Intraocular Delivery of miR-146 Inhibits Diabetes-Induced Retinal Functional Defects in Diabetic Rat Model

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Submitted: December 2, 2016
Accepted: February 10, 2017

Citation: Zhuang P, Muraleedharan CK, Xu S. Intraocular delivery of miR-146 inhibits diabetes-induced retinal functional defects in diabetic rat model. *Invest Ophthalmol Vis Sci*. 2017;58:1646–1655. DOI:10.1167/iovs.16-21223

Purpose. Previously, we showed that microRNA-146 (miR-146) is a pivotal negative feedback regulator of multiple nuclear factor kappa-B (NF-κB) activation pathways in retinal endothelial cells (RECs). We hypothesized that miR-146 plays an important role in diabetic retinopathy (DR) by inhibiting diabetes-induced inflammatory response in the retina. The purpose of the current study is to test this hypothesis in vivo.

Methods. Lentiviruses expressing rno-miR-146a, lenti-miR-146a, and negative control oligonucleotide with scrambled sequence, lenti-miR-neg ctl, were produced. Young male Sprague-Dawley rats were injected with a single dose of streptozotocin ([STZ] 65 mg/kg) to induce diabetes. One week after diabetes, animals were injected with lentivirus intravitreally (4 µl, ~10^6 CFU/mL). Three months after diabetes, retinal microvascular leakage was tested by Evans blue assay; retinal function by electroretinogram (ERG). Total RNA and protein lysate were isolated from the retina for quantitative (q)RT-PCR and Western blot analyses.

Results. Lenti-miR-146a robustly transduced human retinal endothelial cells (HRECs) and increased the expression of miR-146a in vitro. In vivo, intravitreal injection of lenti-miR-146a increased the expression of miR-146a in the retina, while its key downstream target genes, including CARD10, IRAK1, and TRAF6, were downregulated. Intravitreal delivery of miR-146 inhibited diabetes-induced upregulation of NF-κB downstream gene, Intercellular Adhesion Molecule 1 (ICAM1), as well as microvascular leakage and retinal functional defects.

Conclusions. Intravitreal delivery of miR-146 inhibited diabetes-induced NF-κB activation and retinal microvascular and neuronal functional defects in a diabetic rat model.

Keywords: microRNA-146, diabetic retinopathy, NF-κB, retinal endothelial cell, microvascular leakage

Diabetic retinopathy (DR) is the leading cause of blindness in people between ages of 25 and 74 in the industrialized world.[1] Diabetes affects 200 million people worldwide, and 20 million in the United States alone.[2] Nearly all individuals who have had type I diabetes (T1D) for more than 15 years develop DR; approximately 50% to 80% of type II diabetic (T2D) patients also develop retinopathy after 20 years of diabetes.[3] Diabetic retinopathy is a result of multiple pathogenetic processes caused by hyperglycemia and abnormalities of insulin signaling pathways,[4,5] leading to retinal microvascular defects[6] and neuroretinal dysfunction and degeneration.[7] Although significant progress has been made, especially with recent advances involving blocking VEGF pathway,[8–10] there is still no efficient treatment. Development of novel therapy to prevent and treat DR is of great urgency to improve the quality of life of patients and alleviate mounting economic burden.[11]

MicroRNAs (miRNAs) are small, noncoding, regulatory RNAs.[12] Since their discovery in 1993, miRNAs have been proven to be an important mechanism of fine-tuning of gene expression[12–15] and play regulatory roles in almost all aspects of normal biological functions[14–34] and diseases.[35] However, roles of miRNAs in DR and its treatment are still largely unknown. Previously, we reported one of the first miRNA transcriptomes of the retina and retinal endothelial cells (RECs) of diabetic rats, and identified a series of miRNAs involved in early DR.[36] Among DR-related miRNAs, we demonstrated that miR-146 is a pivotal negative feedback regulator of nuclear factor kappa-B (NF-κB) activation.[36,37] Nuclear factor kappa-B is a master regulator of inflammatory responses, and plays critical roles in inflammatory damages to RECs and retinal microvasculature during development of DR.[38–44] Nuclear factor kappa-B induces expression of proinflammatory molecules, including intercellular adhesion molecule 1 (ICAM1),[45] a key endothelial adhesion molecule to recruit leukocytes onto endothelial cell surface, and facilitate leukostasis and propagation of inflammatory responses, contributing to REC cell death and DR development.[46–49] We showed that miR-146 inhibited IL-1R/Toll-like receptor (TLR)-mediated NF-κB activation pathway by targeting key adaptor molecules, interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6),[36,50] and prevented IL-1β–induced damage to retinal endothelial barrier function in vitro.[37] Furthermore, we showed that miR-146 also inhibited G protein-coupled receptor (GPCR)-mediated NF-κB activation pathway by targeting a key adaptor molecule, Caspase Recruitment Domain Family, Member 10 (CARD10),[37,51] and decreased thrombin-induced leukocyte adhesion to HRECs in vitro.[37] These data suggest that miR-146 plays an important role in DR through modulating NF-κB activation and inflammatory responses.

Therefore, we hypothesize that overexpression of miR-146 in retinal microvasculature inhibits diabetes-induced NF-κB activation and prevents and/or slows down DR development. To test this hypothesis in vivo, we produced lentivirus expressing rno-miR-146a, lenti-miR-146a, and performed intravitreal injection of lentivirus in diabetic rats 1 week after streptozotocin (STZ)-induced diabetes. Here, we provide evidence that intraocular delivery of miR-146a inhibited diabetes-induced retinal microvascular and neuronal functional defects in vivo.

