of delivery of cholesterol to the liver and results in the release of cholesterol-depleted HDL particles that are recycled to further promote cholesterol efflux. The level of SR-BI expression is controlled at the transcriptional level by nuclear hormone receptor transcription factors such as PPAR γ and LXR, and at the post-transcriptional level by alternative splicing of the mRNA. The additional post-transcriptional control of this pathway by microRNAs was recently demonstrated using small interfering RNA (siRNA) silencing of the miRNA-processing enzymes Drosha and Dicer, which resulted in a marked increase in SR-BI mRNA and protein in HEPG2 cells⁸⁶. Bioinformatic prediction algorithms, such as Targetscan and MiRanda, indicate that up to 50 microRNAs may target the 3'UTR of human SR-BI. Among

these, miR-185, miR-96, and miR-223 were validated as strong repressors of SR-BI mRNA and cell surface expression, and their inhibition in HEPG2 cells increased SR-BI expression and selective HDL-C uptake⁸⁶. Notably, when two or three of these miRNAs were combined, there was greater repression of SR-BI than that conferred by any single miRNA, suggesting that these miRNAs may coordinately repress SR-BI mRNA by simultaneously binding to different regions of the SR-BI 3'UTR. Interestingly, miR-185, miR-96 and miR-223 have all been reported to regulate genes involved in the proliferation of various tumor cell lines, although changes in proliferation were not observed in the HEPG2 cells used for the study of SR-BI. While the factors regulating expression of these miRNAs have not yet been explored, miR-185 is located within the first intron of a gene of unknown function, *C22orf25*, whereas miR-96 and miR-223 are intergenic miRNAs encoded on chromosomes 7 and X, respectively. Of these miRNAs, only miR-223 does not have conserved target sites in the rodent SR-BI 3'UTR. An analogous screen of miRNAs targeting the 3'UTR of mouse SR-BI identified miR-125a and miR-455 as potent regulators of SR-BI expression in murine steroidogenic and hepatic cell lines⁸⁷. Overexpression of these miRNAs reduced both SR-BI-mediated selective HDL uptake and HDL-stimulated progesterone production. The miR-125a binding site is conserved in the human SR-BI 3'UTR, however studies of its function in human cells have yet to be performed. While *in vivo* studies demonstrating that inhibition of mouse and primate SR-BI targeting microRNAs can increase HDL-C uptake are still lacking, these hold promise as a therapeutic approach to hold to increase RCT through this pathway.

During its journey to the liver, HDL undergoes numerous remodeling events that affect its size and composition. Cholesteryl ester transfer protein (CETP) mediates the exchange of HDL-cholesteryl esters for triglycerides from apoB-containing lipoproteins, thus shifting HDL's cholesterol cargo for uptake by the LDL receptor in the liver. Inhibitors of CETP have been actively pursued by the pharmaceutical industry as a means to raise plasma HDL-C and RCT^{88, 89}. Although no microRNAs have yet been described to target the CETP gene, and its relatively small UTR (<200 nucleotides) is only predicted to contain few miRNA binding sites, it is likely that the expression of this and other enzymes that modulate HDL composition and function, such as lecithin-cholesterol acyltransferase (LCAT), hepatic lipase, and endothelial lipase, will be found to be under microRNA control.

Hepatic cholesterol delivered via either SR-BI or the LDL receptor can be oxygenated, converted into bile acids, and secreted into the intestine via canalicular transporters. While the majority of bile acids are reabsorbed in the intestines, a proportion is eliminated in the feces, thereby ridding the body of excess cholesterol. In addition to its roles in regulating HDL biogenesis and macrophage cholesterol efflux, miR-33 has also been shown to regulate hepatic bile metabolism⁸⁰. miR-33 targets the 3'UTRs of ABCB11 and ATP8B1, transporters that reside in hepatic canalicular membranes and play essential roles in regulating biliary output (Figure 1). Using locked nucleic acid (LNA)-mediated silencing of miR-33, Allen et al showed that miR-33 inhibition increased sterols in the bile and enhanced reverse cholesterol transport *in vivo*⁸⁰. Similar studies in *Ldlr*^{-/-} mice by Rayner et al using 2'F/MOE oligonucleotide inhibitors of miR-33 noted a step-wise increase in RCT to the

serum, liver and feces -30, 50 and 85%, respectively⁷⁹ – reinforcing the notion that miR-33 coordinates reverse cholesterol transport at multiple levels.

