Pathogenic Role of microRNA-21 in Diabetic Retinopathy Through Downregulation of PPARα

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Fenofibrate, a specific agonist of peroxisome proliferator–activated receptor-α (PPARα), displays robust therapeutic effects on diabetic retinopathy (DR) in patients with type 2 diabetes. Our recent studies have shown that PPARα is downregulated in the diabetic retina, which contributes to the pathogenesis of DR. However, the mechanism for diabetes-induced downregulation of PPARα remains unknown. We investigated the role of microRNA-21 (miR-21) in regulating PPARα in DR. miR-21 was overexpressed, while PPARα levels were decreased in the retina of db/db mice, a model of type 2 diabetes. Such alterations were also observed in palmitate-treated retinal endothelial cells. miR-21 targeted PPARα by inhibiting its mRNA translation. Knockout of miR-21 prevented the decrease of PPARα, alleviated microvascular damage, ameliorated inflammation, and reduced cell apoptosis in the retina of db/db mice. Intravitreal injection of miR-21 inhibitor attenuated PPARα downregulation and ameliorated retinal inflammation in db/db mice. Further, retinal miR-21 levels were increased, while PPARα levels were decreased in oxygen-induced retinopathy (OIR). Knockout of miR-21 prevented PPARα downregulation and ameliorated retinal neovascularization and inflammation in OIR retinas. In conclusion, diabetes-induced upregulation of miR-21 in the retina is at least partly responsible for PPARα downregulation in DR. Targeting miR-21 may represent a novel therapeutic strategy for DR.

Diabetic retinopathy (DR) is a common microvascular complication of diabetes and a leading cause of blindness among the working age population in developed countries (1). The pathology of DR is characterized by pericytes loss, endothelial cell death, formation of acellular capillaries, thickening of the basement membrane, formation of microaneurysms, and later, retinal neovascularization (2). The current therapeutic approaches for DR, such as antivascular endothelial growth factor (VEGF) therapy and laser photocoagulation, are not effective for all patients with DR and are associated with significant adverse effects (3).

Fenofibrate is a peroxisome proliferator–activated receptor-α (PPARα) agonist that is used clinically to lower blood lipid levels in patients with dyslipidemia and cardiovascular disease (4). Recently, the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study reported that fenofibrate monotherapy reduced the cumulative need for laser therapy for DR by 37% in patients with type 2 diabetes (5). The ACCORD (Action to Control Cardiovascular Risk in Diabetes) study reported that simvastatin/fenofibrate combination therapy reduced the progression of proliferative DR in patients with type 2 diabetes by 40% over simvastatin alone (6). The therapeutic effects of fenofibrate on DR are not correlated with plasma lipid concentrations (5). The mechanism underlying beneficial effects of fenofibrate on DR is still under investigation. PPARα is a ligand-activated nuclear receptor that functions as a transcription factor and regulates the expression of multiple genes involving lipid metabolism, insulin signaling, and energy homeostasis (7,8). Our recent study showed that fenofibrate had therapeutic effects on DR via...
a PPARα-dependent mechanism (9). Our previous studies also showed that PPARα was significantly decreased in the retina of both type 1 and type 2 diabetic animal models (10). Knockout of PPARα exacerbated and overexpression of PPARα ameliorated diabetes-induced retinal inflammation (10). In addition, PPARα showed protective effects against hyperglycemia-induced endothelial inflammation and retinal cell apoptosis through blocking the nuclear factor-κB pathway and alleviating oxidative stress in the retina (10–12). The mechanism for the downregulation of PPARα in the diabetic retina, however, remains to be elucidated.

MicroRNAs (miRNAs) are small (~22 nucleotide), single-stranded, noncoding RNA molecules that negatively regulate gene expression by binding to the 3’ untranslated region (UTR) of target mRNAs (13,14). miRNAs regulate a variety of biological and pathological processes, such as cell proliferation, migration, and apoptosis (13,14). miRNAs play important roles in the pathogenesis of various diseases, including cancer, diabetes, and diabetic complications (15–17). Recent studies have revealed that several miRNAs are involved in the pathogenesis of DR (18,19). In addition, studies have shown that miRNAs may regulate PPARα in liver cells and endothelial cells under some stress or disease conditions (20–22). However, whether miRNAs play a role in regulating PPARα in the diabetic retina was still unknown.

