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miRNA Modulation of Cholesterol Homeostasis

Carlos Fernández-Hernando and **Kathryn J. Moore**
Departments of Medicine and Cell Biology, Leon H. Charney Division of Cardiology and the Marc and Ruti Bell Vascular Biology and Disease Program, New York University School of Medicine, New York, NY 10016, USA

Abstract

Although the roles of the SREBP1 and SREBP2 transcription factors in regulating fatty acid and cholesterol synthesis and uptake have been known for some time, it was recently discovered that two related microRNAs, *miR-33a* and *miR-33b*, are embedded in these genes. Studies indicate that *miR-33a* and *miR-33b* act with their host genes, *Srebp2* and *Srebp1*, respectively, to reciprocally regulate cholesterol homeostasis and fatty acid metabolism in a negative feedback loop. miR-33 has been shown to post-transcriptionally repress key genes involved in cellular cholesterol export and HDL metabolism (*Abca1*, *Abcg1*, *Npc1*), fatty acid oxidation (*Crot*, *Cpt1a*, *Hadhb*, *Ampk*), and glucose metabolism (*Sirt6*, *Irs2*). Delivery of inhibitors of miR-33 in vitro and in vivo relieves repression of these genes resulting in up-regulation of the associated metabolic pathways. In mouse models, miR-33 antagonism has proven has proven to be an effective strategy for increasing plasma HDL cholesterol and fatty acid oxidation, and protecting from atherosclerosis. These exciting findings have opened up promising new avenues for the development of therapeutics to treat dyslipidemia and other metabolic disorders.

Unraveling the role of microRNAs (miRNAs) in the regulation of gene pathways is an exciting new frontier in many different areas of biological research. This class of short (22 nt) non-coding RNAs post-transcriptionally represses gene expression through binding to complementary target sites in the 3′untranslated regions (3′UTRs) of messenger RNA (mRNA)[1-4]. Since their discovery in Caenorhabditis elegans[5, 6], our understanding of miRNA processing and action has increased tremendously through the work of many investigators in this field. MicroRNAs are encoded in intergenic or intronic regions of the genome of metazoan animals, plants and viruses, and are processed from primary transcripts through the sequential actions of Drosha and Dicer enzymes[1, 3, 4]. Mature miRNAs are then incorporated into the RNA-induced silencing complex (RISC) in the cytoplasm and bind to partially complementary target sites in mRNAs, thereby inhibiting their expression through mRNA destabilization, repression of translation or a combination of both processes[1-4]. Bioinformatic predictions and experimental approaches indicate that a single microRNA may simultaneously target more than 100 mRNAs that function in the same or related pathways, thus providing a mechanism of “fine-tuning” entire gene networks involved in a physiological process or biological pathway.

SREBP-miR-33a/b

The elegance of this mechanism of post-transcriptional gene control is exemplified by the recent identification of *microRNA-33a* and *b* (*miR-33a/b*) as intronic microRNAs located within the sterol response element binding protein (SREBP) genes *Srebp2* and *Srebp1*[7-9].

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

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These loci code for the membrane bound transcription factors SREBP1 and SREBP2 that activate the synthesis of fatty acid and the synthesis and uptake of cholesterol[10-12]. Coincident with the transcription of *Srebp1* and *Srebp2*, the embedded *miR-33a* and *miR-33b* are transcribed and these negative regulators act to repress a number genes involved in fatty acid oxidation and cholesterol export[7-9, 13-15]. This cleverly designed negative feedback loop helps to boost intracellular cholesterol and fatty acid levels by simultaneously balancing transcriptional induction and post-transcriptional repression of lipid metabolism genes.

