Macrophage Mitochondrial Energy Status Regulates Cholesterol Efflux and Is Enhanced by Anti-miR33 in Atherosclerosis

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Rationale: Therapeutically targeting macrophage reverse cholesterol transport is a promising approach to treat atherosclerosis. Macrophage energy metabolism can significantly influence macrophage phenotype, but how this is controlled in foam cells is not known. Bioinformatic pathway analysis predicts that miR-33 represses a cluster of genes controlling cellular energy metabolism that may be important in macrophage cholesterol efflux.

Objective: We hypothesized that cellular energy status can influence cholesterol efflux from macrophages, and that miR-33 reduces cholesterol efflux via repression of mitochondrial energy metabolism pathways.

Methods and Results: In this study, we demonstrated that macrophage cholesterol efflux is regulated by mitochondrial ATP production, and that miR-33 controls a network of genes that synchronize mitochondrial function. Inhibition of mitochondrial ATP synthase markedly reduces macrophage cholesterol efflux capacity, and anti-miR33 required fully functional mitochondria to enhance ABCA1-mediated cholesterol efflux. Specifically, anti-miR33 derepressed the novel target genes PGC-1α, PDK4, and SLC25A25 and boosted mitochondrial respiration and production of ATP. Treatment of atherosclerotic Apoe−/− mice with anti-miR33 oligonucleotides reduced aortic sinus lesion area compared with controls, despite no changes in high-density lipoprotein cholesterol or other circulating lipids. Expression of miR-33a/b was markedly increased in human carotid atherosclerotic plaques compared with normal arteries, and there was a concomitant decrease in mitochondrial regulatory genes PGC-1α, SLC25A25, NRF1, and TFAM, suggesting these genes are associated with advanced atherosclerosis in humans.

Conclusions: This study demonstrates that anti-miR33 therapy derepresses genes that enhance mitochondrial respiration and ATP production, which in conjunction with increased ABCA1 expression, works to promote macrophage cholesterol efflux and reduce atherosclerosis. (Circ Res. 2015;117:266-278. DOI: 10.1161/CIRCRESAHA.117.305624.)

Key Words: atherosclerosis ■ cholesterol ■ macrophages ■ mitochondria ■ microRNA-33, mouse

The accrual of modified lipoproteins and macrophages in the vessel wall drives the progression of atherosclerosis.1 Excess circulating lipoproteins, in particular low-density lipoprotein (LDL), become trapped beneath the protective endothelial layer and become modified in the oxidant-rich environment, recruiting monocytes that differentiate into inflammatory macrophages. In an attempt to restore the lipid-balance within the vessel wall, excess intracellular cholesterol is removed from macrophage foam cells into the reverse cholesterol transport (RCT) pathway by the interaction of ABC cholesterol transporter proteins (eg, ABCA1) and apolipoprotein A-I (apoA1), a component of high-density lipoprotein (HDL). Although HDL-cholesterol (HDL-C) has been widely used as a surrogate for HDL function, it is now appreciated that capacity for HDL to promote cholesterol efflux from macrophages is a more predictive measure of the antiatherosclerotic abilities of HDL.2–4 However, to date, there are no therapies that specifically enhance macrophage RCT in the vessel wall.

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Mitochondria are widely recognized as the powerhouses of the cell, yet, relatively little is known about how mitochondrial function is regulated in macrophages, especially as it relates to foam cell formation in atherosclerosis. Mitochondrial metabolism encompasses a complex series of oxidizing processes that ultimately produce the cell’s energy currency, adenosine triphosphate (ATP), which are tightly regulated by both nuclear and mitochondrial genes. Macrophages can have a high demand for cellular energy and require the efficient use of either glycolysis or fatty acid metabolism to maintain necessary levels of ATP to meet the demands during inflammation and, in the atherosclerotic plaque, increased rates of cholesterol efflux. Although the plasticity of macrophage energy metabolism is becoming evident during acute inflammation, less is known about how macrophage mitochondrial energy metabolism is controlled during atherosclerosis.

Excitement has grown for the potential of microRNA (miRNA)-based therapeutics for preventing and regressing atherosclerosis. In particular, strategies that inhibit miR-33 have shown promise in both small and large preclinical models to raise HDL-C and promote RCT and the regression of existing atherosclerotic plaques. miR-33 (and its human equivalents miR-33a and miR-33b) is an intronic miRNA located within the gene coding for the cholesterol transcription factor SREBP-2 (and in humans, also SREBP-1) where they cooperate to increase cholesterol synthesis and decrease cholesterol elimination, in part through targeting cholesterol transport proteins ABCA1 and ABCG1. In addition, miR-33 represses genes involved in fatty acid oxidation (ie, HADHB, CROT, Cpt1α) and high levels of miR-33 activity can limit the oxidation of fatty acids. Thus, although miR-33 has been primarily studied for its role in regulating LDL biogenesis and circulating LDL levels, it is becoming increasingly evident that miR-33 post-translationally modifies several genes that control cellular energy status.

Given the importance of mitochondrial metabolism in maintaining cellular energy status, we hypothesized that mitochondrial function can regulate cholesterol efflux from foam cells, and that miR-33 can fine-tune cellular energetic pathways to regulate this process. Microarray analysis of plaque macrophages from anti-miR-33–treated Ldlr−/− mice revealed a series of novel miR-33 targets genes that were derepressed on miR-33 inhibition, including peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1α (PPARGC1A, or PGC-1α), a gene that plays a central role in regulating cellular energy homeostasis and mitochondrial metabolism. As miRNAs are known to target entire functional pathways, we tested whether miR-33 targets a network of genes controlling mitochondrial metabolism and identified additional novel targets in this pathway. Furthermore, using gain and loss of function approaches, we show that this regulation contributes to the regulation of cholesterol efflux in atherosclerosis in mice and is associated with the presence of advanced atherosclerosis in humans.

Methods
An expanded Materials and Methods is included in the Online Data Supplement and provides details on the identification of novel miR-33 target genes using bioinformatics, the delivery of anti-miRNAs in vitro and in vivo, assays measuring mitochondrial respiration and ATP production, as well as details about the analysis of human atherosclerotic plaque tissue for miR-33a/b and target gene expression.

Results
Mitochondrial Activity Controls Cholesterol Efflux in Macrophages
Excess cholesterol removal from macrophage foam cells is mediated through ABCA1 transporters on the cell surface, which account for a significant portion of both phospholipid and cholesterol efflux from the cell. Although the exact mechanism by which these lipids are transported across the cell membrane onto apoA1 remains incompletely understood, it is known to be an ATP-dependent process. Therefore, we hypothesized that mitochondrial function was important for efficient cholesterol removal from macrophages to apoA1. To test this hypothesis, we treated THP-1 macrophages with the mitochondrial respiratory inhibitor oligomycin, which blocks the production of ATP from oxidative phosphorylation in the mitochondria. This resulted in a significant reduction in cholesterol efflux to apoA1, diminishing the percentage of cholesterol efflux back to those found the absence of an acceptor, similar to what had been observed previously in mouse macrophages (Figure 1A). Similarly, macrophages from Pgc-1α−/− mice, which have reduced mitochondrial function and a reduced capacity for oxidative phosphorylation, showed impaired cholesterol efflux to apoA1, in both cholesterol-loaded and unloaded conditions (Figure 1B). Taken together, these results confirm that mitochondrial production of ATP via oxidative phosphorylation is important for efficient cholesterol efflux from macrophages, and confirm the notion that enhancing mitochondrial function may serve to enhance cholesterol removal from foam cells.

