MiR-34c Participates in Diabetic Corneal Neuropathy Via Regulation of Autophagy

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RESULTS. The expression of miR-34c was significantly increased in TG tissue of type I diabetic mice by real-time PCR. Western blot showed that autophagy-related proteins Atg4B and LC3-II were significantly down-regulated in diabetes TG compared with normal control. Trigeminal neuron immunofluorescence showed that the length of the trigeminal ganglion cell synapses was significantly increased after miR-34c antagonim treatment compared with normal cultures. Subconjunctival injection of miR-34c antagonim can significantly promote corneal epithelium healing of diabetic mice and appreciably promote the regeneration of corneal nerve. At the same time, it can significantly increase the expression of autophagy in TG tissue of type I diabetic mice.

CONCLUSIONS. In this study, miR-34c was found to affect the growth of trigeminal sensory neurons and the repair of diabetic corneal nerve endings by acting directly on Atg4B.

Keywords: miR-34c, Atg4B, autophagy, diabetic corneal neuropathy, nerve regeneration, trigeminal ganglion

Diabetes mellitus (DM) is a major disease worldwide, and people with diabetes develop various types of corneal lesions that can cause irreversible visual impairment.1,2 The cornea is rich in nerve fibers, and corneal fibers are sensitive to cold, heat, pain, and touch pressure. Corneal innervation is derived from branching of the trigeminal ganglia.3 The corneal nerve supports corneal epithelial nerve function and plays an important role in corneal epithelial wound healing through interactions with TG sensory neurons.5 Corneal nerve injury can cause corneal hypoesthesia, leading to neurotrophic corneal epithelial lesions.6 A previous study7 has shown that diabetes is a risk factor for corneal neuropathy, which can lead to corneal sensory, nutritional, and metabolic dysfunction, causing keratitis and delayed healing of corneal ulcers.

Autophagy is an important cellular pathway in which lysosomes degrade large quantities of protein and organelles, such as mitochondria.8 Autophagy maintains intracellular balance by removing protein aggregates and damaged or excess organelles.9 At present, studies on autophagy in the field of diabetes are increasing. Fang et al.10 reported that high glucose can inhibit basal autophagy in cultured podocytes by reducing the expression of Beclin1, Atg5-12, and LC3, and rapamycin can recover high-glucose-induced autophagy defects, thereby protecting cultured podocytes from high-glucose-induced injury. It is currently believed that diabetic peripheral neuropathy is associated with ROS-mediated mitochondrial autophagy defects. Mitochondria are important capacity organelles in cells and are also the main source of reactive oxygen species (ROS). In a diabetic state, neuronal glucose metabolism is impaired and oxidative stress is enhanced, leading to mitochondrial function disorder. Impaired mitochondria cannot be discharged in time due to defects in mitochondrial autophagy, causing neuronal apoptosis or even death. Axonal retrograde transport disorders interfere with neuronal protein synthesis, causing axonal degeneration, atrophy, trigger diabetic peripheral neuropathy (DPN).11 The long-term neurological tissue of diabetic patients is in the state of ischemia, hypoxia, and nutrient and neurotrophic deficiency. In addition, persistent oxidative stress damage constitutes the premise and basis of abnormal autophagy. Autophagy in spinal dorsal root neurons of STZ-induced diabetic rats significantly increases, and autophagy could also occur in human neuroblastoma cells (SH-SY5Y) cultured in vitro by patients with diabetic peripheral neuropathy. Increase in autophagy is closely related to the increase in IgG and IgM in the serum of these patients. The FADD-caspase-8 death signaling pathway...
is involved in activation of autophagy, and when autophagy is mediated by autoimmune globulin, it is significantly reduced by prior treatment with Fas receptor blockers. Autophagy has the potential to protect against apoptosis through activation of the FAS autoantibodies. 12,13

Atg4B is a cysteine protease that cleaves the C-terminal amino acid of pro-Atg8, thereby exposing a C-terminal glycine. Atg4B is a key member of autophagy core proteins and affects autophagic membrane extension, closure, and maturation. Studies have found that Atg4B is the strongest activator of LC3, an autophagosome marker. During autophagy, LC3 is cleaved by Atg4B to the 5 amino acid residues at the C-terminus, forming an activated form of LC3-I. LC3-I is modified and processed by a ubiquitin-like system, including Atg7 and Atg3, to produce LC3-II and then localize to autophagosomes. Thus, LC3-II was used as autophagy markers for autophagy, and the amount of LC3-II is directly proportional to the degree of autophagy.

