

# YAP and TAZ mediate steroid-induced alterations in the trabecular meshwork cytoskeleton in human trabecular meshwork cells

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**Abstract.** Primary open angle glaucoma is an important type of glaucoma as it is one of the most common causes of blindness. Previous studies have demonstrated that in glaucomatous patients, the human trabecular meshwork (HTM) is markedly stiffened. The purpose of the present study was to determine the regulatory role of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) in HTM cells. Primary HTM cells were cultured with different concentrations of dexamethasone (DEX), and the expression levels of YAP and TAZ were evaluated using reverse transcription-quantitative polymerase chain reaction and western blotting. The results revealed that DEX increased the expression of YAP and TAZ in a dose-dependent manner. In addition, the western blot analysis of cytoskeleton-associated proteins revealed that the inhibition of YAP and/or TAZ using small interfering RNA resulted in the increased expression of collagen I, and decreased expression of fibronectin, laminin and collagen IV. The expression of  $\beta$ -catenin, a key protein in the Wnt pathway, was also observed to be regulated by YAP and TAZ. A 5-ethynyl-2'-deoxyuridine staining assay indicated that YAP and TAZ induced the proliferation of HTM cells. The investigation of cross-linked actin network formation by the HTM cells demonstrated that the knockdown of YAP and TAZ genes rescued HTM cells from cytoskeletal reorganization. Furthermore, functional evaluation of a HTM

cell monolayer using a permeability assay demonstrated that the inhibition of YAP and TAZ attenuated the DEX-induced impairment of permeability. These findings suggest that YAP and TAZ play pivotal roles in the DEX-induced cytoskeletal changes of HTM cells, and reveal novel potential mechanisms for the development and progression of glaucoma.

## Introduction

Glaucoma is the second most common cause of blindness worldwide, and ~47% of the individuals with glaucoma are of Asian origin (1). Notably, primary open angle glaucoma (POAG) is one of the most prevalent types of glaucoma, and its main clinical manifestations are high intraocular pressure (IOP) and visual function damage (2). Although the mechanism of POAG development is unclear, the main pathological change is considered to be the reduced outflow of aqueous humor through the trabecular meshwork (TM), the major site of IOP regulation (3). A previous study has demonstrated that an increased amount of sheath-derived plaque material in the TM is associated with an increased severity of optic nerve damage in POAG (4). In addition, the TM from glaucomatous ocular tissue has been found to be stiffer than that from normal ocular tissue (5), indicating the significance of the morphological and biophysical changes of the TM in glaucoma. Therefore, primary cultured human trabecular meshwork (HTM) cells have been studied *in vitro* in order to investigate the pathogenic mechanism of POAG. Corticosteroids have long been recognized as a cause of glaucoma (6,7). Thus, a commonly used *in vitro* model for glaucoma is the treatment of HTM cells with dexamethasone (DEX), a synthetic glucocorticoid (8-11).

The Hippo signaling pathway, first discovered in *Drosophila*, is a signaling pathway that controls organ size by promoting cell death and cell differentiation (12,13) and inhibiting cell proliferation (14-17). Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are two Yorkie homologs in the Hippo pathway that have been identified in mammals and are highly conserved (18). It has been reported that YAP and TAZ exist at all levels

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of the TM (19), and are influenced by the stiffness of the extracellular matrix (ECM) (20). Studies conducted by Raghunathan *et al.* (19) demonstrated the expression of YAP and TAZ in HTM cells. In these studies, polyacrylamide hydrogels mimicking the normal (5 kPa) and glaucomatous (75 kPa) meshworks were used in the culture of HTM cells, and the researchers observed that YAP and TAZ mRNA expression levels were upregulated on the 75-kPa hydrogels in comparison with the 5-kPa hydrogels. However, to the best of our knowledge, no previous studies concerning the involvement of YAP and TAZ in corticosteroid-induced glaucoma have been reported. Therefore, the present study aimed to elucidate the roles of YAP and TAZ in DEX-induced glaucoma. The knockdown and overexpression of YAP and TAZ genes was conducted in order to investigate their roles in the regulation of DEX-induced glaucoma. In addition, the regulatory roles of YAP and TAZ in the cell proliferation and cytoskeletal structure of HTM cells were determined.

## Materials and methods

**Chemicals.** DEX, fluorescein isothiocyanate (FITC)-labeled phalloidin (P5282), Triton X-100 (T9284), bovine serum albumin (BSA; V900933), Bradford reagent (B6916) and paraformaldehyde (PFA; 158127) were all purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Small interfering RNAs (siRNAs) against human YAP (sc-38637) and human TAZ (sc-38568), and scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Click-iT 5-ethynyl-2'-deoxyuridine (EdU) Alexa Fluor 488 Imaging kit (C10337), SYBR-Green PCR Master mix, NuPAGE 10% Bis-Tris protein gel (NP0315BOX), NuPAGE transfer buffer (NP0006) and 4',6-diamidino-2-phenylindole (DAPI; D1306) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Polyvinylidene fluoride membranes were purchased from Pall Corporation (Port Washington, NY, USA).

Constitutively activated YAP (GST-YAP, Addgene plasmid 38105), TAZ (HA-TAZ, Addgene plasmid 32839) and empty vector (pcDNA3.1, Addgene plasmid 62803) were all purchased from Addgene, Inc. (Cambridge, MA, USA).