Cholesterol efflux is tightly controlled by both transcriptional and post-transcriptional mechanisms. miR-33 has been shown to modulate cholesterol efflux pathways by reducing the expression of the cholesterol transporters ABCA1 and ABCG1, however, relatively little is known about its impact on other energy metabolism pathways. As mitochondria are central regulators of cellular energy homeostasis, we sought to determine whether miR-33 target genes involved in maintaining mitochondrial function. We interrogated a robust list of miR-33 predicted target genes, as determined using 5 prediction algorithms, and performed bioinformatic pathway analysis using the DAVID tool. In addition to PGC-1α, we identified several other genes encoding mitochondrial proteins predicted to be targeted by miR-33, including genes involved in oxidation of pyruvate (pyruvate dehydrogenase kinase 4 or

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<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>ABCA1</td>
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<tr>
<td>apoA1</td>
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<td>ATP</td>
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Figure 1. Mitochondria are required for cholesterol efflux in macrophages and are predicted to be regulated by miR-33. A, Human THP-1 macrophages transfected with control anti-miR or anti-miR33 were cholesterol-loaded for 24 hours before pretreatment with oligomycin for 1 hour, and subsequently incubated with apoA1 for 6 hours. Percentage cholesterol efflux is shown as a proportion of total radiolabeled cholesterol in the cell. B, Peritoneal macrophages from wild-type C57BL6 or Pgc-1α−/− mice were loaded with or without cholesterol for 24 hours, and cholesterol efflux to apoA1 was measured for 6 hours. Percentage cholesterol efflux is shown as a proportion of total radiolabeled cholesterol in the cell. C, Bioinformatic pathway analysis using the DAVID gene tool and Gene Set Enrichment Analysis predicted that miR-33 regulates multiple mitochondrial genes. Predicted miR-33 targets are depicted as yellow circles; interacting downstream genes are shown as violet circles; genes outlined in blue are previously confirmed miR-33 target genes, and dotted lines are novel miR-33 target genes confirmed in this study.
PDK4), solute carrier proteins (SLC25A25, SLC25A23) and previously confirmed targets involved in fatty acid oxidation (HADHB, CROT)\(^1\) (Table 1). Molecular interaction analysis using Cytoscape revealed that many of the miR-33 targets, both predicted and validated, interact with other mitochondrial genes, suggesting that miR-33 may regulate mitochondrial function by both direct and indirect mechanisms (Figure 1C).

**Increasing Mitochondrial Gene Expression via miR-33 Pathway Inhibition**

Given their established role in metabolism in vivo,\(^20\)\(^{-}\)\(^22\) we next sought to confirm the putative target genes PGC-1\(\alpha\), SLC25A25, and PDK4 as direct miR-33 targets. Overexpression of miR-33 mimics in conjunction with candidate 3′UTR-luciferase reporter constructs confirmed that miR-33 directly represses the 3′UTR of human PGC-1\(\alpha\),\(^23\) PDK4, and SLC25A25 and disrupting these binding sites in these genes by site-directed mutagenesis with these sites abolishes the inhibitory effects of miR-33 on these genes (Figure 2A; Online Figure 1). miR-33 binding sites are also conserved in the 3′UTR of these genes in mice, indicating that miR-33 can repress gene expression in both species (Online Figure 1). To confirm whether miR-33 endogenously regulates mitochondrial gene expression in macrophages, we transfected mouse peritoneal and human THP-1 macrophages with anti-miR33 or control anti-miRs and examined the expression of target genes. We observed a significant derepression of PGC-1\(\alpha\), PDK4, SLC25A25, and SLC25A23, as well as the previously established miR-33 target genes ABCA1 and HADHB, at the mRNA level (Figure 2B). Moreover, anti-miR33 treatment increased the protein expression of PGC-1\(\alpha\), PDK4, and SLC25A25 as observed by Western blot analysis, in both mouse and human macrophages (Figure 2C). These data confirm that in macrophages, miR-33 directly regulates the expression of PGC-1\(\alpha\), SLC25A25, and PDK4 via binding to complementary sites in the 3′UTR, in addition to its previously described targets ABCA1 and HADHB.

Our pathway interaction analysis suggests that miR-33 has the capacity to regulate the expression of multiple mitochondrial genes, both directly (ie, by 3′UTR binding) and indirectly (ie, via interaction with direct miR-33 targets; Figure 1C). In particular, PGC-1\(\alpha\) directly activates important activators of mitochondrial biogenesis, including nuclear respiratory factor 1 (NRF1) a transcription factor that activates expression of nuclear-encoded mitochondrial genes and is essential for mitochondrial respiration. We therefore measured the expression of NRF1 and show that indeed miR-33 inhibition leads to an upregulation of NRF1 mRNA (Figure 3A) and protein expression (Figure 3B). As the 3′UTR of NRF1 in both human and mouse does not contain any predicted miR-33 binding sites, we conclude that the upregulation of NRF1 on miR-33 inhibition is an indirect consequence of the derepression of other direct miR-33 target genes that control mitochondrial biogenesis, primarily PGC-1\(\alpha\). The increase in PGC-1\(\alpha\) and NRF1 resulted in a significant increase in mitochondrial DNA copy number, a readout of mitochondrial biogenesis, in macrophages treated with anti-miR33 compared with controls (1.7-fold anti-miR33 versus cont anti-miR, \(P \leq 0.01\); Figure 3C). To further explore the indirect effects of anti-miR33 on macrophage mitochondrial gene expression, we used a pathway-focused polymerase chain reaction arrays, which contain 84 genes involved in mitochondrial metabolism and compared control anti-miR and anti-miR33 treated macrophages. In agreement with our network interaction analysis, mitochondrial pathway arrays demonstrated that miR-33 regulates the expression of several genes involved in mitochondrial function (Online Figure IIIB; Table 1). Of the genes analyzed in the mitochondrial pathway, some genes containing miR-33 binding sites in their 3′UTR showed derepression on miR-33 inhibition (ie, Slc25a25, Slc25a23), whereas others lacking miR-33 binding sites also showed upregulated and downregulated expression (ie, Ucp-2, Bcl-2). We next measured levels of the mitochondrial oxidative phosphorylation machinery (OXPHOS), a complex of proteins

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**Table 1. GO Analysis of Predicted miR-33 Target Genes Involved in Mitochondrial Function**

