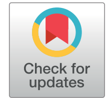

Pathogenic Role of *microRNA-21* in Diabetic Retinopathy Through Downregulation of PPARα

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Diabetes 2017;66:1671–1682 | https://doi.org/10.2337/db16-1246

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 overexpression 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

COMPLICATIONS

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Received 12 October 2016 and accepted 1 March 2017.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-1246/-/DC1.

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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-*Dock7*$^{m+/+}$ *Lepr*^{db}/J (*db/db*), C57BLKS/J (nondiabetic control), B6;129S6-Mir21a^{tm1Yoli}/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*^{rd1}), retinal degeneration 8 (*Crb1*^{rd8}), and retinal degeneration 10 (*Pde6b*^{rd10}) 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* isolectin 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

1 (VCAM-1; cat# SC-8304) was purchased from Santa Cruz Biotechnology. Antibodies for glial fibrillary acidic protein (GFAP; cat# G3893) and β-actin (cat# A5441) were purchased from Sigma-Aldrich.

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 mm^2) 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 nondiabetic control mice (Fig. 1*A* 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. 1*C*). 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 nondiabetic mice (Fig. 1*D*).

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 SEVEC4-10 cells treated with palmitate relative to vehicle-treated cells (Fig. 2*A* and *B*). In addition, *miR-21* levels were elevated in palmitate-treated

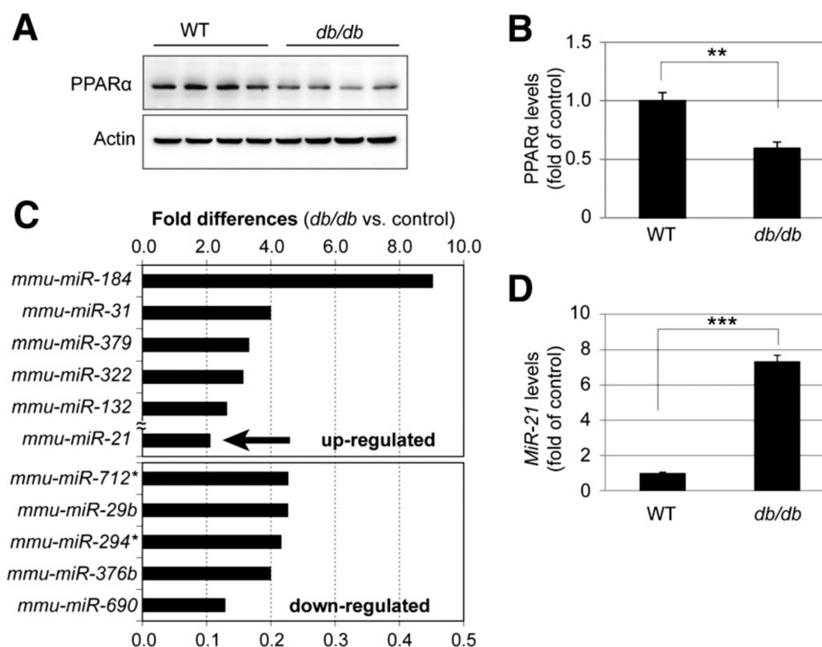

Figure 1—Altered levels of PPARα and *miR-21* in *db/db* retinas. *A:* Western blot analysis of PPARα in the retinas from 6-month-old *db/db* mice and nondiabetic mice (WT) matched for age and genetic background. *B*: Protein levels of PPARα were semiquantified by densitometry and normalized by actin levels (n = 8). **P < 0.01. *C*: A miRNA microarray was performed using retinal RNA from 6-month-old *db/db* and WT mice (n = 5). The most significantly changed miRNAs are shown. *D*: Retinal levels of *miR-21* in *db/db* mice were analyzed by qRT-PCR and expressed as fold changes over that in the age-matched WT controls (n = 10). ***P < 0.001.

