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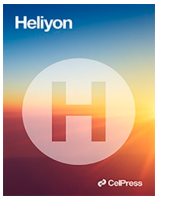

Knockdown of lncRNA PVT1 protects human trabecular meshwork cells against H_2O_2-induced injury *via* the regulation of the miR-29a-3p/VEGF/MMP-2 axis

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ABSTRACT

Purpose: Human trabecular meshwork cell (HTMC) dysfunction results in imbalanced aqueous humor inflow and outflow, leading to an increase in intraocular pressure (IOP). Uncontrolled high IOP can promote the occurrence of glaucoma, an irreversible optic neuropathy. Here, we explored whether the long non-coding RNA plasmacytoma variant translocation 1 (lncRNA PVT1)/microRNA-29a-3p (miR-29a-3p) axis could ameliorate HTMC dysfunction under oxidative stress by modulating the expression of the proangiogenic factor vascular endothelial growth factor (VEGFA) and the profibrotic factor metalloproteinase-2 (MMP-2).

Methods: HTMCs were cultured under H_2O_2-induced oxidative stress for 48 h. The expression of lncRNA PVT1, miR-29a-3p, VEGFA, MMP-2, intracellular adhesion molecule-1 (ICAM-1), and alpha-smooth muscle actin (α-SMA) was detected by reverse transcription quantitative real-time polymerase chain reaction, western blotting, and immunofluorescence. Interference experiments were conducted *via* the transfection of HTMCs with small interfering RNA (siRNA) targeting lncRNA PVT1 or miR-29a-3p mimics. A luciferase reporter assay was undertaken to identify the presence of a miR-29a-3p binding site in lncRNA PVT1. Flow cytometry and Transwell and Cell Counting Kit-8 assays were employed to evaluate HTMC functions under oxidative stress with different treatments.

Results: In HTMCs, the expression of lncRNA PVT1 was induced by H_2O_2 treatment, whereas that of miR-29a-3p was inhibited. The levels of angiogenic factors (VEGFA, ICAM-1) and fibrosis-associated mediators (MMP-2, α-SMA) were upregulated in HTMCs under oxidative stress. The siRNA-mediated suppression of lncRNA PVT1 or the upregulation of miR-29a-3p significantly suppressed the expression of VEGFA, MMP-2, ICAM-1, and α-SMA. A luciferase reporter assay confirmed that lncRNA PVT1 directly targeted miR-29a-3p and acted as a miR-29a-3p sponge. The knockdown of lncRNA PVT1 restored the level of miR-29a-3p in H_2O_2-treated HTMCs, thereby inhibiting VEGFA and MMP-2, its target mRNAs. HTMC dysfunction, including increased

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apoptosis and decreased cell mobility and viability, could be effectively ameliorated by lncRNA PVT1 downregulation or miR-29a-3p overexpression under oxidative stress.
Conclusion: LncRNA PVT1 has potential as a therapeutic target for inhibiting VEGFA and MMP-2, thus protecting HTMCs, suppressing the progression of fibrosis, and, consequently, improving the outcome of glaucoma filtration surgery.

1. Introduction

Glaucoma is a progressive and irreversible optic neuropathy, which is one of the leading reasons for blindness worldwide [1]. Glaucoma is characterized by the atrophy of the optic nerve head, visual field defects, and vision loss, mostly due to long-term detrimental high intraocular pressure (IOP) [2]. The balance between aqueous humor inflow and outflow is the key determinant of IOP, and reducing IOP is an effective therapy for glaucoma [3]. Human trabecular meshwork cells (HTMCs), situated in the iridocorneal angle, are primarily responsible for the drainage of aqueous humor. Elevated IOP is a consequence of HTMC dysfunction or the resistance of HTMCs to aqueous humor outflow [4]. Increasing evidence supports that oxidative stress plays an essential role in the pathophysiological changes in HTMCs [5,6] that lead to the suppression of aqueous humor outflow, thus upregulating the IOP. Recent cell culture-based studies have also identified the deleterious role of oxidative stress in HTMC abnormalities [7–9]. Therefore, it is crucial to explore the mechanisms underlying oxidative stress-induced HTMC impairment and identify novel targets for protecting HTMCs and ameliorating the progression of glaucoma.

Acute primary angle closure (APAC) refers to the onset of acute glaucoma usually induced by an abrupt closure of the trabecular meshwork (TM) in the anterior chamber angle [10]. Frequent attacks of APAC will result in TM impairment, and, consequently, uncontrolled high IOP [11]. Patients with APAC often require a trabeculectomy to sustain IOP at a relatively low level. However, filtering bleb failure, which is a poor outcome of trabeculectomy, is associated with some risks, including increased vascularization [12]. Huang et al. [13] observed that in the aqueous humor of patients with APAC and senile cataracts, the level of vascular endothelial growth factor (VEGF) was significantly increased and was positively correlated with IOP. Meanwhile, a series of studies have demonstrated that APAC patients demonstrate an early ‘‘acute inflammatory’’ condition, characterized by an increase in the levels of matrix metalloproteinases (MMPs) [14]. MMPs comprise a family of proteolytic enzymes that degrade components of the extracellular matrix (ECM), such as collagen and exert crucial effects on kinds of biological processes. In the eye, ECM turnover mediated by MMP in the juxtacanalicular region of the TM decreases outflow resistance in the conventional outflow pathway and helps sustain IOP homeostasis. MMPs also participate in tissue repair, and their levels increase after acute injury. These observations suggest that targeting VEGF and MMPs may represent an effective therapeutic strategy for protecting against APAC and reducing the severe damage associated with glaucoma.

