mir33 Inhibition Overcomes Deleterious Effects of Diabetes on Atherosclerosis Plaque Regression in Mice

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ABSTRACT

Rationale: Diabetes increases cardiovascular disease risk in humans and remains elevated despite cholesterol-lowering therapy with statins. Consistent with this, in mouse models diabetes impairs atherosclerosis plaque regression after aggressive cholesterol-lowering. miR33 is a key negative regulator of the reverse cholesterol transport factors, ABCA1 and HDL, 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 LDL receptor and in which hypercholesterolemia is reversed by conditional inactivation of the microsomal triglyceride transfer protein (Mttp) gene, were placed on an atherogenic diet for 16 weeks, then either made diabetic by STZ injection or kept normoglycemic. Lipid-lowering was induced by Mttp inactivation and mice were treated with anti-miR33 or control oligonucleotides. Whereas 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 in atherosclerosis regression in mice, which suggests a therapeutic strategy in diabetic patients, who remain at elevated cardiovascular disease risk despite plasma cholesterol lowering.

Keywords: Atherosclerosis, diabetes mellitus, regression, miR33, high-density lipoprotein, microRNA

Nonstandard Abbreviations and Acronyms:
- ABCA1: ATP-binding cassette transporter A1
- ABCG1: ATP-binding cassette transporter G1
- ASO: Anti-sense oligonucleotides
- BM: Bone marrow
- CMP: Common myeloid progenitor
- GMP: Granulocyte-macrophage progenitor
- LSK: Lin<sup>−</sup>, Sac1<sup>+</sup>, ckit<sup>+</sup> stem cells
- LCM: Laser capture micro-dissection
- miR33: microRNA 33
- Mttp: Microsomal triglyceride transfer protein
- pIpC: Polyinosinic polycytidylic
- STZ: Streptozotocin
INTRODUCTION

Diabetes increases the clinical risk of cardiovascular morbidity and mortality. This risk remains elevated with conventional LDL-cholesterol lowering therapies, such as statins. In the FIELD study, type 2 diabetic patients with mixed dyslipidemia (high triglycerides and low HDL-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 dramatically accelerates the progression of atherosclerosis and we have shown that it inhibits atherosclerosis regression following aggressive LDL reduction. This was due to increased monocyte infiltration and macrophage burden in the plaque compared to non-diabetic 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 non-diabetic 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 progenitor cells.

In non-diabetic 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 cholesterol efflux from macrophages in the plaque. This process is mediated by the ABC transporters, ABCA1 and 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-/- mice with anti-miR33 up-regulated ABCA1 in the liver and macrophages and resulted in plaque regression. Taken with our findings that both Abca1 and Abcg1 are down-regulated 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-/-) 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, our protocol was to first establish atherosclerosis by placing mice on a western diet for 16 weeks, and then inducing diabetes 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 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 bone marrow 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>100/100</sup>; Mttp<sup>fl/fl</sup>; Mx1-Cre<sup>+/+</sup>)<sup>14</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 4-5 weeks and placed on a western diet (21% (wt/wt) fat, 0.15% cholesterol (Research Dyes)) to allow the development of atherosclerotic plaques. After 15 weeks, mice were injected i.p. daily with STZ (50 mg/kg, Sigma-Aldrich) or citrate buffer for 5 days to induce diabetes or serve as a control. One week later, all mice were switched to a chow diet. Following the diet switch Reversa mice were injected i.p. with polyninosinic polycytidylic RNA (pIpC) (Sigma-Aldrich 15 mg/kg) every other day for a total of four injections<sup>4</sup>. Mice were then treated subcutaneously 4 times with the anti-sense oligonucleotides (ASO) anti-miR33 or the ASO control (Regulus Therapeutics, 10mg/kg) as previously described<sup>9</sup>. Regulus Therapeutics provided 2' fluoro/methoxyethyl (2F/MOE) modified, phosphorothioate backbone modified anti-miR33 (TGCAATGCAACTACAATGCAC) and mismatch control (TCCAATCCAACTTCAATCATC) anti-miR. At sacrifice, 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 OCT 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-cholesterol (HDL-C), and triglyceride (TG) 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 OCT, 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 picrosirius 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<sup>15</sup>.