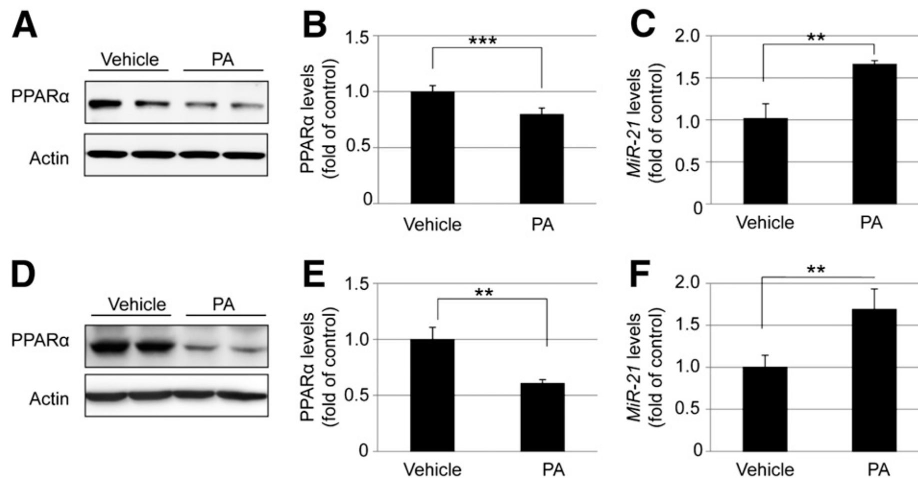

Figure 2—Palmitate-induced downregulation of PPARα and upregulation of *miR-21* in retinal endothelial cells. *A* and *B*: SEVEC4-10 cells were treated with palmitate (PA) or vehicle. Protein levels of PPARα were measured by Western blot analysis (*A*) and semiquantified by densitometry (*B*). *C*: *miR-21* levels in palmitate-treated SVEC4-10 cells and vehicle-treated cells were measured by qRT-PCR. Similarly, PPARα protein levels were measured by Western blot analysis (*D*) and semiquantified using densitometry (*E*) in HRMECs treated with palmitate or vehicle. *F*: *miR-21* levels in palmitate-treated or vehicle-treated HRMECs were measured by qRT-PCR. Data are representative of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

SEVEC4-10 cells (Fig. 2*C*). Similarly, PPARα protein levels were decreased (Fig. 2*D* and *E*), and *miR-21* levels were elevated in palmitate-treated HRMECs (Fig. 2*F*).

miR-21 Targets *PPARα* in Retinal Cells

The *miR-21* sequence is conserved across species, including the human, mouse, and rat mRNA (Fig. 3*A*). 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. 3*B*). 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. 3*C*, *D*, *G*, and *H*), and transfection of *miR-21* inhibitor significantly increased protein levels of PPARα (Fig. 3*E*, *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 preformed to investigate and compare diabetes-induced acellular capillaries between *db/db* mice and *dKO* mice (Fig. 4*A*). The *dKO* mice had fewer acellular capillaries in the retina compared with *db/db* mice (Fig. 4*B*), 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. 4*C*). The results showed that retinal levels of extravasated albumin were significantly lower in the *dKO* mice compared with *db/db* mice (Fig. 4*D*), 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. 4*E*), 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. 5*A*). Quantification of adherent leukocytes in flat-mounted retinas showed that *dKO* mice had fewer adherent leukocytes per retina compared with control *db/db* ($miR\text{-}21^{+/+}$) mice (Fig. 5*B*). In addition, PPARα levels were significantly higher in the retinas of *dKO* mice compared with control *db/db* mice (Fig. 5*C* and *D*), suggesting that knockout of *miR-21* prevented diabetes-induced