<table>
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<th>Term</th>
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<tr>
<td>GO:0005739-mitochondrion</td>
<td>BID, CYB5R1, ABCA9, ADHFE1, SGPP1, ALDOC, ACN9, ALDH3A2, HADHB*, SLC25A25*, LACE1, ATP5S, SLC25A23, CSDE1, CABC1, GUF1, HIGD1A, SC01, MRPS25, PDK4†, COO9, SLC3A1, HERC2, XPNPEP3, CLPX, MSRB3, TMEM65, DUSP26, CLIC4, ALDH2, CROT*, DUT, ACADSB</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>BID, ADHFE1, MRPS25, PDK4†, COO9, ACN9, CLPX, HADHB*, MSRB3, MTC2, SLC25A25, CLJC4, PGAM5, SLC25A23, ATP5S, ALDH2, ELOVL6, CABC1, SC01</td>
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<tr>
<td>GO:0044429-mitochondrial part</td>
<td>ACADSB, MRPS25, PDK4†, HERC2, SLC3A1, ALDH3A2, ACN9, CLPX, HADHB*, MTC2, SLC25A25†, SLC25A25†, ATP5S, CSDE1, ALDH2, SC01</td>
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<tr>
<td>GO:0005740-mitochondrial envelope</td>
<td>BID, PDK4†, HERC2, SLC3A1, ALDH3A2, ACN9, CLPX, HADHB*, SLC25A25†, MTC2, SLC25A23†, ATP5S, CSDE1, SC01</td>
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<tr>
<td>GO:0031966-mitochondrial membrane</td>
<td>BID, PDK4†, HERC2, SLC3A1, ALDH3A2, CLPX, HADHB, SLC25A25†, MTC2, SLC25A23†, ATP5S, CSDE1, SC01</td>
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<tr>
<td>GO:0005743-mitochondrial inner membrane</td>
<td>PDK4†, HERC2, SLC3A1, ALDH3A2, CLPX, HADHB*, SLC25A25†, MTC2, SLC25A22†, ATP5S, CSDE1, SC01</td>
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<tr>
<td>Transit peptide: mitochondrion</td>
<td>ACADSB, ADHFE1, PDK4†, COO9, ACN9, CLPX, MSRB3, HADHB*, ATP5S, ALDH2, CABC1, SC01</td>
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<tr>
<td>GO:0031980-mitochondrial lumen</td>
<td>ACADSB, MRPS25, PDK4†, ALDH2, HADHB*</td>
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<tr>
<td>GO:0005759-mitochondrial matrix</td>
<td>ACADSB, MRPS25, PDK4†, ALDH2, HADHB*</td>
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<tr>
<td>Mitochondrion inner membrane</td>
<td>SLC25A25†, MTC2, ATP5S, SLC25A23†</td>
</tr>
<tr>
<td>IPR018108: mitochondrial substrate/solute carrier</td>
<td>SLC25A25†, MTC2, SLC25A23†</td>
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*Previously identified miR-33 targets. †miR-33 targets of interest.
that are found on the inner membrane of the mitochondria and together produce the majority of cellular ATP. The OXPHOS complex is comprised of complexes I to V, which together use the reducing equivalents from oxidized fuels to produce the proton motive force across the membrane, which then drives the conversion of ADP to ATP by ATP synthase. We quantified the levels of the OXPHOS machinery in macrophages transfected with anti-miR33 or control oligonucleotides and show that complexes I, III, IV, and V are significantly upregulated with miR-33 inhibition (Figure 3D). Together, these data reveal that in addition to directly targeting mitochondrial genes PGC-1α, PDK4, and SLC25A25, anti-miR33 indirectly modifies the expression of additional mitochondrial metabolism genes that may have important functional consequences on mitochondrial metabolism.

**Anti-miR33 Treatment Increases Mitochondrial Respiration and ATP Production**

Given its ability to alter mitochondrial gene expression and augment oxidative phosphorylation machinery, we tested whether anti-miR33 can specifically enhance mitochondrial function. We measured the outcome of miR-33 inhibition on oxygen consumption rates in macrophages, under ADP phosphorylating and nonphosphorylating conditions. Cellular respiration was quantified in macrophages transfected with control anti-miR or anti-miR33 using the Seahorse XF Extracellular Flux Analyzer. Under basal conditions, anti-miR33 treatment...
increased oxygen consumption rates compared with control cells (Figure 4A). When oxidative phosphorylation is blocked using oligomycin to induce the nonphosphorylating (proton leak) condition, anti-miR33 had no effect on oxygen consumption rates. Finally, anti-miR33 enhanced maximal cellular respiration rates after treatment with uncoupling agent, FCCP, suggesting inhibition of miR-33 promotes electron transport chain activity (or substrate delivery) in the mitochondria (Figure 4A). To further confirm that miR-33 alters mitochondrial energy metabolism, we measured the intracellular ATP production as a measure of optimal mitochondrial function and activity. As shown in Figure 4B, macrophages overexpressing miR-33 had decreased ATP production relative to control (−45.83%; \( P \leq 0.05 \)). In contrast, the inhibition of miR-33 resulted in increased production of ATP relative to controls (+28.01%; \( P \leq 0.05 \)). Collectively, these data demonstrate that the inhibition of miR-33 positively drives mitochondrial aerobic respiration and activity, which in turn results in an increase in ATP production, likely through its direct and indirect modulation of multiple mitochondrial genes.

Increased oxygen consumption rates compared with controls (Figure 4C). Inhibition of mitochondrial ATP production blocked the ability of anti-miR33 to promote cholesterol efflux. In the absence of optimally respiring mitochondria, macrophages treated with anti-miR33 had reduced cholesterol efflux compared with controls, suggesting that the salutary effects of miR-33 inhibition absolutely require mitochondrial respiration and energy production (Figure 4C). These experiments highlight an essential role for mitochondrial respiration and ATP production in miR-33 regulation of cholesterol homeostasis in macrophages and represent a novel mechanism by which cholesterol efflux can be augmented in atherosclerosis.

Given the ability of miR-33 to control mitochondrial gene expression and function, we next asked whether the regulation of cholesterol efflux by anti-miR33 was dependent on PGC-1\( \alpha \) and other newly identified mitochondrial target genes. Using mice deficient in Pgc-1\( \alpha \), which have with impaired mitochondrial metabolism, we measured ATP production on inhibition of miR-33. In wild-type macrophages, anti-miR33 robustly augments ATP production, but this effect is lost in the absence of Pgc-1\( \alpha \) (Figure 4D). We next tested the dependency of PGC-1\( \alpha \) on anti-miR33 regulation of cholesterol efflux. Although anti-miR33 treatment results in a robust 50% increase in cholesterol efflux in macrophages from wild-type mice, anti-miR33 has no effect on cholesterol efflux in macrophages from Pgc-1\( \alpha \)−/− mice (Figure 4E). In contrast, anti-miR33 could still augment efflux in Pdk4−/− macrophages, albeit to a lesser extent that wild-type macrophages (Figure 4E). Anti-miR33 could equally increase cholesterol efflux in both Slc25a25−/− and wild-type cells (Figure 4E).
Taken together, these data reveal that miR-33 inhibition depends on functional Pgc-1α to regulate ATP production, as well as cholesterol efflux from macrophages, highlighting the essential role for mitochondrial metabolism in the salutary effects of anti-miR33.

Anti-miR33 Protects From Atherosclerosis and Increases Mitochondrial Gene Expression in Apoe<sup>−/−</sup> Mice

Previous studies using miR-33 inhibition have shown beneficial effects on RCT and atherosclerosis progression and regression. Part of the atheroprotective mechanism of miR-33 inhibition may be attributable to raising of HDL-C, however, studies in *Ldlr<sup>−/−</sup>* mice on a Western diet have shown reductions in atherosclerosis in the absence of HDL-raising. We wondered if the mitochondrial pathways regulated by anti-miR33 could promote HDL-independent cholesterol efflux pathways in lesions from the highly inflamed *Apoe<sup>−/−</sup>* mice, which have little to no circulating HDL. Eight-week-old *Apoe<sup>−/−</sup>* mice were simultaneously fed a Western diet and administered control anti-miR or anti-miR33 oligonucleotides via weekly injections for 8 weeks. Quantification of the aortic sinus lesion area shows a reduction in lesion burden in mice treated with anti-miR33 compared with their controls (Figure 5A; *P*≤0.05). However, the anti-miR33 dose used in this study neither affects circulating HDL levels (Figure 5B) nor alters total plasma cholesterol or LDL cholesterol (Table 2). Visualization of lipid droplets, elastin, and

**Figure 4. Inhibition of miR-33 increases mitochondrial respiration and ATP production that contributes to macrophage cholesterol efflux potential.**