In this study, we tested the hypothesis that miRNAs contribute to retinal inflammation in DR by decreasing PPARα expression. We have identified that microRNA-21 (miR-21) was significantly upregulated in the retina of leptin receptor-deficient (db/db) mice and that PPARα was a target gene of miR-21. To further elucidate the role of miR-21 in DR, we generated miR-21 knockout db/db mice and investigated the role of miR-21 in retinal inflammation and retinal cell apoptosis induced by diabetes. We also used a mouse model of oxygen-induced retinopathy (OIR) to further demonstrate the role of miR-21 in retinal neovascularization and inflammation through regulating PPARα. Our findings suggest a pathogenic role of miR-21 in DR through modulating PPARα levels in the diabetic retina and that miR-21 could be a potential therapeutic target for DR.

RESEARCH DESIGN AND METHODS

Animals
Male homozygous BKS.Cg-Dock7mvs/+ Leprdb/J (db/db), C57BLKS/J (nondiabetic control), B6;129S6-Mir21atm1Yoli/J (miR-21−/−), and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). miR-21−/− mice and wild-type (WT) littermates were obtained from breeding of miR-21−/− mice. Leptin receptor-deficient (db/db) miR-21−/− mice (dKO) were generated by crossing miR-21−/− male mice with db/+ female mice. Littermates db/db (miR-21−/−) were used as control mice for dKO mice. The mouse strains have been tested for retinal degeneration 1 (Pde6b−/−), retinal degeneration 8 (Crb1−/−), and retinal degeneration 10 (Pde6b−/−) mutations, and they do not carry any of these mutations. Care, use, and treatment of mice was approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Oklahoma Health Sciences Center, and all of the experiments were performed following the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

Cell Culture
A human telomerase reverse transcriptase (hTERT)–immortalized retinal pigment epithelial (RPE) cell line (hTERT–RPE-1) and a simian virus 40–transformed mouse endothelial cell line (SVEC4-10) were purchased from American Type Culture Collection (Manassas, VA). Primary human retinal microvascular endothelial cells (HRMECs) were obtained from Cell Systems Corporation (Kirkland, WA). hTERT-RPE-1 cells were maintained in DMEM/F-12 culture medium (Cellgro, Manassas, VA) supplemented with 10% FBS. SVEC4-10 cells were cultured in low-glucose DMEM supplemented with 20% FBS. HRMECs were grown in Endothelial Basal Medium-2 supplemented with SingleQuots kit (Lonza Group, Basel, Switzerland).

Real-time Quantitative RT-PCR for miRNA
Total RNA, including miRNA, was extracted from retinas or cultured cells using the mirNeasy Mini kit (Qiagen, Valencia, CA). Levels of miRNAs were measured using TaqMan quantitative (q)RT-PCR (Applied Biosystems, Carlsbad, CA). The detailed procedure of detecting miRNA was described previously (23).

OIR Model and Quantification of Retinal Neovascularization
OIR was generated as described previously (24). Retinas were isolated at postnatal day (P)16 for RNA or protein analyses. Flat-mounted retinas were stained with fluorescent Griffonia simplicifolia isoelectrin B4 (Invitrogen, Carlsbad, CA). Vaso-obliteration and neovascularization in the retina were quantified by Adobe Photoshop and ImageJ software, according to a documented method (25).

Transfection of miR-21 Mimic or Inhibitor
miR-21 mimic, miR-21 inhibitor, and their controls (mirVana miRNA; Ambion, Austin, TX) were separately transfected into hTERT–RPE-1 cells and HRMECs. The procedure of transfection of miRNA mimics or inhibitors was described previously (23).