miR-33 regulates cholesterol metabolism

The presence of *miR-33a* in the intron of *Srebp2* is remarkably conserved in many species, including large and small mammals, chickens and frogs, suggesting a critical function[7-9]. By contrast, there is a gap in the evolutionary conservation of *miR-33b*, which is present in the *Srebp1* gene of mammals, with the exception of rodents. Interestingly, *miR-33* and *Srebp* gene are conserved in some cholesterol auxotroph animals, where the regulation of SREBP protein and its transcriptional targets are related to fatty acid and phospholipid metabolism. This observation suggests that the function of ancestral miR-33 may have been more related to miR-33b than miR-33a. The two isoforms of *miR-33* differ by only 2 nucleotides in their mature sequences, but are identical in their seed sequence, which dictates target recognition. Thus, *miR-33a* and *b* are likely to repress a similar subset of target genes. However the metabolic conditions that regulate their induction are quite different. In a series of parallel studies, it was reported that *miR-33a* is co-regulated with its host gene *Srebp2* under low sterol conditions[7-9]. SREBP2 activates the transcription of genes involved in cholesterol homeostasis, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMGCR) reductase, which catalyzes a rate-limiting step in cholesterol biosynthesis, and the low-density lipoprotein receptor (LDLr), which imports cholesterol from the blood[10, 12, 16]. Like *SREBP2*, *miR-33a* is widely expressed and is induced 2-3 fold under low sterol conditions suggesting that these two regulatory elements are co-transcribed[9]. Similar studies have recently been performed demonstrating that conditions that induce *SREBP1* transcription, such as insulin and liver X receptor (LXR) activation, also induce expression of *miR-33b*[13]. Notably, the amplitude of *SREBP1*, and thus *miR-33b*, induction can be quite large compared to *SREBP2* under these conditions, and thus in humans, this isoform may be more abundant.

Recent studies identified a role for *miR-33a/b* in the repression of genes involved in cholesterol export, including the adenosine triphosphate binding cassette (ABC) transporters, ABCA1 and ABCG1, and the endolysosomal transport protein, NPC1[9]. Of these targets, the most prominent is ABCA1, a cholesterol transporter that promotes the movement of excess free cholesterol out of the cell[17, 18]. In the liver, ABCA1 is essential for efflux of cholesterol to apolipoprotein AI (apoAI), the building block of high-density lipoprotein (HDL)[17, 18]. In peripheral cells, ABCA1 effluxes excess cholesterol to these nascent HDL particles for delivery to the liver for excretion to the bile and feces[17-20]. This process, termed reverse cholesterol transport, plays an essential role in both cellular and whole body cholesterol homeostasis.

The 3′UTR of mouse and human *Abca1* contain three binding sites for miR-33a/b (Figure 1) and transfection of miR-33 mimics strongly represses *ABCA1* mRNA and protein in a variety of cell types[7-9]. Functionally, miR-33 overexpression in hepatocytes and macrophages results in decreased cellular cholesterol efflux to apoAI[7-9]. Furthermore, inhibition of endogenous miR-33 in both these cell types promotes an increase in the expression of ABCA1 protein and a concomitant increase in cholesterol efflux to apoA1, indicating a physiologically relevant role for this microRNA in regulating ABCA1[7-9]. In mouse cells, miR-33 was also shown to target a second member of the ABC transporter

family, ABCG1[9]. ABCG1 also mobilizes excess free cholesterol from the cell, but uses more mature HDL particles as its acceptor[17, 18]. This pathway appears to be most active in peripheral cells, particularly macrophages in atherosclerotic lesions, where ABCA1 and ABCG1 are thought to act in sequence to lipidate nascent and then mature HDL to generate larger α-HDL particles destined for clearance by the liver[17, 18]. Notably, miR-33 repression of ABCG1 is not conserved in human cells due to the loss of miR-33 binding sites in the 3′UTR of human *Abcg1*. However, an additional component of this pathway, Neimann Pick C1 (NPC1), is targeted in human cells. NPC1 is a lysosomal protein that facilitates the transport of cholesterol to other parts of the cell in need and acts in concert with ABCA1 to promote cholesterol efflux to apoA1[9]. The 3′UTR of human *Npc1* contains 2 miR-33 binding sites, resulting in the repression of NPC1 protein in hepatocytes and macrophages[9]. Thus, miR-33 targets multiple genes in the pathway regulating efflux of cholesterol to HDL.

