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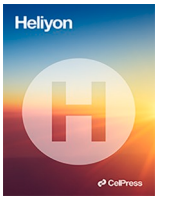

MicroRNA-93 promotes the pathogenesis of glaucoma by inhibiting matrix metalloproteinases as well as up-regulating extracellular matrix and Rho/ROCK signaling pathways

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ABSTRACT

Objective: To investigate the effect and potential molecular mechanism of microRNA-93 (miR-93) on retinal ganglion cells (RGCs) apoptosis as well as retinal damage in acute glaucoma mice.
Methods: RGCs apoptosis were induced by oxygen-glucose deprivation and reperfusion (OGD/R). The pro-apoptotic effect of miR-93 was evaluated by transfecting miR-93 mimics or miR-93 inhibitor into OGD/R-induced RGCs. The viability and apoptosis of RGCs were determined by MTT assay and flow cytometry. Mouse model of acute glaucoma were successfully induced via high intraocular pressure (IOP), and then these model animals were randomly divided into vehicle group, miR-93 mimics group or miR-93 inhibitor group (n = 10), using healthy mice as normal control. Histopathologic changes of retinal tissue were evaluated by Hematoxylin and Eosin (H&E) staining method. Moreover, cell counts of retinal ganglion cell layer and mean thickness of different layers were also determined. Quantitative real-time PCR (qPCR) and western blotting analysis were used to detect the mRNA and protein expression levels of extracellular matrix (ECM), matrix metalloproteinases (MMPs) and Rho/ROCK signaling pathway.
Results: miR-93 mimics significantly decreased or promoted the viability and apoptosis of OGD/R-induced RGCs, respectively. In addition, miR-93 mimics significantly exacerbated the degree of retinal tissue damage in mice with acute glaucoma, which was accompanied by a decrease in the number of ganglion cell layer (GCL) cells and the thickness of different tissue layers. Moreover, miR-93 mimics significantly increased IOP in mice with acute glaucoma. Significantly, miR-93 inhibitors significantly reversed the above changes. In addition, results of Western blot analysis showed that miR-93 mimics increased and decreased the expression of ECM-associated and MMP-associated proteins, respectively, by activating the Rho/ROCK signaling pathway. In contrast, miR-93 significantly decreased and increased the expression of ECM-associated and MMP-associated proteins, and suppressed the expression of Rho/ROCK signaling pathway-related proteins.
Conclusion: miR-93 can promote the development of glaucoma by activating Rho/ROCK signaling pathway to mediate the accumulation of ECM-related proteins as well as the down-regulation of MMP-related proteins.

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1. Introduction

Glaucoma is one of the leading blinding eye diseases worldwide [1,2]. Apoptosis of retinal ganglion cells (RGCs) is the final pathway in glaucomatous neuropathy [3,4]. The key factor causing glaucomatous optic neuropathy is elevated intraocular pressure [1]. Current treatments of Glaucoma are mainly to reduce intraocular pressure by drugs or surgery [2,5]. However, lowering intraocular pressure alone does not completely prevent the progressive degeneration and damage of ganglion cells and axons [6]. Hence, there is still an urgent need to seek glaucoma therapy that protect retinal ganglion cells and axons. Currently, many animal experiments have been carried out in related fields, which have achieved meaningful research results, including neurotrophic factors, apoptosis inhibitors, glutamate antagonists, NO synthase inhibitors, calcium channel blockers, antioxidants, vaccines, etc [7,8]. However, there is still a lack of persuasive evidence to prove clinical effectiveness.

MicroRNAs (miRNAs) are endogenous single-stranded small RNAs (about 22 nucleotides in length) of non-coding proteins ubiquitous in biological genomes discovered in recent years [9]. miRNAs regulate physiological and pathological processes by silencing gene expression at the post-transcriptional and translational levels [10,11]. miRNAs play an important role in cell proliferation, apoptosis, differentiation and organogenesis [12]. In recent years, significant progress has been made in miRNA research related to the pathogenesis and treatment of glaucoma [13]. The biological behavior of miRNAs may provide a theoretical basis for elucidating the pathogenesis of glaucoma and can also be used as one of the specific drug targets for the diagnosis and treatment of glaucoma, and their application has attracted much attention [14].

Wang et al. demonstrated that miRNA-93 (miR-93) promotes apoptosis in glaucomatous trabecular meshwork (TM) cells [15]. Meanwhile, transfection of TM cells with lentiviruses containing their specific inhibitors inhibited the expression of miR-93 and revealed a significant increase in viability of TM cells [15]. These previous findings suggest that miR-93 may be a potential target for glaucoma therapy. Nevertheless, apoptosis of RGCs is a key factor in the pathogenesis of glaucoma [3]. The effect of miR-93 on RGCs apoptosis and its role in glaucoma animal models remains unknown. Therefore, the aim of this study was to elucidate the role of miR-93 in promoting OGD/R-induced apoptosis of RGCs and retinal damage in mice with acute glaucoma and its underlying molecular mechanisms.

2. Materials and methods

2.1. Cell culture and transfection

Primary RGCs purification and culture were performed according to Winzeler et al. [16]. Briefly, C57BL/6 mice (within 7 days of age) were euthanized by direct decapitation, and retinas were removed to prepare single cell suspensions, incubated with negative selection dishes (goat anti-mouse IgG + IgM, Jackson ImmunoResearch) to remove most of the impurity cells, and positive selection dishes (mouse Thyl.2 antibodies, Sigma Aldrich) specifically screened out RGCs. RGCs were inoculated in Neurobasal medium containing glutamine, brain-derived neurotrophic factor, ciliary neurotrophic factor, B27 supplement (all from Sigma Aldrich) and cultured at 37 °C in a 5 % CO_2 incubator. The purified RGCs were divided into four groups: control, OGD/R group, miR-93 mimics group as well as miR-93 inhibitor group (all n = 6). For OGD/R, after the cell condition was stable, glucose-free and serum-free DMEM culture medium (Gibco) was replaced and placed in a hypoxic chamber (95 % N_2 + 5 % CO_2) and sealed at 37 °C. After 2 h incubation, the medium was replaced with high-glucose and DMEM culture medium (Gibco) containing 10 % fetal bovine serum (Sigma Aldrich), and incubated in a 37 °C and 5 % CO_2 incubator for 6 h. Both 50 nM miR-93 mimics (RiboBio) or 50 nM miR-93 inhibitor (RiboBio) were transfected into RGCs 24 h before OGD/R in miR-93 mimics group or miR-93 inhibitor group. Transfection was started after the fusion rate of primary RGCs reached 50–70 %. The miR-93 mimics and miR-93 inhibitor were transfected into RGCs using lipofectamine 2000 (Invitrogen) for 48 h. RGCs viability was determined by MTT cell proliferation assay kit (Invitrogen). In addition, apoptosis of RGCs was assessed by flow cytometry using propidium iodide (PI) -PE and Annexin V-FITC detection kits according to the manufacturer 's instructions (Keygen Biotech). Anti-mouse TGF-β1 antibody (Cat No. SAB4502954), anti-mouse Smad2 antibody (Cat No. SAB5701203), anti-mouse Smad3 antibody (Cat No. SAB5701204), anti-mouse MMP1 antibody (Cat No. BS62563), anti-mouse MMP3 antibody (Cat No. BS90872), anti-mouse α-SMA antibody (Cat No. A5228), anti-mouse RhoA antibody (Cat No. SAB4501661), anti-mouse ROCK1 antibody (Cat No. SAB5700923), and anti-mouse ROCK2 antibody (Cat No. SAB4301564) were obtained from Sigma Aldrich or Bioworld Technology, Inc.