Noncoding RNAs (ncRNAs) are RNAs with little or no protein-coding potential that play important roles under normal physiological conditions. However, their dysregulation has been implicated in diseases such as cancers and several genetic disorders [15,16]. Long ncRNAs (lncRNAs) refer to non-coding transcripts longer than 200 nucleotides that have been detected to modulate the expression of genes at the epigenetic, transcriptional, post-transcriptional, and splicing levels. They are enrolled in a wide variety of physiological and pathological processes, including oxidative stress, cell proliferation, and apoptosis [17]. MicroRNAs (miRNAs) are a group of short (approximately 21–23 nucleotides long) endogenous RNAs with highly conserved sequences that modulate gene expression by combining with the seed region of the 3′-untranslated region (3′-UTR) of target mRNAs, resulting in their degradation or the inhibition of their translation. MiRNAs are expressed in all human cell types and take part in biological processes such as cell growth and apoptosis [18]. LncRNAs and miRNAs are the major subtypes of ncRNAs and function as critical regulatory elements. Moreover, they can interact with each other under both physiological and pathological conditions [19,20], and there is evidence implicating their interaction in glaucoma pathophysiology [21,22]. Using Target. Scan, we identified VEGFA and MMP-2 as targets of miR-29a-3p. Additionally, starBase predicted the presence of a putative complementary sequence for miR-29a-3p on lncRNA plasmacytoma variant translocation 1 (PVT1). LncRNA PVT1 is highly expressed and acts as an oncogene in multiple cancers [23], and also functions in ophthalmic diseases such as diabetic retinopathy [24], retinoblastoma [25], and diabetic cataract [26]. However, the mechanism underlying the role of lncRNA PVT1 in glaucoma remains unknown. Similarly, it is not known whether interaction between lncRNA PVT1 and miR-29a-3p is involved in regulating VEGFA and MMP-2 in HTMCs under oxidative stress and whether this plays a role in the pathophysiology of glaucoma.

Hydrogen peroxide (H_2O_2) stimulation is a widely used method for simulating the induction of oxidative stress *in vitro*. In this study, we explored how the interaction between lncRNA PVT1 and miR-29a-3p affected HTMCs under H_2O_2-induced oxidative stress to uncover potential novel therapeutic targets for ameliorating glaucoma.

2. Materials and methods

2.1. Cell line and cell culture

HTMCs, extracted from the juxtacanalicular and corneoscleral regions of the human eye, were provided by ScienCell Research Laboratories (#6590, Carlsbad, CA, USA). The cells were cultured in TMC medium (#6591, ScienCell) consisting of 5 % fetal bovine serum (FBS), 1 % TMC growth supplement, and 1 % penicillin/streptomycin at 37 °C in an incubator with 5 % CO_2.

2.2. H_2O_2 treatment

Hydrogen peroxide so lution (3 %) was purchased from Sigma-Aldrich (#323381, Waltham, MA, USA). The reagent was diluted to 200 μM in serum-free medium before being added to the cells. HTMCs were treated with H_2O_2 for 24 h and the reaction was stopped by washing three times with 1 × phosphate-buffered saline (PBS).

2.3. Transfection of small interfering RNA (siRNA) and miRNA mimics

MiR-29a-3p mimics and three siRNAs for knocking down lncPVT1 were synthesized by GenePharma (Shanghai, China). HTMCs were seeded in 6-well plates and cultured under normal or H_2O_2-containing medium for 24 h (until 70%–80 % confluence). The cells were then transfected with lncPVT1 siRNA, miR-29a-3p mimics, or the corresponding negative controls (NC) using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). 6 h later, the medium was changed with fresh TMC medium, and incubation was continued.

2.4. Total RNA isolation and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from HTMCs treated with different conditions by the TaKaRa Mini BEST Universal RNA Extraction Kit (TaKaRa Bio, Dalian, China) referring to the manufacturer's protocol. The RNA (1 μg) was reverse transcribed into cDNA using the Perfect Real Time RT Reagent Kit (TaKaRa Bio) and the qPCR was performed by SYBR Premix Ex Taq (TaKaRa Bio). The qPCR mixture involoved 2 μL of cDNA, 10 mmol primers (forward and reverse mixed), and 10 μL of 2 × SYBR Green Master Mix. For the miRNA, qPCR was carried out by TransScript Green miRNA Two-Step qRT-PCR SuperMix (TransGen Biotech, Beijing, China). GAPDH was applied as an internal control for lncRNA or mRNA and U6 was used as the internal control for miRNA. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 1.

2.5. Western blotting

Protein was extracted from cultured cells using radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 1 mM; Beyotime, Jiangsu, China). Protein lysates were electrophoresed on 10 % SDS polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA), blocked with 5 % skimmed milk for 1 h, and then incubated with primary antibodies including VEGF (1:1,000, ab52917), MMP-2 (1:1,000, ab92536), ICAM1 (1:1,000, ab222736) (all from Abcam, USA), α-SMA (1:1,000, #19245), or GAPDH (1:1,000, #2118 (both from CST, USA) at 4 °C overnight. After washing with TBST buffer, the membranes were treated with the secondary antibodies for 1 h at room temperature. Finally, the bands were demonstrated using an Enhanced Chemiluminescence (ECL) Plus Kit (Millipore) and the gray values were calculated through ImageJ software.

2.6. Immunofluorescence staining

HTMCs were plated on polylysine-coated glass coverslips and cultured under different treatments. After rinsing, the cells were fixed in 4 % paraformaldehyde, permeabilized with 0.5 % Triton X-100 in PBS for 15 min at room temperature, blocked in 1 % bovine serum albumin (BSA) for 30 min at 37 °C, and then incubated overnight at 4 °C with primary antibodies, including rabbit monoclonal anti-VEGF (1:500), rabbit monoclonal anti-MMP-2 (1:500), rabbit monoclonal anti-ICAM1 (1:500), and rabbit polyclonal anti-α-SMA (1:500). Subsequently, the cells were incubated with Cy3-conjugated goat anti-rabbit IgG (Bioss, Beijing, China) or DyLight 488-conjugated goat anti-mouse IgG (Thermo, IL, USA) secondary antibodies for 1 h at 37 °C. Nuclei were counterstained with DAPI (1:600; Solarbio, Beijing, China). The cells were analyzed and imaged under a fluorescent microscope (Olympus IX71, Tokyo, Japan).

