Hsa_circ_0023826 protects against glaucoma by regulating miR-188-3p/MDM4 axis

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Objective: Circular RNAs (circRNAs) are characterized as a class of covalently closed circRNA transcripts and are associated with various cellular processes and neurological diseases by sponging microRNAs. The most common feature of glaucoma, a form of retinal neuropathy, is the loss of retinal ganglion cells. Although the pathogenesis of glaucoma is not fully understood, elevated intraocular pressure is undoubtedly the only proven modifiable risk factor and elevated intraocular pressure is undoubtedly the only proven modifiable risk factor. Therefore, further elucidation of the pathogenesis of glaucoma is still required.

Circular RNAs are a new class of non-coding RNAs characterized by closed-loop structures (Ebbesen et al., 2017). With bioinformatics analysis and high-throughput sequencing, circRNA may participate in the pathogenesis and progression of various eye diseases, including glaucoma, diabetic retinopathy, and retinoblastoma (Guo et al., 2019). Therefore, an in-depth understanding of circRNAs involved in glaucoma will help to open up new avenues for early diagnosis and clinical treatment.

INTRODUCTION

Glaucoma, a retinal neurodegenerative disease characterized by progressive and irreversible degeneration of retinal ganglion cells (RGCs) and optic nerve, is the second leading cause of blindness (Quigley et al., 2006). Elevated intraocular pressure (IOP) is a risk factor and RGC apoptosis is the core cause of glaucoma (Geva et al., 2021). At present, the treatment methods of glaucoma mainly include surgery and drugs (Hooshmand et al., 2022). Despite effective medical and surgical treatment to lower IOP, many patients will continue to lose vision due to the death of RGCs and degeneration of the optic nerve. Therefore, further elucidation of the pathogenesis of glaucoma is still required.

CircRNAs serve as gene modulators of microRNAs (miRNAs) (Yang et al., 2021), thereby participating in the progression of glaucoma. have found that circ_0023826 is upregulated in glaucoma-related retinal neurodegeneration, and is capable of regulating neurodegeneration by acting as a miR-615 sponge (Wang et al., 2018). In another report, circZRANB1 directly regulates Müller cell function and RGC function through miR-217 (Wang et al., 2018). TENM4-encoded circ_0023826 has been identified as a diagnostic biomarker for glaucoma (Chen et al., 2020), but rare studies have reported its action in glaucoma.

The present study hypothesized that circ_0023826 protects against retinal neurodegeneration by regulating the miR-188-3p/MDM4 axis. First, we constructed a glaucoma model by chamber injection of microbeads and isolated RGCs to study the expression patterns of circ_0023826 in glaucoma-induced retinal neurodegeneration. Then, we confirmed the downstream miRNA (miR-188-3p) and target gene (MDM4) to elucidate the regulation of circ_0023826/miR-188-3p/MDM4 in glaucoma, targeting to develop a new theoretical basis for glaucoma therapy.

MATERIALS AND METHODS

Collection of patient tissues

From January 2017 to March 2018, 12 pairs of human fascia tissue and normal human fascia tissue (3 mm from the conjunctival edge) from glaucoma patients were collected at Muping District Hospital of Traditional Chinese Medicine. Tissue samples were frozen in liquid nitrogen and then stored at -80°C. All procedures were performed under the review of the Ethics Committee of...
Muping District Hospital of Traditional Chinese Medicine. All patients provided written consent.

Establishment of a glaucoma model

All animal experiments were performed with the approval of the Animal Ethics Committee of Muping District Hospital of Traditional Chinese Medicine. Forty-eight Sprague Dawley (SD) rats (male, 200–250 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. After one week of adaptive feeding, 42 rats were randomly selected to construct a glaucoma model by intra-chamber injection of microbeads. In short, anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (75 mg/kg), the rats were injected with sterile microbeads into one eye and dropped with 0.5% moxifloxacin hydrochloride in each eye. IOP was measured after 24 h. At 4 weeks post-injection, the second injection of microbeads was performed. IOP was measured every 4 days using a digital tonometer. An equal volume of phosphate-buffered saline (PBS) served as a sham operation procedure.

