MicroRNA-Management of Lipoprotein Homeostasis

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Although HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme)-reductase inhibitors (statins) have significantly contributed to the reduction in major adverse cardiac events observed in primary and secondary prevention trials, in part, by lowering circulating low-density lipoprotein (LDL)-cholesterol, ischemic cardiovascular disease still remains the number 1 cause of death in the United States, suggesting that additional therapies that address patient residual risk are desperately needed.1,2 Recent epidemiological studies in patients treated with statins suggest a marked association of levels of circulating high-density lipoprotein-cholesterol (HDL-C) and future cardiovascular risk independent of LDL-cholesterol.3 Indeed, an inverse association has been implicated in regulating HDL-C and cholesterol metabolism, or facilitating reverse cholesterol transport.5–7

MicroRNAs (miRNAs) are small, evolutionarily conserved noncoding RNAs that inhibit target genes by binding to the 3′-untranslated region of mRNAs to promote mRNA degradation or translational repression. Several miRNAs have been implicated in regulating HDL-C and cholesterol metabolism, including microRNA-33 (miR-33).8–10 Human miR-33a and miR-33b are located in the introns of the sterol-response-element-binding protein (SREBP) genes, SREBP-2 and SREBP-1, respectively. MiR-33a and miR-33b differ by 2 nucleotides in their mature form with the same seed sequence. MiR-33a is expressed across different species, including rodents, primates, and humans; however, miR-33b is not expressed in rodents. Several groups identified miR-33 as a key regulator in cholesterol metabolism by targeting the adenosine triphosphate-binding cassette transporter A1 (ABCA1) in 2010.11–15 Antisense inhibition or genetic deletion of miR-33 resulted in increased ABCA1 expression, HDL-C, and macrophage cholesterol efflux,11,12,14,15 whereas lentiviral or adenoviral delivery of miR-33 had the opposite effects.12,15 Emerging studies in nonhuman primates have also demonstrated increased levels of HDL-C in response to miR-33 inhibition.16,17 Recent studies, however, examining the role of miR-33 inhibition in the context of atherosclerotic progression have revealed conflicting effects in Ldlr–/– mice.18,19 Work by Marquart et al18 showed no effect on atherosclerotic lesion size, composition, or HDL-C, rather, surprisingly, miR-33 inhibition induced a marked increase in circulating triglycerides. In this issue of Circulation Research, work from the same group extend these observations and identified a novel mechanism by which miR-33 controls cholesterol metabolism by regulating the secretion of very low-density lipoprotein-triglyceride (VLDL-triglyceride; Figure).20 Prolonged miR-33 inhibition ≥11 weeks increased plasma levels of VLDL-triglyceride in chow-fed C57BL/6 mice because of increased hepatic N-ethylmaleimide-sensitive factor (NSF) expression, which they identified as a direct target of miR-33 using a combination of complementary gain- and loss-of-function approaches. MiR-33 inhibition also increased the secretion of other hepatic-associated proteins, including apolipoprotein B (apoB), apoA1, and albumin. Knockdown of NSF expression decreased VLDL-triglyceride secretion, which phenocopied the effects of miR-33 overexpression. In contrast, NSF overexpression enhanced VLDL-triglyceride and secretion of other hepatic proteins, apoB, apoA1, and albumin, and rescued the effect of miR-33. The study demonstrates that the miR-33-NSF axis regulates the overall secretory pathway in both mice and human hepatocytes. Whether similar operative mechanisms exist for miR-33 inhibition in insulin-resistant mice, for example, on high-fat diet (HFD) or in the background of LDLR-deficiency, remains unclear. Furthermore, whether the increased plasma VLDL-triglyceride because of miR-33 inhibition in this study exerts any detrimental functional effects on atherosclerotic progression or regression warrants further examination. Nevertheless, the findings by Allen et al20 add another layer to the complexity of miR-33’s effects in regulating lipoprotein metabolism and shed light to explain the discrepancies about the role of miR-33 in the progression of atherosclerosis in Ldlr–/– mice, as discussed below. Moreover, the study enforces the notion that additional studies may be required for a better understanding of anti–miR-33 therapy before translating this promising therapeutic approach from the bench to clinic for ameliorating atherosclerotic cardiovascular disease.