2.2. Establishment of acute glaucoma animal models

All animals were purchased from Shanghai SLAC Laboratory Animal Co. LTD (Shanghai, China), maintained and used in compliance with the "Eye & Vision Research Association Statement for the Use of Animals". C57BL/6 mice (6–8 weeks) were randomly divided into normal group, vehicle group, miR-93 mimics group, and miR-93 inhibitor group (n = 10). Mouse models of acute glaucoma were constructed as previously described [17]. C57BL/6 mice were anesthetized intraperitoneally with topical anesthesia and mydriasis of the eyeball. Disposable sterile infusion sets were connected with 0.9 % normal saline solution and 30G insulin needle to form anterior chamber perfusion devices, and anterior chamber puncture was performed to maintain the perfusion height of 1 m. Intraocular pressure was measured by Tonolah tonometry and remained at approximately 110 mmHg, then maintaining the anterior chamber fluid tight for 50 min. Glaucoma models were assessed by monitoring IOP and retinal ischemia. IOP maintenance at 110 mmHg was verified with a tonometer. Microscopic examination confirmed whitening of the anterior segment of the mouse eye and retinal arteries, suggesting retinal ischemia. Before acute glaucoma surgery induction, mice in the miR-93 mimics group or miR-93

inhibitor group were intravitreally injected with 2 μL miR-93 mimics (5 nM/μL) or miR-93 inhibitor (5 nM/μL), respectively. Mice in the vehicle group were injected intravitreally with saline. Operational protocol for animal experiments were reviewed by the Affiliated Hospital of Southwest Medical University Ethics Committee with the approval number of IAEC/AH-SMU/2022/07.

2.3. Histopathology analysis

On the 7th day after reperfusion, the mice were euthanized, and some retinal tissues were taken to make paraffin sections and stained with H&E. Images of the retina were captured by a Zeiss inverted microscope. Axiovision software was used to measure the thickness of ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL) and outer nuclear layer (ONL), and the mean values were calculated.

2.4. RT-qPCR analysis

RNA in retinal tissues were extracted using TRIzol (Takara), and cDNA was prepared using the PrimeScript reverse transcription kit (Takara) according to the instructions. PCR was performed using the real-time PCR kit (Takara), and glyceraldehyde phosphate dehydrogenase was selected as an internal reference for mRNA determination of miR-93, fibronectin (FN), lamininm (LN), and type IV collagen (COL-IV). Primer sequences used were as follows: miR-93: F: 5′-AGTCTGGCTGACTACATCACAG-3′, R: 5′-CTACTCACAAAACAGGAGTGGAATC-3’; FN: F: 5′-ACAGTGCAAGCAGCAAGC-3′, R: 5′-TGGTGGTGGTCACTGTAGCCTGTC-3′; LN F: 5′-TGCTCAAGACGGCGAATAAGAC-3′, R: 5′-ATCGTGAGGCCTTGGCTCTTCCTC-3′; COL-IV: F: 5′-TAGAGAGAGGAGCGAGATGTTG-3′, R: 5′-TGCTAGAGAGCTCTAATTCAGG-3′.

2.5. Western blotting analysis

Total proteins were extracted from retinal tissues or cells, subjected to polyacrylamide gel electrophoresis, polyvinylidene difluoride membrane transfer, blocked with 5 % bovine serum albumin, incubated with mouse primary antibodies, and incubated overnight at 4 °C. After incubation with horseradish peroxidase-labeled secondary antibodies (Sigma Aldrich), the exposure was developed, and the protein bands were compared using Image software for gray scale analysis and statistical processing.

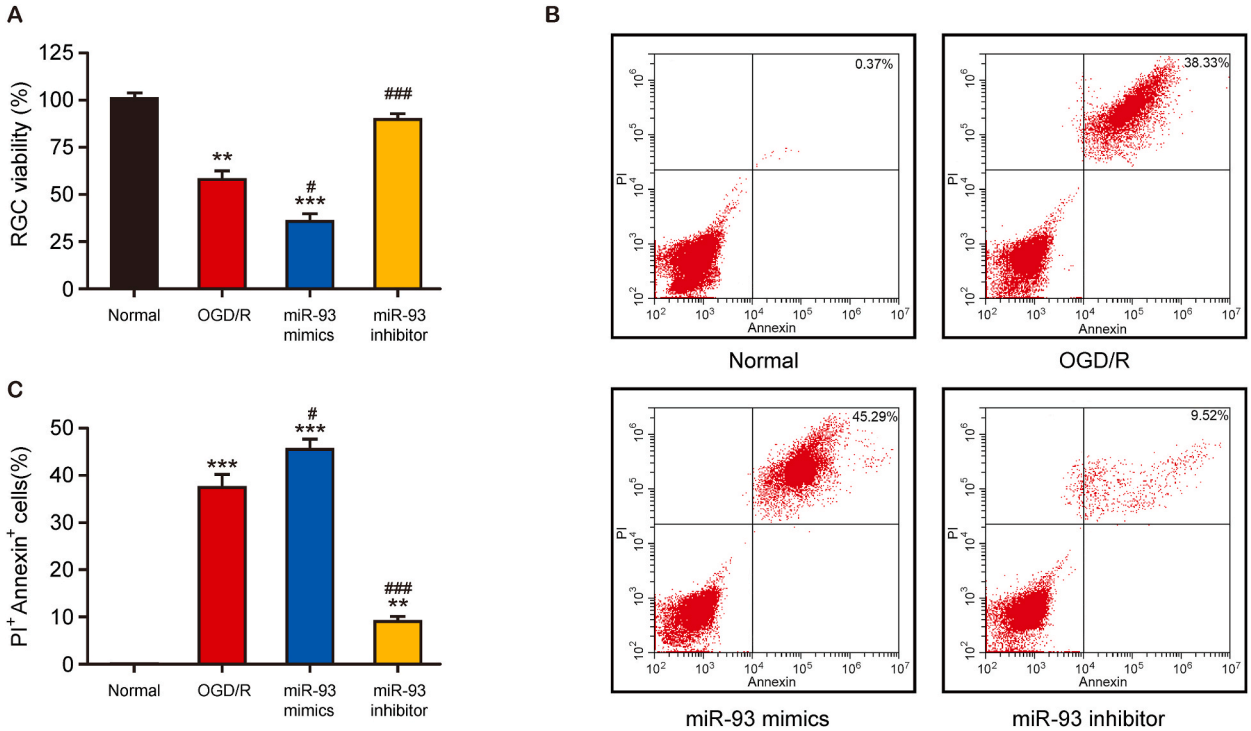

Fig. 1. Effects of miR-93 on OGD/R-induced apoptosis of RGCs. (A) Cell viability of RGCs determined by MTT assay. (B–C) OGD/R-induced apoptosis of RGCs determined by flow cytometry analysis. Data presented as Mean ± SD, n = 6. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs.* OGD/R group.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 and SPSS 20.0 software. The data were firstly subjected to Shapiro-Wilk test and presented normal distribution. Independent sample *t*-test was used for comparison between the two groups, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-93 promotes OGD/R-induced apoptosis of RGCs

