miR33 Inhibition Overcomes Deleterious Effects of Diabetes Mellitus on Atherosclerosis Plaque Regression in Mice

Emilie Distel,* Tessa J. Barrett,* Kellie Chung, Natasha M. Girgis, Saj Parathath, Christine C. Essau, Andrew J. Murphy, Kathryn J. Moore, Edward A. Fisher

Rationale: Diabetes mellitus increases cardiovascular disease risk in humans and remains elevated despite cholesterol-lowering therapy with statins. Consistent with this, in mouse models, diabetes mellitus impairs atherosclerosis plaque regression after aggressive cholesterol lowering. MicroRNA 33 (miR33) is a key negative regulator of the reverse cholesterol transport factors, ATP-binding cassette transporter A1 and high-density lipoprotein, which suggested that its inhibition may overcome this impairment.

Objective: To assess the effects of miR33 inhibition on atherosclerosis regression in diabetic mice.

Methods and Results: Reversa mice, which are deficient in the low-density lipoprotein receptor and in which hypercholesterolemia is reversed by conditional inactivation of the microsomal triglyceride transfer protein gene, were placed on an atherogenic diet for 16 weeks, then either made diabetic by streptozotocin injection or kept normoglycemic. Lipid-lowering was induced by microsomal triglyceride transfer protein gene inactivation, and mice were treated with anti-miR33 or control oligonucleotides. Although regression was impaired in diabetic mice treated with control oligonucleotides, anti-miR33 treatment decreased plaque macrophage content and inflammatory gene expression in these mice. The decreased macrophage content in anti-miR33 treated diabetic mice was associated with a blunting of hyperglycemia-induced monocytosis and reduced monocyte recruitment to the plaque, which was traced to an inhibition of the proliferation of bone marrow monocyte precursors associated with the upregulation of their Abca1.

Conclusions: miR33 inhibition overcomes deleterious effects of diabetes mellitus in atherosclerosis regression in mice, which suggests a therapeutic strategy in diabetic patients, who remain at elevated cardiovascular disease risk, despite plasma cholesterol lowering. (Circ Res. 2014;115:759-769.)

Key Words: atherosclerosis ■ diabetes mellitus ■ high-density lipoprotein ■ microRNA ■ regression

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In nondiabetic atherosclerotic mice, increasing the plasma level of HDL by providing apoA1 (by transgenesis or infusion) or by anti-miR33 treatment promotes plaque regression with an enrichment of macrophages expressing anti-inflammatory M2 markers. An important component of this beneficial effect of HDL is ascribed to its ability to promote

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Diabetes mellitus increases the clinical risk of cardiovascular morbidity and mortality. This risk remains elevated with conventional low-density lipoprotein (LDL) cholesterol–lowering therapies, such as statins. In the Fenofibrate Intervention and Event Lowering in Diabetes study, type 2 diabetic patients with mixed dyslipidemia (high triglycerides and low high-density lipoprotein cholesterol [HDL-C]) showed the most benefit of combination fenofibrate/statin therapy, suggesting the importance of raising HDL-C in these patients. In mouse models, diabetes mellitus dramatically accelerates the progression of atherosclerosis, and we have shown that it inhibits atherosclerosis regression after aggressive LDL reduction. This was caused by increased monocyte infiltration and macrophage burden in the plaque compared with nondiabetic mice. The plaque macrophages from diabetic mice also exhibited an M1 inflammatory phenotype, unlike the M2 inflammation-resolving/tissue remodeling macrophages we have typically found in regressing plaques in nondiabetic mice. These impairments were largely driven by hyperglycemia, as glucose reduction improved the response to lipid lowering. Notably, this restored regression was associated with reduced circulating monocyte levels as a result of the loss of the proliferative stimulus that hyperglycemia has on monocyte bone marrow (BM) progenitor cells.

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette transporter G1</td>
</tr>
<tr>
<td>ASO</td>
<td>Anti-sense oligonucleotides</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
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<tr>
<td>GMP</td>
<td>Granulocyte–macrophage progenitor</td>
</tr>
<tr>
<td>LSK</td>
<td>Lin−, Sac1+, ckit+ stem cells</td>
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<tr>
<td>miR33</td>
<td>microRNA 33</td>
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<tr>
<td>Mttp</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>plpC</td>
<td>Polynucleosin polycytidylic RNA</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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cholesterol efflux from macrophages in the plaque. This process is mediated by the ATP-binding cassette (ABC) transporters, ABC transporter A1 (ABCA1) and ABC transporter G1 (ABCG1), which provide cholesterol to lipid-poor and spherical HDL particles, respectively. In the liver, ABCA1 also provides phospholipids to newly secreted apoA1 to generate nascent lipid-poor HDL particles. We have previously shown that Abca1 and Abcg1 mRNAs are both targets of miR33, and that treatment of Ldlr<sup>−/−</sup> mice with anti-miR33 upregulated ABCA1 in the liver and macrophages and resulted in plaque regression. Taken with our findings that both Abca1 and Abcg1 are downregulated by hyperglycemia, which may contribute to impaired regression of atherosclerosis in diabetic mice after plasma lipid lowering, this made anti-miR33 treatment an attractive candidate for overcoming this impairment.