Accumulating studies among both human subjects and animals reveal a strong association with oversecretion of hepatic apoB100-enriched VLDL particles into the circulation and the development of insulin resistance, type 2 diabetes mellitus, or atherosclerosis.21–25 VLDL-triglyceride particles, secreted by the liver, deliver triglycerides to other tissues and are subsequently hydrolyzed and converted into intermediate-density lipoprotein and LDL, which are eventually cleared by...
the liver.26,27 Dysregulation of this process may contribute to excessive accumulation of atherogenic lipoprotein particles in the circulation.28 As such, pharmacological therapies that reduce VLDL-triglyceride secretion from the liver may offer a complementary approach at limiting hyperlipidemia and, possibly, coronary artery disease. Indeed, an inhibitor to microsomal triglyceride transfer protein (Mttp), a critical protein for effective hepatic assembly of VLDL, has shown efficacy in lowering LDL in patients with familial homozygous hypercholesterolemia,29,30 one of the most severe forms of hyperlipidemia (often because of deficiency or absence of the LDL receptor) that can lead to premature coronary artery disease, myocardial infarction, and death.31 In the study by Allen et al,32 although miR-33 inhibition increased secretion of VLDL-triglyceride and apoB100, it did not alter the expression of Mttp or apoB or regulate their 3′-untranslated region reporter activities, suggesting other potential mechanisms underlying the increased VLDL-triglyceride secretion.

Mobilization of apoB-enriched cargo from the endoplasmic reticulum to Golgi to the cell outer membrane is thought to rely on an intricate series of proteins, termed soluble NSF attachment protein receptor (SNARE) complexes, which facilitate vesicular membrane fusion and may also bind apoB100. Using a deductive approach for exploring potential proteins involved in VLDL vesicular trafficking in primary mouse and human hepatocytes, they found that miR-33 overexpression reduced NSP mRNA and protein, whereas expression of others (Napa, Sec22b, Gosp1, Stx5, or Bet1) was not altered. Furthermore, in the presence of miR-33 overexpression, NSF was dynamically recruited into the RNA-induced silencing complex (where miRNAs associate with target mRNA in association with Argonaute-2) and bound to the NSF 3′-untranslated region, strongly implicating that it is a bona fide miR-33 target in hepatocytes. Interestingly, NSF activity is needed for resolution of the SNARE complexes formed during this vesicular trafficking stage. As such, miR-33 inhibition may, in theory, enhance nascent VLDL trafficking and hepatic VLDL-triglyceride mobilization and secretion by altering a component of this multistep SNARE-dependent process.33 Because SNARE-regulated vesicular trafficking occurs in a variety of extrahepatic cell types, including cells involved in innate and adaptive immunity or coagulation, future investigation should consider the tantalizing possibility that miR-33 may alter SNARE-dependent events, such as phagocytosis, endocytosis, or the secretion of inflammatory or thrombotic mediators.

Given that miR-33 inhibition has been extensively studied from rodents to nonhuman primates, the findings from Allen et al32 also beg the larger question—how do we still reconcile their observations from the other miR-33 inhibition studies,17–19,33,34 in which no inductive effects on triglyceride or VLDL-triglyceride were observed? After early studies demonstrated that miR-33 inhibition increased HDL-C levels and promoted reverse cholesterol transport, subsequent studies examined its role in regulating circulating lipoproteins and the regression or progression of atherosclerosis in mice.17–19,33,34 Specific influential factors in these miR-33 inhibitor studies include percentage of cholesterol in the diet, chemical modification of the anti-miR-33 oligonucleotides, delivery dose/route, length of treatment, and animal strains used. Factors that may influence anti-miR-33 effects on lipoprotein metabolism: (1) oligo length, chemistry (e.g. LNA vs 2′F-MOE); (2) dose (e.g. 5–10 mg/kg); (3) delivery (e.g. subcutaneous versus intraperitoneal); (4) diet (e.g. 1.25% vs 0.3% cholesterol); (5) diurnal food consumption (e.g. ad libitum vs fixed time). G6PC indicates glucose-6-phosphatase; PCK1, phosphoenolpyruvate carboxykinase; RORγt, retinoic acid receptor (RAR)-related orphan receptor alpha; SRC1, steroid receptor coactivator 1; and SREBP-1, the sterol regulatory element-binding protein 1.35