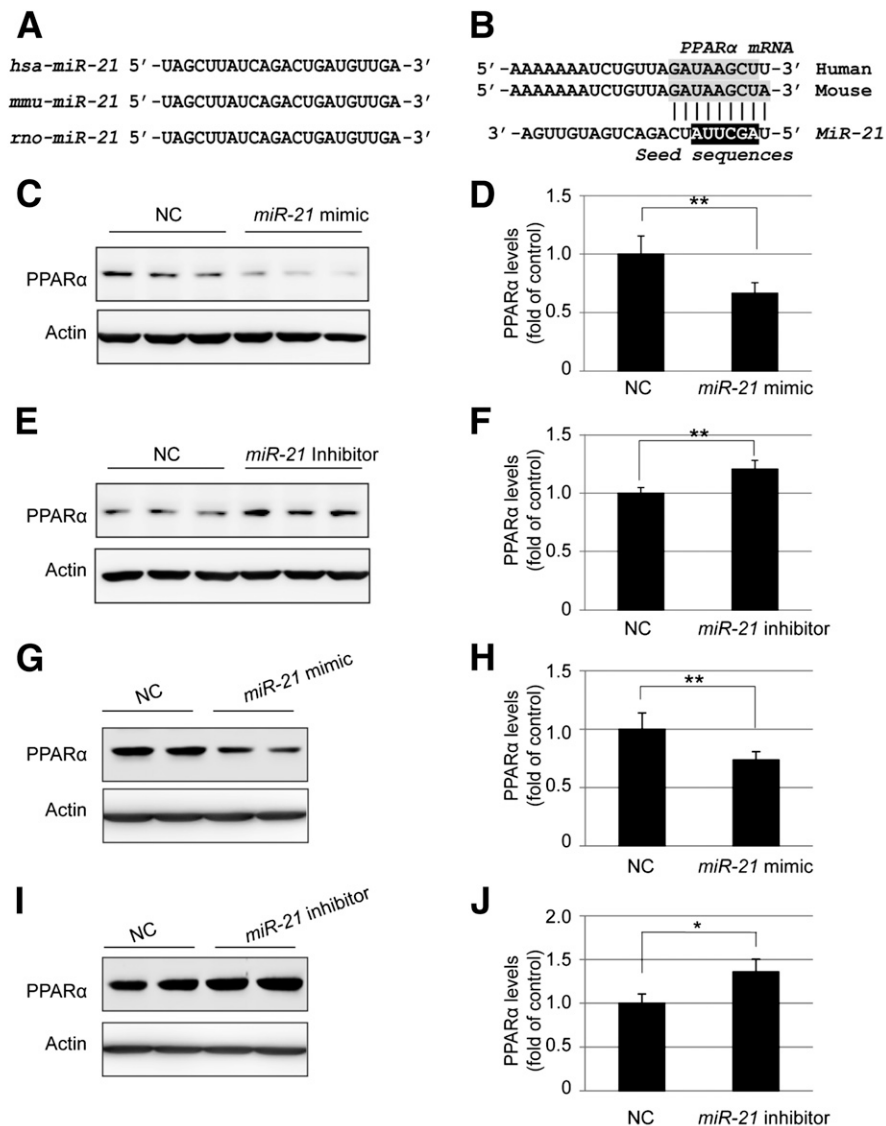

Figure 3—*miR-21* targeted *PPARα* in retinal cells. *A*: The *miR-21* sequence is conserved among human, mice, and rat. *B*: Alignment of 3′ UTR of *PPARα* mRNA with mature *miR-21* based on bioinformatics predictions (www.TargetScan.org and www.microrna.org). The seed sequence of mature *miR-21* (highlighted) has perfect complementarity with the nucleotides of the 3′ UTR of the mouse and human *PPARα* mRNAs. *miR-21* mimic (*C* and *D*), *miR-21* inhibitor (*E* and *F*), and their negative controls (NC) were separately transfected into hTERT–RPE-1 cells. Protein levels of PPARα were measured by Western blot analysis (*C* and *E*) and semiquantified by densitometry (*D* and *F*). Similarly, *miR-21* mimic (*G* and *H*) and *miR-21* inhibitor (*I* and *J*) were transfected into HRMECs, and PPARα levels were measured by Western blot analysis (*G* and *I*) and semiquantified (*H* and *J*). Data are representative of three independent experiments. *$P < 0.05$; **$P < 0.01$.

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. 5*C* 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. 6*A*), 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. 6*B* 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. 6*B* and *D*–*F*), indicating that *miR-21* could be a therapeutic target in DR.

