The Role of MicroRNAs in Cholesterol Efflux and Hepatic Lipid Metabolism

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Abstract

MicroRNAs (miRNAs) represent an elegant mechanism of posttranscriptional control of gene expression that serves to fine-tune biological processes. These tiny noncoding RNAs (20–22 nucleotide) bind to the 3′ untranslated region of mRNAs, thereby repressing gene expression. Recent advances in the understanding of lipid metabolism have revealed that miRNAs, particularly miR-122 and miR-33, play major roles in regulating cholesterol and fatty acid homeostasis. miR-122, the most abundant miRNA in the liver, appears to maintain the hepatic cell phenotype, and its inhibition decreases total serum cholesterol. miR-33, an intronic miRNA located with the sterol response element-binding protein (SREBP)-2 gene, regulates cholesterol efflux, fatty acid β oxidation, and high-density lipoprotein metabolism. These findings have highlighted the complexity of lipid homeostasis and the important role that miRNAs play in these processes, potentially opening new avenues for the treatment of dyslipidemias.

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CHOLESTEROL AND CARDIOVASCULAR DISEASE

Complications of atherosclerosis are the most common causes of death in Western societies (33). Among the many genetic and environmental risk factors that have been identified by epidemiologic studies, elevated levels of serum cholesterol are probably unique in being sufficient to drive the development of atherosclerosis in human and experimental animals, even in the absence of other known risk factors (33). Serum cholesterol is carried by several lipoprotein particles that perform the complex physiologic tasks of transporting dietary and endogenously produced lipids. Low-density lipoproteins (LDLs) have an essential role as a vehicle for the delivery of cholesterol to the peripheral tissues. Increased LDL levels are associated with increased risk of cardiovascular disease. LDL is taken up by cells via LDL receptors that recognize an N-terminal domain of apoB-100. Lack of functional LDL receptors leads to a massive accumulation of LDL in the artery wall (33). Atherosclerotic lesions begin as fatty streaks underlying the endothelium of large arteries. Recruitment of macrophages and their subsequent uptake of LDL-derived cholesterol are the major cellular events contributing to fatty streak formation (33).

To maintain cholesterol homeostasis, excess cholesterol is effluxed from cells to apoA-1 acceptors via the ATP-binding cassette transporters, ABCA1 and ABCG1 (89). Lipid-poor prebeta high-density lipoproteins (HDLs), rich in apoA-1, are synthesized by the liver or intestinal mucosa and released into circulation. ABCA1 facilitates the efflux of phospholipids and cholesterol to apoA-1 to generate nascent, discoidal HDL particles. Lecithin-cholesterol acyl transferase (LCAT) esterifies free cholesterol from prebeta HDL, resulting in mature HDL, which acts as an acceptor of ABCG1-effluxed cholesterol. Ultimately HDL transports cholesterol esters to the liver by two pathways (89). The cholesteryl esters are either directly delivered to the liver by scavenger receptor class B type 1 (SR-B1) or transferred to apoB-containing lipoproteins (VLDL/LDL) through cholesteryl ester transfer protein (CETP) and then transported to the liver through the LDL receptor (LDLr). In both cases, the cholesterol ester that is transported to the liver is secreted as bile acid. CETP exchanges cholesteryl ester from HDL for triglycerides from apoB-containing lipoproteins; these triglycerides are then hydrolyzed by hepatic lipases, regenerating small HDL particles and prebeta HDL, which, in turn, are released into circulation as acceptors of free cholesterol. The whole process is termed reverse cholesterol transport (RCT) and is believed to contribute to the protective role of HDL in the development of atherosclerosis.

LDL: low-density lipoprotein

ABCA1: ATP-binding cassette transporter A1

ABCG1: ATP-binding cassette transporter G1

HDL: high-density lipoprotein

REGULATION OF CELLULAR CHOLESTEROL METABOLISM

Cellular cholesterol levels are maintained through a tightly regulated and complex mechanism that involves de novo biosynthesis, internalization of exogenous cholesterol, and efflux of excess cholesterol. This coordinated process is regulated by ER-bound sterol regulatory element-binding proteins (SREBPs) (9, 48). The SREBP family of transcription factors consists of SREBP-1a, SREBP-1c, and SREBP-2 proteins that are encoded by two unique genes, *Srebp-1* and *Srebp-2*. The *Srebf-1* gene generates two alternative spliced transcripts, *Srebp-1a* and *Srebp-1c*, which are transcribed from different promoters (9, 48).

The SREBPs differ in their tissue-specific expression, their target gene selectivity, and the relative potencies of their *trans*-activation domains. SREBP1c regulates the transcription of genes involved in fatty acid metabolism, such as fatty acid synthase (FASN) (5–7). SREBP2 and SREBP1a regulate transcription of cholesterol-related genes, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), which catalyzes a rate-limiting step in cholesterol biosynthesis, and the LDLr, which imports cholesterol from the blood (9, 41, 69). The other major transcription factors that regulate cholesterol homeostasis are the liver X receptors (LXR), which regulate the response to cholesterol excess (96). LXRα and LXRβ are ligand-activated transcription factors of the nuclear hormone receptor family that are activated by endogenous oxidized metabolites of cholesterol (oxysterols). LXRs are activated in response to elevated cholesterol levels and induce the expression of proteins involved in cholesterol absorption, transport, excretion, and efflux, including ABCA1, ABCG1, ABCG5/8, and apoE (96). As described above, ABCA1 promotes cellular cholesterol efflux to apoA-1 and is also primarily responsible for initiating HDL formation in the liver (89, 96). ABCG1 mediates the cellular cholesterol efflux to HDL, and ABCG5/8 promotes cholesterol excretion into bile (89, 96).