Primary antibodies to the following proteins were purchased from Santa Cruz Biotechnology, Inc.: YAP (sc-15407), TAZ (sc-48805), fibronectin (sc-53285), laminin  $\alpha$ 1 (sc-6016),  $\beta$ -catenin (sc-65480) and  $\beta$ -actin (sc-47778). Primary antibodies to collagen I (ab34710) and collagen IV (ab19808) were both purchased from Abcam (Cambridge, MA, USA). The secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG (cat. no. 11-035-003) and goat anti-mouse IgG (cat. no. 11-035-003) were both purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

**Cell culture.** HTM cells were purchased from Bolise Co., Ltd. (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 U/ml penicillin (all from Thermo Fisher Scientific, Inc.), and maintained at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. All studies were conducted using cells prior to the eighth passage.

**Cell treatment, proliferation assay and morphological analysis.** HTMC cells were treated with PBS or DEX (1x10<sup>-4</sup>, 1x10<sup>-5</sup>, 1x10<sup>-6</sup> and 1x10<sup>-7</sup> M) at 37°C for 48 h. Cell proliferation was analyzed using a Click-iT EdU cell proliferation assay kit according to the manufacturer's instructions. In brief, 3x10<sup>5</sup> HTM cells were plated on coverslips and incubated with 10  $\mu$ M EdU solution at 37°C for 16 h. The cells were then fixed with 4% buffered PFA and incubated with 0.5 ml Click-iT reaction mixture for 30 min at room temperature. The coverslips were examined using a Nikon Eclipse 80i microscope system (Nikon, Tokyo, Japan). DAPI was used as a nuclear counterstain (blue) at room temperature for 5 min. EdU-positive cells (green) in 15-20 randomly selected fields were manually counted and the proportion of all cells that were EdU-positive was determined.

For the immunofluorescence analysis of cell morphology, 3x10<sup>5</sup> HTM cells plated on coverslips were fixed with 4% PFA and permeabilized with Triton X-100. Following the removal of the remaining Triton X-100, the cells were incubated with 5  $\mu$ g/ml FITC-labeled phalloidin for 1 h. Nuclear staining of the cells was conducted by staining with DAPI at room temperature for 5 min. The coverslips were examined using the Nikon Eclipse 80i microscope system.

**Transfection and gene silencing.** HTM cells were plated in 60-mm dishes with DMEM/F-12 containing 10% FBS and 1% penicillin-streptomycin at a density of 1x10<sup>6</sup> cells/dish. The plated HTM cells were continuously cultured in DMEM/F-12 without 10% FBS and without 1% penicillin-streptomycin for a further 2 h. The YAP, TAZ and empty plasmids were mixed with 25X diluent Lipofectamine LTX with PLUS reagent, and incubated for 20 min at room temperature to allow plasmid-Lipofectamine LTX complexation. Transfections were then conducted in OptiMEM reduced-serum medium (cat. no. 31985070; Thermo Fisher Scientific, Inc.) containing 1 mg/ml plasmid DNA, 0.1% PLUS reagent and 0.3% Lipofectamine LTX transfection reagent, where HTM cells were cultured in the medium with plasmid in 60-mm dishes for 48 h (21). Finally, the transfection efficiency was detected using western blot analysis. For the knockdown of YAP and TAZ genes, HTM cells were transfected with YAP siRNA, TAZ siRNA or scrambled siRNA at 33 nM using DharmaFect transfection reagent with SMARTpool ON-TARGET siRNA (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) according to the manufacturer's instructions (22). The transfected cells were used for subsequent experiments 24 h later.

**Western blot analysis.** The cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology, Suzhou, China) and the protein lysates were centrifuged at 13,400 x g for 30 min. The supernatants were collected and quantified using the Bradford protein assay method with BSA as the standard. The protein was then denatured by heating to 95°C for 5 min. Approximately 20  $\mu$ g protein was loaded into each well of the precast NuPAGE 10% Bis-Tris protein gel. Following electrophoresis, the proteins were transferred onto a polyvinylidene fluoride membrane using the NuPAGE transfer buffer. The membranes were blocked with 5% BSA in TBST at room temperature for 1 h, and then incubated overnight at 4°C with primary antibodies at the dilutions recommended

by the manufacturer (1:500). The blots were then incubated with HRP-conjugated IgG secondary antibodies (1:5,000 dilutions) for 1 h at 37°C. Bands were detected using Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500; EMD Millipore, Billerica, MA, USA) and imaged using an ImageQuant 350 system (GE Healthcare Life Sciences, Piscataway, NJ, USA) (23).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RNA was extracted from the HTM cells using an RNeasy Isolation kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA, USA), and then reverse transcribed. The reaction mixture comprised 1  $\mu$ g total RNA, 1  $\mu$ l random primer (50  $\mu$ mol/l, cat. no. 48190011; Thermo Fisher Scientific, Inc.), 1X reverse transcription buffer and 10 units reverse transcriptase (A5003; Promega, Madison, WI, USA) in a total volume of 20  $\mu$ l. The RNA and primer were heated to 72°C and slowly cooled prior to reverse transcription at 42°C for 1 h (24). When cooled to room temperature, the reaction was diluted to 100  $\mu$ l with RNase-free water. qPCR, was carried out with SYBR-Green PCR Master mix in a total reaction volume of 20  $\mu$ l using the following amplification steps: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec; and then elongation at 55°C for 30 sec. The expression levels were normalized to those of the internal standard 18S rRNA. The following primer sequences were used: YAP sense, 5'-ACCCACAGCTCAGCATCTTCG-3' and antisense, 5'-TGGCTTGTTCATCCATCAG-3'; TAZ sense, 5'-GTCACCAACAGTAGCTCAGATC-3' and antisense, 5'-AGTGATTACAGCCAGGTTAGAAAG-3'; 18S rRNA sense, 5'-GGGCATTCGTATTCATAGTCAGAG-3' and antisense, 5'-CGGTTCTTGATTAATGAAAACATCCT-3'. The experiment was performed in triplicate. The YAP and TAZ mRNA expression levels of the DEX-treated groups (treated with 10  $\mu$ M dexamethasone at 37°C for 48 h) were normalized relative to those of the DMSO (DEX-free) group, which was assigned a value of 1.0. In order to control for slight variations in the amount of RNA loaded for PCR, the difference in cycle threshold ( $\Delta$ Cq) between the gene of interest and the average cycle threshold (Cq) of the house keeping gene 18S (also loaded in triplicate wells) were calculated. By calculating the difference in  $\Delta$ Cq between the DEX-treated group and the control group, the experimental data was normalized to the control data using the  $2^{-\Delta\Delta Cq}$  method (25).