A, Human macrophages were transfected with control anti-miR or anti-miR33, and the oxygen consumption rate was determined using the Seahorse XF24 Extracellular Flux Analyzer. Data depicted demonstrate mean±SEM of n=4 experiments. B, Peritoneal macrophages were transfected with control miR, miR-33 mimics, control anti-miR, or anti-miR33 for 48 hours before the addition of apolipoprotein A-I (apoA1) for 6 hours. Intracellular ATP levels were measured and are shown as either μmol/L [ATP]/μg protein. C, THP-1 macrophages transfected with control anti-miR or anti-miR33 for 24 hours were incubated with 1 μg/mL 3[H]-cholesterol and 37.5 μg/mL AcLDL for 24 hours before the addition of 25 μg/mL apoA1 for 6 hours in the presence or absence of 10 μmol/L oligomycin. Percentage cholesterol efflux was determined relative to control, and data show mean±SD of 6 replicates and are representative of at least 3 independent experiments. D, Peritoneal macrophages from either wild-type (WT) or *Pgc-1α<sup>−/−</sup>* mice were transfected with control anti-miR or anti-miR33 and intracellular ATP was measured as described in (B) above. Data shown are % change relative to control±SD from 3 replicates and are representative from at least 3 independent experiments. E, Peritoneal or BMDM macrophages from either WT, *Pgc-1α<sup>−/−</sup>*, *Pdk4<sup>−/−</sup>* or *Slc25a25<sup>−/−</sup>* mice were transfected with anti-miRs, and cholesterol efflux was measured as in (B). Data are shown as % increase in efflux to apoA1 by anti-miR33 compared with control anti-miR of 6 replicates and are representative of n=3 independent experiments (*P*≤0.05, **P*≤0.01, ****P*≤0.0001).
collagen was performed using coherent anti-Stokes Raman scattering (CARS), 2-photon fluorescence (TPF), and second harmonic generation (SHG), respectively (Figure 5C). Anti-miR33–treated mice have reduced lipid aggregation and overall lesion area (Figure 5D), both of which are consistent with an increased macrophage cholesterol efflux capacity induced by anti-miR33 therapy. Given that miR-33 inhibition resulted in increased expression of PGC-1α and other mitochondrial target genes in vitro, we measured the expression of these targets in the lesions of Apoe−/− mice treated with control-anti-miR and anti-miR33. Dashed outlines depict lesion/lumen border. D, Quantification of lipid content from CARS signal (total CARS area) in the aortic sinus from cont anti-miR and anti-miR33 treated Apoe−/− mice. P<0.01, n=3 per group. E, Representative images of immunohistochemistry staining for PGC-1α and PDK4 in aortic sinus sections of Apoe−/− mice treated with control-anti-miR or anti-miR33. H&E indicates hematoxylin and eosin.

Figure 5. Anti-miR33 therapy decreases atherosclerosis in Apoe−/− mice independently of high-density lipoprotein (HDL) cholesterol. Apoe−/− mice were simultaneously fed Western diet (0.2% cholesterol) and an administered anti-miR33 (or control anti-miR) oligonucleotides for 8 weeks before euthanize. A, Quantification of atherosclerotic lesion areas in aortic sinus sections. Mean±SEM for each treatment group is shown, *P<0.05, n=7–8 per group, Student t test. B, Plasma HDL cholesterol levels at the end of study showed no difference between control and anti-miR33. C, Coherent anti-Stokes Raman scattering (CARS), 2-photon fluorescence (TPF), and second harmonic generation (SHG) microscopy were used to visualize lipid droplets (CARS, red), elastin (TPF, green), and collagen (SHG, blue) in atherosclerotic lesions of Apoe−/− mice treated with control-anti-miR and anti-miR33. Dashed outlines depict lesion/lumen border. D, Quantification of lipid content from CARS signal (total CARS area) in the aortic sinus from cont anti-miR and anti-miR33 treated Apoe−/− mice. P<0.01, n=3 per group. E, Representative images of immunohistochemistry staining for PGC-1α and PDK4 in aortic sinus sections of Apoe−/− mice treated with control-anti-miR or anti-miR33. H&E indicates hematoxylin and eosin.

Table 2. Body Weight and Serum Cholesterol Measurements in Apoe−/− Mice

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<th>Cont Anti-miR</th>
<th>Anti-miR33</th>
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<tr>
<td>Body weight, g</td>
<td>31.6±5.8</td>
<td>28±4.8</td>
<td>ns</td>
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<tr>
<td>TC, mg/dL</td>
<td>658.8±73.4</td>
<td>767.7±64.6</td>
<td>ns</td>
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<tr>
<td>LDL, mg/dL</td>
<td>129.1±26.0</td>
<td>129.3±21.2</td>
<td>ns</td>
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<tr>
<td>HDL, mg/dL</td>
<td>7.12±1.5</td>
<td>7.10±1.9</td>
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HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and ns, not significant.
proatherogenic milieu, we isolated peritoneal macrophages from hypercholesterolemic mice, which are considered a surrogate for plaque macrophages. In vivo formed foam cells from Apoe−/− mice treated with anti-miR33–treated mice showed significantly increased expression of target genes Abca1, Pgc-1α, and Slc25a25 (Figure 6A; P≤0.05), in parallel with the observed increases in of Pgc-1α and Pdk4 protein in the vessel wall. We also measured the corresponding levels of ATP in the aortas of Apoe−/− mice, and mice treated with anti-miR33 tended to have increased levels of aortic ATP compared with controls (968.8±225.5 μmol/L ATP versus 2930±1367 μmol/L ATP/total lesion area, P=0.15; Online Figure IIIA). We analyzed plaque macrophage gene expression using laser-capture microdissection in a related model of atherosclerosis progression, Western diet fed Ldlr−/− mice treated with anti-miR33 or control anti-miR for 8 weeks. Quantitative polymerase chain reaction on LCM-isolated macrophages revealed a trend toward increased expression of miR-33 target genes Pgc-1α and Slc25a23, and a significant increase in Pdk4 mRNA (Online Figure IIIC). Nrf1, a Pgc-1α downstream target and a marker of mitochondrial biogenesis, was robustly increased in lesional macrophages from anti-miR33–treated mice compared with controls (Online Figure IIIC). Finally, in an attempt to translate our findings to humans, we examined whether miR-33 and its mitochondrial target genes are dysregulated in human atherosclerosis. Indeed, miR-33 (both copies, miR-33a and miR-33b) are significantly elevated in atherosclerotic plaques from patients with carotid atherosclerosis compared with control arteries (Figure 6B). This was associated with a parallel decrease in miR-33 target gene expression of PGC-1α, SLC25A25, and SLC25A23, as well as in the indirect markers of mitochondrial biogenesis NRF1 and TFAM (Figure 6B). These data are the first to show that miR-33a/b expression is dysregulated in atherosclerosis in humans, and that genes with known roles regulating mitochondrial biogenesis (ie, PGC-1α, NRF1, TFAM) are significantly lower in atherosclerotic versus healthy arteries. Together these data suggest that anti-miR33 therapy would be predicted to increase the expression of mitochondrial gene expression in the plaque to promote mitochondrial respiration and cholesterol.

Figure 6. Novel miR-33 mitochondrial target gene expression is regulated in vivo. A. Gene expression analysis of peritoneal macrophages isolated from Western-fed Apoe−/− mice treated with control anti-miR or anti-miR33 for 8 weeks (from Figure 5). B. Relative expression of miR-33a (red) and miR-33b (blue), as well as PGC-1α (PPARGC1A), SLC25A25, SLC25A23, NRF1, and TFAM in healthy arteries (Normal) or carotids from patients with atherosclerosis (Plaque) from the Biobank of Karolinska Endarterectomy (BiKE) (*P≤0.05, **P≤0.01, ***P≤0.0001).
efflux capacity, which may contribute to the ability of miR-33 inhibitors to reduce atherosclerotic lesion size in the absence of changes in HDL-C, and that this pathway may be active in human atherosclerosis development.

