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Original Article

MiR-184 prevents chronic oxidative stress induced human trabecular meshwork cells apoptosis and cytotoxicity in vitro by targeting hypoxia-inducible factor 1α

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Abstract: Crucial regulatory roles of miRNAs in processing of glaucoma had been identified. This study was conducted to investigate the effect of miR-184 in chronic oxidative stress (COS) mediated human trabecular meshwork (HTM) cells dysfunctions *in vitro*. HTM cells were generated from cadaver eyes tissues and incubated at 40% O_2 and 5% CO_2 for 5 days to induce COS stressed cell model. Cell apoptosis, cytotoxicity, and extracellular matrix (ECM) changes were studied in cells transfected with miR-184 mimic, inhibitor, silenced (si-)HIF-1α, or controls. The decreased miR-184 was detected, companied with the significantly reduced HTM cell apoptotic percentage, cytotoxicity, and ECM upregulation. The HTM cell dysfunction, induced by COS, was attenuated and enhanced by the mR-184 mimic and miR-184 inhibitor transfection, respectively. Moreover, si-HIF-1α was found as a direct target for miR-184. Furthermore, shRNA plasmids transfection of a predicted miR-184 target, si-HIF-1α, cancelled the miR-184 inhibitor effect on apoptosis, cytotoxicity, and ECM upregulation in HTM cells. Taken together, we concluded that miR-184 inhibited HTM cells cytotoxicity, apoptosis and ECM proteins expression via targeting HIF-1α *in vivo*. We suggest that miR-184 could be used as a potential target for glaucoma management.

Keywords: Extracellular matrix, chronic oxidative stress, human trabecular meshwork cells, miR-184, HIF-1α

Introduction

Glaucoma is characterized by visual field loss and irreversible blindness [1]. The progressive degeneration of optic nerve is a progressive nature of glaucoma and it is usually characterized by the progressive degeneration of the optic nerve structure and the loss of optic nerve function, including retinal oxidative stress, retinal ganglion cells (RGCs) apoptosis and loss, extracellular matrix (ECM) changes, optic atrophy, and visual field loss, which are induced by the increased intraocular pressure (IOP) in the eyes [2-5].

The reduced trabecular meshwork (TM) outflow facility results in IOP, whereas the increased TM outflow facility reduced IOP [6, 7], suggesting that TM plays important roles in preventing optic nerve damage. Accordingly, human TM (HTM) cells are commonly used as *in vitro* glaucoma models for glaucoma progression and management [8, 9]. Notably, the immerging evidences indicate that the reactive oxygen species (ROS), induced by oxidative stress, a common pathologic pathway in neurodegenerative diseases [10], takes crucial roles in glaucomatous damage [11-13]. The oxidative stress-induced mitochondrial DNA damage and dysregulation of genetic factors in human retinal pigment epithelial cells partially account for mechanism of optic nerve diseases including the age-related macular degeneration [14], such as oxidative stress induced ECM degrada-

tion [15], chronic oxidative stress (COS) mediated dysregulation of ECM proteins, and microRNA (miR)-29b in the TM [16].

The crucial regulatory roles of miRNAs in the biology of glaucoma had been identified, such as miR-29 family [14, 16, 17], miR-204 [18], miR-24 [19], and miR-184 [20, 21]. MiR-184 acts as a potential oncogenic miRNA for human cancers, such as squamous cell carcinoma of tongue [22], malignant gliomas [23], and exhibits angiostatic properties through regulating signaling pathways including Akt, TNF-α, and VEGF [24]. Specifically, down-regulation of miR-184 had been demonstrated in ocular diseases [20]. MiR-184 specifically highly expressed in cornea and HTM of keratoconus patients, as compared to ciliary body and retina [25], this might suggest the important roles of miR-184 in preventing optic nerve diseases.

In the present study, to investigate the effect of miR-184 in glaucoma and gain more information on the roles of miR-184 in HTM cells, we established the HTM cell model *in vitro*, using COS conditions of 40% oxygen (O_2) treatment for 5 days. Subsequently, the cell apoptosis, cytotoxicity, and ECM proteins accumulation were detected. Moreover, model cells would be transfected with miR-184 mimic/inhibitor to analysis the potential of therapeutic management by alternating miR-184 expression for COS in HTM cells.