**Permeability assay.** The *in vitro* permeability assay was performed as previously reported (26). Briefly, HTM cells were plated onto the cell culture inserts of Transwell plates (cat. no. 353104, BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA). When the cells reached subconfluence, they were transfected with the aforementioned siRNA or plasmids. At 3 days after transfection, the cells were subjected to an *in vitro* permeability assay. On the day of the assay, the medium in the upper and lower chambers was replaced with fresh DMEM, and 150  $\mu$ l FITC-Dextran (1:30 dilution, D1845; Thermo Fisher Scientific, Inc.) was added to the medium in the upper chamber of each insert. After 5 min at room temperature, 100  $\mu$ l solution from the lower chamber was transferred to a 96-well plate. The plate was read using

an Envision 2103 Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at an excitation wavelength of 480 nm and emission wavelength of 530 nm, with a bandwidth of 10.

**Statistical analysis.** All data are presented as mean  $\pm$  standard error of the mean from at least three independent experiments. Statistical significance was determined by one-way analysis of variance followed by Bonferroni correction when three or more groups were compared, and by Student's t-test when two groups were compared.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### *DEX induces elevated YAP and TAZ expression in HTM cells.*

A previous study has demonstrated a marked stiffness of the HTM in glaucomatous patients (5). In addition, the HTM substratum stiffness closely correlates with the expression and activity of the YAP and TAZ proteins (27). Thus, it was hypothesized that DEX-induced substratum stiffness is accompanied by elevated expression levels of YAP and TAZ. Staining of the HTM cells with the F-actin probe FITC-phalloidin, as shown in Fig. 1A-E, indicates that DEX altered the F-actin architecture in a concentration-dependent manner, and promoted the formation of a cross-linked actin network (CLAN) in the HTM cells (10). To determine whether YAP and TAZ transcription was associated with the DEX induced-CLAN formation, RT-qPCR was performed. It was observed that  $1 \times 10^{-5}$  M DEX induced a significant increase in the transcription level of the YAP gene (Fig. 1F). In addition, the transcription level of the TAZ gene was also significantly elevated in the presence of  $1 \times 10^{-5}$  M DEX (Fig. 1G). These results suggest an association of the DEX-induced cytoskeletal changes in the cultured HTM cells with the increased transcription levels of YAP and TAZ. To further validate the correlation of the expression levels of YAP and TAZ with the DEX-induced cytoskeletal reorganization, western blotting was performed. As shown in Fig. 1H, the expression levels of the YAP and TAZ proteins in the HTM cells were observed to increase as the concentration of DEX increased. These data indicate that DEX has a dose-dependent association with the transcription and expression levels of YAP and TAZ.

**YAP and TAZ regulate actin-associated proteins.** Previous studies have demonstrated that in response to DEX treatment, several actins and actin-associated proteins are involved in the development of a CLAN (28). Furthermore, among those proteins, fibronectin (29) and laminin (30) have been shown to be increased in DEX-treated HTM cells. Therefore, in order to further elucidate the effects of YAP and TAZ on actin-associated proteins, YAP and TAZ genes were knocked down using siRNA (Fig. 2A), and the expression levels of fibronectin and laminin were measured. As shown by the western blotting results in Fig. 2B, DEX markedly increased the expression levels of fibronectin and laminin. However, the elevated expression levels were attenuated by the addition of YAP and TAZ siRNA. The expression of ECM-associated molecules, including collagen types I and IV, is known to be associated with DEX-induced CLAN formation (31,32). Thus, the expression levels of types I and IV collagen were