Materials and Methods

Lentiviral Production

Lenti-miR-146a and negative control construct expressing an oligonucleotide with scrambled sequence (lenti-miR-neg ctl; Fig. 1A) were purchased from Genecopoeia (Rockville, MD, USA). These constructs are built in the pEZX-MR03 vector (in the public domain, http://www.genecopoeia.com), a third generation HIV-based lentiviral vector system.[52,53] Lentivirus was packaged and titered following manufacturer's instructions. Briefly, 1.5×10^6 of the lentiviral packaging cells, 293Ta (Genecopoeia), were plated in a 10-cm dish in Dulbecco's Modified Eagles Medium (DMEM; HyClone Laboratory, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone). When the cells were 70% to approximately 80% confluent, 2.5 μg of plasmid DNA of lentiviral construct mixed with Lenti-Pac HIV and EndoFectin Lenti (Genecopoeia) was added to the medium. Twenty-four hours later, the medium was replaced with fresh DMEM + 5% FBS + penicillin (100 IU/mL) and streptomycin (100 μg/mL; HyClone). Then, 1/500 volume of the TiterBoost reagent (Genecopoeia) was added to the culture medium to enhance viral production. Subsequently, the medium was collected 48 hours posttransfection, and centrifuged at 500 *g* for 10 minutes to get rid of cell debris. The supernatant (lentiviral solution) was filtered through 0.45-μm polyethersulfone low protein-binding filters (Research Products International, Mt. Prospect, IL, USA), and stored in 100-μL aliquots at −80°C.

The lentivirus was titered in human primary retinal endothelial cells (Passage 4-6; Cell Systems, Kirkland, WA, USA). Briefly, 2×10^4 HRECs/well were plated in a 24-well plate in 500 μL of Endothelial Basal Medium-2 (EBM-2; Lonza, Basel, Switzerland) with 5% FBS and penicillin-streptomycin. Before infection, fresh media with 4 μg/mL of polybrene (Sigma-Aldrich Corp., St. Louis, MI, USA) was added. Then, 10 or 50 μL of lentivirus was used to infect HRECs. The plate was incubated at 37°C, 5% CO_2 for 5 days, with the medium changed every other day. The numbers of total and GFP-positive cells were counted in five random fields of view under fluorescent microscope. The titer of the lentiviral production was calculated as the number of colony forming units per milliliter of lentiviral solution.

Rats

Male Sprague-Dawley rats (~250 g) were purchased from Harlan Laboratory (Indianapolis, IN, USA). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A single dose of STZ (65 mg/kg in 50 mM citrate buffer [pH 4.0]; Sigma-Aldrich Corp.) was injected intraperitoneal injection to induce diabetes as we described previously.[36] Nondiabetic control rats were injected with equal amount of citrate buffer. Blood glucose level was detected using a FreeStyle *Lite* glucose meter

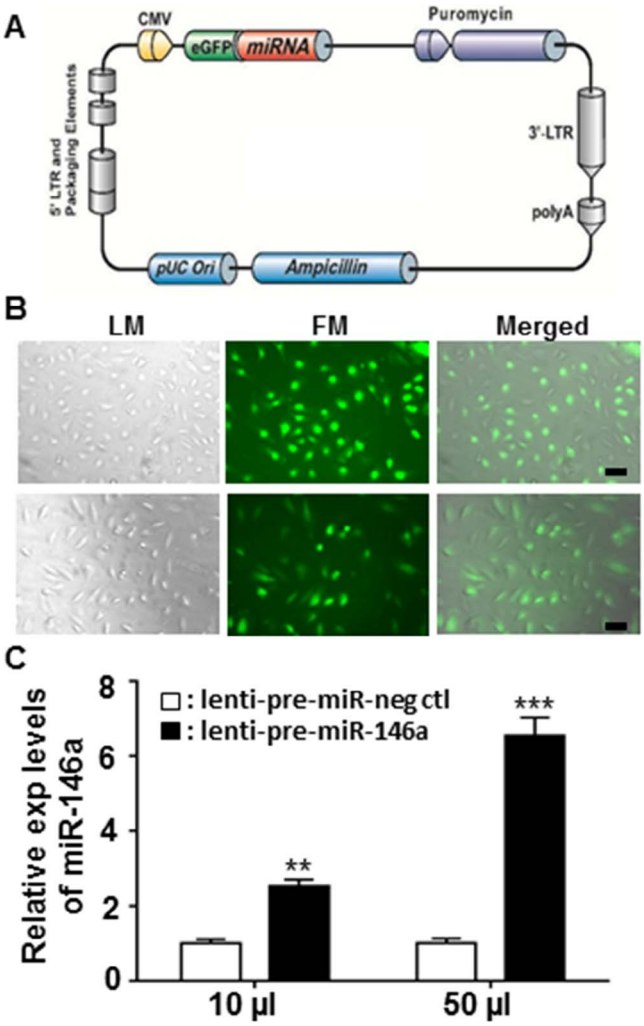

Figure 1. Lenti-miR-146a robustly infected and delivered miR-146a in HRECs in vitro. (**A**) Lentiviral construct. (**B**) Five days after transduction with 50 μL of lentivirus, HRECs were robustly infected and expressed GFP. *Upper row*: Lenti-miR-neg ctl (1.3×10^6 cfu/mL); *lower row*: lenti-miR-146a (1.0×10^6 cfu/mL). *Scale bar*: 100 μm. (**C**) Quantitative RT-PCR of miR-146a in HERCs 5 days after viral transduction. LM, light microscopy; FM, fluorescent microscopy.

(Abbott Diabetes Care, Inc., Alameda, CA, USA). Rats with blood glucose level greater than 250 mg/dL were deemed as diabetes (diabetes mellitus, DM).

One week after diabetes, rats were anesthetized with a ketamine (80 mg/kg)/xylazine (10 mg/kg) cocktail (Butler Schein, Dublin, OH, USA). Then, 4 μL of lenti-miR-146a (1.0×10^6 cfu/mL) was injected intravitreally into one eye; and 4 μL of lenti-miR-neg ctl (1.3×10^6 cfu/mL) into the other eye. For non-DM control rats, 4 μL of lenti-miR-neg ctl was injected intravitreally to serve as negative controls.