Glaucoma is a progressive optic neuropathy characterized by apoptosis of RGCs, optic atrophy, and visual field defect changes. To verify the pro-apoptotic effect of miR-93 on RGCs, miR-93 mimics or miR-33 inhibitor were transfected into OGD/R-induced RGCs to mimic the apoptosis of RGCs upon glaucoma development. The viability of RGCs was detected by MTT assay and the results are shown in Fig. 1A. OGD/R treatment could induce a decrease in RGCs activity, while miR-93 mimics could further significantly reduce RGCS activity on the basis of OGD/R treatment, and the difference was statistically significant ($P < 0.01$). In addition, treatment of miR-93 inhibitor effectively reversed the change of RGCs viability ($P < 0.01$), and there was no significant difference compared with the normal group ($P > 0.05$). Flow cytometry results showed that OGD/R-induced apoptosis in RGCs was significantly increased ($P < 0.001$, Fig. 1B and C). Interestingly, miR-93 mimics and miR-93 inhibitor significantly promoted and inhibited the apoptosis of RGCs, respectively, compared with the OGD/R group ($P < 0.05$ or $P < 0.001$, respectively).

3.2. miR-93 induces apoptosis of RGCs by up-regulating the expression of ECM-associated proteins and down-regulating MMP-associated proteins

To investigate the mechanism of miR-93 promoting OGD/R-induced apoptosis of RGCs, the expression of related ECM proteins were detected by western blotting analysis. As shown in Fig. 2A–D, ECM-associated proteins, including TGF-β1, Smad2 as well as Smad3, were significantly up-regulated after OGD/R induction (all $P < 0.001$). In addition, the expression levels of TGF-β1, Smad2, and Smad3 increased to 1.34-, 1.31-, and 1.66-fold, respectively, after treatment with miR-93 mimics. In contrast, treatment of miR-93 inhibitor significantly reversed the up-regulation of the expression levels of ECM-associated proteins (all $P < 0.05$). Due to the degradation of ECM proteins require the activity of MMPs, the expression of MMP-1 and MMP-3 in RGCs were further determined. As shown in Fig. 2E and F, the expression of MMP-1 and MMP-3 were significantly down-regulated in OGD/R-treated group compared

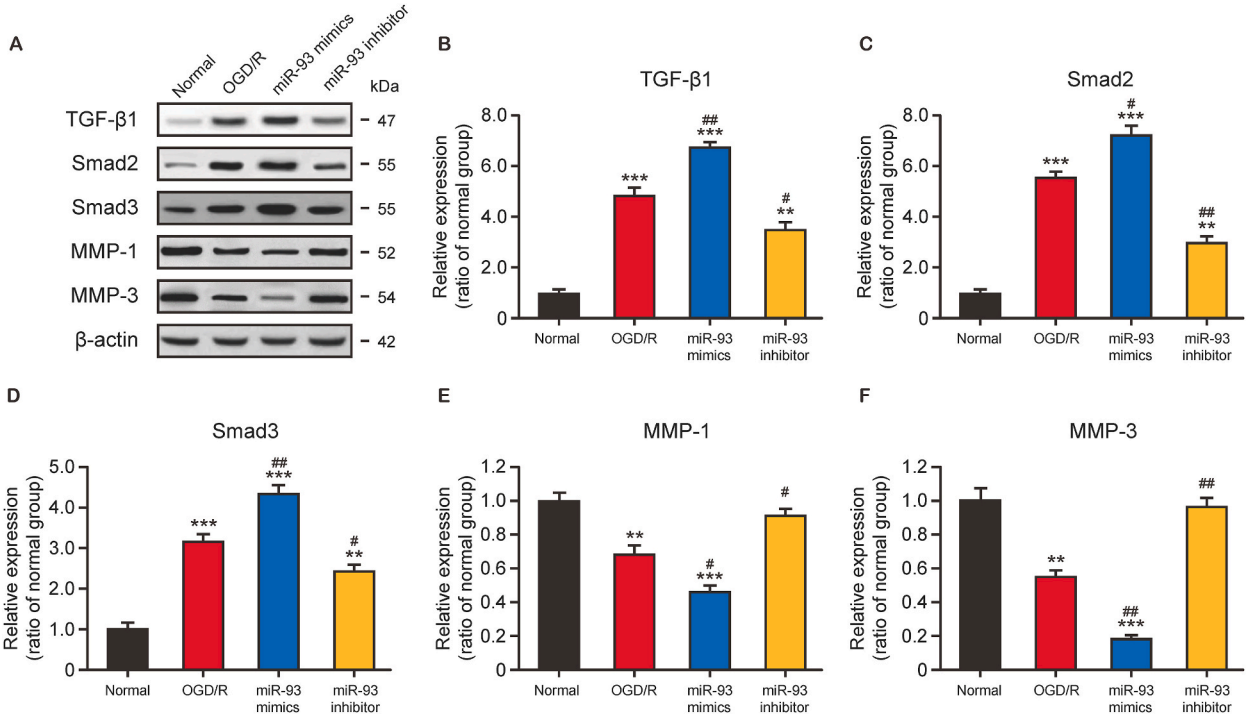

Fig. 2. Effect of miR-93 on the expression of ECM-associated proteins and MMP-associated proteins. (A) Representative images and quantification analysis of (B) TGF-β1, (C) Smad2, (D) Smad3, (E) MMP-1 and (F) MMP-3 of OGD/R-induced RGCs by Western blot analysis. Data presented as Mean ± SD, n = 6. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* OGD/R group.

with normal group (both $P < 0.01$). However, the expression of MMP-related proteins in RGCs were significantly down-regulated and up-regulated in the presence of miR-93 mimics or miR-93 inhibitor, respectively (both $P < 0.05$). Current results suggested that miR-93 induces apoptosis in RGCs by up-regulating ECM-associated proteins and down-regulating the expression of MMP-associated proteins.