MicroRNAs (miRNAs or miRs) are a class of small, endogenous, non-coding single-stranded RNAs that are widely expressed in vivo. miRNAs regulate the expression of their target genes at the post-transcriptional level by specifically binding to the 3′ untranslated region (3′UTR) of the target mRNA. 14,15 It is well known that miRNAs play an important role in multiple biological processes, including growth, cell death, central nervous system function, and tumor formation. 16–19 A number of studies using microRNA microarray analyses in mouse and model cells have shown that expression of the miR-34 family increases with age. 20 Recent studies have shown that miR-34 overexpression accelerates the aging process of human diploid cells 21 and endothelial progenitor cells. 22 Moreover, the latest research has shown that some miR-34 target genes (such as SIRT1 and Bcl-2) are also important regulators of autophagic degradation. 23–24 Previously, Yang et al. 25 found that miR-34 represses autophagy by directly inhibiting the expression of autophagy-related protein Atg9 in mammalian cells. All these studies support the hypothesis that miR-34 may have a role in regulating autophagy.

Taken together, these previous findings suggest a possible regulatory association among miR-34c, autophagy, and diabetes. Therefore, we sought to determine whether miR-34c is involved in the pathology of diabetic corneal neuropathy and whether miR-34c contributes to diabetic corneal neuropathy by regulating autophagy. Herein, we have investigated this hypothesis using in vivo and in vitro models.

**Materials and Methods**

**Experimental Animals**

C57BL/6 mice (6–8 weeks old, male) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animal care and procedures were carried out according to the Principles of Laboratory Animal Care. All animals were treated in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research during the study. We developed mice with type 1 diabetes through intraperitoneal injection of low-dose streptozotocin (STZ, 50 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA) in ice-cold citrate-citric acid buffer (pH 4.5) for 5 consecutive days. Twelve weeks after the streptozotocin injections, animals with HbA1c values higher than 6.5% and blood glucose levels higher than 16.7 mmol/L were considered to have DM and were used for experiments.

**Conical Epithelial Wound Healing**

Diabetic mice and normal mice were anesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (70 mg/kg), followed by topical anesthesia of 2% xylocaine. The central corneal epithelium (2.5 mm) was scraped with an AlgerBrush II rust ring remover (Alger Co., Lago Vista, TX, USA), and fluoro olofacin ointment was then applied to avoid infection. The sizes of the corneal epithelium defects were assessed by staining with 0.25% fluorescein sodium and photographed under a BX900 slit-lamp (Haag-Streit, Bern, Switzerland) at 0, 24, 48, and 72 hours. The residual epithelial defect percentage was calculated by analyzing the staining area with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The miRNA-specific antagonim was used to downregulate the expression of miR-34c (RiboBio Co. Ltd., Guangzhou, China).

The mice were randomly divided into 4 groups, and each group contained 10 mice: group 1 included untreated control mice; group 2 included untreated diabetic mice; group 3 included 2.5 nmol miR-34c antagonist-treated diabetic mice; and group 4 included 2.5 nmol miR-34c antagonist native-control (NTC)-treated diabetic mice. The left eyes of all mice in the groups remained uninjured and were also untreated in the normal controls. In the untreated control and diabetic mice, the right eyes were untreated after injury; for miR-34c treatment, the miR-34c antagonist or antagonist NTC was injected into the right eye’s subconjunctival site on the same day of the corneal epithelial injury. This experiment was performed three times.