Figure. The multifaceted regulation of lipoprotein metabolism by miR-33 and the potential effect on cardiovascular disease. MiR-33 inhibition may influence high-density lipoprotein (HDL), very low-density lipoprotein-triglyceride (VLDL-TG), and hepatic-secreted proteins to affect cardiovascular disease risk. Inhibition of miR-33 in hepatocytes leads to increased HDL synthesis and hepatic secretion of VLDL-TG and other hepatic proteins because of derepression of adenosine triphosphate-binding cassette transporter A1 (ABCA1) and N-ethylmaleimide-sensitive factor (NSF), respectively. In macrophages, miR-33 targets ABCA1/ABCG1; however, a role for NSF as a direct target of miR-33 remains unexplored in extrahepatic cell types. Increased VLDL-TG secretion may adversely affect cardiovascular disease (CVD). Inhibition of miR-33 promotes reverse cholesterol transport in macrophages, an effect that may confer protection against CVD. Multiple factors in miR-33 inhibitor studies may account for differences observed across studies on lipoprotein metabolism and atherosclerosis: percentage of cholesterol in the diet, length or chemical modification of the anti-miR-33 oligonucleotides, delivery dose/route, length of treatment, and animal strains used. Factors that may influence anti-miR-33 effects on lipoprotein metabolism: (1) oligo length, chemistry (e.g. locked nucleic acid vs 2′-fluoro/ methoxymethyl); (2) dose (e.g. 5–10 mg/kg); (3) delivery (e.g. subcutaneous versus intraperitoneal); (4) diet (e.g. 1.25% vs 0.3% cholesterol); (5) species (e.g. rodent vs non-human primate); and (6) diurnal food consumption (e.g. ad libitum vs fixed time). G6PC indicates glucose-6-phosphatase; PCK1, phosphoenolpyruvate carboxykinase; RORγt, retinoic acid receptor (RAR)-related orphan receptor alpha; SRC1, steroid receptor coactivator 1; and SREBP-1, the sterol regulatory element-binding protein 1.35
genetic loss of miR-33; however, it did not reach significance. Interestingly, bone marrow transplant studies revealed that chimeric mice with miR-33 myeloid deficiency did not increase HDL-C levels or reduce atherosclerotic plaque size although lipid accumulation was reduced in atherosclerotic plaques. These data suggest that the protective effect of miR-33 inhibition may not be the result of loss of miR-33-mediated effects in macrophages (eg, cholesterol efflux) but rather hepatic or extrahepatic effects. To assess the efficacy of anti–miR-33 inhibitors on the progression of atherosclerosis, Rotllan et al injected 9-week-old Ldlr−/− mice (Jackson Laboratories) subcutaneously with 2′F/MOE phosphorothioate anti–miR-33 oligonucleotides (21 nucleotides in length; 10 mg/kg), and fed a HFD (0.3% cholesterol) for 12 weeks. Anti–miR-33 treatment reduced lesion size and macrophage content but not collagen content and necrotic areas. Surprisingly, hepatic ABCA1 protein expression was not increased by miR-33 inhibition. In agreement with this observation, total cholesterol, HDL-C, and triglyceride levels were not different among treatments. Nevertheless, HDL from these anti–miR-33-treated mice had, intriguingly, improved functionality evidenced by its ability to promote cellular cholesterol efflux and to protect endothelial cells from cytokine-induced activation. In addition, anti–miR-33 increased ABCA1 expression in aortas from mice fed a HFD, suggesting that these anti-miRs may penetrate the vessel wall. In chow-fed mice for 4 weeks, anti–miR-33 led to increased plasma HDL, increased hepatic expression of miR-33 targets, such as ABCA1 in mice, but plasma triglyceride levels were similar. These data suggest that HFD may blunt effects on circulating HDL-C in response to 2′F/MOE anti–miR-33 therapy in mice. Finally, Marquart et al examined 10-week-old Ldlr−/− mice injected intraperitoneally with locked nucleic acid (LNA) anti–miR-33 (15 nucleotides in length; 7 mg/kg) and fed a Western diet (1.25% cholesterol) for 12 weeks after 2 weeks on chow. The anti–miR-33 group exhibited a trend for increased body weight gain. HDL-C was not increased by miR-33 inhibition at the end of the Western diet feeding although in the first 2 weeks when the mice were chow-fed HDL-C significantly increased. Mice that received LNA-anti–miR-33 exhibited increased plasma triglycerides both on chow and after Western diet, with similar atherosclerotic lesion size, despite the increase in hepatic expression of miR-33 targets, such as ABCA1. Notably, the expression of ABCA1 in aortas was not examined in the study to correlate miR-33 expression, targets, and efficiency of penetration in the vessel wall. Importantly, the level of total cholesterol achieved in this study was significantly higher (≈1200 mg/dL on 1.25% cholesterol diet) when compared with the study by Rotllan (≈900 mg/dL on 0.3% cholesterol diet). In theory, rapid induction of high cholesterol could reduce miR-33 expression to a level beyond the inhibitory effect of LNA-anti–miR-33 in the liver or arterial wall.