MicroRNAs

In addition to classical transcription regulators, a class of noncoding RNAs termed microRNAs (miRNAs) has emerged as a critical regulator of gene expression acting predominantly at the posttranscriptional level. miRNAs are small [~22 nucleotide (nt)] RNA molecules, single stranded in the mature form, that regulate gene expression in metazoan animals, plants, and protozoa (2, 4). Since their discovery in *Caenorhabditis elegans* (53, 77, 94), hundreds of miRNAs have been identified in animals, plants, and viruses (6). The miRNAs identified to date are currently curated and annotated at miRBase, hosted by the Sanger Institute as a publicly available repository (**http://microrna.sanger.ac.uk/**). Bioinformatic predictions and experimental approaches indicate that a single miRNA may simultaneously target more than 100 mRNAs (27). miRNAs thus constitute a new layer of regulatory control over gene expression programs in many organisms. In fact, human miRNAs are predicted to control the activity of more than 30% to 60% of all protein-coding genes (24, 27, 36). miRNAs have been shown to be involved in a broad range of developmental and physiological processes, (2, 4, 10, 13, 22, 51, 59, 88); thus, their deregulation is closely linked to human diseases, including cancer and heart disease, and vascular disorders (1, 11, 13, 28, 90).

In previous decades, much effort and progress has been made in understanding the molecular mechanisms that regulate cholesterol metabolism and participate in the progression of cardiovascular diseases. miRNAs are emerging as important contributors to the molecular mechanisms underlying physiological homeostasis and disease. Further understanding of how miRNAs are integrated into complex genetic networks that regulate lipid homeostasis and dysregulation may potentially reveal novel therapeutic targets in this area.

Sterol response element-binding proteins (SREBPs): transcription factors that regulate expression of lipid metabolism genes

LXR: liver X receptor

Cholesterol efflux: the process whereby free cholesterol is exported from the cell

microRNA (miRNA): small noncoding RNA that posttranscriptionally inhibits gene expression

MicroRNA BIOGENESIS AND FUNCTION

miRNAs are encoded in diverse regions of the genome including both protein-coding and noncoding transcription units. Approximately half of miRNAs are derived from noncoding RNA transcripts; the majority of the remainder are located within introns of protein-coding genes, with a very small portion (~10%) encoded by exons of long nonprotein-coding transcripts (mRNA-like noncoding RNAs) (20, 79, 81). The production of the functional, ~22 nt mature miRNA involves multiple processing steps (**Figure 1**). The majority of animal miRNAs are transcribed by RNA polymerase II, as long (thousands of nucleotides) primary transcripts generating a stem-loop containing the primary miRNA (pri-miRNA) (2, 49). In addition, RNA polymerase III has been demonstrated to generate the transcript of a subset of miRNAs (7). The resultant pri-miRNA is then processed within the nucleus by the Microprocessor complex, which consists of a ribonuclease III (RNase III), called Drosha (54), and an RNA-binding protein, DGCR8/Pasha (38). The Microprocessor complex requires a variety of accessory cofactors, including DEAD box RNA helicases p68 and p72, as well heterogeneous nuclear ribonucleoproteins (35) that may function to promote the fidelity, specificity, and/or activity of Drosha cleavage. The product of the Drosha cleavage event is a 70–100 nt hairpin-shaped precursor RNA (premiRNA) that is transported to the cytoplasm via an Exportin-5 and Ran-GTP-dependent mechanism (58). In most mammalian miRNAs, including the majority of those encoded within introns, Drosha processing occurs cotranscriptionally, before splicing of host RNA (canonical pathway). However, there is a small subset of intronic miRNAs called miRtrons that circumvent the Drosha pathway (50, 65, 80). The lariat-debranching enzyme resolves the branch point to generate a premiRNA-like hairpin that can be exported from the nucleus and further processed in the cytoplasm. The premiRNA is further processed to produce the ~22-nt-long miRNA duplex (miRNA:miRNA*) by Dicer, another RNase III enzyme, which removes the terminal loop, yielding a ~22 nt RNA duplex (15, 42, 55).

Cleavage by Drosha and Dicer are not the only RNA-processing events that miRNAs can go through during maturation. Adenosine deaminases that act on RNA (ADAR) catalyze the conversion of adenine to inosine in dsRNA. Several studies have demonstrated that pri-miRNAs and premiRNAs are substrates of ADAR enzymes, which prevents Dicer processing and results in the accumulation of pre-miRNA hairpins. This editing process occurs in a tissue-specific manner and may underlie tissue-specific expression of some miRNAs (46, 95). It has been very recently shown that a subset of miRNAs, typified by the miR-144/451, is processed independently of Dicer in the cytoplasm. Instead, the secondary cleavage is carried out by Ago2, an Argonaute protein that is part of the complex that aligns the miRNA and messenger RNA (14, 16, 75). The precursor of these miRNAs possesses a stem that is uncommonly short (17 nt); the mature miRNA (23 nt) spans not only the length of the 5′ arm of the stem but covers all of the loop sequence. These features are predicted to be insufficient for recognition by Dicer.

The fully processed miRNA duplex obtained via Dicer or Ago2 processing is unwound into the mature single-stranded form (guide strand) and its complementary strand (passenger strand or miRNA*). One of them will be selected and will associate with other members of the Ago protein family within the effector ribonucleoprotein complex RISC (RNA-induced silencing complex) (34, 35, 61, 70). The intrinsic features of the duplex will determine which strand is incorporated into RISC and will become a mature miRNA (49, 86). The thermodynamic asymmetry of the miRNA duplex contributes to the strand selection (47, 82). The strand, whose 5′ end is less stably base-paired, will be more frequently selected as the guide, while the other strand (miRNA*) will be excluded from the RISC and is generally thought to be degraded. However,

in some instances, the passenger strand may be selected and used in gene regulation (67). In fact, recent deep-sequencing efforts indicate that a large number of miRNAs* are not degraded but rather associate to Ago1 or Ago2 (17, 31, 66, 67, 83). In this case, both strands may be functional, indicating that miRNA precursors can also be bifunctional (17, 66, 67).

