MicroRNAs regulating lipid metabolism in atherogenesis

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MicroRNAs have emerged as important post-transcriptional regulators of lipid metabolism, and represent a new class of targets for therapeutic intervention. Recently, microRNA-33a and b (miR-33a/b) were discovered as key regulators of metabolic programs including cholesterol and fatty acid homeostasis. These intronic microRNAs are embedded in the sterol response element binding protein genes, SREBF2 and SREBF1, which code for transcription factors that coordinate cholesterol and fatty acid synthesis. By repressing a variety of genes involved in cholesterol export and fatty acid oxidation, including ABCA1, CROT, CPT1, HADHB and PRKAA1, miR-33a/b act in concert with their host genes to boost cellular sterol levels. Recent work in animal models has shown that inhibition of these small non-coding RNAs has potent effects on lipoprotein metabolism, including increasing plasma high-density lipoprotein (HDL) and reducing very low density lipoprotein (VLDL) triglycerides. Furthermore, other microRNAs are being discovered that also target the ABCA1 pathway, including miR-758, suggesting that miRNAs may work cooperatively to regulate this pathway. These exciting findings support the development of microRNA antagonists as potential therapeutics for the treatment of dyslipidaemia, atherosclerosis and related metabolic diseases.

Introduction

The discovery of microRNAs and their role in regulating gene expression in humans is one of the most exciting scientific breakthroughs in the last decade. These short (22 nt) non-coding RNAs repress gene expression through sequence-specific hybridization to complementary target sites in the 3’untranslated regions (3’UTRs) of messenger RNA (mRNA) (1–4). By altering mRNA stability and/or repressing miRNA translation, microRNAs represent an additional layer above transcriptional control for both fine-tuning and dramatically altering cell behaviour. Since their original discovery in Caenorhabditis elegans (5, 6), more than 700 microRNAs have been identified in the human genome, and over one-third of all human genes are predicted to be regulated by these small RNAs. Many of these microRNAs are highly conserved across species, highlighting the evolutionary importance of these molecules as modulators of gene expression.

A single miRNA can simultaneously regulate the expression of multiple target genes, thereby providing a mechanism to regulate entire networks of genes (3). To date, microRNAs have been shown to play an integral role in numerous biological processes, including the immune response, development, stem cell differentiation and most recently, lipid metabolism. Recent work from our groups and others identified two intronic miRNAs, miR-33a and miR-33b, present within the sterol response element binding protein genes SREBF2 and SREBF1, that act as key regulators of metabolic pathways (7–9). The SREBF2 and SREBF1 genes code for the transcription factors, SREBP1 and SREBP2 respectively, which have well-established roles in regulating fatty acid and cholesterol metabolism (10, 11). However, the discovery of miR-33a and miR-33b embedded in these genes illuminated a clever feedback loop that helps to boost cellular fatty acid and cholesterol levels during times of need. Under conditions that initiate transcription of the SREBPs, miR-33a/b are co-expressed with their host genes and reciprocally regulate genes involved in cholesterol export/high-density lipoprotein (HDL) synthesis (ABCA1, ABCG1 and NPC1) (7–9), fatty acid oxidation (HADHB, CROT, CPT1a) (12, 13), and very low density lipoprotein (VLDL) triglyceride metabolism (AMPKa, SREBP-1) (12, 14) (Fig. 1). These genetic regulatory elements thus work together to fine tune the levels of cholesterol and fatty acids in the cell.

Although the genomic location of miR-33a was reported in 2004 (15), it was not until 2010 that a series of parallel studies established the significance and the functional consequences of this location (7–9). Using a microarray screening approach, Rayner et al identified miR-33a as one of 20 microRNAs altered by cellular cholesterol content in macrophages and showed that its expression paralleled that of SREBF2 (9). Two other groups, Najafi-Shoushtari et al. and Marquardt et al., uncovered the presence of miR-33 through in silico bioinformatic analysis of SREBF loci (7, 8). Each
of the studies confirmed that miR-33a is co-transcribed along with its host gene, SREBF2, under conditions of sterol depletion. All three groups noted that target prediction algorithms placed a very interesting candidate at the top of the list of potential miR-33 target genes (Table 1): the ATP-binding cassette transporter ABCA1 that is responsible for the movement of free cholesterol out of the cell and for the generation of nascent HDL particles. This observation underscored the potential importance of miR-33 in the regulation of cholesterol homeostasis. Indeed, 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, in particular hepatocytes (7–9). Although the role for ABCA1 in the liver is essential for its role in HDL biogenesis, ABCA1 expression in macrophages is critical for transport of excess cholesterol out of the cell, a process known as reverse cholesterol transport (16). miR-33 overexpression in macrophages was found to decrease ABCA1 expression and thus dampen apoA1-mediated cholesterol efflux (7–9). Conversely, inhibition of endogenous miR-33 in macrophages increased ABCA1 expression and enhanced cholesterol efflux from lipid-laden macrophages, highlighting the physiological importance of miR-33 in this pathway (7–9).