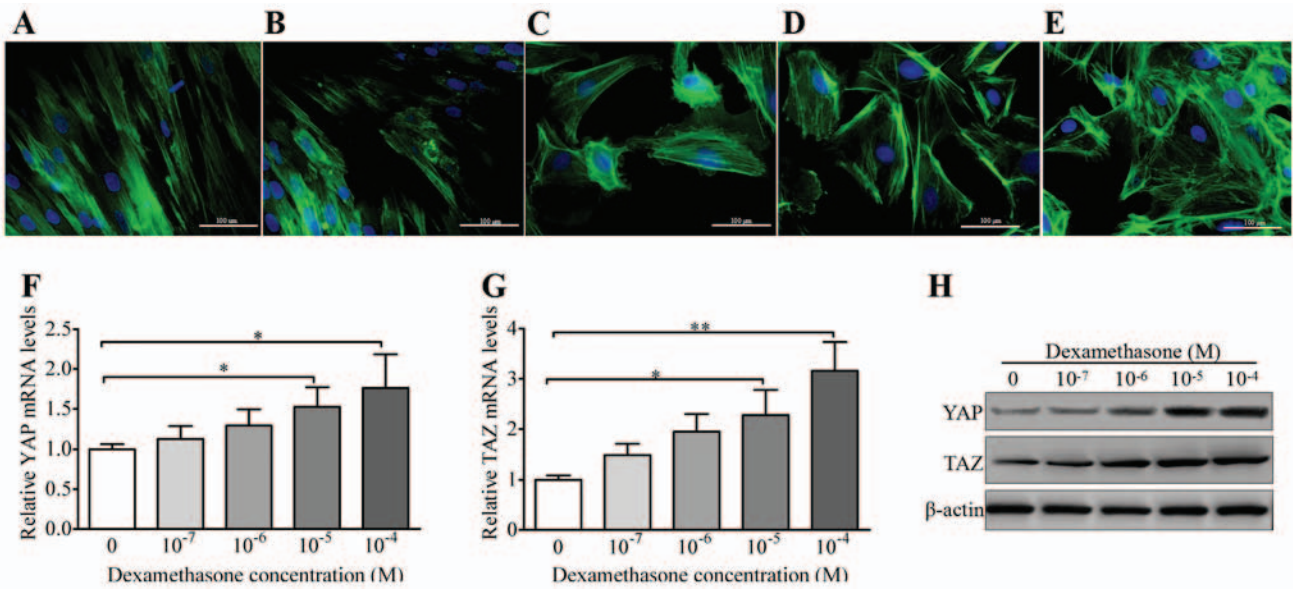


Figure 1. DEX increases the expression of YAP and TAZ. (A-E) Observation of DEX-induced cross-linked actin network formation by HTM cells. The cells were treated with (A) DMSO, (B)  $1 \times 10^{-7}$  DEX, (C)  $1 \times 10^{-6}$  DEX, (D)  $1 \times 10^{-5}$  DEX and (E)  $1 \times 10^{-4}$  DEX. Green, F-actin stain; blue, nuclear stain (DAPI). Scale bar, 100  $\mu$ m. (F) YAP mRNA levels and (G) TAZ mRNA levels in HTM cells treated with different concentrations of DEX. (H) Western blot analysis of YAP and TAZ proteins in the DEX-treated HTM cells. Protein bands shown are representative of three independent experiments with similar results. Data are presented as the mean  $\pm$  standard error of the mean from three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ . DEX, dexamethasone; YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; HTM, human trabecular meshwork.

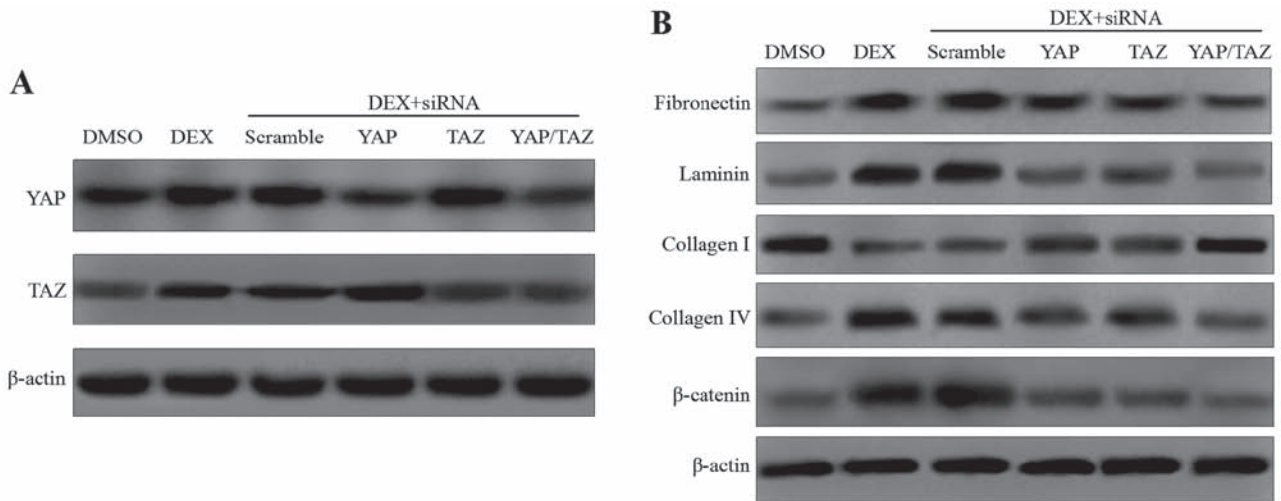


Figure 2. Knockdown of YAP and TAZ mRNA results in the inhibition of actin-related proteins in DEX-stimulated HTM cells. (A) Western blot analysis demonstrating the efficiency of YAP siRNA knockdown and/or TAZ siRNA knockdown. (B) Western blot analysis of fibronectin, laminin, collagen I, collagen IV and  $\beta$ -catenin following transfection with YAP siRNA and/or TAZ siRNA. Protein bands shown are representative of three independent experiments with similar results. YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; DEX, dexamethasone; HTM, human trabecular meshwork; siRNA, small interfering RNA.

determined in the present study. Increased expression levels of type I collagen and decreased expression levels of type IV collagen were observed in the HTM cells treated with DEX plus YAP and/or TAZ siRNA concurrently compared with those treated with DEX alone (Fig. 2B). In addition, a previous study by the present research team demonstrated that the expression of  $\beta$ -catenin was induced by DEX in cultured HTM cells (33). The present study confirmed this, and revealed that  $\beta$ -catenin expression was decreased in the DEX-treated cells transfected with YAP and/or TAZ siRNA compared with that in the DEX control.