Materials and methods

Cells and culture conditions

As previously reported [16], human trabecular meshwork (HTM) cells cultures were generated from isolated tissue of cadaver eyes within 48 h post mortem, without history of eye disease. Cell cultures were maintained in low glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich Biotechnology, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, and 0.25 μg/ml amphotericin B (Sigma-Aldrich) at 37°C in 5% CO_2 [16]. Induction of COS was generated by incubating HTM cells at 40% O_2 5% CO_2 for 5 days. Control cells were incubated at 5% O_2. Procedures involving human tissues were conducted according to the tenets of the Declaration of Helsinski.

Cell transfections

Mature miR-184 mimic, miR-184 inhibitor and their corresponding scrambles, small interference RNA against HIF-1α (si-HIF-1α), and negative control were purchased from GenePharma (Shanghai, China). Lentivirus-si-HIF-1α plasmids were constructed using lentivirus vector pLKO.1 puro (AgeI and EcoR, Sigma-Aldrich). For cell transfection, HTM cells were seeded into 6-well plates (5×10^5 cells/per well) and then transiently transfected with miR-184 mimic, miR-184 inhibitor, or lentivirus-si-HIF-1α using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instruction for 48 h. Then cells were harvested for further analysis. Untreated cells, cells transfected with PLKO.1-puro lentiviral vector without shRNA, and scrambles were considered as the control groups.

Dual-luciferase reporter analysis

Potential target HIF-1α of miR-184 was predicted using TargetScan Human: http://www.targetscan.org/vert_71/) [25]. Reporter vectors of pGL3-HIF-1α-3'-UTR were synthesized and purchased from Sangon Biotech (Shanghai, China). Dual-luciferase (Firefly and Renilla) reporter plasmids pGL3-HIF-1α-WT (containing putative 3'-UTR-binding site for miR-184) and pGL3-HIF-1α-Mut (containing the mutated sequence in the complementary site for miR-184) were constructed. The luciferase assay was performed according to the manufactures' introductions (Invitrogen).

Cell cytotoxicity assay

Cell viability or cell cytotoxicity induced by COS was assayed by measuring the released lactate dehydrogenase (LDH) in cell cultures using the Promega cytotoxicity assay kit (Promega) as previously described [16].

Apoptosis analysis

Apoptosis was detected using flow cytometry with annexin V Apoptosis Detection Kit (BD Biosciences) by adding into FACS tubes and mixing with annexin V and propidium iodide (PI) to incubate for 15 min at room temperature in the dark. Early apoptotic cells percentage was counted by Annexin V-PE positive and PI-negative cells (Annexin V+/PI-).