As part of the RISC, the mature miRNA guides the complex to its RNA target by Watson-Crick base-pairing interactions regulating protein synthesis (5). When there is a perfect or near-perfect complementarity to a site(s) in the 3′ untranslated region (3′UTR) of an mRNA, the target can be cleaved and degraded. However, in animals, most studied miRNAs pair imperfectly with their mRNA targets in the 3′UTR and inhibit expression of their targets mainly through translational repression and/or mRNA decay (71). Recent studies have shown that miRNAs can also repress mRNA targets through binding to other regions, including 5′UTRs or protein-coding exons (25, 60, 68, 78), and in some cases may even activate translation (39, 68, 91, 92). However, the mechanistic details of protein synthesis inhibition by miRNAs are not well understood. Potential pathways include sequestration from ribosomes (by relocation into P bodies), blockage of translational initiation, translational repression after initiation, and target deadenylation coupled to transcript degradation (57, 71) (**Figure 1**). Several recent reviews have covered these topics (5, 10, 13, 23, 24, 49, 73, 87).

The computational identification of miRNA targets and the validation of miRNA-target interactions represent fundamental steps in understanding the contribution of miRNAs in cell functions. Several bioinformatic tools have been developed to predict miRNA targets (5), and these algorithms collectively rely on several criteria (5). In mammals, the most important requirement of miRNA-target interaction, although not always essential, is a contiguous base pairing of the miRNA nt 2–8, representing the seed region (56). In many cases, the seed region seems to determine recognition. However, in certain cases additional determinants are required, such as reasonable complementarity to the miRNA 3′ half to stabilize the interaction (nt 13–16) to compensate for inconsistent binding in the seed region (3, 5, 37, 84). In addition, certain features of the 3′UTR sequences surrounding the target site may boost miRNA efficacy, such as AU-rich sequences and positioning at least 50 nt from the stop codon and away from the center of long UTRs (5, 37). Given the many criteria that influence miRNA targeting, it has become clear that the validation of predicted miRNA targets is a crucial step in understanding potential (85) miRNA functions. As the understanding of miRNA binding biology increases, it can be expected that existing algorithms will become progressively more accurate.

3′ untranslated region (3′UTR): region of mRNA that frequently contains microRNA-binding sites

CONTROL OF CHOLESTEROL METABOLISM BY MIRNAS

The elegance of miRNAs comes from their ability to simultaneously regulate the expression of many targets, allowing for the synchronized control of multiple genes in the same biological pathway. One such pathway is cholesterol metabolism, which has been shown to be regulated in different ways by the following miRNAs: miR-122, miR-370, miR-378/378*, miR-335, miR-125a-5p and miR-33. Among these, miR-122 was the first to be described for its role in regulating total serum cholesterol and liver metabolism and has been the most widely studied liver miRNA. More recently, however, other miRNAs have been shown to control a variety of aspects of lipid metabolism, from adipocyte differentiation to HDL biogenesis and cholesterol efflux.

miR-122 and Liver Metabolism

miR-122 is highly expressed by the liver, and it is estimated to account for approximately 70% of all liver miRNA (68). It was first identified owing to an unusual genetic rearrangement of c-myc found in a woodchuck liver tumor, which gave rise to a noncoding, hairpin structure that was later revealed to be the precursor for

Anti-microRNA: antisense oligonucleotides that inhibit miRNA expression

HCV: hepatitis C virus

miR-122 (39). miR-122 is highly conserved, from humans to frogs, suggesting an important role for this miRNA that has been under selection pressure throughout evolution (32). Despite its high degree of conservation and its abundance in the liver, the targets of miR-122 were initially described to be those involved in the cellular response to stress and amino acid starvation, and at first did not appear to regulate normal hepatic physiology (12). It was only in using inhibition strategies to target miR-122 in vivo that the physiological relevance of miR-122 was revealed. Esau et al. (21) employed 2′-O-methoxyethyl phosphorothioate antisense oligonucleotides in both normal and high-fat diets fed to C57BL/6 mice for a period of five weeks and examined the expression of predicted targets of miR-122, showing that miR-122 inhibition upregulated a number of predicted miR-122 targets, including ALDOA, GYS1, and CCNG1. More importantly, using gene expression array analysis, the study revealed that silencing of miR-122 resulted in an upregulation of more than 100 genes containing 7–8 nt seed matches for miR-122 in their 3′UTR, many of which had not been previously predicted based on bioinformatic prediction algorithms. Similarly, Elmen et al. (19) used a complementary strategy to identify miR-122 target genes, using locked-nucleic acid (LNA)-mediated antagonism of miR-122 in normal mice. Using gene expression arrays, they identified 199 genes that were upregulated by the anti-microRNA antimiR122 and that contained at least one miR-122 recognition site. Taken together, these results imply that endogenous miR-122 is repressing a large number of genes that become derepressed when miR-122 is silenced. Functionally speaking, the consequences of silencing miR-122 in mice are a sustained reduction in total plasma cholesterol, observed in both the LDL and HDL fractions (19, 21). Furthermore, miR-122 antagonism in mice fed a high-fat diet resulted in a significant improvement in liver steatosis, as evidenced by the reductions in liver triglyceride content, and an increase in the rate of fatty acid β oxidation (21). These results were consistent with the observation that miR-122 controls the expression of FASN and antioxidant acetyl-cysteine 1 and 2—all genes involved in fatty acid synthesis and oxidation. The promising results from mouse studies prompted the use of LNA antagomirs directed against miR-122 in tests in nonhuman primates (18, 52). Similar to what was observed in mice, silencing of miR-122 in African green monkeys (18) and chimpanzees (52) resulted in substantial reductions in total plasma cholesterol, ranging from 20% to 30%, with no apparent toxicity or histopathological changes in the liver.