2.7. Luciferase reporter assay

The putative binding sites (wild-type [WT] or mutated [Mut]) for miR-29a-3p on lncPVT1 were cloned into the pGL3 plasmid (Promega, Madison, WI, USA). The resulting constructs were confirmed by sequencing. Human embryonic kidney 293 (HEK293) cells were co-transfected with LncPVT1-WT, LncPVT1-Mut, or control vector and miR-29a-3p mimics or scramble miRNA by Lipofectamine

Table 1
The sequence of mRNAs used in RT-qPCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
Lnc-PVT1	CCCAGCTTGGGGCTGTCAAA	CCACCACTTCTGGCTGGGTG
VEGFA	TCAGCGCAGCTACTGCCATC	GTGCTGGCCTTGGTGAGGTT
MMP2	TCTGGAGCGATGTGACCCCA	GCATGAGCCAGGAGTCCGTC
GAPDH	GCGGGGCTCTCCAGAACATC	TCCACCACTGACACGTTGGC
miR-29a-3p RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTTTTTTTTTTTTTAG	
miR-29a-3p	TAGCACCATCTGAAATCGGTTA	TGGTGTCGTGGAGTCG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

2000 (Invitrogen). After 5 h of transfection, the medium was refreshed. 48 h later, luciferase assays were performed by the Dual-Glo Luciferase Assay System (E2920; Promega) following the manufacturer's protocol. Luciferase activity was detected with a GloMax 96 Microplate Luminometer (Promega). Firefly luciferase activity was compared to that of *Renilla* luciferase for each sample.

2.8. Cell Counting Kit-8 (CCK-8) assay

Cell viability was measured using a CCK-8 assay (#E606335, Sangon Biotech, Shanghai, China) following the protocol. HTMCs were cultured and treated with different conditions and then plated in 96-well plates with a density of 2×10^4 cells/well for 24, 48, and 72 h. Then, the medium was changed with 100 μL of fresh medium, and 10 μL of CCK-8 reagent was added per well. After incubation for 1 h, absorbance at 450 nm was detected using a Varioskan Flash multimode reader (Thermo Fisher Scientific).

2.9. Analysis of apoptosis

After 48 h of transfection, HTMCs were subjected to apoptosis analysis using an APC Annexin V kit (#561012, BD Biosciences, USA). Briefly, the cultured cells were resuspended, and 1×10^5 cells were added to 100 μL of 1 × binding buffer, treated with 5 μL of FITC Annexin-V for 20 min, and finally with 3 μL of propidium iodide (PI) for 10 min in the dark at room temperature. Then, 400 μL of binding buffer was added to the cells, and the rate of apoptosis was detected by a FACSAria Flow Cytometer (BD Biosciences).

2.10. Transwell assay

Transwell assays were performed to evaluate the migratory ability of HTMCs. Suspensions of 5×10^3 cells were seeded in the top chambers containing 200 μL of medium with 5 % FBS (with 8-μm pore polycarbonate membrane inserts (Corning)). The bottom chambers consisted 500 μL of medium supplemented with 20 % FBS. Culturing for 24 h, the cells in the top chamber (non-migrated

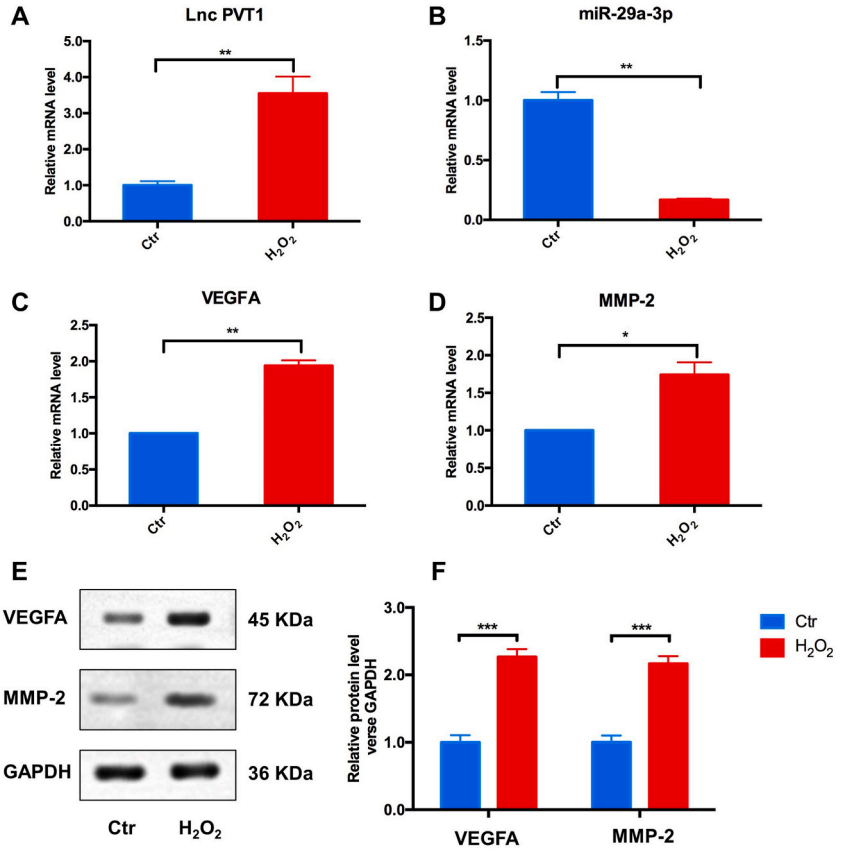

Fig. 1. LncRNA PVT1 is upregulated while miR-29a-3p is downregulated in H_2O_2-treated human trabecular meshwork cells (HTMCs). (A, B) RT-qPCR analysis of the expression of lncRNA PVT1 and miR-29a-3p in HTMCs under H_2O_2 treatment. (C, D) The mRNA expression of VEGFA and MMP-2 was increased in H_2O_2-treated HTMCs. (E, F) Western blots and quantification of the protein levels of VEGFA and MMP-2 in H_2O_2-treated HTMCs. Ctr, control group; H_2O_2, H_2O_2 treatment group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *t*-test was used; $n = 3$. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; VEGFA, vascular endothelial growth factor A; MMP-2, matrix metalloproteinase-2. The uncropped and non-adjusted blots were shown in the Supplemantary Material 3.

cells) were removed, and the transmigrated cells were fixed in 4 % paraformaldehyde and stained with 0.1 % crystal violet. Images were obtained and cells were counted in five fields. Quantification was performed using ImageJ software.

