Therapeutic Inhibition of miR-33 Promotes Fatty Acid Oxidation but Does Not Ameliorate Metabolic Dysfunction in Diet-Induced Obesity

Denuja Karunakaran, Laura Richards, Michele Geoffrion, Danyk Barrette, Ryan J. Gotfrit, Mary-Ellen Harper, Katey J. Rayner

Objective—miR-33 has emerged as an important regulator of lipid homeostasis. Inhibition of miR-33 has been demonstrated as protective against atherosclerosis; however, recent studies in mice suggest that miR-33 inhibition may have adverse effects on lipid and insulin metabolism. Given the therapeutic interest in miR-33 inhibitors for treating atherosclerosis, we sought to test whether pharmacologically inhibiting miR-33 at atheroprotective doses affected metabolic parameters in a mouse model of diet-induced obesity.

Approach and Results—High-fat diet (HFD) feeding in conjunction with treatment of male mice with 10 mg/kg control anti-miR or anti-miR33 inhibitors for 20 weeks promoted equivalent weight gain in all groups. miR-33 inhibitors increased plasma total cholesterol and decreased serum triglycerides compared with control anti-miR, but not compared with PBS-treated mice. Metrics of insulin resistance were not altered in anti-miR33–treated mice compared with controls; however, respiratory exchange ratio was decreased in anti-miR33–treated mice. Hepatic expression of miR-33 targets Abca1 and Hadhb were derepressed on miR-33 inhibition. In contrast, protein levels of putative miR-33 target gene SREBP-1 or its downstream targets genes Fasn and Acc were not altered in anti-miR33–treated mice, and hepatic lipid accumulation did not differ between groups. In the adipose tissue, anti-miR33 treatment increased Ampk gene expression and markers of M2 macrophage polarization.

Conclusions—We demonstrate in a mouse model of diet-induced obesity that therapeutic silencing of miR-33 may promote whole-body oxidative metabolism but does not affect metabolic dysregulation. This suggests that pharmacological inhibition of miR-33 at doses known to reduce atherosclerosis may be a safe future therapeutic.

Key Words: cholesterol ◼ diet, high-fat ◼ microRNAs ◼ obesity ◼ therapeutics

The risk factors that promote the development of atherosclerosis are inextricably linked with those that promote type 2 diabetes, including dyslipidemia, insulin resistance, and adiposity. Together, this collection of risk factors known as the metabolic syndrome increases the likelihood of developing heart disease and type 2 diabetes by 2- and 5-fold, respectively. Therefore, understanding the molecular mechanisms that are shared between and promote metabolic syndrome is of tremendous therapeutic importance.

Recently, miR-33 was discovered as a post-transcriptional modulator of genes involved in cholesterol transport and fatty acid oxidation. Two copies of the intronic microRNAs (miRNAs) located within the genes encoding sterol regulatory element–binding proteins 1 and 2 (SREBP-1 and SREBP-2), miR-33a/b (miR-33 in mice) collectively repress the expression of cholesterol efflux proteins, ATP-binding cassette A1 (ABCA1) and ABCG1, and genes involved in fatty acid oxidation CPT1α, CROT, and HADHB. In mice and nonhuman primates, miR-33 inhibition by oligonucleotide inhibitors or gene deletion led to a derepression of hepatic expression of ABCA1, the main protein responsible for high-density lipoprotein (HDL) biogenesis and an accompanying increase in circulating HDL. In models of atherosclerosis progression and regression, blockade of miR-33 reduced atherosclerotic lesion size and promoted the removal of cholesterol from macrophages into the reverse cholesterol transport pathway, largely owing to its regulation of the cholesterol transporter ABCA1. Studies in nonhuman primates also suggested that blockade of miR-33 derepresses hepatic genes involved in fatty acid oxidation, leading to a reduction in very low-density lipoprotein (VLDL) triglyceride, and in vitro, miR-33 targets genes involved in insulin signaling, such as IRS2 and SIRT6. Based on this collection of miR-33 regulatory targets, it was assumed that blocking miR-33 would have favorable outcomes on the development of insulin resistance.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette A1</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HFD</td>
<td>high-fat diet</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>sterol regulatory element–binding protein-1</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
</tbody>
</table>

in vivo. However, these findings were not recapitulated in miR33−/− mice, where deletion of miR-33 in conjunction with HFD feeding resulted in increased total plasma cholesterol, increased hepatic lipid accumulation, and worsening of insulin resistance associated with diet-induced obesity.13 The effects of genetic deletion of miRNAs have not always been replicated using pharmacological miRNA inhibitors, yet the latter are in clinical development for a number of diseases, including cardiovascular disease.14 Given the potential promise of miR-33 inhibitors for the promotion of reverse atherosclerosis, we tested whether or not pharmacologically inhibiting miR-33 at doses that protect from atherosclerosis could also influence the development of hyperlipidemia, obesity, and insulin resistance in a mouse model of obesity.