Certainly the targeting of miR-122 shows promise for the treatment of lipid metabolism disorders, but currently antagonism of miR-122 is being actively investigated for a therapeutic strategy in patients with hepatitis C infection. In addition to the target genes described above, miR-122 binds two positions in the 5′ noncoding region in the hepatitis C viral genome, and this binding is essential to viral accumulation and propagation in infected hepatocytes (44, 45, 74). In nonhuman primates, silencing of miR-122 resulted in sustained reductions in hepatitis C virus (HCV) viremia and improvement in liver pathology, with no evidence of viral resistance (52). The safety and efficacy of antimiR122 demonstrated in nonhuman primates prompted the first use of a miRNA-based therapeutic to enter a phase II clinical trial. Miravirsen, by Santaris Pharma A/S, is an LNA-antagomir against miR-122 and is currently being tested in patients with hepatitis C infection (26). Phase I results in healthy subjects showed that Miravirsen is well tolerated, and the double-blind placebo-controlled phase II trial will likely yield exciting results for the potential for miRNA-based therapeutics for the treatment of chronic diseases.

miR-370 and Its Multifaceted Regulation of Lipid Metabolism

Another miRNA, miR-370, has been described for its role in regulating cholesterol metabolism. miR-370 was first studied in a model of dominant-negative cJun

overexpression, which was found to upregulate miR-370 2.2-fold and concomitantly downregulate carnitine palmitoyl transferase (Cpt)1α—the rate-limiting step in the fatty acid β-oxidation pathway (43). miR-370 directly targets Cpt1α by binding target sequences in the 3′UTR, which results in downregulation of Cpt1α expression and consequently reduced fatty acid β oxidation in cultured hepatic cell lines. However, in addition to directly regulating genes involved in fatty acid metabolism, miR-370 regulates the expression of miR-122, which as described above, controls the expression of lipid and fatty acid metabolism. The authors demonstrate that overexpression of miR-370 leads to an upregulation of miR-122 and a resulting upregulation of lipogenic genes such as SREBP-1c and diacylglycerol O-acyltransferase 2 (43). Although the mechanism of this reciprocal control of miRNA expression and the consequent upregulation of certain genes is still unclear, the study highlights the complexity and the multiple layers of miRNA-mediated modulation of lipid metabolism and the incompleteness of our current understanding.

miR-378/378* in Adipocyte Lipid Biogenesis

More recently, another miRNA was found to play a role in the regulation of lipid metabolism: miR-378/378*. Gerin et al. (29) began by investigating the effect of Ago2 knockdown on adipocyte differentiation and surprisingly found that Ago2 had very little effect on this process, which suggests that miRNAs may not strongly influence this pathway. In contrast, during lipid droplet formation, Ago2 deletion had profound effects on the accumulation of triacylglycerol and phospholipids. This prompted the screening of miRNAs that are altered during adipogenesis in vitro; of the few miRNAs that were altered by lipid content, miR-378/378* was most highly upregulated. Interestingly, miR-378/378* is located within the peroxisome proliferator-activated receptor gamma coactivator-1 beta gene, and the two transcripts are cotranscribed during adipocyte differentiation (29, 87). Overexpression of miR-378/378* led to increased lipid droplet size and accumulation of triacylglycerol in 3T3-L1 adipocytes, without demonstrable effects on fatty acid β oxidation. Moreover, overexpression of miR-378/378* resulted in increased triacylglycerol synthesis, whereas knockdown of miR-378/378* caused a decrease in triacylglycerol synthesis. In searching for possible targets of miR-378/378*, the authors performed a gene expression array, but surprisingly found that in response to overexpression of miR-378/378*, when target genes would be expected to be downregulated, eight sets of genes were upregulated in response to miR-378/378*. These included genes involved in fatty acid synthesis (i.e., FAS, SCD-1, and resistin), which would help to explain the increased triacylglycerol synthesis in the presence of miR-378/378*. Interestingly, none of the more than 20 predicted targets of miR-378/378* that were tested were found to be true targets of this miRNA, and there was an unanticipated increase in the transcriptional activity of C/EBPα and β. The authors speculate that this could be an indirect effect, through the miR-378/378*-mediated downregulation of a transcriptional repressor of the C/EBPs, or else could suggest that miR-378/378* is acting to enhance transcriptional activity of C/EBPα and β by an atypical mechanism. Although this hypothesis requires further study, it is clear that the miRNA-mediated regulation of adipocyte differentiation and adipogenesis is multifaceted and not as straightforward as it may first appear, representing another layer of complexity of miRNA regulation.

miR-33 REGULATES LIPID METABOLISM

Recently, several groups independently reported that the SREBP-2 locus transcribes not only the mRNA for this transcriptional regulator of cholesterol uptake and synthesis, but also a highly conserved microRNA, miR-33a, that represses multiple genes involved in cellular cholesterol trafficking (63, 64, 76). miR-33a has

NPC1: Neimann Pick C1

been highly conserved during evolution and is present in the genomes of flies to vertebrates. Notably, a second isoform of miR-33, *miR-33b*, is found within *Srebf-1* in a more limited number of species (64). Although present in primates, miR-33b is absent in rodents and to date has been less well studied than miR-33a. As has been reported for several intronic miRNAs, miR-33a and miR-33b are cotranscribed with their host genes under conditions that regulate SREBP-2 and SREBP-1, respectively. Interestingly, despite a two-nucleotide variation in the mature forms of miR-33a and miR-33b, these miRNAs similarly target a variety of lipid metabolism–associated genes including *Abca1*, *Crot*, *Cpt1a*, *Hadhb*, and *Ampkα*, suggesting overlapping functions (17a).

Although the genomic location of miR-33a was reported in 2004, a number of years passed before the significance and the functional consequences of this location were established. Yet in a series of parallel studies published in 2010, miR-33 was identified as a key post-transcriptional regulator of cellular cholesterol homeostasis by three independent laboratories (63, 64, 76). Using an unbiased microarray approach, our group identified miR-33a as one of twenty miRNAs altered by cholesterol content in macrophages (76). Under cholesterol-depleted or cholesterol-enriched conditions, expression of miR-33 paralleled that of SREBP-2, suggesting that these genetic elements are coregulated. Furthermore, we showed that dietary cholesterol alters hepatic miR-33 expression levels in vivo, in both normal and hypercholesteromic mice. Two other groups, Najafi-Shoushtari et al. (64) and Marquardt et al. (63) uncovered the presence of miR-33 through in silico bioinformatic analysis of SREBP loci. These investigators also showed that *miR-33a* is cotranscribed along with *Srebf-2* in both hepatocytes and macrophages and that the expression of *miR-33a* and *Srebf-2* is comparable across many tissues (63, 64).