Body weight and blood glucose levels of the rats were checked biweekly. One-third Linplant (LinShin Canada, Toronto, Ontario) was implanted to the rats subcutaneously when their blood glucose levels were higher than 500 mg/dL so as to keep their blood glucose level at 300 to 500 mg/dL to avoid severe weight loss and ketoacidosis. Three months after lentiviral injection, electroretinogram (ERG) and Evans blue assays were performed to determine retinal function and the integrity of retinal microvasculature. The retina was harvested for RNA and protein preparation.

RNA Preparation and Quantitative RT-PCR

Total RNA from HRECs and the retina was prepared using miRVana miRNA isolation kit (Life Technologies, Carlsbad, CA, USA) as described previously.[36,37,54,55] Quantitative (q)RT-PCR of miRNAs was performed using TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA), with small nuclear (sn)RNA U6 as a normalization control. Quantitative RT-PCR of mRNAs was performed using QuantiTect primer assays and QuantiFast SYBR Green RT-PCR kit (Qiagen, Germantown, MD, USA), with 18s rRNA as a normalization control as described previously.[36,37,54,55]

Antibodies and Western Blot Analysis

The protein lysate from the retina was homogenized using a pellet pestle motor (Fisher Scientific, Chicago, IL, USA) in RIPA buffer with a protease inhibitor cocktail, including 0.5 μM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF); 0.4-μM aprotinin, 10-μM leupeptin, 20-μM bestatin, 7.5-μM pepstatin A, and 7.0-μM E-64 (Sigma-Aldrich Corp.). Western blot was performed following a standard protocol as we described previously.[36,56,57] Antibodies against rat CARD10 (1:200), TRAF6 (1:200), IRAK1 (1:200), and β-actin (1:500) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish Peroxidase (HRP)-conjugated secondary antibodies (1:5000) and enhanced chemiluminescence (ECL) detection reagents (GloBrite ECL Reagent Kit PLUS) were purchased from Detroit R&D (Detroit, MI, USA). Enhanced chemiluminescence signals were detected using a FluorChemE detector (ProteinSimple, San Jose, CA, USA). ImageJ 1.50e software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used to quantify the intensity of the bands. Relative quantity of protein of interest was normalized to β-actin.

ELISA Assay

The expression of ICAM1 was detected using ELISA kit (Cloud-Clone Corp, Houston, TX, USA) per manufacturer's instruction. Twenty micrograms of protein lysate of each sample was added to the precoated well. The detection range of the kit is 0.312 to 20 ng/mL.

Electroretinogram

Electroretinogram was performed using a hmsERG machine (OcuScience, Henderson, NV, USA) as we described previously[54] with modifications. Rats were dark-adapted overnight. Before the test, rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg; Butler Schein) and kept on a heat pad during the entire procedure to maintain body temperature. One percent tropicamide (Bausch & Lomb, Rochester, NY, USA) was applied to both eyes for 5 to 10 minutes for pupil dilation before ERG tests. Electroretinogram thread electrodes (OcuScience) were used for ERG recording. Electroretinogram tests were carried out sequentially at 10, 100, 1000 mcd s/m^2 with 5-minute interval between different intensities. Each response was recorded for 500 ms. Responses to 20 light flashes were averaged to produce one ERG recording at each light intensity.

Evans Blue Assay

Evans blue assay was performed as described previously.[58] Briefly, the animals were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg; Butler Schein), and injected with Evans blue (45 mg/kg body weight; Sigma-Aldrich Corp.) through the tail vein. Peripheral blood was drawn at 0.1 mL from the carotid artery every 20 minutes up to 2 hours after injection to obtain the time-averaged Evans blue plasma concentration. Two hours after Evans blue injection, the rats were perfused via the left ventricle with 0.05 M citrate buffer (pH 3.5) for 2 minutes. Eyes were enucleated after the perfusion; and the retinas were carefully dissected. The weight of each retina was measured after drying for 4 hours in a Vacufuge (Eppendorf, Hamburg, Germany). Evans blue in the retina was extracted by incubating the retina in 50 μL of formamide for 18 hours at 72°C. The extract was filtered through a 30-kDa Nanosep centrifugal filter (VWR International, Radnor, PA, USA) at 12,000 *g* for 120 minutes at 4°C. The absorbance of the filtrate was measured with a Nanodrop (Themo Scientific, Waltham, MA, USA) at 620 and 740 nm as the absorption maximum and minimum for Evans blue in formamide. Retinal microvascular permeability was calculated as nanograms of Evans blue per gram of retinal dry weight per hour, (ng/g retinal dry wt/hr) using the following formula:

$$\text{Concentration of Evans blue in retina} / (\text{time-averaged Evans blue concentration in the plasma} \times \text{retinal dry weight} \times \text{circulation time}). \quad (1)$$

Statistical Analysis

All data are shown as mean ± SEM. The statistical analysis between nondiabetic animals and diabetic animals was performed using 2-way ANOVA followed by Bonferroni posttest as appropriate; *t*-test was used to analyze the difference between the eyes injected with lenti-miR-146a and the ones injected with lenti-miR-neg ctl of the diabetic rats. Pearson correlation coefficient was used to analyze the correlation between Evans blue results and ERG results.

Results

Lenti-miR-146a Robustly Infected and Delivered rno-miR-146a in HRECs

To test whether the lentivirus that we produced can effectively transduce retinal endothelial cells, we first infected HRECs with lenti-miR-146a as well as negative control lentivirus in vitro. Five days after transduction, greater than 75% HRECs are infected with lentivirus, which coexpresses green fluorescent protein (GFP; Figs. 1A, 1B). To determine whether lenti-miR-146a delivered miR-146a in infected HRECs, we harvested RNA and performed qRT-PCR. The results showed that miR-146a was significantly upregulated in cells infected with lenti-miR-146a in a dosage-dependent manner, compared with the ones infected with lenti-miR-neg ctl (Fig. 1C).

