MicroRNA-483-3p Inhibits Extracellular Matrix Production by Targeting Smad4 in Human Trabecular Meshwork Cells

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PURPOSE. This study investigated the effects of microRNA-483-3p (miR-483-3p) on extracellular matrix (ECM) production, and clarified the regulatory mechanism of microRNA-483-3p in human trabecular meshwork cells (HTMCs) under oxidative stress.

METHODS. The expression levels of ECM (fibronectin, laminin, collagen I) in HTMCs under oxidative stress were measured by Western blot. Changes of miR-483-3p expression in HTMCs were evaluated by quantitative polymerase chain reaction (qPCR). After using lentivirus stably expressing pri-miR-483, the effects of miR-483-3p on the ECM were assessed by qPCR and Western blot. Smad4, the potential target of miR-483-3p according to mRNA target-predicting algorithms, was confirmed by luciferase assay and Western blot. Furthermore, the effects of Smad4 knockdown on ECM expression were investigated by qPCR and Western blot.

RESULTS. The mRNA and protein levels of ECM (fibronectin, laminin, collagen I) were upregulated in HTMCs induced by oxidative stress. The expression level of miR-483-3p decreased in HTMCs under oxidative stress, and the ectopic expression of miR-483-3p decreased the levels of ECM. In addition, miR-483-3p targeted Smad4 through two binding sites, resulting in a decrease of Smad4 expression. Furthermore, knockdown of Smad4 reduced the levels of ECM in HTMCs.

CONCLUSIONS. MicroRNA-483-3p has an inhibitory effect on ECM production in HTMCs through downregulating Smad4, which indicates that miR-483-3p may serve as a potential therapeutic target in glaucoma.

Keywords: microRNA-483-3p, Smad4, extracellular matrix, trabecular meshwork cells, glaucoma

G lobally, glaucoma is the second leading cause of blindness. Recent studies estimated that the number of patients with glaucoma will increase to 79.6 million until 2020, with 74% of patients becoming open-angle glaucoma (OAG) patients.1 Meanwhile, the number of glaucoma patients suffering from blindness may increase.2,3 As is well known, elevated intraocular pressure (IOP) is regarded as the main risk factor for primary open-angle glaucoma (POAG) patients.1,3 The trabecular meshwork (TM), located in the anterior segment of the eye, plays a vital role in regulating the outflow of AH. It has been proposed that excessive deposition of extracellular matrix (ECM) at the deepest portion of the TM could be a main site of outflow resistance.4,5 Thus, it is necessary to clarify the cause of excessive deposition of ECM from glaucomatous TM cells.

The transforming growth factor-beta (TGF-β) family regulates various cellular activities, including proliferation, differentiation, wound healing, and ECM synthesis.6,7 The TGF-β family are also known as key profibrotic mediators in fibrotic diseases. Ligand-induced activation of TGF-β family receptors triggers the phosphorylation of receptor-regulated Smads (R-Smads), which can form heteromeric complexes with common-partner Smads (Co-Smads) and regulate transcription.9,10 It has been widely reported that the TGF-β/Smad4 pathway has an essential effect on regulating ECM production in the fibrotic process of various diseases.8,11,12 In glaucoma, TGF-β/Smad pathway regulates the deposition of the key ECM components, such as fibronectin, collagen, and laminin in TM, which leads to IOP elevation.4,13 Moreover, knockdown of Smad7 blocks the effects of TGF-β2 on promoting expression of several ECM components.14 Hence, it is necessary to find a potential target in TGF-β/Smad pathway to suppress ECM production from TM, which inhibits the elevated IOP.