To further substantiate the association of YAP and TAZ with the proteins shown in Fig. 2B, the HTM cells were cotransfected with YAP and/or TAZ expression plasmids (Fig. 3A). The western blotting results presented in Fig. 3B indicate that the expression levels of fibronectin and laminin were increased in the presence of YAP and TAZ overexpression, and the expression was comparable to that in the DEX-treated cells. In addition, the expression of type I collagen was inhibited in the YAP and TAZ overexpression groups, whereas the expression of type IV collagen was clearly increased. Increased expression of  $\beta$ -catenin was also observed in the YAP and TAZ overexpressing

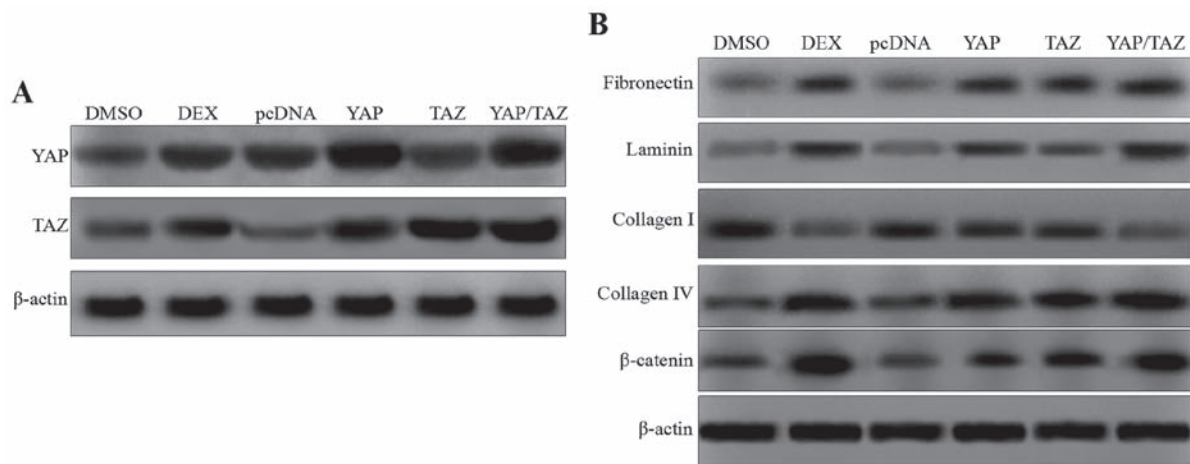


Figure 3. Overexpression of YAP and TAZ mRNA results in the activation of actin-related proteins in HTM cells. (A) Western blot analysis demonstrating the efficiency of YAP overexpression and/or TAZ overexpression. (B) Western blot analysis of fibronectin, laminin, collagen I, collagen IV and  $\beta$ -catenin following transfection with plasmids. Protein bands shown are representative of three independent experiments with similar results. YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; HTM, human trabecular meshwork; DEX, dexamethasone; pcDNA, empty vector.