Intravitreal Injection of Lenti-miR-146a Increased the Level of miR-146a Expression in Rat Retina

To test whether lenti-miR-146a can deliver miR-146a in the retina in vivo, we performed intravitreal injection of lenti-miR-146a in one eye and lenti-miR-neg ctl in the other eye of STZ-induced diabetic rats and non-DM negative control animals 1 week after STZ injection. Three months later, we harvested their retina and performed qRT-PCR analysis. Our result showed that, like we reported previously,[36] miR-146a is upregulated in the retina of diabetic rats injected with lenti-miR-neg ctl, compared with non-DM rats injected with negative control lentivirus (Fig. 2), suggesting that diabetes induced moderate upregulation of endogenous miR-146a. More importantly, our result showed that lenti-miR-146a injection resulted in a further increase of miR-146a expression in the retina of

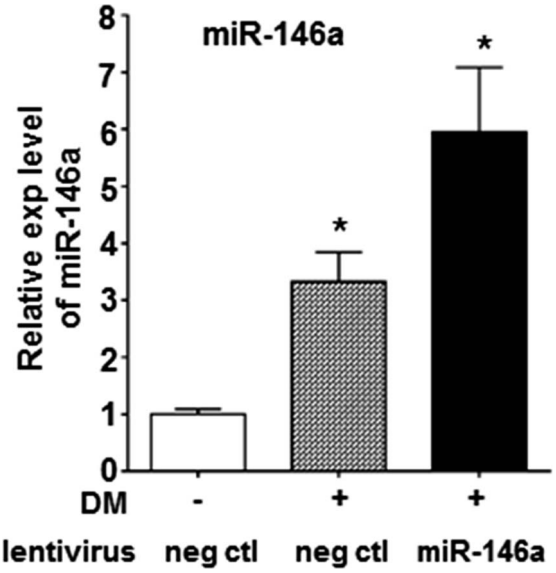

FIGURE 2. Intravitreal injection of lenti-miR-146a resulted in increased expression of miR-146a in the retina 3 months after diabetes. $^{*}P < 0.05$ versus non-DM rats injected with lenti-miR-neg ctl. $n = 4$/group.

diabetic rats, compared with lenti-miR-neg ctl–injected diabetic rats, suggesting that intravitreal injection of lenti-miR-146a delivered miR-146a in the retina in vivo for at least 3 months after viral injection.

Overexpression miR-146 Downregulated Key Downstream Target Genes in NF-κB Activation Pathways in the Retina of Diabetic Rats

Previously, we and others showed that miR-146 is a negative feedback regulator of IL-1R/TLR- and GPCR-mediated NF-κB activation pathways by targeting key adaptor molecules in these pathways, including IRAK1, TRAF6,[36,50] and CARD10.[37,51] To test whether lentivirus-delivered miR-146a regulates the expression of these molecules in vivo, we harvested retinal protein lysate 3 months after lentiviral injection. Western blot analysis showed that IRAK1, TRAF6, and CARD10 were increased in the retina in lenti-miR-neg ctl–injected diabetic animals compared to negative control lentivirus-injected non-DM control rats (Fig. 3), consistent with NF-κB activation in diabetic retina. However, in the eyes injected with lenti-miR-146a, IRAK1 and CARD10 were significantly decreased compared with the eyes of diabetic rats injected with negative control lentivirus, suggesting that lentivirus-delivered miR-146a inhibited the expression of endogenous target genes.

Overexpression of miR-146 Inhibited Diabetes-Induced NF-κB Downstream Proinflammatory Factor ICAM1

Nuclear factor kappa-B, a key regulator of inflammatory responses, is known to be activated in the retina as early as 2 months after the onset of diabetes and plays important roles in the pathogenesis of DR through its downstream proin-