MicroRNAs (miRNAs) are small noncoding RNAs (containing approximately 22 nucleotides) that modulate gene expression at the posttranscriptional level and regulate many cellular functions. MicroRNAs are also regarded as a vital and evolutionarily ancient component of genetic regulation.15,16 However, different cells and tissues have different miRNA expression profiles, participating in their own developmental and biological processes.17,18 More and more studies have focused on the miRNAs regulating the cellular functions of TM
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under physiological and pathological conditions. It has been demonstrated that miR-200c could regulate trabecular contraction and modulate IOP in vivo,19 and miR-146a contributed to the senescence of TM.20 Meanwhile, it has been reported that miRNAs could regulate and maintain ECM homeostasis.21-23 In TM cells, miR-29b targets bone morphogenetic protein 1 (BMP1), ADAM12, and NKIRAS2 and downregulates multiple ECM components, including collagens, LAMC1, and FBN.24-25 However, abnormal deposition of ECM is still not fully explored.

Here, we explored the function of miR-483-3p and its potential mechanism in regulating ECM production in human trabecular meshwork cells (HTMCs). Ectopic expression of miR-483-3p decreased the expression level of Smad4, which blocked TGF-β/Smad signaling and hindered ECM production. Thus, miR-483-3p acts as therapeutic target in HTMCs.

Materials and Methods

Human Trabecular Meshwork Cell Culture

Human TMCS were purchased from Sciencell Research Laboratories (San Diego, CA, USA). Cells were cultured at 37°C in 5% CO2 in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine, 110 mg/mL sodium pyruvate, 10% fetal bovine serum, 100 μM nonessential amino acids, 100 units/mL penicillin, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B. All reagents were obtained from Gibco (San Diego, CA, USA).

To establish an oxidative stress model, cells were treated with 300 μM H2O2 (Beyotime Institute of Biotechnology, Shanghai, China) in serum-free medium for 2 hours and then the medium was replaced with serum-free media without H2O2 cultured for another 2 hours.

Quantitative Real-Time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 4°C. After extensive washing with PBS, Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 555-conjugated goat anti-rabbit IgG (Cell Signaling, Danvers, MA, USA) was added in blocking buffer for 30 minutes at room temperature.

Lentiviral Expression Vector Generation and Cell Line Selection

To overexpress miR-483-3p, a DNA fragment carrying pri-miR-483 was amplified using sense and antisense Pri-483 primers. Then, the amplified sequence was cloned into the PCDH-CMV-MCS-EF1-Puro lentiviral vector (Addgene, Cambridge, MA, USA) between the EcoRI and BamHI restriction sites.

The Smad4 knockdown oligonucleotide sequences were obtained from The RNAi Consortium/Public TRC portal (construct ID: TRCN0000040028 [No. 1] and TRCN000165027 [No. 2]). A scrambled sequence (Addgene) was used as a control. The oligonucleotides were annealed and cloned into the AgeI/EcoRI sites of the shRNA vector plKO1-1-puro (Addgene) according to the instructions.

To produce lentiviral particles, 293T cells were seeded into 10-cm cell culture dishes and cultured for 24 hours before transfection. The PCDH/pri-miR-483 overexpression plasmid or Smad4-shRNA plasmid with psPAX2 packing plasmid and pMD2.G envelope plasmid were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 12-hour transfection, the cell culture medium was replaced with fresh complete medium. After 48- and 72-hour transfection, the culture medium was collected and centrifuged at 4°C for 5 minutes to remove any cellular debris. The biological titer of the purified virus was determined in 293T cells using the serial dilution method.

To select stable HTMC cell lines, 20 μL concentrated viral supernatant (MOI [multiplicity of infection] = 108 TU/mL) was added to 10-cm dishes and infected cells for 24 hours, and then replaced with fresh medium for an additional 24 hours. Next, puromycin (2 μg/mL) was used to select stable cell lines for 3 days; then the cells were lysed and analyzed.

3’-UTR Luciferase Assay

The 3’-UTR of gene of interest was amplified by PCR using cDNA from 293T cells and cloned into a p-mirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The miR-483-3p precursor expression vector and p-mirGLO Dual-Luciferase 3’-UTR vector were cotransfected into 293T cells using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were harvested and lysed at 48 hours after transfection. The interaction between miR-483-3p and 3’-UTR of Smad4 was measured by Dual-Luciferase Assay system (Promega).