Several reasonable explanations may, therefore, underlie why miR-33 depletion has different effects on circulating lipoproteins, and in particular, in the progression of atherosclerosis (Figure). Collectively, diet (1.25% versus 0.3% cholesterol), oligonucleotide length (23 versus 16 nt), and chemistries (LNA versus 2′F/MOE), delivery route (intraperitoneal versus subcutaneous), and anti-miR dose (7 mg/kg versus 10) may have contributed to the discrepancies for the effects of miR-33 inhibition on circulating HDL, VLDL, triglycerides, and atherosclerosis. In earlier studies when miR-33 was acutely depleted in mice, no changes of VLDL and triglycerides levels were reported. Different target profiles may be exposed in response to LNA-anti–miR-33 and 2′F/MOE anti–miR-33 treatment, an effect also influenced by acute and prolonged depletion of miR-33. NSF expression may also be differentially regulated on miR-33 knockdown in different studies. Genetic depletion of miR-33 did not cause an increase of plasma VLDL and triglycerides in mice, which may reflect the activation of compensatory mechanisms or derepression of factors that counter the increase of NSF expression in the context of complete absence of miR-33.

In addition to its role in cholesterol metabolism, miR-33 also regulates fatty acid and glucose metabolism. MiR-33 inhibits fatty acid oxidation and degradation, and regulates insulin signaling. Interestingly, the passenger strand of miR-33 (miR-33*) shares a similar target gene network as the guide strand in different cell types, suggesting both arms of the miR-33/miR-33* duplex regulate lipid metabolism. Surprisingly, genetic deletion of miR-33 aggravated HFD-induced obesity and liver steatosis by targeting the SREBP-1 pathway in mice. Although there were no changes in circulating triglyceride in wild-type and miR-33−/− mice, the HFD groups actually had lower circulating triglyceride than the chow-fed groups making interpretation unclear.