HDL transport of microRNAs: a predictor of functionality?

Although miRNAs act intracellularly, microRNAs have been shown to be exported from both healthy and diseased tissues and cells. Extracellular microRNAs can be transported in membrane-derived vesicles (exosomes and microparticles), on lipoproteins, or bound to proteins like Argonaute2, and these circulating microRNAs are remarkably stable in plasma. As a result, extracellular miRNAs are being studied as novel biomarkers of disease states, including CVD, and distinct circulating miRNA signatures are beginning to be identified in health and disease⁹⁰. For example, recent studies have identified miRNA-208b and miR-499 as promising biomarkers of acute myocardial infarction, however these remain to be validated in larger populations^{91, 92}. Perhaps more exciting is the recognition that extracellular miRNAs represent a novel class of signaling molecules that may mediate cell-to-cell communication. A key role for such extracellular miRNA signaling was demonstrated in the artery wall between endothelial and smooth muscle cells and was shown to mediate atheroprotection⁹³. Dimmeler et al showed that atheroprotective shear stress regulates the expression of multiple miRNAs in endothelial cells via the transcription factor KLF2, which are exported in extracellular vesicles⁹³. Most prominent among these were miR-143 and miR-145, which can regulate smooth muscle cell phenotype prevent de-differentiation. Indeed, extracellular vesicles derived from KLF2-overexpressing endothelial cells, injected intravenously in *ApoE*^{-/-} mice for 6 weeks, protected from atherosclerotic lesion formation, and this could be reversed by inhibiting miR-143/145. Endothelial functions such as migration have also been shown to be regulated by such cell-to-cell miRNA-based communication from monocyte-derived (miR-150)⁹⁴ and apoptotic cell-derived (miR-126)²¹ miRNAs.

The discovery that HDL can transport miRNAs in the plasma, and stably deliver these to cells for uptake, suggests that HDL may participate in extracellular miRNA signaling^{95, 96}. Vickers et al demonstrated that several species of RNAs, including miRNAs and tRNA- and RNase P-derived RNA fragments, are carried on plasma HDL. Although miRNAs can also be isolated from LDL, for reasons that are unclear they appear to be more highly enriched on HDL. For example, miR-223 is one of the most abundant miRNAs on both HDL and LDL, yet it is approximately 7-fold higher on HDL (10,000 copies per μg of HDL)⁹⁶. Other miRNAs with defined roles in vascular biology and inflammation are also present on HDL, albeit at lower levels than miR-223: the endothelial enriched miRNAs, miR-126 and miR-92a, are present at 3,000 copies per μg of HDL, whereas the inflammation-associated miRNAs miR-146a and miR-155, and the metabolically controlled miR-378 are present at fewer than 120 copies/ μg of HDL⁹⁶. Notably, of these microRNAs, only the pro-inflammatory miR-155 was found to be greater quantities on LDL than HDL, a distinction that bears further investigation given the finding that miR-155 is proatherosclerotic. Similar to what has been reported for exosomes and microvesicles, HDL-derived miR-223 was shown to be delivered to cells, including hepatocytes and a kidney cell line overexpressing the HDL receptor SR-BI, which was shown to be required for HDL-mediated delivery of miRNAs to recipient cells⁹⁵. Notably, HDL-transferred miR-223 reduced target gene

expression in the recipient cells, including the miR-223 target SR-BI^{86, 95}. However, a second study of HDL-transported miRNAs in which the *Caenorhabditis elegans* miRNA, cel-miR-39, was used to track delivery of HDL-derived miRNAs reported that only a small number of cel-miR-39 copies could be detected in recipient cells such as endothelial cells, monocytes and smooth muscle cells⁹⁶. Thus, further investigation will be need to understand the functional relevance and physiological impact of HDL-derived miRNAs, as well as such basic questions as how such miRNAs are selected for export and associate with HDL.

In addition to its critical role in RCT, HDL can exert anti-inflammatory, anti-oxidant, and anti-thrombotic effects—and these functions appear to vary among individuals. It is thus possible that these functions of HDL may be mediated in part by, or altered by, the subset of microRNAs that it carries. Indeed, the microRNA cargo of HDL has been shown to be altered in both mice and humans by hypercholesterolemia and atherosclerosis⁹⁵. An analysis of HDL from subjects with familial hypercholesterolemia revealed approximately 22 miRNAs that were significantly altered as compared to HDL from normal subjects. Moreover, HDL from familial hypercholesterolemia subjects was found to impact target gene expression in recipient hepatoma cells, as compared to normal HDL. These findings offer insight into new potential mechanisms by which HDL may mediate pleiotropic effects, however whether microRNAs are responsible for some of the observed variations in HDL's anti-inflammatory, antioxidant, and antithrombotic effects await further testing.

