Article

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Sirtuin1 Promotes the Migration of Cells and Reduces the Accumulation of Extracellular Matrix in Trabecular Meshwork Cells Induced by Transform Growth Factor-β via Smads System

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Background: Primary open-angle glaucoma (POAG) is one of the common types of glaucoma, an eye disease that causes irreversible blindness. Fibrosis of the trabecular meshwork (TM) caused by the accumulation of extracellular matrix (ECM) induced by transform growth factor-β (TGF-β) is closely related to high intraocular pressure (IOP). Deacetylase Sirtuin1 (Sirt1) plays an anti-oxidation and anti-fibrosis role in many diseases, including glaucoma; however, its mechanisms have not been fully revealed. In this study, we analyzed the anti-fibrotic role of Sirt1 in TM fibrosis induced by TGF-β to investigate potential mechanisms.

Methods: Transcriptome sequencing of trabecular meshwork cells (TMCs) was performed after transfection with the adenovirus-Sirt1-green fluorescent protein (Adv-Sirt1-GFP). Then, 5 ng/mL TGF-β was used to induce overexpression of ECM in TMCs *in vitro*. The expression of target proteins was detected by Western blot and immunofluorescence, and cytokine expression was detected by enzyme-linked immunosorbent serologic assay (ELISA). At the same time, we detected the functional changes in cell proliferation, adhesion, and migration.

Results: After treatment with TGF-β, we found that the accumulation of ECM was increased (fibronectin (FN), collagen I (COL I), laminin (LN), $p < 0.05$), and the phosphorylation (activation) of Smad2/3 and the expression of Smad4 were increased ($p < 0.001$). The results of transcriptome sequencing suggested that Sirt1 inhibits the expression of ECM by regulating the functions of co-Smad and co-COL binding proteins, thus participating in the regulation of cell adhesion. Finally, we confirmed that: (1) Sirt1 reduced the accumulation of ECM in TMCs by inhibiting the phosphorylation of Smad2/3 ($p < 0.05$) and the expression of Smad4 ($p < 0.05$), and (2) Sirt1 decreased the adhesive ability of TMCs by reducing the secretion of integrins (integrin-α3 (ITGα3), $p < 0.01$; integrin-β1 (ITGβ1), $p < 0.001$) and cadherins (E-cadherin, $p < 0.01$; N-cadherin, $p < 0.01$), and promoted cell migration ($p < 0.05$).

Conclusion: Sirt1 promotes the migration of cells and reduces the accumulation of ECM in TMCs induced by TGF-β by inhibiting the activation of Smad2/3 and the expression of Smad4.

Keywords: Sirt1; Smad; TGF-β; TMCs; ECM; cell adhesion

Introduction

Glaucoma, a disease of optic neuropathy, is characterized by progressive death of retinal ganglion cells (RGCs) and visual field defects. Primary open-angle glaucoma (POAG) is the most common type of glaucoma, in which the angle formed by the iris and cornea remains open [1]. The increase in intraocular pressure (IOP) is due to the increase of extracellular matrix (ECM) secretion and the dysfunction of migration and adhesion of the trabecular meshwork cells (TMCs), resulting in trabecular meshwork (TM) tissue fibrosis and reduced outflow of aqueous humor (AH). Elevated IOP is the main risk factor for RGC death and vision loss in glaucoma [2].

A previous clinical study found that, compared with the control group, the expression of transform growth factor-β (TGF-β) in patients' AH with POAG [3] was significantly higher, and that TGF-β could induce TM morphological changes and ECM deposition in patients with POAG. As we know, TGF-β upregulates ECM expression by activating the Smads signaling pathway, which is a classic fibrosis regulatory pathway. It was shown *in vitro* that TGF-β induces ECM deposition in cultured Tenon's capsule fibroblasts and TMCs from glaucoma patients through the Smads signaling pathway [4]. These growth factors increase the expression of fibronectin and collagen in a concentration-dependent manner as shown in human and pig TMCs [5]. TGF-β also promotes ECM deposition by

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changing the matrix metalloproteinase (MMP)/tissue inhibitor of MMP (TIMP) balance, thus inducing ECM remodeling in TM [6].

Deacetylase Sirtuin1 (Sirt1), a nicotinamide adenine dinucleotide NAD (+)-dependent deacetylase, is the closest mammalian homolog of yeast silencing information regulator 2 protein [7]. Sirt1 regulates many cell functions by L-lysine deacetylation, including fibrosis. Smad2/3 has been shown to regulate TGF-β signal transduction by changing their transcriptional activity through acetylation or deacetylation of specific lysine residues [8]. Sirt1 may suppress the activation of fibroblasts by inhibiting the phosphorylation of Smad to p-Smad3, ultimately reducing liver [9], cardiac [10], and renal fibrosis [11].

Sirt1, therefore, may be a therapeutic target for fibrosis of tissues and organs. In this paper, we will explore the role and mechanism of Sirt1 in TM fibrosis, and provide an experimental basis for a new treatment target of glaucoma.