Despite their different approaches, all three groups quickly honed in on the top predicted gene target of miR-33, ABCA1, which is responsible for the movement of free cholesterol out of the cell. The 3′UTR of *Abca1* contains three highly conserved binding sites for miR-33a and/or miR-33b, and the expression of ABCA1 mRNA and protein is strongly repressed by miR-33 overexpression in a variety of cell types (63, 64, 76). Analysis of the 3′UTR of *Abca1* revealed that the targeting by miR-33 is direct and specific, as mutations in these sites result in the relief of the repression of *Abca1* by miR-33 (63, 64, 76). Functionally, overexpression of miR-33 in macrophages and hepatocytes decreased cholesterol efflux to apoA1—a key step in the generation of HDL and RCT. Moreover, the inhibition of endogenous miR-33 results in an increase in expression of ABCA1 protein and a concomitant increase in cholesterol efflux to apoA1, indicating a physiologically relevant role for this miRNA in regulating ABCA1 (63, 64, 76).

In addition to ABCA1, our study identified two other genes involved in cholesterol mobilization as targets of miR-33: ABCG1 (63, 76), which mobilizes cellular free cholesterol to more lipidated HDL particles, and Neimann Pick C1 (NPC1) (76), which transports cholesterol from lysosomal compartments to other parts of the cell in need (**Figure 2**). Interestingly, miR-33 targeting of these two genes differs in mice and humans. The 3′UTR of the mouse *Abcg1* gene contains two miR-33 binding sites; however, these sites are not conserved in the human 3′UTR. miR-33 overexpression in macrophages confirmed that miR-33 inhibits ABCG1 expression in cells of mouse but not human origin, indicating species-specific regulation of this gene by miR-33 (63, 76). The functional consequence of ABCG1 targeting in cells of mouse origin was demonstrated by a decrease in cholesterol efflux to HDL after overexpression of miR-33 (76). Furthermore, the 3′UTR of human *Npc1* contains two miR-33 binding sites, resulting in repression of NPC1 protein expression by miR-33 in human macrophages and hepatocytes. NPC1 acts in concert with ABCA1 to efflux cholesterol to apoA1 (93), which indicates that miR-33 represses a second part of the cellular cholesterol export pathway in humans. However, the 3′UTR of mouse

Npc1, which contains only one miR-33 binding site, is not repressed (76). Together, these results indicate that miR-33 targets multiple genes in the pathway regulating efflux of cholesterol to HDL.

miR-33 REGULATES HIGH-DENSITY LIPOPROTEIN LEVELS IN VIVO

HDL plays a key role in the removal of excess cholesterol from the body through the RCT pathway. ABCA1 and ABCG1 have important functions in the RCT pathway. (*a*) In the liver, ABCA1 mediates the initial lipidation of apoA1 to form nascent HDL particles. (*b*) In peripheral tissues, particularly macrophages in atherosclerotic lesions, ABCA1 mediates the efflux of cellular cholesterol to this nascent HDL, while ABCG1 contributes to the further lipidation of HDL. The observations that miR-33 could reciprocally control ABCA1 and ABCG1 expression upon activation of SREBP-2 thus lead to the hypothesis that miR-33 may control HDL levels in vivo. Using a variety of approaches, from viral delivery of antisense oligonucleotides to LNA inhibitors, it was demonstrated that modulation of miR-33 levels in vivo alters hepatic ABCA1 protein expression and circulating HDL (63, 64, 76) (**Figure 3**). In vivo overexpression of miR-33 decreased HDL levels by 25%, whereas its inhibition increased circulating HDL by 25%–30%. Notably, these experiments were performed in both normal and high-fat-diet-fed mice, indicating that inhibition of miR-33 results in a functional increase in HDL even when miR-33 (and SREBP-2) levels would be predictably low. Together these data indicate that anti-miR-33 treatment to elevate ABCA1 levels and increase HDL levels holds promise for the treatment and/or prevention of coronary artery disease, in which an underlying risk factor is low levels of HDL.

Shortly after the demonstration that miR-33 regulates HDL levels in vivo, this finding was confirmed in a molecular genetic causation study by Horie et al. (40). These investigators generated mice in which miR-33 was deleted from the intron of SREBP-2 without affecting the expression of the host gene. These mice, which were viable and fertile, showed increased hepatic ABCA1 expression and a 25% to 40% increase in circulating HDL levels (40). Notably, whereas no differences in male and female mice were observed in studies using synthetic inhibitors, female miR33$^{-/-}$ mice show larger increases in circulating HDL than do their male counterparts. The molecular mechanisms of this difference are currently unknown. Interestingly, the increase in HDL in miR33$^{-/-}$ mice appears to be in the larger, more lipidated HDL fractions, with no changes observed in the smaller HDL fractions (40). This increase in HDL particle size may be as a result of efflux of cholesterol to HDL from extrahepatic tissues into the RCT pathway, where ABCA1 and ABCG1 play important roles.

miR-33 TARGETS GENES INVOLVED IN FATTY ACID METABOLISM

In addition to cholesterol transport, miR-33a and miR-33b were recently reported to target genes involved in the β oxidation of fatty acids. We and others showed that miR-33 binding sites are highly conserved in the 3′UTR of *Cpt1a*, *Crot*, and *Hadhb*, from humans to chicken (17a, 30). Each of these genes plays a distinct role in the fatty acid oxidation pathway: CPT1a is required for coupling of acetyl CoA to carnitine, allowing for transport of medium- and long-chain fatty acids to the mitochondria for β oxidation (8, 62, 72). CPT1a is the rate-limiting step for this process and surprisingly is not one of the most highly regulated members of the β-oxidation pathway (8, 62, 72). Similarly, CROT is a peroxisomal enzyme that is required for the coupling of short-chain fatty acids to carnitine, also transporting them into the mitochondria. Finally, HADHB is required for the last three steps in the β-oxidation pathway in the mitochondria (8, 62, 72). Analysis of the 3′UTR activity of *Cpt1a*, *Crot*, and *Hadhb* revealed that miR-33 targets these genes in

the fatty acid oxidation pathway, and overexpression of miR-33 in hepatocytes decreases CPT1a and HADHB protein expression.