**Trigeminal Ganglion Neuron Culture and Treatment**

Trigeminal ganglion (TG) neurons were isolated and dissociated from TG tissue of diabetic or normal mice, as described previously. 26 In brief, trigeminal ganglia were harvested from diabetic and normal control mice and then digested with papain and collagenase/dispase, followed by centrifugation at 55-33 Hz for 30 min in a Percoll gradient to separate the trigeminal nerve cells. The isolated cells were seeded on a plate coated with poly-D-lysine and laminin and then incubated in neurobasal medium supplemented with 1% B27. Cultured neurons from diabetic animals were treated with 5 mmol/L D-glucose (considered normal glucose [NG]) or 25 mmol/L D-glucose (considered high glucose [HG]). The osmotic pressure of the NG medium was adjusted to the same level of the HG medium by adding 20 mmol/L L-glucose.

**Cell Transfection**

All miRNA antagonist and agonim sequences are fully complementary to the miRNA and are single-stranded. The non-targeting negative controls were from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Trigeminal nerve cells were seeded on 6-well plates in completed KSFM medium to 80% confluence. The cells were then shifted to KSFM medium with no EGF and BPE overnight for starvation. Next, the cells were transiently transfected with a miRNA antagonist or non-targeting negative control using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) for 6 hours according to the manufacturer’s protocol. The cells were then transferred to KSFM medium and were collected for further analysis 48 hours after transfection. The expression of miR-34c was detected by qRT-PCR and the expression of autophagy protein were detected by Western blot.
Real-Time PCR Analysis

Total RNA was isolated from cultured cells or mouse TG tissues using Nucleospin RNA Kits (Macherey-Nagel, Duren, Germany). All specific primers were designed and synthesized by Guangzhou RiboBio, Co., Ltd. The expression levels of endogenous control U6 (RiboBio) were used to normalize the expression levels of each miRNA. Comparisons of multiple changes in miRNA expression levels were performed by the CT method.

Luciferase Assay

miRNA target prediction algorithms (http://www.targetscan.org/) were used to predict the possible miRNAs that regulate the expression of Atg4b. The mouse Atg4b 3’-UTR containing the putative target site for miR-34c was amplified from genomic DNA by PCR amplification and inserted into the pMIR-REPORT (RiboBio). A mutant reporter plasmid of the miR-34c complementary locus was created with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer’s instructions. SH-SY5Y neuroblastoma cells are representative of nerve cells. Compared with trigeminal nerve cells, they have a higher transfection rate during transfection and are easier to culture. SH-SY5Y neuroblastoma cells were transiently transfected with the wild-type or mutant reporter plasmid and miRNA mimic/agomir using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 24 hours after transfection. Three independent experiments were performed in triplicate.

Corneal Nerve Staining

Corneal whole-mount staining was performed as previously described.27 Briefly, full-thickness corneal flat mounts were fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature, incubated for 30 minutes at 37°C in 20 mM EDTA (Sigma-Aldrich Corp.) and permeabilized in 10% Triton X-100 for 1 hour at room temperature. To block nonspecific staining, all samples were incubated with 5% bovine serum albumin (Sigma-Aldrich Corp.) in PBS for 1 hour. Subsequently, they were stained with an anti-III tubulin antibody (ab15708A; Millipore Sigma, Billerica, MA, USA) for 24 hours at 4°C. Finally, the flat mounts were examined under an Eclipse TE2000-U microscope (Nikon; Tokyo, Japan).

Corneal Nerve Density Calculation

Using a modified version of a method from previous studies,28,29 we evaluated the corneal nerve fiber density by taking images of the corneal nerve staining. The peripheral and central cornea nerve densities were calculated as the total length per unit area (mm/mm2) of the observed areas. The images were then converted into 8-bit grayscale images. The length of each fiber, consisting of the subbasal nerve plexus, was measured by tracing the fiber with Neuron J software (plug-in for ImageJ), and the total length of the subbasal nerve plexuses in the observed field was presented. Seven image fields were sampled in each corneal whole mount: one at the center cornea and the rest at midway between the central and limbal cornea for each of six corneal quadrants.