Nanoparticle Formulation and Intravitreal Delivery of miR-21 Inhibitor
miR-21 inhibitor and its negative control miRNA (miRIDIAN Inhibitor; Thermo Fisher Scientific, Chicago, IL) were separately packed into liposome-based nanoparticles, as described by Rajala et al. (26). Then, 1.5 μL of the prepared nanoparticles (15 pmol of miR-21 inhibitor) was injected into the vitreous space of 5-month-old db/db mice, and the retinas were isolated 4 weeks later for further experiments.

Western Blot Analysis
Western blot analysis was performed as described previously (23). Antibodies for PPARα (cat# ab8934), VEGF (cat# ab46154), albumin (cat# ab19196), and tumor necrosis factor-α (TNF-α; cat# ab9739) were obtained from Abcam. An antibody for vascular cell adhesion molecule
Cell Death ELISA
DNA cleavage was measured in the retina using an ELISA-based kit (Roche, Indianapolis, IN), as described previously with a few minor modifications (27). Relative DNA fragmentation was expressed as absorbance (405/490 nm) normalized by total protein concentrations.

Retinal Leukostasis Assay
The leukostasis assay was performed by staining adherent leukocytes in the vasculature in flat-mounted retinas, as described previously (10).

Retinal Trypsin Digestion Assay
The retinal trypsin digestion assay was performed as described previously (10). Images of retinal vasculature were captured at eight random fields (1.08 mm²) in each retina. Acellular capillaries in the field were counted and averaged within each group.

Statistical Analysis
To determine statistical significance, at least three independent measurements were conducted for each assay, and data were entered into Microsoft Excel and analyzed by Student t test. Significance was denoted at a P value of <0.05. All of the values are expressed as the mean ± SEM.

RESULTS
Upregulated miR-21 in the Retina of db/db Mice
To determine the expression of PPARα in the diabetic retina, we selected db/db mice, a genetic model of type 2 diabetes, as an experimental model. Protein levels of PPARα were significantly decreased in the retina of 6-month-old db/db mice compared with age-matched non-diabetic control mice (Fig. 1A and B). Next, to study whether the alterations of miRNAs may correlate to the downregulation of PPARα in the diabetic retina, we conducted a miRNA-specific microarray using retinal RNA from db/db mice and nondiabetic control mice, which demonstrated a significant upregulation of miR-21 expression in the retina of db/db mice (Fig. 1C). We further verified levels of miR-21 in the retina of db/db mice by qRT-PCR. miR-21 was upregulated more than sevenfold in the retina of db/db mice compared with age-matched non-diabetic mice (Fig. 1D).

Upregulated miR-21 and Downregulated PPARα in Palmitate-Treated Retinal Endothelial Cells
To study whether miR-21 regulates PPARα expression in diabetic conditions, we treated endothelial cells (SVEC4-10 cells and HRMECs) with palmitate, a commonly used diabetic stressor, to induce cellular oxidative stress, inflammation, and cell apoptosis (11,28,29). PPARα protein levels were significantly decreased in SVEC4-10 cells treated with palmitate relative to vehicle-treated cells (Fig. 2A and B). In addition, miR-21 levels were elevated in palmitate-treated...
miR-21 Targets PPARα in Retinal Cells

The miR-21 sequence is conserved across species, including the human, mouse, and rat mRNA (Fig. 3A). Bioinformatics analysis predicts that the seed sequence of miR-21 is complementary to the sequence of the 3’ UTR of mouse and human PPARα mRNAs (Fig. 3B). To verify whether PPARα was a target gene of miR-21, we transfected miR-21 mimic or miR-21 inhibitor into hTERT–RPE-1 cells and HRMECs and measured PPARα levels. Transfection of miR-21 mimic into hTERT–RPE-1 cells and HRMECs caused significant reductions of PPARα protein levels (Fig. 3C, D, G, and H), and transfection of miR-21 inhibitor significantly increased protein levels of PPARα (Fig. 3E, F, I, and J). These results, consistent with previous studies in other tissues (22,30), suggest that PPARα is a target of miR-21. To check the specificity of PPARα regulation by miR-21, we separately transfected HRMECs with miR-184 mimic, miR-31 mimic, and miR-21 mimic into HRMECs and measured PPARα protein levels. Only miR-21 mimic is capable of decreasing PPARα protein levels, suggesting a specific regulatory effect of miR-21 on PPARα (Supplementary Fig. 1). To elucidate the mechanism by which miR-21 downregulated PPARα levels, we measured PPARα mRNA levels in hTERT–RPE-1 cells and HRMECs transfected with miR-21 mimic, miR-21 inhibitor, or their controls by qRT-PCR. PPARα mRNA levels were not significantly changed (Supplementary Fig. 2), suggesting that miR-21 regulated the expression of PPARα by inhibiting its translation rather than by destabilizing its mRNA.