Gerin et al. (30) proposed that when SREBP-2 is activated, miR-33a would also target genes involved in cellular β oxidation of fatty acids, which is known to occur during states of cholesterol depletion. Indeed, they demonstrated that overexpression of miR-33a reduced cellular fatty acid β oxidation. Although this group focused its studies on miR-33a, perhaps of greater interest is the role of miR-33b in this process, which is encoded within *Srebp-1*. SREBP1 is upregulated during states of insulin excess, when cellular fatty acid β oxidation is reduced, possibly as a result of upregulation of miR-33b. Indeed, work from our group showed that activation of SREBP-1 with an LXR agonist leads to increased expression of miR-33b (17a). Furthermore, we found that miR-33a and miR-33b similarly target the 3′ UTR activity of *Crot*, *Cpt1a*, *Hadhb*, and *Ampkα*, indicating that both isoforms likely play a role in the regulation of these genes (17a). Taken together, these data suggest that anti-miR-33 treatment may be a viable therapy for metabolic syndrome, in which both low HDL and hypertriglyceridemia are key contributors to the disease. The data to date indicate that inhibition of miR-33 would increase HDL levels and fatty acid oxidation; however, further in vivo testing of this in animal models will be challenging due to the lack of conservation of miR-33b in lower mammals such as mice.

CONCLUSIONS

It has become increasingly recognized that microRNAs play a prominent role in regulating biological processes. Although this field is still in its infancy, in the area of lipid metabolism there is little doubt that additional miRNAs will be identified in the near future that regulate lipid metabolism pathways. Many of these may have potential as targets for the treatment of metabolic disorders, but the development of such therapeutics faces numerous challenges, including target identification, specificity, mode of delivery, and length of action. Perhaps most critical will be understanding the full range of targets that a specific miRNA or anti-miRNA affects. As an example, anti-miR-122 is in phase II clinical trials for the treatment of chronic HCV infection (**Figure 3**); however, it remains unclear which direct targets of miR-122 account for its profound effects in the liver. Such an understanding may take years of basic research to untangle. Anti-miR-33 appears to hold promise as a therapy to raise HDL in the treatment of cardiovascular disease and metabolic syndrome. Studies of the effects of anti-miR-33 in animal models of atherosclerosis will undoubtedly be revealed shortly and will likely provide valuable information on the efficacy of such a treatment. The success of phase I clinical trials of anti-miR-122 and studies of this drug in primates are encouraging, and similar studies of anti-miR-33 are eagerly awaited.

SUMMARY POINTS

Two miRNAs have been identified as key regulators of lipid metabolism pathways: miR-122 and miR-33.

1. miR-122

- miR-122 is the most highly expressed microRNA in the liver and appears to maintain the hepatic cell phenotype.
- Inhibition of miR-122 decreases total serum cholesterol in mice and primates.
- Anti-miR-122 is in phase II clinical trial development for the treatment of chronic HCV.

2. miR-33

- miR-33a and miR-33b are intronic microRNAs located within the SREBP-2 and SREBP-1 genes, respectively.
- miR-33a/b are coregulated with their host genes.
- miR-33a/b coordinately target genes involved in cellular cholesterol export and fatty acid β oxidation.
- Inhibition or targeted deletion of miR-33 increases circulating HDL levels in mice.

FUTURE ISSUES

1. Identification of novel miRNAs controlling lipid metabolism. It is clear from the work to date that miRNAs can play a key role in regulating lipid metabolism pathways, and there remains much to be discovered. In the coming years it is likely that the identification of novel miRNAs and their targets will provide insight into the complex network that regulates lipid homeostasis. One of the many challenges will be to fully characterize these emerging candidates and confirm their physiological relevance.
2. Translation of basic research on miRNAs into therapeutics. The development of miRNA-based therapeutics faces numerous challenges, including identification of the full spectrum of targets and a better understanding of their specificity, persistence, and length of action. The success of phase I clinical trials of anti-miR-122, along with the studies of this drug in primates, are encouraging and suggest that it is not unlikely that anti-miRNA therapies may become viable therapeutics in the not-so-distant future.
3. Determination of the therapeutic potential of antagonizing miR-33. Anti-miR-33 appears to hold promise as a therapy to raise HDL; however, several key questions remain to be answered. For example, is the HDL generated by anti-miR-33 treatment functional (i.e., can it promote cholesterol efflux)? Also, what is the impact of anti-miR-33 on atherosclerosis? Finally, as with anti-miR-122, understanding the full complement of miR-33 targets will be necessary before this can be tested therapeutically.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Shows that miR-33a and miR-33b act in concert with their SREBP host genes to regulate expression of ABCA1 and cholesterol efflux to apoA1. The authors show that LNA-mediated silencing of miR-33 in vivo increases plasma HDL levels, and together with references 32 and 33, establish miR-33 as a regulator of cholesterol homeostasis.

Reports that miR-33a, an intronic miRNA located within the SREBF-2 gene, is regulated by cellular and dietary cholesterol and coordinately inhibits a number of key genes involved in cellular cholesterol transport.