Immunofluorescence

The TG samples were fixed in OCT gel and then sliced with a frozen microtome. The frozen sections were fixed in 4% paraformaldehyde for 15 minutes. The samples were then perforated with 0.1% Triton X-100 for 5 minutes, followed by incubation with 5% BSA (Boster Biological Technology Ltd., Wuhan, China) for 1 hour to block nonspecific binding. The samples were stained with primary antibodies overnight at 4°C. After three washes with PBS, the cells were incubated with biotinylated rabbit anti-goat IgG (1:100) for 1 hour. The stained samples were photographed with a commercial confocal microscope (Eclipse CI-si Spectral Imaging Confocal Microscope; Nikon Instruments). According to a previous method, all stained samples were observed under a microscope and photographed.30

Western Blot Assay

The total protein from mouse TG or cultured neurons was extracted with RIPA lystate. The homogenates, which contained 20 μg of protein, were run on 12% SDS-PAGE gels and then transferred to a PVDF membrane (Millipore Sigma). The PVDF membrane was blocked with 5% non-fat dry milk for 1 hour at room temperature on a shaker and incubated overnight at 4°C with primary antibodies, including LC3 (1:300; Abcam), Atg4b (1:1000; GTX), and GAPDH (1:3000; Abcam).

Statistical Analysis

All experiments were repeated three times and all measured data are expressed as the means ± SD. Statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA) for an unpaired t-test or 1-way ANOVA. A P value of less than 0.05 was considered statistically significant.

RESULTS

MiR-34c Is Upregulated in Trigeminal Sensory Neurons From Diabetic Mice

Twelve weeks after streptozotocin injection, we measured the body weight and blood glucose concentrations of diabetic mice and normal mice. As expected, diabetic mice had significantly higher blood glucose levels and lower body weights than those of the normal mice. First, we determined the expression level of miR-34c in diabetic TG neurons and found that miR-34c expression was increased in diabetic TG neurons compared with normal TG neurons (Fig. 1A). Consistently, the expression levels of miR-34c were also increased in TG cells with HG treatment versus those in the group receiving NG treatment (Fig. 1B).

Weakened Autophagy in TG Tissue of Type I Diabetes

In diabetic TG neurons, the expression of Atg4b was significantly downregulated at the protein levels compared with that in the normal controls. What’s more, we found that the expression of LC3-II was more reduced in the diabetic TG neurons than in the normal mice. The decreased LC3-II indicated that diabetes inhibited the formation of autophagosomes. The protein expression levels of Atg4b and LC3-II were also downregulated in TG cells in the high glucose condition (Figs. 2A, 2B).

Atg4b Is a Direct Target of miR-34c

To study the molecular mechanism of miR-34c in diabetic TG sensory neurons and diabetic corneal neuropathy, we employed a bioinformatics approach using TargetScan software to predict the target genes of miR-34c. Notably, Atg4b was identified as a potential miR-34c target. To test the possibility of
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Atg4B is directly targeted and regulated by miR-34c. Overall, these results suggest the suppressive effect of miR-34c by disrupting the interaction between miR-34c and Atg4B. Next, we tested whether the inhibition of miR-34c affects corneal epithelial wound healing. We examined the effects of the miR-34c antagonist on corneal epithelial wound healing after injury by comparing type 2 diabetic mice to normal mice. After 3 days of subconjunctival injections in diabetic mice, the expression level of miR-34c was five-fold lower in TG sensory neurons treated with the miR-34c antagonist than in those treated with the miR-34c antagonist NTC (Fig. 4A). Fluorescence staining revealed a significant difference in the corneal epithelial healing rate between miR-34c antagonist-injected and miR-34c antagonist NTC-injected diabetic mice 48 hours after corneal epithelium debridement. The defect area of the corneal epithelium in the miR-34c antagonist-injected diabetic mice was remarkably enhanced compared with that of the diabetic mice and the miR-34c antagonist NTC-injected diabetic mice (Figs. 4B, 4C). Western blotting analysis revealed that the LC3-II and Atg4B expression were significantly higher after subconjunctival injection of the miR-34c antagonist (n = 4 for each group; Fig. 4D). Thus, we speculated that the downregulation of miR-34c may boost corneal epithelial wound healing in diabetic mice via autophagy.