Apoptosis analysis.
Apoptosis was analyzed in aortic roots from Reversa mice by TUNEL method using an in situ cell detection kit (Roche Diagnostics). Nuclei were labeled with Vectashield Mounting media with DAPI (Vector labs). 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 micro-dissection (LCM)<sup>16,17</sup>. All LCM procedures were performed under RNase-free conditions. Aortic root sections were stained with hematoxylin-eosin and captured from 36 frozen sections. After LCM, 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 5ng 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 described18, 19. Briefly, 1μm Fluoresbrite FITC-dyed (YG) plain microspheres (Polysciences Inc.) were diluted in PBS (1:4), and 250μl of the solution was injected into the retro-orbital vein of mice to label circulating Ly6Clo monocytes. For the egress study, beads were injected at week 14 before any treatment. For the recruitment study, beads were injected 24h prior to harvesting. For both protocols, bead labeling efficiency was assessed by flow cytometry, 24h after bead injection18, 19.

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 anti-mouse CD16/CD32 (eBioscience). Monocytes were identified by staining with PE anti-mouse CD115 (Biolegend) and APC anti-mouse Ly-6G/Ly-6C (Biolegend)18, 19. Neutrophils were identified as CD45hiCD115loLy-6-C/Ghi cells. Flow cytometry was performed using a LSRII analyzer.

Hematopoietic stem and progenitor cells: Hematopoietic stem and progenitor cells were analyzed by flow cytometry as previously described5, 20. Briefly, bone marrow 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, all FITC) and markers to identify the stem and progenitor cells that were identified as LSK (lin–, Sca1+, and ckit–), CMP (lin–, Sca1–, ckit+, CD34int, and FcγRIIint/FcγRIIIint), and GMP (lin–, Sca1–, ckit+, CD34int, and FcγRIIia/FcγRIIIa). Cell cycle analysis was performed using DAPI (Sigma Aldrich) to measure cells in the S-G2M phase, G0 and sub-G0 (apoptotic). Flow cytometry was performed using an LSR II (for analysis) or MoFlo (for sorting). All flow cytometry data were analyzed using FlowJo X software (Tree Star). RNA from sorted cells was extracted with RNaseasy Micro kit (Qiagen) and cDNA was obtained with SuperScript VILO (Life Technologies).

Statistical analysis.
For atherosclerosis and immunohistochemical analyses, all comparisons were made using a 1-way ANOVA (P ≤ 0.05), and data are expressed as mean ± SEM.

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 (WD; 21% fat, 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 (i.p.) injections for 5 consecutive days with citrate buffer or 50 mg/kg streptozotocin (STZ) to induce diabetes4. At 16 weeks, all mice were switched to a standard chow diet (6% fat) and injected i.p. four times every other day with pIpC to conditionally delete the Mttp gene and induce lipid lowering. Mttp deletion resulted in effective cholesterol lowering compared to baseline levels (baseline: 863±47 mg/dL to 83-101 mg/dL in the experimental groups; Table 1). The reduction in total cholesterol levels was independent of STZ or anti-miR (control or miR33) treatment.
Following pIpC injections, mice were treated weekly by subcutaneous injection with either miR33 or control ASOs. After 4 weeks, anti-miR33 treatment increased HDL-C by 30% (p<0.05) and 42% (p<0.05) in the normoglycemic and in the diabetic groups, respectively (Table 1). Anti-miR33 treatment did not affect the blood glucose levels of either the normoglycemic or diabetic mice when compared to the corresponding ASO control-treated mice (Table 1). Consistent with previous observations in mouse models and diabetic patients, plasma triglyceride levels were increased by hyperglycemia; no effect of anti-miR33 on triglyceride levels was observed when compared to control ASO treated mice (Table 1).

The efficacy of miR33 inhibition was evaluated by measuring the 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 to 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 WD 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 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 non-diabetic 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 to baseline (Figure 2; Baseline 52% vs. 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 towards 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 (e.g. increased Il1β, 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, Nos2) and increased those of anti-inflammatory M2 macrophage-
related genes (Ym1, 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 monocyte recruitment, but does not change macrophage egress.

A decrease in macrophage content in the regressing plaque could be due to 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 24h before the harvesting of aortic roots and the number of beads in the plaques counted. Monocyte labeling was not affected by either diabetes 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≤0.01; Figure 3B), consistent with our previous observations in another model of regression. Notably, anti-miR33 treatment prevented this diabetes-induced increased recruitment of monocytes (p≤0.01; 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 & IIIB).