To test this hypothesis, we turned to the Reversa mouse, which we have reported to be a model of atherosclerosis regression. To create a regression environment after plaques develop, the hyperlipidemia of the LDL receptor knockout (Ldlr<sup>−/−</sup>) mouse is suppressed by the conditional inactivation of the microsomal triglyceride transfer protein gene (Mttp), which shuts down the secretion by the liver of VLDL (the precursor of LDL). As clinical studies show that atherosclerotic plaque formation starts before the onset of diabetes mellitus, our protocol was to first establish atherosclerosis by placing mice on a Western diet for 16 weeks and then inducing diabetes mellitus with streptozotocin (STZ) in a subset of mice. We then tested the effects of anti-miR33 (or control oligonucleotides) in both normoglycemic and diabetic mice after plasma lipid normalization.

Consistent with our previous results, regression was impaired by diabetes mellitus in control oligonucleotide-treated mice, but inhibition of miR33 caused a significant decrease in the content and inflammatory state of plaque macrophages. These beneficial effects of anti-miR33 therapy were associated with changes in the population of circulating monocytes and their BM precursors, as will be presented. Overall, the results suggest that targeting of miR33 may improve cardiovascular risk reduction by statins and other lipid-lowering therapies in patients with hyperglycemia.

**Methods**

**Animals**

Reversa mice (Ldlr<sup>−/−</sup>; ApoB<sup>1000k</sup>; Mttp<sup>60</sup>; Mx1-Cre<sup>−/−</sup>) and C57Bl6J were cared for in accordance with the National Institutes of Health guidelines and the New York University Institutional Animal Care and Use Committee (Protocol 090908). Reversa pups were weaned at 4 to 5 weeks and placed on a western diet (21% [wt/wt] fat, 0.15% cholesterol [Research Dyets]) to allow the development of atherosclerotic plaques. After 15 weeks, mice were injected IP daily with STZ (50 mg/kg, Sigma-Aldrich) or citrate buffer for 5 days to induce diabetes mellitus or serve as a control. One week later, all mice were switched to a chow diet. After the diet switch Reversa mice were injected IP with polyinosinic polycytidylic RNA (Sigma-Aldrich 15 mg/kg) every other day for a total of 4 injections. Mice were then treated subcutaneously 4x with the antisense oligonucleotides (ASO) anti-miR33 or the ASO control (Regulus Therapeutics, 10 mg/kg) as previously described. Regulus Therapeutics provided 2′fluoro/methoxyethyl (2′F/MOE)–modified, phosphorothioate backbone–modified anti-miR33 (TCCATGCAAATCTACATGCAC) and mismatch control (TCCATGCAAATCTCAATCATC) anti-miR. At euthenize, mice were anesthetized with xylazine/ketamine, and blood was collected via cardiac puncture for plasma analyses. Mice were perfused with PBS, followed by 10% sucrose in PBS. Aortic roots were embedded in optimal cutting temperature compound medium and frozen immediately, and tissues were snap-frozen under liquid nitrogen and stored at −80°C until further use.

**Plasma Lipoprotein Analyses**

Total cholesterol, HDL-C, and triglyceride concentrations were measured using colorimetric assays (all kits from Wako Diagnostics, Richmond, VA). Glycemia was measured after 4 hours of fast with a blood glucometer (Freestyle lite, Roche).

**Histochemical Analyses**

Aortic roots were harvested, frozen in optimal cutting temperature compound, and serial-sectioned at a thickness of 6 μm onto glass slides. For immunostaining of CD68 (macrophage marker), slides were fixed in 100% acetone and exposed to primary anti-CD68 antibody (Serotec), followed by biotinylated secondary antibody (Vector Laboratories), with visualization using a Vectastain ABC kit (Vector Laboratories). Microscopic images of aortic root sections were digitized, and morphometric measurements were performed using Image Pro Plus software (Micro Optical Solutions).

For collagen content, tissues were stained with picrosinus red and quantified with Image Pro Plus software using polarizing light microscopy.

**Necrosis Analysis**

Necrosis, an indicator of the effectiveness of efferocytosis, was quantified in aortic roots from Reversa mice by measuring the acellular areas of plaques with Image Pro Plus software.

**Apoptosis Analysis**

Apoptosis was analyzed in aortic roots from Reversa mice by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using an in situ cell detection kit (Roche Diagnostics). Nuclei were labeled with Vectashield Mounting media with 4,6-diamidino-2-phenylindole (DAPI; Vector laboratories). Only TUNEL-positive cells that colocalized with DAPI-stained nuclei were considered apoptotic.