Knockout of miR-21 Attenuates Microvascular Damage and Reduces Cell Apoptosis in the Retina of db/db Mice

To investigate the role of miR-21 in the retina under diabetic conditions, we generated dKO mice. A retina trypsin digestion assay was performed to investigate and compare diabetes-induced acellular capillaries between db/db mice and dKO mice (Fig. 4A). The dKO mice had fewer acellular capillaries in the retina compared with db/db mice (Fig. 4B), suggesting that knockout of miR-21 protected db/db mice against diabetes-induced retinal capillary degeneration. In addition, we used Western blot analysis to evaluate retinal vascular leakage by measurement of extravasated albumin in the retina (Fig. 4C). The results showed that retinal levels of extravasated albumin were significantly lower in the dKO mice compared with db/db mice (Fig. 4D), indicating that knockout of miR-21 ameliorated diabetes-induced retinal vascular leakage. Furthermore, results from cell death ELISA demonstrated that retinal cell apoptosis was reduced in dKO mice compared with control db/db mice (Fig. 4E), suggesting that knockout of miR-21 reduced diabetes-induced retinal cell apoptosis.

Knockout of miR-21 Attenuates PPARα Downregulation and Ameliorates Inflammation in the Retina of db/db Mice

A retinal leukostasis assay was performed to investigate the role of miR-21 in retinal inflammation in the diabetic retina (Fig. 5A). Quantification of adherent leukocytes in flat-mounted retinas showed that dKO mice had fewer adherent leukocytes per retina compared with control db/db (miR-21−/−) mice (Fig. 5B). In addition, PPARα levels were significantly higher in the retinas of dKO mice compared with control db/db mice (Fig. 5C and D), suggesting that knockout of miR-21 prevented diabetes-induced
downregulation of retinal PPARα. In addition, retinal levels of inflammatory factors (TNF-α, VCAM-1, and VEGF) were decreased in dKO mice relative to db/db mice (Fig. 5C and E–G). Taken together, these results suggested that ablation of miR-21 attenuated PPARα downregulation and reduced retinal inflammation in an animal model of type 2 diabetes.

**Intravitreal Delivery of miR-21 Inhibitor Attenuates PPARα Downregulation and Suppresses Retinal Inflammation in db/db Mice**

To investigate the therapeutic potential of miR-21 inhibitor in DR, we intravitreally delivered nanoparticles containing miR-21 inhibitor or control miRNA into db/db mice. Retinal levels of miR-21 were decreased in db/db mice injected with miR-21 inhibitor nanoparticles (Fig. 6A), suggesting a successful delivery of the inhibitor into the retina. Retinal levels of PPARα were increased in db/db mice injected with miR-21 inhibitor nanoparticles compared with those injected with the control nanoparticles (Fig. 6B and C). Moreover, expression of inflammatory factors (TNF-α, VCAM-1, and VEGF) was decreased in the retina of db/db mice injected with miR-21 inhibitor nanoparticles (Fig. 6B and D–F), indicating that miR-21 could be a therapeutic target in DR.
Upregulated miR-21 and Downregulated PPARα in OIR
To explore the role of miR-21 in retinal neovascularization, we measured retinal miR-21 and PPARα levels in a mouse model of OIR (Fig. 7A), a commonly used model for proliferative retinopathy (31). Flat-mounted OIR retinas showed areas of vaso-obliteration and neovascularization compared with normoxic control retinas (Fig. 7B). Retinal miR-21 levels were upregulated in OIR mice compared with normoxic mice (Fig. 7C), and protein levels of PPARα were decreased in OIR retinas (Fig. 7D and E). In addition, the retinal stress marker GFAP was substantially upregulated in OIR retinas compared with the normoxic controls (Fig. 7F and G), demonstrating retinal stress in OIR retinas.