Lentiviral intervention

The glaucoma rats were randomly divided into 7 groups: Glaucoma group, sh-NC group, Lenti-circ_0023826 group, Lenti-NC group, Lenti-circ_0023826 + Lenti-miR-188-3p group, Lenti-circ_0023826 + sh-MDM4 group. Except for the Glaucoma group, the other rats were additionally injected with a single injection of shRNA lentiviral vector or lentiviral overexpression vector targeting circ_0023826/miR-188-3p/MDM4. After 3 weeks, the rats were subjected to visual behavioral testing and subsequently euthanized by inhalation of excess CO₂ and the eyeball tissue was collected. The above lentiviral vectors were prepared by GenePharma.

Visual behavior test

An infrared light-illuminated black room (0.3 m×0.5 m×0.5 m) and a larger infrared light-illuminated white room (0.5 m×0.5 m×0.5 m) were used for visual behavior testing. There is a 10 cm×12 cm hole between the two rooms, allowing rats to freely shuttle from one room to another. The activity of the rats was recorded by a camera and the time spent by the rats in the dark room was calculated using Nordx etvision XT 8.0 software (Li et al., 2018).

Hematoxylin-eosin (H&E) staining

Rats’ eyeballs were fixed with 4% paraformaldehyde (Solarbio). The cornea was incised along the sclera, the iris and lens were obtained, and the retinas were frozen in 30% sucrose and sectioned into 5 μm. Frozen tissues were stained with H&E solution (Solarbio) routinely and observed under an optical microscope (BX-51, Olympus, Tokyo, Japan) in 5 fields. The thickness of the ganglion cell layer (GCL) and the inner plexiform layer (IPL) was measured with SE IPS image analyzer Each group was measured with SE IPS image analyzer Each group.

Isolation and culture of RGCs

The retinas were isolated from 10 normal rats using a dissecting microscope, digested with 0.125% trypsin, and centrifuged at 179g. The retinal pellet was centrifuged with 0.25% trypsin inhibitor (Sigma Aldrich), rinsed with Kreb solution containing magnesium and 1% bovine serum albumin (BSA), and made into a single-cell suspension. The cell suspension (1×10⁶ cells) was treated with 0.1 mg/ml porcine-l-ornithine (Sigma Aldrich) and 1 g/ml laminin (Sigma Aldrich), cultured in basal medium Eagle (BME) medium (Nanjing SenBeijia Biological Technology) containing 10% FBS, 25 mol/l glutamine and 0.1 mg/ml gentamicin.

Retinal single-cell suspension was incubated with goat anti-mouse IgG antibody (ab-6785; 1:1,000; Abcam) and rat anti-mouse Thy-1.1 antibody (ab-44898; 1:1,000; Abcam). The adherent cells were digested with 0.125% trypsin, centrifuged at 1000 rpm, and cultured with polylysine. Every three days, half of the medium was renewed (Xu et al., 2021). The isolated RGCs were immunocytochemically stained for Thy-1 (Kong et al., 2014). Briefly, antigen retrieval treatments were performed in citrate buffer in the microwave on medium heat for 5 min and then cooled to room temperature. After washing 3 times with PBS buffer, the slides were sealed with goat serum (ZSBG Bio, Beijing, China) for 30 min and incubated with anti-Thy-1 (Abcam, ab29574) overnight at 4°C. After 30 min incubation with HRP-conjugated secondary antibody solution (PV-6001, ZSBG Bio) at room temperature, staining was performed using DAB (ZSBG Bio). RGCs specific markers Bm3a (Abcam, ab245230, 1/1000), Thy-1 (Abcam, ab29574, 1/1500) and NF-L (Abcam, ab223343, 1/1000) were all analyzed by Western blot.

To establish an in vitro glaucoma model, RGCs were treated with N-methyl-D-aspartic acid (NMDA, 100 μmol/L) and glycine (10 μmol/L) for one hour. PBS-treated RGCs served as a negative control.