Western Blot Analysis

Human TMCS were washed twice in cold PBS. Total protein was extracted using RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 5 mM EDTA, pH 8.0) with 10X protease inhibitor cocktail (Roche). Total protein extracts (20–100 μg) were separated by 8% to 12% SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Membranes were blocked with 5% nonfat dry milk and incubated overnight with the primary antibodies, anti-fibronectin (Abcam, Cambridge, UK), anti-laminin-5 (Chemicon, Hampshire, UK), anti-collagen I (Abcam), anti-Smad4 (Abcam), and anti-β-actin (Santa Cruz, Carlsbad, CA, USA) at 4°C. After incubation with secondary antibodies, the antibody-antigen complexes were detected using the Chemiluminescent HRP Substrates (Millipore, Billerica, MA, USA).

Immunofluorescence

Human TMCS, treated with 300 μM H2O2 in serum-free medium for 2 hours followed by 2-hour incubation with serum-free medium, were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Cells were subsequently blocked with 5% BSA in PBS for 30 minutes at room temperature and incubated with primary antibodies overnight at 4°C. After extensive washing with PBS, Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 555-conjugated rabbit IgG (Cell Signaling, Danvers, MA, USA) was added in blocking buffer for 30 minutes at room temperature in the dark. After extensive washing with PBS, DNA was counterstained with DAPI (eBioscience, Carlsbad, CA, USA). Then, the fluorescent cells were observed and photographed using an Olympus fluorescence microscope (Guangzhou, China).
All results were derived from at least three independent experiments. Statistical analysis of data was performed with the Student’s t-test using Microsoft Office Excel 2007 software (Redmond, WA, USA). The data were expressed as the mean ± SD using the GraphPad Prism statistical program (La Jolla, CA, USA). Differences with $P < 0.05$ were statistically significant.

### RESULTS

**Oxidative Stress Upregulates ECM and Downregulates miR-483-3p Expression**

To identify the expression levels of ECM in HTMCs under oxidative stress, HTMCs were treated with H$_2$O$_2$ for 2 hours followed by 2-hour incubation with FBS-free culture media. The
expression levels of fibronectin, laminin, and collagen I were all upregulated after H2O2 treatment (Fig. 1A). Furthermore, low concentration of H2O2 can significantly increase the protein levels of laminin and collagen I, while high concentration of H2O2 can increase the level of fibronectin (Fig. 1A). The variation tendency was also shown on the mRNA level (Fig. 1B). Immunofluorescence analysis of H2O2-treated cells further showed the increase in cytoplasmic ECM components in HTMCs (Figs. 1C–E). Taken together, these results indicated that oxidative stress activated the expression of ECM genes in HTMCs.

**MiRNAs Possibly Targeting Smad Family Are Downregulated in HTMCs Under Oxidative Stress**

The expression levels of ECM genes were elevated in HTMCs induced by oxidative stress. MicroRNAs play an important role in regulating the biological behavior of TM.26–28 To determine the differential expression of miRNAs under oxidative stress, seven miRNAs, which may target the Smad family, were chosen, and the expression levels were detected by quantitative PCR (qPCR) (Fig. 2A). As expected, the expression levels of miR-483-3p, miR-485-3p, miR-425-3p, and miR-3679-3p were decreased under oxidative stress (Fig. 2B). In particular, the expression of miR-483-3p was significantly higher than that of the other miRNAs (Fig. 2B). Therefore, we chose miR-483-3p for further investigation in subsequent research. The expression level of miR-483-3p gradually decreased with increasing H2O2 concentration (200, 400, 600 μM) (Fig. 2).

**MiRNA-483-3p Downregulates ECM Expression**

To investigate the effect of miR-483-3p on regulating ECM expression in HTMCs, we constructed the stably expressed miR-483-3p using a lentiviral delivery system. The transcript level of miR-483-3p was approximately 200 times higher than scramble control as detected by qPCR (Fig. 3A). The expression of ECM genes, such as fibronectin, laminin and collagen I, was found to be downregulated by overexpression of miR-483-3p (Fig. 3B). Also, ECM proteins decreased (Fig. 3C). These results suggested that miR-483-3p downregulated ECM expression in HTMCs.