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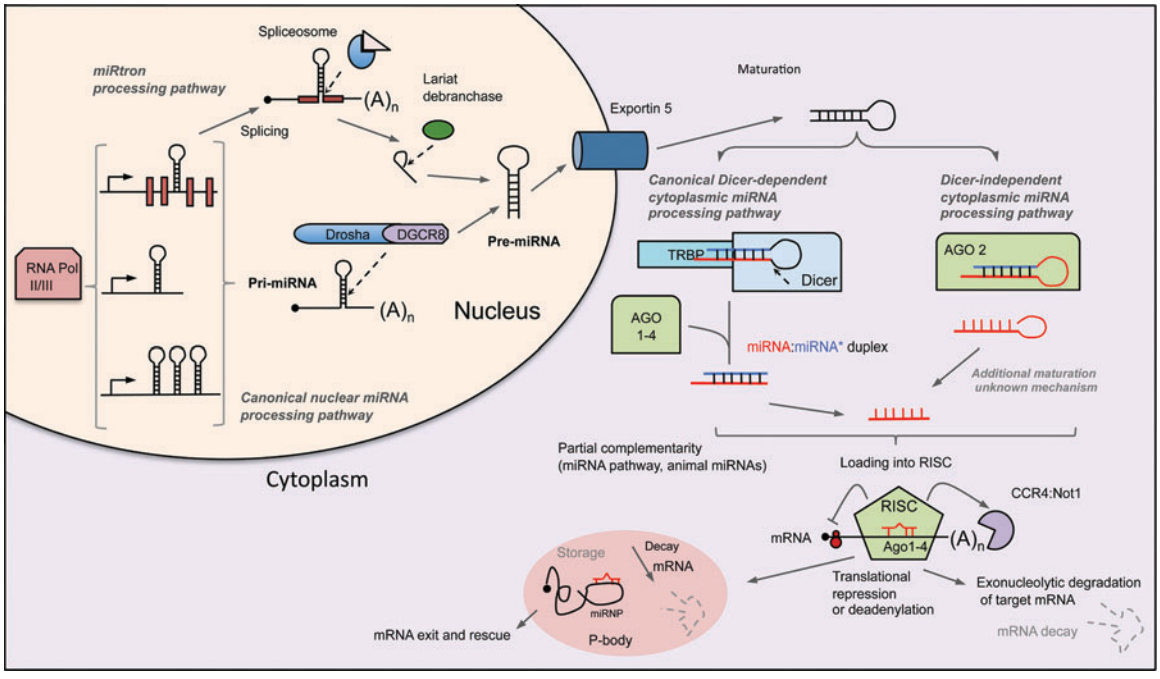

Figure 1

The microRNA (miRNA) biogenesis pathway. miRNAs are transcribed in the nucleus by RNA polymerase from independent miRNA genes located in polycistronic transcripts or introns of protein-coding genes into primary transcripts (pri-miRNAs). Pri-miRNAs are processed in two steps in the nucleus and cytoplasm by the RNase III–type endonucleases Drosha and Dicer, respectively, in complexes with dsRNA-binding domain proteins DGCR8 and TRBP, as indicated. In the canonical pathway, Drosha-DGCR8 processes the transcript to a stem-loop-structured precursor (premiRNA). Alternatively, miRtrons, a subset of miRNAs derived from introns, may be processed into premiRNAs by the spliceosome and the debranching enzyme. Both canonical miRNAs and miRtrons are exported to the cytoplasm via Exportin 5, where they are further processed by Dicer-TRBP to yield ≈20-bp miRNA duplexes. One strand is selected to function as the mature miRNA and loaded into the RNA-induced silencing complex (RISC) containing components of the Argonaute family (Ago 1–4), while the partner miRNA* strand is preferentially degraded. A subset of miRNAs, exemplified by miR-451, is produced independently of Dicer through recognition by Ago2. The mature miRNA produced by these two mechanisms leads to translational repression or degradation of the target mRNA. Animal miRNAs usually show only partial complementarity to the target mRNA promoting translational repression (initiation and postinitiation steps) or deadenylation coupled to exonucleolytic degradation of target mRNA. mRNAs repressed by deadenylation or at the translation-initiation step can be moved to P-bodies for either degradation or storage.