Materials and Methods

Cell Culture and Treatments

Human trabecular meshwork cells (HTMCs) used in this study were purchased from ScienCell (6590, Carlsbad, CA, USA). The cell line was confirmed by short tandem repeat (STR) identification. No potential biological pollutants, including mycoplasma, were detected in the cells. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (10-013-CV, Corning Inc., Corning, NY, USA) culture medium with 10% fetal bovine serum (04-010-1A, Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin (MA0110, Meilunbio, Dalian, China) at 37 °C and 5% CO_2. 5 ng/mL TGF-β was used to induce ECM expression [4].

Western Blot

Western blot was performed using Radio Immunoprecipitation Assay (RIPA) lysis buffer (0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 1 mM MgCl2 and 10 mM Tris, PH 8.0) containing 1× proteinase inhibitor. 30 µg of protein were dissolved in 5× SDS loading buffer and boiled for 5 min at 100 °C. Proteins were separated on 6% or 10% SDS-polyacrylamide gels, then transferred onto Polyvinylidene fluoride (PVDF) membranes (0.22 µm) which were blocked in 5% bovine serum albumin (BSA) in 1× Tris-Borate-Sodium Tween (TBST). Primary antibodies were obtained (Cell Signaling Technology, Beverly, MA, USA) against the following antigens: fibronectin (FN) (26836), collagen I (COL I) (72026), laminin (12255), Smad2/3 (8685), p-Smad2/3 (8828), and Smad4 (46535). β-actin (8457s, Cell Signaling Technology, Beverly, MA, USA) was diluted at 1:1000. Antibodies were diluted at 1:1000 and incubated overnight at 4 °C. After washing with TBST, horseradish peroxidase (HRP)-conjugated anti-rabbit (ab6721, Abcam, Cambridge, UK) was diluted at 1:5000 and incubated for 1 h at room temperature. Reactions were visualized using an Enhanced Chemiluminescence (ECL) detection kit (RPN2135, GE Healthcare, Buckinghamshire, UK). Quantification of bands was done with Quantity One software (Bio-Rad, Hercules, CA, USA). ImageJ (1.8.0, US National Institutes of Health, Bethesda, MD, USA) was used to obtain strip gray values for statistical analysis.

Adenovirus Transfection

Adenovirus-NC-green fluorescent protein (Adv-NC-GFP) and Adenovirus-Sirt1-green fluorescent protein (Adv-Sirt1-GFP) were purchased from Hanbio (Shanghai, China). After the replacement of the fresh medium, the adenovirus original solution was added into the wells to infect cells according to the volume of the medium, selecting the MOI = 30. Samples were mixed and then incubated at 37 °C for 4 hours. The culture medium containing adenovirus was replaced with fresh culture medium, and cultured for 48 hours. GFP was detected by fluorescence microscopy and the expression of target protein was detected by western blot.

Immunofluorescence

Cells were grown in 48-well plates. After treatments, cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 (Sigma Aldrich, 9036-19-5, St. Louis, MO, USA) at room temperature. To reduce nonspecific antibody binding, cells were blocked in 3% BSA for 30 min at room temperature. Primary antibodies (Cell Signaling Technology, MA, USA) against FN (26836), COL I (72026), and laminin (12255) were diluted at 1:1000 and used to stain the samples; and nuclei were visualized with 4′,6-Diamidino-2′-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA) diluted at 1:1000 in 0.1% BSA in phosphate buffer saline (PBS) at room temperature for 2 h. Cells were washed with 0.1% BSA in PBS and incubated with Alexa Fluor secondary antibodies (A-21244, Invitrogen, CA, USA) which were diluted at 1:2000 for 1 h in the dark. Cells were washed and observed with a fluorescence microscope (CKX41, Olympus, Tokyo, Japan). ImageJ was used to calculate the average fluorescence intensity (Average Fluorescence Intensity = Integrated Density/Area) for statistical analysis.

Microarray Assay

The detailed experimental methods of RNA sequencing are described in our previously published article [12].

Crystal Violet Staining Assay

Proliferation was determined by crystal violet staining assay. The cells were washed 3 times with PBS, then fixed by 4% polyformaldehyde (PFA), and stained with 0.2% crystal violet solution (Sigma Aldrich). The images of stained cells were obtained by microscopy. ImageJ was used for automatic cell counting.

Cytoskeleton Staining

The culture medium was removed, and cells were washed 3 times with PBS. Cells were washed after being fixed by 4% PFA for 20 min. Cells were then treated with 0.3% Triton for 10 min and washed. Alexa Fluor488-Phalloidin (5 µg/mL) was used to label F-actin, and vinculin was labeled with red fluorescence. Cells were incubated at room temperature for 40 min in the dark and then observed with a fluorescence microscope. ImageJ was used for automatic counting of adhesion spots.

Cell Migration Assays

The Transwell® cell migration system with polycarbonate membranes (8 µm pore size; Corning Life Sciences; Tewksbury, MA, USA) was used to perform cell migration assays. 200 µL of cell suspension (4×10^5 cells/mL) was added into each upper chamber, treated with TGF-β and adenovirus or not; and 300 µL of normal culture medium was added into each lower chamber as a basic chemoattractant. After 12 hours, cells above the membrane were removed with cotton swabs. The chambers were washed 3 times with PBS, then fixed by 4% PFA, and stained with 0.2% crystal violet solution. The images of stained cells on the outer side of the membrane were obtained by microscopy. ImageJ was used for automatic cell counting.