**Laser Capture Microdissection**

CD68<sup>−</sup> cells were isolated from atherosclerotic plaques by laser capture microdissection. All laser capture microdissection procedures were performed under RNase-free conditions. Aortic root sections were stained with hematoxylin-eosin and captured from 36 frozen sections. After laser capture microdissection, RNA was isolated using the PicoPure Kit (Molecular Devices), and quality and quantity were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was converted to cDNA and amplified using the WT-Ovation Pico RNA Amplification Kit (NuGEN). Real-time PCR was performed with 5 ng of amplified cDNA using the ABI PRISM 7300 sequence detection system (Applied Biosystems). Gene expression was assessed using the ΔΔCt calculation method.
Monocyte Tracking
Monocytes were labeled as previously described. Briefly, 1 μm Fluoresbrite fluorescein isothiocyanate-dyed (YG) plain microspheres (Polysciences Inc.) were diluted in PBS (1:4), and 250 μL of the solution was injected into the retro-orbital vein of mice to label circulating Ly6C<sup>+</sup> monocytes. For the egress study, beads were injected at week 14 before any treatment. For the recruitment study, beads were injected 24 hours before harvesting. For both protocols, bead labeling efficiency was assessed by flow cytometry 24 hours after bead injection. 

Flow Cytometry
Blood Leukocytes
Blood was collected via retro-orbital bleeding with EDTA-coated capillaries. Red blood cells were lysed with RBL buffer (Sigma Aldrich) and blocking achieved with antimouse CD16/CD32 (eBioscience). Monocytes were identified by staining with PE antimouse CD115 (Biolegend) and APC antimouse Ly-6G/Ly-6C (Biolegend). Neutrophils were identified as CD45<sup>+</sup>CD115<sup>+</sup>Ly6-C/G<sup>+</sup> cells. Flow cytometry was performed using an LSRII analyzer.

Hematopoietic Stem and Progenitor Cells
Hematopoietic stem and progenitor cells were analyzed by flow cytometry as previously described. Briefly, BM was harvested from femurs and tibias, and red blood cells were lysed with BD Pharm Lyse (BD Biosciences). Cells were incubated with a cocktail of antibodies against lineage-committed (lin-) cells (B220, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly6-C/Ly6-G, and all fluorescein isothiocyanate) and markers to identify the stem and progenitor cells. Flow cytometry was performed using a LSRII analyzer.

Results
Anti-miR33 Increases HDL-C and Target Gene Expression in Livers of Diabetic Mice
To assess the effects of miR33 inhibition on atherosclerosis regression in a hyperglycemic environment, Reversa mice were fed a western diet (21% fat and 0.15% cholesterol) for 16 weeks to allow complex plaques to develop (see Figure 1A for a schematic depiction of the overall protocol). At 15 weeks, mice were given daily (IP) injections for 5 consecutive days with citrate buffer or 50 mg/kg STZ to induce diabetes mellitus. At 16 weeks, all mice were switched to a standard chow diet (6% fat) and injected IP every other day (4 injections total) with polynosinic polycytidylic RNA (pIpC) 15 mg/kg every other day for a total of 4 injections to initiate lipid lowering. Mice then received weekly (i.e., 4 weeks post the final pIpC injection). Hepatic expression of Abca1 (B), Abcg1 (C), Cpt1 (D), and Hmgcr (E) was determined by qRT-PCR using either control anti-miR or anti-miR33 2′F/MOE oligonucleotides (10 mg/kg) for 4 weeks, before euthenizing (i.e., 4 weeks post the final pIpC injection).
The efficacy of miR33 inhibition was evaluated by measuring expression of known target mRNAs in the liver. As previously reported, anti-miR33 treatment significantly increased Abca1 mRNA expression (P ≤ 0.05) in normoglycemic mice (Figure 1B). Furthermore, although Abca1 mRNA was reduced by almost 50% (P ≤ 0.05) in the livers of hyperglycemic mice, anti-miR33 treatment restored Abca1 expression to the level seen in normoglycemic anti–miR33 treated mice (Figure 1B). Abcg1 expression in the livers of diabetic mice was modestly reduced compared with normoglycemic mice (Figure 1C). Anti-miR33 treatment increased Abcg1 mRNA levels by 1.5-fold (P ≤ 0.05) in the diabetic mice, whereas no effect of anti-miR33 was observed in normoglycemic mice. Levels of Cpt1 mRNA, another miR33 target gene, were also increased by anti-miR33 treatment in both normo- and hyperglycemic mice (P ≤ 0.05; Figure 1D). Non-miR33 target genes, such as HMG-CoA reductase, were not affected by anti-miR33 treatment (Figure 1E), although its expression was increased in the diabetic mice (P ≤ 0.05; Figure 1E), as previously reported.

Anti-miR33 reduces plaque macrophage content in diabetic mice after plasma lipid lowering