Lentiviral transfection

NMDA- and glycine-treated RGCs were collected. Transfection was performed when cells reached 70-80% confluence. shRNA targeting circ_0023826/MDM4 lentiviral vector (sh-circ_0023826/sh-MDM4), lentiviral vector overexpressing circ_0023826/miR-188-3p (Lenti-circ_0023826/Lenti-miR-188-3p), sh-NC and Lenti-NC were from GenePharma. RGCs were transfected using Lipofectamine 2000 reagent (Invitrogen). The medium was changed 24 h after transfection (Shen et al., 2020).

Subcellular localization analysis

Cytoplasmic and nuclear RNAs of cells were isolated using the PARIS Kit (Invitrogen) and allowed to quantitatively analyze circ_0023826 expression.
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RT-qPCR

Based on the Trizol reagent (Invitrogen), total RNA was extracted for reverse transcription of circRNA/mRNA and miRNA using PrimeScript RT reagent kit (Takara, Tokyo, Japan) and miRNA First Strand Synthesis kit (Takara), respectively. SYBR Green kit (Thermo Fisher Scientific, Waltham, MA, USA) in combination with the Mx3005P QPCR system (Agilent Technologies, CA, USA) was employed for PCR. U6 and GAPDH were used as internal controls. The primer sequences are shown in Table 1.

Western blot

Total protein was extracted with RIPA lysis buffer (Beyotime, China), loaded on 8% SDS-PAGE gels (Solarbio), transferred to PVDF membranes (Invitrogen), and blocked with 5% skim milk. It was incubated with primary antibodies MDM4 (ab39470, Abcam), Bax (ab32503, Abcam), Bel-2 (ab196495, Abcam), cleaved caspase-3 (ab2302, Abcam), GAPDH (ab8245, Abcam) and with the HRP-conjugated goat anti-rabbit IgG secondary antibody (Beyotime, China). Developed by ECL kit (34080, Thermo Fisher Scientific), signals were analyzed using ImageJ software (Li et al., 2022).

Cell viability assay

After 24 h of different treatments, transfected or untransfected RGCs (1×10⁶ cells/well) were added with 1 mL of MTT solution (Beyotime) for 2 h, and with 1 mL DMSO for 10 min. Finally, optical density (OD) values were recorded with a microplate reader (PerkinElmer) (Zhang et al., 2021).

Flow cytometry

RGCs were centrifuged at 1000×g, resuspended in 195 μL Annexin V-FITC binding buffer, and added with 5 μL Annexin V-FITC and 10 μL propidium iodide. The apoptosis of RGCs was detected by a FACS-Calibur flow cytometer (BD Biosciences).

ELISA

ELISA kits TNF-α (Abcam, ab181421), IL-6 (Abcam, ab178013) and IL-1β (Sigma-Aldrich, RAB0273) were employed to measure inflammation in the culture supernatants (Gao et al., 2020).

Dual-luciferase reporter assay

Potential binding sites for hsa_circ_0023826 and miR-188-3p were predicted by https://circinteractome.nia.nih.gov, while those for miR-188-3p and MDM4 were predicted by https://cm.jefferson.edu/rna22/. Based on the above wild-type sequence of hsa_circ_0023826 containing the miR-188-3p binding site, a mutant sequence was designed. RGCs were transfected with PGL4 containing hsa_circ_0023826/MDM4 wild-type sequence (hsa_circ_0023826/MDM4-WT) or hsa_circ_0023826/MDM4 mutant sequence (hsa_circ_0023826/MDM4-mut) together with miR-188-3p mimic or miR-NC. A dual-luciferase reporter assay system (Promega, Madison, WI, USA) was implicated to measure luciferase activity (Sui et al., 2020).

RNA pull-down experiment

A biotin-labeled RNA (Roche, Shanghai, China) was used. After RGCs were lysed using RIPA lysis buffer (Beyotime, China), biotin miR-188-3p-labeled streptavidin magnetic beads were added for 1-h incubation. After elution, the RNA levels were analyzed (Wei et al., 2021).