Therapeutic targeting of miRNAs to increase HDL abundance and function

MicroRNA-based therapeutics represent a new class of drugs that hold promise for the treatment of cardiovascular and other diseases. The recent FDA approval of Kynamro (previously known as Mipomersen), a first-in-class antisense oligonucleotide inhibitor that targets apolipoprotein B-100 to reduce LDL cholesterol for the treatment of homozygous familial hypercholesterolemia⁹⁷, represents a giant leap forward for oligonucleotide-based therapies, including miRNA therapeutics. The first anti-microRNA therapy has yet to reach the clinic, yet anti-sense oligonucleotides against miR-122 (known as Miravarsen) have shown efficacy in patients with hepatitis C infection, where the Miravarsen-treated group showed prolonged dose-dependent reductions in hepatitis C viral RNA levels without evidence of viral resistance⁹⁸. These results have generated considerable excitement for the possibility of using microRNA-based therapies to treat cardiovascular disorders. miR-33 inhibition is thought to be particularly promising as a therapeutic for atherosclerosis, as it would enhance multiple components of the reverse cholesterol transport pathway, including HDL biogenesis, cholesterol efflux from plaque macrophages, and cholesterol excretion to the bile. Indeed preclinical studies of miR-33 inhibition in mice and nonhuman primates for up to 12 weeks showed sustained increases in HDL-C (on the order of 40-50%)^{39,40}. Furthermore, miR-33 deletion or inhibition has now been tested in several different mouse models of atherosclerosis progression and regression. The first study, performed in *Ldlr*^{-/-} mice with established atherosclerotic plaques, showed that a 4 week regimen of anti-miR-33 treatment led a 40% increase in HDL-C, greater RCT and a marked regression of atherosclerotic lesions⁷⁹. Characterization of the plaques in anti-miR-33 treated mice revealed 35% reductions in plaque size, lipids and macrophages, and an accompanying increase in plaque collagen content. Of note, the 2/F/MOE modified anti-miR-33

oligonucleotides used in that study were shown to penetrate the plaque, where they accumulated in lesional macrophages to directly upregulate ABCA1 mRNA. Microarray expression profiling of plaque macrophages isolated by laser capture microdissection revealed an overall decrease in inflammatory gene expression, as well as a polarization of macrophages to the reparative M2 macrophage phenotype which has been shown to characterize regressing atherosclerotic plaques^{99, 100}.

The beneficial effects of miR-33 targeting on atherosclerosis were confirmed in *miR33*^{-/-} mice crossed onto the *Apoe*^{-/-} background, which showed 20-25% reductions in plaque size and lipid content compared to control *Apoe*^{-/-} mice after 14 weeks on a 0.15% cholesterol-containing western diet¹⁰¹. Peritoneal macrophages isolated from *miR33*^{-/-}*Apoe*^{-/-} mice showed enhanced cholesterol efflux to apoA-I and HDL-C compared to macrophages from *Apoe*^{-/-} mice, reinforcing the concept that miR-33 targeting enhances RCT at the level of both HDL biogenesis and macrophage cholesterol efflux. However, studies in mice treated with miR-33 inhibitors for 12 weeks during the progression of atherosclerosis have been less clear. One study by Rotlan et al in *Ldlr*^{-/-} mice fed a western diet (0.3% cholesterol) together with treatment with 2'F/MOE modified anti-miR-33 oligonucleotides showed 20% reductions in both plaque size and macrophage content, and a decrease in miR-33 target genes in the aorta¹⁰², while a second study by Marquart et al in *Ldlr*^{-/-} mice fed a western diet (1.25% cholesterol) together with treatment with an anti-miR33 LNA failed to show any benefit¹⁰³. Notably, both groups reported that although miR-33 inhibitors increased plasma HDL-C in mice fed a chow diet, this effect was lost when the mice were switched to the atherogenic western diets. This absence of effect of miR-33 inhibition on HDL-C in mice fed a diet enriched in cholesterol may be due to low levels of hepatic miR-33 under these conditions: miR-33 is co-regulated with its host gene SREBF2, whose transcription in the liver is decreased by dietary cholesterol²⁰. However, the reasons for the divergent outcomes of miR-33 inhibition on atherosclerosis are less clear, but may relate to differences in the cholesterol contents of the different western diets (0.3 and 1.25%) or the bioavailability of the miR-33 inhibitors used (i.e. LNA vs. 2'F/MOE). While the ability of the anti-miR33 LNA used by Marquart et al to reach macrophages in the plaque has not been tested, this could potentially account for the efficacy of the 2'F/MOE modified anti-miR-33 in reducing atherosclerosis in the absence of an increase in plasma HDL. The study by Rotlan et al used the same 2'F/MOE modified anti-miR-33 oligonucleotides that had proven to be effective at increasing ABCA1 in plaque macrophages and regressing atherosclerosis in the previous study by Rayner et al, however miR-33 target gene expression in these cells was not evaluated. Future studies will be needed to determine whether anti-miR-33 targeting of plaque macrophages is responsible for its atheroprotective effects in the absence of increased plasma HDL. These have further underscored the importance of studying how miRNAs modulate *cholesterol flux* through the HDL pathway and not HDL *cholesterol* alone.