Anti-miR33 treatment has been shown to promote plaque regression in Ldlr−/− mice fed western diet for 16 weeks and then switched to chow for the duration of treatment (4 weeks). This beneficial effect of anti-miR33 was associated with increased HDL-C and reverse cholesterol transport, presumably as a consequence of greater Abca1 expression in liver and plaque macrophages. Consistent with our previous findings, we observed that diabetes mellitus impaired plaque regression after lowering of plasma cholesterol as reflected by the decrease in plaque macrophage content (CD68+ cells) in the normoglycemic group (P ≤ 0.001), but not in the diabetic group (Figure 2). Anti-miR33 treatment restored plaque regression in the diabetic group and reduced macrophage content to the same level observed in normoglycemic mice. Interestingly, anti-miR33 treatment did not further reduce plaque macrophage content in the nondiabetic group, suggesting that, in the absence of hyperglycemia, the reduction in plasma cholesterol level alone was sufficient to drive this process, as shown by the >50% decrease of plaque CD68 content compared with baseline (Figure 2; baseline 52% versus Regression+anti-miR33 26±±2.2%; P ≤ 0.05). As we have observed previously, reduction in macrophage content did not result in changes in total plaque area (Online Figure I), which has been shown to be from increases in plaque collagen content in the regressing plaque. Consistent with this, anti-miR33 treatment increased the plaque collagen content in the diabetic group, restoring it to the same level observed in normoglycemic mice (Figure 2B). Together, these data suggest that anti-miR33 treatment promotes a remodeling of the diabetic plaque toward a more stable-appearing phenotype.

Anti-miR33 treatment favorably alters plaque macrophage inflammatory phenotype in diabetic mice after plasma lipid lowering

We next assessed the effects of anti-miR33 on the phenotype of diabetic plaque macrophages by analyzing mRNA expression of laser-captured cells. Consistent with our previous findings, macrophages in the plaques from control anti-miR-treated diabetic mice showed a pattern of gene expression predominantly of the inflammatory M1 phenotype (eg, increased Il1β and Tnfα) and also had reduced expression of Abca1 (Table 2). Notably, anti-miR33 treatment of diabetic mice reduced mRNAs of M1 macrophage-related genes (Il1β, Tnfα, and Nos2) and increased those of anti-inflammatory M2 macrophage-related genes (Ym1 and Cd206). Furthermore, treatment with anti-miR33 restored levels of macrophage Abca1 mRNA in the diabetic group to that observed in normoglycemic mice (Table 2). These findings suggest that anti-miR33 treatment dampened the persistent inflammation in the plaque associated with hyperglycemia.

Anti-miR33 treatment reduces plaque macrophage recruitment, but does not change macrophage egress

A decrease in macrophage content in the regressing plaque could be because of reduced recruitment of monocytes to the plaque, loss of macrophages through egress (regulated by retention and chemotaxis), or macrophage death. To investigate the mechanisms underlying the reduction in macrophages in diabetic plaques by anti-miR33 treatment, we used an in vivo bead-labeling technique to assess monocyte recruitment and macrophage egress. To measure the recruitment of
monocytes, mice were injected with fluorescent latex beads 24 hours before the harvesting of aortic roots and the number of beads in the plaques counted. Monocyte labeling was not affected by either diabetes mellitus or anti-miR treatment, indicating that neither condition had a significant effect on the phagocytic ability of circulating monocytes (Online Figure II). Hyperglycemia was associated with a 60% increase in monocyte recruitment to the plaque \( (P \leq 0.01; \text{Figure 3B}) \), consistent with our previous observations in another model of regression.\(^5\) Notably, anti-miR33 treatment prevented this diabetes mellitus–induced increased recruitment of monocytes \( (P \leq 0.01; \text{Figure 3B}) \). Although these results could be attributed in large part to changes in the circulating number of monocytes (see below), studies in vitro suggested that anti-miR33 treatment could also have caused an intrinsic decrease in monocyte/macrophage chemotaxis in a hyperglycemic environment (Online Figure IIIA and IIIB).\(^{24}\)

To study macrophage egress, we injected fluorescent beads before the induction of diabetes mellitus or anti-miR treatment (week 14; Figure 3A). One week after bead injection, a group of mice was euthanized to obtain baseline measurements of the content of labeled macrophages (ie, CD68\(^+\) cells colocalized with beads) in aortic root plaques. As in the experiment described above, additional mice were then treated with STZ, polyinosinic polycytidylic, and anti-miR (control or miR33; Figure 3A), and the beads remaining in the aortic root plaques when euthanized were quantified. Reversal of hyperlipidemia was associated with a reduction in plaque

\[ \text{Figure 2. } \alpha\text{-miR33 treatment restores regression in diabetic mice. Aortic roots from baseline and the regression groups were sectioned, fixed, and stained for CD68 (A) and collagen (B). Representative pictures of CD68 immunostaining (A, magnification } \times20) \text{ and picrosirius red staining (B, under white and polarized light) of collagen (magnification } \times10) \text{ are shown for each group. The areas of the plaques occupied by CD68}\(^+\text{ cells and collagen (the latter as detected by polarized light) were quantified by Image Pro Plus Software and displayed in the graphs. Results are expressed as the percentage of plaque area. } \ ^{\text{P}} \leq 0.05 \text{ vs baseline, } ^{\text{P}} \leq 0.05, ^{\text{***}} \leq 0.001 \text{ vs con } \alpha\text{-miR normoglycemic; } ^{***} \leq 0.001 \text{ vs } \alpha\text{-miR33 diabetic.} \]

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lipid lowering induced a significant decrease in the level of monocytes (expressed as their percent of circulating leukocytes) compared with baseline (P≤0.001; Figure 4A and 4B). Lipid lowering, however, had no effect on the monocyte levels in the control anti–miR treated diabetic group, likely because of the increased monocyte precursor proliferation via glucose-dependent mechanisms.5 In contrast, anti-miR33 treatment decreased monocyte levels in the diabetic mice to levels comparable to the nondiabetic mice (P≤0.001; Figure 4A and 4B).