Statistical analysis

SPSS 21.0 statistical software was used to analyze the data, and the Kolmogorov-Smirnov test showed that the data were normally distributed, and the results were expressed as mean ± standard deviation (S.D.). One/Two-
way ANOVA analysis of variance and the least significant difference t test were suitable for comparing data. 

RESULTS

Hsa_circ_0023826 is downregulated during glaucoma

A previous study showed that circ_0023826 was downregulated in chronic glaucoma rats. It was speculated that circ_0023826 may have an important relationship with the development of glaucoma. Circ_0023826 expression was assessed in glaucoma patients. As shown in Fig. 1A, circ_0023826 expression in the eye fascia of glaucoma patients was lower than that of normal human eye fascia. Then, a rat model of glaucoma was constructed and the IOP was tested before and after modeling. The results showed that the IOP of the glaucoma rats began to increase after the microbead injection, and the IOP was higher than that of the rats in the Sham group until the end of the modeling (Fig. 1B). The visual behavior test manifested that the rats treated with microbead surgery stayed longer in the dark room than the rats in the sham-operated group (Fig. 1C), indicating that the increase of IOP caused by the injection of microbeads impairs the visual function of rats. These results indicated that a rat model of glaucoma was successfully constructed.

Circ_0023826 expression pattern in retinal tissue was checked by RT-qPCR, manifesting a decline in glaucoma rats (Fig. 1D). Subsequently, RGCs were isolated from rat eyeball tissues. The isolated cells were confirmed to be positive for Thy-1 expression by IHC staining (Fig. 1E). In addition, the expression of RGCs markers (Brn3a, Thy-1, and NF-L) in the isolated cells was higher than that in the rat eyeball tissue (Fig. 1F). This indicates that the collected cells were high-purity RGCs. Subsequently, an in vitro model of glaucoma was established by NMDA induction. As shown in Fig. 1G, the expression of circ_0023826 in the NMDA-treated RGCs was significantly higher than that in the PBS-treated RGCs. Subcellular isolation test further confirmed that circ_0023826 was a cytoplasmic RNA (Fig. 1H). All the above data suggest that circ_0023826 may be involved in the development of glaucoma at the cellular post-transcriptional translational level.

Hsa_circ_0023826 improves glaucoma symptoms and survival of RGCs

To explore the role of circ_0023826 in glaucoma, circ_0023826 was upregulated and downregulated in glaucoma rats (Fig. 2A). Visual behavioral test showed that knockdown of circ_0023826 increased the dark room dwell time of rats, while overexpression of circ_0023826 decreased the dark room dwell time of rats (Fig. 2B). H&E staining observed the morphological changes of the retinas, showing that IPL and GCL...
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were reduced in the Glaucoma group, while knockdown of circ_0023826 resulted in further reduction of IPL and GCL, but overexpression of circ_0023826 restored the levels of IPL and GCL (Fig. 2C). In addition, the number of RGCs in glaucoma rats was significantly reduced, knockdown of circ_0023826 further reduced the number of RGCs, while overexpression of circ_0023826 was able to restore the number of RGCs (Fig. 2D). Subsequently, the effect of circ_0023826 on the biological behavior of RGCs was examined. As shown in Fig. 2E, MND induced apoptosis of RGCs, while knockdown of circ_0023826 resulted in a further decrease in the cell viability of RGCs, but overexpression of circ_0023826 restored the cell viability of RGCs. In addition, MNDA promoted the apoptosis rate of RGCs and increased the expression of apoptosis-related proteins Bax and cleaved caspase-3, which was further enhanced by knockdown of circ_0023826, but attenuated by overexpression of circ_0023826 (Fig. 2F, G). These data suggest that overexpression of circ_0023826 improves glaucoma by promoting the cell viability of RGCs.

Circ_0023826 is a sponge for miR-188-3p

To reveal the underlying mechanism by which circ_0023826 works, a bioinformatics website was used to predict the downstream miRNAs of circ_0023826. miR-188-3p was found to have a targeted binding site of circ_0023826 (Fig. 3A).