Inhibition of miR-33, as well as other ABCA1-targeting microRNAs, may also prove advantageous in a number of other conditions in which increased cholesterol efflux is thought to be beneficial. For example, to reduce islet cholesterol levels which impair β -cell function and glucose tolerance¹⁰⁴, and to reduce the secretion of β -amyloid in the brain

which is inversely correlated with ABCA1 -mediated cholesterol efflux to apoE¹⁰⁵. Studies of these approaches are eagerly awaited. Furthermore, although the pre-clinical studies of miR-33 inhibitors in mice and monkeys appear promising, many questions remain to be addressed, such as the effects of long-term suppression of miR-33, and whether compensatory mechanisms may become activated during miR-33 inhibition such as the upregulation of other ABCA1 targeting miRNAs. Finally, as the miRNA networks that regulate hepatic and systemic lipid homeostasis are unraveled, this will no doubt be paralleled by the identification of additional targets for therapeutic intervention.

Summary and Future Directions

Since the original hypothesis that HDL may be cardioprotective, HDL's halo has become somewhat tarnished as a series of HDL-C raising therapies have failed to confer protection from cardiovascular disease¹⁰⁶. This has prompted a reconsideration of the “HDL cholesterol hypothesis” and its evolution into the “HDL flux hypothesis”, owing to the renewed interest in understanding the mechanisms controlling HDL flux and functionality. The discoveries of microRNAs that control HDL biosynthesis, function and uptake, have greatly expanded our understanding of the molecular mechanisms regulating plasma levels of HDL-C and components of the RCT pathway, and have identified new therapeutic targets to regulate HDL flux. The list of microRNAs targeting lipoprotein metabolism pathways continues to grow at a rapid pace, and will no doubt expand to include microRNAs targeting other genes involved in HDL biogenesis (eg. apoAI), remodeling (eg. CETP and LCAT) and functionality. There remains much to understand about how microRNAs contribute to HDL functionality in health and disease. For example, whether dysregulation of miRNA activity contributes to the pathogenesis of cardiovascular disease or HDL dysfunction remains to be ascertained. The prospect that HDL-carried microRNAs contribute to the heterogeneous effects of HDL on endothelial cells, macrophages, and other cell types that influence vascular health, is intriguing and may provide insight into how the protective effects of HDL may be altered in disease or enhanced for therapeutic purposes. As our knowledge of these points of post-transcriptional control of HDL increases, so too will the potential for translating these discoveries from animal models to humans and, eventually, new therapies to treat and prevent cardiovascular disease burden.

Acknowledgments

Sources of funding: Research on microRNAs in the Moore Lab is supported by the NIH (R01 HL108182). K.J.R. is supported by a CIHR Operating Grant and Salary Award.