Materials and Methods

A detailed Materials and Methods section is available in the online-only Data Supplement.

Results

Inhibition of miR-33 With Antisense Oligonucleotides Does Not Alter Body Weight or Circulating Lipids

To test whether long-term miR-33 silencing alters metrics of the metabolic syndrome, we used a mouse model of diet-induced obesity, where HFD feeding over 5 months results in increased body weight, elevated blood glucose, and impaired glucose tolerance.15 We used an established dosing regimen of 10 mg/kg anti-miRNA oligonucleotides that maximize mature miRNA inhibition while limiting toxicity, ultimately resulting in the effective derepression of miR-33 targets in mouse models.16,17 C57BL6/J mice were fed a HFD in conjunction with anti-miR treatment (10 mg/kg of control anti-miR or anti-miR33) weekly the first 2 weeks, then every second week thereafter for a period of 20 weeks. Mice gained considerable body weight over the course of the study, but no differences in body weight were observed between control anti-miR, anti-miR33, and PBS groups throughout the study period (Figure 1A). miR-33 has previously been shown to regulate circulating HDL-cholesterol levels in mice and nonhuman primates, and recent reports have emerged about the potential of miR-33 in controlling triglyceride-rich lipoproteins, LDL and VLDL.12 Therefore, we examined total serum cholesterol, apoB-associated (LDL and VLDL), and nonapoB-associated (HDL) cholesterol levels in mice treated with control anti-miR, anti-miR33, or PBS. No differences were observed in LDL/VLDL or HDL cholesterol between groups, although there was significant increase in total cholesterol in anti-miR33–treated mice compared with control anti-miR–treated (128.3±71.29 versus 104.2±51.1 mg/dL, respectively, P≤0.0001), but not compared with PBS (118.5±57.8 mg/dL, P=0.12) (Figure 1B). Surprisingly, there was a statistically significant decrease in circulating serum triglyceride levels in anti-miR33–treated mice compared with control anti-miR–treated (24.6±5.3 versus 34.7±5.8 mg/dL respectively, P≤0.01), but once again this was not significant when compared with PBS control mice (27.5±8.7 mg/dL, P=0.18; Figure 1C). Similarly, unlike what was reported for mice with genetic deletion of miR-33, long-term miR-33 silencing did not result in increased liver weight nor did it increase epididymal or inguinal adipose tissue weight (Figure 1D). Therefore, long-term therapeutic silencing of miR-33 using an established dosing regimen does not promote weight gain or adiposity nor does it increase serum lipid levels in either the HDL or the VLDL/LDL fractions.

Long-Term Inhibition of miR-33 Does Not Improve Insulin Resistance

Given its ability to promote insulin signaling in vitro, we next tested whether or not miR-33 inhibition could improve blood glucose and glucose tolerance in vivo after prolonged high-fat feeding.6 Following glucose challenge, intraperitoneal glucose tolerance test revealed no differences in blood glucose levels in mice treated with or without anti-miR33 oligonucleotides (Figure 2A; Figure IA in the online-only Data Supplement). Similarly, blood glucose returned to normal levels at the same rate after intraperitoneal insulin injection in all 3 groups (Figure 2B; Figure IB in the online-only Data Supplement), indicating that insulin resistance is equal between all treatments. miR-33 has previously been reported to impair insulin secretion from pancreatic β-cells in vitro by reducing Abca1 expression and promoting cholesterol build-up.16 We therefore measured circulating levels of insulin to determine whether anti-miR33 treatment could affect insulin secretion in obese mice. There were no differences between fasting serum insulin levels in PBS, control anti-miR, and anti-miR33–treated groups (Figure 2C), suggesting that miR-33 inhibition is not sufficient to overcome the islet cell dysfunction associated with prolonged HFD feeding.