Knockout of miR-21 Attenuates PPARα Downregulation and Suppresses the Neovascularization and Inflammation in OIR Retinas
We further investigated the role of miR-21 in PPARα downregulation in OIR retinas. Under normoxic conditions, miR-21−/− mice showed no detectable retinal vasculature changes compared with WT mice at P16 (Supplementary Fig. 3). However, miR-21−/− OIR mice showed smaller areas of vaso-obliteration and retinal neovascularization compared with WT OIR mice at P16 (Fig. 8A–C), indicating an antiangiogenic effect of miR-21 knockout in OIR retinas. Moreover, PPARα was upregulated in miR-21−/− OIR retinas relative to WT OIR retinas, confirming that miR-21 played a role in PPARα downregulation in the retinas under ischemia (Fig. 8D and E). Furthermore, retinal levels of inflammatory factors GFAP, TNF-α, and VEGF (Fig. 8D and F–H) and retinal cell apoptosis (DNA fragmentation) (Fig. 8I) were decreased in miR-21−/− OIR retinas compared with WT OIR retinas. Taken together, these results indicated that knockout of miR-21 attenuated ischemia-induced PPARα decreases and ameliorated retinal inflammation, apoptosis, and neovascularization.

DISCUSSION
PPARα is an important transcription factor that regulates lipid metabolism and energy homeostasis (7,8). The PPARα agonist fenofibrate has shown robust beneficial effects on DR in patients with type 2 diabetes (5,6). Our previous
study showed that PPARα levels were decreased in the retina of human donors with diabetes and diabetic animal models (10). Diabetes-induced PPARα downregulation has been shown to play a key role in retinal inflammation in DR (10,11). To understand the mechanism for PPARα downregulation in the diabetic retina, we investigated the roles of miRNAs in the regulation of PPARα and identified that miR-21 was overexpressed in the retina of a type 2 diabetic mouse model, in a mouse model of OIR, and in retinal endothelial cells exposed to oxidative stress, which was correlated with the decreased PPARα expression. We have also verified that PPARα is a target gene of miR-21 in retinal cells. Knockout of miR-21 alleviated microvascular damage, attenuated diabetes-induced PPARα downregulation, ameliorated inflammation, and reduced cell apoptosis in the retina of db/db mice and OIR mice. Consistently, intravitreal delivery of miR-21 inhibitor attenuated PPARα downregulation and suppressed retinal inflammation in db/db mice. These findings provide the first evidence that overexpression of miR-21 is at least partly responsible for PPARα downregulation and retinal inflammation in DR. Our study has also revealed that miR-21 overexpression is a novel pathogenic mechanism and a potential therapeutic target for DR.

Because fenofibrate was reported to have benefits on DR in patients with type 2 diabetes (5,6), we chose the db/db mouse, a type 2 diabetic model, to study the mechanism responsible for diabetes-induced PPARα downregulation. The db/db mice display pathological changes of DR, including overexpression of VEGF, blood–retinal barrier breakdown, leukostasis, loss of pericytes, retinal capillary degeneration, and retinal neuron apoptosis (32,33). Our results showed that PPARα levels were significantly decreased in db/db retinas, which is consistent with our recent study demonstrating that PPARα levels were decreased in the retina of diabetic animal models (10). To define whether miRNAs play roles in the regulation of PPARα, we performed a miRNA microarray analysis in 6-month-old db/db retinas. We analyzed all of the upregulated miRNAs with twofold or more changes in the microarray. Bioinformatics analysis shows that miR-21 is one of only two miRNAs that have conserved binding sites in the 3′ UTR of both human and mouse PPARα mRNAs (Supplementary Table 1). As verified by qRT-PCR, miR-21 was significantly upregulated in the retina of db/db mice relative toagematched nondiabetic controls. In addition, miR-21 has been implicated in a variety of diseases, especially cancer and cardiovascular disease (34,35); however, the implication
of miR-21 in ocular disorders has not been documented previously. Here, we showed for the first time that miR-21 is upregulated in the retina of db/db mice and OIR mice, a model of proliferative retinopathy. These findings were consistent with a recent study showing that miR-21 was increased in the vitreous humor of patients with proliferative DR, supporting that miR-21 may play a role in DR (36).