Whether similar mechanisms exist for the miR-33-NSF axis regulating VLDL secretion in human subjects remains unknown. However, 2 studies performed in nonhuman primates—African green monkeys—demonstrated no such effects on VLDL-triglyceride. Systemic delivery of 2′F/MOE anti–miR-33 oligonucleotide (21 nucleotides in length; 5 mg/kg SC twice weekly for 2 weeks, then weekly for 10 weeks) in African green monkeys increased hepatic expression of miR-33 target genes, including ABCA1, and increased plasma HDL-C levels (≥50%) ≥12 weeks. Monkeys were fed a normal chow diet for 4 weeks, then switched to a high carbohydrate, moderate cholesterol diet for 8 weeks. HDL from anti–miR-33-treated monkeys maintained anti-inflammatory properties in endothelial cells and had similar capacity for cholesterol efflux in macrophages. MiR-33 inhibition surprisingly reduced the plasma levels of VLDL-triglyceride ascribed, in part, to decreased gene expression involved in fatty acid synthesis (eg, Srebp1 by derepression of Prkaa1) and increased those involved in fatty acid oxidation (eg, Hadh, Cpt1a, and Crot). In a more recent study using African green monkeys, long-term treatment with the 8-mer LNA-anti–miR-33a/b increased HDL (≤25% on HFD; ≤39% on high carbohydrate diet) and hepatic ABCA1 expression without evidence of off-target effects or toxicity. No change in triglyceride levels was detected in response to the 8-mer LNA-anti–miR-33a/b treatment. Thus, how do we reconcile why miR-33 inhibition in monkeys had no effect or even decreased levels of plasma VLDL-triglyceride? Potential explanations
also highlighted in the discussion by Allen et al\(^\text{20}\) include (1) the clearance of VLDL-triglyceride from circulation is efficient in African green monkeys, an effect leading to significantly lower baseline circulating VLDL-triglyceride levels (by $\pm$15%-30\%) when compared with VLDL-triglyceride levels in humans.\(^{40,41}\) Because of this high turnover, hepatic VLDL-triglyceride secretion may be difficult to monitor by a static measurement of plasma triglyceride concentration accurately;\(^{42}\) (2) both nonhuman primate studies did not have important additional control groups; the study by Rayner et al\(^{16}\) lacked a vehicle control group, whereas the study by Rottiers et al\(^{17}\) lacked an oligonucleotide control group (but had a vehicle control group). Thus, data interpretation are unclear about whether control oligonucleotides raised VLDL-triglyceride in the former, and whether the lack of control oligonucleotides minimized any VLDL-triglyceride effects in the latter; and (3) the study by Rottiers et al\(^{17}\) reported no difference in triglyceride levels, but triglyceride lipoprotein distribution was not shown. In addition, VLDL and triglycerides levels measured in circulation in nonhuman primate studies may need to be carefully interpreted in the absence of blocking the clearance pathway in an analogous manner in mice. Finally, mice, but not monkeys, consumed food ad libitum in the above studies,\(^{18,20}\) which might need to be taken into consideration for effects of diurnal feeding patterns on lipoprotein metabolism.

The study by Allen et al\(^{20}\) also raises important issues not only for miR-33 in particular but also for the field of miRNA therapeutics in general. Relevant points to appreciate include (1) differences of miRNA expression across species (eg, miR-33a and miR-33b in humans and primates; only 1 miR-33 in rodents) may result in differential miRNA regulation and target gene expression; (2) differences may exist in bioavailability, potency, penetration efficiency, and pharmacokinetic properties of 2'F/MOE- and LNA-anti–miR-33 in relevant tissues and cell types (eg, hepatic and extrahepatic tissues including the vessel wall [endothelial cells and smooth muscle cells], peripheral blood mononuclear cells, and other insulin-responsive depots, eg, visceral fat and skeletal muscle); (3) diurnal fluctuations of blood lipid, insulin, and glucose levels across rodents, monkeys, and humans can be vastly different; (4) how anti–miR-33 treatment regulates functionality of lipoproteins, including HDL and VLDL-triglyceride (eg, HDL may transfer miRNAs to endothelial cells to exert anti-inflammatory properties)\(^{34}\); and (5) whether miR-33, ABCA1, and NSF are dynamically regulated in patients with cardiovascular or metabolic disease.

The role of miR-33 in cholesterol efflux, and fatty acid and glucose metabolism, makes it an attractive therapeutic target for the treatment of cardiovascular disease. The study by Allen et al\(^{20}\) however, provides scientific pause to investigate miR-33’s potential effects on VLDL-triglyceride secretion further, other hepatic-secreted proteins, and NSF-mediated effects in relevant extrahepatic tissues that may affect health and disease before this therapy inches closer to the clinic.

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None.

### References


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