In an attempt to understand whether anti-miR33 derepression of fatty acid oxidation genes would promote differences in energy utilization, we evaluated the energy utilization of the treatment groups by indirect calorimetry. We housed the mice in indirect calorimetry chambers and measured the O2 consumption and CO2 release to calculate the respiratory exchange ratios (RERs). Throughout both dark (maximum activity) and light (minimum activity) periods, anti-miR33–treated mice had decreased RER compared with both control groups (Figure 2E–2F). A lower RER is indicative of the preferred metabolism of lipids compared with carbohydrates, possibly as a result of increased fatty acid oxidation.17 Mice consumed nearly equivalent amounts of HFD in all 3 groups (Figure 2D). These data suggest that miR-33 inhibition may drive expression of fatty acid oxidation genes toward increased whole-body fatty acid utilization.
Anti-miR33 Increases Hepatic Expression of ABCA1 but Not SREBP-1

ABCA1 is a well-established gene target of miR-33 and has been validated by our group and others as being derepressed on inhibition or deletion of miR-33. To ensure that anti-miR33 oligonucleotides were efficiently inhibiting miR-33 activity, we assessed ABCA1 protein levels in both the liver and adipose tissue in treated mice. Anti-miR33 significantly upregulated the expression of ABCA1 protein in the liver (Figure 3A). Although there was a slight trend toward increased ABCA1 expression in the adipose tissue in treated mice, the adipose levels of ABCA1 were too low to reliably assess (Figure 3B). These results confirm that our dosing regimen of anti-miR33 oligonucleotides were indeed able to reach the liver and inhibit miR33-mediated repression of target gene protein expression. In agreement with this, we found reduced levels of miR-33 detected in the livers of anti-miR33–treated mice compared with controls (Figure 3C). These results suggest that the miR-33 may repress SREBP-1 expression via a conserved (6-mer) seed sequence in the 3' untranslated region of both mouse and human SREBF-1. Unlike what was found with whole-body miR-33 deletion, anti-miR33 treatment did not result in increased expression of SREBP-1 protein levels in the liver (Figure 3A) and in fact significantly reduced adipose tissue expression of SREBP-1 (Figure 3B). To investigate this mechanism further, we evaluated the activity of SREBP-1 by measuring expression of its major downstream transcriptional targets, fatty acid synthase (Fasn), and acetyl-CoA carboxylase (Acc). We did not find any increases in Fasn or Acc mRNA transcript levels in the liver of anti-miR33–treated mice compared with PBS or control anti-miR–treated mice, indicating that miR-33 inhibition does not alter the expression of SREBP-1 target genes (Figure 3D). Established miR-33 target genes, Abca1 and Hadh, were derepressed at the mRNA level in livers of anti-miR33–treated mice, whereas other validated miR-33 target genes (ie, Cpt-1α, Nfyc, Nsf) were not. Notably, after 4 weeks of anti-miR33 treatment, there was already a significant upregulation of target genes, Cpt1α and Hadh, in the livers of anti-miR33–treated mice, indicating that this dosing regimen of miR-33 inhibitors promotes fatty acid oxidation pathways at early time points (Figure IIA in the online-only Data Supplement). Histological analysis and quantification of hepatic lipids revealed no differences in lipid accumulation between PBS, control anti-miR, or anti-miR33–treated mice (Figure 3D). Taken together, these data demonstrate that miR-33 inhibition leads to a derepression of ABCA1 in the liver.
liver, without accompanying changes in SREBP-1c expression, and does not result in the accumulation of hepatic lipid nor the activation of downstream SREBP-1c target genes.