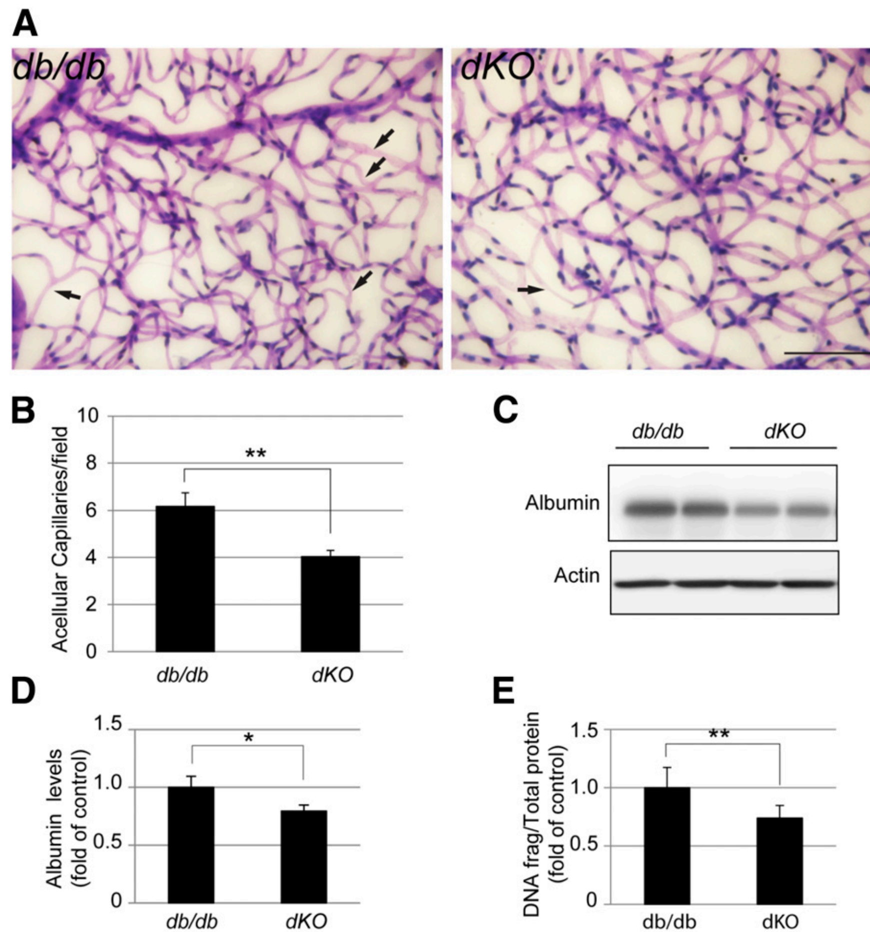

Figure 4—Knockout of *miR-21* attenuates microvascular damage in the retina of *db/db* mice. *A*: Representative images of retinal trypsin digestion assay in *dKO* mice and age-matched control *db/db* mice. Black arrows indicate acellular vessels. Scale bar: 100 μm. *B*: Acellular vessels were quantified in flat-mounted retinas of *dKO* and *db/db* mice (n = 8). $^{**}P < 0.01$. *C*: Protein levels of extravasated albumin were measured by Western blot analysis. *D*: Levels of extravasated albumin were quantified by densitometry (n = 10). $^{*}P < 0.05$. *E*: Apoptosis in the retina of *dKO* and *db/db* mice was measured using cell death ELISA (n = 8). $^{**}P < 0.01$.

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. 7*A*), 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. 7*B*). Retinal *miR-21* levels were upregulated in OIR mice compared with normoxic mice (Fig. 7*C*), and protein levels of PPARα were decreased in OIR retinas (Fig. 7*D* and *E*). In addition, the retinal stress marker GFAP was substantially upregulated in OIR retinas compared with the normoxic controls (Fig. 7*F* 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. 8*A*–*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. 8*D* and *E*). Furthermore, retinal levels of inflammatory factors GFAP, TNF-α, and VEGF (Fig. 8*D* and *F*–*H*) and retinal cell apoptosis (DNA fragmentation) (Fig. 8*I*) 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