In addition to the monocytosis, hyperglycemia induced neutrophilia, as we previously observed,5 but this was not affected by cholesterol lowering in the regression period or by anti-miR33 treatment (Figure 4C).

**Anti-miR33 Treatment Reduces Bone Marrow Monocyte Progenitors in Diabetic Mice**

We previously showed that diabetes mellitus induces myelopoesis in the BM,5 and it has also been shown that the membrane content of cholesterol, as influenced by cholesterol efflux–related genes and plasma cholesterol levels, positively regulates myeloid cell production by increasing BM progenitor cell proliferation.20,28 Because the anti-miR33 treatment is given concurrent with plasma lipid lowering, to study the effects of anti-miR33 on BM progenitors, we used normolipidemic C57BL/6J (WT) mice, to avoid the confounding effects of hypercholesterolemia.20

A group of mice were made diabetic with STZ and treated with control anti–miR or anti–miR33 weekly for 4 weeks as earlier. Treatment with the control anti–miR or anti–miR33 had no effect on blood glucose levels (Figure 5A) or on plasma levels of total cholesterol and triglycerides (Online Figure IVA and IVB). As expected, anti-miR33 significantly increased HDL-C in both diabetic and normoglycemic mice (Figure 5B and Online Figure IVB). Consistent with our previous findings,5 hyperglycemia induced an increase in the abundance and proliferation of both CMP and GMP, without affecting the stem cells (LSK; Figure 5C and 5D). This was accompanied by a reduction of CMP and GMP, but not LSK in the G0 phase, confirming entry into the proliferative cell cycle (Online Figure VA). Importantly, the reduction in the abundance and proliferation of myeloid precursors with anti-miR33 in the diabetic mice was not caused by enhanced apoptosis (Online Figure VB) or senescence (Online Figure VC).

Treatment with anti-miR33 reduced the expansion and proliferation of the CMP and GMP in the diabetic mice (Figures 5C and 6D), indicating that anti-miR33 acts via glucose-independent mechanisms to inhibit the production of monocytes in diabetes mellitus. This effect of anti-miR33 treatment on proliferation of monocyte progenitors, however, was not observed in macrophages in vitro; although there was evidence of their increased proliferation in a hyperglycemic environment, it was not suppressed by anti-miR33 treatment (Online Figure IIC).

**Anti-miR33 Treatment Restores Abca1 and Abcg1 Gene Expression in Bone Marrow Monocyte Progenitor Cells**

ABCA1 and ABCG1 have been shown to play a crucial role in hematopoietic stem cell proliferation.29,30 We
hypothesized that hyperglycemia-induced BM progenitor proliferation was caused by a defect of Abca1/Abcg1 expression in the different progenitor subsets, which would increase the plasma membrane cholesterol content of these cells, stimulating their proliferation.29 Indeed, as observed in the liver and in plaque macrophages, Abca1 and Abcg1 gene expression was decreased by hyperglycemia in the CMP (Figure 6B) and GMP (Figure 6C), without affecting expression in the LSK population (Figure 6A). Notably, anti-miR33 treatment restored Abca1 expression in both the CMP and GMP subset (Figure 6B and 6C). These results suggest that anti-miR33 may prevent monocytosis at the
level of BM progenitor cells, through its upregulation of Abca1 expression in those cells.

**Discussion**

Diabetic patients after cholesterol lowering remain at higher risk of CVD relative to nondiabetics. Consistent with this is our finding in 2 independent mouse models that after cholesterol lowering, atherosclerosis regression is impaired by hyperglycemia. Because anti-miR33 treatment regressed plaques in nondiabetic mice, we reasoned that a similar approach may overcome this impairment. Indeed, our results show that miR33 inhibition confers several atheroprotective benefits in the context of hyperglycemia, chief among them being the reduction in the content and inflammatory state of plaque macrophages. This was found to be because of the ability of anti-miR33 to reduce the monocytopsis associated with diabetes mellitus, which decreased the continued recruitment of monocytes into plaques that occurs even in the face of lipid lowering. These findings, then, expand the atheroprotective mechanisms of anti-miR33 to the diabetic setting, an area of great clinical importance.

Although we observed, as before, egress of macrophages after plasma lipid lowering in Reversa mice, this was not further increased by anti-miR33 treatment in either normo- or hyperglycemic mice. The lack of increase in egress associated with anti-miR33 treatment was consistent with the absence of changes in the gene expression of previously identified factors affecting this pathway, including the chemotactic factor Ccr7 or the retention factor Netrin-1. We also observed no changes in plaque macrophage apoptosis and senescence or in macrophage proliferation in vitro with anti-miR33 treatment, but did find evidence for increased efferocytosis, as judged by necrotic core analysis. Thus, the basis for the reduction in plaque macrophage content in the diabetic mice by anti-miR33 seems to derive primarily from decreased recruitment of monocytes into the plaques and increased clearance of apoptotic cells.