3.3. miR-93 induced apoptosis of RGCs via activation of Rho/ROCK signaling pathway

The α-smooth muscle actin (α-SMA) is a cytoskeletal protein that is an important component of RGCs, and also affects ECM deposition. RhoA is a central modulator of stress fiber formation that acts on α-SMA and upregulates its expression. We therefore evaluated the expression levels of the Rho/ROCK signaling pathway in RGCs following exposure to OGD/R. As shown in Fig. 3, OGD/R exposure significantly up-regulated the expression levels of α-SMA as well as RhoA in RGCs compared with the normal group ($P < 0.01$). Similarly, ROCK1 and ROCK2, downstream targets of RhoA, showed the same trend ($P < 0.001$). Interestingly, miR-93 mimics further up-regulated the expression levels of α-SMA, RhoA, ROCK1 and ROCK2. In contrast, miR-93 inhibitor significantly reversed the expression of these proteins compared with the OGD/R group. The above results indicate that miR-93 can promote the apoptosis of RGCs by up-regulating the expression of α-SMA as well as activating the Rho/ROCK signaling pathway.

3.4. miR-93 promotes retinal tissue damage in mouse models of acute glaucoma

Subsequently, the effect of miR-93 on retinal damage in mouse model of IOP-induced acute glaucoma were explored. The results of H&E staining showed that retinal injury in mice with acute glaucoma was characterized by retinal edema, vacuolation, and nuclear chromatin condensation (Fig. 4A). Moreover, the number of GCL cells in the retina of glaucoma mice were significantly decreased compared with the normal group ($P < 0.01$) (Fig. 4B). In addition, the thickness of GCL, inner plexiform layer (IPL), inner nuclear layer (INL) and outer nuclear layer (ONL) also all decreased significantly (all $P < 0.05$) (Fig. 4C–F). Compared with vehicle group, miR-93 mimics treatment group further aggravated retinal injury in glaucoma mice, accompanied by a significant decrease in the number of GCL cells ($P < 0.01$), as well as a synchronous decrease in the thickness of GCL, IPL, INL and ONL (all $P < 0.05$) (Fig. 4B–F). As expected, miR-93 inhibitor significantly attenuated the degree of retinal injury, while significantly reversing the decrease in the number of GCL cells as well as the decrease in the thickness of different retinal tissue layers compared with vehicle group ($P < 0.05$).

3.5. miR-93 promotes elevation of IOP and accumulation of ECM-associated proteins in a mouse model of acute glaucoma

Increased intraocular pressure is an early pathognomonic marker of glaucomatous neurodegeneration. As shown in Fig. 5A, mean

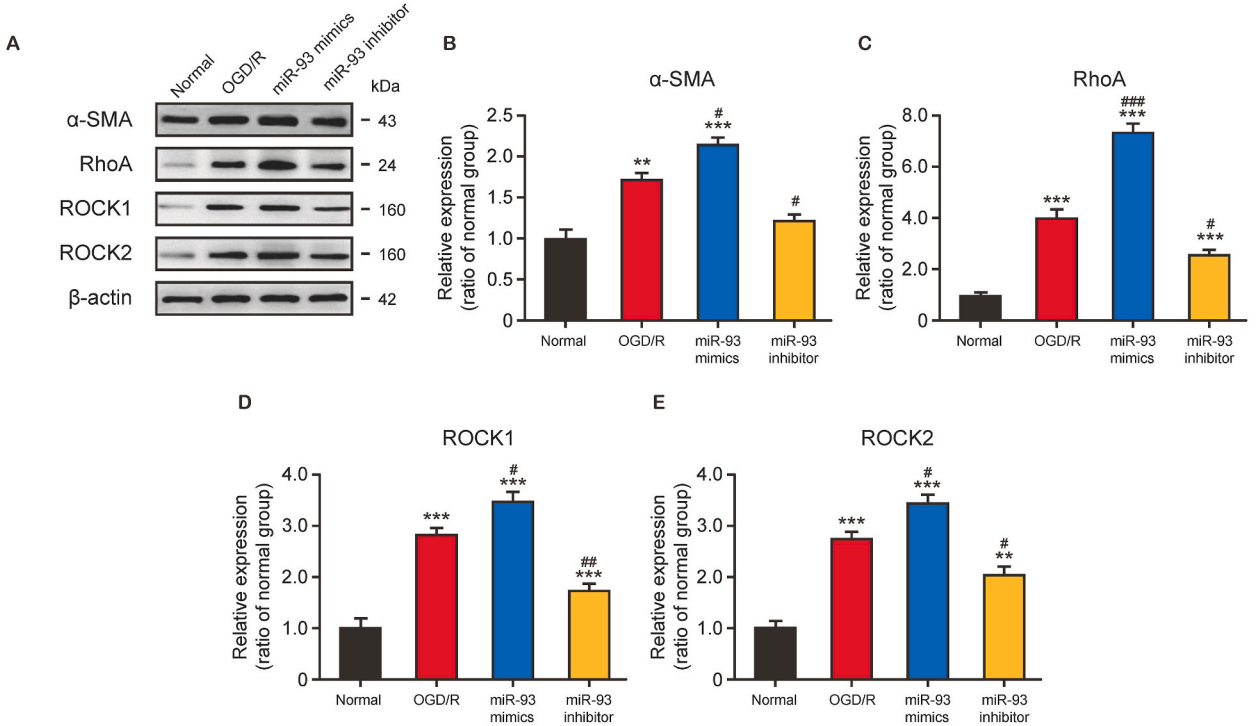

Fig. 3. Effect of miR-93 on the expression of Rho/ROCK signaling pathway. (A) Representative images and quantification analysis of (B) α-SMA, (C) RhoA, (D) ROCK1 and (E) ROCK2 of OGD/R-induced RGCs by Western blot analysis. Data presented as Mean ± SD, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* OGD/R group.