To study macrophage egress, we injected fluorescent beads prior to the induction of diabetes or anti-miR treatment (week 14, Figure 3A). One week after bead injection, a group of mice was sacrificed to obtain baseline measurements of the content of labeled macrophages (i.e. CD68+ cells co-localized with beads) in aortic root plaques. As in the experiment described above, additional mice were then treated with STZ, pIpC and anti-miR (control or miR33, Figure 3A), and the beads remaining in the aortic root plaques at sacrifice were quantified. Reversal of hyperlipidemia was associated with a reduction in plaque bead content to ~50% of baseline independent of the treatment (p≤0.001; Figure 3C). These results suggest that though macrophages were actively leaving the plaque during regression, this process was not differentially affected by diabetes or anti-miR33 treatment. Consistent with this was the lack of significant changes in the plaque macrophage expression of the chemotactic factor Ccr7 or of the retention factor Netrin-1 and its receptor, Unc5b (Table 2).

Anti-miR33 treatment does not affect plaque macrophage apoptosis, but does increase efferocytosis.

Plaque macrophage content can also be modulated by apoptosis, but we did not observe any significant differences in this among the groups as measured by TUNEL analysis (Figure 3D). Diabetes, however, promoted an increase in the area occupied by necrotic cores, which negatively reflects the level of efferocytotic activity, compared to plaques from regressing normoglycemic mice (Figure 3E, p≤0.05), similar to its effects in progression. Notably, anti-miR33 treatment of diabetic mice significantly reduced the plaque necrotic area to that of normoglycemic mice (Figure 3E, p≤0.05), suggesting that diabetes-associated deficiencies in efferocytosis were corrected.

Anti-miR33 treatment reduces hyperglycemia-induced monocytosis, but not neutrophilia.

The increased recruitment of monocytes in the diabetic mice after plasma lipid reduction may be a consequence of the higher numbers of circulating monocytes observed in mice that have hyperglycemia, which are also elevated with hypercholesterolemia. Indeed, in the present study, plasma lipid lowering induced a significant decrease in the level of monocytes (expressed as their % of circulating leukocytes).
compared to baseline ($p \leq 0.001$; Figure 4A, B). Lipid lowering, however, had no effect on the monocyte levels in the control anti-miR treated diabetic group, likely due to the increased monocyte precursor proliferation via glucose-dependent mechanisms. In contrast, anti-miR33 treatment decreased monocyte levels in the diabetic mice to levels comparable to the non-diabetic mice ($p \leq 0.001$; Figure 4A, B).

In addition to the monocytosis, hyperglycemia induced neutrophilia, as we previously observed, 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 induces myelopoiesis in the bone marrow (BM), 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. 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.

A group of mice were made diabetic with STZ and treated with control anti-miR or anti-miR33 weekly for 4 weeks as before. 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, Online Figure IVB). Consistent with our previous findings, hyperglycemia induced an increase in the abundance and proliferation of both common myeloid (CMP) and granulocyte-macrophage progenitor cells (GMP), without affecting the stem cells (LSK) (Figure 5C & D). 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 due to 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 (Figure 5C, 6D), indicating that anti-miR33 acts via glucose-independent mechanisms to inhibit the production of monocytes in diabetes. This effect of anti-miR33 treatment on proliferation of monocyte progenitors, however, was not observed in macrophages in vitro; while there was evidence of their increased proliferation in a hyperglycemic environment, it was not suppressed by anti-miR33 treatment (Online Figure IIIIC).

**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. We hypothesized that hyperglycemia-induced BM progenitor proliferation was due to 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. 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, 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 non-diabetics. Consistent with this, is our finding in two independent mouse models that after cholesterol-lowering, atherosclerosis regression is impaired by hyperglycemia. Because anti-miR33 treatment regressed plaques in non-diabetic mice, we reasoned that a similar approach may overcome this impairment. Indeed, our results show that miR33 inhibition confers a number of atheroprotective benefits in the context of hyperglycemia, chief among them being the reduction in the content of macrophages. This was found to be due to the ability of anti-miR33 to reduce the monocytosis associated with diabetes, 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 appears to derive primarily from decreased recruitment of monocytes into the plaques and increased clearance of apoptotic 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 due to 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 Figure 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 and colleagues showed that deficiency of Abca1 and Abcg1, even in the non-hyperlipidemic 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 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 bone marrow.