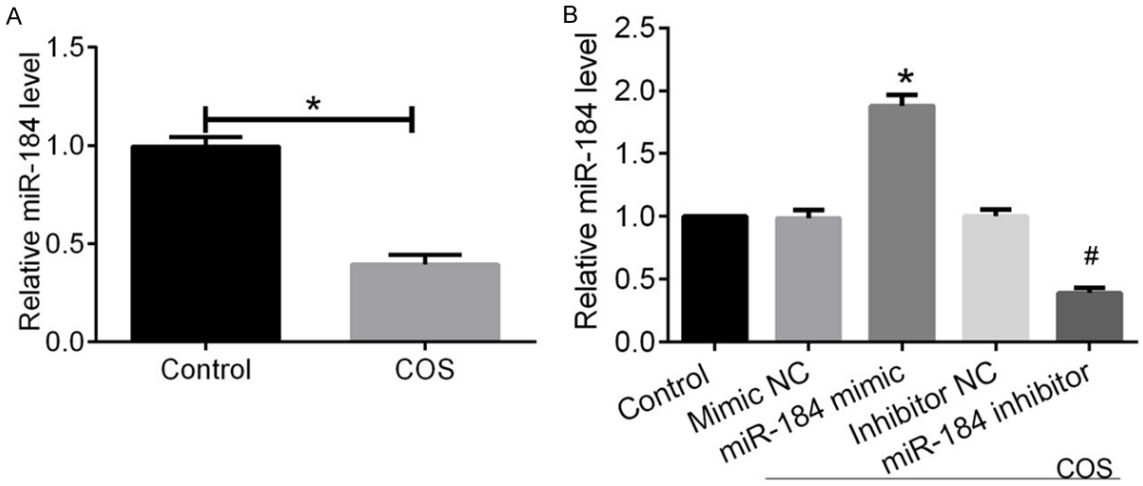

Figure 1. Expression of miR-184 in HTM cells. A. HTM cells were treated with 40% (chronic oxidative stress, COS) or 5% CO_2 (normal conditions, Control) for 5 days. B. HTM cells transfected with control mimic, or miR-29b mimic or nothing were subjected to 5% O_2 (normal conditions). * and #indicates statistical significance at $P < 0.05$ level, in comparison with control and mimic, respectively.

Quantitative reverse transcription PCR

Total RNA extracted from cells using Trizol reagent (Sigma-Aldrich) was reverse-transcripted into the first strand cDNA using an M-MLV Reverse Transcriptase kit (Invitrogen). DNA template for miRNA and mRNA detection was reverse-transcripted with and without specific stem-loop primers (Invitrogen), respectively, supplemented with 500 ng of olig (dT) (Promega). The qPCR reaction mixtures generated by a Toyobo SYBR Green PCR kit (Toyobo, Osaka, Japan) was performed on a Rotor-Gene RG-3000A system (Corbett Life Science, Sidney, Australia) following the cycling parameters: Initial activation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s. Relative quantification of gene expression levels were calculated by normalizing to endogenous reference genes GAPDH or U6 using the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

HCM cellular proteins were extracted lysed in RIPA Lysis Buffer (Sigma-Aldrich), quantified, and separated on the 10% 12% SDS polyacrylamide gel electrophoresis (Solarbio, Beijing, China). Separated proteins were transferred to PVDF membrane (Promega), which were then blocked and probed with specific antibodies overnight at 4°C, and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Antibodies against Bcl-2, Bax, cleaved and pro-Caspase-3, COL1A1, COL1A2, COL3A1, COL5A1, SPARC, HIF-1α, and GAPDH were purchased from Abcam Inc., (Cambridge, MA, USA, 1:1000 in dilution). Enhanced chemiluminescence (ECL, Beyotime, Shanghai, China) western blotting detection system was used for band intensity analysis. Data were analyzed using a Bio-Rad Quantity One software (Bio-Rad) normalizing to the band with GAPDH control.

Statistical analysis

Each experiment was performed in triplicate. All the quantitative data were expressed as mean value ± standard deviation (SD) in three independent experiments. Statistical analysis was performed using SPSS 19.0 (SPSS Inc., USA), and statistical graphs were made using Graphpad Prism 6.0 (GraphPad Software Inc, La Jolla, CA). Differences between groups were analyzed by Tukey's test, and among four groups were analyzed using ANOVA, respectively. $P < 0.05$ was considered as the statistically significant.

Results

COS decreases miR-184 expression in HTM cells

The significantly decreased expression of miR-184 in COS stressed HTM cells were detected, as compared to control ($P < 0.05$, **Figure 1A**).