**Discussion**

The metabolic status of a cell is a strictly regulated process. In cells of the innate immune system, whose main purpose is to offer rapid protection against invading nonself and modified-self antigens, the availability of energy substrates is vital to mount an appropriate response in times of stress. For macrophages in the atherosclerotic plaque, this stress comes in the form of excess cholesterol accumulation, and macrophages need to boost the removal and detoxification of cholesterol via efflux pathways. We demonstrate that macrophage cholesterol efflux is dependent on functionally respiring mitochondria, as inhibiting mitochondrial ATP production and function by pharmacological (ie, oligomycin) or genetic (ie, deletion of Pgc-1α) means significantly blunts the ability of cells to efflux cholesterol to apoA1. Moreover, we show that miR-33, a miRNA with reported roles in controlling cholesterol efflux, can also regulate production of ATP by the mitochondria and can limit ATP availability for ABCA1-dependent cholesterol efflux. These data indicate that boosting energy metabolism pathways may be a novel method to enhance cholesterol efflux in macrophages and positively affect atherosclerosis development.

miRNAs are small but potent post-transcriptional modulators of gene expression that can regulate entire genetic networks. Previous studies from our group and others have shown that miR-33 (miR-33a/b in humans) controls the expression of genes that influence cellular metabolism, including cholesterol transport (eg, ABCA1, ABCG1) and fatty acid β-oxidation (eg, CPT1α, CROT, HADHB) pathways. Using bioinformatic analysis, we have now identified another network of genes targeted by miR-33 to regulate mitochondrial respiration and metabolism. We confirmed several novel mitochondrial genes (ie, PGCG-1α, PDK4, and SLC25A25) that are direct and specific targets of miR-33, with conserved binding sites in the 3'UTR of both human and mouse transcripts. Importantly, inhibition of endogenous miR-33 in mouse and human macrophages increases the protein expression of PGC-1α, PDK4, and SLC25A25, as well as the mitochondrial fatty acid oxidation genes HADHB and CROTO, which are also direct targets of miR-33. In addition to the direct miRNA targets of miR-33, we also found that anti-miR33 can indirectly increase the expression of NRF1 and OXPHOS complexes, key factors that promote mitochondrial biogenesis and efficient production of ATP, respectively. Both NRF1 and OXPHOS are downstream of PGCG-1α, and miR-33 inhibition could result in an increase in mitochondrial biogenesis when PGCG-1α levels are derepressed. Indeed, we observed a significant increase in mitochondrial DNA copy number, a readout of mitochondrial biogenesis, in anti-miR33–treated macrophages. Although the changes in expression exerted by miR-33 on any 1 target gene may be small, the cumulative functional outcome of fine-tuning many genes in the same pathway or network can be large, as evidenced by the significant changes in ATP synthesis on manipulation of miR-33 levels. Thus, these data support the notion that miR-33 exerts control over genetic networks that regulate cellular energy homeostasis (ie, lipid, fatty acid, mitochondrial), with miR-33 serving as a regulatory hub of energy metabolism.

ABCA1 is a member of the ABC transporter protein family, with 2 ATP-binding cassettes in its inner membrane domain, which hydrolyze and transport ATP molecules during efflux. During the transport of cholesterol and phospholipids, a steady supply of ATP is required for ABCA1 (and other ABC transporters, such as ABCG1) to move substrate across the membrane to an acceptor, making ATP central to the efficient removal of cholesterol. In addition to directly exerting post-transcriptional control over ABCA1 expression, we now show that miR-33 regulates the availability of ATP for cholesterol efflux via repressing a network of genes that control mitochondrial respiration and ATP production. miR-33 expression is activated during times of nutrient depletion (ie, sterol, fatty acid) where it serves to repress the expression of cholesterol efflux and fatty acid oxidation pathways, to conserve energy. Our data are consistent with miR-33 conserving cellular energy by regulating multiple pathways, for example, repression of sterol and fatty acid utilization, and concomitantly dampening of ATP production. Further, our data also support the hypothesis that under conditions of excess cholesterol (ie, in atherogenic macrophage foam cells), enhancing mitochondrial function can promote cholesterol efflux in part by increasing ATP production. Indeed, derepression of PGCG-1α, PDK4, and SLC25A25 on inhibition of miR-33 corresponds to an increase in mitochondrial respiration and ATP production—both of which contribute to the increased cholesterol efflux capacity seen in anti-miR33 transfected macrophages. We found that inhibition of mitochondrial function (ie, on treatment with oligomycin) markedly reduced macrophage cholesterol efflux to apoA1, and blocking normal mitochondrial function blunted the beneficial effects of anti-miR33 on macrophage cholesterol efflux, suggesting respiring mitochondria are necessary for the favourable effects of anti-miR33 on efflux. Similarly, in the absence of PGCG-1α, anti-miR33 can no longer exert its effects on ATP production or cholesterol efflux, once again confirming that intact mitochondrial ATP production enhances anti-miR33 regulation of cholesterol efflux. Mitochondrial dysfunction has been mechanistically linked to the progression of atherosclerosis in Apoe−/− mice, independent of reactive oxygen species production, due in part to defects in oxidative phosphorylation. Furthermore, it has been suggested that mitochondrial distress can reduce the efficiency of cholesterol efflux from macrophages, which could be a consequence of reduced ATP production by the mitochondria. Here, we show enhanced mitochondrial respiration in anti-miR33–treated macrophages by derepression of key mitochondrial genes to promote ATP production and in conjunction with the major miR-33 target gene ABCA1, enhances cholesterol efflux, indicating that miR-33 dampens cholesterol efflux pathways via its direct effects on the mitochondria. Interestingly, we observed the greatest increase in mitochondrial respiration by anti-miR33 in the presence of the uncoupling agent, FCCP. In uncoupled states, when the mitochondria are in overdrive to respire, we think that miR33 represses mitochondrial biogenesis and respiration through its multitude of mitochondrial gene targets.
Anti-miR33 derepresses many of these gene targets simultaneously, amplifying their additive effects on an overdriven respiratory system, which when translated in vivo, could have beneficial outcomes in the plaque where defective oxidative phosphorylation and mitochondrial function are present. Similarly, although we measured ATP production at 1 fixed time point, blocking miR-33 continuously over time would expect to have a sustained effect on mitochondrial respiration and thus contribute to increased cholesterol efflux and reduced atherosclerotic lesion area in vivo. These data support the concept that improving mitochondrial function in the vessel wall could be a novel therapeutic approach for reducing atherosclerosis.

PGC-1α is a well-characterized master regulator of mitochondrial biogenesis and metabolism, and promotes fatty acid oxidation, gluconeogenesis and browning of white adipose tissue. In macrophages, anti-miR33 therapy increases PGC-1α expression, which in turn increases cellular respiration and ATP production, 2 fundamental roles of the mitochondria. Clinical evidence suggests that promoting the activity of PGC-1α has positive outcomes on metrics of the metabolic syndrome, including insulin resistance and obesity. However, until recently, PGC-1α activity in macrophages had not been well studied, and its role in atherogenesis was unknown. To investigate the function of PGC-1α in atherogenesis, McCarthy et al recently reported that deletion of Pgc-1α in hematopoietic cells accelerated the progression of atherosclerosis in Ldlr−/− mice. Our current study supports the idea that increasing PGC-1α expression has salutary effects on the development of atherosclerosis, as mice treated with anti-miR33 oligonucleotides had increased vascular Pgc-1α protein expression, increased foam cell expression of Pgc-1α transcripts, and a concomitant decrease in aortic lesion area. Indeed in human atherosclerotic lesions, the expression of PGC-1α in macrophages was inversely correlated to the severity of disease, and overexpression of macrophage PGC-1α in vitro reduced foam cell formation. In agreement with these observations, we show that in arteries from patients having atherosclerosis, PGC-1α levels are significantly downregulated compared with healthy arteries, and there is a concomitant increase in miR-33a/b expression. Similarly, the downstream target of PGC-1α, NRF1, is also decreased in atherosclerotic lesions, suggesting an impairment of mitochondrial biogenesis in atherosclerosis. Thus, augmenting PGC-1α expression and function in macrophages could have protective effects on the progression of atherosclerosis in both mice and humans.