2.11. Statistical analysis

Statistical analysis was carried out by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Data are presented as means ± standard deviation (SD) of at least three independent experiments. Adhoc test was applied in all experiments. Two-tailed Student's *t*-tests were employed for comparisons between two groups. One-way analysis of variance (ANOVA) was applied for comparisons among multiple groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. The level of lncRNA PVT1 was upregulated while that of miR-29a-3p was decreased in H_2O_2-treated HTMCs

To detect the roles of lncRNA PVT1 and miR-29a-3p in glaucoma, the expression levels of both RNAs were measured in HTMCs treated with H_2O_2. We found that the expression of lncRNA PVT1 was significantly increased (approximately 4-fold compared with control cells) in HTMCs under H_2O_2-induced oxidative stress (Fig. 1A). Conversely, the expression of miR-29a-3p in HTMCs was inhibited by H_2O_2 treatment (Fig. 1B). Bioinformatics analysis indicated that VEGFA and MMP-2 were potential targets of miR-29a-3p (Fig. S1). The binding sites for miR-29a-3p on the 3′-UTRs of VEGFA and MMP-2 mRNA have been previously reported [27]. Here, we

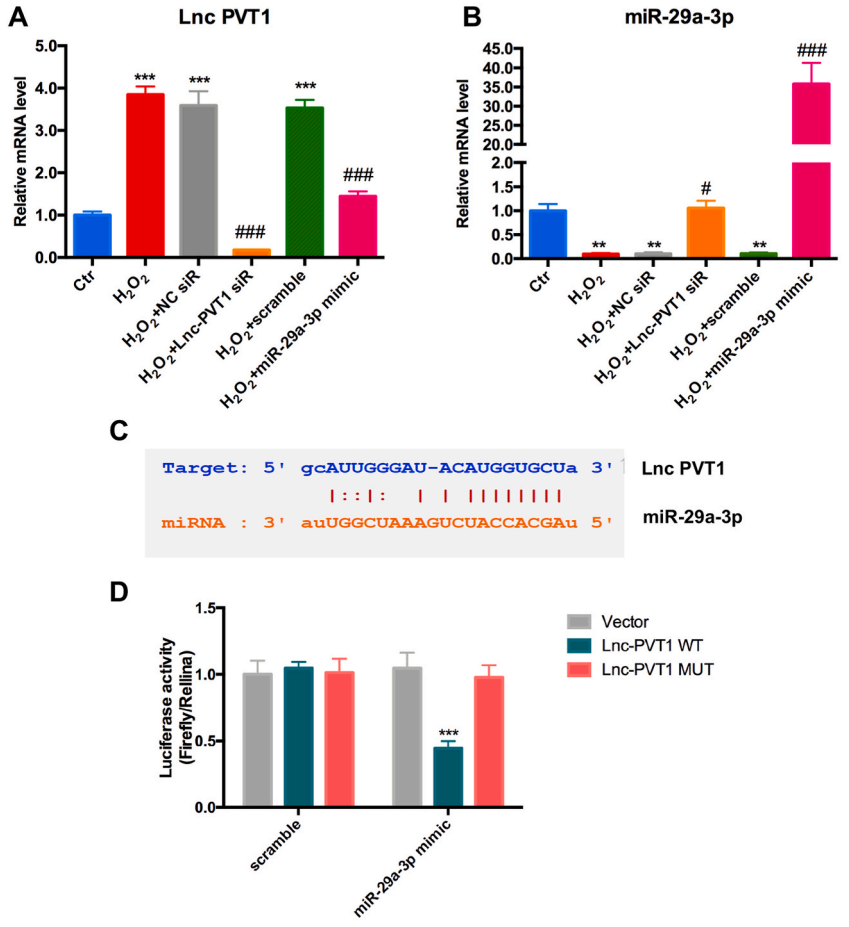

Fig. 2. LncRNA PVT1 directly interacts with and acts as a sponge of miR-29a-3p. (A) The H_2O_2 treatment-mediated increase in the levels of lncRNA PVT1 in human trabecular meshwork cells (HTMCs) was inhibited when the cells were transfected with small interfering RNA targeting lncRNA PVT1 or when miR-29a-3p was overexpressed. (B) Suppressing lncRNA PVT1 reversed the loss of miR-29a-3p expression and the transfection of miR-29a-3p mimics significantly upregulated the expression of miR-29a-3p in H_2O_2-treated HTMCs. (C) The potential target site for miR-29a-3p on lncRNA PVT1. (D) A luciferase assay confirmed that lncRNA PVT1 and miR-29a-3p directly interacted. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control (ctr) group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, compared with the H_2O_2 group, ANOVA was applied; $n = 3$. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1.

measured the expression of VEGFA and MMP-2 in H_2O_2-treated HTMCs to explore the regulatory role of miR-29a-3p in glaucoma. The results showed that, compared with the control condition, the expression of VEGFA and MMP-2 was significantly increased (>2-fold) in HTMCs under oxidative stress at both the mRNA and protein levels (Fig. 1C–F). This initial observation of target genes in H_2O_2-exposed HTMCs hinted further mechanistic exploration.

3.2. LncRNA PVT1 targets miR-29a-3p directly and acts as a miR-29a-3p sponge

To explore the biological roles of lncRNA PVT1 in HTMCs, we transfected the cells with three siRNAs targeting PVT1 to silence its expression (Fig. S2). We found that, compared with NC siRNA, lncPVT1 siRNA significantly inhibited the oxidative stress-induced upregulation of lncRNA PVT1 expression (Fig. 2A). Additionally, the knockdown of lncRNA PVT1 restored the level of miR-29a-3p in H_2O_2-treated HTMCs to normal levels (Fig. 2B). The expression of miR-29a-3p was significantly enhanced, whereas that of lncRNA PVT1 was suppressed, in H_2O_2-treated HTMCs overexpressing miR-29a-3p *via* the transfection of miR-29a-3p mimics. These results suggested that lncRNA PVT1 and miR-29a-3p compete with each other for target sites. Bioinformatics analysis showed a putative target site for miR-29a-3p on lncRNA PVT1 (Fig. 2C). In a luciferase reporter assay, luciferase activity was decreased when LncPVT1-WT and miR-29a-3p mimics were co-transfected into HEK293 cells, suggestive of direct binding between miR-29a-3p and the 3′-UTR of lncRNA PVT1 (Fig. 2D). The fact that the expression of lncRNA PVT1 and miR-29a-3p was negatively correlated in H_2O_2-treated HTMCs suggested that lncRNA PVT1 acted as a sponge for miR-29a-3p.