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 at least two 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 (TLRs). That the down-regulation of Abca1 by hyperglycemia and the restoration of its expression by anti-miR33 modulated plaque macrophage inflammatory state through this mechanism is supported by finding that increases in Ccr2, Vcam1 and Icam1, all NF-kB targets downstream of TLR-signaling, were reversed by anti-miR33. Despite Abcg1 also being a NF-kB target, its gene expression was not changed in plaque macrophages or in CMP precursors, suggesting that under our experimental conditions, ABCG1 may be more likely in these cell types to be regulated by post-transcriptional mechanisms.

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 de-repressed by the anti-miR33 treatment. A previous report has also implicated the eicosanoid pathway in the inflammatory process of diabetes-accelerated atherosclerosis. In the present studies, 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 (Acsil, 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% vs. 1.25%), which resulted in a wide range of plasma cholesterol levels. It appears 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 to <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 further 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 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 co-morbidities.

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 that 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
ED, TJB, KG, SP, AMJ: none
CCE: At the time of these studies, was a full-time employee at Regulus Therapeutics
KJM, EAF: Past members of Regulus Therapeutics Clinical Advisory Board

REFERENCES


Table 1: Plasma characteristics of Reversa mice after 5 weeks of α-miR control or α-miR33 treatment.

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Glycemia, total plasma cholesterol, HDL-C and triglyceride levels were obtained after 16 weeks of diet (baseline) and at sacrifice. All data are expressed as mean ± SEM (n>10). # p≤0.05 vs. con α-miR normoglycemic; * p≤0.05, ***p≤0.001 vs. con α-miR diabetic.

Table 2: Gene expression analysis of laser-captured plaque CD68+ cells.

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CD68+ cells from aortic plaques of Reversa mice were isolated using LCM. mRNA was isolated, amplified and analyzed by RT-PCR. mRNA levels are presented as relative levels normalized to HPRT. Data are expressed as mean ± SEM (n≥6 per group). # p≤0.05 vs. con α-miR normoglycemic; * p≤0.05 vs. con α-miR diabetic.
FIGURE LEGENDS

Figure 1. α-miR33 treatment derepresses its target genes in the liver of Reversa mice. (A) Experimental design: Reversa mice were placed on a western diet for 16 weeks. At 15 weeks, the mice received 5 injections of citrate (control) or 50mg/kg streptozotocin (diabetic). At 16 weeks, all mice were switched to a chow diet and received polynucleosin polycytidylic RNA (plpC) 15 mg/kg every other day for a total of four injections to initiate lipid lowering. Mice then received weekly (s.c) injections of either control anti-miR or anti-miR33 2′F/MOE oligonucleotides (10mg/kg) for 4 weeks, prior to sacrifice (i.e 4 weeks post the final plpC injection). Hepatic expression of (B) Abca1, (C) Abcg1, (D) Cpt1 and (E) Hmgcr. # p≤0.05 vs. con α-miR normoglycemic; * p≤0.05, *** p≤0.001 vs. con α-miR diabetic.

Figure 2. α-miR33 treatment restores regression in diabetic mice. Aortic roots from baseline and the regression groups were sectioned, fixed and stained for (A) CD68 and (B) collagen. Representative pictures of (A) CD68 immunostaining (magnification x20) and (B) picrosirius red staining (under white and polarized light) of collagen (magnification x10) are shown for each group. The areas of the plaques occupied by CD68+ 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 ±SEM (n=4 per group).

Figure 3. α-miR33 treatment affects monocyte trafficking to the plaque. Experimental design for monocyte trafficking. (A) Reversa mice were injected with fluorescent latex beads either 24h prior to harvesting (week 21) for the recruitment protocol or at week 14 of western diet for the egress protocol. (B) To assess monocyte recruitment mice were injected with fluorescent latex beads 24h prior to sacrifice, and beads counted in the aortic root. Data are expressed as mean ±SEM (n≥6 per group). # p≤0.05 vs. con α-miR normoglycemic ** p≤0.01 vs. con α-miR diabetic. (C) To assess macrophage retention mice were injected with fluorescent latex beads at week 14, and the number of beads remaining at sacrifice was used to assess macrophage retention (baseline was established 1 week post bead injection). Data are expressed as mean ±SEM (n≥6 per group). ^^p≤0.001 vs. baseline. (D) Quantitative analysis of TUNEL+DAPI+ cells per section in the aortic root, after regression. Results are expressed as the percentage of total plaque area ±SEM (n≥6 per group). (E) Quantitative analysis of necrotic core area as a percentage of total plaque area ±SEM (n≥6 per group) # p≤0.05 vs. baseline, *** p≤0.001 vs. con α-miR diabetic.