In addition to controlling ABCA1 expression, our group identified two other proteins involved in cholesterol transport in the cell that are controlled by miR-33: ABCG1, which effluxes cholesterol to HDL, and NPC1, which transports cholesterol from lysosomal compartments to other parts of the cell in need (9). 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 (7, 9). The functional consequence of ABCG1 targeting in cells of mouse origin was demonstrated by a decrease in cholesterol efflux to HDL after over-expression of miR-33 (9). 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 (17), indicating that miR-33 represses a second part of the cellular cholesterol export pathway in humans. These studies highlight how a single miRNA can simultaneously control several genes of the same pathway to regulate cellular homeostasis in a coordinated fashion.

The identification of the miR-33/SREBF/ABCA1 axis exemplified the elegant nature of miRNA-mediated post-transcriptional gene regulation. However, these early studies primarily focused on miR-33a, and little was known about miR-33b. Notably, miR-33a has been highly conserved throughout evolution, whereas miR-33b is present only in the SREBF1 gene of medium and large mammals. Although miR-33a and b differ by two of 19 nucleotides in their mature form, they have identical seed sequences (nucleotides 1 through 8 or 2 through 9), the most critical sequence for targeting and function. Bioinformatics analyses predict that miR-33a and miR-33b largely repress the same subset of genes, and to date, there have been no genes identified that are specifically targeted by miR-33a versus miR-33b. However, as these microRNAs are co-transcribed with their host genes, the relative abundance of miR-33a and b is likely to be regulated by conditions that induce SREBF2 and SREBF1, and thus may be quite different. Interestingly, the relative abundance of SREBF2 mRNA in the liver is significantly less than that of SREBF1 (10); it would be predicted that miR-33a would also be less abundant than miR-33b. Moreover, in conditions that cause SREBF1 transcription to be elevated, such as hyperinsulinaemia, miR-33b levels may also be dramatically increased, and would be predicted to influence its target genes dramatically (10). Indeed, hallmarks of the metabolic syndrome include high plasma insulin levels and low circulating HDL (18) – perhaps in part due to increased hepatic transcription of SREBF1/miR-33b, and a resulting decrease in ABCA1 expression. While this hypothesis remains to be tested in humans, it is an example of how one miRNA may have profound consequences in both physiological and pathological states.

Table 1: Target genes of miR-33 and miR-758 and their biological functions.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Confirmed targets</th>
<th>Biological effect</th>
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<tbody>
<tr>
<td>miR-33a, miR-33b</td>
<td>ABCA1 (7–9), ABCG1 (9), NPC1 (9)</td>
<td>Cellular cholesterol efflux</td>
</tr>
<tr>
<td></td>
<td>CROT, HADHB, CPT1A (12, 13), SIRT6, PRKAA1 (12)</td>
<td>Fatty acid oxidation</td>
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<td></td>
<td>IRS2 (12)</td>
<td>Insulin signaling</td>
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<tr>
<td>miR-758</td>
<td>ABCA1 (24)</td>
<td>Cellular cholesterol efflux</td>
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<tr>
<td></td>
<td>SLC38A1 (24), NTM (24), EPHA7 (24)</td>
<td>Amino acid synthesis, neurite outgrowth, neuronal migration</td>
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Figure 1: miR-33 simultaneously targets proteins involved in multiple metabolic pathways in the liver. When miR-33a or b is expressed in the liver, there is a decrease in ABCA1, resulting in decreased cholesterol efflux and HDL. miR-33 also decreases fatty acid oxidation and increases VLDL by targeting CROT, CPT1A, HADHB and AMPKα2. Finally, miR-33 inhibits expression of IRS2, resulting in impaired insulin signalling.
miR-33 inhibition in animal models increases HDL cholesterol