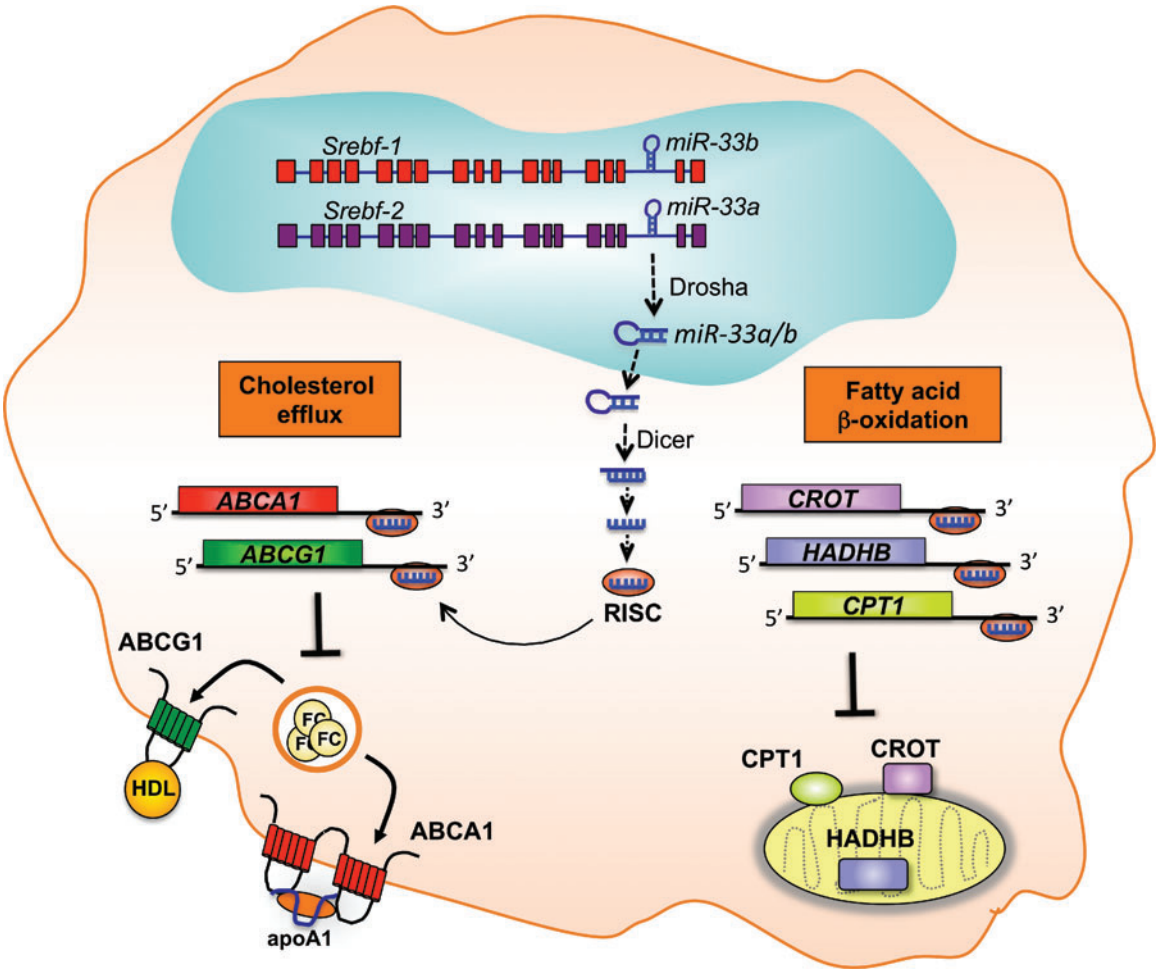

Figure 2

MicroRNA miR-33 regulation of fatty acid metabolism and cholesterol efflux. Cellular stimuli that activate transcription of sterol response element-binding factor (*SREBF*)*-1* or *SREBF-2* (e.g., insulin or low sterol, respectively) induce the cotranscription of miR-33b and miR-33a, respectively. These pri-miRNAs are sequentially processed by Drosha and Dicer and loaded into the RNA-induced silencing complex (RISC). As a result of binding target sites in the 3′UTR, miR-33a/b simultaneously inhibit the expression of genes involved in fatty acid metabolism (CROT, CPT1a, and HADHB) and cholesterol transport (ABCA1, ABCG1, and NPC1). The outcome of miR-33 targeting of these genes is reduced fatty acid β oxidation, reduced cholesterol efflux, and reduced lipidation of apoA1 and high-density lipoprotein particles.

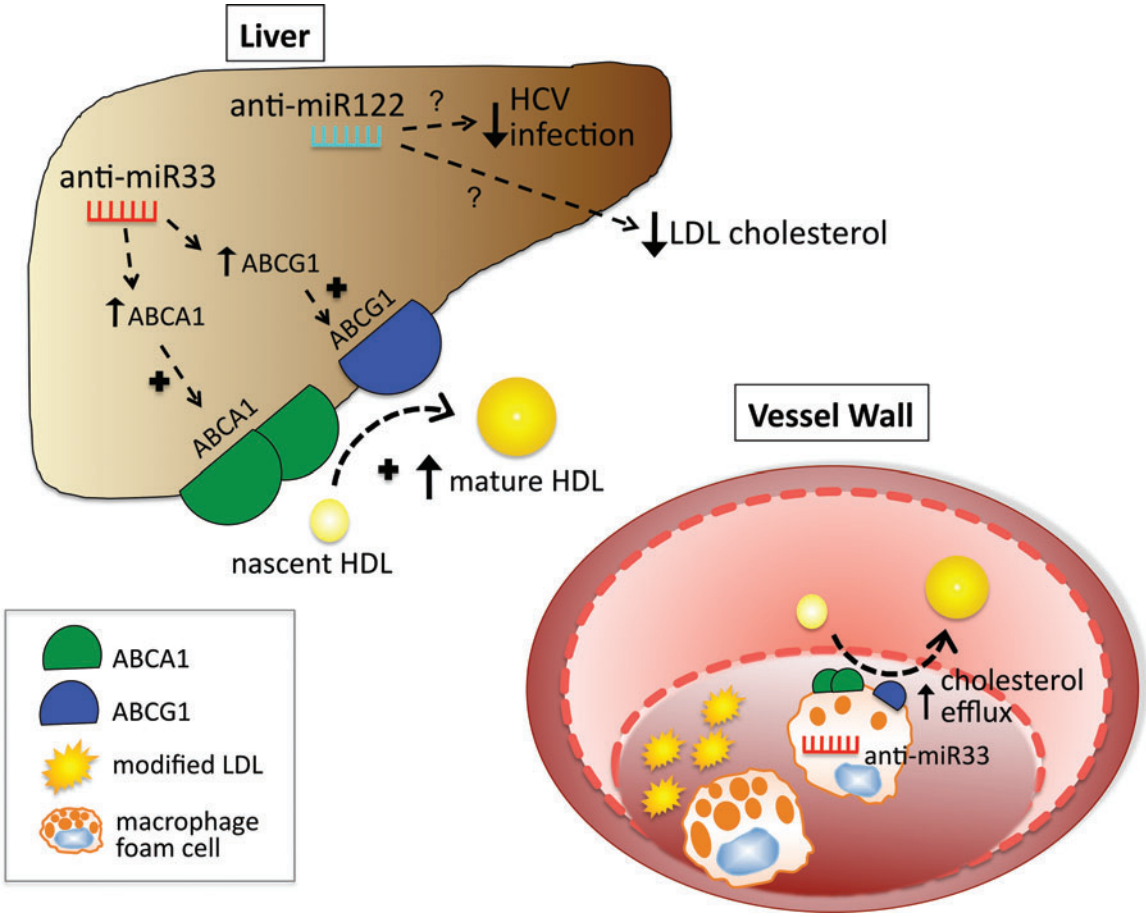

Figure 3

Therapeutic targeting of microRNAs miR-33 and miR-122 in atherosclerosis. Upon treatment with anti-miR-33 inhibitors, hepatic miR-33 levels are reduced, resulting in increased expression of target genes ABCA1 and ABCG1 in the liver. This results in enhanced high-density lipoprotein biogenesis and an increase in circulating HDL, which can promote reverse cholesterol transport from tissues, including from lesional macrophage foam cells. In addition, anti-miR-33 may directly target these lesional macrophages, upregulating ABCA1 and ABCG1 expression, and further enhancing cholesterol removal from the plaque. Antagonism of miR-122, the most highly expressed miRNA in the liver, decreases circulating cholesterol, particularly low-density lipoprotein, via as yet unspecified mechanisms. Notably, anti-miR-122 also decreases propagation of hepatitis C virus in the liver and is currently in a clinical trial for this application.

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