3.3. VEGFA and MMP-2 are regulated by the lncRNA PVT1/miR-29a-3p axis

Given that VEGFA and MMP-2 are known to influence IOP, we next investigated the regulatory mechanism underlying this effect. Because VEGFA and MMP-2 were found to be targets of miR-29a-3p, we first measured the expression levels of VEGFA and MMP-2 in H_2O_2-treated HTMCs overexpressing miR-29a-3p *via* the knockdown of lncRNA PVT1 or the transfection of miR-29a-3p mimics. We found that the transcriptional levels of both VEGFA and MMP-2 were significantly inhibited in H_2O_2-treated HTMCs following the upregulation of miR-29a-3p compared with that seen with H_2O_2 treatment alone (Fig. 3A and B). No difference in VEGFA or MMP-2 expression was detected with the transfection of NC lncPVT1 siRNA or scramble miRNA. The inhibitory effects of miR-29a-3p on VEGFA and MMP-2 were further confirmed by western blotting (Fig. 3C). The results revealed that the H_2O_2-mediated increase in the levels of VEGFA and MMP-2 in HTMCs was significantly suppressed by lncRNA PVT1 inhibition or miR-29a-3p upregulation (Fig. 3D and E). These findings supported that VEGFA and MMP-2 could be negatively modulated by miR-29a-3p.

Fibration is the initiating factor and primary mediator of wound healing and fibrotic scar formation post-trabeculectomy, which is

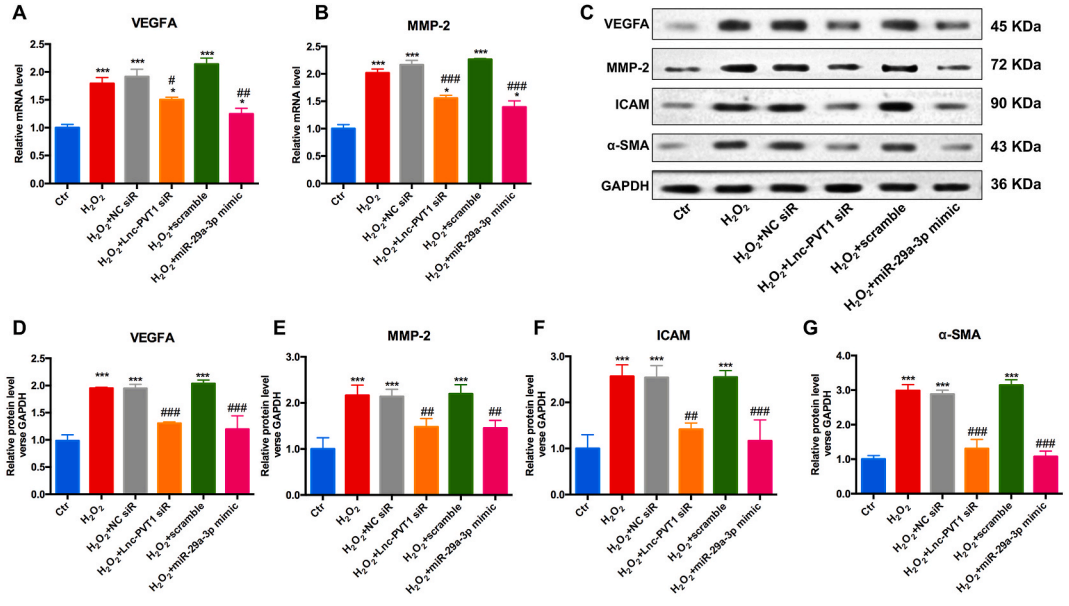

Fig. 3. LncRNA PVT1/miR-29a-3p regulates the VEGFA/MMP-2 axis in human trabecular meshwork cells (HTMCs). (A, B) The mRNA expression of VEGFA and MMP-2 was significantly inhibited by the knockdown of lncRNA PVT1 and the overexpression of miR-29a-3p. (C–E) Western blots analysis confirmed that VEGFA and MMP-2 were regulated by lncRNA PVT1 and miR-29a-3p. (C, F, G) ICAM-1 and α-SMA expression was also induced by oxidative stress in HTMCs, an effect that was suppressed by the downregulation of lncRNA PVT1 or the upregulation of miR-29a-3p. $^{*}p < 0.05$, $^{***}p < 0.001$, compared with the control (ctr) group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the H_2O_2 group, ANOVA was applied; $n = 3$. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; VEGFA, vascular endothelial growth factor A; MMP-2, matrix metalloproteinase-2; ICAM-1, intracellular adhesion molecule-1; α-SMA, alpha-smooth muscle actin. The uncropped and non-adjusted blots were shown in the Supplemantary Material 3.

as well the main factor of trabeculectomy failure [28]. Following their differentiation from fibroblasts, myofibroblasts produce alpha-smooth muscle actin (α-SMA), a profibrotic protein that has been implicated in ECM contraction and remodeling [29]. Meanwhile, intracellular adhesion molecule-1 (ICAM-1), a proinflammatory factor, plays a promotive role in fibrosis development [30]. These suggest that reducing α-SMA and ICAM-1 levels might mitigate fibrotic scar formation and development after trabeculectomy in patients with primary angle-closure glaucoma (PACG). In the present study, we found that H_2O_2 treatment increased the expression levels of ICAM-1 and α-SMA in HTMCs (Fig. 3C), whereas the knockdown of lncRNA PVT1 or miR-29a-3p upregulation exerted the opposite effect (Fig. 3F and G). These findings suggested that the inhibition of lncRNA PVT1 might suppress H_2O_2-induced fibrotic scarring in HTMCs by downregulating ICAM-1 and α-SMA expression.