To further confirm the roles of miR-483-3p under oxidative stress, HTMCs stably expressing miR-483-3p were treated with H2O2. After H2O2 treatment, the levels of ECM expression were higher than in the H2O2-untreated groups. However, overexpression of miR-483-3p led to the decrease in ECM expression in both the H2O2-treated and untreated groups (Supplementary Fig. S1). Immunofluorescence analysis further showed the same effects of miR-483-3p under oxidative stress (Figs. 3D–F). Therefore, miR-483-3p could downregulate the expression of ECM in HTMCs under oxidative stress.
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Figure 3. MiRNA-483-3p downregulates ECM genes expression in HTMCs. (A) qPCR analysis of miR-483-3p in HTMCs stably expressing miR-483-3p. (B) qPCR analysis of fibronectin, laminin, and collagen I in HTMCs stably expressing miR-483-3p. (C) Western blot analysis of fibronectin, laminin, and collagen I in HTMCs stably expressing miR-483-3p. (D–F) Immunofluorescence detection of fibronectin (D), collagen I (E), and laminin (F) in HTMCs stably expressing miR-483-3p with the stimulation of 300 μM H₂O₂ for 2 hours. Scale bars: 100 μm. Data represent mean ± SD. *0.01 < P < 0.05; **0.001 < P < 0.01.
MicroRNA-483-3p Directly Binds to the 3'-UTR of Smad4

To select and identify the downstream targets of miR-483-3p, we used mRNA target-predicting algorithms (TargetScan, TargetRank, and miRDB), which are based on the presence of binding sites in the 3'-UTR. Of the hundreds of genes that overlapped among these algorithms (Fig. 4A), five genes (Smad3, Smad4, Smad7, Furin, and Mapkapk2) were selected that were possibly associated with ECM production (Fig. 4B).

To confirm whether a direct relationship exists between miR-483-3p and the predicted target genes, the 3'-UTRs of key target genes were cloned into a dual-luciferase UTR vector between the firefly and renilla luciferase coding sequence (Fig. 4B). Notably, 3'-UTR of Smad4 was found to be uniquely repressed by miR-483-3p (Fig. 4B). At the mRNA and protein levels, Smad4 significantly decreased due to miR-483-3p in HTMCs (Figs. 4C, 4D). Analysis of the 3'-UTR sequence of Smad4 revealed two binding sites that perfectly matched with miR-483-3p (Fig. 4E). Next, we generated the mutations in the binding site to abrogate the miR-483-3p-Smad4–3'-UTR interaction (Fig. 4E). As expected, a reporter with an intact Smad4 3'-UTR was effectively suppressed by miR-483-3p, whereas the reporter with a Smad4 mutated binding site in 3'-UTR had no
response to the suppression of miR-483-3p (Fig. 4F). In addition, with increased concentration of H₂O₂ (100, 200, 300, 400 μM), the expression level of miR-483-3p was found to gradually decreased, but Smad4 expression presented the opposite trend (Fig. 4G). Taken together, these results indicate that miR-483-3p directly regulates Smad4 expression through binding to its 3'-UTR.

Knockdown of Smad4 Decreased ECM Expression Induced by Oxidative Stress in HTMCs

Smad4 can function as a positive transcriptional regulator of laminin-5 genes and influence the structure of basement membrane.29 As above, we confirmed the inhibitory function of miR-483-3p on ECM production and identified Smad4, a known cofactor in TGF-β signaling pathway, as a direct target of miR-483-3p. Then we further explored the effect of Smad4 on regulating ECM induced by oxidative stress in HTMCs. Human TMCs were infected with Smad4 knockdown or scramble lentivirus, and the expression of ECM components (fibronectin, laminin, and collagen I) in HTMCs was analyzed. Knockdown of Smad4 in HTMCs led to decreased expression of laminin and fibronectin (Fig. 5A). However, no inhibitory effect on collagen I was observed (Fig. 5A). Western blot analysis also revealed that knockdown of Smad4 in HTMCs resulted in decreased protein levels of laminin and fibronectin, but not collagen I (Fig. 5B). Furthermore, knockdown of Smad4 led to a decrease in laminin and fibronectin expression in both H₂O₂-treated and -untreated groups (Supplementary Fig. S2). From the above, silencing Smad4 in HTMCs may inhibit or delay ECM accumulation.