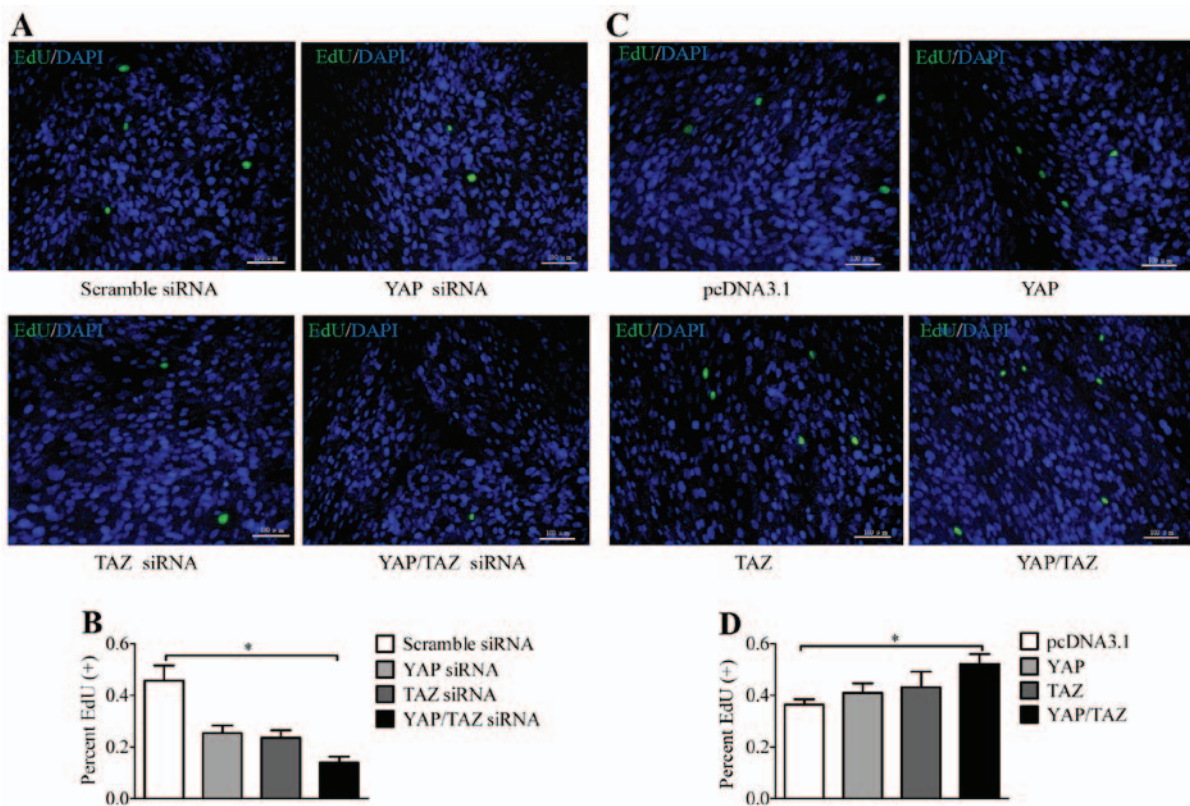


Figure 4. YAP and TAZ induce the proliferation of HTM cells. (A) EdU fluorescent staining of HTM cells following transfection with YAP siRNA and/or TAZ siRNA and (B) quantified results. (C) EdU fluorescent staining of HTM cells following transfection with YAP and/or TAZ expression plasmids and (D) quantified results. (A and C) Blue staining indicates the DAPI-labeled cell nuclei, and green staining indicates the EdU-labeled nuclei of proliferating cells. Magnification,  $\times 100$ . (B and D) Quantification was performed by manually counting the EdU-positive HTM cells, and calculating the proliferation percentages. Data are the mean  $\pm$  standard error of the mean from three independent experiments. \* $P < 0.05$ . YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; HTM, human trabecular meshwork; siRNA, small interfering RNA; EdU, 5-ethynyl-2'-deoxyuridine.

cells. These data demonstrate that YAP and TAZ regulate actin-associated proteins and the expression of  $\beta$ -catenin.

*YAP and TAZ induce the proliferation of HTM cells.* The Hippo/Yap pathway is a well-conserved signaling cascade that regulates cell proliferation and differentiation, and

controls organ size (34,35). In the present study, the proliferation of HTM cells was investigated by labeling the cells with EdU to fluorescently stain the replicating cells. Notably, the fluorescence images indicate that the cells in which the YAP and TAZ genes were both knocked down possessed significantly reduced proliferation capability (Fig. 4A and B),

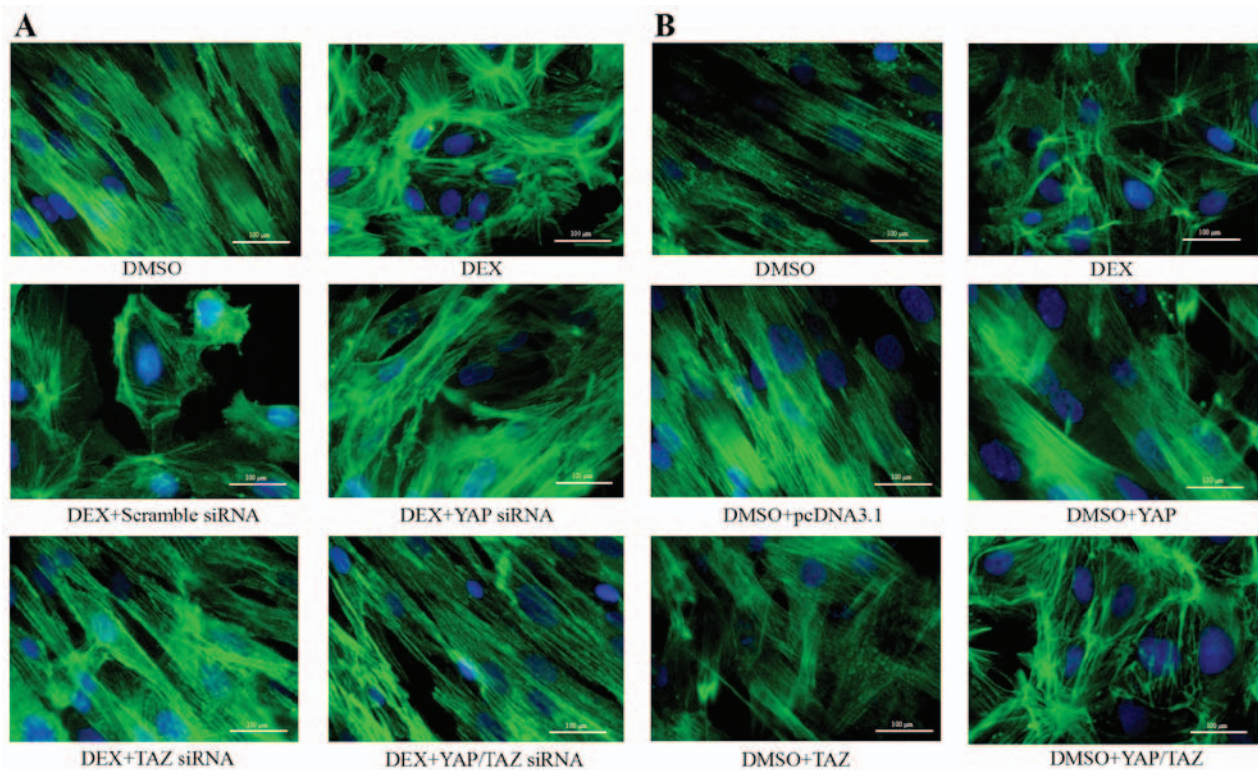


Figure 5. Inhibition of YAP and TAZ rescues HTM cells from cross-linked actin network formation. (A) Immunofluorescent staining of the cytoskeleton of HTM cells in the presence of YAP siRNA and/or TAZ siRNA. (B) Immunofluorescent staining of the cytoskeleton of HTM cells in the presence of YAP and/or TAZ plasmids. Blue staining indicates the DAPI-labeled cell nuclei, and green staining indicates cytoskeletal staining with fluorescein isothiocyanate-labeled phalloidin. Magnification, x400. YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; HTM, human trabecular meshwork; siRNA, small interfering RNA; DEX, dexamethasone; pcDNA, empty vector.

indicating that YAP and TAZ may have a pivotal role in the regulation of HTM cell proliferation. By contrast, as depicted in Fig. 4C and D, when expression vectors for YAP and TAZ were employed, the HTM cells cotransfected with YAP and TAZ plasmids exhibited significantly increased proliferation ability. These results indicate the involvement of YAP and TAZ in the regulation of the proliferation of HTM cells.