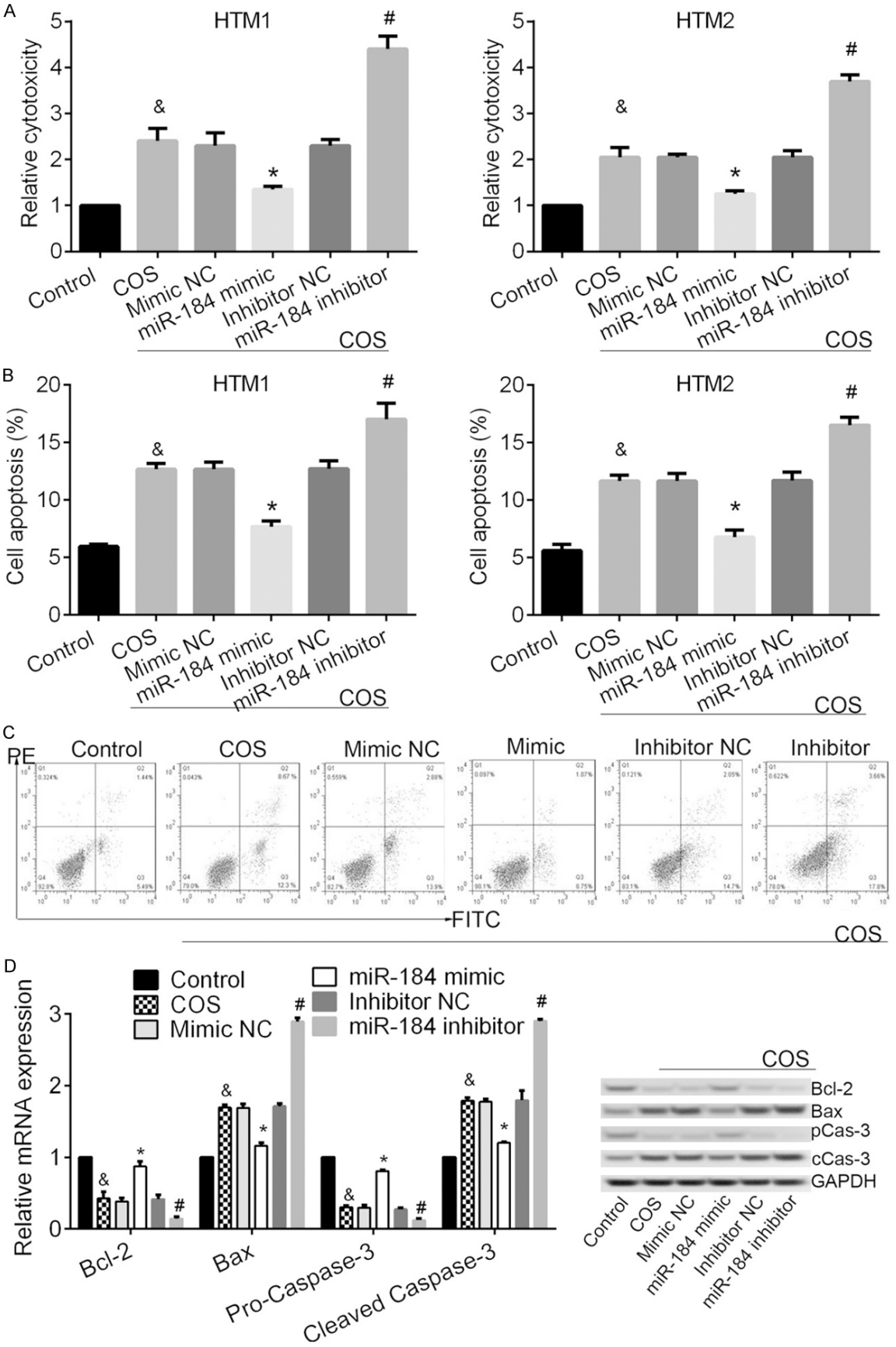
A
HTM1
HTM2
Relative cytotoxicity
Control
COS
Mimic NC
miR-184 mimic
Inhibitor NC
miR-184 inhibitor
COS
B
HTM1
HTM2
Cell apoptosis (%)
C
PE
FITC
Control
COS
Mimic NC
Mimic
Inhibitor NC
Inhibitor
D
Relative mRNA expression
Bcl-2
Bax
Pro-Caspase-3
Cleaved Caspase-3
GAPDH
pCas-3
cCas-3

Figure 2. MiR-184 inhibits chronic oxidative stress (COS) induced human trabecular meshwork (HTM) cells cytotoxicity and apoptosis. Transfected HTM cells were treated with 40% O_2 (COS) or 5% O_2 (normal conditions, Control) for 5 days. A. Cytotoxicity levels were measured by released lactate dehydrogenase (LDH) in cell cultures. B and C. Cell apoptosis was analysis using FACS. D. mRNA and proteins expression of cell apoptosis related proteins in COS treated and transfected HTM cells. &, *, and #indicates statistical significance at $P < 0.05$ level, in comparison with control, COS, and mimic, respectively.