**Anti-miR33 Promotes M2 Macrophage Polarization in Adipose Tissue**

Diet-induced obesity is known to promote inflammatory macrophage accumulation in the adipose tissue, and anti-miR33 has previously shown to promote M2 macrophage polarization. We therefore tested whether anti-miR33 therapy could penetrate the adipose tissue and alter gene expression. We first assessed the expression of known miR-33 target genes, *Abca1* and *Ampk*, and found that indeed adipose tissue mRNA expression of these genes was upregulated in anti-miR33–treated mice compared with controls (Figure 3F). We next assessed macrophage accumulation and M2 macrophage expression in the adipose tissue of anti-miR33–treated mice and found that M2 markers, *Arg1* and *Fizz1*, were significantly increased in mice treated with anti-miR33 compared with controls (Figure 3F). There was a trend toward decreased CD68+ crown-like structures in anti-miR33 mice compared with control mice, although this did not achieve statistical significance (Figure IIIA in the online-only Data Supplement). There was also an increase in *Arg1* expression in the liver of anti-miR33–treated mice (Figure IIIB in the online-only Data Supplement). Therefore, anti-miR33 treatment targets the adipose tissue where it results in increased expression of pro-oxidative *Ampk* and M2 macrophage gene
Figure 3. Anti-miR33 increases hepatic expression of ABCA1 but not SREBP-1. Western blot analysis of hepatic (A) and subcutaneous adipose tissue (B) lysates from treated mice. Anti-miR33 significantly upregulated the expression of ABCA1 protein but not SREBP-1 protein in the liver, and although there was a slight trend toward increased ABCA1 expression in the adipose tissue, adipose levels of ABCA1 were too low to reliably assess. *P<0.05 Student t test, n=8 mice/group. C, miR-33 expression in the liver of control anti-miR and anti-miR33-treated mice. *P<0.05 Student t test. D, mRNA expression analysis of miR-33 target genes and downstream Srebp-1 transcriptional targets, fatty acid synthase (Fasn) and acetyl-CoA carboxylase (Acc). *P<0.05, 1-way ANOVA. E, Histological assessment of hepatic lipid accumulation using Oil Red O (ORO) reveals no difference in lipid levels between all 3 treated groups. n=8 mice/group; *P<0.05. F, mRNA expression in adipose tissue of miR-33 target genes, Abca1 and Ampk, and M2 markers, Arg1 and Fizz1. *P<0.05 Student t test. All analyses were performed using n=8 mice per group. ABCA1 indicates ATP-binding cassette A1; H&E, hematoxylin and eosin; and SREBP-1, sterol regulatory element–binding protein-1.
expression, which may overall increase whole-body oxidative metabolism.

**Discussion**

Antagonists of miR-33 have been touted for their potential as a therapy for atherosclerosis and its related complications. In most scenarios, therapeutic and genetic inhibition of miR-33 reduces atherosclerosis burden in various mouse models, either dependent or independent of levels of HDL cholesterol. Although many of these models examined miR-33 inhibition over a relatively short time (ie, 4–8 weeks), few if any adverse consequences were reported. Therefore, we sought to determine whether long-term treatment of therapeutic doses of anti-miR33 known to protect from atherosclerosis would have any outcome on whole-body metabolism, as genetic deletion or lower doses of anti-miR33 had been reported to adversely affect glucose and lipid parameters in mice. After treatment of anti-miR33 at 10 mg/kg for 20 weeks, there were no differences in body weight, liver, or adipose tissue weight, and no differences in LDL cholesterol in mice treated with anti-miR33 or control anti-miR. In the liver, there was an upregulation of miR-33 target genes, *Abacl* and *Hadh3*, and no changes in the expression of SBREPs-1 or its downstream targets, *Fasn* or *Acc*. Notably, although there were no differences in glucose tolerance test or intraperitoneal insulin injection in anti-miR33–treated mice compared with controls, there was an increase in fatty acid utilization (as measured by RER), an increase in *Ampk* expression, and M2 macrophage polarization markers in the adipose tissue of anti-miR33–treated mice. Taken together, our results suggest that overall the pharmacological inhibition of miR-33 does not adversely affect hepatic metabolic parameters yet may promote whole-body oxidative metabolism after long-term treatment at doses known to prevent atherosclerosis.