3.4. The knockdown of lncRNA PVT1 suppressed H_2O_2-induced fibering in HTMCs

Next, we performed double immunofluorescence staining to detect the localization and expression intensity of VEGFA, MMP-2, ICAM-1, and α-SMA in HTMCs under oxidative stress. As shown in Fig. 4A, ICAM-1 and VEGFA were detected in the membrane and cytoplasm of HTMCs. Moreover, the fluorescence intensity of both molecules was significantly enhanced in HTMCs under H_2O_2 treatment compared with that seen in untreated cells. Meanwhile, transfection with lncRNA PVT1 siRNA or miR-29a-3p mimics reduced the fluorescent intensity of both ICAM-1 and VEGFA in H_2O_2-treated HTMCs compared with that detected with H_2O_2 treatment alone. MMP-2 was mainly expressed in the nucleus and only weakly in the cytoplasm. As expected, α-SMA clearly labeled the cytoskeleton of HTMCs (Fig. 4B). H_2O_2 exposure significantly enhanced the fluorescence associated with MMP-2 and α-SMA in HTMCs, an effect that was inhibited by the knockdown of lncRNA PVT1 or the upregulation of miR-29a-3p. A quantitative analysis of fluorescence intensity confirmed this observation. Compared with the normal condition, oxidative stress induced an increase in VEGFA (~3-fold), MMP-2 (~4-fold), ICAM-1 (~3.5-fold), and α-SMA (~5-fold) expression in HTMCs (Fig. 4C–F). These findings indicated that silencing lncRNA PVT1 or overexpressing miR-29a-3p could reverse the oxidative stress-induced upregulation of the levels of genes involved in fibrosis and fibrotic scar formation. The data further suggested that the knockdown of lncRNA PVT1 may help ameliorate the progression of fibrotic scarring after trabeculectomy, thereby helping to control IOP.

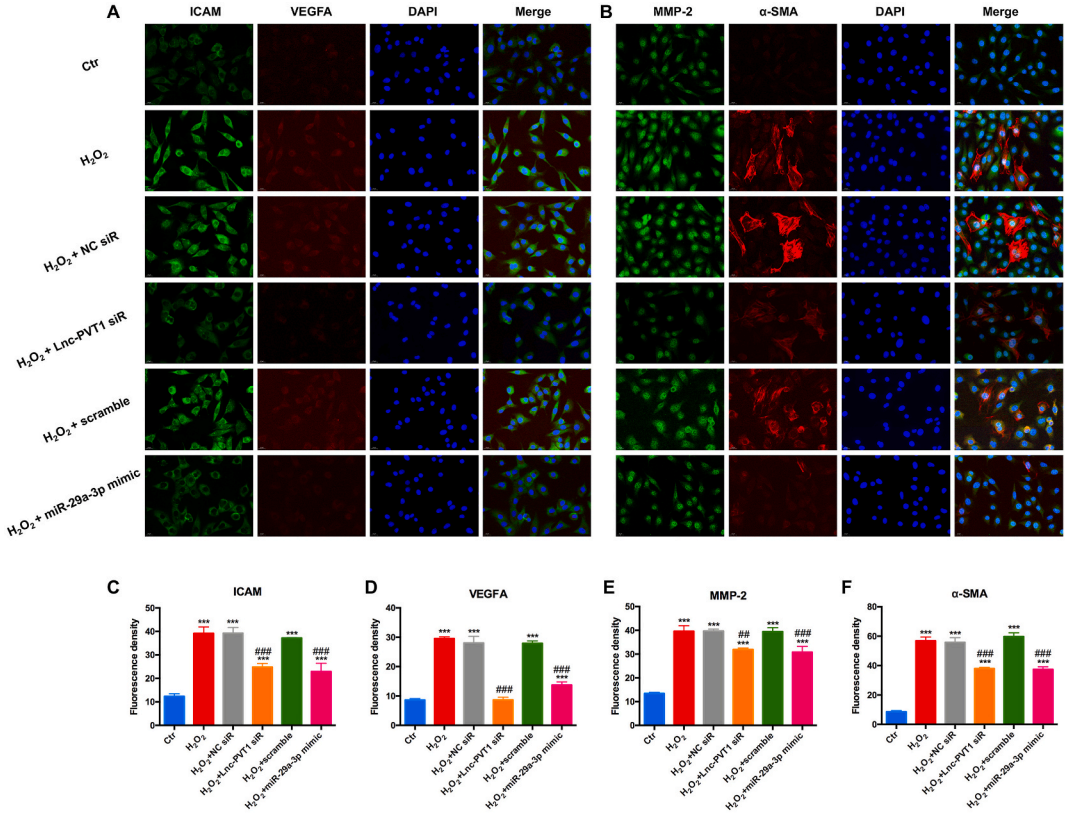

Fig. 4. Double immunofluorescence staining of VEGFA, ICAM-1, MMP-2, and α-SMA in human trabecular meshwork cells (HTMCs) under different conditions. (A) The localization of VEGFA and ICAM-1 in HTMCs. (B) The localization of MMP-2 and α-SMA in HTMCs. (C–F) Quantification of the fluorescence intensity of VEGFA, ICAM-1, MMP-2, and α-SMA; the H_2O_2 treatment-induced increase in their expression levels could be alleviated by lncRNA PVT1 knockdown or miR-29a-3p overexpression. $^{***}p < 0.001$, compared with the control (ctr) group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the H_2O_2 group, ANOVA was applied; $n = 3$. VEGFA, vascular endothelial growth factor A; MMP-2, matrix metalloproteinase-2; ICAM-1, intracellular adhesion molecule-1; α-SMA, alpha-smooth muscle actin.

3.5. Suppressing lncRNA PVT1 exerted protective effects against H_2O_2-induced HTMC dysfunction

The pathological change seen in uncontrolled glaucoma is commonly characterized by abnormally functioning TM cells. Here, we assessed the effect of H_2O_2 treatment on the apoptosis, migration, and viability of HTMCs. Flow cytometry-based analysis of apoptosis demonstrated that H_2O_2 administration significantly increased the apoptotic rate in HTMCs. However, this effect was mitigated by lncRNA PVT1 silencing or miR-29a-3p overexpression (Fig. 5A and B). Changes in the migratory ability of the cells under oxidative stress were evaluated by Transwell assay. The results demonstrated that H_2O_2 inhibited the migratory ability of HMTCs; nevertheless, this inhibition could be alleviated by downregulating the expression of lncRNA PVT1 or upregulating that of miR-29a-3p (Fig. 5C and D). CCK-8 assays further indicated that the proliferative capacity of HTMCs was inhibited under H_2O_2 exposure. However, when HTMCs were transfected with lncPVT1 siRNA or miR-29a-3p mimics, the loss of proliferative ability was reduced, with proliferation attaining normal levels after 72 h (Fig. 5E). No difference in the rate of apoptosis, migratory ability, or proliferative capacity was observed with the transfection of NC lncPVT1 siRNA or scramble miRNA. These findings indicated that the knockdown of lncRNA PVT1 exerted a protective effect against apoptosis, motility, and viability in HTMCs under conditions of oxidative stress. Combined, these results demonstrated that inhibiting VEGFA and MMP-2 activity by regulating the expression of lncRNA PVT1 improved the H_2O_2-induced changes in HTMC morphology and has potential as a strategy in clinical therapy.