*YAP and TAZ regulate CLAN formation in HTM cells.* Since CLAN formation in glaucomatous and DEX-treated HTM cells has been indicated to contribute to reduced outflow facility (36), CLAN formation in the presence of DEX, YAP siRNA and/or TAZ siRNA was investigated in the present study. From the images presented in Fig. 5A, it is clear that DEX-treated HTM cells formed unusual geodesic dome-like cross-linked actin networks. The majority of these cells exhibited a CLAN, which was composed of a series of interconnected F-actin bundles that radiated outward from central vertices in a geodesic network. However, YAP siRNA and TAZ siRNA appeared to markedly alter overall cell spreading, and induced reorganization of the actin skeleton. Thus, it appears that CLAN formation in the HTM cells cotransfected with YAP siRNA and TAZ siRNA was attenuated. The actin cytoskeleton and actin microfilament patterns were comparable to those of the DMSO-treated control cells. To further evaluate whether YAP and TAZ contribute to rearrangement of the cytoskeleton in HTM cells, YAP and/or TAZ overexpression plasmids were transfected into the HTM cells. As

shown in Fig. 5B, the overexpression of YAP and TAZ induced CLAN formation with polygonal actin arrangements in the HTM cells. The actin filaments were dispersed around the cell, with a few microfilaments in the center, and were similar in appearance to those of the DEX-treated control cells. These observations suggest that YAP and TAZ play a key role in CLAN formation in HTM cells.

*YAP and TAZ modulate the aqueous humor outflow of HTM cells.* It has been suggested that DEX-induced CLAN formation in HTM cells decreases the overall contractility of the tissue by causing the cells to become more rigid and unable to change shape (10). Therefore, the permeability of DEX-treated monolayer HTM cells may be markedly impaired. In order to further investigate the hypothesis that YAP and TAZ regulate the function of HTM cells, a permeability assay was performed. The DEX-treated monolayer HTM cells exhibited impaired permeability, which is consistent with previous studies (26,37). However, when YAP and TAZ expression was downregulated with a combination of YAP and TAZ siRNA, the permeability was increased compared with that in the cells treated with DEX alone and those treated with DEX plus scramble siRNA (Fig. 6A). In addition, cells transfected with a combination of YAP and TAZ expression plasmids exhibited significantly decreased permeability compared with the cells treated with DMSO and transfected with an empty plasmid (Fig. 6B). This observation indicates that YAP and TAZ regulate aqueous outflow and, thereby alter the function of HTM cells.

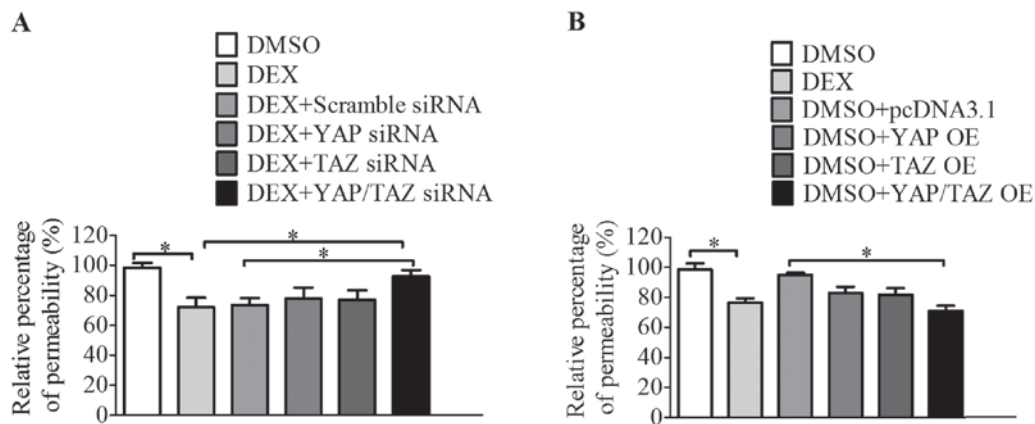


Figure 6. YAP and TAZ modulate aqueous humor outflow via the inhibition of permeability. Relative permeability percentages of HTM cell monolayers transfected with (A) YAP siRNA and/or TAZ siRNA and (B) YAP and/or TAZ expression plasmids. Data are the mean  $\pm$  standard error of the mean from three independent experiments. \* $P < 0.05$ . YAP, Yes-associated protein; TAZ, transcriptional coactivator with PDZ-binding motif; HTM, human trabecular meshwork; siRNA, small interfering RNA; DEX, dexamethasone; pcDNA, empty vector; OE, overexpression.

## Discussion

POAG is a common cause of blindness (1). Although the exact cause of POAG is not yet clearly understood, the main abnormal changes associated with this condition appear to affect the TM (3). TM cells are capable of the phagocytosis, migration, synthesis and secretion of ECM components, and the transduction of signals between ECMs. Dysfunction of the TM and the lining cells of Schlemm's canal, as well as structural abnormalities of the ECM are the main causes of resistance to the outflow of aqueous humor. Changes in ECM components cause narrowing of the outflow tract in adjacent areas, thereby increasing resistance to the outflow of aqueous humor and elevating the IOP (38). In the present study, the signal transduction of YAP and TAZ in HTM cells, and its regulatory role in the cytoskeletal arrangement of HTM cells were investigated.