In addition to PGC-1α, this study also identifies several novel mitochondrial targets of miR-33 and confirms that PDK4 and SLC25A25 are indeed directly repressed by miR-33 via binding to the 3′UTR. In keeping with its role as a regulator of energy homeostasis, these novel targets PDK4 and SLC25A25 have known roles in regulating mitochondrial energy supply. PDK4 phosphorylates and deactivates the pyruvate dehydrogenase complex, switching the primary ATP-generating oxidative reactions in the mitochondria from relying on glucose (via glycolysis) to fatty acid, and the expression of PDK4 pushes the cell’s energy supply from relying on the energy-poor glycolytic to the energy-rich fatty acid β-oxidation. SLC25A25 (and its related gene, SLC25A23, also a target of miR-33) is a member of the SLC25 mitochondrial solute carrier protein family. Both SLC25A25 and SLC25A23 are nuclear encoded ATP-Mg2+/Pi carriers that drive the reversible exchange of substrates, such as ATP-Mg2+, Pi, ATP, and ADP across the mitochondrial inner membrane, tightly regulating mitochondrial metabolic pathways. However, like PDK4, the exact role of SLC25A25 in macrophages is unclear. Here, we present the first evidence of that miR-33 controls PDK4 and SLC25A25 in macrophages, which may potentially regulate mitochondrial respiration, ATP production and cholesterol efflux. In agreement with its known ability to regulate fatty acid oxidation genes our data suggest that anti-miR33 therapy targets additional macropage energy metabolism pathways to redirect energy utilization and production to fatty acid oxidation pathways, contributing to the increased energy demand during the energy-intensive process of macrophage cholesterol efflux. Although it is difficult to decipher the specific contribution of any 1 of the miR-33 target genes on mitochondrial output and cholesterol efflux, miRNAs are designed to work on entire pathways and thus is likely not because of a single target gene isolation. Indeed, our data suggest that inhibition of endogenous miR-33 is able to augment mitochondrial activity via direct derepression of target genes (PGC-1α, PDK4, SLC25A25) and indirect activation of other key mitochondrial genes (ie, NRF1, OXPHOS), cumulatively resulting in increased ATP production and, acting in concert with the central miR-33 target gene ABCA1, enhancing overall cholesterol efflux.

Despite the excitement for HDL-C raising agents as a means to reduce atherosclerosis, their clinical use to treat vascular disease has been called into question. However, agents that promote cholesterol efflux in lesional macrophages are believed to hold tremendous promise, yet no therapies exist that can specifically enhance macrophage efflux and RCT. We and others have previously shown that miR-33 controls the expression of ABCA1, the terminal step in cholesterol efflux to apoA1 and the formation of HDL, making the inhibition of miR-33 an exciting therapeutic strategy. Indeed, in most preclinical models, blocking miR-33 activity results in a decrease in lesion size, with an accompanying increase in HDL-C. We show that anti-miR33 treatment of atherosclerotic ApoE−/− mice can reduce atherosclerotic lesion burden even in the absence of increased HDL-C. This corresponds to decreased lipid content in lesions, and increased expression of the potent mitochondrial regulators Pgc-1α and Pdk4. In plaque macrophages from similarly treated Ldlr−/− mice, anti-miR33 also results in derepression of mitochondrial genes and a robust increase in Nrf1 expression, indicating that anti-miR33 promotes mitochondrial biogenesis in vivo in the plaque. The regulation of mitochondrial energy metabolic pathways corresponds to an increase in cellular respiration and ATP production in vitro, and a trend toward increases aortic ATP production in vivo, all of which work in concert with the known roles of anti-miR33 in promoting macrophage cholesterol efflux via derepression of ABCA1. Despite recent evidence suggests that long-term inhibition of miR-33 leads to increases in VLDL cholesterol and can promote obesity, we did not observe any changes in apoB-containing lipoproteins (VLDL and LDL) or body weight with anti-miR33 treatment compared with controls in this 8-week study. Thus, in the setting of short-term therapeutic...
inhibition, anti-miR-33 can promote overall macrophage mitochondrial metabolism to enhance efflux pathways, protecting from the development of atherosclerosis. This occurs independently from any effects on circulating lipoproteins, and could have important therapeutic value for the treatment of cardiovascular disease. In the clinical setting, a short-term regimen of anti-miR-33 treatment in combination with other established lipid-lowering agents (ie, statins) could promote significant cholesterol removal from advanced lesions, promoting the stabilization and regression of atherosclerotic disease. This work highlights the potential of miRNA-based therapeutics as agents that promote cholesterol efflux via targeting novel mitochondrial genetic networks to reduce atherosclerotic lesion burden.

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Disclosures

None.

References

The cholesterol efflux capacity of HDL is an important metric of
• In plaques from patients with atherosclerosis, miR-33a/b levels are
To promote cholesterol efflux, anti-miR33 augments ATP production
miR-33 controls a network of mitochondrial genes and coordinates
• Although miR-33 antagonism alters HDL levels, it is becoming increas-
MicroRNA-33 (miR-33) has been identified as a post-transcriptional
handschin & moore kJ. microRNAs
• The complete repertoire of miR-33 target genes is unknown.
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Macrophage Mitochondrial Energy Status Regulates Cholesterol Efflux and Is Enhanced by Anti-miR33 in Atherosclerosis

Denuja Karunakaran, A. Brianne Thrush, My-Anh Nguyen, Laura Richards, Michele Geoffrion, Ragnnath Singaravelu, Eleni Ramphos, Prakriti Shangari, Mireille Ouimet, John P. Pezacki, Kathryn J. Moore, Ljubica Perisic, Lars Maegdefessel, Ulf Hedin, Mary-Ellen Harper and Katey J. Rayner

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Materials & Methods

Mice

Pdk4−/− mice and Slc25a25−/− were generously provided by Dr. Robert Harris and Dr. Randy Mynatt respectively. Pgc-1α−/− and C57BL6 mice were purchased from Jackson Laboratories.

Cell culture & transfections

Mouse peritoneal macrophages were isolated by peritoneal lavage with 2 x 5mL sterile PBS from wild-type mice that had intraperitoneal injection of 3% thioglycollate 4 days prior. Cells were centrifuged, resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P-S) and seeded at 1x10⁶ cells/well in a 24-well plate or 3-4x10⁶ cells/well in a 6-well plate. Bone-marrow derived macrophages (BMDMs) were isolated from femurs of adult mice and differentiated into macrophages using DMEM supplemented with 20% L929 conditioned media plus 10% FBS, 1%P-S for 7-10 days. THP-1 cells were maintained in RPMI 1640 media supplemented with 10% FBS, 1% P-S, 1% MEM sodium pyruvate, 40nM β-mercaptoethanol and 2g/L glucose. THP-1 cells were differentiated with 100nM phorbol-12-myristate acetate (PMA) for 72 h prior to transfections. Transfections of 2′F/MOE anti-miR33 (TGCAATGCAACTACAATGCAC) oligonucleotide or mismatch control anti-miR (TCCAATCCACTTCAATCATC) (Regulus Therapeutics) were performed using Lipofectamine RNAiMAX as previously. Briefly, cells were transfected with 120nM control anti-miR or anti-miR33 in the presence of Lipofectamine RNAiMAX in OPTIMEM media overnight. The next day, same volume of DMEM media containing 20% FBS was added and cells were either harvested at 48 or 72h post-transfection.