Cell Adhesion Assay

96-well plates were coated with the ECM proteins. After treatment, cells were suspended in the medium and adjusted to a concentration of 5×10^6 cells/mL. 100 µL of cell suspension labeled by Calcein AM (KGA9501-1000, KeyGEN Biotech, Jiangsu, China) was added to each well (5×10^5 cells). Cells were incubated at 37 °C for 30 minutes and carefully washed 4 times to remove the non-adherent labeled cells. 200 µL PBS was added to each well and cells were examined by fluorescence microscopy. ImageJ was used for automatic cell counting.

Enzyme-Linked Immunosorbent Serologic Assay (ELISA)

Cytokine levels were assessed using the ELISA Kits (JL13185, JL15350, bs-20630R, JK-(a)-E03096, JL45077, JL19971, JL26821, Jianglai Biological, Shanghai, China) according to the manufacturer's instructions. The cell culture medium was centrifuged at 5000 rpm for 4 min to obtain supernatant. 100 µL of standard or cell supernatant was added into the reaction pore, then mixed well and incubated at 37 °C for 60 min. After discarding the liquid, plates were washed with washing liquid 5 times, then dried. 100 µL 1× Biotin was added into each pore and incubated at 37 °C for 60 min. The plate was washed and dried, then 100 µL 1× HRP was added and incubated at 37 °C for 60 min. After washing, 100 µL TMB solution was added and incubated at 37 °C for 20 min. The optical density (OD) at 450 nm was measured after adding 100 µL of terminal solution. A standard curve was established, with the OD value as ordinate and standard concentration as abscissa, and the concentration of target samples was calculated according to the curve formula.

Statistical Analysis

All results were derived from at least three independent experiments. The data were expressed as the mean ± Standard Error of Mean (SEM). The GraphPad Prism statistical program (5.0, La Jolla, CA, USA) was used for data analysis. The two-tailed Student's test was used for comparisons of two groups, and a one-way analysis of variance with a Bonferroni post hoc test was used for multiple comparisons. p values < 0.05 were considered statistically significant.

Results

TGF-β Activates TGF-β/Smad Signal and Induced ECM Expression in TMCs

In order to study the anti-fibrosis effect of Sirt1 in TMCs, we treated TMCs with TGF-β to induce overexpression of ECM-associated proteins. After 24 hours, we found that there was a significant phosphorylation of Smad2/Smad3 and the expression of Smad4 significantly increased (Fig. 1A,B, $p < 0.001$), and the expression of ECM-associated proteins COL I, FN, and laminin (LN) were significantly increased (Fig. 1A–E, $p < 0.05$). These results are consistent with Fan Y's research findings [4], which suggest that TGF-β can induce the accumulation of ECM in TMCs by activating the TGF-β/Smad signaling pathway.

Sirt1 Inhibits the Expression of ECM Induced by TGF-β

Sirt1 plays an anti-fibrosis role in many other nonocular diseases as described in the Introduction. In this study, we investigated the anti-fibrosis role of Sirt1 in TMCs treated with TGF-β. We used GFP to label the negative control gene fragment or the overexpressed Sirt1 gene fragment and integrated Adv-NC-GFP or Adv-Sirt1-GFP with adenovirus as the vector. After transfection with the adenovirus, we found that the GFP fluorescence and the expression of Sirt1 increased significantly in TMCs (Fig. 2A–C, $p < 0.05$), which confirmed that the transfection method was feasible and efficient. Then, after treating TMCs with TGF-β, we found that the expression of ECM-associated proteins in TMCs with overexpressed Sirt1 decreased significantly (Fig. 2D–K, $p < 0.05$). The results suggested that Sirt1 could inhibit fibrosis induced by TGF-β in TMCs.

The Possible Functions of Sirt1 Analysed by RNA-seq

To explore the functions of Sirt1 and its possible regulatory mechanisms, we performed the transcriptome se-