The effects of whole-body genetic miR-33 deletion were recently evaluated, and it was reported that *miR33*−/− mice on a normal diet gain slightly more weight than WT mice, and this was exacerbated significantly if mice were placed on a HFD for 20 weeks. In contrast, our study and another performed using lower doses of anti-miR33 did not show increases in body weight or total serum cholesterol compared with control anti-miR–treated mice after 20 weeks of treatment. *miR33*−/− mice showed worsened insulin resistance compared with WT controls, whereas no differences in glucose tolerance test, intraperitoneal insulin injection, or fasting insulin were observed with long-term pharmacological inhibition in this study. The differences between genetic deletion and pharmacological inhibition of miR-33 can likely be attributed to the tissue biodistribution of anti-miRNA oligonucleotides, which primarily penetrate the liver, kidneys, and the adipose tissue, with little to no uptake of the anti-miR by the pancreas or the intestine. In contrast, genetic deletion of miR-33 occurs in all tissues, and the absence of miR-33 in some tissues may greatly affect the overall metabolic phenotype of the animals. For example, *miR33*−/− mice on a HFD are reported to have high circulating levels of insulin, possibly as a result of pancreatic miR-33 deletion, which may contribute to the insulin resistance and hepatic steatosis observed in these animals. Similar to what has been previously reported, we did not observe any significant increases in circulating HDL levels in this study; however, this is likely because of the relatively low hepatic expression of miR-33 in the presence of high levels of sterols in conjunction with the observation that C57BL6 mice have high baseline levels of HDL compared with other mouse models under similar conditions. Nonetheless, ABCA1 protein expression was significantly upregulated in the livers of anti-miR33–treated mice, confirming that miR-33 levels were effectively inhibited with the anti-miR oligonucleotide dosing regimen used in this study.

Pharmacological miR-33 inhibition in mice using lower doses of anti-miR33 has previously been reported to result in hepatic lipid accumulation and elevated serum triglycerides in conjunction with high-fat feeding. In this study, analysis of lipid content within the livers of treated mice showed no difference in lipid accumulation in PBS, control anti-miR, or anti-miR33–treated mice. Different doses of antisense oligonucleotides could likely explain the divergent findings between the studies, as the dose of anti-miR33 used in this study (10 mg/kg) was higher than that used by Goedeke et al (5 mg/kg). The choice of 10 mg/kg was made because of previous work by our group and others showing this dose of anti-miR33 effectively derepresses miR-33 target genes in mouse models and protects against atherosclerosis, and indeed we demonstrate that ABCA1 was effectively derepressed in the liver in our treated mice. Although 2′F/MOE anti-miRNAs can be taken up by the liver, kidney, and adipose tissue, it is likely that higher doses of oligonucleotides are necessary to completely abolish miR-33 activity in all tissues, resulting in incomplete derepression of miR-33 target genes when lower doses are used. Supporting this, we demonstrate that the expression of miR-33 target genes, *Abacl* and *Ampk*, are derepressed at the mRNA level in the adipose tissue of anti-miR33–treated mice, indicating that miR-33 was successfully inhibited in the adipose tissue at this dose. Furthermore, we show that adipose tissue from anti-miR33–treated mice has elevated expression of M2 macrophage markers, which are likely a result of elevated miR-33 target gene, *Ampk*, which is known to promote M2 polarization. Adipose tissue was not evaluated using lower doses of anti-miR33, therefore it is unclear if miR-33 target gene expression was affected using a lower dose of oligonucleotide treatment. In addition to differences in dosing regimen of anti-miR, another key difference in this study and that of Goedeke et al is the fasting time before metabolic measurements. The mice in this study were fasted for 6 hours before all analyses and tissue harvest, whereas those the study by Goedeke were fasted overnight. It has been reported that differences in fasting time can have profound differences in the metrics of insulin resistance, and prolonged fasting leads to hepatic steatosis. After overnight fasting, hepatic triglyceride accumulation can increase up to 6-fold compared with 4 to 5 hours of fasting because of the increased release of free fatty acids from adipose tissue and subsequent uptake and storage in the liver as triglyceride. Under HFD feeding conditions, when the liver may be under stress, prolonged fasting could lead to the expression of a distinct complement of miR-33 target genes that might otherwise not be expressed, resulting in divergent outcomes on hepatic lipid accumulation upon miR-33 inhibition after short-term versus long-term...
fasting. Therefore, caution must be taken when analyzing the outcome of therapeutic miR-33 inhibition in different models of diet-induced obesity where variation in fasting as well as dosing may contribute to divergent outcomes, and highlights the complexity and potentially limited therapeutic window when modulating energy metabolism pathways that are tightly regulated by multiple intricate mechanisms.