miR-33 regulates fatty acid metabolism

miR-33a is found within the same intron of *Srebp2* from many animal species, including large and small mammals. Interestingly, the fruit fly *Drosophila melanogaster* also has a highly conserved mature form of miR-33a, but these organisms do not synthesize sterols. This observation pointed to broader roles for miR-33 and lead to the identification of additional targets of miR-33a/b. Of note, several genes involved in fatty acid metabolism including *Crot*, *Cpt1a*, *Hadhb* and *Ampk* contain predicted binding sites for miR-33a/b[13, 14]. Overexpression of miR-33a/b reduces fatty acid oxidation and leads the accumulation of triglycerides in human hepatic cells and in the fat body of miR-33 transgenic flies[13, 14]. Of particular interest is the inhibitory effect of miR-33 on AMPK protein expression[13]. The role of AMPK in regulating cellular energy places this enzyme at a central point in maintaining energy homeostasis[21]. AMPK promotes hepatic fatty acid β-oxidation and inhibits cholesterol and triglyceride (TAG) synthesis. In this way, miR-33 inhibition of AMPK also increases cellular cholesterol and TAG content. Altogether, these data implicate miR-33a/b as central regulators of multiple aspects of lipid metabolism by limiting cholesterol efflux and fatty acid degradation as SREBP boosts their production.

miR-33 regulates glucose metabolism and insulin signaling

miR-33 has also been implicated in regulating insulin signaling via targeting of insulin receptor substrate 2 (IRS2), an essential signaling molecule that mediates the effects of insulin[13]. miR-33a/b over-expression reduces IRS2 levels and inhibits the activation of downstream messenger cascades, including AKT[13]. Moreover, miR-33a/b also target FSR-2 which has been suggested to participate in insulin signaling by recruiting Src-homology-phosphatase 2 (SHP2) and to function as a docking molecule similar to IRS2[13]. In addition with IRS2 and FRS2, miR-33 also regulates the expression of other genes involved in glucose metabolism such as sirtuin-6 (SIRT6)[13]. Interestingly, hepatic-specific disruption of SIRT6 in mice results in fatty liver formation because of enhanced glucolysis and triglyceride synthesis[22] which correlates with the increased triglyceride content observed in human hepatic cell lines transfected with miR-33.

Other functions of miR-33

A recent report has suggested a role for miR-33 in regulating stem cell self-renewal via down regulation of p53[23]. p53 has two putative miR-33 binding sites in the 3′UTR region and miR-33 transfection represses p53 expression and p53-mediated apoptosis[23]. This study suggests that miR-33 may promote the repopulation capacity of hematopoietic stem cell (HSC). Interestingly, SREBP-1 and cellular cholesterol content have also been shown to regulate cell cycle progression[24-27]. Thus, miR-33a/b might cooperate with their host genes in regulating cell proliferation and cell cycle progression. Indeed, it has been recently

reported that miR-33 over-expression reduces cell proliferation by direct targeting the serine/threonine-protein kinase Pim-1[28].

Pre-clinical studies with miR-33 inhibitors

The physiological relevance of miR-33 targeting of cellular cholesterol efflux has been demonstrated by short-term overexpression or silencing of miR-33 in mice using strategies such as viral delivery of miR-33 mimics or hairpin inhibitors[7, 9] or parenteral administration of modified anti-sense oligonucleotides[8, 29]. In vivo overexpression of miR-33 reduced expression of ABCA1 in the liver and decreased plasma HDL levels by 25%[9]. Conversely, various methods of miR-33 inhibition increased hepatic ABCA1 expression, resulting in up to 40% increases of plasma HDL cholesterol[7-9]. The results of these miR-33 antagonism studies were recently confirmed by the generation of a miR-33 knock-out mouse[15]. Targeted deletion of miR-33a from the intron of SREBP2 generated mice that were viable and fertile and showed no disruption of SREBP-2 function[15]. These miR-33 deficient mice had circulating HDL cholesterol levels that were 25-40% higher than wild type C57BL/6 mice[15]. Notably, whereas no differences in male and female mice were observed in studies using pharmacologic inhibitors of miR-33, female miR-33 knock-out mice showed larger increases in plasma HDL than their male counterparts[15]. The molecular mechanisms of this difference are currently being investigated.