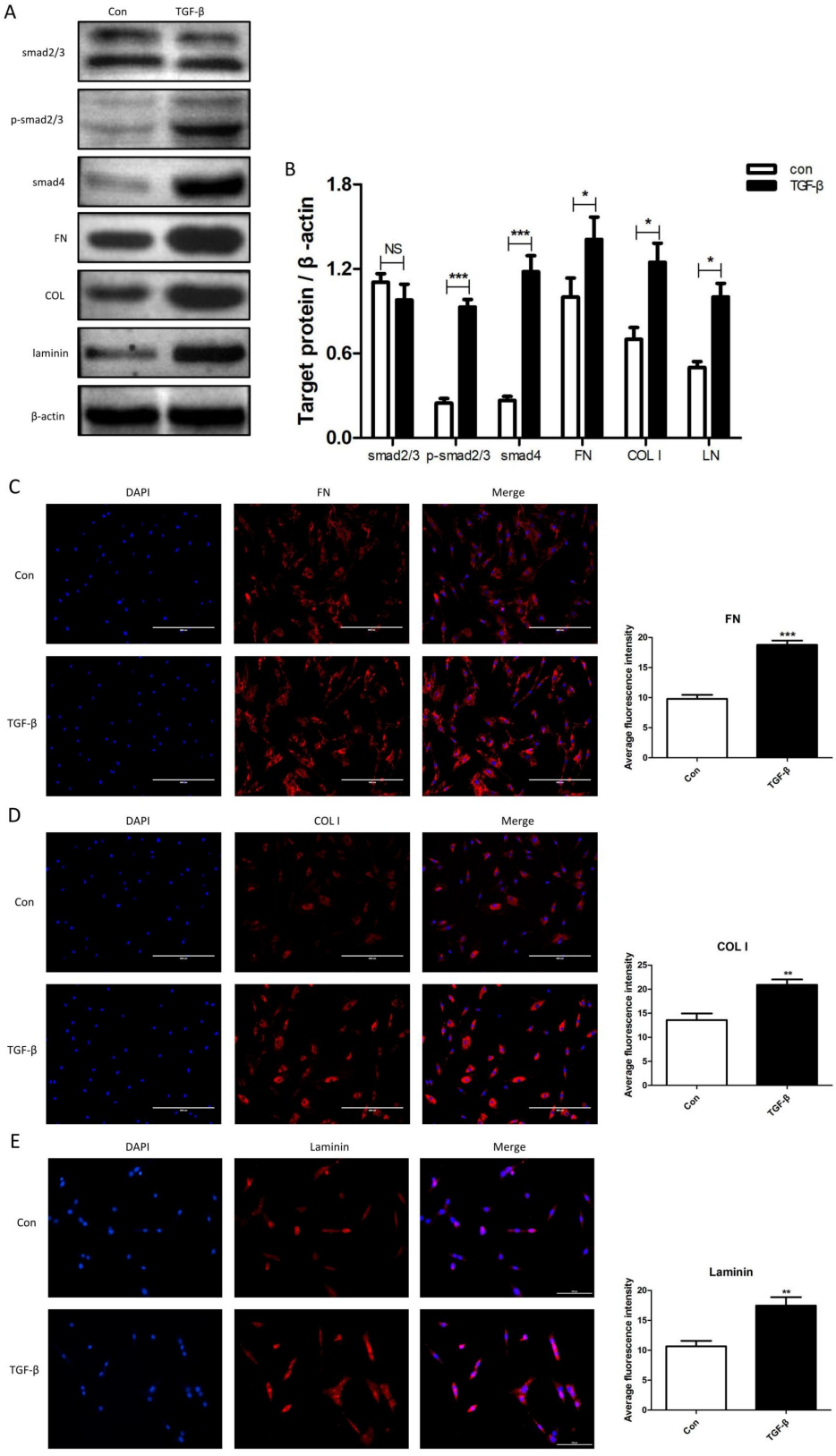

Fig. 1. Transform growth factor-β (TGF-β) activated the Smads system and induced extracellular matrix (ECM) expression in trabecular meshwork cells (TMCs). (A,B) Western blotting images for target protein expression. (C–E) Photographs were taken by fluorescence microscope for fibronectin (FN), collagen I (COL I), and laminin (LN) stained by immunofluorescence (Scale bar: (C,D) 400 µm, (E) 100 µm) (NS, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) (n = 3 in each experiment, and each experiment was repeated 3 times).