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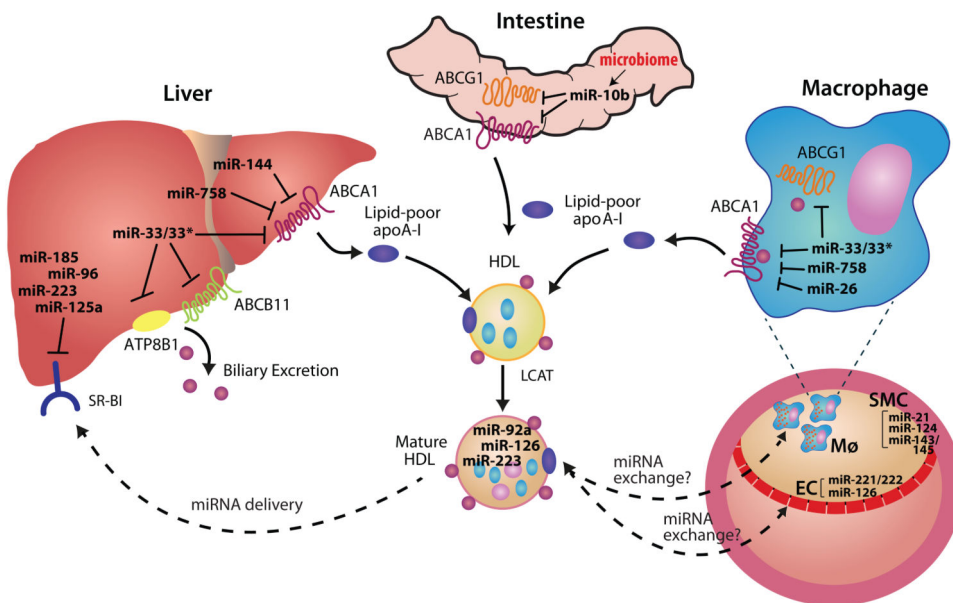


Figure 1. microRNA coordination of HDL homeostasis

miRNAs have been shown to regulate genes involved in cholesterol efflux and HDL homeostasis in various tissues, including the liver, intestine and macrophage. These microRNAs repress mRNA and/or protein expression of their target genes as indicated. In the liver, ABCA1 is a target of a number of microRNAs that reduce cholesterol efflux to lipid-poor apolipoprotein A-I (apoA-I), which generates nascent HDL. Free cholesterol (FC) on nascent HDL particles is then esterified to cholesteryl esters (CE) by lecithin-cholesterol acyltransferase (LCAT), converting nascent HDL to mature HDL. In peripheral tissues, ABCG1 mediates cholesterol transfer to mature HDL, and is a target of miR-33, as well as miR-10b, whose expression is impacted by the microbiome. Secreted microRNAs, such as miR-223, miR-92a and miR-126 are carried on circulating HDL particles and may mediate extracellular signaling by repressing genes in target tissues. HDL interaction with macrophages and endothelial cells may result in miRNA exchange (ie. pick-up or delivery), although this remains an area of open investigation. Upon its return to the liver, HDL's cholesterol and microRNA cargo is selectively taken up by scavenger receptor B-I (SR-BI), and excess cholesterol is excreted from the liver into the bile. Targeting of SR-BI and the ABC11 and ATP8B1 transporters reduce cholesterol hepatic clearance and/or excretion.

Table 1
MicroRNAs targeting the HDL pathway and their regulation

miRNA	Target gene(s)	Regulation of miR Expression	Effects in vivo?	Refs
miR-33a/b	ABCA1, ABCG1, NPC1, ABCB11, ATP8B1, CROT, CPT1a, HADHB, IRS2, PRKAA1, R1P140, SRC3	Sterols, LXR ligands, insulin	↓HDL, ↑atherosclerosis, ↓RCT	20, 33-35, 39, 79, 80
miR-33*	NPC1, CROT, IRS2, SRC3, NFYC, RIP140	Sterols, LXR ligands	Not yet tested	42
miR-758	ABCA1	Sterols	Not yet tested	47
miR-144	ABCA1	LXR & FXR ligands	↓HDL	50,51
miR-26	ABCA1.ARL7	LXR ligands	Not yet tested	48
miR-106b	ABCA1	Not known	Not yet tested	49
miR-10b	ABCA1, ABCG1	Dietary anthocyanins (i.e.Cy-3-G metabolites)	Reduced expression ↓associated with atherosclerosis, ↑RCT	84
miR-185	SR-61	Not known	Reduced expression associated with ↑SR-BI in Apoe ^{-/-} liver	86
miR-96	SR-BI	Not known	Reduced expression associated with ↑SR-BI in Apoe ^{-/-} liver	86
miR-223	SR-BI	Carried on HDL	Reduced expression associated with ↑SR-BI in Apoe ^{-/-} liver	86, 95, 96

* refers to miR-33 passenger strand