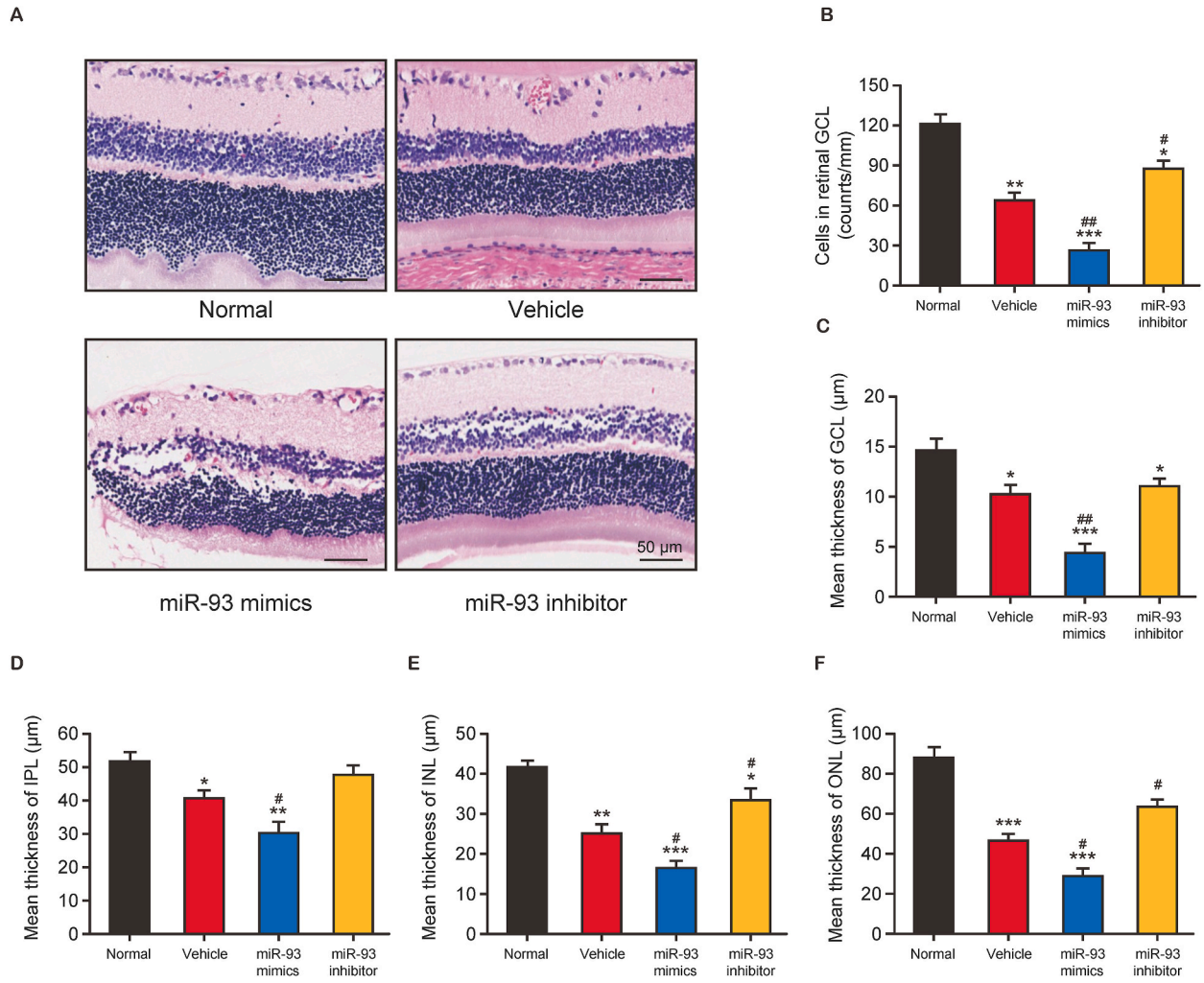

Fig. 4. Effect of miR-93 on retinal tissue damage in mouse models of acute glaucoma. (A) Representative images of H&E stain (Scale bar: 50 μm, 400 ×), (B) cell counts in retinal GCL, and mean thickness of (C) GCL, (D) IPL, (E) INL and (F) ONL of retinal tissue in mouse models of acute glaucoma. Data presented as Mean ± SD, n = 10. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* vehicle group.

IOP was significantly elevated in the glaucoma mouse model compared to the normal group. In addition, treatment with miR-93 mimics or miR-93 inhibitor significantly increased or decreased mean IOP in glaucoma mice, respectively ($P < 0.05$ or 0.01), indicating that miR-93 promotes the pathogenesis of glaucoma. In addition, the expression of miR-93 and related ECM proteins in different groups by RT-qPCR were evaluated. As shown in Fig. 5B–F, the expression of miR-93 in both peripheral blood (PB) and retinal tissue were significantly increased in glaucoma mice compared with the normal group (both $P < 0.05$). In addition, the mRNA levels of ECM-related proteins including FN, LN and COL-IV were significantly up-regulated in the retinal tissue of glaucoma mice ($P < 0.05$). Compared with vehicle group, miR-93 mRNA levels were significantly increased after miR-93 mimics treatment, while mRNA levels of FN, LN and COL-IV were further up-regulated ($P < 0.05$). In addition, miR-93 inhibitor treatment significantly reduced miR-93 mRNA levels, while partially reducing mRNA levels of ECM-associated proteins ($P < 0.05$).

3.6. miR-93 promotes glaucoma development by up-regulating the expression of ECM-associated proteins and down-regulating MMP-associated proteins

Accumulation of ECM and imbalance of MMP-associated proteases are key pathogenic mechanisms of retinal neuropathy in glaucoma. Western blot results showed that the expression levels of TGF-β1, Smad2 as well as Smad3 were significantly up-regulated (all $P < 0.01$), while the expression of MMP-1 and MMP-3 were significantly down-regulated (both $P < 0.05$) in glaucoma mice (Fig. 6A–F). In addition, the expression levels of TGF-β1, Smad2, and Smad3 in the miR-93 mimics treatment group were significantly higher than those in the vehicle group (all $P < 0.05$), while the expression levels of MMP-1 and MMP-3 were significantly lower than those in the glaucoma group (both $P < 0.01$) (Fig. 6A–F). In addition, miR-93 inhibitor treatment significantly down-regulated the