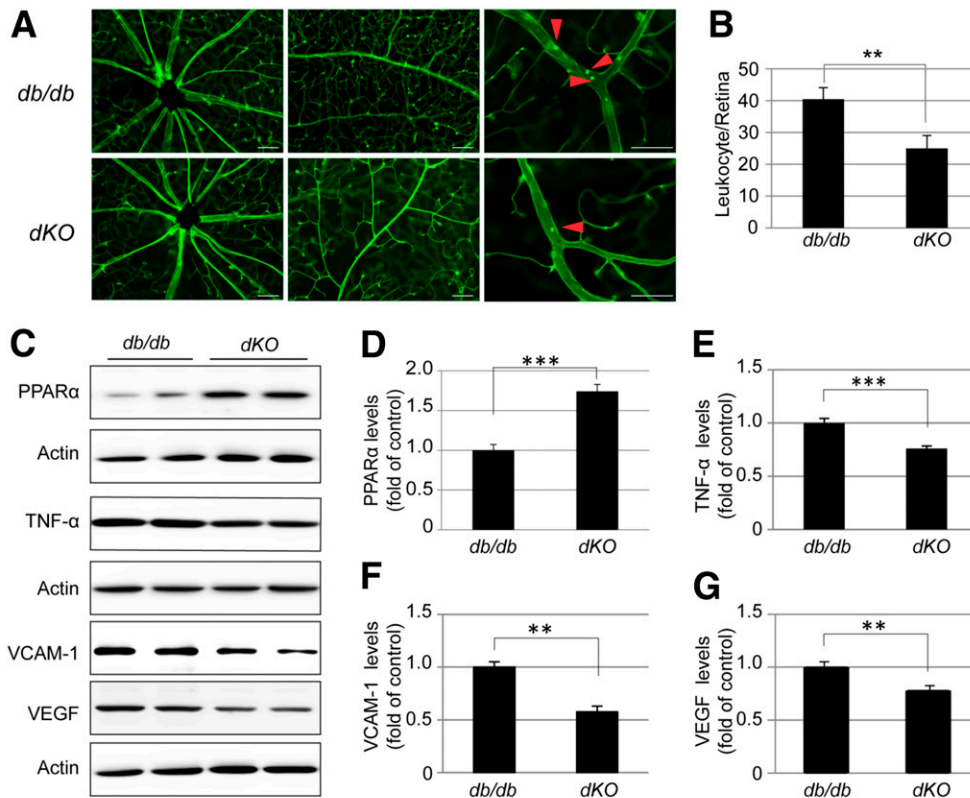

Figure 5—Knockout of *miR-21* attenuated PPARα downregulation and ameliorated retinal inflammation and retinal cell apoptosis in *db/db* mice. *A*: Representative images of retinal leukostasis assay in *dKO* mice and age-matched control *db/db* mice. Red arrowheads indicate adherent leukocytes. Scale bars: 100 μm. *B*: Adherent leukocytes were quantified in flat-mounted retinas of *dKO* and *db/db* mice (n = 4). $^{**}P < 0.01$. *C*: Protein levels of PPARα, TNF-α, VCAM-1, and VEGF were measured by Western blot analysis. Levels of PPARα (*D*), TNF-α (*E*), VCAM-1 (*F*), and VEGF (*G*) were quantified by densitometry (n = 6–8). $^{**}P < 0.01$; $^{***}P < 0.001$.

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 to age-matched nondiabetic controls. In addition, *miR-21* has been implicated in a variety of diseases, especially cancer and cardiovascular disease (34,35); however, the implication