**Figure 5.** α-miR33 treatment decreases bone marrow progenitors in diabetic mice. C57Bl6/J mice were injected with citrate (normoglycemic) or 50 mg/kg streptozotocin (diabetic). Mice were then injected 4× with control α-miR or α-miR33 2′F/MOE oligonucleotides (10 mg/kg). Plasma blood glucose (A) and high-density lipoprotein-cholesterol (B) 4 weeks post treatment. C. Hematopoietic stem and progenitor cell populations in the bone marrow were determined by flow cytometry and expressed as a percentage of cells in the BM. D. Hematopoietic stem and progenitor cell cycle (G,M phase) was assessed by flow cytometry using DAPI (4',6-diamidino-2-phenylindole). Data are expressed as mean±SEM, n≥7/group. *P≤0.05, **P≤0.01 vs con α-miR normoglycemic; ##P≤0.05 vs con α-miR diabetic. CMP indicates common myeloid progenitor; GMP, granulocyte–macrophage progenitor; and LSK, Lin−, Sac1+, ckit+ stem cells.

**Figure 6.** α-miR33 treatment restores Abca1 expression in bone marrow progenitors of diabetic mice. C57Bl6/J mice were injected with citrate (normoglycemic) or 50 mg/kg streptozotocin (diabetic). Mice were then injected 4× with control α-miR or α-miR33 2′F/MOE oligonucleotides (10 mg/kg). Gene expression for Abca1 and Abcg1 was assessed by quantitative polymerase chain reaction in the different bone marrow progenitor subsets LSK (A), CMP (B), and GMP (C), obtained by cell sorting. Data are expressed as mean±SEM, n≥7/group. *P≤0.05, **P≤0.01 vs con α-miR normoglycemic; ##P≤0.05 vs control α-miR diabetic. CMP indicates common myeloid progenitor; GMP, granulocyte–macrophage progenitor; and LSK, Lin−, Sac1+, ckit+ stem cells.
That recruitment continues in a regression environment has been previously established, and the present results represent another demonstration that its reduction can contribute to decreased plaque content of macrophages after cholesterol lowering.

As we recently reported in another mouse model, hyperglycemia induces monocytosis because of BM progenitor proliferation, and this in turn results in increased recruitment of these cells to plaques after reduction in the plasma lipid level. As shown in Figures 4 and 6, anti-miR33 treatment reduced monocytosis and BM progenitor proliferation, despite persistence of the hyperglycemia, thereby preventing the expansion of the pool of recruitable cells by hyperglycemia. These findings are related to the report from Yvan-Charvet et al that links cholesterol efflux-related genes in the BM progenitors to their proliferation and subsequent production of monocytes. Yvan-Charvet et al showed that deficiency of Abca1 and Abcg1, even in the nonhyperlipidemic setting, led to monocytosis and leukocyte infiltration into tissues, presumably through changes in plasma membrane cholesterol that affected responsiveness to proliferative factors. In the present study, hyperglycemia induced a decrease in Abca1 and Abcg1 expression in the BM progenitors (Figure 6), which would be expected to be a proliferative stimulus. Restoration of Abca1 and Abcg1 expression with anti-miR33 treatment, therefore, would be predicted to normalize BM proliferation and the level of circulating monocytes, just as observed. Because monocytosis is also observed in people with diabetes mellitus and correlates with their risk of cardiovascular disease, it is tempting to speculate that anti-miR33 therapy would be effective in that population to reduce this risk, in part, through similar effects in human BM.

We previously reported that when plasma lipids are lowered, in addition to the reduction in the plaque content of macrophages, the inflammatory state of these cells is also lowered in normoglycemic, but not hyperglycemic, mice. As also previously reported, anti-miR33 treatment induces an enrichment in anti-inflammatory M2 macrophages. In the present study, consistent with this, and shown in Table 2, miR33 inhibition reversed this adverse effect of hyperglycemia on plaque macrophage inflammation. There are ≥2 potential bases for this. One could be related to previous findings from the laboratories of Alan Tall and John Parks that showed that free cholesterol enrichment of the plasma membrane, particularly in lipid-rafts in macrophages with ABCA1 deficiency, leads to enhanced inflammatory signaling via toll-like receptors. That the downregulation of Abca1 by hyperglycemia and the restoration of its expression by anti-miR33 treatment, therefore, would be predicted to normalize BM proliferation and the level of circulating monocytes, just as observed. Because monocytosis is also observed in people with diabetes mellitus and correlates with their risk of cardiovascular disease, it is tempting to speculate that anti-miR33 therapy would be effective in that population to reduce this risk, in part, through similar effects in human BM.

The other potential basis, particularly for the enrichment in M2 features, is that direct or indirect targets that promote the M2 phenotypic state of macrophages were derepressed by the anti-miR33 treatment. A previous report has also implicated the eicosanoid pathway in the inflammatory process of diabetes mellitus–accelerated atherosclerosis. In the present study, however, we found no significant evidence to suggest this pathway as a mechanism for the effects of anti-miR33 treatment, as judged by the lack of change in the gene expression of the major related enzymes (Acsl1, Pges1, Ptges; data not shown).