3′-UTR luciferase reporter assays
These assays were performed as previously described\(^\text{14}\). In short, HEK293 cells were transfected with the full-length 3'-UTR of PGC-1\(\alpha\), PDK4 or SLC25A25 (SwitchGear Genomics) downstream of renilla luciferase in the presence of either miR-33 mimic or control miRNA mimic and co-transfected with a pGL3-Basic control plasmid (firefly luciferase). After 24 h, cells were lysed and luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to firefly luciferase activity for transfection efficiency. Site-directed mutagenesis was performed according to Liu et al (BMC Biotechnology, 2008), where point mutations were made within the seed sequences of the miR-33 binding sites for all 3'UTR constructs. Briefly, using a modified protocol from the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) primers were designed to contain extended non-overlapping sequences at the 3' end, which has a melting temperature 5 to 10°C higher than that of primer-primer complementary sequences at the 5’ end (Tm pp). The PCR cycles were initiated at 95°C for 5 minutes, followed by 25 amplification cycles. Each cycle includes 95°C for 1 minute, (Tm no -5°C) for 1 minute and 72°C for 15 minutes. The cycles were finished with an annealing step at (Tm pp -5°C) for 1 minute and an extension step at 72°C for 30 minutes. The remaining steps were performed according to the instructions of the QuickChange site-directed mutagenesis kit. Mutations were verified by sequencing, and mutated nucleotides are shown in Supplemental Figure 1 as underlined.

**RNA isolation and quantitative real-time PCR**

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.
Mitochondrial DNA copy number was measured as described, using either mouse or human primers to mitochondrial tRNA$^{\text{Leu}}$.

**Western blot analysis**

Cells were lysed in ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Roche) for 30 min before centrifugation to remove triton-insoluble pellet. Samples normalized with equal protein concentration (50-70 µg) were subjected to SDS-PAGE and western blot analysis$^{14,23}$. PVDF membranes were incubated with the following primary antibodies overnight: ABCA1 (Novus; 1:1000), PGC-1α (Calbiochem, clone 4C1.3, 1:250), SLC25A25 (Novus; 1:250), PDK4 (Novus, 1:500), OXPHOS (Abcam; 1:250), NRF1 (Abcam; 1:1000), TFAM (Aviva Systems Biology; 1:1000), tubulin (Sigma-T6074; 1:1000) or HSP90 (Santa Cruz Biotechnology, 1:1000). Goat anti-mouse (1:2500) or anti-rabbit (1:5000) IRDye® secondary antibodies (Rockland) were utilized. The protein bands were visualized using Odyssey Infrared Imaging System (LI-COR Biotechnology).

**Seahorse mitochondrial respiration assays**

The Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) was utilized to determine the oxygen consumption rate. THP-1 cells were seeded at 1x10$^6$ cells/mL and differentiated with 100nM PMA for 72hrs in 24-well Seahorse plates. Cells were then transfected with control-anti-miR or anti-miR33 for 48 h. On the day of the experiment, cells were pre-incubated for 30 min at 37°C in low-glucose media in situ oxygen consumption rate were determined using fluorimetric sensors. Basal respiration was first determined by measuring OCR for 2 min at 3 intervals in triplicates. Next, cells were exposed to oligomycin or FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) or antimycin A for 30 min prior to measurement
of OCR, facilitating the determination of proton leak, maximal respiration and non-mitochondrial oxygen consumption. OCR were corrected for total protein and expressed relative to control-anti-miR basal OCR.

**Cholesterol Efflux**

Cholesterol efflux experiments were performed as previously described\textsuperscript{14}. Differentiated THP-1 or peritoneal macrophage cells were transfected with control-anti-miR or anti-miR33 for 24h, followed by cholesterol-loading with 37.5\textmu g/mL acetylated LDL (Biomedical Technologies) and labeled with 0.5 mCi/mL \textsuperscript{3}H cholesterol (Perkin Elmer) for an additional 24 h. Cells were washed extensively with PBS and equilibrated in 2mg/mL fatty acid-free BSA in FBS-free RPMI media for 1 h prior to being treated with 25\textmu g/mL apoA1 (or BSA alone, where indicated) overnight. Medium and cellular \textsuperscript{3}H were counted and expressed as a percentage of total cellular \textsuperscript{3}H cholesterol content.

**ATP assays**

Peritoneal macrophages were transfected with 100nM control mimic, miR-33 mimic, control anti-miR or anti-miR33 for 48 h and then treated with 50 \textmu g/mL apoA1 for 6 h. Cells were washed extensively with PBS and lysed with ATP lysis buffer. Intracellular ATP levels were determined using the ATP bioluminescence assay kit HS II (Roche) as per manufacturer's instructions and normalized to total protein content.

**Animal Studies**

Experiments were performed in accordance with the Animal Care and Use Committee, University of Ottawa, Canada. Eight week old Apoe\textsuperscript{−/−} mice were simultaneously fed an adjusted calories diet (21% fat; 0.2% cholesterol Harlan
Karunakaran, CIRCRES/2014/305624D

Teklad) and injected with either control-anti-miR or anti-miR33 as previously established. Briefly, mice received 2 subcutaneous injections of 5mg/kg anti-miR for the first week, followed by weekly injections of 5mg/kg for 8 weeks prior to sacrifice, where they were subjected to isoflurane anesthetic and exsanguination by cardiac puncture. Mice were perfused with saline and aortic roots were embedded in OCT medium and frozen. Aortas were sectioned (8µm) and stained with hematoxylin and eosin for lesion area quantification, and a minimum of 10 sections per animal were measured across the length of the entire aortic root. Total plasma cholesterol, LDL and HDL levels were measured using the Cholesterol E Assay kit (Wako) as per manufacturer’s instructions. Coherent anti-Stokes Raman scattering (CARS), two-photon fluorescence (TPF) and second harmonic generation (SHG) microscopy was used to perform label-free visualization of lipid droplets, collagen and elastin in aortic sinus sections\textsuperscript{4, 6}. Immunohistochemistry was performed using antibodies against CD68 (rat anti-mouse, 1:100, ABD Serotec), PGC-1α and PDK4 (rabbit anti-mouse, 1:200, Abcam).

**Human atherosclerotic lesion analysis**

Human arterial and plaque samples were part of the Biobank of Karolinska Endarterectomy (BiKE) at the Centre for Molecular Medicine, Karolinska Institute\textsuperscript{6, 7}. Atherosclerotic plaques and clinical data were obtained from patients undergoing surgery for stable or unstable carotid stenosis. Control normal arteries (undiseased macroscopically atherosclerosis free-arteries, iliac and one aorta) were obtained from organ donors without any current or history of cardiovascular disease. All samples are collected with informed consent from patients, organ donors or their guardians. The study is approved by the Ethical Committee of Northern Stockholm. Details regarding clinical variables have been published previously\textsuperscript{6, 7}. Plaque tissues were ruptured with a Tissue Rupturer/Homogenizer (Omni Inc) and RNA isolated. Gene
expression profiles were analyzed by Affymetrix HG-U133 plus 2.0 Genechip microarrays in n=127 patient plaque tissues and n=10 normal arteries. Robust multi-array average (RMA) normalization and correction for batch effect was performed and processed gene expression data was returned in log2-scale. Here, Student’s T-test was used for statistical analyses of microarray data. For genes with multiple probe sets, probes with the highest sensitivity and specificity according to GeneAnnot were chosen. miR-33a/b levels were analyzed by Taqman MicroRNA assays (Life Technologies) on n=20 atherosclerotic plaque samples and n=5 control arteries. Statistical analyses were done with the GraphPad Prism 6 software and a p-value <0.05 was considered significant.

Bioinformatics
To identify miR-33 target genes, we first employed 9 miRNA search tools, including DIANAmT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR5, PITA, RNA22, Targetscan. As many of these prediction algorithms yield numerous false positives, we only selected miR-33 target genes that were predicted by at least 5 search tools. From that, a total of 504 genes were identified. We investigated the functional interaction between the identified miR-33 target genes (504) and mitochondrial genes using String-db and only considering the functional interactions with confidence score > 0.4. We then used Cytoscape software to display functional interaction in a network format, which is displayed in Figure 1C.