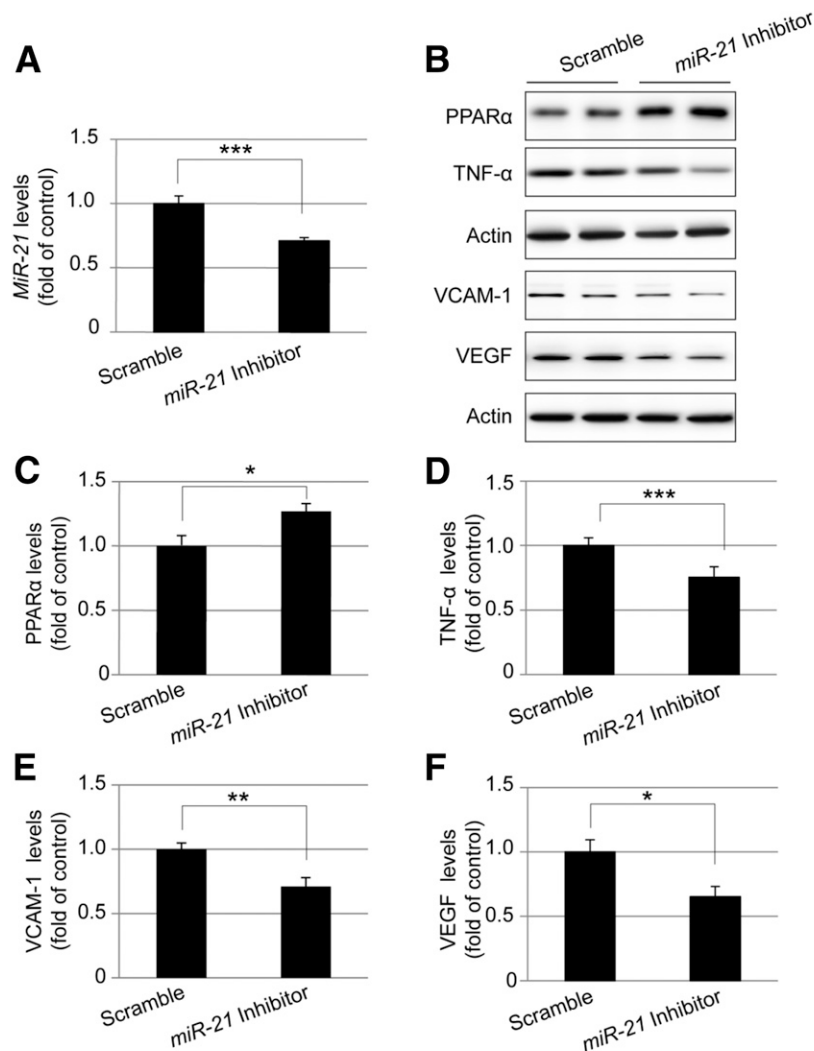

Figure 6—Delivery of *miR-21* inhibitor attenuated PPARα downregulation and suppressed retinal inflammation in *db/db* mice. *A*: Retinal levels of *miR-21* were measured in *db/db* mice injected with negative control nanoparticles or *miR-21* inhibitor nanoparticles (n = 6). ***P < 0.001. *B*: Protein levels of PPARα and inflammatory factors (TNF-α, VCAM-1, and VEGF) were measured by Western blot analysis in the retina of *db/db* mice injected with negative control nanoparticles or *miR-21* inhibitor nanoparticles. Levels of PPARα (*C*), TNF-α (*D*), VCAM-1 (*E*), and VEGF (*F*) were quantified by densitometry (n = 10–12). *P < 0.05; **P < 0.01; ***P < 0.001.