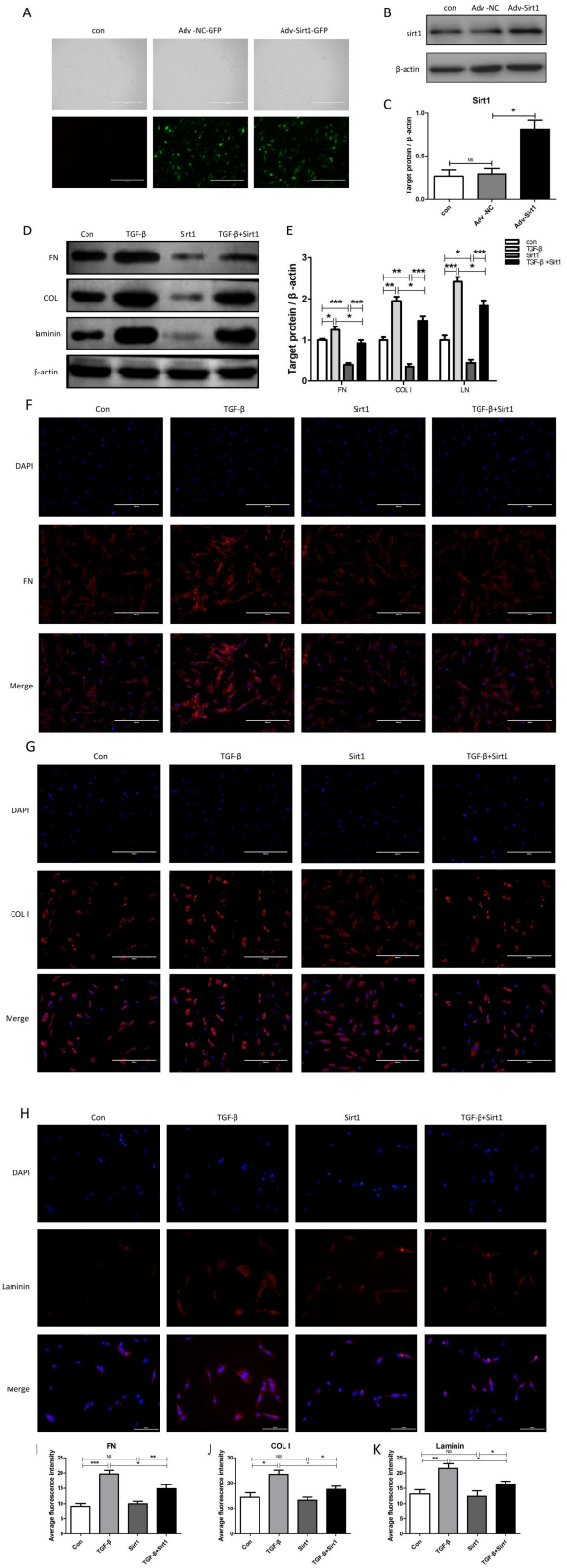

Fig. 2. Deacetylase Sirtuin1 (Sirt1) inhibits the expression of ECM induced by TGF-β. (A) Photographs were taken by fluorescence microscope for TMCs transfected by Adenovirus-NC-green fluorescent protein (Adv-NC-GFP) or adenovirus-Sirt1-green fluorescent protein (Adv-Sirt1-GFP) (Scale bar: 1000 um). (B,C) Western blotting images for Sirt1 expression in TMCs transfected with Adv-NC-GFP or Adv-Sirt1-GFP (n = 3, $p < 0.05$). (D–K) Western blotting image and photographs taken by fluorescence microscope for FN, COL I, and LN (Scale bar: (F,G) 400 µm, (H) 100 µm) (n = 5, NS, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

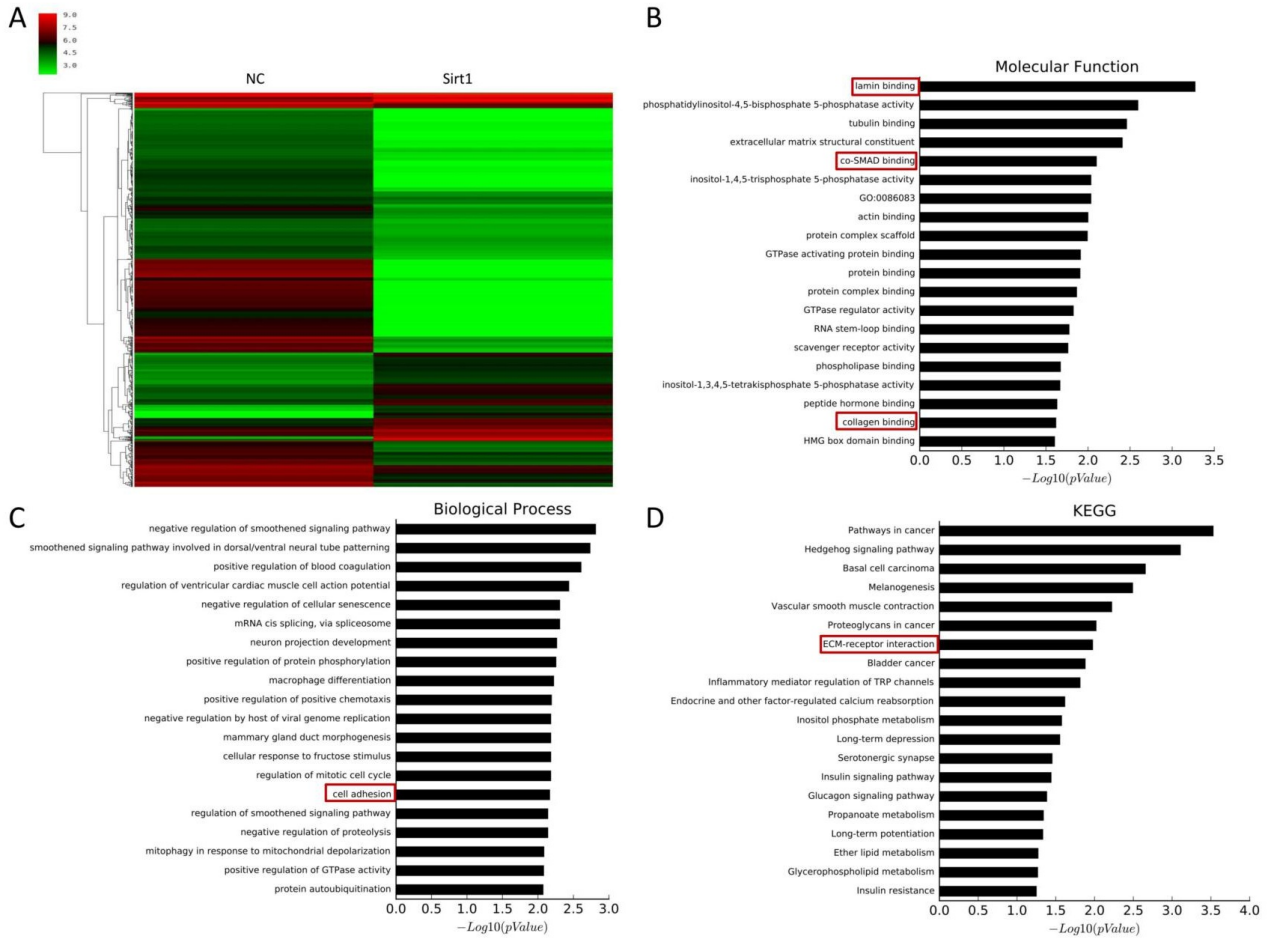

Fig. 3. Transcriptome-sequencing of TMCs and the bioinformatics analysis. (A) Heatmap of mRNA in TMCs transfected with Adv-NC-GFP or Adv-Sirt1-GFP. (B–D) Bioinformatics analysis of differentially expressed mRNAs. (B) SIRT1 can regulate the molecular function of lamin binding, co-SMAD binding and collagen binding. (C) Sirt1 can affect the process of cell adhesion. (D) Sirt1 is involved in the pathway of ECM-receptor interaction (n = 3).