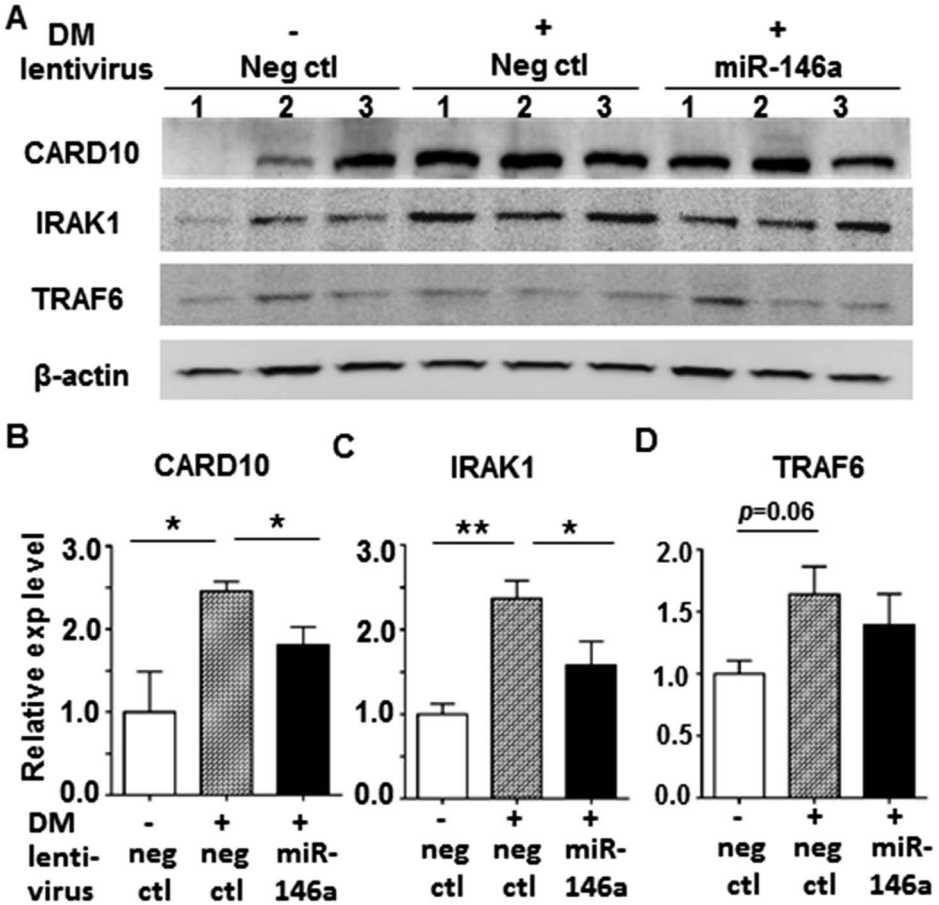

FIGURE 3. Intravitreal delivery of miR-146a prevented diabetes-induced increased expression of its downstream target genes, CARD10 (**A**, **B**), IRAK1 (**A**, **C**) and TRAF6 (**A**, **D**) in the retina by Western blot analysis. $n = 3$/group. $^{*}P < 0.05$; $^{**}P < 0.01$.

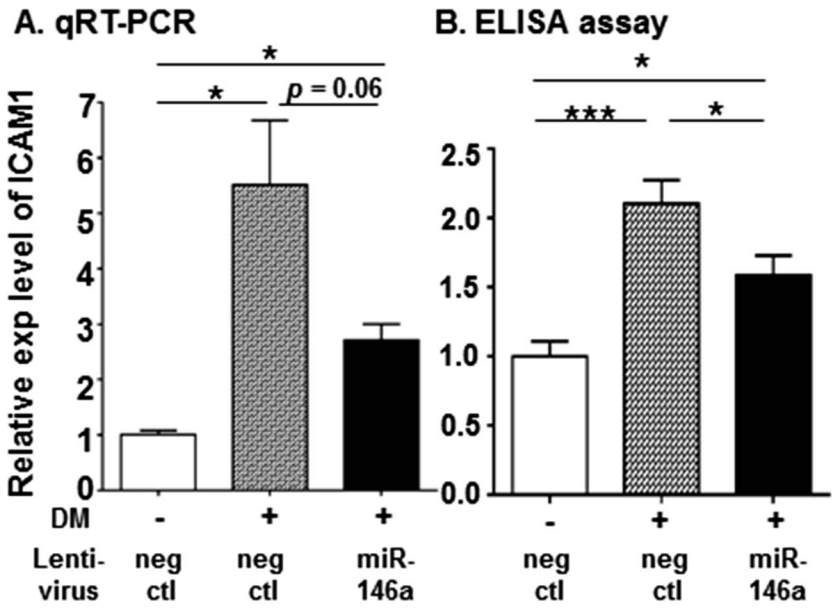

FIGURE 4. Intravitreal delivery of miR-146a inhibited diabetes-induced expression of NF-κB downstream inflammatory factor, ICAM1, in the retina 3 months after diabetes. (**A**) Quantitative RT-PCR analysis. $n = 3$/group. (**B**) ELISA analysis in retinas. All relative expression levels are normalized to non-DM rats injected with lenti-miR-neg ctl. $^{*}P < 0.05$, $^{***}P < 0.001$.

flammatory factors.[38,39,41] Nuclear factor kappa-B downstream gene, ICAM-1, is a key adhesion molecule to recruit leukocytes onto endothelial-cell surface to facilitates leukostasis and propagate inflammatory responses, contributing to subsequent REC cell death, microvascular defects, and DR development.[45–49] To test whether lentivirus-delivered miR-146a inhibits NF-κB activation-induced inflammatory response, we performed qRT-PCR and ELISA assays on ICAM-1. Our results showed that intravitreal injection of lenti-miR-146a significantly inhibited diabetes-induced increased expression of ICAM1 in the retina at both mRNA and protein levels (Fig. 4), suggesting that lentivirus-delivered miR-146a limited diabetes-induced NF-κB activation in the retina.

Overexpression of miR-146 in the Retina Is Protective From Diabetes-Induced Microvascular and Neuroretinal Functional Defects

To test whether intraocular delivery of miR-146a protects the retina from diabetes-induced damages, we performed scotopic ERG 3 months after lentiviral injection. Our result showed that the b-wave amplitude was significantly decreased in diabetic rats compared with non-DM control rats, with approximately 42%, 40%, and 38% decrease at 10, 100, and 1000 mcd s/m^2 light intensities, respectively (Figs. 5A, 5B), suggesting diabetes-induced functional defect of the retina. Intravitreal injection of lenti-miR-146a partially rescued diabetes-induced decrease of b-wave amplitude (Figs. 5A, 5B).

To test the effect of overexpression of miR-146a on the integrity of retinal microvasculature, we performed Evens blue assays. Our result showed that, in negative control lentivirus-injected eyes, the leakage of Evens blue was significantly increased by approximately 64% after 3 months of diabetes, compared with nondiabetic control rats (Fig. 5C). Lenti-miR-146a injection prevented diabetes-induced increase of Evans blue leakage (Fig. 5C); no significant difference was detected between nondiabetic rats (14.96 ± 1.98 ng/retina dry wt/hr) and the eyes of diabetic animals injected with lenti-miR-146a (18.25 ± 0.99 ng/retina dry wt/hr; Fig. 5C).

In the rats subjected to both Evans blue assay and ERG test, correlation analysis showed that improved retinal neuronal function is significantly correlated to the decreased retinal microvascular leakage in lenti-miR-146a–injected eyes of diabetic rats (Fig. 5D), suggesting that intraocular delivery of miR-146a protected the retina from both diabetes-induced microvascular and neuroretinal functional defects.