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

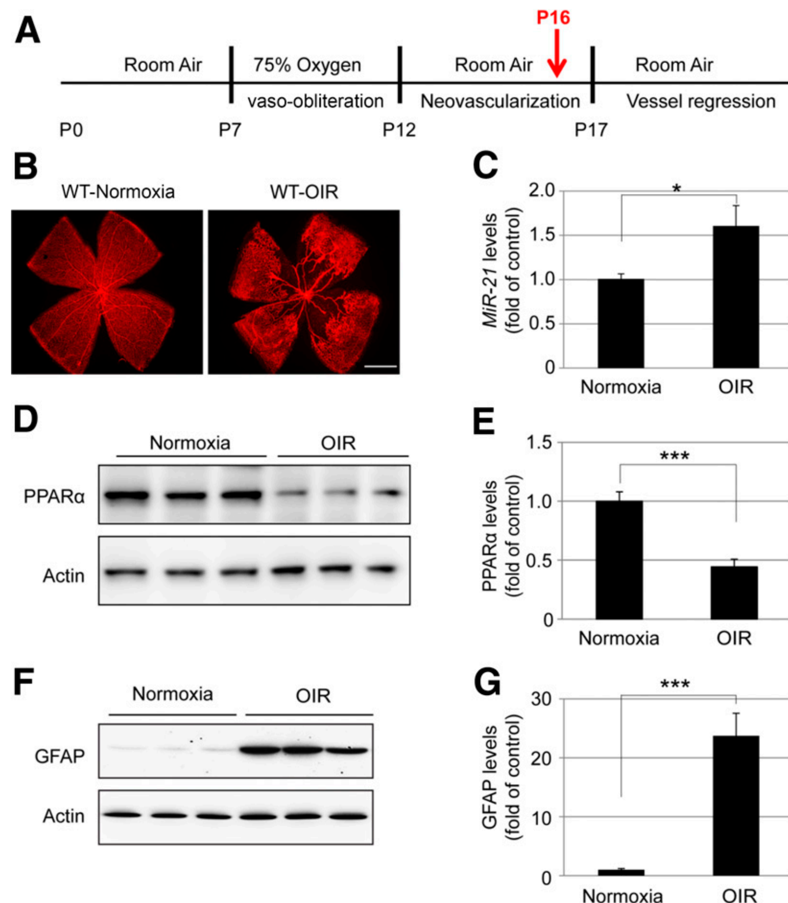

Figure 7—Upregulated *miR-21* and downregulated PPARα in OIR. *A*: Diagram of OIR. C57BL/6J mice were exposed to 75% oxygen from P7 to P12 to induce retinal vaso-obliteration and returned to room air at P12 to induce pathological neovascularization. At P16, the retinas were isolated for further analysis. *B*: Retinas from OIR mice and normoxic controls at P16 were flat-mounted and stained by isolectin B4 (red). A representative image from OIR mice or normoxic mice is shown ($n = 8$). Scale bar: 1,000 μm. *C*: *MiR-21* levels were measured by qRT-PCR in normoxic control retinas and OIR retinas ($n = 8$). $^{*}P < 0.05$. *D*: Retinal levels of PPARα were measured by Western blot analysis in normoxic control and OIR mice at P16. *E*: PPARα levels were quantified by densitometry and normalized by actin levels ($n = 6$). $^{***}P < 0.001$. Retinal levels of GFAP in normoxic controls and OIR mice were measured by Western blot analysis (*F*) and quantified by densitometry (G) ($n = 6$). $^{***}P < 0.001$.

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 *miR-21* regulates PPARα expression by translational repression of its mRNA, which is consistent with the studies by Zhou et al. (22) and Kida et al. (30).

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. 4*A* 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. 4*C* 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