quencing of TMCs transfected with Adv-Sirt1-GFP that overexpressed Sirt1. Compared to TMCs treated with Adv-NC-GFP, we found 854 mRNAs with differential expression (FC $\geq$2, $p \leq 0.05$), of which 212 mRNAs were up-regulated and 642 mRNAs were down-regulated (Fig. 3A). Bioinformatics analysis of sequencing results showed that the molecular functions of differential mRNAs were significantly enriched in co-Smad binding and collagen-binding (Fig. 3B), and the KEGG signal pathways and cell processes were significantly enriched in ECM-receptor interaction and cell adhesion respectively (Fig. 3C,D). ECM has been proven to be an important regulator of cell migration and adhesion. Sirt1 may affect the interaction between ECM and its protein receptor by regulating the expression of Smad and COL and may participate in the conduction process of the cell adhesion signal pathway.

Sirt1 Improves the Dysfunction of TMCs Induced by TGF-β

We carried out a series of experiments to confirm the bioinformatics analysis of sequencing results. The proliferation assays showed that the TMCs treated with Adv-Sirt1-GFP did not proliferate significantly (Fig. 4A); the cytoskeleton staining showed that TGF-β promoted hypertrophy of TMCs, decrease in intercellular space, and an increase in adhesion spots, whereas Sirt1 significantly improved these phenomena (Fig. 4B, $p < 0.05$); the adhesion assays showed that Sirt1 could inhibit the enhanced adhesion function of TMCs caused by TGF-β (Fig. 4C, $p < 0.05$) and promote cell migration (Fig. 4D, $p < 0.05$). These results indicated that Sirt1 could improve the dysfunctions of TMCs migration and adhesion induced by TGF-β.