The selective modulation of microRNAs through *in vivo* delivery of miR-mimics (double stranded oligonucleotides that replace microRNA function) or anti-miRs (single stranded oligonucleotide inhibitors) allows for the investigation of miRNA function and physiological relevance (19). Such studies in mice have shown that silencing of miR-33a using modified anti-sense oligonucleotides (8, 14), or viral delivery of hairpin inhibitors (7, 9), increased hepatic ABCA1 and circulating HDL by as much as 40%. These studies were critical in substantiating the physiological importance of miR-33 in the regulation of circulating HDL levels in mice, and established the potential for miR-33 inhibition as a therapeutic target for raising HDL and potentially offering protection from atherosclerosis.

The results of these miR-33 antagonism studies were recently confirmed by the generation of a miR-33 knockout mouse (20). The targeted deletion of miR-33a from the intron of Srebf2 in mice had no effects on fertility or viability, and the targeting strategy used did not disrupt SREBP2 function. The resulting miR-33 deficient mice had 25% to 40% higher plasma HDL cholesterol levels compared to wild-type C57BL/6 mice. Surprisingly, whereas no differences on the effects on HDL have been noted in male and female mice using pharmacological inhibitors of miR-33, female miR-33 knockout mice were reported to have larger increases in plasma HDL than their male counterparts. The molecular mechanisms of this disparity are currently unknown, but may point to potential differences in miR-33 biology in males and females.

As the static measurement of HDL has inherent limitations in extrapolating to its functionality, it was important to verify that the increase in HDL cholesterol had functional consequences. Growing evidence suggests that the absolute levels of plasma HDL are less important than the ability of this HDL to promote removal of cholesterol from peripheral tissues (such as macrophages in atherosclerotic plaques) into the feces for excretion— a process known as reverse cholesterol transport (21). Using an *in vivo* assay to measure the efficiency of reverse cholesterol transport, Rayner et al. showed that the HDL generated by miR-33 inhibition was functional and increased the transport of cellular radiolabelled cholesterol to the plasma, liver, and faeces (14). Notably, the atheroprotective properties of the HDL was retained in anti-miR-33-treated mice, particularly its ability to promote macrophage cholesterol efflux and to protect endothelial cells from cytokine induced inflammation (14).

These studies suggested that miR-33 inhibition may be a promising therapeutic modality for the treatment of atherosclerosis. In mouse models of atherosclerosis, overexpression of apoA1 to increase HDL has been shown to hinder plaque progression and to promote regression. Furthermore, direct infusion of HDL in apolipoprotein E-deficient mice, cholesterol-fed rabbits, or human subjects with established atherosclerosis reduces plaque size. Similarly, in mice with established atherosclerosis, inhibition of miR-33 resulted in a marked regression of atherosclerotic lesions that was characterised by a reduction in plaque size, decreased lipid and macrophage content, and a transition to a more stable plaque phenotype (22). Remarkably, anti-miR-33 oligonucleotides penetrated macrophages within the atherosclerotic plaque and directly increased ABCA1 expression, likely increasing cholesterol efflux from these cells and reducing overall plaque cholesterol content. Furthermore, gene expression analysis of plaque macrophages isolated by laser capture microdissection showed a reduction in inflammatory gene expression. Thus, the benefit of anti-miR-33 therapy is two-fold: it increases hepatic ABCA1 expression, circulating HDL and reverse cholesterol transport, as well as increases macrophage cholesterol efflux from the plaque, resulting in lesion regression and transition to a more stable phenotype.

Effect of miR-33 inhibition on fatty acid metabolism in mice

Interestingly, the fruit fly *Drosophila melanogaster* has a highly conserved mature form of miR-33a, yet these organisms do not synthesise sterols. This observation pointed to broader roles for miR-33 and lead to the identification of additional targets of miR-33a/b. Guerin et al. and Davalos et al. demonstrated that in addition to targeting genes involved in cholesterol transport, miR-33 also controls genes involved in the fatty acid oxidation pathway (12, 13). Over-expression of miR-33 in hepatocytes down regulates expression of CPT1a, CROT, HADHB and PRKAA1 (AMP Kinase α; AMPKα), decreasing β-oxidation of fatty acids and increasing triglyceride accumulation. Importantly, inhibition of endogenous miR-33 resulted in de-repression of CPT1a, CROT and HADHB, and increased fatty acid oxidation (12, 13). These studies expanded our understanding of miR-33 in the biological regulation of lipid homeostasis, and suggested that miR-33 may have important effects that extend beyond its role as an HDL-regulator.