It has been suggested that SREBP-1 is a direct target of miR-33. Horie et al showed that specific miR-33–binding sequences in the 3′ untranslated region of human and mouse SREBP-1 were responsible for the observed increases in SREBP-1 protein levels in miR-33−/− mice. Supporting this, when Srebp-1 was partially deleted, the effects of miR-33 inhibition on hepatic lipid accumulation, body weight, and insulin resistance were reversed. However, other groups have not found functional miR-33–binding sites within the 3′ untranslated region of SREBP-1. Similarly, we did not observe increases in SREBP-1 protein expression on inhibition of miR-33, suggesting that SREBP-1 may not be a direct target of miR-33. Unlike miRNAs, which often inhibit translation of target genes while leaving mRNA expression intact, transcription factors like SREBP-1 induce the transcriptional activation and thus mRNA expression of downstream targets.

We did not observe any changes in SREBP-1 downstream target genes, Acc and Fasn, upon inhibition of miR-33, confirming that SREBP-1 was not activated in these mice nor did we observe an increased accumulation of hepatic lipids mice treated with anti-miR33 compared with controls. Of note, we found that mice treated with anti-miR33 had decreased RER compared with controls, indicative of a preference for fatty acid oxidation as a fuel source. This may be a result of increased gene expression of miR-33 target genes Cpt1α and Hadhb as early as 4 weeks after treatment, as well as oxidative programming (i.e., increased Ampk and M2 macrophage polarization), in the adipose tissue. These pro-oxidative pathways may serve to offset any untoward increase in SREBP-1 that may occur during fasting and with varying activity levels of miR-33, resulting in the net effect of no changes in hepatic lipid metabolism. Taken together, this data indicate that upregulation of SREBP-1 and its downstream activation of lipogenesis were not present in anti-miR33–treated mice, possibly as a result of increased fatty acid oxidation, in contrast to what was observed with whole-body miR-33 deletion.

Studies in nonhuman primates allow the evaluation of both copies of miR-33, given that primates harbor both miR-33a and miR-33b embedded within the introns of SREBP-2 and SREBP-1, respectively, whereas miR-33b is lacking in rodents. When African green monkeys were treated for 12 weeks with pharmacological inhibitors to miR-33 identical to those used in this study, there was a derepression of miR-33 target genes involved in fatty acid oxidation and a marked reduction of serum triglycerides. Similarly, here we show that 20 weeks of miR-33 silencing in mice reduces serum triglycerides compared with control anti-miR–treated animals, and this is associated with an increase in hepatic HADHB expression and a switch from carbohydrate to fat utilization (decreased RER). However, this reduction in triglycerides does not reach significance when comparing with PBS-treated mice, which were not evaluated in the nonhuman primate study. In another model of miR-33 inhibition in nonhuman primates, where shorter 8-mer antisense oligonucleotides were used, obese and insulin-resistant African green monkeys treated with anti-miR33 inhibitors for 15 weeks were reported to have a small but nonsignificant decrease in circulating triglycerides compared with saline-treated animals. Similar to what is observed in mice, this study did not report any increases in body weight in anti-miR33–treated monkeys compared with controls. Therefore, analogous to what is observed in mice, long-term pharmacological miR-33 inhibition in a model that closely resembles humans does not result in adverse effects on obesity or circulating triglycerides and is in stark contrast to what was found on genetic deletion of miR-33 in mice.

In conclusion, we report that long-term inhibition of miR-33 using antisense oligonucleotides does not alter the development of obesity, insulin resistance, or hepatic lipid accumulation in a mouse model of diet-induced obesity. Mice receiving anti-miR33 oligonucleotides at doses known to prevent atherosclerosis showed no differences in body weight, glucose tolerance, serum insulin levels, or hepatic lipid accumulation after 20 weeks of high-fat feeding compared with either control- or placebo-treated mice and showed a trend toward decreased circulating triglycerides. There was an increase in the expression of fatty acid oxidation genes in the liver, Ampk expression and macrophage M2 markers in the adipose tissue, and decreased RER suggesting that anti-miR33 promotes a whole-body oxidative phenotype. These data confirm that at doses found to protect against atherosclerosis and without major fasting stressors, anti-miR33 therapy does adversely affect liver lipid metabolism, yet highlights the complexity of designing miRNA-based therapeutics for cardiometabolic diseases with overlapping mechanisms.

Acknowledgments

We gratefully acknowledge Christine Esau for her contribution to the design of the current study, and Regulus Therapeutics for the antisense oligonucleotides. We also thank Vivian Franklin and Jian Xuan for technical assistance.