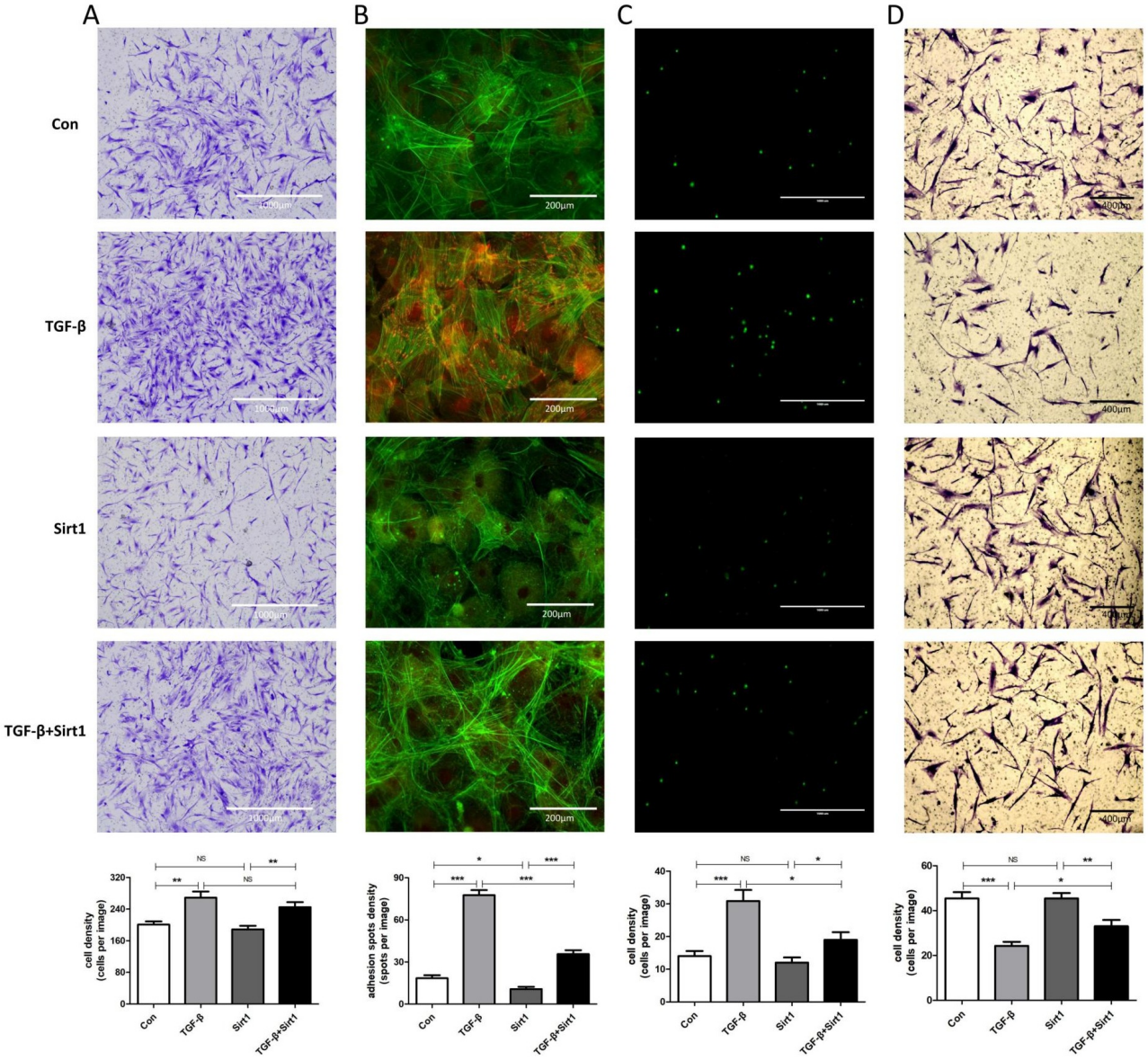

Fig. 4. Sirt1 improves the dysfunction of TMCs induced by TGF-β. (A) The proliferation assays of TMCs (Scale bar: 1000 μm). (B) Cytoskeleton staining of TMCs, F-actin marked in green and vinculin marked in red, orange or yellow dots indicated adhesive spots (Scale bar: 200 μm). (C) Adhesion assays of TMCs (Scale bar: 1000 μm). (D) Migration of TMCs (Scale bar: 400 μm) (n = 5, NS, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Sirt1 Performs its Function by Regulating the Smads System

Because the Smads system is the upstream regulation signal of the ECM, combining the results of the bioinformatics analysis mentioned above, we can reasonably speculate that Sirt1 may inhibit the expression of ECM in TMCs induced by TGF-β by regulating the activation and expression of Smads. Additional experiments have been performed to confirm this mechanism. As the results showed, Sirt1 did not cause a change in the total expression level of smad2/3, but it suppressed the phosphorylation (activation) of Smad2/3 and the expression of Smad4 (Fig. 5A,B, $p < 0.05$), and inhibited the secretion of some cytokines, like integrins (integrin-α3 (ITGα3), integrin-β1 (ITGβ1)) (Fig. 5C,D, ITGα3, $p < 0.01$; ITGβ1, $p < 0.001$) and cadherins (E-cadherin, N-cadherin) (Fig. 5E,F, E-cadherin, $p < 0.01$; N-cadherin, $p < 0.01$). But other cytokines such as NCAM and CD31 showed no significant expression changes (Fig. 5G,H), and CD44 expression increased (Fig. 5I, $p < 0.001$). It was confirmed that Sirt1 downregulated the expression of ECM induced by TGF-β in TMCs by inhibiting the activation of Smad2/3 and the expression of Smad4, and improved the dysfunction of adhesion and migration by reducing the secretion of adhesion molecules.