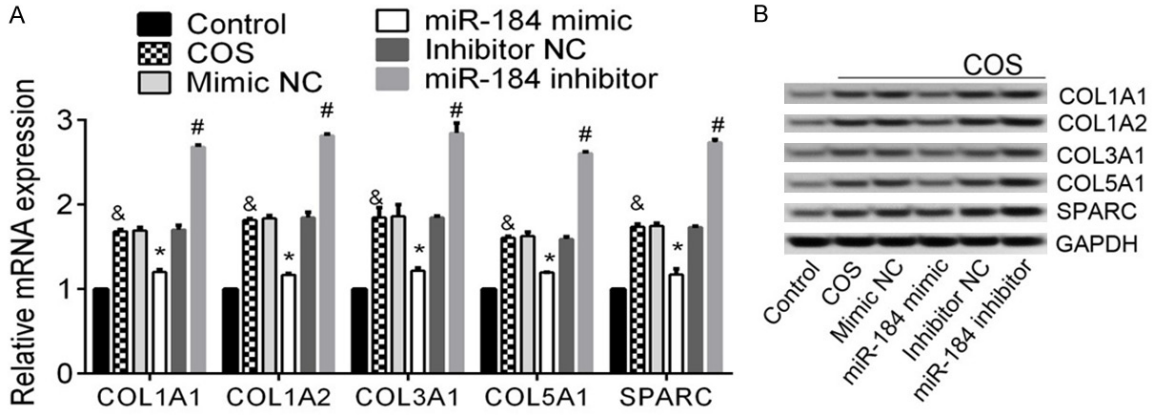

Figure 3. MiR-184 inhibits chronic oxidative stress (COS) induced extracellular matrix (ECM) proteins expression in human trabecular meshwork (HTM) cells. Transfected HTM cells were treated with 40% O_2 (COS) or 5% O_2 (normal conditions, Control) for 5 days. The mRNA (A) and protein (B) expression level was determined using qRT-PCR and western blot analysis. &, *, and #indicates statistical significance at $P < 0.05$ level, in comparison with control, COS, and mimic, respectively.

The expression of miR-184 in HTM cells was significantly upregulated (~180%) and down-regulated (~70%), respectively, by mimic and inhibitor ($P < 0.05$, **Figure 1B**), demonstrating the efficiency of using miR-184 mimic and inhibitor for the following transfection experiments.

Effects of miR-184 on cytotoxicity and apoptosis in HTM cells

Two individual HTM cell lines (HTM1 and HTM2) transfected with control sequences, miR-184 inhibitor, or miR-184 mimic were subjected to COS conditions and incubated for 5 days for cytotoxicity analysis. In both HTM cell lines, a significant decrease and enhance in COS-induced cytotoxicity was detected in HTM cells transfected with miR-184 mimic and miR-184 inhibitor compared to the COS ($P < 0.05$, **Figure 2A**), showed a significant decrease in cytotoxicity. Similarly, increased cell apoptotic percentage in COS treated HTM cells was inhibited and enhanced by miR-184 mimic and miR-184 inhibitor, respectively ($P < 0.05$, **Figure 2B** and **2C**). As expected, cell apoptosis related proteins (i.e. Bax and cleaved Caspase-3) were up- and down-regulated by miR-184 mimic and inhibitor, while Bcl-2 and pro Caspase-3 were down- and up-regulated by miR-184 mimic and inhibitor, respectively ($P < 0.05$, **Figure 2D**). That is to say, miR-184 protects HTM cells against COS induced cytotoxicity and cell apoptosis.

MiR-184 inhibits COS induced ECM proteins expression in HTM cells

Increased ECM in the to be a very important contributor in glaucoma [26, 27]. We detected the mRNA and protein expression of ECM structural proteins in COS treated HTM cells and determined miR-184 down-regulated several ECM structural proteins, as collagens (i.e. COL1A1, COL1A2, COL3A1, and COL5A1) and an ECM formation modulator protein SPARC, which were up-regulated by COS stress ($P < 0.05$, **Figure 3**). In contrast, miR-184 inhibitor enhanced the COS induced dysregulation of ECM proteins, suggesting miR-184 modulate ECM changes.