Plasma HDL cholesterol levels bear a strong inverse correlation with cardiovascular disease risk, and thus the finding that HDL levels can be modulated by manipulating miR-33 has generated considerable interest in its therapeutic potential. In mouse models of atherosclerosis, overexpression of apoA1 to increase HDL has been shown to hinder plaque progression[30-33], and to promote regression[34-36]. Furthermore, direct infusion of HDL in apolipoprotein E (apoE) deficient mice, cholesterol-fed rabbits[37], or human subjects[38] with established atherosclerosis, reduces plaque size. To test whether miR-33 inhibition might have a similar impact, *Ldlr*$^{-/-}$ mice were treated with an oligonucleotide inhibitor of miR-33 for 4 weeks following establishment of atherosclerotic plaques by western diet feeding for 14 weeks. Notably, in this mouse model of atherosclerosis, miR-33 inhibition increased HDL by 35% as previously seen in wild type mice, and this was associated with a 35% reduction in both plaque size and lipid content[29]. Using an in vivo assay to measure the efficiency of reverse cholesterol transport, it was shown that the HDL generated by miR-33 inhibition was functional and increased the transport of cellular radiolabelled cholesterol to the plasma, liver and feces[29]. The atheroprotective effects of HDL have been largely attributed to its function in reverse cholesterol transport and in line with this, atherosclerotic lesions in anti-miR33 treated mice showed increased markers of plaque stability, including reduced macrophage accumulation and inflammatory gene expression, as well as an increase in collagen content[29]. Notably, in addition to raising ABCA1 in the liver, anti-miR33 oligonucleotides were also detected within macrophages of the atherosclerotic plaque. Isolation of these macrophages by laser capture microdissection showed an increase in ABCA1 expression of anti-miR-33 treated mice, as well as a decrease in inflammatory gene expression. Thus, oligonucleotide inhibitors of miR-33 may promote the reverse cholesterol transport pathway in two ways: by directly increasing HDL biogenesis in the liver and by increasing cellular cholesterol efflux from plaque macrophages.

While the preclinical studies of miR-33 inhibition in mice are encouraging, extrapolation of these findings to humans is complicated by the fact that mice lack miR-33b. This difference between mice and humans may be particularly relevant under conditions in which the transcription of *Srebp1* is highly up-regulated, such as hyperinsulinemia[11], which would lead to profound increases in miR-33b expression. Not only would such a condition lead to greater downregulation of cellular cholesterol efflux and plasma HDL levels, but the

increased miR-33b/Srebp1 transcription in insulin-resistant states would be predicted to promote hypertriglyceridemia by inhibiting fatty acid oxidation and promoting fatty acid synthesis. Thus, inhibitors of miR-33a/b may relieve repression of both of these metabolic pathways resulting in reduced plasma triglycerides as well as increased plasma HDL. However, a comprehensive understanding of the effects of inhibiting both miR-33a and miR-33b awaits translational studies in animal models containing both isoforms of miR-33, such as a non-human primate model.

Future aspects

The therapeutic manipulation of miRNA-regulated pathways is emerging as a promising avenue for the treatment of dyslipidemia and other metabolic disorders. Given the role of miR-33a/b in repressing cholesterol efflux, fatty acid oxidation and insulin signaling (Figure 2), pharmacologic targeting of miR-33a/b may be a promising strategy to treat metabolic syndrome. Cardinal features of this syndrome include dyslipidemia, characterized by an increase in plasma triglycerides and a decrease in plasma high-density lipoproteins (HDL), as well as obesity and insulin resistance[39]. The metabolic syndrome is a growing public health concern worldwide, with complex and interrelated risk factors for both cardiovascular disease (CVD) and diabetes. Despite widespread use of statins to lower levels of low density lipoproteins (LDL) and apolipoprotein B-containing lipoproteins, considerable residual CVD risk persists in this patient population. Major goals in the pursuit of novel therapies to target this residual risk have focused on raising levels of HDL to exploit its atheroprotective functions, lowering triglycerides and improving insulin signaling. Whether miR-33 could be such a panacea awaits future studies.