DISCUSSION

Nuclear factor kappa-B is a key regulator of immune and inflammatory responses.[59,60] Diabetes-induced NF-κB activation contributes to REC cell death, and plays an important role in the pathogenesis of DR.[38–44] Prevention of NF-κB activation is a viable approach for treatment of DR.[38,43] Previously, we showed in vitro that miR-146 inhibited IL-1β- and thrombin-induced NF-κB activation and prevented subsequent functional defects, including compromised endothelial barrier function and increased leukocyte adhesion.[36,37] Here, we show that intravitreal injection of lenti-miR-146a in diabetic rats resulted in increased expression of miR-146a, decreased expression of its key target genes, including IRAK1, TRAF6, and CARD10, which are important adaptor molecules of NF-κB activation pathways, and led to downregulation of NF-κB downstream gene, ICAM1, a proinflammatory factor attracting leukocytes docking on endothelial cells. More importantly, intraocular delivery of miR-146a prevented diabetes-induced retinal microvascular leakage, a key pathological changes during the development of DR,[61,62] and inhibited diabetes-induced retinal functional defects. These data provides an in vivo proof-of-principle evidence that overexpression of miR-146 in the retina is protective from diabetes-induced retinal damage.

MiR-146a was originally identified as negative feedback regulator of IL-1R/TLR-mediated NF-κB activation pathway in a macrophage cell line.[50] Increasing studies have shown that miR-146a is expressed in a wide range of tissues and cell types, and plays important roles in innate[50] and adaptive immunity,[63–66] and many tissue-specific functions in different cell types and biological context.[36,37,51,67,68] MiR-146a itself can be regulated by different pathways in different cell types in

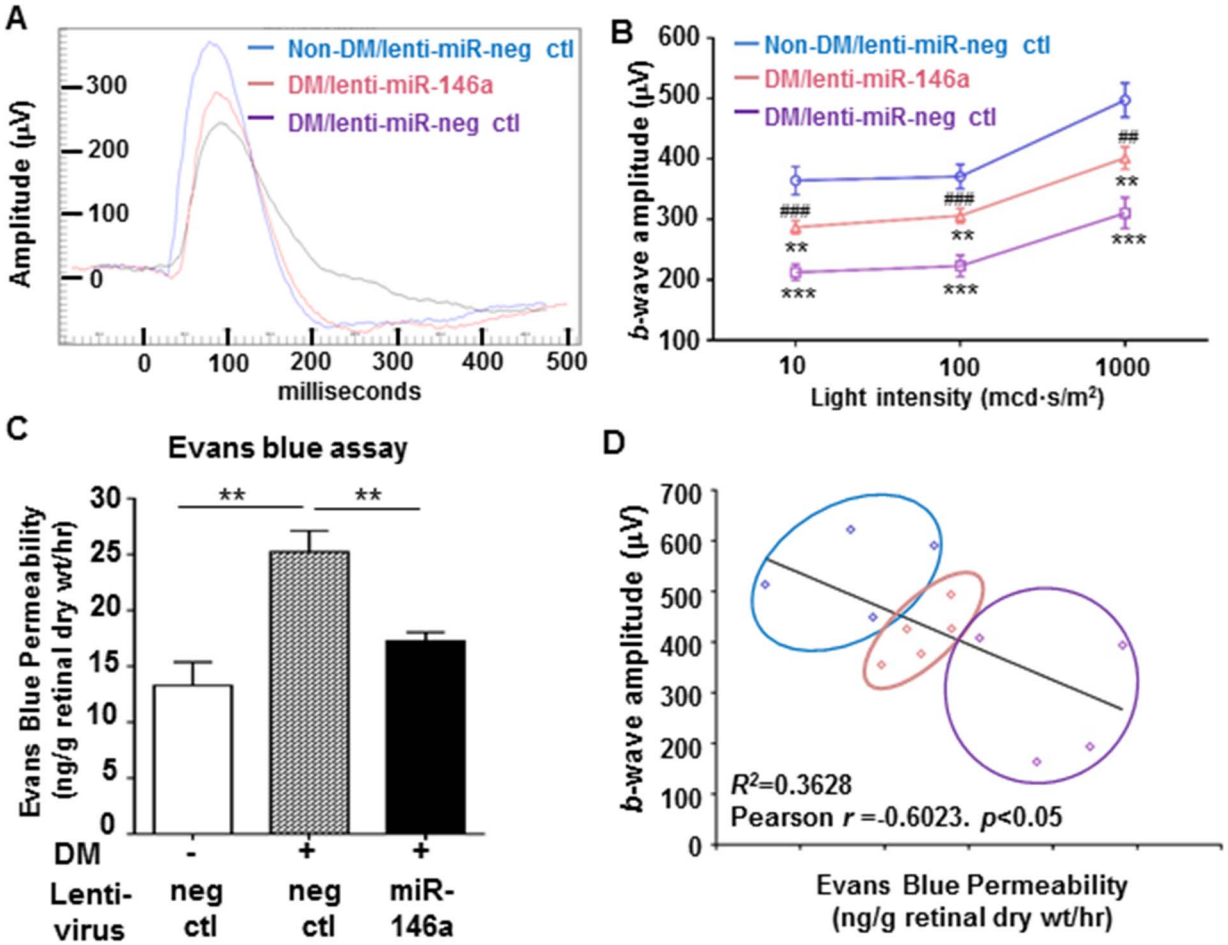

Figure 5. Intraocular delivery of miR-146a inhibited diabetes-induced functional damages to the retina 3 months after diabetes. (**A**) Representative scotopic ERG recording; (**B**) b-wave amplitudes of ERG at different light intensities. $n = 8$/group. (**C**) Evaluation of retinal microvascular leakage by Evans blue assays. $n = 4$/group. $^{**}P < 0.01$; $^{***}P < 0.001$ versus non-DM rat eyes injected with lenti-miR-neg ctl; $\#\#P < 0.01$; $\#\#\#P < 0.001$ versus DM rat eyes injected with lenti-miR-neg ctl. (**D**) Correlation analysis between ERG b-wave amplitude and Evans blue leakage in the rats subjected to both assays.