Mitochondrial PCR Pathway Array Analysis
The purity and integrity of RNA isolated from peritoneal macrophages transfected with control anti-miR or anti-miR33 was confirmed using the Agilent Bioanalyzer (Agilent Technologies) as per manufacturer’s instructions. A total of 250 ng was reverse transcribed with the RT² First Strand kit (Qiagen) and used for the RT² mouse mitochondrial Profiler PCR Array kit (Qiagen) as per manufacturer’s
instructions. Using the manufacturer's PCR Array Data Analysis software (www.SABiosciences.com/pcrarraydataanalysis.php), average data was normalized and used to determine the $\Delta \Delta C_T$. A full table of genes found on this PCR array can be found at http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-087A.html as well as in Supplemental Table I.

**Laser capture microdissection**

Laser capture microdissection (LCM) was performed on *Ldlr/-* mice treated with 10mg/kg control or anti-miR33 and fed a high-fat diet (21% fat; 0.2% cholesterol, Harlan Teklad) for 8 weeks, as previously described (Rayner JCI 2011). Isolated and amplified RNA was analyzed by quantitative PCR using SYBR green (BioRad) and primers as indicated.

**Statistics**

Data shown is either mean ± SD or mean ± SEM of experiments performed in triplicate, and is representative of at least 3 independent experiments, and is indicated in the corresponding figure legend. Comparison between control anti-miR and anti-miR33 treatments was made using Student’s t-test ($p \leq 0.05$) or comparison between groups by One-way ANOVA ($p \leq 0.05$) using Prism GraphPad.
Supplemental Figure Legends

**Supplemental Figure 1:** (A) Alignment of the miR33 binding sites in the 3’UTR of *PPARGC1A*, (3 sites) SLC25A25 (1 site) or PDK4 (1 site) and the species conservation for each miR-33 binding site. Point mutations used in Figure 2A are indicated by the underline (A→T, T→A). miRNA binding sites were aligned according to the Miranda Prediction algorithm and species conservation was determined using PicTar and Ensembl Genome Browser.

**Supplemental Figure 2:** Indirect regulation of mitochondrial gene expression by miR-33. (A) Tfam protein expression in peritoneal macrophages transfected with 120nM anti-miR33 for 48h. (B) Mitochondria RT2 Profiler PCR array was performed using RNA isolated from peritoneal macrophages treated with control anti-miR or anti-miR33 and normalized to housekeeping genes using the ΔΔCt method. Graph depicts gene expression of control anti-miR (x-axis) versus anti-miR33 (y-axis). Blue and red symbols represent the up- and down-regulated genes (greater than 2-fold) in anti-miR33 transfected cells compared to controls, respectively. Statistically significant differences are denoted by an * (p≤0.05).

**Supplemental Figure 3:** ATP levels and novel miR-33 mitochondrial target gene expression are regulated in Apoe<sup>−/−</sup> mice administered anti-miR33 therapy. (A) Aortic arches were dissected and cleaned from Apoe<sup>−/−</sup> mice administered anti-miR33 therapy (shown in Figure 5). The aortas were lysed in ATP lysis buffer using the Bullet Blender Tissue Homogenizer (Next Advance) and ATP levels were determined as per manufacturer’s instructions (Roche) and normalized to total lesion area. Graph shows data generated from n = 6 mice/group. (B) Plaque macrophages were isolated using LCM from Ldlr<sup>−/−</sup> mice administered anti-miR33 therapy and mRNA expression of select novel mitochondrial genes were determined. Data are
representative of n = 4 animals/group and were analyzed using a t-test (*p≤0.05, **p<0.001). (C) Representative images of immunohistochemistry staining for CD68 and IgG control in aortic sinus sections of Apoe<sup>-/-</sup> mice treated with control-anti-miR or anti-miR33 (shown in Figure 5E).

**Supplemental Table I: List of mitochondrial genes profiled in Figure 2B.**

The full list of genes profiled on the Mitochondrial PCR Array (Qiagen) 

**PPARGC1A**

3' acGUUACGUUGAU-GUUACGUg 5' miR-33a

1147:5' agCUAUGC-ACUGUAAAUGCAg 3' PPARGC1A

3' acguuacguugauGUUACGUg 5' miR-33a

2312:5' uucacccccagccCAAUGCAg 3' PPARGC1A

3' acguuAGUUGAUGUUACGUg 5' miR-33a

2537:5' uguucUGUAGGUUAAAUGCAg 3' PPARGC1A

**SLC25A25**

3' acgGUUACGUUGAU-GUUACGUg 5' miR-33a

1666:5' uagGAUGCAAAGAUCAAUGCAa 3' SLC25A25

3' acguuACGUUGAUGUUACGUg 5' miR-33a

2740      2750      2760      2770      2780      2790

**PDK4**

3' acGUUACGUUGAU-GUUACGUg 5' miR-33a

1170:5' augAAUGACACCUGAAUGCAu 3' PDK4

| Human AAGATGGATTTCACCCCCAGGCCAATGCAGCTAATTTTGATAGCTGCATTCATTT |
| Chimp AAGATGGATTTCACCCCCAGGCCAATGCAGCTAATTTTGATAGCTGCATTCATTT |
| Mouse AAAATGGGTTTCACCCCCAGGCCAATGCAGCTAATTTTGACAGCTGCATTCATTT |
| Rat AAAATGGGTTTCACCCCCAGGCCAATGCAGCTAATTTTGACAGCTGCATTCATTT |
| Dog AAAACGGATTTCACCCCCAGGCCAATGCAGCTAATTTTTGAGCTGCATTCATTT |

2740      2750      2760      2770      2780      2790

**SLC25A25**

3' acgGUUACGUUGAU-GUUACGUg 5' miR-33a

1666:5' uagGAUGCAAAGAUCAAUGCAa 3' SLC25A25

3' acguuACGUUGAUGUUACGUg 5' miR-33a

2740      2750      2760      2770      2780      2790

**PDK4**

3' acGUUACGUUGAU-GUUACGUg 5' miR-33a

1170:5' augAAUGACACCUGAAUGCAu 3' PDK4

| Human AGAGATAATGAATGACACCTGAAATGCAATAT |
| Chimp AGAGCTAATGAATGACACCTGAAATGCAATAT |
| Mouse TTTGCAATGCAAAATGACTCCTGACTGTTTC |
| Rat TTTGCAATGCAAAATGACTGACTGTTTC |
| Chicken TATCAGAACGCAAAAATCAATGCAAAATGACAACTGTCTTTGTA— |
| 2000      2010      2020      2030      2040 |

2740      2750      2760      2770      2780      2790

**SLC25A25**

3' acgGUUACGUUGAU-GUUACGUg 5' miR-33a

1666:5' uagGAUGCAAAGAUCAAUGCAa 3' SLC25A25

3' acguuACGUUGAUGUUACGUg 5' miR-33a

2740      2750      2760      2770      2780      2790

**PDK4**

3' acGUUACGUUGAU-GUUACGUg 5' miR-33a

1170:5' augAAUGACACCUGAAUGCAu 3' PDK4

| Human AGAGATAATGAATGACACCTGAAATGCAATAT |
| Chimp AGAGCTAATGAATGACACCTGAAATGCAATAT |
| Mouse TTTGCAATGCAAAATGACTCCTGACTGTTTC |
| Rat TTTGCAATGCAAAATGACTGACTGTTTC |
| Chicken TATCAGAACGCAAAAATCAATGCAAAATGACAACTGTCTTTGTA— |
| 2000      2010      2020      2030      2040 |

2740      2750      2760      2770      2780      2790
Supplemental Figure II

A

cont anti anti-miR33

Tfam

HSP90

B

Mitochondrial Gene Expression

Anti-miR33 Expression level

Cont anti-miR Expression level

Log 2^(-Ave ΔCt)

Log 2^(-Ave ΔCt)
Aorta ATP content

Supplemental Figure III

Plaque macrophages

C

cont anti-miR  anti-miR33

CD68

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