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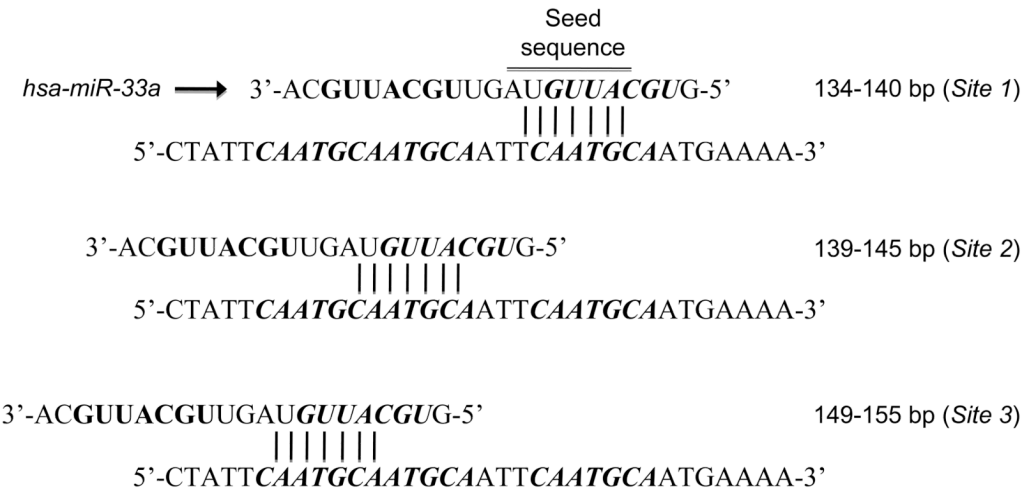

Figure 1.
Evolutionary conserved sequences in the 3′UTR of ABCA1 is partially complementary to miR-33. Annealing of miR-33 to some sequences are shown

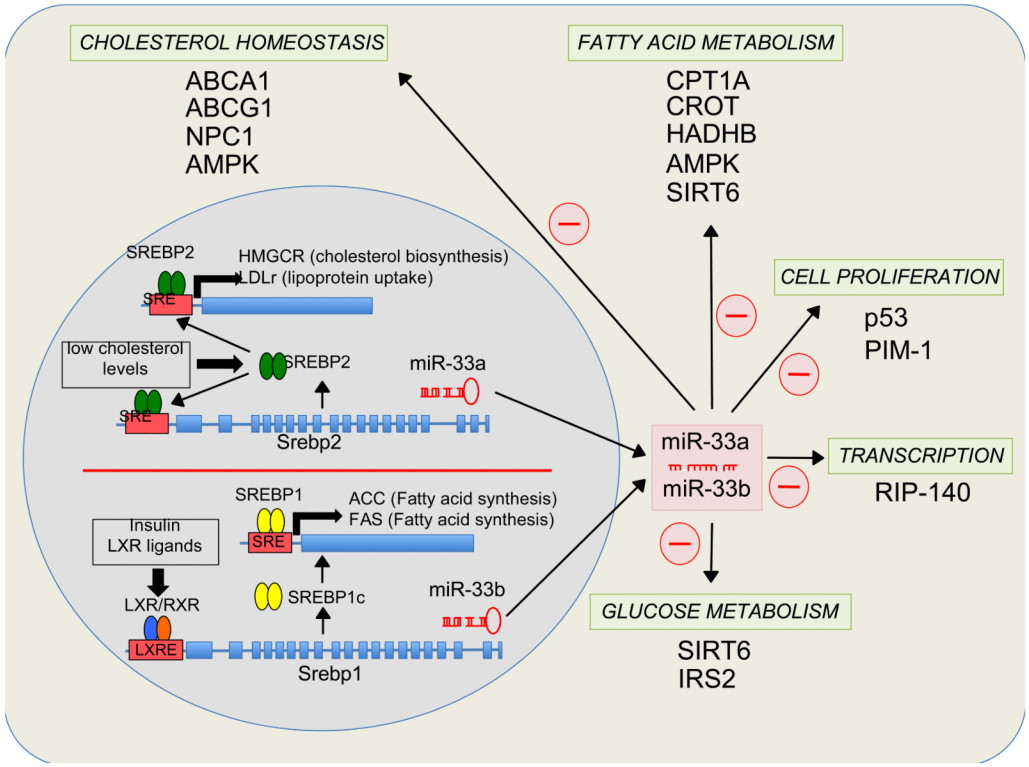

Figure 2.

Pathways by which activated miR-33 may contribute to regulating cholesterol, fatty acid and glucose metabolism, cell proliferation and transcription. Transcriptional activation of SREBP-2 under low intracellular cholesterol levels and SREBP-1 upon insulin-LXR stimulation leads the co-transcription of miR-33a and miR-33b respectively. miR-33a/b target several genes involved in the regulation of cholesterol efflux and trafficking, fatty acid oxidation, cell proliferation and cell cycle progression, and glucose metabolism.