Fig 3. circ_0023826 is a sponge for miR-188-3p. (A) The binding site (https://circinteractome.nia.nih.gov/) between circ_0023826 and miR-188-3p. (B–C) Dual-luciferase reporter assay and RNA pull-down assay to evaluate the interaction between circ_0023826 and miR-188-3p. (D) Changes of miR-188-3p in RGCs after MND induction (E) miR-188-3p expression after downregulating circ_0023826 in isolated RGCs. Data are presented as mean ± S.D. (n=3). ***p<0.001, **p<0.01, *p<0.05.

Fig 4. miR-188-3p directly targets MDM4.

(A) The bioinformatics website (https://cm.jefferson.edu/rna22/) predicts the binding site between MDM4 and miR-188-3p. (B–C) Dual-luciferase reporter assay and RNA pull-down assay to assess the interaction between MDM4 and miR-188-3p. (D–E) Effects of MND treatment on MDM4 expression in RGCs detected by RT-qPCR or western blot. (F–G) Effects of down-regulation of circ_0023826 or up-regulation of miR-188-3p on MDM4 expression detected by RT-qPCR or western blot. Data are presented as mean ± S.D. (n=3). *p<0.05, **p<0.01, ***p<0.001.
pull down circ_0023826 (Fig. 3C). Furthermore, MNDA promoted the expression of miR-188-3p in RGCs (Fig. 3D); knockdown of circ_0023826 increased the expression of miR-188-3p (Fig. 3E). The above data indicated that miR-188-3p was the target gene of circ_0023826.

**MiR-188-3p directly targets MDM4**

There are multiple complementary binding sites in miR-188-3p and MDM4 based on bioinformatics analysis (Fig. 4A). The results of dual-luciferase reporter gene assay confirmed that co-transfection of miR-188-3p-mimic with MDM4-WT reduced luciferase activity, while co-transfection with MDM4-MUT had no effect on luciferase activity (Fig. 4B). Meanwhile, the results of RNA pull-down experiment detected that Bio-miR-188-3p enriched MDM4 (Fig. 4C). Furthermore, MNDA treatment decreased MDM4 expression in RGCs (Fig. 4D, E). Knockdown of circ_0023826 or overexpression of miR-188-3p also inhibited the expression of MDM4 (Fig. 4F, G). The above data indicate that MDM4 is a downstream target gene of miR-188-3p.

**Circ_0023826 alleviates glaucoma and the survival of RGCs by miR-188-3p targeting MDM4**

To investigate the effect of circ_0023826 on glaucoma via the miR-188-3p/MDM4 axis, Lenti-NC, Lenti-circ_0023826, Lenti-circ_0023826 + Lenti-miR-188-3p, Lenti-circ_0023826 + sh-MDM4 were injected into the eyes of rats, respectively. As shown in Fig. 5A, overexpression of circ_0023826 promoted the expression of MDM4, but this effect was reversed by overexpression of miR-188-3p or knockdown of MDM4 (Fig. 5A). The results of visual behavioral test showed that up-regulation of circ_0023826 could improve visual function in rats, while up-regulation of miR-188-3p or down-regulation of MDM4 could reverse the improvement of visual function in rats by circ_0023826 addition (Fig. 5B). H&E staining found that Lenti-circ_0023826 significantly protected the reduction of GCL and IPL, and significantly increased the number of RGCs. However, Lenti-miR-188-3p or sh-MDM4 could reverse the effects of Lenti-circ_0023826 (Fig. 5C, D). As shown in Fig. 5E–G, Lenti-circ_0023826 can increase the cell viability and inhibit apoptosis of RGCs. However, Lenti-miR-188-3p or sh-MDM4 could reverse the effects of Lenti-circ_0023826. Taken together, circ_0023826 alleviates glaucoma symptoms and promotes the survival of RGCs by regulating the miR-188-3p/MDM4 axis.