4. Discussion

Irreversible vision loss due to glaucoma is characterized by progressive optic neuropathy [31]. Uncontrolled elevated IOP represents the main risk factor for glaucoma, while oxidative stress is a key mechanism underlying the pathophysiology of this condition [32,33]. An imbalance in aqueous humor inflow and outflow leads to elevated IOP, and the oxidative stress-mediated impairment of the TM is responsible for the suppression of aqueous humor outflow. Thus, in this study, we explored the potential molecular mechanisms involved in oxidative stress-induced injury in HTMCs *via* the administration of H_2O_2.

LncRNA/miRNA interactions influence a wide variety of processes, including oxidative stress, cell viability, and differentiation, among other processes. However, their dysregulation has been enrolled in the pathology of different types of diseases, and some have been proposed to act as biomarkers for glaucoma. LncRNAs and miRNAs interact with each other through the following mechanism: (1) MiRNAs can modulate target lncRNAs *via* the association with RNA-induced silencing complexes (RISCs), which reduces the structural and functional stability of lncRNAs by imperfect base-pairing [34]; (2) lncRNAs play as 'miRNA sponges' that degrade target miRNAs through miRNA response elements (MREs) and subsequently decrease the degradation of target mRNAs; this has been designated as the miRNA–mRNA–lncRNA pathway [35,36]; (3) lncRNAs can produce specific miRNAs through intracellular RNA splicing and promote the posttranscriptional regulation of target mRNAs [37]; and (4), lncRNAs can compete with miRNAs for binding

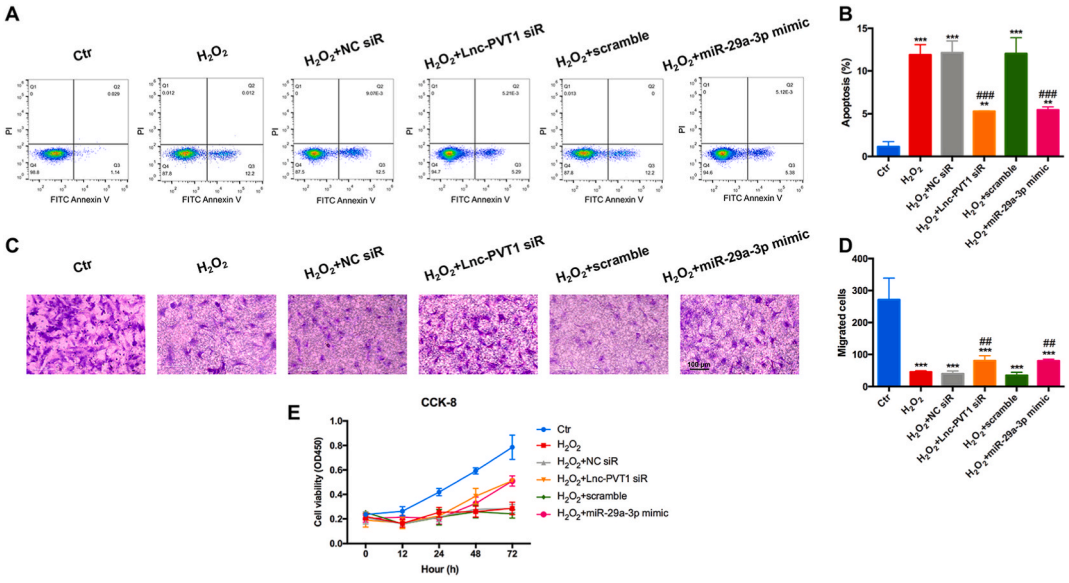

Fig. 5. The functions of human trabecular meshwork cells (HTMCs) were modulated by the lncRNA PVT1/miR-29a-3p/VEGFA/MMP-2 axis under oxidative stress. (A, B) The rate of apoptosis in H_2O_2-treated HTMCs was reduced by lncRNA PVT1 knockdown or miR-29a-3p overexpression. (C, D) H_2O_2 inhibited the migratory ability of HTMCs under H_2O_2 treatment, an effect that was reversed when lncRNA PVT1 was knocked down. (E) The H_2O_2 treatment-mediated decrease in HTMC viability was mitigated by lncRNA PVT1 upregulation or miR-29a-3p overexpression. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control (ctr) group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the H_2O_2 group, ANOVA was applied; $n = 3$. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; VEGFA, vascular endothelial growth factor A; MMP-2, matrix metalloproteinase-2; PI, propidium iodide. Scale bar, 100 μm. Magnification, × 200.