The symptoms of POAG include increased resistance to the outflow of aqueous humor, increased TM hardness, hardening and degeneration of the trabecular tissue, reduced mesh density, irregularity or injury of the trabecular plate layer, increased endothelial cell density, the degeneration of collagen or elastic fibers, and the narrowing of spaces in the TM (5). These changes in TM properties are indicative of a biophysical mechanism for the regulation of IOP and glaucoma (39-43). However, the molecular mechanism by which the biophysical properties affect cell behaviour is not yet completely understood. YAP and TAZ have been identified as mechanical signaling factors, which are affected by ECM hardness (20). YAP and TAZ are the main effectors of the classical Hippo pathway in the regulation of organ size and tissue topology; however, they are considered to exert actions beyond those of the classical pathway due to their dynamic transduction effects as nuclear transcription factors in mechanical signaling (44). As mechanotransducers of the extracellular-microenvironment and coactivators of transcription, YAP and TAZ have been shown to be upregulated by the elastic hardness of the TM in glaucoma (19). In addition, previous studies have demonstrated that YAP serves important roles in the regulation of cell proliferation and apoptosis,

the control of organ size, the contact inhibition of cells and tumorigenesis (13-17,44).

Glucocorticoids have been shown to cause progressive changes in the organization of the microfilaments in TM cells (31,45). Therefore, in the present study, the effects of DEX on HTM cells were investigated. It was observed that as the concentration of DEX increased, the expression levels of YAP also significantly increased, and the cytoskeleton was damaged. TAZ and YAP genes are both conjugated with 14-3-3 $\sigma$  protein, and have the same transcription activation function (46). The similarity of the TAZ and YAP genes was reflected in the dose response data for DEX, which demonstrated that the expression levels of TAZ were also elevated as the concentration of DEX increased. It has been reported that in HTM cells cultured under high pressure, the expression levels of YAP and TAZ greatly exceed than those of HTM cells cultured at normal pressure (19). Their study demonstrated that numerous proteins are involved in the dynamic induction and transduction process, and identified that YAP and TAZ are dynamic transduction and transcription factors. YAP and TAZ have been confirmed to exist in all layers of the TM, including adjacent tissue (14), which is considered to be important in the generation of resistance to the outflow of aqueous humor. Therefore, the further exploration of YAP and TAZ genes in glaucoma is merited.

The fluorescence microscopic analysis conducted in the present study demonstrated that the knockdown of YAP and TAZ rescued HTM cells from the morphological changes induced by DEX. Furthermore, it revealed that the overexpression of YAP and TAZ contributed to the reorganization of HTM cells to form geodesic-dome-like polygonal lattices. Since reorganization of the TM cytoskeleton alters cell function (8,47), the normalization of HTM cell morphology by silencing the expression of YAP and TAZ was observed to restore the functionality of the HTM cells in the presence of DEX in the cell permeability assay.

The glucocorticoid treatment of cultured TM cells has been reported to inhibit TM cell migration and proliferation (45). Therefore, the present study investigated the effect of YAP and TAZ knockdown on the proliferation of HTM cells.

It has been reported that  $\beta$ -catenin binds with E-cadherin to bridge between the cytoplasmic domain of cadherin and the actin cytoskeleton, thus realizing a connection between cells and affecting the adhesion and motility of cells (48). The Wnt signaling pathway is considered a new target for intervention in the treatment of glaucoma, and the Wnt/ $\beta$ -catenin signaling pathway has been demonstrated to be modulated by YAP and TAZ, with TAZ exhibiting antagonistic effects (49,50). In the present study, it was demonstrated that the knockdown of YAP and TAZ decreased the expression levels of  $\beta$ -catenin, whereas YAP and TAZ overexpression increased them. In addition, the present study indicated that YAP and TAZ overexpression stimulated cell proliferation.

The present study on the mechanisms underlying the effects of YAP and TAZ in HTM cells indicates that YAP and TAZ serve critical roles in glaucoma. The expression of YAP and TAZ in the cultured TM cells of normal human eyes was demonstrated by western blotting and RT-qPCR. Notably, when the HTM cells were treated with DEX, the expression levels of YAP and TAZ were elevated, indicating the involvement of YAP and TAZ in the pathogenesis of glaucoma. The treatment of cultured TM cells with DEX is a classical model with which to simulate the state of TM cells in glucocorticoid-induced glaucoma. In the present study, the treatment of TM cells with DEX was demonstrated to markedly increase the expression levels of fibronectin, laminin and collagen IV in the ECM, reduce the expression of collagen I, induce the restructuring of actin microfilaments, promote the formation of classical cross-linked actin networks, and increase the expression of  $\beta$ -catenin. These changes are likely to result in increased TM hardness, restructuring of the TM and dynamic changes in the microenvironment.

In conclusion, in the present study, the effects of the knock-down and overexpression of YAP and TAZ on the expression levels of fibronectin, laminin, collagen I, collagen IV and  $\beta$ -catenin were analyzed, and the effects of YAP and TAZ on the ECM and the actin microfilaments and cytoskeletons of DEX-treated HTM cells were observed. The findings improve our understanding of the involvement of YAP and TAZ in glaucoma pathogenesis, and suggest new mechanisms for the development and progression of glaucoma.

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