Studies in non-human primates

While these early studies in mice highlighted the therapeutic promise of miR-33 inhibitors for raising plasma HDL, extrapolation of these findings to humans was complicated by the fact that mice lack miR-33b. Although miR-33b is absent in rodents, its presence in the SREBF1 gene of non-human primates allowed for the investigation of whether this isoform of miR-33 also contributes to the regulation of HDL and fatty acid metabolism in a model highly relevant to humans. Systemic delivery of an anti-miR-33 oligonucleotide designed to inhibit both miR-33a and miR-33b to African green monkeys over 12 weeks increased hepatic expression of ABCA1 and induced a sustained increase in plasma HDL (23). In this model, miR-33 inhibition induced a maximal HDL increase of 50% after eight weeks of treatment that was sustained throughout the remainder of the study. Notably, miR-33 antagonism in this non-human primate model also resulted in a striking reduction in plasma triglycerides. This decrease
was apparent as early as four weeks, and reached a maximum reduction of 50% at the termination of the study. Fractionation of plasma lipoproteins revealed that this derived from reduced VLDL-associated triglycerides, primarily in large VLDL particles that are newly secreted from the liver.

Hepatic gene expression analysis of the treated monkeys revealed that anti-miR-33 increased expression of ABCA1 mRNA and protein, as well as key members of the fatty acid oxidation pathway, CPT1a, CROT and HADHB (23). In addition, after 12 weeks of anti-miR-33 treatment, there was also a marked decrease of SREBP-1 at both the mRNA and protein level. The reduction of this key regulator of fatty acid synthesis and host gene of miR-33b was surprising as it is not a direct target of miR-33. However, further analysis revealed an increase in hepatic expression of AMPKα (encoded by PRKA1), which is a negative regulator of the SREBP-1 pathway and also a direct target of miR-33. Notably, in addition to decreased SREBP-1, the livers of anti-miR-33 treated monkeys showed a decrease in expression of key genes involved in fatty acid synthesis downstream of this transcription factor, including fatty acid synthase (FASN), ATP citrate lyase (ACLY) and acetyl-CoA carboxylase alpha (ACACA). Thus, by simultaneously increasing fatty acid oxidation via derepression of HADHB, CPT1A and CROT, and decreasing fatty acid synthesis via inhibition of the SREBP-1 pathway (SREBF1, FASN, ACLY, ACACA), anti-miR-33 treatment results in a pronounced reduction in plasma VLDL triglyceride.

This ground-breaking study established, in a model highly relevant to humans, that pharmacological inhibition of miR-33a and b is a promising therapeutic strategy to raise plasma HDL and lower VLDL triglycerides for the treatment of dyslipidaemias that increase cardiovascular disease risk (Fig. 2). As low HDL and high VLDL triglycerides are commonly associated with metabolic syndrome (18), miR-33 inhibitors may have clinical utility for the treatment of this growing health concern. Notably, in both mice and monkeys (14, 23), inhibition of miR-33 also increased hepatic expression of IRS2, a key component of insulin signalling which also becomes dysfunctional in metabolic syndrome (18). miR-33a/b over-expression reduces IRS2 levels and inhibits the activation of downstream messenger cascades, including AKT (12). Moreover, miR-33a/b also target PPARγ which has been suggested to participate in insulin signalling by recruiting Src-homology-phosphatase 2 (SHP2) and to function as a docking molecule similar to IRS2 (12). While the animals used in studies to date were normoglycaemic, future studies in animal models of obesity/diabetes will be important to fully understand the impact of miR-33 on insulin signalling and diabetes.

Other miRNAs regulating lipid metabolism

miR-758

In addition to miR-33, a recent study identified miR-758 as a second microRNA targeting ABCA1 (24). Like miR-33, miR-758 inhibits the expression of ABCA1 in human and mouse macrophages and decreases cellular cholesterol efflux to apoA1. Furthermore, miR-758 is highly expressed in the liver and was shown to modulate the expression of ABCA1 in human and mouse hepatic cell lines overexpressing miR-758, suggesting that it may play a role in regulating HDL biogenesis. Unlike miR-33, miR-758 is an intergenic miRNA and the factors mediating its regulation are largely unknown. Consistent with its role in regulating ABCA1, miR-758 is down-regulated after cholesterol loading in macrophages and in most tissues from mice fed with high cholesterol diet. Thus, both miR-33 and miR-758 may cooperate to down-regulate ABCA1 under cholesterol-depleted conditions, however the presence of three miR-33 binding sites at the beginning of the ABCA1 3’UTR suggests that this miR may target ABCA1 with higher efficacy (Fig. 3). Together, these findings suggest that the post-transcriptional regulation of ABCA1 expression may involve several different miRNAs and the physiological relevance of each miRNA will be determined by its relative tissue expression.