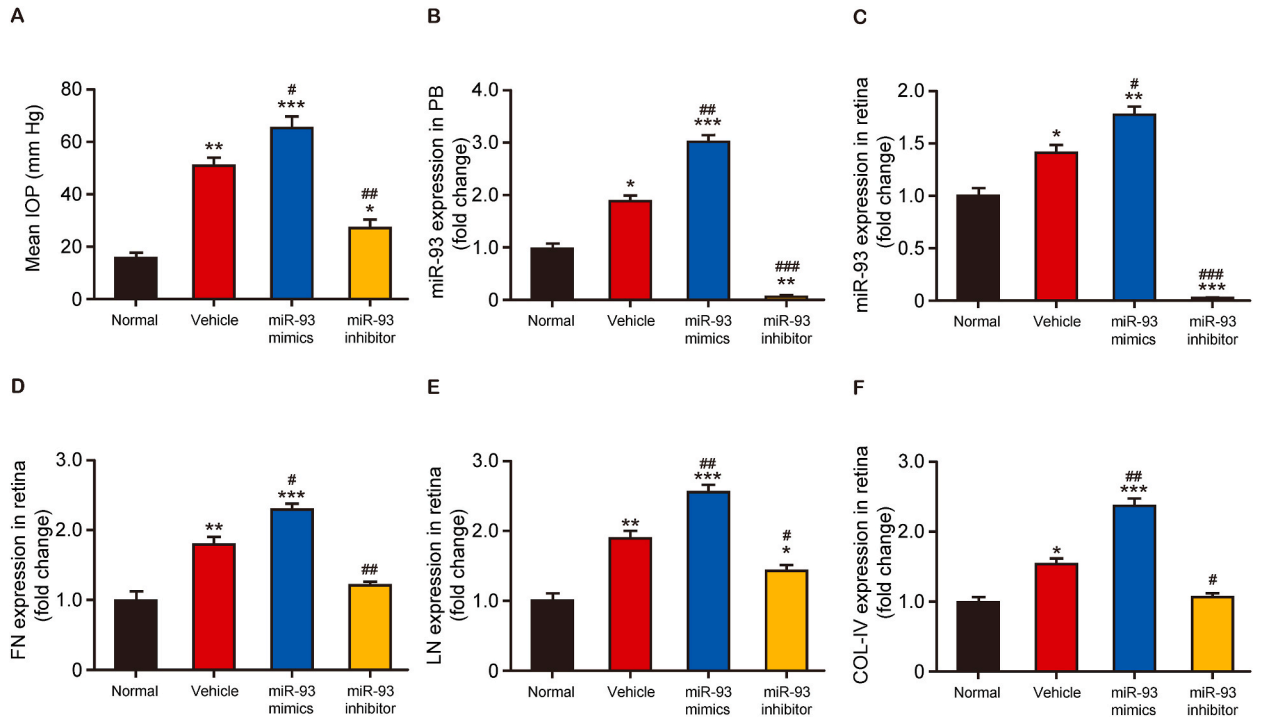

Fig. 5. Effect of miR-93 on IOP and mRNA levels of ECM-associated proteins in mouse models of acute glaucoma. (A) Mean IOP levels in mouse models of acute glaucoma. mRNA levels of miR-93 in (B) PB and (C) retinal tissue in mouse models of acute glaucoma. mRNA levels of (D) FN, (E) LN and (F) COL-IV of retinal tissue in mouse models of acute glaucoma. Data presented as Mean ± SD, n = 10. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* vehicle group.

expression levels of TGF-β1, Smad2 as well as Smad3 (all $P < 0.05$) as up-regulated the expression levels of MMP-1 and MMP-3 (both $P < 0.05$) compared with vehicle group (Fig. 6A–F). Current results suggested that miR-93 could contribute to glaucoma development by promoting the accumulation of ECM proteins in retinal tissues.

3.7. miR-93 promotes the development of glaucoma by activating the Rho/ROCK signaling pathway

To further elucidate the potential mechanism of miR-93-mediated glaucoma pathogenesis, the expression of Rho/ROCK signaling pathway-related proteins in retinal tissues was analyzed by western blotting analysis. As shown in Fig. 7A and B, expression level of α-SMA was significantly up-regulated in the retinal tissues of glaucoma mice compared with the normal group ($P < 0.001$). Moreover, Rho/ROCK signaling pathway-related proteins, including RhoA, ROCK1, and ROCK2, were synchronously and significantly up-regulated (all $P < 0.05$) (Fig. 5A and 5C-E). In addition, miR-93 mimics treatment before IOP induction further up-regulated the expression levels of α-SMA, RhoA, ROCK1 as well as ROCK2 (all $P < 0.05$) (Fig. 5A–E). In contrast, miR-93 significantly down-regulated the expression of α-SMA and Rho/ROCK signaling pathway-related proteins compared with vehicle group (all $P < 0.05$) (Fig. 5A–E). Current results further confirmed that miR-93 promotes glaucoma development through activation of Rho/ROCK signaling pathway.

4. Discussion

About 90 % of human eye miRNAs are unequally expressed in the cornea, lens, retinal pigment epithelium, and choroid [18]. Ryan et al. demonstrated by NCode™ miRNA microarrays that at least 31 miRNAs were expressed in adult mouse corneas and at least 17 miRNAs were expressed in adult mouse lenses [19]. Shu et al. used RT-PCR to detect miRNA expression in two human lens cell lines, HLE-B3 and SRA01/04, and found that 19 miRNAs were differentially expressed in human lens cell lines compared with mouse lenses [20]. Of these, 13 miRNAs showed up-regulated expression and 6 miRNAs showed down-regulated expression [20]. The miRNAs expressed in the eye are not only diverse, but also highly tissue-specific [20]. McArthur et al. found that miRNA-200b expression was decreased in retinal and vascular endothelial cells of diabetic rats, while its target gene vascular endothelial growth factor (VEGF) mRNA and protein expression was increased [21]. Endothelial cell transfection and intravitreal injection of miRNA-200b analogues prevented diabetes-induced increases in VEGF mRNA and protein expression, and also prevented hyperglycemia-induced increases in vascular permeability and neovascularization, while antagonists transfected with miRNA-200b resulted in increases in VEGF expression [21]. In addition, studies have shown that miRNA expression in the eye has dual specificity for developmental stages and

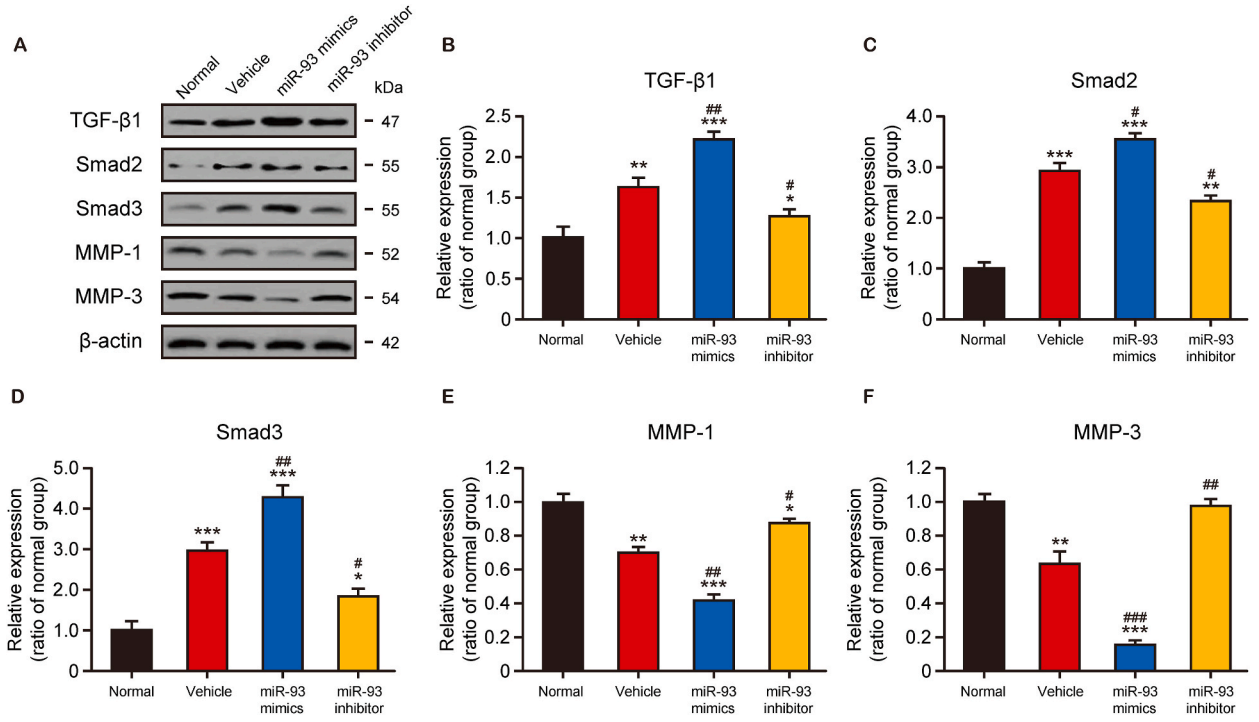

Fig. 6. Effect of miR-93 on the expression of ECM-associated proteins and MMP-associated proteins in retinal tissue of mouse models of acute glaucoma. (A) Representative images and quantification analysis of (B) TGF-β1, (C) Smad2, (D) Smad3, (E) MMP-1 and (F) MMP-3 in retinal tissue of IOP-induced acute glaucoma mice by Western blot analysis. Data presented as Mean ± SD, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* OGD/R group.