Inhibition of miR-34c Promotes Diabetic TG Neurite Outgrowth

Because corneal innervation originates from the ophthalmic division of the TG, we investigated the role of miR-34c on diabetic TG neuron growth in vitro. The primary cultured TG sensory neurons of the diabetic and normal mice were transfected with the miR-34c antagonist or miR-34c antagonist NTC (200 umol/L). As shown in Figure 5A, after 5 days, the miR-34c antagonist treatment significantly accelerated neurite growth compared with that of the neurons transfected with the miR-34c antagonist NTC. Statistical analysis showed that the total neurite length of TG neurons treated with the miR-34c antagonist was increased greater than five-fold (for miR-34c antagonist-treated diabetic mice) and four-fold (for miR-34c antagonist-treated control mice) than that of the mRNA antagonist NTC-treated diabetic or control mice (Fig. 5B). These data indicated that the inhibition of miR-34c promotes the growth of neurites in diabetic TG sensory neurons in vitro.

Inhibition of miR-34c Promotes Innervations of the Corneal Nerve in Diabetic Mice

Hyperglycemia-damaged corneal nerve fibers regenerate and reinnervate in diabetic animals and humans with injured corneal epithelium. To study the effect of miR-34c on diabetic corneal nerve regeneration, the miR-34c antagonist or miRNA antagonist NTC was subconjunctivally injected immediately after the corneal epithelium was scraped. The density of regenerated subbasal corneal nerve fibers was examined 7 days after the corneal epithelium was scratched. As previously reported, the inhibition of miR-34c promoted subbasal nerve fiber regeneration in diabetic mice. Moreover, the diabetic mice that were treated with the miR-34c antagonist had a significantly increased neuronal density in both the central and peripheral regions of the cornea compared to that of the miR-34c antagonist NTC-treated diabetic mice (Fig. 6).

Together, our data shows that in diabetic TG sensory neurons, overexpression of miR-34c silences Atg4B and inhibits Atg4B-activated autophagy, contributing to diabetic corneal neuropathy. However, the loss of miR-34c induces Atg4B expression and enhances autophagy, therefore promoting diabetic corneal nerve regeneration.

Discussion

The increasing prevalence of diabetic peripheral neuropathy (DPN) indicates the need to develop new agents that can
stimulate nerve regeneration. MicroRNAs are known to play important roles in the occurrence and development of diabetes mellitus\(^{31,32}\). In contrast, the roles of microRNAs in the regulation of diabetic peripheral neuropathy, including axon growth and regeneration, are much less studied. In this study, by using an STZ-induced diabetic mouse model, we found that miR-34c was significantly overexpressed in diabetic TG neurons and that the downregulation of miR-34c promoted diabetic corneal nerve regeneration and epithelial wound healing via the autophagy pathway.

Several studies have reported that some miRNAs—such as miR-210 and let-7 miRNAs—can be used as potential therapeutic approaches for peripheral nerve regeneration. MiR-210 overexpression is able to promote sensory axon regeneration and inhibit apoptosis by inhibiting the expression of its downstream target gene—ephrin-A3 (EFNA3).\(^{33}\) It has been reported that let-7 miRNAs can regulate peripheral nerve regeneration by the robust phenotypic modulation of neural cells.\(^{34}\) In this study, miR-34c inhibited corneal sensory nerve growth in a chronic diabetic neuropathy mouse model. Furthermore, miR-34c antagonim treatment significantly promotes the growth of neurites in diabetic TG sensory neurons. The trigeminal nerve is believed to play an important role in maintaining corneal epithelial integrity and function. Previous studies have found that interruption of the ophthalmic TG pathways can cause delayed healing of corneal wounds, which is clinically difficult to control.\(^{35}\) Diabetic patients exhibit a wide variety of corneal complications (diabetic keratopathy) and have low corneal sensitivity if they have sterile neurotrophic corneal ulcers or neuronal abnormalities.\(^{36}\) In the present study, using subconjunctival injections of the miR-34c antagonim in diabetic mice, the miR-34c antagonim promoted a significant increase in the corneal nerve density and epithelial wound healing compared with that of the antagonim NTC-treated diabetic mice. These results also suggest that the miR-34c antagonim can induce the activation of multiple signaling pathways needed for nerve regeneration.