Sources of Funding

This work was supported by operating grants from the Canadian Institutes for Health Research (MOP130365 and OCN126572; K.J. Rayner). D. Karunakaran was supported by an Endowed Cardiovascular Genetics Postdoctoral Fellowship from the University of Ottawa Heart Institute.

Disclosures

None.

References


**Significance**

microRNA-based inhibitors are being developed for the treatment of many chronic diseases, including atherosclerosis. miR-33 has been touted as a novel therapeutic target to improve reverse cholesterol transport and atherosclerosis, thus understanding the breadth of metabolic effects upon miR-33 inhibition is of therapeutic importance. In this study, we show that pharmacological inhibition of miR-33 is safe at doses known to prevent atherosclerosis, which underscores the important differences when inferring miRNA function from whole-body genetic deletion versus pharmacological inhibition strategies.
Therapeutic Inhibition of miR-33 Promotes Fatty Acid Oxidation but Does Not Ameliorate Metabolic Dysfunction in Diet-Induced Obesity
Denuja Karunakaran, Laura Richards, Michele Geoffrion, Danyk Barrette, Ryan J. Gotfrit, Mary-Ellen Harper and Katey J. Rayner

Arterioscler Thromb Vasc Biol. 2015;35:2536-2543; originally published online October 1, 2015;
doi: 10.1161/ATVBAHA.115.306404
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/35/12/2536

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2015/10/01/ATVBAHA.115.306404.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Materials and Methods

Animals
Six- to eight-week old male mice were purchased from Jackson Laboratories (C57BL6/J). Mice were placed on a high-fat diet (D12492, Research Diets Inc) containing 60% kcal from fat. A total of 8 mice per group were randomized and then treated with PBS or 2′F/MOE anti-miR oligonucleotides (Regulus Therapeutics) with the following sequences: 5′-TGCAATGCAACTACAATGCAC-3′ anti-miR33, 5′-TCCAATCCAACTTCAATCATC-3′ control anti-miR (mismatch) at a dose of 10mg/kg delivered subcutaneously once weekly for the first two weeks, then once every two weeks for the duration of the study (20 weeks) as previously described. Body weight was measured weekly. Food intake was measured daily for 7 days during the 20-week study. At sacrifice, mice were fasted for 6hrs and anaesthetized with isofluorane prior to cardiac puncture, exsanguination and perfusion with PBS. Tissues were collected and snap-frozen before storage at -80 for future analysis.

Serum cholesterol and triglyceride measurements
Mice were fasted for 6 h prior to blood collection and serum separation by centrifugation. HDL (non-apoB) was precipitated from LDL (apoB-containing) using the HDL-Cholesterol E kit (Wako, 431-52501) and cholesterol in each fraction was measured using the Total Cholesterol E kit (Wako, 439-17501). Serum triglycerides were measured with the L-Type Triglyceride M kit (465-09791 & 461-09891, Wako).

Glucose and insulin tolerance tests
Mice were fasted for 6h then injected intraperitoneally with either 1 g/kg of D-glucose (D16, Fisher Scientific) or 0.75 units/kg of insulin (HI0213, Eli Lilly Canada Inc). Blood glucose was measured from the tail vein at various intervals (t=0, 15, 30, 60, 120 minutes) with an Accu-Check Aviva Nano glucose meter (Roche Diagnostics). For the GTT, blood glucose levels in each group were averaged and plotted for each time point (Figure 2A) and area under of the curve was determined for each individual mouse and the mean ± SD for each group is represented within the GTT graph (Supplemental Figure 1A). For the ITT, percent glucose (compared to baseline) was determined for each mouse and the average blood glucose for each group was plotted for each time point (Figure 2B). Similar to GTT, area under the curve for each mouse was determined and plotted as a bar graph (Supplemental Figure 1B).
Serum insulin
Serum insulin was measured with the Mouse Ultrasensitive Insulin ELISA kit (80-INSMSU-E01, Alpco).