**DISCUSSION**

Glaucoma is a major cause of irreversible vision loss and is characterized by retinal neurodegeneration (Van et al., 2022). Directly or indirectly promoting the survival of RGCs can delay or prevent the progression of glaucoma. Here, we reported that circ_0023826 was downregulated during glaucoma and demonstrated that circ_0023826 alleviated visual impairment in glaucoma rats and promoted the survival of RGCs. Mechanistically, circ_0023826 worked through sponge-absorbing miR-188-3p, which affected its downstream gene MDM4. Regarding study novelty, we reported for the first time that circ_0023826 was involved in the pathogenesis of glaucoma through the downstream miR-188-3p/MDM4 axis.
CircRNAs are abundant in the eukaryotic transcriptome (Jek et al., 2013) and significantly enriched in human brain and retinal tissues (Akhter et al., 2018). It has been reported that circRNAs may drive changes that lead to glaucoma progression through one or more of these pathophysiological processes and lead to RGC death (Rong et al., 2021). Studies have revealed the significance of circRNAs in glaucoma, including circZNF609 and circZRNAB1. circ_0023826 suggests a diagnostic value in glaucoma. We first studied circ_0023826’s biological function in glaucoma. Apoptosis and inflammation of RGCs are important pathological features of eye diseases such as glaucoma (Ou et al., 2021). We found that circ_0023826 in glaucoma-induced rats could alleviate the visual impairment, increase the number of RGCs, and inhibit the apoptosis of RGCs.

It has been demonstrated that circRNAs can act as miRNA sponges to regulate targeted downstream gene expression. Therefore, we predicted the targeted miRNA (miR-188-3p) of circ_0023826. miRNAs are important regulators of the complex biological progression of various neurodegenerative diseases (Mead et al., 2022). Recently, miRNAs have been widely reported to be involved in the regulation of cell growth, differentiation, metabolism and apoptosis in the biological progression of glaucoma (Greene et al., 2022). MiR-126 promotes retinal ganglion cell apoptosis in glaucoma rats (Wang et al., 2020) and miR-223 induces retinal ganglion cell apoptosis. miR-188-3p has been reported to be associated with various diseases, such as cancer (Luo et al., 2021), cardiovascular disease (Mi et al., 2020), and diabetic nephropathy (Jin et al., 2021). miR-188-3p has been reported to disrupt gemmarone-mediated podocyte protection in a mouse model of type 1 diabetic nephropathy by triggering mitochondrial damage (Wang et al., 2021). Despite accumulating evidence that miR-188-3p exerts anti-apoptotic roles in several cell types, its function in retinal ganglion cell apoptosis and glaucoma progression is unclear. We first found that miR-188-3p was upregulated in RGCs isolated from retinal tissue of glaucoma rats and confirmed that circ_0023826’s effect on the survival of RGCs could be reversed by overexpressing miR-188-3p.

Subsequently, we further predicted downstream factors of miR-188-3p and found that MDM4 was downregulated during glaucoma. MDM4 protein, also known as MDMX, was first discovered in 1996 by screening for p53-binding proteins (Shvarts et al., 1996). It has been reported that MDMX is often amplified and highly expressed in human cancers, promoting cancer cell growth, and inhibiting apoptosis (Yu et al., 2020). However, the function of MDMX in retinal ganglion cell apoptosis and glaucoma progression is currently unclear. To further verify the regulatory effect of circ_0023826/miR-188-3p/MDM4 axis on NSCLC, we performed rescue experiments to further verify the molecular mechanism of circ_0023826 protecting glaucoma in vitro and in vivo.

CONCLUSION

Our study reports the protective effect of circ_0023826 in glaucoma-induced neuroretinal degeneration. It was confirmed that upregulating circ_0023826 expression in glaucoma-induced rats can alleviate the damage to visual function in rats, increase the number of RGCs, and inhibit the apoptosis of RGCs. Mechanistically, our results suggest that circ_0023826 acts in glaucoma-induced neuroretinal degeneration by regulating the miR-188-3p/MDM4 axis and defines circ_0023826 as a new target for neuroprotective therapy of glaucoma.

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