To confirm whether the altered miR-21 and PPARα levels also occurred in diabetic conditions in vitro, we treated retinal endothelial cells (RECs) with palmitate, a saturated fatty acid commonly used as a diabetic stressor. Palmitate induces oxidative stress and causes cell dysfunction and apoptosis in many cell types, including pericytes and endothelial cells (28,29). Our results demonstrated that palmitate induced miR-21 upregulation and decreased expression of PPARα in RECs, suggesting a role of miR-21 overexpression in downregulating PPARα levels in RECs under diabetic conditions (22).

miRNAs are known to regulate target genes at the posttranscriptional level through two mechanisms: translational repression and mRNA destabilization (13). Zhou et al. (22) reported that protein levels of PPARα were altered by transfection of miR-21 mimic or miR-21 inhibitor in cultured cells and that the deletion of miR-21 binding sites abolished its regulatory effects on PPARα, suggesting miR-21 regulates PPARα by targeting its 3′ UTRs. Meanwhile, they have also shown no detectable alteration in PPARα mRNA levels in the transfected cells. Using pull-down assay, they have found that the association of miR-21 with miRNA-induced silencing complexes (miRISCs) was reduced by miR-21 inhibitor and induced by miR-21 mimic. In addition, Kida et al. (30) reported that levels of PPARα protein, but not its mRNA, were
decreased by the transfection of miR-21 mimic in liver cells. To define whether miR-21 targets PPARα in the retina, we transfected miR-21 mimic or miR-21 inhibitor into RPE cells and HRMECs. miR-21 mimic decreased and miR-21 inhibitor increased the PPARα levels in the transfected cells, suggesting that PPARα is a target gene of miR-21 in the retina. In addition, we found that PPARα mRNA levels were not changed in these cells. Our results supported that PPARα is a target gene of miR-21 in the retina. In addition, we found that PPARα mRNA levels were not changed in these cells. Our results supported that PPARα is a target gene of miR-21 in the retina.

A recent study reported that miR-21 indirectly upregulated hypoxia inducible factor 1α (HIF-1α) expression in the human prostate cancer DU145 cells (37). In addition, PPARα was also reported to be regulated by HIF-1α in intestinal epithelial cells (38). To investigate whether HIF-1α mediated downregulation of PPARα in DR, we measured HIF-1α protein levels in the retina of db/db mice and control mice. However, HIF-1α levels were not significantly changed (Supplementary Fig. 4A and B), although miR-21 levels were significantly higher and PPARα levels were significantly lower in db/db retinas compared with nondiabetic control retinas. In addition, we measured HIF-1α levels in db/db retinas injected with miR-21 inhibitor or control inhibitor, and no alteration of HIF-1α levels was found in these retinas after the injection (Supplementary Fig. 4C and D). These results suggest that the regulation of PPARα may be cell-type specific, and HIF-1α is unlikely to play a critical role in the regulation of PPARα in the diabetic retina.

To establish the regulatory role of miR-21 in PPARα in the diabetic retina, we have used two different models: miR-21–deficient db/db mice and OIR mice. Knockout of miR-21 attenuated PPARα downregulation in the retina of db/db mice and OIR mice. These results demonstrated a causative role for miR-21 overexpression in PPARα downregulation in DR. We also measured retinal levels of PPARα in miR-21−/− mice under normal conditions and found that retinal levels
of PPARα were not changed in miR-21<sup>−/−</sup> mice compared with WT control mice (Supplementary Fig. 5), suggesting that miR-21–mediated PPARα regulation in the retina may be a dominant process only under diabetic conditions.