tissues, suggesting that miRNAs may have unique functions in ocular tissues [21].

miRNAs can regulate the development, differentiation, regeneration and apoptosis of ocular cells, and affect the function and circadian rhythm of ocular cells. In recent years, with the continuous discovery of new miRNAs in ocular tissues, the relationship between miRNAs and glaucoma has gradually attracted the attention of researchers. Nevertheless, the specific pathogenesis of glaucoma has not been fully clarified. Ischemia theory, mechanical compression theory, immunological theory and endothelin-1 damage can not completely explain all the clinical problems of glaucoma [22]. To date, most of treatments for glaucoma in clinical practice still depend on lowering intraocular pressure [23]. miRNAs play an important role in the regulation of aqueous humor secretion, intraocular pressure maintenance, trabecular meshwork structural remodeling, filtering tract scar formation after glaucoma surgery, and promoting nerve regeneration and other glaucoma lesions and intermediate links in the treatment process [24]. Taking miRNA as the starting point, analyzing the occurrence and development of glaucoma, exploring the diagnosis and treatment of glaucoma, and further revealing the pathophysiological process of the disease can provide new ideas for the prevention and treatment of the disease.

Zou et al. collected 75 diabetic retinopathy (DR) patients, 65 non-DR diabetic patients, and 127 healthy people, and found that the AUC of plasma miR-93 levels for the diagnosis of DR was 0.866, and the diagnostic sensitivity and specificity reached 73.33 % and 89.24 %, respectively [25]. The results of this study suggest that miR-93 is associated with a high risk of developing type 2 diabetic retinopathy [25]. In addition, it has been shown that miR-93 expression is increased in aqueous humor and trabecular meshwork cells (TMC) in patients with primary open-angle glaucoma (POAG) compared with cataract patients [24]. Further studies revealed that inhibition of miR-93 could promote TMC proliferation and inhibit apoptosis [15]. This suggests that miR-93 expression is elevated in glaucoma and promotes TMC apoptosis. However, the signaling mechanisms by which miRNA-93 promotes apoptosis in RGCs and the interaction between miR-93 and glaucoma development have not been explored.

While the clinical manifestations and treatment methods varied between various types of glaucoma, it is always the focus and difficulty of glaucoma research to achieve the purpose of protecting the optic nerve by delaying the apoptosis or progressive death of RGCs [26]. Ischemia-hypoxia injury is the main cause of most visual nervous system damage. Recombinant adenovirus-mediated miR-30b transfection was found to resist glucose-oxygen deprivation damage to RGCs and to have a similar protective effect on the survival of glucose-oxygen deprived RGCs [27]. In this study, we transfected miR-93 mimics or miR-93 inhibitor into RGCs and performed OGD/R induction. The results showed that co-incubation of miR-93 mimics significantly reduced RGCs viability. In contrast, miR-93 inhibitor significantly increased RGCs viability. Consistent with this, flow cytometry results showed that miR-93 mimics or miR-93 inhibitor promoted or inhibited OGD/R-induced RGCs apoptosis, respectively. Subsequently, we validated the effect of miR-93 on retinal tissue damage in a mouse model of acute glaucoma. MiR-93 mimics treatment significantly decreased the

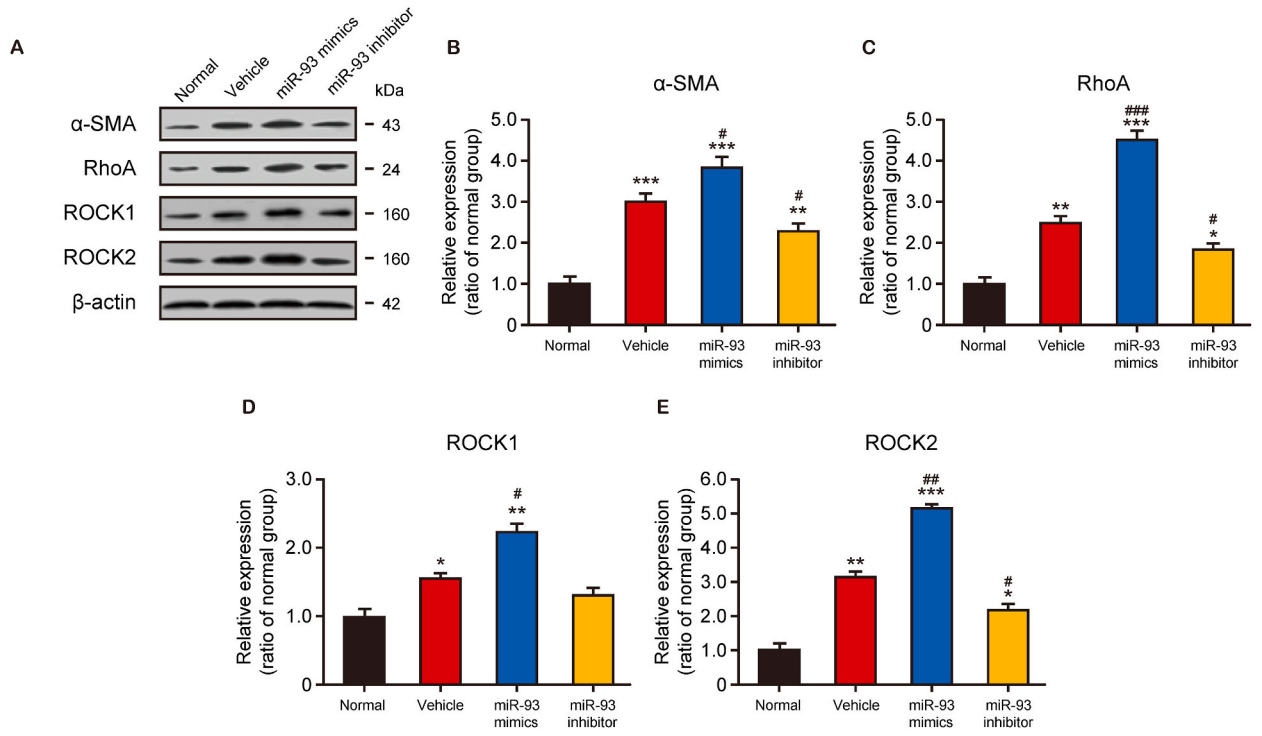

Fig. 7. Effect of miR-93 on the expression of Rho/ROCK signaling pathway in retinal tissue of mouse models of acute glaucoma. (A) Representative images and quantification analysis of (B) α-SMA, (C) RhoA, (D) ROCK1 and (E) ROCK2 in retinal tissue of IOP-induced acute glaucoma mice by Western blot analysis. Data presented as Mean ± SD, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* OGD/R group.

number of GCL cells as well as the thickness of different tissue layers in the retinal tissue of mice with acute glaucoma. In contrast, miR-93 inhibitor significantly ameliorated retinal injury in mice with acute glaucoma. The above results showed that miR-93 could promote the pathogenesis of glaucoma.