MiR-184 targets to HIF-1α

Binding sites of the potential target HIF-1α of miR-184 predicted using TargetScan is showed

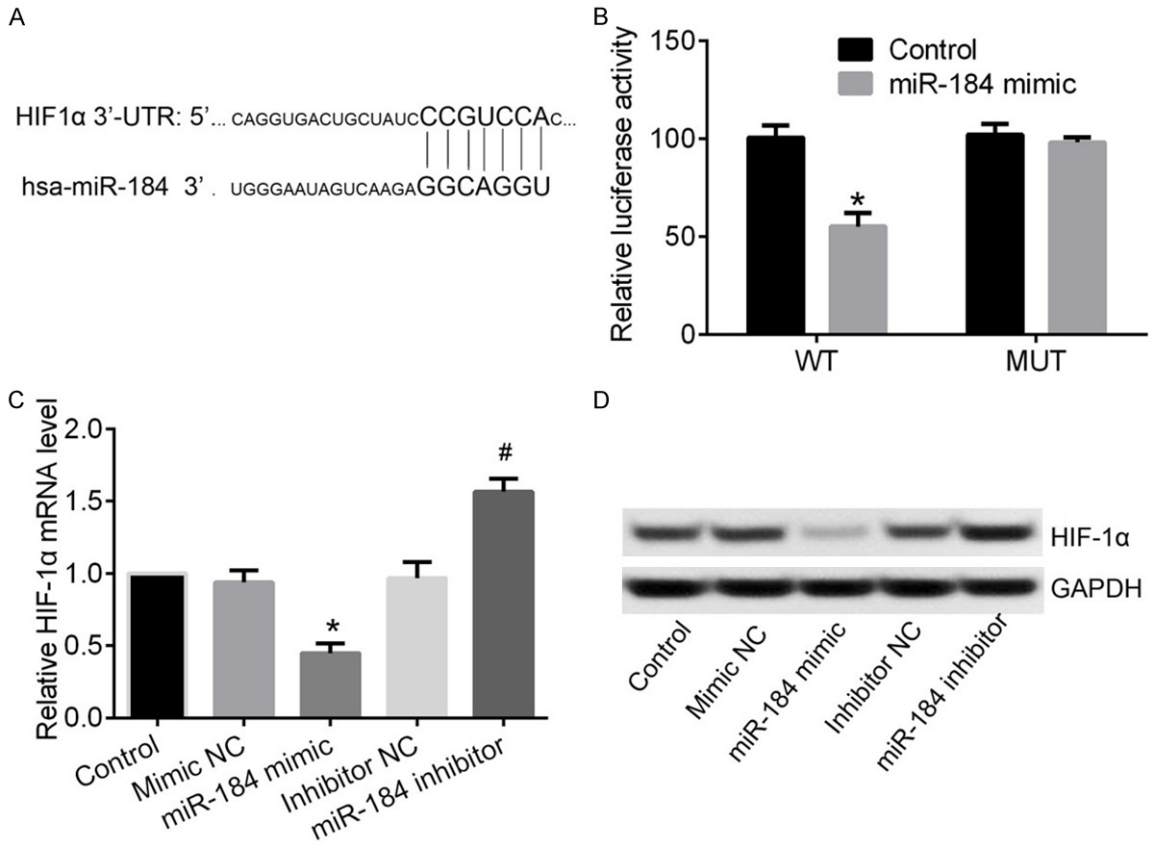

Figure 4. MiR-184 targets to HIF-1α. HIF-1α is a predictive target of miR-184 using Target Scan Human (http://www.targetscan.org/vert_71/) (A), and dual-luciferase reporter analyses were performed to testify predictive target relationship (B). (C) Expression of HIF-1α in miR-184 transfected cells using qRT-PCR (left) and (D) Western blot analyses (right). * and #indicates statistical significance at $P < 0.05$ level, in comparison with control and mimic, respectively.

in **Figure 4A**, which were then validated by dual-luciferase reporter analysis (**Figure 4B**). Dual-luciferase reporter analysis showed cotransfection of miR-184 mimic and pGL3-HIF-1α-3'-UTR-WT vector significantly quenched relative firefly luciferase activity, as compared to control and Mut vector ($P < 0.05$). Notably, significantly decreased or increased HIF-1α level was observed in miR-184 mimic or inhibitor transfected cells, respectively ($P < 0.05$, **Figure 4C**), suggesting the negative regulatory relationship between miR-184 and its target HIF-1α.