Metabolic outputs and energy expenditure
Mice were placed individually in a customized 4-chamber Oxymax open-circuit indirect calorimeter equipped with laser beam sets (Columbus Instruments, Columbus, OH) and allowed to acclimatize, as described\(^2\). The 2.5-L Plexiglass chambers were supplied with air at 0.5 L/min, and were maintained at 24°C throughout a 12h light/dark cycle. In each chamber, concentrations of O\(_2\) and CO\(_2\) in dry air were measured for 60 sec every 9 min, with a sample line-purge time of 2 min. Whole body O\(_2\) consumption (VO\(_2\)), and CO\(_2\) production (VCO\(_2\)) data were collected over a 24h period and are represented as respiratory exchange ratio (RER).

Western blotting
At sacrifice, tissues were weighed immediately upon isolation then snap frozen and stored at -80°C. Tissues were lysed in ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Roche) using the Bullet Blender homogenizer (NextAdvance Inc) then incubated on ice for 30 min before centrifugation to remove triton-insoluble pellet. Samples normalized with equal protein concentration were subjected to SDS-PAGE and western blot analysis. PVDF membranes were incubated with the following primary antibodies overnight: ABCA1 (Novus; 1:1000), SREBP1 (Santa Cruz Biotechnology; 1:1000) and GAPDH (Millipore; 1:1000).

RNA isolation and quantitative real-time PCR
Tissues were placed in Trizol reagents and homogenized using the Bullet Blender homogenizer (NextAdvance Inc). Total RNA was isolated using TRIzol reagent (Invitrogen) as per manufacturer's instructions and cDNA was synthesized using iScript Reverse Transcription kit (Biorad). Quantitative real-time PCR was performed in triplicate using the Sso Advanced Universal SYBR Green Supermix (Biorad) and mRNA level of target genes was normalized to either HPRT or GAPDH house keeping genes.
**Oil Red O staining**
10mm thick frozen liver sections were fixed in formalin, stained with Oil Red O (ORO) for 12 minutes at 60°C, then counterstained with Harris hematoxylin (SH30, Fisher Scientific) and mounted in Aquatex (108562, Merck). Quantification of ORO staining was performed using ImageJ software.

**Hematoxylin and eosin staining**
Paraffin-embedded 8mm thick sections were deparaffinized and rehydrated with graded alcohols, fixed with 4% paraformaldehyde, stained with hematoxylin for 1 minute, counterstained with 1% eosin (E511, Fisher Scientific) for 2 minutes, then dehydrated with graded alcohols and mounted in Vectamount (H-5000, Vector Labs).

**Statistical Analysis**
Groups were compared using a one-way ANOVA for single parameters, or two-way ANOVA for multiple parameters. Comparisons between two groups was done using a Student’s t-test. All statistical analyses were done using GraphPad Prism, and statistically significant differences are denoted by an * for \( p \leq 0.05 \) or ** for \( p < 0.01 \).
References


Supplemental Figure I

A

GTT

ns

PBS
cont antimiR
Anti-miR33

B

ITT

ns

PBS
cont antimiR
Anti-miR33
Supplemental Figure I: Area under curve (AUC) for GTT and ITT. Following glucose or insulin challenge, glucose levels were measured over time (Figure 2) and the average AUC was determined using GraphPad Prism software. AUC for both GTT and ITT was analyzed using one-way ANOVA, n=8 mice/group.
Supplemental Figure II

Liver

Cpt1α

Hadhb

Relative expression

cont anti
anti-miR33

*
Supplemental Figure II: Hepatic gene expression with anti-miR33 treatment.
(A) C57BL6/J mice were treated with 10mg/kg control anti or anti-miR33 (n=5/group) for 4 weeks prior to sacrifice. Hepatic gene expression of CPT1α and Hadhb were measured by qPCR and analyzed by Student’s t-test, *p≤0.05.
**Supplemental Figure III**

**A**

![Image of adipose tissue CD68+ area](cont anti anti-miR33)

**Adipose tissue CD68+ area**

- Cont anti
- Anti-miR33

Relative Expression

**B**

![Liver](Liver)

- cont anti
- anti-miR33

Relative Expression

*Arg1*
Supplemental Figure III: Tissue macrophage content and Arg1 expression. (A) Sub-cutaneous adipose tissue from mice treated with 10mg/kg control anti or anti-miR33 were sectioned and stained for CD68+, a macrophage marker. Area positively stained for CD68+ was quantified using Image J and average area is presented. Statistical significance was determined using Student’s t-test. (B) Hepatic gene expression of Arg1 in mice treated with anti-miR33 for 20 weeks. Student's t-test, *p≤0.05.