Figure 4. α-miR33 treatment decreases monocytosis in diabetic mice. Total monocytes and neutrophils were analyzed by flow cytometry in the blood at 16 weeks of diet (baseline) or after treatment. (A) Representative flow cytometry plots. Quantification of (B) monocytes and (C) neutrophils. Data are expressed as mean ±SEM (n≥10 per group). ^p≤0.01, *** p≤0.001 vs. baseline.

Figure 5. α-miR33 treatment decreases bone marrow progenitors 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). (A) Plasma blood glucose and (B) HDL-C 4 weeks post treatment. (C) Hematopoietic stem and progenitor cell populations in the BM were determined by flow cytometry and expressed as a percentage of cells in the BM. (D) Hematopoietic stem and progenitor cell cycle (G2:M phase) was assessed by flow cytometry using DAPI. 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.

Figure 6. α-miR33 treatment restores Abca1 expression in bone marrow progenitors of 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). Gene expression for Abca1 and Abcg1 was assessed by qPCR in the different bone marrow progenitor subsets (A) LSK, (B) CMP and (C) GMP, 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. con α-miR diabetic.
Novelty and Significance

What Is Known?

- Compared to non-diabetics, diabetic patients have high rates of cardiovascular disease (CVD), low plasma levels of HDL, and attenuated risk reduction after plasma LDL-lowering therapies.

- Diabetic mice have impaired atherosclerotics plaque regression after plasma LDL lowering compared to non-diabetic mice, in part from monocytosis associated with hyperglycemia.

- miR-33 controls HDL metabolism in mice and non-human primates through regulation of ABCA1, and is considered to be a potential anti-atherosclerosis 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 (BMP) most likely through upregulation of ABCA1, a factor known to regulate their proliferation.

The overwhelming cause of death in type 1 and 2 diabetic patients is CVD. 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 impairs atherosclerosis regression after aggressive lowering of high LDL levels. This impairment was traced to monocytosis, which drives more monocytes into diabetic plaques. In pre-clinical studies by others, the cholesterol content of the monocyte BMP was positively associated with their proliferation. We now show, for the first time, that anti-miR-33 therapy reduced cellular cholesterol content by increasing ABCA in the diabetic monocyte BMP and decreased BMP 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. 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.
**Figure 1**

A. Timeline of treatments: STZ (Wk 15) → pIpC (Wk 16) → Con α-miR → α-miR33 → Chow 5 weeks → WD 16 weeks → Diabetic

B. **Abca1**

C. **Abcg1**

D. **Cpt1**

E. **Hmgcr**

**Relative expression**

- **Normoglycemic**
- **Diabetic**

Significance levels:

- *p < 0.05
- **p < 0.001
- #p < 0.0001
Figure 2

A

Baseline
Con a-miR
a-miR33

%CD68

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<th>α-miR33</th>
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B

Baseline
Con a-miR
a-miR33

%collagen

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Diabetic
Normoglycemic
Con α-miR α-miR33

Monocyte recruitment

Macrophage retention

Apoptosis

Necrotic Area

Figure 3
**Figure 4**

**A**

Toxicologica Research Peer Review. Do not distribute. Destroy after use.

**B**

**C**

**Figure 4**

**A**

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**C**

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<td>a-miR33</td>
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**Figure 5**

**A**

![Graph A](image1.png)

**B**

![Graph B](image2.png)

**C**

![Graph C](image3.png)

**D**

![Graph D](image4.png)
Figure 6

(A) LSK
(B) CMP
(C) GMP

Relative expression

Abca1 Abcg1

Con α-miR
α-miR33

Normoglycemic

Con α-miR
α-miR33

Hyperglycemic
mir33 Inhibition Overcomes Deleterious Effects of Diabetes on Atherosclerosis Plaque Regression in Mice
Emilie Distel, Tessa Barrett, Kellie Wing Ki Chung, Natasha M Girgis, Sajesh Parathath, Christine C Esau, Andrew J Murphy, Kathryn J Moore and Edward A Fisher

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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\textsuperscript{lo} monocytes. Diabetic and normoglycemic mice treated with anti-miR33 or control ASO were injected 24h prior to harvesting with fluorescent (FITC\textsuperscript{+}) beads to label Ly6C\textsuperscript{lo} monocytes. Labeling efficiency was assessed by flow cytometry. Results are presented as the percentage of CD115\textsuperscript{+}Ly6C\textsuperscript{lo}FITC\textsuperscript{+} 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 chemotactic 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.