MiR-184 affects cells function and ECMs expression by targeting HIF-1α

We determined the transfection of lentivirus-si-HIF-1α plasmids inhibited mRNA and protein expression of HIF-1α by almost 60% ($P < 0.05$, **Figure 5A**). The inverse effect of si-HIF-1α on miR-184 mediated cytotoxicity, cell apoptosis, expression of ECM proteins was detected. Results showed that cotransfection of lentivirus-si-HIF-1α vector with miR-184 inhibitor attenuated cytotoxicity, cell apoptosis, and accumulation of ECM proteins induced by miR-184 absence (**Figure 5B-D**), suggesting MiR-184 modulates HTM cells function and ECMs expression by targeting HIF-1α.

Discussion

Oxidative damage results in the dysfunction of outflow tissue through apoptotic cell loss [16], and plays a major role in ocular diseases including glaucoma, cataract, and macular degeneration [13, 28, 29]. In this study, we demonstrated that COS with 40% O_2 for 5 days induced HTM cells apoptosis, cytotoxicity, down-regulation of miR-184 and accumulation of ECM proteins. In contrast, miR-184 mimic transfection

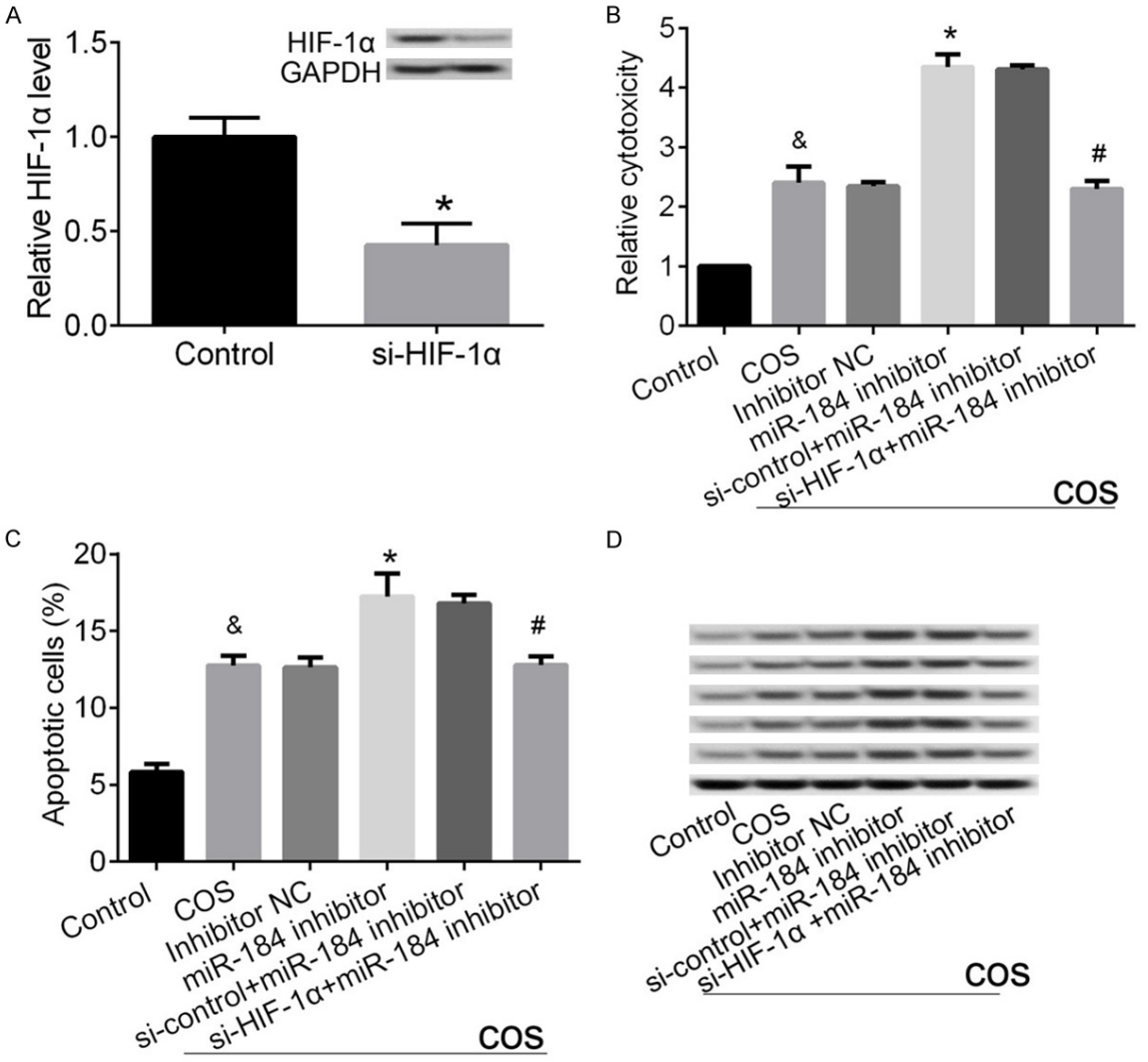

Figure 5. MiR-184 affects HTM cells function by targeting HIF-1α. (A) HIF-1α expression is a decreased in si-HIF-1α transfected cells. Cotransfection of lentivirus-si-HIF-1α vector with miR-184 inhibitor reduced cytotoxicity (B), cell apoptosis (C), and expression of ECM proteins (D). HTM cells transfected with miR-184 inhibitor and lentivirus-si-HIF-1α vector were subjected to 40% or 5% O_2 (Control). &, *, and #indicates statistical significance at $P < 0.05$ level, in comparison with control, COS, and mimic, respectively.

attenuated COS induced HTM cell apoptosis, cytotoxicity, and up-regulation of ECM proteins. These data suggested that miR-184 might be used as an inhibitor for COS induced glaucoma.

Previously, miR-184 acts as a potential oncogenic miRNA for human cancers [22, 23]. The down-regulation of miR-184 in ocular diseases [20], and specifically high expressed of miR-184 in cornea and HTM of keratoconus patients, as compared to ciliary body and retina, had been demonstrated [25]. In our study, we revealed that miR-184 expression was down-regulated in COS treated HTM cells, companied with the increased apoptotic cells percentages, and the increased cytotoxicity, whereas miR-181 mimic transfection attenuated these changes induced by COS, indicating the antiapoptotic potential roles of miR-184 in HTM cells.