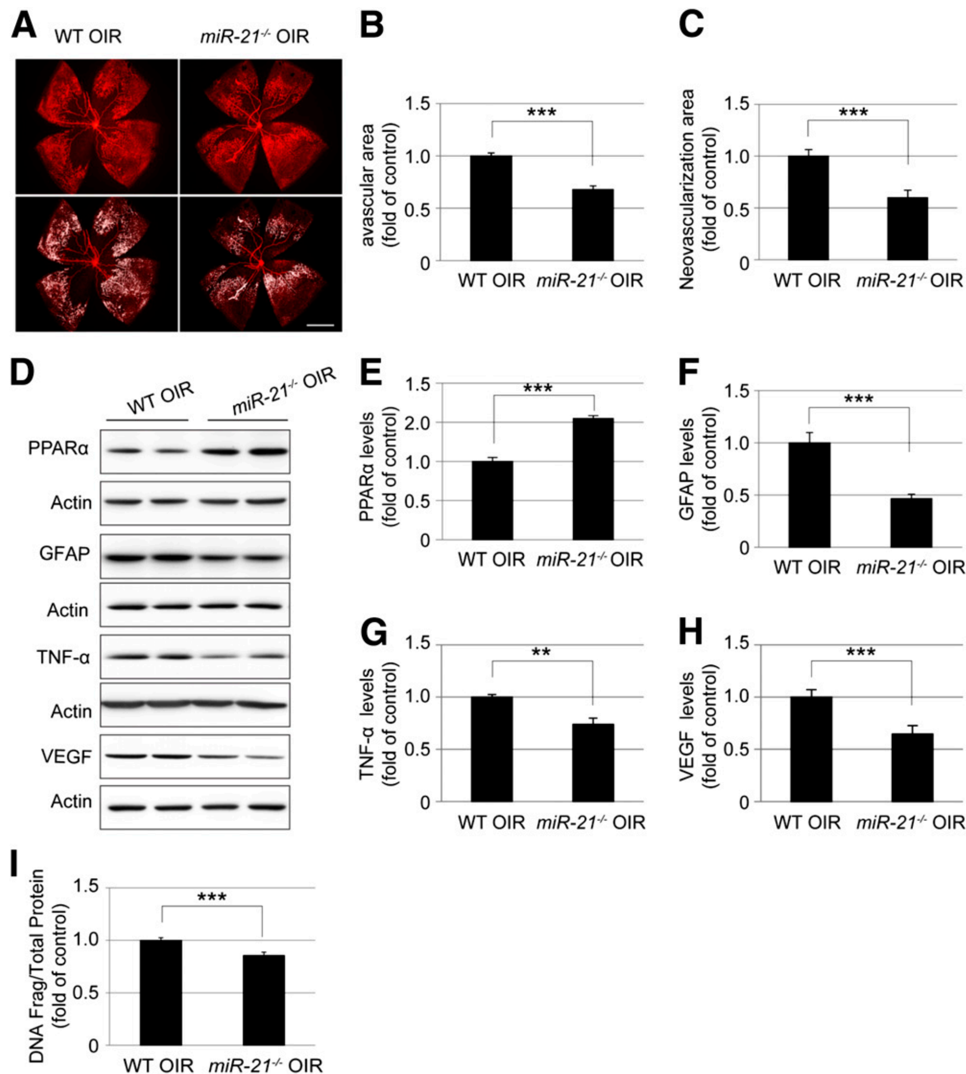

Figure 8—Knockout of *miR-21* attenuated PPARα downregulation and suppressed the retinal neovascularization and inflammation in OIR mice. *A*: Flat-mounted retinas of WT OIR mice and *miR-21*$^{-/-}$ OIR mice at P16 were stained with isolectin B4 (red). Areas of pathological neovascularization were labeled (white highlight). Scale bar: 1,000 μm. Areas of retinal vaso-obliteration (*B*) and neovascularization (*C*) in the OIR retina of WT and *miR-21*$^{-/-}$ mice were quantified (n = 6–8). ***P < 0.001. *D*: Retinal levels of PPARα, GFAP, TNF-α, and VEGF were measured by Western blot analysis in WT OIR mice and *miR-21*$^{-/-}$ OIR mice at P16. Retinal levels of PPARα (E), GFAP (*F*), TNF-α (*G*), and VEGF (*H*) were quantified by densitometry (n = 6). **P < 0.01; ***P < 0.001. *I*: Retinal cell apoptosis in WT and *miR-21*$^{-/-}$ OIR retinas was measured by cell death ELISA (n = 12). ***P < 0.001.

of PPARα were not changed in *miR-21*$^{-/-}$ 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 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.

Acknowledgments. The authors thank Dr. Xiang Wang, Centers for Biomedical Research Excellence (COBRE) Image Core Facility at the University of Oklahoma Health Sciences Center, for his assistance in image acquisition.

Funding. This study was supported by the National Institutes of Health (EY-012231, EY-018659, EY-019309, and GM-104934), a grant from the Oklahoma Center for the Advancement of Science & Technology (HR16-041), a grant from Harold Hamm Diabetes Center (Seed Grant), and a fellowship from American Heart Association (14PRE20460229).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. Q.C. designed research studies, conducted experiments, acquired and analyzed data, and wrote the manuscript. F.Q., 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.

Prior Presentation. Parts of this study were presented in abstract form at the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, Seattle, WA, 1–5 May 2016.

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