DISCUSSION

Cellular responses to oxidative stress are believed to play an important role in the physiology of the outflow pathway. In
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this study, we found that miR-483-3p decreased in HTMCs under oxidative stress; however, ECM increased and accumulated. Furthermore, we demonstrated that miR-483-3p targeted Smad4 and knockdown of Smad4 significantly decreased ECM expression (Fig. 6). Transforming growth factor/Smad signaling is important for ECM production and fibrotic progress, which is commonly found in various kinds of diseases or pathological conditions. Idiopathic pulmonary fibrosis is characterized by excessive deposition of collagen, other ECM components, and excessive fibroblast proliferation.30,31 Ascher et al.32 revealed that receptor-type protein tyrosine phosphatase α (PTP-α) promoted profibrotic signaling pathway in fibroblasts by modulating cellular responsiveness to TGF-β. Several studies have indicated that TGF-β could significantly increase the expression of collagen-I and fibronectin.33–35 The dominating intracellular signaling pathway of TGF-β is the Smads system. Additionally, other pathways can react to TGF-β signaling and induce ECM synthesis through Smads-dependent or -independent pathways, such as PI3K/Akt35 and p38 MAPK.35,36

Transforming growth factor/Smad signaling also plays important role in TM.35,36 Which frequently is overactive in glaucoma. Both Smad and non-Smad signaling pathways participated in TGF-β-induced LOX (lysyl oxidase) production, which is partially responsible for elevated IOP.37 Smad3, a member of the Smad family, was responsive as a negative factor for TGF-β-induced fibronectin deposition in TM and ocular hypertension.38 In addition, secreted protein acidic and rich in cysteine (SPARC), BMP1, and more related findings were proved responsible for ECM protein production and IOP upregulation.39–41 To our knowledge, only several previous studies have taken advantage of miRNAs in regulating the biological behaviors of TM and offered a potential opportunity to identify biomarkers for diagnosis and prognosis of glaucoma.27,42–45 MiR-24 can directly target 3’-UTR of the subtilisin-like proprotein convertase Furin, which could activate TGF-β.42 There is one binding site in 3’-UTR of Furin for miR-483-3p, but we found that Furin was not a novel target of miR-483-3p by 3’-UTR luciferase assay. The miRNA-23b/24-1/27b cluster has been demonstrated to regulate TGF-β/bone morphogenetic protein signaling by different mechanisms including the direct targeting of Smads.44 In pancreatic cancer, miR-483-3p was found to target DPC4/Smad4, and their expression levels are inversely correlated in human pancreatic cancer tissues.45 Given the potential involvement of miR-483-3p in the response to oxidative stress in HTMCs and the relevance of TGF-β/Smad signaling in the pathogenic responses induced by oxidative stress, we analyzed the influence of miR-483-3p on gene expression in HTMC, and found that miR-483-3p induced significant changes in expression of several genes involved in ECM deposition, including fibronectin, collagen, and laminin.

Laminin, a basement membrane component, is a direct target of Smad4.29,46 Our data also supported that laminin production is dependent on Smad4. The feeble effect of knockdown Smad4 on collagen I may be due to other intracellular signaling pathways that regulate collagen I, requiring further study.

In summary, oxidative stress induced significant alterations in the expression of miRNAs that could contribute to the regulation of some of the responses to oxidative stress in HTMCs. Specifically, we showed that miR-483-3p limited ECM accumulation by directly targeting Smad4 in HTMCs under oxidative stress. Moreover, we confirmed that the production of fibronectin and laminin was Smad4 dependent. Thus, upregulation of miR-483-3p and subsequent downregulation of its target Smad4 may serve as a mechanism to limit the amount of ECM activated by oxidative stress and prevent some potentially pathogenic effects of these ECM genes in the AH outflow pathway.

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