Trabecular meshwork (TM) is a key tissue regulating anterior chamber outflow, and abnormal regulation often leads to elevated intraocular pressure [28]. ECM has a highly dynamic structure of specific physical and biochemical properties that impacts the phenotype of all resident cells and regulates many cellular activities, such as migration, proliferation, differentiation, survival, and immune system signaling, ultimately affecting development, homeostasis, and adhesion to tissue repair [29]. Dynamic balance between synthesis and outcome determines the final composition and maintenance of normal function of the ECM [29]. ECM deposition in TM, narrowing or obliteration of trabecular meshwork apertures, increased aqueous outflow resistance, and increased intraocular pressure [28]. In addition, MMPs are a group of zinc- and calcium-dependent endopeptidases involved in ECM homeostasis and remodeling [30]. MMPs can degrade and alter ECM molecules, and affect cellular activity and maintain homeostasis of epithelial cell membranes [30]. In the development of glaucoma, an imbalance between MMPs and their inhibitors may impair ECM outcome in TM, thereby increasing the resistance of aqueous humor outflow channels, causing elevated IOP and ultimately glaucoma [30]. In the present study, miR-93 mimics significantly upregulated the expression of ECM-related proteins and simultaneously downregulated the expression of MMPs in RGCs after OGD/R induction. Furthermore, both ECM and MMP-associated protein expression in RGCs were significantly reversed upon inhibition of miR-93. In acute glaucoma mice, miR-93 mimics significantly increased IOP levels, which was accompanied by a significant up-regulation of ECM-related protein expression levels and a significant down-regulation of MMP-related protein expression levels. In addition, IOP was also decreased in acute glaucoma mice after inhibition of miR-93 expression, and both ECM and MMP-related protein expression levels were significantly down-regulated and up-regulated, suggesting that miR-93 exacerbates glaucoma pathogenesis by promoting ECM accumulation and MMP imbalance.

Rho belongs to the small molecule monomeric GTPases superfamily and is a mammalian gene homologue of the Ras superfamily [31]. Rho regulates the reorganization of the actin skeleton through its most important downstream effector, Rho kinase or ROCK, thus widely participating in a series of biological processes such as cell mitosis, cytoskeletal adjustment, muscle cell contraction, nerve regeneration, tumor cell infiltration, and regulation of apoptosis [32]. More and more studies have shown that Rho/ROCK kinase signaling pathway is involved in the pathogenesis of glaucoma and shows good prospects for the treatment of glaucoma in reducing intraocular pressure, protecting retinal ganglion cells and improving ocular blood flow, and specific blocking of RhoA/ROCK may become a new target for glaucoma treatment [33,34]. Western blot analysis showed that miR-93 mimics significantly activated the Rho/ROCK signaling pathway in RGCs and upregulated the expression level of α-SMA protein. Similarly, miR-93 mimics significantly upregulated the expression of α-SMA and Rho/ROCK signaling pathway-related proteins in retinal tissue of mice with acute glaucoma.

Interestingly, miR-93 inhibitor significantly reversed OGD/R-induced expression of α-SMA as well as Rho/ROCK signaling pathway-related proteins in RGCs and retinal tissue of mice with acute glaucoma. The above results suggest that miR-93 promotes the pathogenesis of glaucoma by activating the Rho/ROCK signaling pathway.

As a hot spot of research at home and abroad in recent years, miRNAs have been widely penetrated into various medical disciplines, and their role in the occurrence and development of ocular diseases has gradually been concerned. The mechanism, regulatory characteristics, and accurate location of aqueous humor outflow resistance in glaucoma are not fully understood. It has been shown that a variety of miRNAs affect the synthesis and outcome of ECM, as well as the expression of cytoskeletal proteins, thereby regulating aqueous humor outflow resistance and causing IOP changes. However, there are still some unstudied miRNAs that should attract the attention of researchers, and these miRNAs are expressed in TM and are associated with proliferation and fibrosis, which may be associated with the mechanism of glaucoma and are the direction of further research. In this paper, we investigated the effect of miR-93 on the mechanism of the development of acute glaucoma, but it also needs to be further explored in what way miR-93 should be applied in clinical practice to play a role in disease prevention and treatment. In addition, miR-93 can be used as a biological marker for glaucoma diagnosis and is expected to be a candidate marker for analyzing its pathogenesis. We believe that for the study of miR-93, it is important in the near future for both the pathogenesis of glaucoma and the treatment of the disease.

The limitations of the current study include that the exact targets of miR-93 are currently unknown. Both ECM- and MMP-related factors and related proteins of the Rho/ROCK signaling pathway are downstream acting proteins of miR-93, which could explain our observations. Nevertheless, this study only demonstrated changes in the expression levels of MMPs, but did not determine their activity. It is still necessary to reflect the function of MMPs by measuring their activity. In addition, other biological targets have not been intensively studied. Alternatively, manipulation of miR-93 expression may have unknown adverse effects. Future research in these areas is needed to help provide valuable insights in glaucoma treatment strategies.

In summary, miR-93 mimics can effectively mediate the accumulation of ECM-related proteins as well as the down-regulation of MMP-related proteins by activating the Rho/ROCK signaling pathway in both OGD/R-induced RGCs model and IOP-induced acute glaucoma mouse model, thereby promoting the development of glaucoma. The present study first identified a novel microRNA-93//ECM/MMP axis that regulated the development of glaucoma, and providing in vivo and in vitro evidence. As an important regulatory molecule in the pathogenesis of glaucoma, miR-93-based advanced therapy is expected to be a new therapeutic target for glaucoma.

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Ethics statement

All the experimental procedures were performed in accordance with the Southwest Medical University animal welfare guidelines and approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Southwest Medical University with the approval number of IAEC/AH-SMU/2022/07.

Data availability statement

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?

Response: No.

Please select why. Please note that this statement will be available alongside your article upon publication.

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Additional information

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CRediT authorship contribution statement

Manhua Xu: Writing – original draft, Methodology, Data curation, Conceptualization. **Yanxi Wang:** Writing – review & editing, Methodology, Data curation. **Juan Zhou:** Writing – review & editing, Methodology, Data curation. **Xun Zhang:** Writing – review & editing, Methodology. **Yinggui Yu:** Writing – review & editing, Data curation. **Kaiming Li:** Writing – review & editing, Software, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

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Appendix A. Supplementary data

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