response to different stimuli under various physiological as well as pathological conditions.[50,66–70] Under diabetic condition, numerous reports have showed that, at different disease stages, miR-146a has different responses and functions in different cell types, including endothelial cells, retinal and renal tissues in both human and animal models. Wang and colleagues[71] showed that miR-146a expression in the retina had a rhythmic oscillation in nondiabetic rats; however, the rhythmic pattern was lost in diabetic rats 6 weeks after diabetes. MiR-146a expression in HRECs from nondiabetic donors exhibited circadian rhythm for up to 48 hours in culture[71]; while the ones from diabetic donors lost this rhythmicity.[71] Intriguingly, in HRECs of diabetic donors in culture, the levels of miR-146a expression appeared to be decreased when compared with the ones of nondiabetic donors.[71] This seems to contradict our report that miR-146a was increased in the RECs and/or the retina 3 months after STZ-induced diabetes[36] (Fig. 2). We speculate that the decreased expression of miR-146a in HRECs of diabetic donors in culture[71] is possibly a result of loss of in vivo diabetic environment, in which many proinflammatory factors activate NF-κB and promote miR-146a expression. Our previous[36] and current observations (Fig. 2) were made on primary RECs and/or retina acutely isolated from diabetic rats 3 months after diabetes; and therefore, they reflected in vivo status of miR-146a in the retina at this stage of disease development. Our unpublished data in human retinas shows that miR-146a is increased in the retina of diabetic donors, supporting our findings in diabetic animal models (data not shown).

In contrast to our findings,[36] Feng et al.[72] reported that miR-146a expression was downregulated in the retina, heart and kidney of STZ-induced diabetic rats 1 month after diabetes, and of T2D *db/db* mice 2 months after poorly controlled diabetes. The difference in miR-146a expression in STZ-induced diabetic rats compared with nondiabetic controls could be a result of different durations of diabetes. At different time-points after diabetes, different pathological pathways may dominate miR-146a expression regulation in the retina and other tissues. As a matter of fact, Feng et al.[72] suggested that transcriptional regulator, p300, played a major role in decreased expression of miR-146a in RECs and the retina 1 month after diabetes; while we demonstrated that increased NF-κB activation and proinflammatory factors in diabetic retina contributed to the increased expression of miR-146a in RECs and the retina 3 months after STZ-induced diabetes.[36,37] Therefore, these seemingly contradictory results may not argue against one another; rather, they underscore the dynamic changes of miR-146a in the retina at different stages under diabetes.

Feng et al.[72] also showed that, under high glucose culture (HG; 25 mM), miR-146a was downregulated in human

umbilical-vein endothelial cells (HUVECs) and bovine retinal microvascular endothelial cells (BRMECs) 24 hours after HG culture. However, in a recent report on HUVECs, Kamali et al.[73] reported an opposite result that, under similar condition, miR-146a expression was significantly upregulated in HUVECs, when NF-κB activity was significantly increased. The down-regulation of miR-146a in BRMECs under HG culture reported by Feng et al.[72] appears to contradict to our finding that miR-146a was increased in RECs and retina of diabetic rats 3 months after diabetes.[36] We speculate whether this difference is a result of species difference (bovine versus rat) or their in vitro condition, which could not simulate the complex environment in diabetic retina in vivo. However, the result reported by Feng et al.[72] is also in contradiction to an observation by Wang et al.[71] on HRECs in which miR-146a expression was not affected by HG culture in vitro.[71] This discrepancy may be arisen from different culture conditions and possibly the purity of endothelial cells, because it has been shown that pure HRECs do not have an inflammatory response to HG culture in vitro.[71,74]

The complexities of the roles of miR-146a under diabetic conditions are also reflected in studies of diabetic nephropathy (DN). Similar to the report by Feng et al.,[72] Lee et al.[75] showed that miR-146a expression was decreased in the glomeruli of T2D patients and of a T2D mouse model, BTBR *ob/ob* mice. However, several other groups reported opposite observations.[76–78] Huang et al.[76] showed that miR-146a was significantly increased in kidney tissue from renal biopsy of DN patients as well as in the renal cortex of STZ-induced T1D rats (1, 4, and 8 weeks after diabetes), and a T2D rat model induced by high-fat diet followed by multiple low dose of STZ (MLDS; before and 8 and 16 weeks after diabetes induction). Alipour et al.[77] also reported increased expression of miR-146a in renal tissue of STZ-induced diabetic rats 2 months after diabetes when NF-κB activation was increased. Bhatt et al.[78] showed that miR-146a was significantly upregulated in renal cortex in STZ-induced diabetic mice at 7 and 16 weeks after diabetes, when many proinflammatory factors were induced. Furthermore, Bhatt et al.[78] showed that miR-146a knockout mice had significantly exacerbated signs of DN and increased proinflammatory cytokines in the kidney after STZ-induced diabetes, compared with wild-type mice, suggesting that miR-146a inhibits diabetes-induced inflammatory response in the kidney. This is consistent with our observation in the diabetic retina[36] and our hypothesis that, under physiological condition, miR-146a maintains the homeostasis of NF-κB activation through its negative feedback regulation; it protects diabetes-induced damage by inhibiting NF-κB activation and subsequent inflammatory responses.[36,37] The difference between our observation in diabetic retina and the ones in diabetic kidney could be a result of different molecular pathways in different tissues and cell types. However, we could not fully explain the discrepancies among different reports in renal tissues of DN patients and animal models. We speculate that different animal models at different stages of disease development may have contributed to these discrepancies. Experimental details (e.g., the timing of tissue harvesting) may also influence the outcomes of the observation, as miR-146a expression may have a circadian rhythm in the kidney, like in the retina.[71]