Notably, one tissue in which miR-758 is more highly expressed than miR-33 is the brain (24). Levels of ABCA1 in the brain have been shown to correlate inversely with amyloid load, and thus therapeutic targeting of ABCA1 is being actively studied in Alzheimer’s disease (25). In the brain, ABCA1 effluxes cholesterol to apolipoprotein E, which is the major cholesterol carrier in the brain and an established genetic risk factor for Alzheimer’s disease (25). In mouse models of Alzheimer’s disease, ABCA1 deficiency...
miR-122

Cholesterol metabolism has also been shown to be broadly regulated by miR-122, the most abundant miRNA in the liver. miR-122 accounts for 70% of total liver miRNA expression and has been associated with the regulation of liver metabolism as well as hepatitis C infection and with hepatocellular carcinoma (HCC). In vivo inhibition strategies targeting miR-122 revealed the physiological relevance of this microRNA in the liver. Silencing of miR-122 in mice resulted in a sustained reduction in total plasma cholesterol, observed in both the low-density lipoprotein (LDL) and HDL fractions (26, 27). Gene expression array analysis revealed that inhibition of miR-122 down-regulated several genes implicated in liver metabolism and produced an increase in expression of hundreds of genes that are normally repressed in hepatocytes, suggesting an important function for this microRNA in maintaining the liver phenotype. Furthermore, miR-122 antagonism in mice fed a high-fat diet resulted in a significant improvement in liver steatosis, as seen by the reductions in liver triglyceride content, and an increase in the rate of fatty acid β-oxidation (26). These results were consistent with the observation that miR-122 controls the expression of FASN, ACC1 and ACC2— all genes involved in fatty-acid synthesis and oxidation. Subsequent studies of LNA-antagonists directed against miR-122 in non-human primates showed that, similar to what was observed in mice, silencing of miR-122 in African green monkeys (28) and chimpanzees (29) caused substantial reductions in total plasma cholesterol, ranging from 20–30%, with no apparent toxicity or histopathological changes in the liver.

In addition to regulating lipid metabolism, miR-122 has been shown to bind the 5'UTR noncoding region in the hepatitis C viral genome, and this binding is essential to viral accumulation and propagation in infected hepatocytes (30–32). Notably, silencing of miR-122 in non-human primates infected with HCV results in sustained reductions in HCV viremia and improvement in liver pathology, with no evidence of viral resistance (29). The safety and efficacy of anti-miR122 demonstrated in these non-human primate studies prompted the advancement of Miravirsen, a miRNA-based therapeutic against miR-122, to clinical trials (33). Phase I results in healthy subjects showed that Miravirsen is well tolerated, offering promise for the advancement of miRNA-based therapeutics for the treatment of chronic diseases.

Future directions

miRNA studies in the field of lipid metabolism and atherosclerosis are in their infancy, and thus there is tremendous opportunity for discovery in this understudied area. The discovery of miR-33a and miR-758 embedded in the SREBP genes, and their activation of a negative feedback loop that helps regulate lipid metabolism, has considerably advanced our understanding of the mechanism controlling lipid homeostasis. At the same time, these seminal studies have broadened our imagination of the potential discoveries to be made of other miRNAs that may similarly regulate lipid metabolism. Cardiovascular disease remains the leading cause of mortality in westernised countries, despite optimum medical therapy to lower LDL cholesterol. The ability to target microRNAs in vivo through delivery of miR-mimics to enhance microRNA function, or anti-miRs which inhibit microRNAs, has opened new avenues for the development of therapeutics for dyslipidemias and offers a unique approach to treating disease by modulating entire biological pathways.

Conflicts of interest
None declared.

References


32. Franciscus A. Hepatitis C Treatments in Current Clinical Development. HCV Advocate 2010 (October 25, 2010); Available at: http://www.hcadvocate.org/hepatitis/HCVDrugs.html.