ECM components conduct retina and optic nerve degeneration [30], and mutations, abnormal accumulations, or remodelling of ECM-related genes are reported to be associated with glaucoma [30-32]. For instance, mutations in Fibrillin 2 are associated with

degeneration of macular [32], and COL1A1 mutation resulted in ocular hypertension or blindness [33, 34]. The matricellular protein SPARC promotes the deposition of ECM [35, 36]. Overexpression of SPARC in HTM cells increases intraocular pressure, while SPARC null mice showed lower intraocular pressure [37-39]. Luna and Li showed that the expressions of ECM proteins including COL1A1, COL1A2, COL3A1, COL5A1, and SPARC were increased COS-treated HTM cells [16]. In this study, our results showed that the expression of COS proteins including COL1A1, COL1A2, COL3A1, COL5A1, and SPARC, are up-regulated, companied with HTM cell apoptosis and cytotoxicity, whereas miR-184 mimic transfection attenuated these dysregulation *in vivo*, suggesting that miR-184 mimic might be used as a potential target for glaucoma management.

The neurodegenerative protection roles of HIF-1α in retinal ganglion cells and in glaucoma had been indicated in previous studies [40-43]. The elevated HIF-1α level had been detected in diabetic retina [42], diabetic retinopathy [43], and retinal ganglion cells in glaucoma [41]. In this present study, our results confirmed that HIF-1α is a direct target of miR-184 with a negative relationship between HIF-1α and miR-184 expression. Moreover, we revealed that the cotransfection of si-HIF-1α with miR-184 inhibitor attenuated effect of miR-184 inhibitor on HTM cell functions, by inhibiting HTM cell apoptosis, cytotoxicity, and ECM proteins expression. Taken together, we concluded that the antiapoptosis effect of miR-184 on HTM cells is mediated by targeting HIF-1α.

In summary, the data presented in this study revealed that miR-184 may play pivotal roles in the development of HTM through inhibiting cell cytotoxicity, apoptosis and ECM protein expression. Using the miR-184 mimic, inhibitor, and si-HIF-1α transfections, we demonstrate that miR-184 exhibits an antiapoptosis effect on COS resulted glaucoma via targeting HIF-1α *in vivo*. These results in the present study showed miR-184 might be used as a potential target for glaucoma management. However, further experimental studies are still needed to confirm and explore the investigate roles of miR-184 in HTM.

Disclosure of conflict of interest

None.

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