The lentiviral construct used in this study, lenti-miR-146a, is a third generation HIV-based lentiviral vector system with advanced safety features[52,53] (in the public domain, http://www.genecopoeia. com). HIV-based vectors are currently the most popular lentiviral-based expression systems and can effectively transduce genes into a wide variety of dividing and nondividing mammalian cells.[52,53] In the current report, a single dose of lenti-miR-146a was administered one week after STZ-induced diabetes; and the effect of intravitreal delivery of miR-146a was studied 3 months after viral injection. Our data suggests that lenti-miR-146a delivered functional miR-146a, which sustained its function for at least 3 months in vivo. Using similar lentiviral constructs, robust transgene expression in vivo has been reported in RPE,[79–81] photoreceptors,[79,80,82] cornea endothelial cells,[81,83] neurons in the brain,[84,85] and so on, as early as 4 days after viral injection,[83] lasting as long as 3 months[82,84]; and the beneficial effect of the transgene can persist as long as 7 months.[80] Whether long-term delivery of miR-146 can be achieved by a single injection of lenti-miR-146a still needs to be determined in future studies. Therapeutic effect may be further optimized by adjustment of the dosage and frequency of injection. In addition, adeno-associated virus (AAV) has been shown to efficiently deliver transgenes in the retina for therapeutic purpose.[86–91] Adeno-associated virus–mediated delivery of miR-146 should also be explored to improve the efficiency and therapeutic effect.

The lentiviral construct in the current study carries a GFP cassette (Fig. 1A) to trace viral transduced cells. Although the GFP cassette was expressed robustly in in vitro transduction of HRECs (Fig. 1B), no apparent GFP expression was observed in the retina and other ocular tissues, including the lens, ciliary body and the iris, of lentivirus-injected eyes (data not shown). This may be a result of unknown epigenetic mechanisms to prohibit long-term expression of GFP in vivo; similar phenomenon has been reported in other gene therapy cases by viral delivery.[92–94] Intriguingly, in spite of the absence of GFP expression, lenti-miR-146a did result in increased expression of mature miR-146a in the retina, suggesting that the inhibition of the GFP expression is possibly on a posttranscriptional level, because pre-miR-146a is cotranscribed with the GFP cassette in the construct (Fig. 1A). One of the hypotheses is that miR-146a processing may have negative impact on the stability of the transcript, leading to the absence of obvious GFP expression; while miR-146a is successfully delivered. Nevertheless, the lack of expression of GFP prevented us from directly observing the cell types transduced in vivo; therefore, the current experiment falls short to gain further insights into viral-transduced cell types and their contribution to the apparent therapeutic effect. In future studies, delivery of miR-146a in specific cell types of the retina by employing cell type-specific expression constructs or cell type-specific transgenic mice will shed new lights into its roles in various cell types of the retina and their contribution to its protective effects.

MiR-146a is widely expressed in various ocular tissues and involved in a wide variety of disease processes in addition to DR. MiR-146a was shown to be increased in the retina of experimental autoimmune uveoretinitis.[95] In the cornea, miR-146a was reported to be enriched in human limbal corneal epithelial cells (LECs)[96,97] and upregulated in LECs from diabetic patients. Overexpression of miR-146a in LECs resulted in delayed cell migration and wound closure, and increased expression of LEC-specific genes.[96,97] These data suggest important roles of miR-146a in LEC maintenance and wound healing in diabetic cornea.[96,97] MiR-146a was also reported to be upregulated in RPE in response to proinflammatory factors.[98] It was shown that miR-146a was increased in RPE and choroid during aging, but not in neuroretina, suggesting age-related, tissue-specific regulation of miR-146.[97,99] Overexpression of miR-146a in RPE inhibited VEGF-A and TNFα-induced IL-6 expression.[97,99] In human trabecular meshwork (HTM) cells, miR-146a was shown to be involved in replicative senescence.[100] Overexpression of miR-146a appeared to inhibit the expression of several proinflammatory cytokines, senescence-associated β-galactosidase activity and the production of intracellular reactive oxidative species.[100] In an experimental autoimmune anterior uveitis rat model, miR-146a was decreased in the iris and ciliary body[101] and

suggested to contribute to NF-κB activation, helper T cell (Th)1 clonal expansion and intraocular inflammation.[101] These data suggest that miR-146a has tissue-specific functions in different ocular tissues, and is involved in many disease processes through different mechanisms. Therefore, when testing the effect of overexpression of miR-146a on one tissue in one disease process, its impact on other ocular tissues should be evaluated to avoid unexpected off-target effects. In this regard, tissue- or cell type–specific delivery may be a safer approach.

Nevertheless, our current study, together with our previous reports,[36,37] underscores important roles of miR-146a in DR. In spite of the differences of its expression levels at different stages of diabetes in different tissues of different species, the consensus message from numerous studies is that miR-146a is protective against diabetes-induced damages, however, through different mechanisms. In retina, miR-146a protects by inhibiting diabetes-induced increased expression of fibronectin[72] and NF-κB activation and subsequent inflammatory responses[36]; while in the kidney, it limits diabetes-induced increased expression of Notch-1 and Ergb4 and their downstream events,[75] besides fibronectin.[72] Additional independent, well-controlled, longitudinal tissue-specific studies are warranted to fully uncover the roles of miR-146a in DR and other diabetic complications, and its potential as a therapeutic target for the treatment of DR and other ocular diseases.

Acknowledgments

Supported by a Basic Science Grant from the American Diabetes Association (7-12-BS-107 to SX; Arlington, VA, USA) and an unrestricted grant from the Research to Prevent Blindness (New York, NY, USA) to the Department of Ophthalmology/Kresge Eye Institute, Wayne State University School of Medicine.

Disclosure: **P. Zhuang**, None; **C.K. Muraleedharan**, None; **S. Xu**, P

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