Studies have shown that chronic inflammation is a major pathogenic factor of DR and that diabetes-induced retinal inflammation may result in endothelium impairment, pericyte loss, increased capillary degeneration, and vascular leakage in DR (39,40). Our previous study demonstrated more severe retinal vascular damage and higher levels of retinal inflammatory cytokines in diabetic PPARα knockout mice relative to diabetic WT mice (10). Overexpression of PPARα reduced retinal microvascular impairment and retinal inflammation in diabetic rats (10). In this study, we demonstrated that ablation of miR-21 attenuated PPARα downregulation, alleviated microvascular damage, and decreased the expression of inflammatory factors (TNF-α, VCAM-1, and VEGF) in the retina under ischemia and diabetic conditions. Because there are no predicted miR-21-binding sites in the 3′ UTR of the mRNAs of the inflammatory factors (TNF-α, VCAM-1, and VEGF) and PPARα is a direct target gene of miR-21, the anti-inflammatory effect of miR-21 knockout in DR is most likely through modulating PPARα levels in the retina, thus attenuating microvascular impairment. This speculation is supported by early studies which have demonstrated that PPARα agonists have anti-inflammatory effects. For instance, a PPARα agonist inhibited
thrombin-induced endothelin-1 biosynthesis in human vascular endothelial cells (41). In RPE cells, upregulation of PPARα decreased Toll-like receptor 4 (TLR4) levels and inhibited the nuclear factor-κB signaling pathway induced by lipopolysaccharide (42).

Indeed, intravitreal delivery of miR-21 inhibitor in db/db mice attenuated PPARα downregulation and reduced the expression levels of inflammatory factors, thus inhibiting retinal inflammation and suggesting that miR-21 could be a therapeutic target for DR. These results also supported that a pathogenic role of miR-21 in DR is through modulating PPARα levels. Interestingly, several studies have also shown the benefits of reduced miR-21 levels in other diabetic complications. Seeger et al. (43) reported that the systemic injection of miR-21 inhibitor reduced body weight, adipocyte size, and serum triglycerides in db/db mice. Moreover, a recent study reported that gene transfer of miR-21 knockdown plasmids into the kidneys of db/db mice reduced microalbuminuria, renal fibrosis, and inflammation (44), further suggesting the benefits of reduced miR-21 levels in suppressing inflammation in diabetes.

In addition to the anti-inflammatory role of miR-21 knockout, our results demonstrated that knockout of miR-21 suppressed retinal neovascularization in OIR, indicating a proangiogenic effect of miR-21 in DR. The proangiogenic effect of miR-21 was supported by a previous study (37) which reported that miR-21 induced tumor angiogenesis in prostate cancer cells by targeting phosphatase and tensin homolog (PTEN), leading to activation of the protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) 1/2 signaling pathways and thus upregulating HIF-1α and VEGF expression. A recent study, however, showed an antiangiogenic effect of miR-21, because miR-21 targeted RhoB, inhibited endothelial cell migration and tubulogenesis, and suppressed angiogenesis in a mouse model of choroidal neovascularization (45). The disparities regarding the role of miR-21 in angiogenesis reveal that miR-21 may play differential roles in different cell types or under different conditions.

In summary, this study showed that downregulation of PPARα in DR is at least partly mediated through the overexpression of miR-21 in the diabetic retina. This study suggested that miR-21–induced PPARα downregulation represents a new pathogenic mechanism for DR and that miR-21 could be a novel therapeutic target for DR.

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Author Contributions. Q.C. designed research studies, conducted experiments, acquired and analyzed data, and wrote the manuscript. F.O., K.Z., H.G.M., and Y.Y. conducted experiments and acquired data. Y.T., R.V.S.R., and E.M. designed research studies and analyzed the data. J.-x.M. designed research studies, analyzed data, and wrote the manuscript. Y.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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