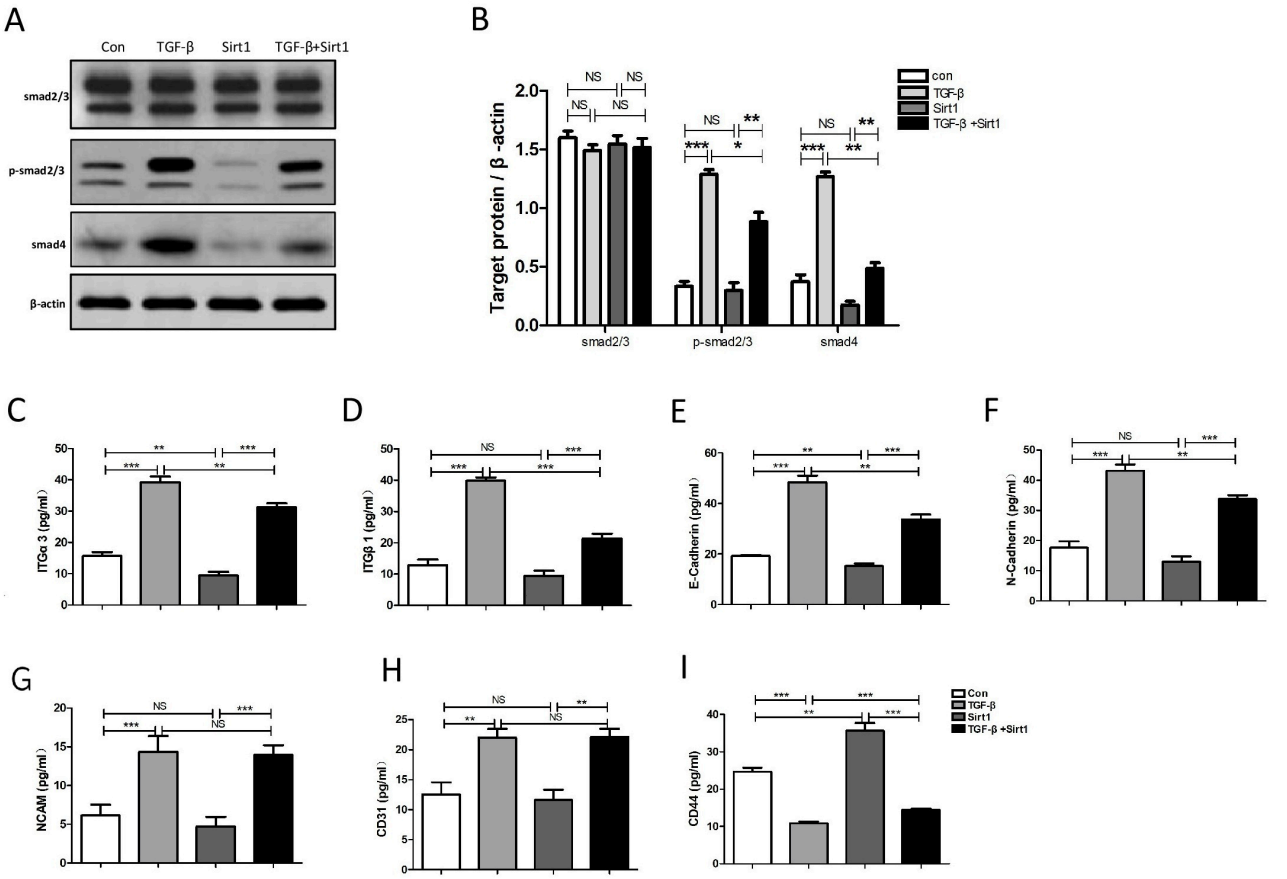

Fig. 5. Sirt1 inhibited the activation of Smad2/3 and the expression of Smad4 and reduced the secretion of adhesion molecules. (A,B) Western blotting images for target protein expression (n = 3, NS, not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (C–I) The enzyme-linked immunosorbent serologic assay (ELISA) results of adhesion molecules in TMCs culture medium supernatant (n = 5, NS, not significant; **$p < 0.01$, ***$p < 0.001$).

Discussion

Glaucoma is a type of irreversible blindness caused by pathologically high IOP, which causes optic nerve damage and visual field defects. According to recent statistics, the incidence rate of the total population is 1%, and 2% after 45 years old. It has become the second leading cause of blindness worldwide [13]. Elevated IOP is the most important risk factor for optic nerve damage in glaucoma [1]. POAG is a common type of glaucoma. The main reason for the increase in IOP in POAG is that the dynamic balance of AH circulation has been altered [14]. Fibrosis and sclerosis of TM caused by the deposition of ECM, and the structural and functional injuries caused by the dysfunction of TMCs migration and adhesion, can lead to increased outflow resistance of AH, thus promoting the elevation of IOP. In this study, we found that ECM increased and accumulated in TMCs under TGF-β stress.

Sirt1 has been shown to play a protective role in glaucoma through various pathways. (1) Sirt1 can inhibit the expression of COL I in TMCs under oxidative stress through activation of the pathway Sirt1-HES1-COL I [15]; (2) Sirt1 can enhance the repair ability of TMCs to promote anti-aging effects [16]; (3) Sirt1 can reduce RGCs apoptosis after retinal ischemia-reperfusion injury [17]. In this study, we demonstrated that Sirt1 significantly decreased the expression of ECM and improved the dysfunctions of TMCs migration and adhesion induced by TGF-β.

As shown in many kinds of diseases or pathological conditions, TGF-β/Smad signaling is important for ECM production and fibrotic progress. It has been reported that TGF-β can significantly increase the expression of COL, FN, and other ECM components, and cause excessive fibroblast proliferation [18–20]. Cellular responses to TGF-β stress have been confirmed to play an important role in the physiology of the outflow pathway. The Smads system is the dominant intracellular signaling pathway of TGF-β. In glaucoma, it has been reported that TGF-β/Smad signaling is frequently overactive and plays an important role in the TM [21]. It has also been reported that both Smad and non-Smad signaling pathways contribute to TGF-β-induced LOX (lysyl oxidase) production, which may lead to elevated IOP [22]. As a key member of the Smad family, Smad3 was found to be a necessary factor for fibronectin

deposition in the TM induced by TGF-β2, which caused ocular hypertension [23]. In the previous study, Shen W's team [24] showed that miR-483-3p limited ECM accumulation by directly targeting Smad4 in HTMCs under oxidative stress. They also confirmed that the production of fibronectin and laminin was Smad4 dependent. Therefore, Smads may be important targets for regulating the amount of ECM in the AH outflow pathway and protecting the TM from potentially pathogenic effects of the ECM. As our RNA-seq results showed, Sirt1 may affect the interaction between ECM and its protein receptor by regulating the expression of Smad and COL and may participate in the conduction process of the cell adhesion signal pathway. Furthermore, we confirmed that Sirt1 suppressed the phosphorylation (activation) of Smad2/3 and the expression of Smad4, and inhibited the secretion of some cytokines, like integrins (ITGα3, ITGβ1) and cadherins (E-cadherin, N-cadherin). All of these results are from cell experiments *in vitro*, and further validation is needed in animal experiments *in vivo*.

Conclusion

In conclusion, Sirt1 promotes the migration of cells and reduces the accumulation of ECM in TMCs induced by TGF-β by inhibiting the activation of Smad2/3 and the expression of Smad4.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

JW designed the research study and provided help and advice on the experiments. JT, JG, and JY performed the research. JT and JG analyzed the data. JT and JG made equal contributions to this research. All authors have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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