It is interesting to compare the benefits of anti-miR33 treatment in atherosclerosis regression to those in progression. Recent studies report conflicting results. Rotllan et al and Horie et al showed that miR33 inhibition, either by therapeutic or by genetic silencing, lead to a delay in plaque progression. Marquart et al, however, did not observe any differences in atherosclerosis progression between anti–miR33 treated and control oligonucleotide–treated mice. The major differences between the studies were the oligonucleotide inhibitors of miR33 used and the amount of cholesterol in the diets (0.15%, 0.3% versus 1.25%), which resulted in a wide range of plasma cholesterol levels. It seems that when plasma cholesterol was above 1200 mg/dL, anti-miR33 was less able to be atheroprotective.

A qualitatively similar phenomenon may also occur in regression. We have seen in another mouse model of atherosclerosis that anti-miR33 treatment was able to improve the content and inflammatory state of the macrophages in the normoglycemic setting, but in that study, the plasma cholesterol levels were ≥250 mg/dL in the treatment groups compared with <100 mg/dL in the present study. The lack of an effect on regression of miR33 inhibition in the normoglycemic group in the present study, then, likely reflects that the plasma cholesterol level was sufficiently low so that no additional treatment benefit would be realized unless there was the concurrent stress of hyperglycemia. Thus, like many therapeutics tested for cardiovascular disease risk reduction, there is a likely a range of plasma cholesterol levels above which the effects of anti-miR33 treatment are attenuated and below which it cannot exert further improvements, with the range potentially dependent on the presence of comorbidities.

In conclusion, the present results show for the first time that miR33 inhibition is able to overcome the deleterious effects of hyperglycemia on plaque regression in atherosclerotic mice after their plasma lipids were aggressively lowered. Because we did not test the effects of miR33 inhibition in a setting of hyperglycemia and insulin resistance, the full relevance of our findings to patients with type 2 diabetes mellitus remains to be established. Nevertheless, given that patients with either type 1 and 2 diabetes mellitus both have hyperglycemia and high rates of cardiovascular disease, it is tempting to suggest anti-miR33 treatment as a promising combination strategy to make statins and other lipid-lowering therapies more effective in these patients to reduce their elevated risk.
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Disclosures
C.C. Essau, at the time of these studies, was a full-time employee at Regulus Therapeutics. K.J. Moore and E.A. Fisher were past members of Regulus Therapeutics Clinical Advisory Board.

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**What Is Known?**

- Compared with nondiabetics, diabetic patients have high rates of cardiovascular disease, low plasma levels of high-density lipoprotein, and attenuated risk reduction after plasma low-density lipoprotein (LDL) lowering therapies.
- Diabetic mice have impaired atherosclerotic plaque regression after plasma LDL lowering compared with nondiabetic mice, in part from monocytosis associated with hyperglycemia.
- miR-33 controls high-density lipoprotein metabolism in mice and nonhuman primates through regulation of ATP-binding cassette transporter A1 and is considered to be a potential antiatherosclerosis therapeutic target.

**What New Information Does This Article Contribute?**

- After plasma LDL lowering, diabetic mice treated with anti-miR33 therapy had improved regression of atherosclerosis as indicated by decreases in the plaque content and inflammatory state of macrophages.
- Decreased plaque content of macrophages was explained by a reduction in hyperglycemia-associated monocytosis as a result of anti-miR33 treatment and reduced recruitment of monocytes to plaques.
- Reduced monocytosis reflected decreased proliferation of bone marrow monocyte precursors most likely through upregulation of ATP-binding cassette transporter A1, a factor known to regulate their proliferation.

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**Novelty and Significance**

The overwhelming cause of death in type 1 and 2 diabetic patients is cardiovascular disease. Though diabetics have shown relative risk reduction in statin intervention trials, their absolute risk remains elevated, making the understanding of this phenomenon an important clinical need. Consistent with the clinical findings, in mouse models, we showed that diabetes mellitus impairs atherosclerosis regression after aggressive lowering of high LDL levels. This impairment was traced to monocytosis, which drives more monocytes into diabetic plaques. In preclinical studies by others, the cholesterol content of the bone marrow monocyte precursors was positively associated with their proliferation. We now show, for the first time, that anti-miR-33 therapy reduced cellular cholesterol content by increasing ATP-binding cassette transporter A in the diabetic bone marrow precursors and decreased bone marrow monocyte precursor proliferation. This prevented diabetic monocytosis and decreased plaque recruitment of monocytes. We previously reported anti-inflammatory effects of anti-miR33 treatment on mouse plaque macrophages and again observed these effects in diabetes mellitus. Thus, by reducing the content and inflammatory state of plaque macrophages after LDL lowering, anti-miR33 treatment was able to improve atherosclerosis regression in diabetic mice.
miR33 Inhibition Overcomes Deleterious Effects of Diabetes Mellitus on Atherosclerosis Plaque Regression in Mice
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miR33 inhibition overcomes deleterious effects of diabetes on atherosclerosis plaque regression in mice

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Short Title: Anti-miR33 attenuates diabetes-impaired regression