sites in the 3′-UTRs of target mRNAs, thus inhibiting the miRNA-mediated negative modulation of target mRNAs [38]. In this work, we analyzed the interdependent regulation of lncRNA PVT1 and miR-29a-3p in H_2O_2-treated HTMCs. Under conditions of oxidative stress, the expression levels of lncRNA PVT1 were enhanced in HTMCs, whereas those of miR-29a-3p were decreased. The knockdown of lncRNA PVT1 significantly suppressed its expression and, conversely, upregulated that of miR-29a-3p. Similarly, the overexpression of miR-29a-3p *via* the transfection of miR-29a-3p mimics led to the upregulation of the expression of miR-29a-3p while inhibiting those of lncRNA PVT1. Furthermore, the luciferase reporter assay confirmed the presence of a miR-29a-3p response element in lncRNA PVT1. This suggested that in H_2O_2-treated HTMCs, lncRNA PVT1 acts as a miR-29a-3p sponge, while miR-29a-3p can also regulate lncRNA PVT1. We also detected whether the miRNA–mRNA–lncRNA pathway was active in HTMCs under oxidative stress. Bioinformatics analysis predicted the presence of miR-29a-3p target sites in the 3′-UTRs of VEGF and MMP-2 mRNA. Here, we found that the upregulation of miR-29a-3p significantly mitigated the increase in the levels of VEGFA and MMP-2 in HTMCs induced by H_2O_2 treatment, with similar results being obtained when lncRNA PVT1 was knocked down. These findings suggested that under oxidative stress, lncRNA PVT1 is upregulated and functions as a miR-29a-3p sponge, thus decreasing the degradation of VEGFA and MMP-2 mRNA, which are targets of miR-29a-3p. The suppression of lncRNA PVT1 restored the level of miR-29a-3p and subsequently inhibited VEGFA and MMP-2 in HTMCs under H_2O_2 exposure. It was previously reported that the upregulation of miR-29a-3p inhibited angiogenesis and invasive motility in human cervical cancer cells by targeting the 3′-UTRs of VEGFA and MMP-2 mRNA, thereby suppressing their expression [27]. These observations were in line with our results.

VEGFA is known to be a central mediator of angiogenesis and vascular permeability and plays key roles in processes such as angiogenesis, inflammatory cell migration, and fibroblast activity [39,40]. VEGFA expression can be induced by oxidative stress, resulting in fibrosis at the operated site post-glaucoma filtration surgery [12,41]. The inhibition of VEGF was reported to advance the outcome of glaucoma surgery through the suppression of angiogenesis and fibrosis in various experimental models [42,43]. This indicates that to develop the outcome of glaucoma filtration surgery, it may be beneficial to suppress VEGF, which would inhibit fibrosis formation. Additionally, MMP-2 is involved in postoperative subconjunctival wound healing of glaucoma [44]. The ideal strategy for improving the success rate of filtration surgery after trabeculectomy involves inhibiting filtration bleb scar formation. Notably, we found that MMP-2 accumulated to significant levels in the nucleus of HTMCs following treatment with H_2O_2, which could impact the apoptotic process. It is known that MMP-2 can induce apoptosis under oxidative stress by activating oxidative DNA damage sensor enzyme poly (ADP-ribose) polymerase 1 (PARP-1) [45,46]. This supports that MMP-2 can not only regulate the fibrotic process but can also modulate apoptosis in HTMCs under H_2O_2-induced oxidative stress. In our study, the suppression of VEGFA and MMP-2 induced by the knockdown of lncRNA PVT1 was mediated by the upregulation of miR-29a-3p in H_2O_2-treated HTMCs. Moreover, the lncRNA PVT1/miR-29a-3p axis also influenced the expression of α-SMA and ICAM-1, two factors with roles in fibrosis and angiogenesis, respectively. Targeting VEGFA and MMP-2 by modulating the levels of lncRNA PVT1 and miR-29a-3p could effectively ameliorate cellular apoptosis, migratory ability, and viability of HTMCs under oxidative stress. It has been documented that antifibrotic therapy in the bleb tissue *in vivo* targeted at inhibiting α-SMA expression significantly suppressed myofibroblast transdifferentiation and reduced IOP after filtration surgery in a rabbit model of glaucoma filtration surgery [47]. The findings of this work supported that targeting the lncRNA PVT1/miR-29a-3p/VEGFA/MMP-2 axis has potential as a strategy for ameliorating fibrosis and scar formation in filtration blebs after trabeculectomy.

Despite the importance of our findings, our study had three key limitations in its experimental design. Firstly, although we detected the levels of VEGFA/MMP-2 in aqueous humor of patients with APAC in our previous study [48,49], we did not evaluate the expression levels of lncRNA PVT1 and miR-29a-3p in such patients in the current study. Secondly, we did not establish an animal model of filtration bleb after trabeculectomy to evaluate the potential effects of the lncRNA PVT1/miR-29a-3p/VEGFA/MMP2 axis in conjunctival tissue. Thirdly, as TM cells are a primary cell line that is isolated from patient tissue, it is better to administer different donor cell lines to run a properly powered experiment and accurately represent the biological variation that is expected in different patient samples. We expect to address these limitations in future research based on the molecular mechanisms identified in the present study.

In the present study, we showed that in HTMCs exposed to H_2O_2, lncRNA PVT1 functioned as a sponge for miR-29a-3p, thereby increasing the expression of VEGFA and MMP-2, which are targets of miR-29a-3p. Our findings indicated that, under oxidative stress, targeting the lncRNA PVT1/miR-29a-3p/VEGFA/MMP-2 axis can protect HTMCs from apoptosis, impaired migratory ability, and decreased cell viability.

Ethics declarations

Informed consent was not required for this study because no human participants and animal experiments was included in this study.

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Consent for publication

Not applicable.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Qiaoyun Gong: Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Danjing Zhou:** Software, Data curation. **Chong Chen:** Formal analysis. **Hangqi Shen:** Data curation. **Xun Xu:** Supervision, Conceptualization. **Tianwei Qian:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Qiaoyun Gong reports financial support was provided by National Natural Science Foundation of China.

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Abbreviations

3′-UTR	3′-untranslated region
APAC	Acute primary angle closure
α-SMA	alpha-smooth muscle actin
BSA	bovine serum albumin
CCK-8	Cell Counting Kit-8
ECM	extracellular matrix
FBS	fetal bovine serum
H_2O_2	Hydrogen peroxide
HEK293	Human embryonic kidney 293
HTMC	Human trabecular meshwork cell
ICAM-1	intracellular adhesion molecule-1
IOP	intraocular pressure
lncRNA PVT1	long non-coding RNA plasmacytoma variant translocation 1
MMP-2	matrix metalloproteinase-2
miRNAs	microRNAs
miR-29a-3p	microRNA-29a-3p
ncRNAs	Noncoding RNAs
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride
PACG	primary angle-closure glaucoma
PI	propidium iodide
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
RIPA	radioimmunoprecipitation assay
siRNA	small interfering RNA
TM	trabecular meshwork
VEGFA	vascular endothelial growth factor A
WT	wild-type
Mut	mutated

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23607.

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