Accumulating evidence implies that autophagy has a critical neuroprotective effect on nerve injury.\(^{37-39}\) Consistent with
this, Kosacka et al.,\textsuperscript{40} reported that autophagy prevents neurodegeneration in the peripheral nervous system (PNS) in animal models of neuropathy. At present, several miRNAs have been reported to regulate autophagy-related proteins, such as the inhibition of autophagy by miR-195 by targeting ATG14, regulating neuroinflammation and neuropathic pain.\textsuperscript{41} Moreover, the previous study found that in the early events of diabetic retinopathy pathogenesis, lysosomes were impaired
FIGURE 4. In vivo downregulation of miR-34c accelerates corneal epithelial wound healing in diabetic mice via autophagy. Diabetic mice received 2 mm central corneal epithelial debridement as noted above and were treated with the miR-34c antagomir or miR-34c antagomir NTC (n = 10 per group). (A) After 3 days of subconjunctival injections, the expression level of miR-34c was significantly decreased in the TG neurons of diabetic mice. *P < 0.05. (B, C) The fluorescein-stained corneas at 0, 24, 48, and 72 hours after injury. *P < 0.05. (D-i, D-ii): After 3 days of subconjunctival injections, the TG sensory neurons were collected and analyzed by PCR and Western blot. PCR and Western blot analysis revealed that the levels of the LC3-II:lc ratio and Atg4B expression were significantly higher after miR-34c antagomir treatment. *P < 0.05.
and autophagy function was diminished. However, the spectrum of miRNA-regulated autophagy in diabetic TG sensory neurons has not been explored in detail.

In fact, miR-34c has many targets, several of which have been reported in previous literature, such as SCF in vascular smooth muscle cell proliferation and neointimal hyperplasia. In this study, we only investigated one target gene of miR-34c, which may appear to be too simplistic. We focused only on the study of Atg4B as a miR-34c target gene for three reasons: (1) Atg4B is an extremely important member of the autophagy core proteins, (2) Atg4B expression is significantly different in diabetic TG tissue than in control group tissue based on PCR and Western blot tests, and (3) Atg4B was present in the miRNA predictions using three methods. As we expected, Atg4B is direct target of miR-34c, and it acts as an important downstream target that is involved in diabetic corneal nerve regeneration; this strongly demonstrates that Atg4B is involved in diabetic corneal neuropathy. The current study revealed the role of autophagic lysosomal clearance mechanisms in diabetic TG neurons. Experimentally, protein expression of LC3-II was used to assess autophagy. We found that high glucose induced a downregulation of LC3-II expression following injury. Aside from the mice treated with the miR-34c antagomir NTC, all diabetic mice treated with the miR-34c antagomir showed an increase in autophagy 3 days after injury. The pathophysiological processes that occur in corneal nerve regeneration, sensation restoration, and epithelial wound healing are likely responsible for autophagy activation.

In summary, our data demonstrated that miR-34c was overexpressed in diabetic TG tissue. Inhibition of miR-34c promoted diabetic corneal nerve fiber regeneration epithelial wound healing. This mechanism was related to the autophagy signaling pathways.

**Figure 5.** Inhibition of miR-34c promotes diabetic TG neuron growth in vitro. (A) The effects of the miR-34c antagomir on neurite growth in the diabetic and normal mice (n = 4 per group). Scale bar = 100 mm. (B) Analysis of the total axonal length in TG sensory neurons of diabetic mice and normal mice treated with the miR-34c antagomir or miR-34c antagomir NTC. *P < 0.05.
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References


FIGURE 6. Inhibition of miR-34c promotes innervation of the corneal nerve in diabetic mice. The miR-34c antagonist or miRNA antagonist NTC was subconjunctivally injected into diabetic mice immediately after the corneal epithelium scrape. (A) Seven days after wounding, corneas were harvested, flat mounted, and stained with anti-βIII tubulin antibody. Representative images of the central zone (top row) and peripheral zone of the subbasal nerves of the cornea (bottom row) from diabetic and normal mice. (B) The percent area occupied by the corneal subbasal nerves in the central cornea at 7 days post-injury (n = 8 per group). (C) The percent area occupied by the corneal subbasal nerves in the peripheral cornea at 7 days post-injury (n = 8 per group). *P < 0.05.

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