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METHODS

Primary mouse macrophages

Male C57BL/6J mice were injected i.p. with 1 mL of sterile 2% Bio-Gel P-100 fine polyacrylamide beads (Biorad Laboratories). Two days later, mice were injected i.p. with either control ASO or anti-miR33 ASO (10mg/kg). Mice were sacrificed 4 days after the initial Bio-Gel injection and the peritoneum washed with 10 mL of ice cold PBS/2mM EDTA. Cells were counted and resuspended at 8x10^6 for 1h at 37°C in glucose-free RPMI media supplemented with 0.2% BSA, 25 mM HEPES and either 5.5 (normal) or 25mM (high) endotoxin-free D-glucose (Sigma Aldrich). The buffer containing 5.5mM D-glucose was supplemented with 19.5 mM L-glucose (Sigma Aldrich) to serve as an osmolite control. To assess the efficiency of ASO treatment (control or anti-miR33), macrophages were collected from the peritoneal cavity and adherence-purified for 1h followed by a wash with PBS to remove non-adherent cells. Trizol purification was used for RNA isolation. Gene expression of miR33-targets was assessed by qPCR.

Chemotaxis

Chemotaxis assays were performed with Bio-Gel elicited macrophages with an xCelligence RTCA-DP instrument (Roche Diagnostics) with CIM-16 well plates (Roche Diagnostics), as previously described^39. MCP-1 and RANTES (R&D Systems) were used at 10nM.

In vitro proliferation

Peritoneal macrophages were elicited and transfected with either ASO control or anti-miR33 (10mg/kg) as described above. Cells were plated at 0.5x10^5 and proliferation was assessed with CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega), according to the manufacturer’s instructions, after incubation in either 5.5 or 25mM glucose for 24hr.
Online Figure I: α-miR33 does not affect plaque size. Aortic roots from baseline and the regression groups were sectioned, fixed and stained for CD68. Plaque size was quantified by Image Pro Plus Software. Results are shown as the plaque size in μm².
Online Figure II: Latex beads were taken up with equal efficiency by Ly6C$^{lo}$ monocytes. Diabetic and normoglycemic mice treated with anti-miR33 or control ASO were injected 24h prior to harvesting with fluorescent (FITC$^+$) beads to label Ly6C$^{lo}$ monocytes. Labeling efficiency was assessed by flow cytometry. Results are presented as the percentage of CD115$^+$/Ly6C$^{lo}$/FITC$^+$ cells.
Online Figure III: α-miR33 treatment decreases macrophage chemotaxis in high glucose, but does not suppress hyperglycemia driven myeloid proliferation.

Peritoneal macrophages from C57Bl6/J mice, injected (i.p.) with con α-miR or α-miR33 2′F/MOE oligonucleotides (10mg/kg) 48h prior to harvesting mice, were incubated under either (A) euglycemic (5.5 mM D-glucose + 19.5 mM L-glucose) or (B) hyperglycemic (25 mM D-glucose) conditions for 1 h prior to assessment of chemotaxic ability. Chemotaxis was assessed using an xCelligence RTCA-DP instrument (Roche Diagnostics) under euglycemia or hyperglycemia. Results are expressed as the percentage of chemotaxis relative to buffer alone for each transfected cell type. * p≤0.05 between control α-miR or α-miR33 under the relevant glucose condition. (C) Peritoneal cells were collected and incubated under euglycemic (5.5 mM D-glucose + 19.5 mM L-glucose) or hyperglycemic (25 mM D-glucose) conditions for 24 h, proliferation was assessed with the CellTiter 96® non-Radioactive cell proliferation assay kit. * p≤0.05 when compared to control cells (5.5 mM glucose). Data is representative of n=3 mice per treatment group.
Online Figure IV: FPLC of α-miR33 and control treated diabetic and citrate treated mice. C57Bl6/J mice were injected with citrate (normoglycemic) or 50mg/kg streptozotocin (diabetic). Mice were then injected 4 times with control α-miR or α-miR33 2′F/MOE oligonucleotides (10mg/kg). Plasma lipoproteins were separated by FPLC and the contents of (A) cholesterol and (B) triglycerides measured. Samples were pooled plasma of control anti-miR and anti-miR33-treated normoglycemic and diabetic mice (n=6 mice per group).
Online Figure V: The effect of α-miR33 treatment on apoptosis and senescence in bone marrow progenitor populations in diabetic mice.
C57Bl6/J mice were injected with citrate (normoglycemic) or 50mg/kg streptozotocin (diabetic). Mice were then injected 4 times with control α-miR or α-miR33 2′F/MOE oligonucleotides (10mg/kg). Hematopoietic stem and progenitor cell cycle was assessed by flow cytometry using DAPI; (A) G0, and (B) apoptosis (sub-G0). (C) Gene expression for p15INK4b (as a marker for senescence) was assessed in the different bone marrow progenitor subsets, obtained by cell sorting. Data are expressed as mean ± SEM, n≥7/group. *p≤0.05, **p≤0.01 vs. con α-miR normoglycemic; *p≤0.05 con α-miR diabetic.